Latitudinal variation in cold tolerance and the role of *vrille* and *Yolk protein* in enhancing cold adaptation in highly cold-tolerant insect species

Running title: Cold adaptation in *Drosophila*

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ABSTRACT

Identifying elements that enhance insect adaptation into changing environmental conditions is challenging, especially for traits affected by multiple factors. We studied latitudinal variation in the basal cold tolerance and body colour of two highly cold-tolerant *Drosophila virilis* group species, *D. montana* and *D. flavomontana*, in climatically diverse locations in North America. *D. montana*, which is generally found on higher latitudes and altitudes than *D. flavomontana*, was darker and more cold-tolerant than *D. flavomontana*. In *D. montana* only fly cold tolerance showed latitudinal variation, while in *D. flavomontana* both traits varied according to latitude and local climatic conditions, but showed no correlation with each other. We also examined the role of a circadian clock gene *vrille* and an insulin-signalling pathway gene *Yolk protein 3 (Yp3)* in the basal cold tolerance and cold acclimation ability of *D. montana* females using RNA interference. Silencing of *vrille* induced expression changes in the *period*, but not *clock*, and decreased flies’ basal cold tolerance and cold acclimation ability, while silencing of *Yp3* affected only cold acclimation. Our study demonstrates that the dependence of insect cold tolerance on latitudinally varying factors and local climatic conditions may vary even between closely-related species. Furthermore, we propose that a functional circadian clock system plays an essential role both in insect basal cold tolerance and cold acclimation ability, and that *Yp3* affects cold acclimation likely through its interactions with other genes in the insulin-signalling pathway.

Keywords: cold tolerance, climate, body colour, RNAi, *Drosophila montana*, *Drosophila flavomontana*
1 | INTRODUCTION

Species’ geographical distribution is largely defined by their ability to tolerate stressful conditions and to respond to rapid environmental changes (e.g. Addo-Bediako, Chown, & Gaston, 2000; Andersen et al., 2015; Kellermann et al., 2012; Kimura, 2004; Overgaard, Kristensen, Mitchell, & Hoffmann, 2011; Sunday, Bates, & Dulvy, 2011). Especially, in temperate, polar and montane regions, ectothermic species and plants have developed a variety of physiological and behavioural strategies, which increase their basal cold tolerance and/or their cold acclimation ability (Angilletta, 2009; Doucet, Walker, & Qin, 2009). To predict how species are able to survive in changing environmental conditions, it is essential to explore how different geographic and climatic factors shape their distributions (Helmuth, Kingsolver, & Carrington, 2005; Kellermann et al., 2012; Pörtner & Farrell, 2008; Somero, 2010) and what mechanisms underlay their ability to respond to daily and seasonal temperature changes. This is especially crucial in the current phase of global warming.

Latitudinal variation in cold tolerance has been observed in a variety of species, including some *Drosophila* species (Hallas, Schiffer, & Hoffmann, 2002; Hoffmann, Anderson, & Hallas, 2002; Overgaard, Hoffmann, & Kristensen, 2011), *Porcellio laevis* woodlice (Castañeda, Lardies, & Bozinovic, 2005), *Myrmica* ants (Maysov, 2014) and *Arabidopsis thaliana* flowers (Zhen & Ungerer, 2008; Zuther, Schulz, Childs, & Hincha, 2012). On the other hand, studies e.g. on some other *Drosophila* (Arthur, Weeks, & Sgrò, 2008) and *Embryonopsis hatticella* moth (Klok & Chown, 2005) have given less clear results. Lack of latitudinal variation in traits such as cold tolerance may be due to low genetic variation, relatively weak latitudinal selection compared to ones induced by local environmental conditions and/or gene flow from conspecific populations (Angilletta, 2009). However, latitudinal variation in cold tolerance may also remain undetected due to a small number of study populations or the robustness of research methods,
or its existence may be covered by local selection pressures varying e.g. according to altitude and closeness to sea. Insect cold tolerance may also be affected by their body colour, as dark cuticle pigmentation can increase solar radiation absorption and enable insects to warm up in colder environments (Clusella-Trullas, van Wyk, & Spotila, 2007; Harris, McQuillan, & Hughes, 2013; Rajpurohit, Parkash, & Ramniwas, 2008). Body colour has been shown to vary latitudinally e.g. in *Drosophila melanogaster* (Parkash & Munjal, 1999), and to get darker towards the cold season through increased melanisation in *Drosophila suzukii* (Shearer et al., 2016; Stephens, Asplen, Hutchison, & Venette, 2015).

Adaptation to seasonally varying environmental conditions can be enhanced by plastic responses. Accordingly, the cold tolerance of many species is increased prior or during the cold season as a response to environmental cues, such as a decreasing temperature and a shortening day length (Denlinger, 1991; Vesala, Salminen, Kankare, & Hoikkala, 2012; Zuther et al., 2012). Cold acclimation can vary from minutes to weeks, although the effects of long-term acclimation are usually more pronounced (Hoffmann, Sørensen, & Loeschcke, 2003; Ransberry, Macmillan, & Sinclair, 2011; Teets & Denlinger, 2013). The extent of species cold acclimation response has been found to correlate strongly with their geographic distribution and climate variability at their home site (Isobe, Takahashi, & Tamura, 2013; Zuther et al., 2012). Moreover, several studies have reported trade-offs between species’ basal cold tolerance and cold acclimation ability (Gerken, Eller, Hahn, & Morgan, 2015; Noh, Everman, Berger, & Morgan, 2017; Nyamukondiwa, Terblanche, Marshall, & Sinclair, 2011).

*Drosophila* flies can sense temperature changes through thermosensory organs in their aristae and legs, and transfer these signals into dorsal clock neurons located in their head (Barber & Sehgal, 2018; Yadlapalli et al., 2018). Signals indicating temperature decrease can induce changes e.g. in ion transport, membrane restructuring, calcium signalling and the synthesis of
cryoprotectants and antifreeze proteins, which can evoke cold acclimation (Teets & Denlinger, 2013) and help the flies to maintain cell homeostasis and functionality at low temperatures (MacMillan et al., 2016). However, the abrupt disruptions in transmembrane ion gradients and ion distribution can also lead to water loss and accumulation of chill injuries, which can eventually lead to death (Findsen, Pedersen, Petersen, Nielsen, & Overgaard, 2014; MacMillan, Andersen, Davies, & Overgaard, 2015).

Several studies have suggested species cold tolerance and cold acclimation ability to be associated with genes involved in the circadian clock system (Fowler & Thomashow, 2002; Magnone, Jacobmeier, Bertolucci, Foa, & Albrecht, 2005; Vesala, Salminen, Laiho, Hoikkala, & Kankare, 2012) and/or metabolic pathways, such as the insulin-signalling pathway (e.g. Sim & Denlinger, 2013; Teets et al., 2012). The role of the circadian clock system in insect cold adaptation, especially in cold acclimation, is logical, as this clocks monitors light and temperature cycles and entrains behavioural and physiological rhythms to match with them (Barber & Sehgal, 2018). In the central circadian clock, described most thoroughly in D. melanogaster, daily rhythms are driven via one or several transcriptional feedback loops involving changes in the expression of core circadian clock genes (studies reviewed e.g. Gunawardhana & Hardin, 2017; Hardin, 2011; Williams & Sehgal, 2001). Briefly, the first core timekeeping loop includes clock (clk) – cycle (cyc) and period (per) – timeless (tim) complexes that activate and repress each other. clock and cycle further activate an interlocked feedback loop that includes vrille (vri) and Par domein protein 1 (Pdp1), which repress and activate clock transcription, respectively. vrille also regulates neuropeptide Pigment-dispersing factor (PDF) expression in clock neurons, which makes it a key regulator of circadian behavioural rhythms (Gunawardhana & Hardin, 2017). Interestingly, vrille expression has been found to increase during cold acclimation e.g. in Drosophila montana (Parker et al., 2015; Vesala, Salminen,
Laiho, et al., 2012). The insulin-signalling pathway, on the other hand, is known to have widespread effects e.g. on insect development, aging, immune function and stress resistance (reviewed in Flatt, Tu, & Tatar, 2005). This pathway involves major hormonal regulators, juvenile hormone (JH) and 20-hydroxy-ecdysone (20E), for which effects are mediated, among other genes, by Yolk proteins (reviewed in Flatt et al., 2005). Yolk protein genes code for a phospholipoglycoprotein vitellogenin, which is primarily an egg-yolk protein synthesized in insect ovarian follicle and fat body cells (reviewed in Flatt et al., 2005), but these genes play a role also in neurogenesis (Neumüller et al., 2011). Under diapause inducing temperature and light conditions, the Yolk protein genes Yp1, Yp2 and Yp3 are dozens of times higher in D. melanogaster head than in ovaries (Zhao et al., 2015), which suggests that the genes have important regulatory functions at neuronal level during diapause. Parker, Ritchie, & Kankare (2016) have suggested that the same could be true during cold acclimation. Moreover, Davis & Moyle (2020) suggest the function of Yolk proteins to extend beyond female reproductive functions and to be associated broadly with stress response.

Kellermann et al. (2012) have shown that Drosophila virilis group species possess very high cold tolerance compared to most other species of the genus. These species differ from more southern species, including D. melanogaster, also in their photoperiodic time-measuring system and in the expression of several daily and seasonal rhythms (Bertolini et al., 2019; Kauranen et al., 2012; Menegazzi et al., 2017). Thus, they offer a good opportunity to complement studies performed on less cold-tolerant southern species and to add new insights on the effects of latitude and local climatic conditions on insect cold tolerance. Both of our study species, Drosophila montana and Drosophila flavomontana, have populations on the western coast and the Rocky Mountains of North America across different latitudes (Fig. 1). D. montana lives generally at higher latitudes and altitudes than D. flavomontana, but in some sites they occur
sympatrically and may hybridize to some degree (Patterson, 1952; Poikela et al., 2019; Throckmorton, 1982). Body colour of *D. montana* is almost black, while that of *D. flavomontana* varies from light to dark brown (Patterson, 1952).

In this study, we traced factors affecting the cold tolerance of *D. montana* and *D. flavomontana* flies originating from diverse climatic conditions and habitats across different latitudes in North America, and disentangled the role of circadian clock system gene *vrille* and insulin-signalling pathway gene *Yolk protein 3* (*Yp3*) in the cold adaptation of *D. montana*. First, we defined the level of basal cold tolerance and the intensity of body colour in several *D. montana* and *D. flavomontana* populations to find out whether their cold tolerance shows latitudinal variation and whether this variation is associated with local climatic conditions and/or fly body colour. Secondly, we tested whether silencing *vrille* or *Yp3* with RNA interference induces expression changes in two circadian clock genes, *clock* and *period*, and whether their silencing decreases female cold tolerance and/or cold acclimation ability in *D. montana*.

2 | MATERIALS AND METHODS

2.1 | Variation in the basal cold tolerance and body colour in *D. montana* and in *D. flavomontana* populations

2.1.1. Study species and populations

*D. montana* and *D. flavomontana* belong to the *montana* phylad of the *virilis* group, and our recent whole-genome analyses have shown their divergence time to be ~1 mya (Poikela et al., in preparation). *D. montana* is distributed around the northern hemisphere across North America, Asia and Europe (Throckmorton, 1982), while the distribution of *D. flavomontana* is restricted to North America (Patterson, 1952; Throckmorton, 1982). In the central Rocky
Mountains *D. montana* is found at altitudes from 1400 m to well over 3000 m, while *D. flavomontana* is found mainly below 2000 m. In the western coast, where *D. flavomontana* has probably invaded only during the last decades, both species live at much lower altitudes (see Patterson, 1952; Poikela et al., 2019). We performed phenotypic assays on the females from 23 *D. montana* and 20 *D. flavomontana* isofemale strains, which were established from the progenies of fertilized females collected from several sites in North America between 2013 and 2015 (Fig. 1). Each site was represented by three isofemale strains per population per species, when possible (Fig. 1; Table S1), and all the strains were maintained since their establishment (15-30 generations) in continuous light at 19 ± 1 °C to prevent females from entering reproductive diapause. For the experiments, we sexed emerging flies under light CO₂ anaesthesia within three days after emergence. Females were changed into fresh malt-vials once a week and used in experiments at the age of 20 ± 2 days, when they all had fully developed ovaries (Salminen & Hoikkala, 2013).

| Site                      | Year | Latitude | Altitude (m) | Longitude |
|---------------------------|------|----------|--------------|-----------|
| Seward, AK, USA           | 2013 | 60° 10’N | 35           | 149° 27’W |
| Terrace, BC, Canada       | 2014 | 54° 27’N | 217          | 128° 34’W |
| McBride, BC, Canada       | 2014 | 53° 07’N | 720          | 120° 18’W |
| Vancouver, BC, Canada     | 2014 | 49° 15’N | 4            | 123° 10’W |
| Cranbrook, BC, Canada     | 2014 | 49° 36’N | 940          | 115° 46’W |
| Ashford, WA, USA          | 2013 | 46° 45’N | 573          | 121° 57’W |
| Livingston, MT, USA       | 2013 | 45° 21’N | 1605         | 110° 36’W |
| Jackson, WY, USA          | 2013 | 43° 26’N | 1875         | 110° 50’W |
| Afton, WY, USA            | 2015 | 42° 43’N | 2000         | 110° 55’W |
| Liberty, UT, USA          | 2015 | 41° 20’N | 1600         | 111° 51’W |

Figure 1. Table shows fly collecting sites and years, and the coordinates for each site. Map contains information on whether we have samples from one or both species in each site in the western coast and in the Rocky Mountains (brown area on the map) in North America (detailed information in Table S1). The map template obtained from [https://d-maps.com/carte.php?num_car=5082&lang=en](https://d-maps.com/carte.php?num_car=5082&lang=en)
2.1.2 | Cold tolerance tests

We investigated latitudinal variation in the cold tolerance of *D. montana* and *D. flavomontana* females using two well-defined and ecologically relevant methods: chill coma temperature (CT\textsubscript{min}; also called critical thermal minimum) and chill coma recovery time (CCRT). CT\textsubscript{min} corresponds to the temperature, at which the fly loses all neurophysiological activity and coordination and falls into a chill coma. In this test, we placed the females individually in glass vials, which were submerged into a 30 % glycol bath. We then decreased the bath temperature from the starting temperature of 19 °C at the rate of 0.5 °C/min and scored the CT\textsubscript{min} for each fly. The second method, CCRT, measures the time taken from a fly to recover from a standardized exposure time at a chill-coma-inducing temperature (reviewed in MacMillan & Sinclair, 2011). In this test, we submerged the females individually in glass vials into a 30 % glycol bath for 16 hours at -6 °C (Vesala, Salminen, Laiho, et al., 2012). After returning the vials into 19 ± 1 °C in fly laboratory, we measured the time required for each female to recover from the chill coma and stand on its legs. CT\textsubscript{min} tests were replicated 21 times and CCRT tests 20 times with Julabo F32-HL Refrigerated/Heating Circulator and Haake k35 DC50 Refrigerating Bath Chiller, respectively. To account for possible variation between replicates, each test included 1-2 females from each strain.

2.1.3 | Fly body colour

We analysed the body colour of the same flies that had been phenotyped in CT\textsubscript{min} or CCRT tests. The flies were photographed under Leica L2 microscope with 5x magnification, using Leica DFC320 R2 camera together with the Leica Application Suite software v4.3.0. Exposure time, zoom factors and illumination level were kept constant, and the photographing arena was surrounded by a plastic cylinder to eliminate glares on the chitinous surface of the fly. All
photographs were taken within 3 months after the cold tolerance tests (females were kept this period in -20 °C). Images were saved in 24-bit (RGB) format and the colour intensity was measured using grayscale image analysis (ImageJ; Schneider, Rasband, & Eliceiri, 2012); linearly scaling from 0 to 255 (0 = black, 255 = white). We took colour measurements from part of thorax (scutum; see Fig. S1), as our preliminary tests showed that it best incorporates the colour variation among flies (Fig. S1).

2.1.4 | Statistical analyses

We used different statistical models to investigate whether variation in fly cold tolerance or body colour was associated with latitude and/or local climatic factors, and whether these traits showed correlation with each other. In these models, we used either $CT_{\text{min}}$ data (chill coma temperatures in Celsius degrees + 10 °C to prevent negative values from affecting the analysis), CCRT data (in minutes) or body colour as response variables. In *D. flavomontana*, the $CT_{\text{min}}$ data were normally distributed (Fig. S2), and they were analysed with linear mixed model (LMM) using *lme* function from nlme package (Pinheiro, Bates, DebRoy, Sarkar, & Team, 2020). Other datasets showed deviation from the normality (Fig. S2) and were analysed using generalized linear mixed model (GLMM) with Gamma distribution, using *glmmTMB* function from glmmTMB package (Brooks et al., 2017). Technical replicates and isofemale strains were handled as crossed random effects.

We extracted climatic information for the fly collecting sites from WorldClim database v2.0 (~340 km$^2$ spatial resolution; current data 1970-2000; Fick & Hijmans, 2017; http://www.worldclim.org) using the latitudinal and longitudinal coordinates of each site (Fig. 1). After extracting 19 bioclimatic variables using raster package v. 2.8-19 (Hijmans & Etten, 2020; Table S2), we performed a principle component analysis (PCA) to summarize climatic
differences on temperature and precipitation of each site (Table S3) and to reduce the number of variables using prcomp function in base R. The variables in the PCA were centered and scaled. We included the first PC in the model comparison of cold tolerance and body colour (see Results). Model comparison was done on three models which included i) Latitude, ii) Latitude + PC1 and iii) Latitude × PC1, and the best-fit model for CT_{min}, CCRT and body colour of both species was chosen for further analysis based on Akaike information criterion (AIC) value and Akaike weight (Table S7) using aictab function from AICcmodavg package (Mazerolle, 2019).

Finally, we investigated whether fly body colour was associated with CT_{min} or CCRT in either species by comparing the cold tolerance and body colour values of individual flies to each other. Here we used cold tolerance measures as response variables (as explained above) and body colour (divided by 100 to scale the variables) as an explanatory variable. All the analyses were conducted in R (v1.2.1335-1) and R studio (v3.6.1).

2.2 | RNA interference (RNAi) and its effects on fly cold tolerance

2.2.1 | Study material

We performed RNAi studies in D. montana, which has become a model species for studying adaption to seasonally varying environments at phenotypic (e.g. Tyukmaeva, Lankinen, Kinnunen, Kauranen, & Hoikkala, 2020) and genetic (Parker et al., 2018) level. We traced the role of vrille and Yp3 genes in cold adaptation using females from a mass-bred D. montana cage population from Seward (Alaska, North America; see Fig. 1). This population has been established from the 4th generation progenies of 20 isofemale strains and maintained in continuous light at 19 ± 1 °C. Malt bottles with freshly laid eggs were transferred from the population cage into a climate chamber in LD 18:6 (Light:Dark cycle) at 19 ± 1 °C to reinforce
flies’ circadian rhythmicity prior to the experiments. The critical day length for the induction of reproductive diapause (CDL; 50 % of the females enter diapause) in 19 °C is LD 17:9 in Seward population (Tyukmaeva et al., 2020), and thus the females emerging in LD 18:6 can be expected to develop ovaries. This is important, as the diaposing females possessing higher cold tolerance than the reproducing ones could induce variation in the results (Vesala, Salminen, Kankare, et al., 2012). After ~4 weeks, we collected newly emerged females (≤ 1 day old) using light CO2 anaesthesia and placed them back in malt-vials into above-mentioned conditions. Females were changed into fresh vials once a week until they were used in the experiments at the age of 21 days.

2.2.2 | Selecting candidate genes and defining their daily expression rhythm

Our earlier transcriptome and DNA microarray studies have identified several candidate genes for cold acclimation in *D. montana* (Parker et al., 2015; Vesala, Salminen, Laiho, et al., 2012). In the present study we used RNAi to get information on the importance of a circadian clock gene *vrille* and an insulin-signalling pathway gene *Yp3* in *D. montana* females’ basal cold tolerance and cold acclimation ability. Both genes have been found to be upregulated in female flies during cold acclimation in our earlier study (Parker et al., in preparation).

To perform RNAi experiments at a right time of the day, we defined the time when the expression of *vrille* and *Yp3* is highest at LD 18:6. To do this, we collected females every four hours (i.e. at six time points / day), starting at 6 am when lights were switched on. At each time point, we stored samples of females into -80°C through liquid N₂, and transferred them later on into RNAlater®-ICE frozen tissue transition solution (Thermo Fisher Scientific). We then checked the size of female ovaries (see Salminen & Hoikkala, 2013) and used only the females with fully developed ovaries (>95 % of the females). For each time point, RNA was extracted
from three pools, each of which consisted of three females, using ZR Tissue & Insect RNA MicroPrep kit with DNase treatment (Zymo Research®). RNA purity was analysed with NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and concentration with Qubit® 2.0 Fluorometer and RNA BR kit (Thermo Fisher Scientific). cDNA was synthesized using equal quantities of RNA (200 ng) with iScript Reverse Transcription kit (Bio-Rad Laboratories®).

We measured the expression levels of vrille and Yp3 with quantitative real time PCR (qPCR; see Method S1). Normalisation of the qPCR data was done with ∆∆(Ct) normalisation method (Livak & Schmittgen, 2001) using Tubulin beta chain (Tub2) and Ribosomal protein L32 (RpL32) as reference genes (these genes had equal expression levels in all samples). Efficiency values of the genes used in the qPCR are given in Table S4.

2.2.3 | Synthesis of double-stranded RNA for RNAi

We generated dsRNA fragments of vrille, Yp3 and LacZ genes, with lengths 347, 419 and 529 bp, respectively. LacZ, which codes a part of a bacterial gene, was used here as a control for dsRNA injections. In brief, DNA was amplified with PCR, ligated into a plasmid, and transformed into E. coli. Inserts from successful transformations were amplified using PCR and transcribed to dsRNA. Finally, the purified dsRNA was suspended in salt buffer. Detailed protocol is given in Method S2.

2.2.4 | RNAi microinjection procedure, response time screening and the expression levels of vrille, Yp3, clock and period at the chosen response time

Injecting dsRNA targeting on vrille or Yp3 genes is expected to cause gene-specific effects, but it may also cause immune responses and physical damage (injection) in the flies. Accordingly,
we used LacZ (encoding for bacterial gene) injections as a control for both immune response to dsRNA and to physical damage of injections and no-injection as a baseline cold tolerance control.

We checked the effectiveness of RNAi treatment on vrille and Yp3 expression 12h, 24h and 48h after injections, at a time of the day when both genes show highest expression (see Results). The females were injected with 138 nl of ~20 µM dsRNA targeting vrille or Yp3 (target genes) or LacZ using Nanoject II Auto-nanoliter Injector with 3.5” Drummond glass capillaries (Drummond Scientific) pulled with P-97 Flaming/Brown Micropipette Puller (Sutter Instrument). No-injection control females were not injected, but were otherwise handled in the same way as the injected females. To prevent CO₂ anaesthesia from inducing variation between these groups, we injected six flies at a time. The samples consisting of three pools, 10 females per pool, for each treatment and response time (12h, 24h or 48h), were transferred into -80 °C through liquid N₂. Then the females were transferred into RNAlater®-ICE solution and their ovaries were checked as explained above. RNA was extracted from the pools using TRIZol® Reagent with the PureLink® RNA Mini kit (Thermo Fisher Scientific). RNA purity was analysed with NanoDrop and concentration with Qubit and RNA BR kit. cDNA was synthesized using equal quantities of RNA (143 ng) using SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific).

Expression levels of vrille and Yp3 were quantified 12h, 24h and 48h after injections, as described in Method S1. Normalised expression values of both genes, analysed with a linear model (ANOVA) in base R, were lowest compared to controls 48h after injections (Fig. S3). Thus, we used this time point to find out whether the silencing of vrille or Yp3 had changed also the expression of two circadian clock genes, clock and period (primer sequences in Table S4). We synthesized cDNA using equal quantities of RNA (150 ng) and quantified the
expression levels of all four genes as described in Method S1, using ΔΔ(Ct) normalisation method (Livak & Schmittgen, 2001) with Tub2 as a reference gene.

2.2.5 | Measuring females’ cold tolerance and cold acclimation ability after RNAi treatment

Prior to performing cold tolerance tests, females were maintained for 16 days in LD 18:6 and at 19 °C, corresponding to summer conditions at their home site (LD 18:6 was used throughout the experiment). Half of the females were then subjected to cold acclimation treatment at 6 °C for 3 days (cold-acclimated females; Vesala, Salminen, Laiho, et al., 2012), while the other half was maintained at 19 °C (non-acclimated females, i.e. basal cold tolerance). At the age of 19 days, both non-acclimated and cold-acclimated females were collected from the chambers, anesthetized with CO2 and injected as described above. They were then placed back to either non-acclimation (19 °C) or cold acclimation (6 °C) conditions for two more days, as the expression levels of target genes had been found to be lowest 48h after RNAi treatment (Fig. S3). At the age of 21 days, females’ cold tolerance was quantified by measuring their chill coma temperature (CTmin) or chill coma recovery time (CCRT) using Julabo F32-HL Refrigerated/Heating Circulator. Sample sizes for CTmin and CCRT tests were 26-38 and 23-30 females per treatment, respectively.

2.2.6 | Statistical analyses

Expression levels of vrille, Yp3, clock and period in LacZ-injected females were compared to three other groups (no-injection control and females injected with dsRNA targeting on either vrille or Yp3) 48h after injections. Log2-transformed normalised expression values were analysed using a linear model (ANOVA) in base R.
To test the effect of cold acclimation on female cold tolerance, we first compared the cold tolerance of non-acclimated and cold-acclimated females with each other within different groups (LacZ injection, no-injection, and vrille and Yp3 injections). We then investigated the effect vrille and Yp3 RNAi on female cold tolerance by comparing these groups to LacZ control. LacZ was also compared to no-injection control to trace possible immune and physical effects of the injections. These analyses were performed separately for non-acclimated and cold-acclimated females. All data showed deviation from normality (Fig. S4) and were analysed with generalized linear mixed model using gamma distribution (GLMM; glmmTMB function from glmmTMB package (Brooks et al., 2017). In the models, response variables were either CTmin (Celsius degrees + 10 to prevent negative values from affecting the results) or CCRT (minutes) data, and the test replicates were used as a random effect. All the analyses were conducted in R (v1.2.1335-1) and R studio (v3.6.1).

3 | RESULTS

3.1 | Variation in the climatic conditions at fly collecting sites and the factors affecting fly cold tolerance and body colour

3.1.1 | Variation in the climatic conditions on fly collecting sites

We performed principal component analysis (PCA) on 19 bioclimatic variables specific for each fly collecting site to describe the patterns of climatic variation along these sites (Table S2, S3). PCA revealed three principal components (PCs) with eigenvalues > 1 (Table S5). The first PC (PC1) explained 63.5 % of the total variation (Fig. 2; Table S5), and it clearly separated the Rocky Mountains populations from the ones on the western coast. The variables with a highest contribution on this separation included temperature seasonality (bio4), annual temperature.
range (bio7), mean diurnal temperature range (bio2) and the minimum temperature of the coldest month (bio6), as well as several precipitation-related variables (Fig. 2; Table S6). Together they showed that the climatic conditions on the high-altitude Rocky Mountains sites are colder and have higher maximum and minimum temperature ranges than the ones on the western coast sites. On the other hand, precipitation was higher on the western coast than on the Rocky Mountains throughout the year. The second PC (PC2) explained only 17.9 % of total variation, and the variables with the highest contribution included the mean temperature of the warmest quarter (bio10), annual mean temperature (bio1) and the maximum temperature of the warmest month (bio5; Fig. 2; Table S6).

Figure 2. Principal component analysis (PCA) on 19 bioclimatic variables on fly collecting sites (Table S2). Direction of the arrows indicate which bioclimatic variables contribute to PC1 and which to PC2. The longer the arrow is, the greater its contribution is.

3.1.2 | Effects of latitude and climatic conditions on fly cold tolerance and body colour

Quantification of variation in fly basal cold tolerance with chill coma temperature ($CT_{\text{min}}$) and chill coma recovery time (CCRT) methods showed that D. montana flies are more cold-tolerant.
than *D. flavomontana* flies, and that the CCRT tests discover variation in this trait better than
the CT<sub>min</sub> tests. Fly body colour showed significant variation only in *D. flavomontana*.

To choose the best-fit linear model explaining the effects of latitude and climatic variation
(PCA) on variation in fly cold tolerance, we performed model selection for different
combinations of latitude and PC1, which explained most variation in PCA (Fig. 2; Table S7).
The best-fit model for CT<sub>min</sub>, CCRT and body colour of *D. montana*, judged from their Akaike
information criterion (AIC) and Akaike weight values, included only latitude (Table S7). CT<sub>min</sub>
and body colour of this species showed only minor variation, which was not significantly
associated with latitude (Fig. 3A, 3C; Table S8), while CCRT showed the fly cold tolerance to
improve towards northern latitudes (Fig. 3B; Table S8). In *D. flavomontana*, best-fit model for
CT<sub>min</sub> included only latitude, that for CCRT latitude, PC1 and their interaction, and the one for
body colour latitude and PC1 (Table S7). CT<sub>min</sub> of this species showed only minor variation
and no significant association with latitude (Fig. 3A; Table S8). However, CCRT values
showed the cold tolerance of *D. flavomontana* to improve towards North and to be strongly
affected by local climatic conditions (PC1) especially at latitudes around 50-55 °N (Fig. 3B;
Table S8). On these latitudes, fly cold tolerance was higher in the humid, low-altitude western
coast populations than in the high-altitude Rocky Mountains populations showing high
temperature variation (Fig. 2, 3B; Table S8). Body colour of *D. flavomontana* became darker
(i.e. its colour intensity decreased) towards North (Fig. 3C; Table S8). This trait was also
affected by climatic conditions (PC1), all western coast populations being equally dark and
darker than the Rocky Mountains populations from the similar latitudes (Fig. 3C; Table S8).
Figure 3. Relationship between latitude and (A) chill coma temperature ($CT_{\text{min}}$), (B) chill coma recovery time (CCRT) and (C) fly body colour in *D. montana* (*D. mon*) and *D. flavomontana* (*D. fla*) populations. Error bars represent bootstrapped 95% confidence intervals. Altitudes (m) are shown in grey scale only for the purpose of illustration. Significant regression lines for latitude with standard errors are shown. Significance levels were obtained from GLMMs or LMM: NS non-significant, * P < 0.05, ** P < 0.01 and *** P < 0.001.

### 3.1.3 Association between fly cold tolerance and body colour

The body colour of *D. montana* and *D. flavomontana* flies showed no significant association with the cold tolerance, measured for the same flies with either $CT_{\text{min}}$ or CCRT method (Fig. 4, Table S9).
Figure 4. Association between body colour and (A) chill coma temperature (CT\textsubscript{min}) and (B) chill coma recovery time (CCRT) in D. montana and D. flavomontana populations. Information on Rocky Mountains and western coast populations is given only for the purpose of illustration. Vertical error bars represent bootstrapped 95% confidence intervals for cold tolerance measurements and horizontal error bars for body colour measurements.

3.2. The role of \textit{vrille} and \textit{Yp3} genes in the cold tolerance and cold acclimation of \textit{D. montana}

3.2.1 Daily rhythms of \textit{vrille} and \textit{Yp3} and effectiveness of RNAi

Expression levels of \textit{vrille} showed clear rhythmicity and was highest at 22 pm and 2 am, while that of \textit{Yp3} showed less variation but was also highest at 22 pm (Fig. 5). Accordingly, all cold tolerance experiments were run between 22 pm and 00 am.
Figure 5. Baseline expression of *vrielle* and *Yp3* at six time points, starting 0 h after lights were switched on, at LD 18:6. Orange represents the time of the day when the lights were on and grey when the lights were off.

### 3.2.2 | Effects of RNAi on the expression levels of *vrielle*, *Yp3*, *clock* and *period*

Checking the expression levels of *vrielle* and *Yp3* 12h, 24h, and 48h after RNAi injection, compared to controls, showed the expression of both genes to be lowest 48h after injections (Fig. S3). Accordingly, this time point was chosen for quantifying the expression levels of *vrielle*, *Yp3*, *clock* and *period* genes as well as for measuring the cold tolerance of RNAi treated flies and their controls.

*vrielle* RNAi induced a significant decrease in the expression of *vrielle* and *period* genes compared to *LacZ* control (approximately 66% and 61%, respectively), while *Yp3* RNAi decreased significantly only *Yp3* expression compared to *LacZ* (approximately 66%; Fig. 6; Table S10). Expression of the *clock* gene showed no significant changes in either of the RNAi treatments (Fig. 6; Table S10).
Figure 6. Expression levels of *vrille*, *Yp3*, *clock* and *period* genes in *LacZ*-injected and no-injection control females, and in females injected with dsRNA targeting on *vrille* or *Yp3*. Error bars represent bootstrapped 95% confidence intervals. Significance levels were obtained from linear model and only significant observations are shown: * P < 0.05, ** P < 0.01 and *** P < 0.001.

3.2.3 | Effects of gene silencing on fly basal cold tolerance and cold acclimation ability

Effects of *vrille* and *Yp3* silencing with RNAi on female basal cold tolerance and cold acclimation ability were studied with CT<sub>min</sub> and CCRT test. In addition to these treatments, all experiments included two control treatments, *LacZ*-injected females (*LacZ*) and females that had not been injected (no-injection). Comparisons of the CT<sub>min</sub> and CCRT data between *LacZ* and *vrille* and *Yp3* enabled us to determine whether the RNAi treatments had affected the studied traits. Comparisons between *LacZ* and no-injection revealed possible immune response to dsRNA and/or physical damage caused by the injection and showed that flies exposed to the harsher CCRT tests are more vulnerable to these kinds of effects than the ones in CT<sub>min</sub> test.

Firstly, we compared the cold tolerances of non-acclimated (basal) and cold-acclimated females within each group. In CT<sub>min</sub> tests, cold acclimation improved female cold tolerance significantly in both control groups (*LacZ* and no-injection), as expected, while it had no effect on the cold
tolerance of vrille-injected females, and it decreased that of Yp3-injected females (Fig. 7A; Table S11). In CCRT tests, cold acclimation improved the cold tolerance of no-injection females, had no effect on the cold tolerance of LacZ- or Yp3-injected females, and decreased that of vrille-injected females (Fig. 7B; Table S11).

Next, we compared the basal cold tolerance of vrille- and Yp3-injected females with that of the LacZ controls. Here the only significant differences were detected between vrille and LacZ-injected females in CT_{min} test (Fig. 7A; Table S12) and between LacZ and no-injection females in CCRT tests (Fig. 7B; Table S12). The first difference suggests that the RNAi treatment on vrille had decreased fly basal cold tolerance in CT_{min} test, and the latter one that the injections could have induced physical damage and/or immune response leading to a decrease in fly cold tolerance in CCRT test.

Silencing of vrille and Yp3 genes decreased the cold tolerance of cold-acclimated females compared to LacZ controls in CT_{min} tests, even though variation among Yp3-injected females was quite high (Fig. 7A; Table S12). The same phenomenon was also detected in the CCRT test, but only for vrille (Fig. 7B; Table S12). In the CCRT test, a significant difference between LacZ and no-injection control implicates physical damage or immune response induced by injection (Fig. 7B; Table S12).
Figure 7. Female cold tolerance before (basal cold tolerance) and after cold acclimation in LacZ and no-injection control groups, and in RNAi treated vrille or Yp3 groups measured with (A) CT_{min} and (B) CCRT tests. Dashed lines indicate significant differences between females’ basal and acclimation cold tolerance within each group, and solid lines significant differences between the LacZ control and other groups among non-acclimated or cold-acclimated females. Significance levels were obtained from GLMMs and only significant observations are shown: * P < 0.05, ** P < 0.01 and *** P < 0.001.

4 | Discussion

Studies on insect cold adaptation in a wide range of environments, combined with information on the geographical location of study populations and the climatic conditions prevailing on these sites, offer a good possibility to predict species potential to survive in changing environmental conditions. We performed this kind of study on two highly cold tolerant species, D. montana and D. flavomontana, which adds a new dimension on studies that have largely concentrated on species with a low or moderate cold tolerance. In addition, RNAi experiments with D. montana on a circadian clock gene vrille and an insulin-signalling pathway gene Yolk
protein 3 (Yp3) enabled us to investigate the role of these genes on fly cold tolerance and cold acclimation ability and get insight on the important molecular mechanisms underlying these traits.

4.1 | Latitudinal variation in fly cold tolerance and its interaction with climatic variables and body colour

Spatially varying selection on insect survival and reproduction along latitudinal clines can be based on photoperiod and/or climatic factors, and sometimes it is difficult to detect the actual targets of selection. In addition, population differentiation generated and maintained by demographic events can be nearly identical to the patterns generated by selection (Bergland, Tobler, González, Schmidt, & Petrov, 2016). Thus, to build a convincing case for clinal adaptation, it is important to gather several independent sources of evidence, including sibling species, multiple populations or geographic regions and environmental correlations that account for population structure (Flatt, 2016). We focused our study on *D. montana* and *D. flavomontana* populations originating from diverse climatic environments on different latitudes on the western coast and in the Rocky Mountains of North America. The cold tolerance of *D. montana* was in all tests higher than that of *D. flavomontana* (see Fig. 3), as could be expected based on its distribution on higher latitudes and altitudes (Patterson, 1952). Nevertheless, both species showed latitudinal variation in fly basal cold tolerance measured with CCRT, but not with CT_{min}. Maysov (2014) has found the same phenomenon in two widespread ant species, *Myrmica rubra* and *Myrmica ruginodis*, and he argues that CCRT is a more useful indicator of adaptation than CT_{min} due to its higher sensitivity to photoperiod and/or climatic conditions.

Latitudinal variation in the basal cold tolerance (as measured with CCRT) of *D. montana* females showed no association with local climatic conditions, which emphasizes its
dependency on photoperiodic and temperature conditions varying according to the latitude in a wider scale. This is interesting, as latitudinal variation in another photoperiodically regulated trait in this species, the critical day length for reproductive diapause (CDL), has been found to be affected also by the climatic conditions prevailing at different altitudes (Tyukmaeva et al., 2020). In *D. flavomontana*, latitudinal variation in basal cold tolerance showed an association with local climatic conditions and was, surprisingly, higher in the humid low-altitude western coast populations than in the high-altitude Rocky Mountains populations. However, *D. flavomontana* flies of the mountain populations could use at least three strategies to enhance their cold tolerance. Firstly, females could enter reproductive diapause at an earlier time of year than the females from the low-altitude populations from the same latitude, as Tyukmaeva et al. (2020) have shown *D. montana* females to do. In *D. montana* diapause improves female cold tolerance considerably (Kauranen et al., 2019; Vesala & Hoikkala, 2011). Secondly, the low basal cold tolerance of the mountain flies could also be compensated by a higher cold acclimation ability, as several studies have detected a linkage between low cold tolerance and high cold acclimation ability (Gerken et al., 2015; Hoffmann et al., 2003; Isobe et al., 2013; Noh et al., 2017; Nyamukondiwa et al., 2011; Stillman, 2003; Zuther et al., 2012). Finally, the flies could avoid freezing injuries by migrating towards warmer habitats and thermally buffered microclimates (Stevenson, 1985). This kind of behaviour has been detected in the Colorado population of *D. flavomontana*, where the flies occupy lower mountain slopes during early spring than in summer (Hoikkala, pers. com.).

Insects’ dark body colour has been suggested to absorb solar radiation, heat up faster and maintain the optimum body temperature better than a light body colour (Clusella-Trullas et al., 2007; Rajpurohit et al., 2008). For example in *Drosophila americana*, darker body colour of the coastal populations, compared to that of the inland populations, (Wittkopp et al., 2011) has
been suggested to be an adaptation to lower solar radiation and smaller temperature variation on the coast (Clusella-Trullas & Terblanche, 2011). On the other hand, several studies have shown the dark body colour to play more important role in desiccation tolerance than in thermoregulation (Parkash, Sharma, & Kalra, 2009; Ramniwas, Kajla, Dev, & Parkash, 2013). 

*D. montana* flies were equally dark in all populations, while in *D. flavomontana* body colour was dark brown in the western coast populations and turned from light to dark brown towards North in the Rocky Mountains. Despite its association with climatic conditions, the body colour of *D. flavomontana* showed no correlation with CT$_{\text{min}}$ or CCRT. However, its variation could reflect species demographic history, as *D. flavomontana* may have distributed from the Rocky Mountains into the coastal area during the last decades (Patterson, 1952; Poikela et al. 2019), most probably through British Columbia in Canada where *D. flavomontana* body colour is dark. 

*D. flavomontana* may also hybridize with *D. montana* to some degree (Patterson, 1952; Throckmorton, 1982; Poikela et al. 2019), which could potentially have led to introgression of dark body colour from *D. montana* to *D. flavomontana*.

### 4.2 | The role of the circadian clock and *Yolk protein 3* genes in cold adaptation in *D. montana*

Cold acclimation has been found to induce shifts e.g. in insect metabolic profile and in the production of cryoprotectants, which helps to maintain osmotic balance and stabilise cell membrane structure during cold stress (Enriquez & Colinet, 2019; Lee, 2010; Storey & Storey, 2012). In addition, cold acclimation has been shown to be accompanied by expression changes in genes involved in stress response, protein folding, repair, transport, signalling, neuronal activity, carbohydrate metabolism and circadian rhythmicity (Des Marteaux, McKinnon, Udaka, Toxopeus, & Sinclair, 2017; Enriquez & Colinet, 2019). Parker et al. (2015) found the
biological processes associated with cold acclimation of *D. montana* flies to cover largely the same classes of genes as in above-mentioned studies, excluding stress response. Furthermore, Parker et al. (2018) found evidence for selection for higher cold tolerance acting on neuronal, membrane-transport and ion-transport related genes in genome-wide comparisons between *D. montana* and several less cold-tolerant *Drosophila* species, as well as between *D. montana* populations. These two studies brought up several candidate genes for cold tolerance and cold acclimation in *D. montana*. Vigoder et al., (2016) used RNAi to silence one of these candidate genes, *myo-inositol-1-phosphate synthase* (Inos), coding the major metabolite of overwintering *D. montana* flies, *myo-inositol* (Vesala, Salminen, Kostál, Zahradníčková, & Hoikkala, 2012), and found this treatment to increase *D. montana* flies’ mortality during cold exposure (5 °C) but not to affect CCRT. We chose for RNAi studies two of the candidate genes, *vrille* and *Yp3*, representing the circadian clock system and functioning in the insulin signalling pathway, respectively.

*Drosophila* flies can monitor light and temperature changes e.g. through their thermosensory organs located in aristae and legs, and to entrain behavioural and physiological changes to fit with environmental conditions, with the aid of the circadian clock system (Sehadova et al., 2009; Yadlapalli et al., 2018). For example in *Gryllus pennsylvanicus* crickets cold acclimation increases the expression levels of two core circadian clock genes, *period* and *clock*, and decreases that of *timeless* (Des Marteaux et al., 2017), while in *D. montana* and *D. virilis* it increases the expression of *vrille* and *period* (Parker et al., 2015; Vesala, Salminen, Laiho, et al., 2012). Blau & Young (1999) have shown that suppressing *vrille* expression decreases the expression levels of *period* and *timeless*, even though a simulation performed by Smolen, Hardin, Lo, Baxter, & Byrne (2004) suggests it to lead to a higher expression of *clock* and *period*. Our study gives support to the finding of Blau & Young (1999), as silencing *vrille*
expression with RNAi decreased the expression of *period*, but not that of *clock*. Moreover, *vrille*-injected acclimated and non-acclimated females had significantly decreased cold tolerance in CT\textsubscript{min} tests, and the same was true for *vrille*-injected acclimated females in CCRT tests. Thus, we propose that circadian clock system is essential both in maintaining insect basal cold tolerance and in increasing it during short cold acclimation.

Neuroendocrine signalling coordinates several processes involved in metabolism, development, reproduction and cold acclimation (Des Marteaux et al., 2017; Hahn & Denlinger, 2011; MacMillan et al., 2016; Torson, Yocum, Rinehart, Kemp, & Bowsher, 2015), and the insulin-signalling pathway, including *Yolk protein* genes, play an important part in this system (Flatt et al., 2005; Gonçalves Santos, Humann, & Hartfelder, 2019). Expression of *Yolk protein* genes has been found to decrease during cold acclimation e.g. in *D. melanogaster* (Colinet, Overgaard, Com, & Sørensen, 2013) and *D. suzukii* (Enriquez & Colinet, 2019). However, the situation is quite opposite in *D. montana*, where these genes are upregulated during cold acclimation (Parker et al., in preparation) and where the silencing of *Yp3* gene with RNAi was found to decrease cold acclimation ability in CT\textsubscript{min} test in the present study. *Yolk protein* genes and other possible genes involved in vitellogenesis (often called *vitellogenins*) seem to affect a wide range of traits involving insect development, immune function, endocytosis and stress resistance through their regulatory and signalling functions in the insulin-signalling pathway (Flatt et al., 2005; Schwerin et al., 2009; Tufail & Takeda, 2008). Moreover, e.g. in *Apis mellifera* honey bees these genes have been shown to function as antifreeze proteins (Qin et al., 2019) and in social hierarchies (Guidugli et al., 2005). However, comparisons of the function of vitellogenins between species and species groups are difficult to be made at gene level, because of the genes’ different evolutionary histories and high sequence divergences (Chen, Sappington, & Raikhel, 1997; Kaulenas, 2012).
5 | Conclusions

Understanding the mechanisms that generate variation in species stress tolerance is a key component for predicting their adaptation ability in the face of global warming. Species, whose cold or heat tolerance is tightly linked with latitude, may encounter more difficulties in adapting to changing environmental conditions than the species whose tolerances are adjusted to local climatic conditions. This study deepens our understanding on how latitudinally varying factors and local climatic conditions shape the evolution of cold tolerance. We show that cold tolerance of *D. montana* and *D. flavomontana* flies relies partly on different environmental cues. While the basal cold tolerance of *D. montana* is tightly linked with latitudinal variation in photoperiod and temperature, that of *D. flavomontana* is also affected by local climatic conditions. Even though variation in the body color of *D. flavomontana* shows no correlation with cold tolerance *per se*, its association with latitude and climatic conditions indicates that it could have thermoregulatory functions e.g. in terms of solar radiation. However, possible effects of species and population phylogenies and interspecific gene flow from *D. montana* on the variation in *D. flavomontana* body colour cannot be ruled out. Our study also propose that a functional circadian clock system is essential both in insect basal cold tolerance and cold acclimation ability. Moreover, *Yp3* gene, and possibly the whole insulin-signalling pathway, may play an important role during cold acclimation.

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REFERENCES

Addo-Bediako, A., Chown, S. L., & Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proceedings of the Royal Society B: Biological Sciences*, 267(1445), 739–745. doi: 10.1098/rspb.2000.1065

Andersen, J. L., Manenti, T., Sørensen, J. G., Macmillan, H. A., Løescheke, V., & Overgaard, J. (2015). How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. *Functional Ecology*, 29, 55–65. doi: 10.1111/1365-2435.12310

Angilletta, M. J. (2009). *Thermal adaptation: a theoretical and empirical synthesis*. Oxford University Press.

Arthur, A. L., Weeks, A. R., & Sgrò, C. M. (2008). Investigating latitudinal clines for life history and stress resistance traits in *Drosophila simulans* from eastern Australia. *Journal of Evolutionary Biology*, 21, 1470–1479. doi: 10.1111/j.1420-9101.2008.01617.x

Barber, A. F., & Sehgal, A. (2018). Cold temperatures fire up circadian neurons. *Cell Metabolism*, 27(5), 951–953. doi: 10.1016/j.cmet.2018.04.016

Bergland, A. O., Tobler, R., González, J., Schmidt, P., & Petrov, D. (2016). Secondary contact and local adaptation contribute to genome-wide patterns of clinal variation in *Drosophila melanogaster*. *Molecular Ecology*, 25(5), 1157–1174. doi: 10.1111/mec.13455

Bertolini, E., Schubert, F. K., Zanini, D., Sehadová, H., Helfrich-Förster, C., & Menegazzi, P. (2019). Life at high latitudes does not require circadian behavioral rhythmicity under constant darkness. *Cell*, 29, 3928–3936. doi: 10.1016/j.cell.2019.09.032

Blau, J., & Young, M. W. (1999). Cycling *vrille* expression is required for a functional *Drosophila* clock. *Cell*, 99, 661–671.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., ... Bolker, B. M. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R Journal*, 9(2), 378–400. doi: 10.32614/rj-2017-066

Castañeda, L. E., Lardies, M. A., & Bozinovic, F. (2005). Interpopulational variation in recovery time from chill coma along a geographic gradient: A study in the common woodlouse, Porcellio laevis. *Journal of Insect Physiology*, 51, 1346–1351. doi: 10.1016/j.jinsphys.2005.08.005

Chen, J., Sappington, T. W., & Raikhel, A. S. (1997). Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *Journal of Molecular Evolution*, 44, 440–451.

Clusella-Trullas, S., van Wyk, J. H., & Spotila, J. R. (2007). Thermal melanism in ectotherms. *Journal of Thermal Biology*, 32, 235–245. doi: 10.1016/j.jtherbio.2007.01.013

Clusella-Trullas, S., & Terblanche, J. S. (2011). Local adaptation for body color in *Drosophila americana*: commentary on Wittkopp et al. *Hereditas*, 106, 904–905. doi: 10.1038/hdy.2010.141

Colinet, H., Overgaard, J., Com, E., & Sørensen, G. J. (2013). Proteomic profiling of thermal acclimation in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, 43(4), 352–365. doi: 10.1016/j.ibmb.2013.01.006

Davis, J. S., & Moyle, L. C. (2020). Constitutive and plastic gene expression variation associated with desiccation resistance differences in the *Drosophila americana* species group. *Genes*, II(2), 146. doi: 10.3390/genes11020146

Denlinger, D. L. (1991). Relationship between cold hardiness and diapause. In *Insects at Low Temperature* (pp. 174–198). Springer, Boston, MA.

Des Marteaux, L. E., McKinnon, A. H., Udaka, H., Toxopeus, J., & Sinclair, B. J. (2017). Effects of cold-acclimation on gene expression in Fall field cricket (*Gryllus pennsylvanicus*) ionoregulatory...
tissues. *BMC Genomics*, 18(1), 1–17. doi: 10.1186/s12864-017-3711-9

676 Doucet, D., Walker, V. K., & Qin, W. (2009). The bugs that came in from the cold: molecular adaptations to low temperatures in insects. *Cellular and Molecular Life Sciences*, 66, 1404–1418. doi: 10.1007/s00018-009-8320-6

680 Enriquez, T., & Colinet, H. (2019). Cold acclimation triggers major transcriptional changes in *Drosophila suzukii*. *BMC Genomics*, 20(413), 1–17.

682 Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology*, 37(12), 4302–4315. doi: 10.1002/joc.5086

685 Flatt, T. (2016). Genomics of clinal variation in *Drosophila*: disentangling the interactions of selection and demography. *Molecular Ecology*, 25(5), 1023–1026. doi: 10.1111/mec.13534

689 Flatt, T., Tu, M., & Tatar, M. (2005). Hormonal pleiotropy and the juvenile hormone regulation of *Drosophila* development and life history. *BioE*, 27, 999–1010. doi: 10.1002/bies.20290

694 Fowler, S., & Thomashow, M. F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *American Society of Plant Biologists*, 14, 1675–1690. doi: 10.1105/tpc.003483. Toward Gerken, A. R., Eller, O. C., Hahn, D. A., & Morgan, T. J. (2015). Constraints, independence, and evolution of thermal plasticity: Probing genetic architecture of long- and short-term thermal acclimation. *Proceedings of the National Academy of Sciences*, 112(14), 4399–4404. doi: 10.1073/pnas.1503456112

700 Gonçalves Santos, C., Humann, F. C., & Hartfelder, K. (2019). Juvenile hormone signaling in insect oogenesis. *Current Opinion in Insect Science*, 31, 43–48. doi: 10.1016/j.cois.2018.07.010

705 Guidugli, K. R., Nascimento, A. M., Amdam, G. V, Barchuk, A. R., Omholt, S., Simões, Z. L. P., & Hartfelder, K. (2005). Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. *FEBS Letters*, 579(22), 4961–4965. doi: 10.1016/j.febslet.2005.07.085

710 Gunawardhana, K. L., & Hardin, P. E. (2017). VRILLE controls PDF neuropeptide accumulation and arborization rhythms in small ventrolateral neurons to drive rhythmic behavior in *Drosophila*. *Current Biology*, 27(22), 3442–3453. doi: 10.1016/j.cub.2017.10.010

715 Hahn, D. A., & Denlinger, D. L. (2011). Energetics of insect diapause. *Annual Review of Entomology*, 56(1), 103–121. doi: 10.1146/annurev-ento-112408-085436

720 Hallas, R., Schiffer, M., & Hoffmann, A. A. (2002). Clinal variation in *Drosophila serrata* for stress resistance and body size. *Genetics Research*, 79(2), 141–148.

725 Hardin, P. E. (2011). Molecular genetic analysis of circadian timekeeping in *Drosophila*. *Advances in Genetics*, 74, 141–173. doi: 10.1016/B978-0-12-387690-4.00005-2.Molecular

730 Harris, R. M., McQuillan, P., & Hughes, L. (2013). A test of the thermal melanism hypothesis in the wingless grass-hopper *Phaulacridium vittatum*. *Journal of Insect Science*, 13(51), 1–18.

735 Helmuth, B., Kingsolver, J. G., & Carrington, E. (2005). Biophysics, physiological ecology, and climate change: Does melanism matter? *Annual Review of Physiology*, 67, 177–201. doi: 10.1146/annurev.physiol.67.040403.105027

740 Hijmans, R. J., & Etten, J. Van. (2020). *raster*: Geographic data analysis and modeling. *R package version* 2.8-19.

745 Hoffmann, A. A., Anderson, A., & Hallas, R. (2002). Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecology*, 5, 614–618.

750 Hoffmann, A. A., Sørensen, J. G., & Loescheke, V. (2003). Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology*, 28, 175–216. doi: 10.1016/S0306-4565(02)00057-8

755 Isobe, K., Takahashi, A., & Tamura, K. (2013). Cold tolerance and metabolic rate increased by cold acclimation in *Drosophila albicans* from natural populations. *Genes & Genetic Systems*, 88, 289–300.
Kaulenas, M. S. (2012). *Insect Accessory Reproductive Structures: Function, Structure, and Development*. Springer Science & Business Media.

Kauranen, H., Kinnunen, J., Hiillos, A., Lankinen, P., Hopkins, D., Wiberg, R. A. W., ... Hoikkala, A. (2019). Selection for reproduction under short photoperiods changes diapause-associated traits and induces widespread genomic divergence. *Journal of Experimental Biology*, 222(20), jeb205831. doi: 10.1242/jeb.205831

Kauranen, H., Menegazzi, P., Costa, R., Helfrich-Förster, C., Kankainen, A., & Hoikkala, A. (2012). Flies in the North: locomotor behavior and clock neuron organization of *Drosophila montana*. *Journal of Biological Rhythms*, 27(5), 377–387. doi: 10.1177/0747730412455916

Kellermann, V., Loeschcke, V., Hoffmann, A. A., Kristensen, T. N., Floijaard, C., David, J. R., ... Overgaard, J. (2012). Phylogenetic constraints in key functional traits behind species’ climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species. *Evolution*, 66(11), 3377–3389. doi: 10.1111/j.1558-5646.2012.01685.x

Kimura, M. T. (2004). Cold and heat tolerance of drosophilid flies with reference to their latitudinal distributions. *Oecologia*, 140, 442–449. doi: 10.1007/s00442-004-1605-4

Klok, C. J., & Chown, S. L. (2005). Inertia in physiological traits: *Embryonopsis halictella* caterpillars (Yponomeutidae) across the Antarctic polar frontal zone. *Journal of Insect Behavior*, 51, 87–97. doi: 10.1016/j.jinsphys.2004.11.011

Lee, R. E. (2010). A primer on insect cold-tolerance. In Lee R. E., Denlinger D. L. (eds). Insects at Low Temperature. *Chapman and Hall: London, UK* (pp. 1–390). doi: 10.1017/CBO9780511675997

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*, 408, 402–408. doi: 10.1006/meth.2001.1262

MacMillan, H. A., Andersen, J. L., Davies, S. A., & Overgaard, J. (2015). The capacity to maintain ion and water homeostasis underlies interspecific variation in *Drosophila* cold tolerance. *Scientific Reports*, 5, 1–11. doi: 10.1038/srep18607

MacMillan, H. A., Knee, J. M., Dennis, A. B., Udaka, H., Marshall, K. E., Merritt, T. J. S., & Sinclair, B. J. (2016). Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. *Scientific Reports*, 6(1), 1–14. doi: 10.1038/srep28999

MacMillan, H. A., & Sinclair, B. J. (2011). Mechanisms underlying insect chill-coma. *Journal of Insect Physiology*, 57(1), 12–20. doi: 10.1016/j.insphys.2010.10.004

Magnone, M. C., Jacobmeier, B., Bertolucci, C., Foa, A., & Albrecht, U. (2005). Circadian expression of the clock gene *Per 2* is altered in the ruin lizard (*Podarcis sicula*) when temperature changes. *Molecular Brain Research*, 133, 281–285. doi: 10.1016/j.molbrainres.2004.10.014

Maysov, A. (2014). Chill coma temperatures appear similar along a latitudinal gradient, in contrast to divergent chill coma recovery times, in two widespread ant species. *The Journal of Experimental Biology*, 217, 2650–2658. doi: 10.1242/jeb.096958

Mazerolle, M. (2019). AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c). *R package version 2.2-2*, https://cran.r-project.org/package=AICcmodavg.

Menegazzi, P., Benetta, E. D., Beauchamp, M., Schlichting, M., Steffan-dewenter, I., & Helfrich-Förster, C. (2017). Adaptation of circadian neuronal network to photoperiod in high-latitude European Drosophilids. *Cell*, 27, 833–839. doi: 10.1016/j.cell.2017.01.036

Neumüller, R. A., Richter, C., Fischer, A., Novatchkova, M., Neumüller, K. G., & Knoblich, J. A. (2011). Genome-wide analysis of self-renewal in *Drosophila* neural stem cells by transgenic RNAi. *Cell Stem Cell*, 8(5), 580–593. doi: 10.1016/j.stem.2011.02.022

Noh, S., Everman, E. R., Berger, C. M., & Morgan, T. J. (2017). Seasonal variation in basal and plastic cold tolerance: Adaptation is influenced by both long- and short-term phenotypic plasticity. *Ecology and Evolution*, 7, 5248–5257. doi: 10.1002/eece.3112

Nyamukondiwa, C., Terblanche, J. S., Marshall, K. E., & Sinclair, B. J. (2011). Basal cold but not heat tolerance constrains plasticity among *Drosophila* species (Diptera: Drosophilidae). *Journal of Evolutionary Biology*, 24, 1927–1938. doi: 10.1111/j.1420-9101.2011.02324.x

Overgaard, J., Hoffmann, A. A., & Kristensen, T. N. (2011). Assessing population and environmental effects on thermal resistance in *Drosophila melanogaster* using ecologically relevant assays.
Journal of Thermal Biology, 36(7), 409–416. doi: 10.1016/j.jtherbio.2011.07.005

Overgaard, J., Kristensen, T. N., Mitchell, K. A., & Hoffmann, A. A. (2011). Thermal tolerance in widespread and tropical Drosophila species: does phenotypic plasticity increase with latitude? The American Naturalist, 178, 80–96. doi: 10.1086/661780

Parkash, R., & Munjal, A. K. (1999). Phenotypic variability of thoracic pigmentation in Indian populations of Drosophila melanogaster. Journal of Zoological Systematics and Evolutionary Research, 37, 133–140.

Parkash, R., Sharma, V., & Kalra, B. (2009). Correlated changes in thermotolerance traits and body color phenotypes in montane populations of Drosophila melanogaster: Analysis of within- and between-population variations. Journal of Zoology, 280(1), 49–59. doi: 10.1111/j.1469-7998.2009.00641.x

Parker, D. J., Ritchie, M. G., & Kankare, M. (2016). Preparing for winter: The transcriptomic response associated with different day lengths in Drosophila montana. G3: Genes, Genomes, Genetics, 6(5), 1373–1381. doi: 10.1534/g3.116.027870

Parker, D. J., Vesala, L., Ritchie, M. G., Laiho, A., Hoikkala, A., & Kankare, M. (2015). How consistent are the transcriptome changes associated with cold acclimation in two species of the Drosophila virilis group? Heredity, 115, 13–21. doi: 10.1038/hdy.2015.6

Parker, D. J., Wiberg, R. A. W., Trivedi, U., Tyukmaeva, V. I., Gharbi, K., Butlin, R. K., … Ritchie, M. G. (2018). Inter and intraspecific genomic divergence in Drosophila montana shows evidence for cold adaptation. Genome Biology and Evolution, 10(8), 2086–2101. doi: 10.1093/gbe/evy147

Patterson, J. T. (1952). Revision of the montana complex of the virilis species group. The University of Texas Publication, 5204, 20–34.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & Team, R. C. (2020). nlme: Linear and nonlinear mixed effects models. R package version 3.1-144, https://CRAN.R-project.org/package=nlme.

Poikela, N., Kinnunen, J., Wurdack, M., Kauranen, H., Schmitt, T., Kankare, M., … Hoikkala, A. (2019). Strength of sexual and postmating prezygotic barriers varies between sympatric populations with different histories and species abundances. Evolution, 73(6), 1182–1199. doi: 10.1111/evo.13732

Pörtner, H. O., & Farrell, A. P. (2008). Physiology and climate change. Science, 322, 690–692.

Qin, M., Wang, H., Liu, Z., Wang, Y., Zhang, W., & Xu, B. (2019). Changes in cold tolerance during the overwintering period in Apis mellifera ligustica. Journal of Apicultural Research, 58(5), 702–709. doi: 10.1080/00218839.2019.1634461

Rajpurohit, S., Parkash, R., & Ramniwas, S. (2008). Body melanization and its adaptive role in thermoregulation and tolerance against desiccating conditions in drosophilids. Entomological Research, 38, 49–60. doi: 10.1111/j.1745-5977.2008.00129.x

Ramniwas, S., Kajla, B., Dev, K., & Parkash, R. (2013). Direct and correlated responses to laboratory selection for body melanisation in Drosophila melanogaster: Support for the melanisation-desiccation resistance hypothesis. Journal of Experimental Biology, 216(7), 1244–1254. doi: 10.1242/jeb.07641

Ransberry, V. E., Macmillan, H. A., & Sinclair, B. J. (2011). The relationship between chill–coma onset and recovery at the extremes of the thermal window of Drosophila melanogaster. Physiological and Biochemical Zoology, 84, 553–559. doi: 10.1086/662642

Salminen, T. S., & Hoikkala, A. (2013). Effect of temperature on the duration of sensitive period and on the number of photoperiodic cycles required for the induction of reproductive diapause in Drosophila montana. Journal of Insect Physiology, 59(4), 450–457. doi: 10.1016/j.jinsphys.2013.02.005

Sehadova, H., Glaser, F. T., Gentile, C., Simoni, A., Giesecke, A., Albert, J. T., & Stanewsky, R. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods, 9(7), 671–675. doi: 10.1038/nmeth.2089

Schwerin, S., Zeis, B., Lamkemeyer, T., Paul, R. J., Koch, M., Madlung, J., … Pirow, R. (2009). Acclimatory responses of the Daphnia pulex proteome to environmental changes. II. Chronic exposure to different temperatures (10 and 20°C) mainly affects protein metabolism. BMC Physiology, 9(1), 1–18. doi: 10.1186/1472-693-9-8
Temperature entrainment of Drosophila’s circadian clock involves the gene noche and signaling from peripheral sensory tissues to the brain. *Neuron*, 64(2), 251–266. doi: 10.1016/j.neuron.2009.08.026

Shearer, P. W., West, J. D., Walton, V. M., Brown, P. H., Svetec, N., & Chiu, J. C. (2016). Seasonal cues induce phenotypic plasticity of Drosophila suzukii to enhance winter survival. *BMC Ecology*, 16(11), 1–18. doi: 10.1186/s12898-016-0070-3

Sim, C., & Denlinger, D. L. (2013). Insulin signaling and the regulation of insect diapause. *Frontiers in Physiology*, 4(189), 1–10. doi: 10.3389/fphys.2013.00189

Smolen, P., Hardin, P. E., Lo, B. S., Baxter, D. A., & Byrne, J. H. (2004). Simulation of Drosophila circadian oscillations, mutations, and light responses by a model with VRI, PDP-1, and CLK. *Biophysical Journal*, 86(5), 2786–2802. doi: 10.1016/S0006-3495(04)74332-5

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine ‘winners’ and ‘losers’. *The Journal of Experimental Biology*, 213, 912–920. doi: 10.1242/jeb.037473

Stephens, A. R., Aspelen, M. K., Hutchison, W. D., & Venette, R. C. (2015). Cold hardiness of winter-acclimated Drosophila suzukii (Diptera: Drosophilidae) adults. *Physiological Ecology*, 44(6), 1619–1626. doi: 10.1093/ee/nvv134

Stevenson, R. D. (1985). The genetics of cold-hardening in insects. *Physiological Entomology*, 38(2), 105–116. doi: 10.1111/phen.12019

Teets, N. M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A., & Denlinger, D. L. (2012). Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiological Genomics*, 44(15), 764–777. doi: 10.1152/physiolgenomics.00042.2012

Throckmorton, L. H. (1982). The virilis species group. *The Genetics and Bioogy of Drosophila*, 3, 227–296.

Torson, A. S., Yocum, G. D., Rinehart, J. P., Kemp, W. P., & Bowsher, J. H. (2015). Transcriptional responses to fluctuating thermal regimes underpinning differences in survival in the solitary bee Megachile rotundata. *Journal of Experimental Biology*, 218(7), 1060–1068. doi: 10.1242/jeb.113829

Tuftail, M., & Takeda, M. (2008). Molecular characteristics of insect vitellogenins. *Journal of Insect Physiology*, 54(12), 1447–1458. doi: 10.1016/j.jinsphys.2008.08.007

Tyukmaeva, V. I., Lankinen, P., Kinnunen, J., Kauranen, H., & Hoikkala, A. (2020). Latitudinal clines in the timing and temperature-sensitivity of photoperiodic reproductive diapause in Drosophila montana. *Ecography*, 43, 1–10. doi: 10.1111/ecog.04892

Vesala, L., & Hoikkala, A. (2011). Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance of Drosophila montana. *Journal of Insect Physiology*, 57(1), 46–51. doi: 10.1016/j.jinsphys.2010.09.007

Vesala, L., Salminen, T. S., Kankare, M., & Hoikkala, A. (2012). Photoperiodic regulation of cold tolerance and expression levels of regucalcin gene in Drosophila montana. *Journal of Insect Physiology*, 58(5), 704–709. doi: 10.1016/j.jinsphys.2012.02.004

Vesala, L., Salminen, T. S., Laiho, A., Hoikkala, A., & Kankare, M. (2012). Cold tolerance and cold-induced modulation of gene expression in two Drosophila virilis group species with different distributions. *Insect Molecular Biology*, 21(1), 107–118. doi: 10.1111/j.1365-2583.2011.01119.x
Vesala, L., Salminen, T. S., Kostál, V., Zahradníčková, H., & Hoikkala, A. (2012). *Myo*-inositol as a main metabolite in overwintering flies: Seasonal metabolomic profiles and cold stress tolerance in a northern drosophilid fly. *Journal of Experimental Biology, 215*(16), 2891–2897. doi: 10.1242/jeb.069948

Vigoder, F. M., Parker, D. J., Cook, N., Tournière, O., Sneddon, T., & Ritchie, M. G. (2016). Inducing cold-sensitivity in the frigophilic fly *Drosophila montana* by RNAi. *PLoS ONE, 11*(11), 1–9. doi: 10.1371/journal.pone.0165724

Williams, J. A., & Sehgal, A. (2001). Molecular components of the circadian system in *Drosophila*. *Annual Review of Physiology, 63*, 729–755.

Wittkopp, P. J., Smith-Winberry, G., Arnold, L. L., Thompson, E. M., Cooley, A. M., Yuan, D. C., … McAllister, B. F. (2011). Local adaptation for body color in *Drosophila americana*. *Heredity, 106*(4), 592–602. doi: 10.1038/hdy.2010.90

Yadlapalli, S., Jiang, C., Bahle, A., Reddy, P., Meyhofer, E., & Shafer, O. T. (2018). Circadian clock neurons constantly monitor environmental temperature to set sleep timing. *Nature, 555*, 98–102. doi: 10.1038/nature25740

Zhao, X., Bergland, A. O., Behrman, E. L., Gregory, B. D., Petrov, D. A., & Schmidt, P. S. (2015). Global transcriptional profiling of diapause and climatic adaptation in *Drosophila melanogaster*. *Molecular Biology and Evolution, 33*(3), 707–720. doi: 10.1093/molbev/msv263

Zhen, Y., & Ungerer, M. C. (2008). Clinal variation in freezing tolerance among natural accessions of *Arabidopsis thaliana*. *New Phytologist, 177*, 419–427.

Zuther, E., Schulz, E., Childs, L. H., & Hincha, D. K. (2012). Clinal variation in the non-acclimated and cold-acclimated freezing tolerance of *Arabidopsis thaliana* accessions. *Plant, Cell and Environment, 35*, 1860–1878. doi: 10.1111/j.1365-3040.2012.02522.x

DATA ACCESSIBILITY

The data will be made available in Dryad.

AUTHOR CONTRIBUTION

NP, VT, AH and MK designed the study. NP and MK performed the research. NP analysed the data and drafted the manuscript, and all authors finalised it.

CONFLICT OF INTEREST

The authors have declared no conflicting interests.