Latitudinal variation in cold tolerance and the role of *vrille* and *vitellogenin* 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 *vitellogenin* 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* gene, and decreased flies' basal cold tolerance and cold acclimation ability, while silencing of *vitellogenin* 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 *vitellogenin* 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 on some other *Drosophila* species (Arthur, Weeks, & Sgrò, 2008; David, Gibert, Moreteau, Gilchrist, & Huey, 2003) and e.g. *Embryonopsis halticella* 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 selection and/or gene flow from conspecific populations, all of which prevent populations from adapting to local environmental conditions (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 has been suggested to increase solar radiation 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 their 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 head (Barber & Sehgal, 2018; Yadlapalli et al., 2018). Signals indicating temperature decrease can induce changes e.g.

91 in ion transport, membrane restructuring, calcium signalling and the synthesis of
92 cryoprotectants and antifreeze proteins, which can evoke cold acclimation (Teets & Denlinger,
93 2013) and help the flies to maintain cell homeostasis and functionality at low temperatures
94 (MacMillan et al., 2016). However, the abrupt disruptions in transmembrane ion gradients and
95 ion distribution can also lead to water loss and accumulation of chill injuries, which can
96 eventually lead to death (Findsen, Pedersen, Petersen, Nielsen, & Overgaard, 2014; MacMillan,
97 Andersen, Davies, & Overgaard, 2015).

98 Several studies have suggested species cold tolerance and cold acclimation ability to be
99 associated with genes involved in the circadian clock system (Fowler & Thomashow, 2002;
100 Magnone, Jacobmeier, Bertolucci, Foa, & Albrecht, 2005; Vesala, Salminen, Laiho, Hoikkala,
101 & Kankare, 2012) and/or metabolic pathways, such as the insulin-signalling pathway (Baker &
102 Russell, 2009; Kankare, Parker, Merisalo, Salminen, & Hoikkala, 2016; Parker et al., 2018;
103 Ramirez et al., 2017). The role of the circadian clock system in insect cold adaptation, especially
104 in cold acclimation, is logical, as this clock monitors light and temperature cycles and entrains
105 behavioural and physiological rhythms to match with them (Barber & Sehgal, 2018). In the
106 central circadian clock, described most thoroughly in *D. melanogaster*, daily rhythms are driven
107 via one or several transcriptional feedback loops involving changes in the expression of core
108 circadian clock genes (studies reviewed e.g. Gunawardhana & Hardin, 2017; Hardin, 2011;
109 Williams & Sehgal, 2001). Briefly, the first core timekeeping loop includes *clock* (*clk*) – *cycle*
110 (*cyc*) and *period* (*per*) – *timeless* (*tim*) complexes that activate and repress each other. *clock* and
111 *cycle* further activate an interlocked feedback loop that includes *vrille* (*vri*) and *Par domein*
112 *protein 1* (*Pdp1*), which repress and activate *clock* transcription, respectively. *vrille* also
113 regulates neuropeptide Pigment-dispersing factor (PDF) expression in clock neurons, which
114 makes it a key regulator of circadian behavioural rhythms (Gunawardhana & Hardin, 2017).

115 Interestingly, *vrille* expression has been found to increase during cold acclimation e.g. in
116 *Drosophila montana* (Parker et al., 2015; Vesala, Salminen, Laiho, et al., 2012). The insulin-
117 signalling pathway, on the other hand, is known to have widespread effects e.g. on insect
118 development, immune function and stress resistance (reviewed in Flatt, Tu, & Tatar, 2005).
119 This pathway involves a major endocrine regulator, juvenile hormone (JH), for which effects
120 are mediated, among other genes, by *vitellogenin* genes that code for a phospholipoglycoprotein
121 vitellogenin (Amdam & Omholt, 2003; Elgendy, Tufail, Mohamed, & Takeda, 2019; Flatt et
122 al., 2005; Guidugli et al., 2005; Sheng, Xu, Bai, Zhu, & Palli, 2011; Tufail & Takeda, 2008).
123 In some species, *vitellogenin* has been found to play an important role in cold adaptation,
124 possibly acting as an antifreeze protein (Crosthwaite, Sobek, Lyons, Bernards, & Sinclair, 2011;
125 Qin et al., 2019).

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

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

149 2 | MATERIALS AND METHODS

150 2.1 | Variation in the basal cold tolerance and body colour in *D. montana* and
151 in *D. flavomontana* populations

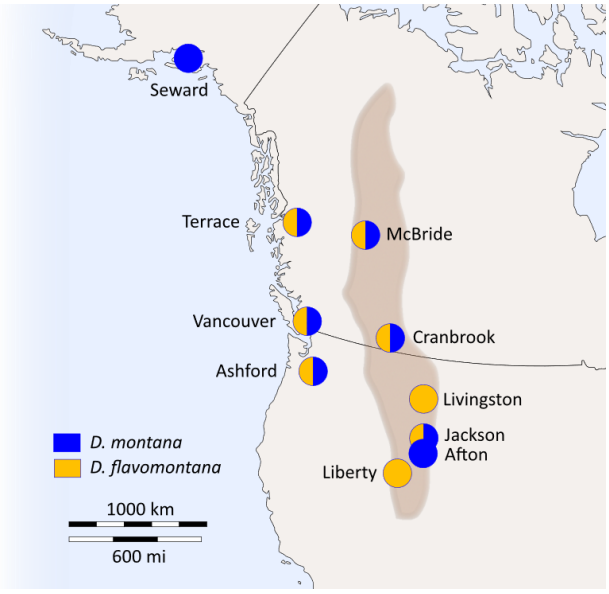
152 2.1.1. | Study species and populations

153 *D. montana* and *D. flavomontana* belong to the *montana* phylad of the *virilis* group, and our
154 recent whole-genome analyses have shown their divergence time to be ~1 mya (Poikela et al.,
155 in preparation). *D. montana* is distributed around the northern hemisphere across North
156 America, Asia and Europe (Throckmorton, 1982), while the distribution of *D. flavomontana* is
157 restricted to North America (Patterson, 1952; Throckmorton, 1982). In the central Rocky
158 Mountains *D. montana* is found at altitudes from 1400 m to well over 3000 m, while *D.*
159 *flavomontana* is found mainly below 2000 m. In the western coast, where *D. flavomontana* has

160 probably invaded only during the last decades, both species live at much lower altitudes (see
161 Patterson, 1952; Poikela et al., 2019).

162 We performed phenotypic assays on the females from 23 *D. montana* and 20 *D. flavomontana*
163 isofemale strains, which were established from the progenies of fertilized females collected
164 from several sites in North America between 2013 and 2015 (Fig. 1). Each site was represented
165 by three isofemale strains per population per species, when possible (Fig. 1; Table S1), and all
166 the strains were maintained since their establishment (15-30 generations) in continuous light at
167 19 ± 1 °C to prevent females from entering reproductive diapause. For the experiments, we
168 sexed emerging flies under light CO_2 anaesthesia within three days after emergence. Females
169 were changed into fresh malt-vials once a week and used in experiments at the age of 20 ± 2
170 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



171

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

176

177 ### 2.1.2 | Cold tolerance tests

178 We investigated latitudinal variation in the cold tolerance of *D. montana* and *D. flavomontana*
179 females using two well-defined and ecologically relevant methods: chill coma temperature
180 (CT_{min}; also called critical thermal minimum) and chill coma recovery time (CCRT). CT_{min}
181 corresponds to the temperature, at which the fly loses all neurophysiological activity and
182 coordination and falls into a chill coma. In this test, we placed the females individually in glass
183 vials, which were submerged into a 30 % glycol bath. We then decreased the bath temperature
184 from the starting temperature of 19 °C at the rate of 0.5 °C/min and scored the CT_{min} for each
185 fly. The second method, CCRT, measures the time taken from a fly to recover from a
186 standardized exposure time at a chill-coma-inducing temperature (reviewed in MacMillan &
187 Sinclair, 2011). In this test, we submerged the females individually in glass vials into a 30 %
188 glycol bath for 16 hours at -6 °C (Vesala, Salminen, Laiho, et al., 2012). After returning the
189 vials into 19 ± 1 °C in fly laboratory, we measured the time required for each female to recover
190 from the chill coma and stand on its legs. CT_{min} tests were replicated 21 times and CCRT tests
191 20 times with Julabo F32-HL Refrigerated/Heating Circulator and Haake k35 DC50 Refrig
192 Circulating Bath Chiller, respectively. To account for possible variation between replicates,
193 each test included 1-2 females from each strain.

194 ### 2.1.3 | Fly body colour

195 We analysed the body colour of the same flies that had been phenotyped in CT_{min} or CCRT
196 tests. The flies were photographed under Leica L2 microscope with 5x magnification, using
197 Leica DFC320 R2 camera together with the Leica Application Suite software v4.3.0. Exposure
198 time, zoom factors and illumination level were kept constant, and the photographing arena was
199 surrounded by a plastic cylinder to eliminate glares on the chitinous surface of the fly. All

200 photographs were taken within 3 months after the cold tolerance tests (females were kept this
201 period in -20 ˚C). Images were saved in 24-bit (RGB) format and the colour intensity was
202 measured using grayscale image analysis (ImageJ; Schneider, Rasband, & Eliceiri, 2012);
203 linearly scaling from 0 to 255 (0 = black, 255 = white). We took colour measurements from
204 part of thorax (scutum; see Fig. S1), as our preliminary tests showed that it best incorporates
205 the colour variation among flies (Fig. S1).

206 2.1.4 | Statistical analyses

207 We used different statistical models to investigate whether variation in fly cold tolerance or
208 body colour was associated with latitude and/or local climatic factors, and whether these traits
209 showed correlation with each other. In these models, we used either CT_{min} data (chill coma
210 temperatures in Celsius degrees + 10 ˚C to prevent negative values from affecting the analysis),
211 CCRT data (in minutes) or body colour as response variables. In *D. flavomontana*, the CT_{min}
212 data were normally distributed (Fig. S2), and they were analysed with linear mixed model
213 (LMM) using *lme* function from nlme package (Pinheiro, Bates, DebRoy, Sarkar, & Team,
214 2020). Other datasets showed deviation from the normality (Fig. S2) and were analysed using
215 generalized linear mixed model (GLMM) with Gamma distribution, using *glmmTMB* function
216 from glmmTMB package (Brooks et al., 2017). Technical replicates and isofemale strains were
217 handled as crossed random effects.

218 We extracted climatic information for the fly collecting sites from WorldClim database v2.0
219 (~340 km^2 spatial resolution; current data 1970-2000; Fick & Hijmans, 2017;
220 http://www.worldclim.org) using the latitudinal and longitudinal coordinates of each site (Fig.
221 1). After extracting 19 bioclimatic variables using raster package v. 2.8-19 (Hijmans & Etten,
222 2020; Table S2), we performed a principle component analysis (PCA) to summarize climatic

223 differences on temperature and precipitation of each site (Table S3) and to reduce the number
224 of variables using *prcomp* function in base R. The variables in the PCA were centered and
225 scaled. We included the first PC in the model comparison of cold tolerance and body colour
226 (see Results). Model comparison was done on three models which included i) Latitude, ii)
227 Latitude + PC1 and iii) Latitude × PC1, and the best-fit model for CT_{min}, CCRT and body colour
228 of both species was chosen for further analysis based on Akaike information criterion (AIC)
229 value and Akaike weight (Table S7) using *aictab* function from AICcmodavg package
230 (Mazerolle, 2019).

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

236 2.2 | RNA interference (RNAi) and its effects on fly cold tolerance

237 2.2.1 | Study material

238 We performed RNAi studies in *D. montana*, which has become a model species for studying
239 adaption to seasonally varying environments at phenotypic (e.g. Tyukmaeva, Lankinen,
240 Kinnunen, Kauranen, & Hoikkala, 2020) and genetic (Parker et al., 2018) level. We traced the
241 role of *vrille* and *vitellogenin* genes in cold adaptation using females from a mass-bred *D.*
242 *montana* cage population from Seward (Alaska, North America; see Fig. 1). This population
243 has been established from the 4th generation progenies of 20 isofemale strains and maintained
244 in continuous light at 19 ± 1 °C. Malt bottles with freshly laid eggs were transferred from the
245 population cage into a climate chamber in LD 18:6 (Light:Dark cycle) at 19 ± 1 °C to reinforce

246 flies' circadian rhythmicity prior to the experiments. The critical day length for the induction
247 of reproductive diapause (CDL; 50 % of the females enter diapause) in 19 °C is LD 17:9 in
248 Seward population (Tyukmaeva et al., 2020), and thus the females emerging in LD 18:6 can be
249 expected to develop ovaries. This is important, as the diapausing females possessing higher cold
250 tolerance than the reproducing ones could induce variation in the results (Vesala, Salminen,
251 Kankare, et al., 2012). After ~4 weeks, we collected newly emerged females (≤ 1 day old) using
252 light CO2 anaesthesia and placed them back in malt-vials into above-mentioned conditions.
253 Females were changed into fresh vials once a week until they were used in the experiments at
254 the age of 21 days.

255 2.2.2 | Selecting candidate genes and defining their daily expression rhythm

256 Our earlier transcriptome and DNA microarray studies have identified several candidate genes
257 for cold acclimation in *D. montana* (Kankare et al., 2016; Parker, Ritchie, & Kankare, 2016;
258 Parker et al., 2015; Vesala, Salminen, Laiho, et al., 2012). In the present study we used RNAi
259 to get information on the importance of a circadian clock gene *vrille* (*vri*) and an insulin-
260 signalling pathway gene *vitellogenin* (*yolk protein 3, Yp3*) in *D. montana* females' basal cold
261 tolerance and cold acclimation ability. Both genes have been found to be upregulated in female
262 flies during cold acclimation in some of the above-mentioned studies.

263 To perform RNAi experiments at a right time of the day, we defined the time when the
264 expression of *vrille* and *vitellogenin* is highest at LD 18:6. To do this, we collected females
265 every four hours (i.e. at six time points / day), starting at 6 am when lights were switched on.
266 At each time point, we stored samples of females into -80˚C through liquid N_2, and transferred
267 them later on into RNAlater®-ICE frozen tissue transition solution (Thermo Fisher Scientific).
268 We then checked the size of female ovaries (see Salminen & Hoikkala, 2013) and used only the

269 females with fully developed ovaries (>95 % of the females). For each time point, RNA was
270 extracted from three pools, each of which consisted of three females, using ZR Tissue & Insect
271 RNA MicroPrep kit with DNAse treatment (Zymo Research®). RNA purity was analysed with
272 NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and concentration with
273 Qubit® 2.0 Fluorometer and RNA BR kit (Thermo Fisher Scientific). cDNA was synthesized
274 using equal quantities of RNA (200 ng) with iScript Reverse Transcription kit (Bio-Rad
275 Laboratories®).

276 We measured the expression levels of *vrille* and *vitellogenin* with quantitative real time PCR
277 (qPCR). qPCR primers for these genes and for reference genes were designed based on *D.*
278 *montana* genomic sequences under NCBI accession number LUVX00000000 (Parker et al.,
279 2018), together with information from *D. virilis* exons using Primer3 (primer3.ut.ee) and
280 NetPrimer (www.premierbiosoft.com/netprimer) programs (gene accession numbers and
281 primer sequences given in Table S4). qPCR mix contained 10 µl of 2x Power SYBR Green
282 PCR Master Mix (Bio-Rad Laboratories), 0.3 µM of each gene-specific primer and 1 µl of
283 cDNA solution. Cycling conditions in Bio-Rad CFX96 instrument were: 3 min at 95 °C, 10 sec
284 at 95 °C, 10 sec at annealing temperature of 56 ºC (except for *vrille* 53 ºC) and 30 sec at 72 °C
285 (repeated 40x), followed by melting curve analysis (65-95 °C) for amplification specificity
286 checking. Each run included three technical replicates for each sample and the final threshold
287 value (Cq) was defined as a mean of the technical replicates that produced good quality data.
288 Normalisation of the data was done with ΔΔ(Ct) normalisation method (Livak & Schmittgen,
289 2001) using *Tubulin beta chain* (*Tub2*) and *Ribosomal protein L32* (*RpL32*) as reference genes
290 (these genes had equal expression levels in all samples). Efficiency values of the genes used in
291 the qPCR are given in Table S4.

292

293 ### 2.2.3 | Synthesis of double-stranded RNA for RNAi

294 We generated fragments of *vrille, vitellogenin* and *LacZ* genes, with the length of 347, 419 and
295 529 bp respectively, with PCR (primer information given in Table S4). *LacZ,* which codes a
296 part of a bacterial gene, was used here as a control for dsRNA injections. PCR products were
297 purified with GeneJET Gel Extraction kit (Thermo Fisher Scientific) and cloned using
298 CloneJET PCR Cloning kit (Thermo Fisher Scientific). PCR products were then ligated into
299 the vector (pJET1.2/blunt Cloning vector), transformed into *E. coli* Zymo JM109 (Zymo
300 Research) cells, which were grown on Luria Broth (LB) ampicillin plates. Individual colonies
301 were picked up after 16 hours and cultivated overnight in LB solution with ampicillin using a
302 Unimax 1010 shaker with incubator (Heidolph Instruments). The samples from the extracted
303 bacterial solutions were analysed for the size of the products in the second PCR, which was
304 carried out with pJET primers from the cloning kit, followed by agarose gel runs. We then
305 selected the initial samples with the right size products for the third PCR using pJET primers,
306 where the R primer contained T7 promoter sequence at the 5' end of the primer (primer
307 sequences given in Table S4). PCR products were first purified with GeneJet Gel Extraction kit
308 and then used in transcription synthesis of the double-stranded RNA (dsRNA), using the
309 TranscriptAid T7 High Yield Transcription kit (Thermo Fisher Scientific). Finally, we purified
310 and precipitated the synthesized products with ethanol, suspended them into a salt buffer and
311 quantified them using NanoDrop and agarose gel.

312

313

314 **2.2.4 | RNAi microinjection procedure, response time screening and the**
315 **expression levels of *vrille*, *vitellogenin*, *clock* and *period* at the chosen response**
316 **time**

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

322 We checked the effectiveness of RNAi treatment on *vrille* and *vitellogenin* expression 12h, 24h
323 and 48h after injections, at a time of the day when both genes show highest expression (see
324 Results). The females were injected with 138 nl of ~20 µM dsRNA targeting *vrille* or
325 *vitellogenin* (target genes) or *LacZ* using Nanoject II Auto-nanoliter Injector (Drummond
326 Scientific) with 3.5" Drummond glass capillaries (Drummond Scientific) pulled with P-97
327 Flaming/Brown Micropipette Puller (Sutter Instrument). No-injection control females were not
328 injected, but were otherwise handled in the same way as the injected females. To prevent CO_2
329 anaesthesia from inducing variation between these groups, we injected groups of six flies at a
330 time. The samples consisting of three pools, 10 females per pool, for each treatment and
331 response time (12h, 24h or 48h), were transferred into -80 ºC through liquid N_2. Then the
332 females were transferred into RNAlater®-ICE solution and their ovaries were checked as
333 explained above. RNA was extracted from the pools using TRIzol® Reagent with the
334 PureLink® RNA Mini kit (Thermo Fisher Scientific). RNA purity was analysed with
335 NanoDrop and concentration with Qubit and RNA BR kit. cDNA was synthesized using equal

336 quantities of RNA (143 ng) using SuperScript IV First-Strand Synthesis System (Thermo Fisher
337 Scientific).

338 Expression levels of *vrille* and *vitellogenin* were quantified 12h, 24h and 48h after injections,
339 as described in section "*Selecting candidate genes and defining their daily rhythm*". Normalised
340 expression values of both genes, analysed with a linear model (ANOVA) in base R, were lowest
341 compared to controls 48h after injections (Fig. S3). Thus, we used this time point to find out
342 whether the silencing of *vrille* or *vitellogenin* had changed also the expression of two circadian
343 clock genes, *clock* and *period* (primer sequences given in Table S4). We synthesized cDNA
344 using equal quantities of RNA (150 ng) and quantified the expression levels of all four genes
345 as described above, using ΔΔ(Ct) normalisation method (Livak & Schmittgen, 2001) with *Tub2*
346 as a reference gene.

347 2.2.5 | Measuring females' cold tolerance and cold acclimation ability after 348 RNAi treatment

349 Prior to performing cold tolerance tests, females were maintained for 16 days in LD 18:6 and
350 at 19 ˚C, corresponding to summer conditions at their home site (LD 18:6 was used throughout
351 the experiment). Half of the females were then subjected to cold acclimation treatment at 6 °C
352 for 3 days (cold-acclimated females; Vesala, Salminen, Laiho, et al., 2012), while the other half
353 was maintained at 19 °C (non-acclimated females, i.e. basal cold tolerance). At the age of 19
354 days, both non-acclimated and cold-acclimated females were collected from the chambers,
355 anesthetized with CO_2 and injected as described above. They were then placed back to either
356 non-acclimation (19 ˚C) or cold acclimation (6 ˚C) conditions for two more days, as the
357 expression levels of target genes had been found to be lowest 48h after RNAi treatment (Fig.
358 S3). At the age of 21 days, females' cold tolerance was quantified by measuring their chill coma

359 temperature (CT_{min}) or chill coma recovery time (CCRT) using Julabo F32-HL
360 Refrigerated/Heating Circulator. Sample sizes for CT_{min} and CCRT tests were 26-38 and 23-30
361 females per treatment, respectively.

362 2.2.6 | Statistical analyses

363 Expression levels of *vrille, vitellogenin, clock* and *period* in *LacZ*-injected females were
364 compared to three other groups (no-injection control and females injected with dsRNA
365 targeting on either *vrille* or *vitellogenin*) 48h after injections. Log_2-transformed normalised
366 expression values were analysed using a linear model (ANOVA) in base R.

367 To test the effect of cold acclimation on female cold tolerance, we first compared the cold
368 tolerance of non-acclimated and cold-acclimated females with each other within different
369 groups (*LacZ* injection, no-injection, and *vrille* and *vitellogenin* injections). We then
370 investigated the effect *vrille* and *vitellogenin* RNAi on female cold tolerance by comparing
371 these groups to *LacZ* control. *LacZ* was also compared to no-injection control to trace possible
372 immune and physical effects of the injections. These analyses were performed separately for
373 non-acclimated and cold-acclimated females. All data showed deviation from normality (Fig.
374 S4) and were analysed with generalized linear mixed model using gamma distribution (GLMM;
375 *glmmTMB* function from glmmTMB package (Brooks et al., 2017). In the models, response
376 variables were either CT_{min} (Celsius degrees + 10 to prevent negative values from affecting the
377 results) or CCRT (minutes) data, and the test replicates were used as a random effect. All the
378 analyses were conducted in R (v1.2.1335-1) and R studio (v3.6.1).

379

380

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).

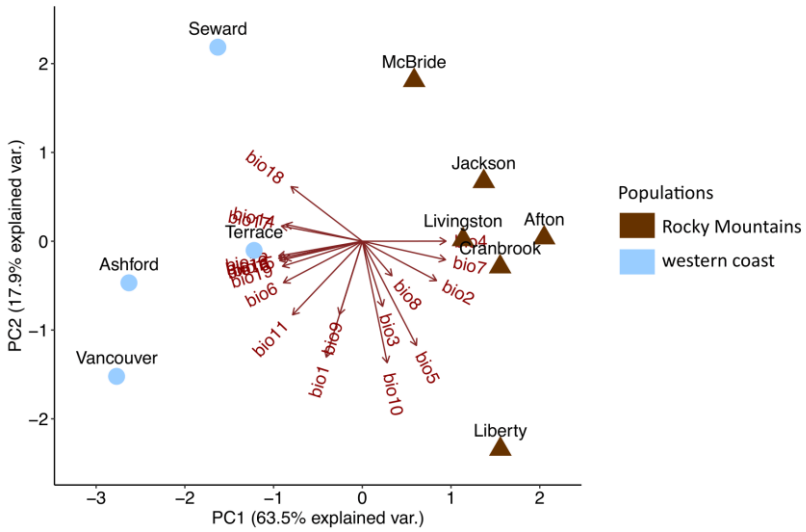


Figure 2. Principal component analysis (PCA) on 19 bioclimatic variables on fly collecting sites (Table S2). Direction of the arrows indicate which bioclimatic variables contribute to PC1 and which to PC2. The longer the arrow is, the greater its contribution is.

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_{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_{min} 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_{min}, CCRT and body colour of *D. montana*, judged from their Akaike information criterion (AIC) and Akaike weight values, included only latitude (Table S7). CT_{min} 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

418 CT_{min} included only latitude, that for CCRT latitude, PC1 and their interaction, and the one for
419 body colour latitude and PC1 (Table S7). CT_{min} of this species showed only minor variation
420 and no significant association with latitude (Fig. 3A; Table S8). However, CCRT values
421 showed the cold tolerance of *D. flavomontana* to improve towards North and to be strongly
422 affected by local climatic conditions (PC1) especially at latitudes around 50-55 ˚N (Fig. 3B;
423 Table S8). On these latitudes, fly cold tolerance was higher in the humid, low-altitude western
424 coast populations than in the high-altitude Rocky Mountains populations showing high
425 temperature variation (Fig. 2, 3B; Table S8). Body colour of *D. flavomontana* became darker
426 (i.e. its colour intensity decreased) towards North (Fig. 3C; Table S8). This trait was also
427 affected by climatic conditions (PC1), all western coast populations being equally dark and
428 darker than the Rocky Mountains populations from the similar latitudes (Fig. 3C; Table S8).

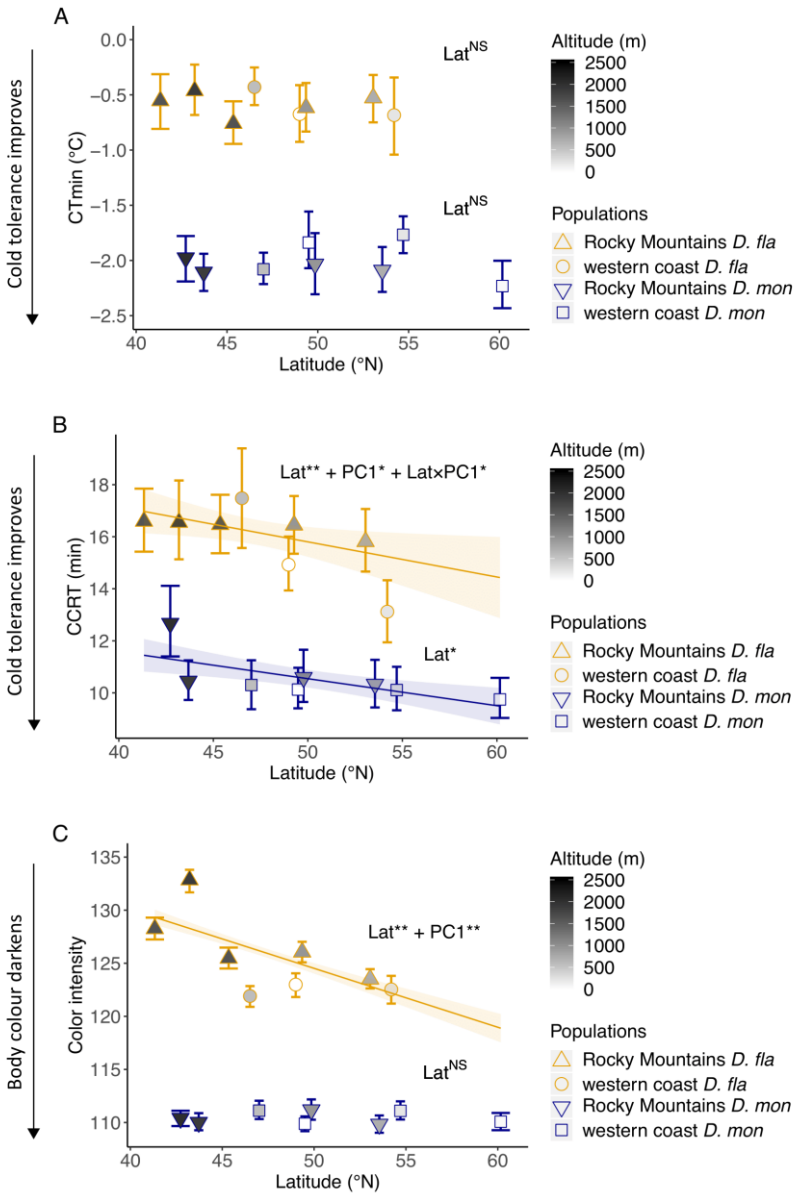


Figure 3. Relationship between latitude and (A) chill coma temperature (CT_{min}), (B) chill coma recovery time (CCRT) and (C) fly body colour in *D. montana* (*D. mon*) and *D. flavomontana* (*D. fla*) populations. Error bars represent bootstrapped 95% confidence intervals. Altitudes (m) are shown in 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_{min} or CCRT method (Fig. 4, Table S9).

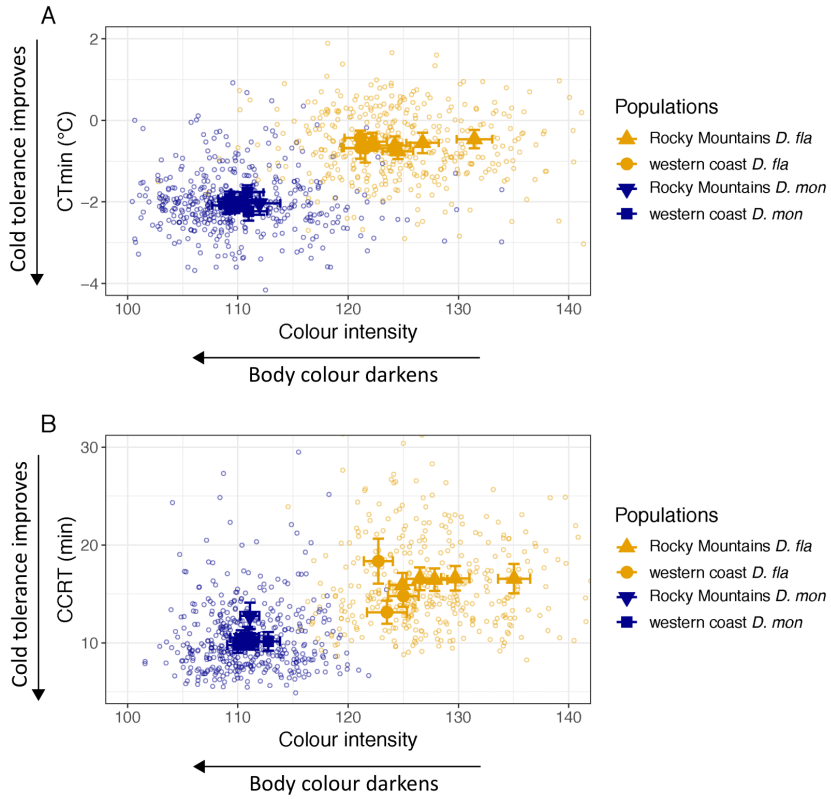


Figure 4. Association between body colour and (A) chill coma temperature (CT_{min}) and (B) chill coma recovery time (CCRT) in *D. montana* and *D. flavomontana* populations. Information on Rocky Mountains and western coast populations is given only for the purpose of illustration. Vertical error bars represent bootstrapped 95% confidence intervals for cold tolerance measurements and horizontal error bars for body colour measurements.

3.2. | The role of *vrille* and *vitellogenin* genes in the cold tolerance and cold acclimation of *D. montana*

3.2.1 | Daily rhythms of *vrille* and *vitellogenin* and effectiveness of RNAi

Expression levels of *vrille* showed clear rhythmicity and was highest at 22 pm and 2 am, while that of *vitellogenin* 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.

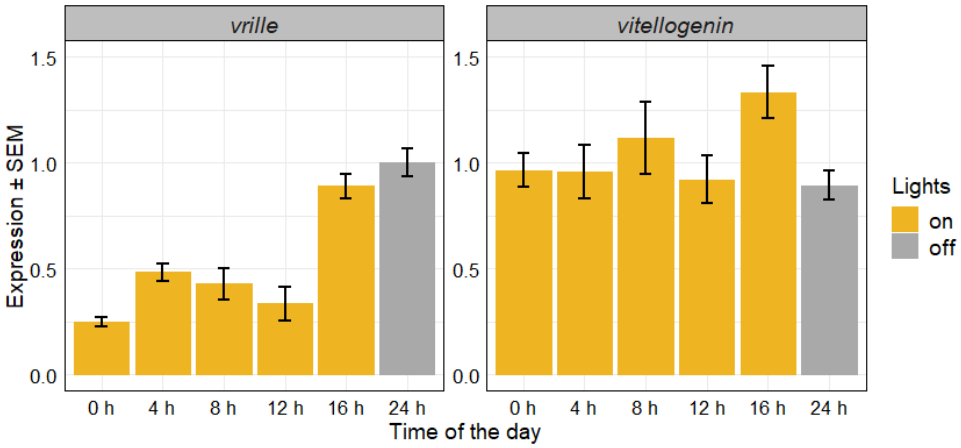


Figure 5. Baseline expression of *vrille* and *vitellogenin* at six time points, starting 0 h after lights were switched on, at LD 18:6. Orange represents the time of the day when the lights were on and grey when the lights were off.

3.2.2 | Effects of RNAi on the expression levels of *vrille*, *vitellogenin*, *clock* and *period*

Checking the expression levels of *vrille* and *vitellogenin* 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 *vrille*, *vitellogenin*, *clock* and *period* genes as well as for measuring the cold tolerance of RNAi treated flies and their controls.

vrille RNAi induced a significant decrease in the expression of *vrille* and *period* genes compared to *LacZ* control (approximately 66% and 61%, respectively), while *vitellogenin* RNAi decreased significantly only *vitellogenin* 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).

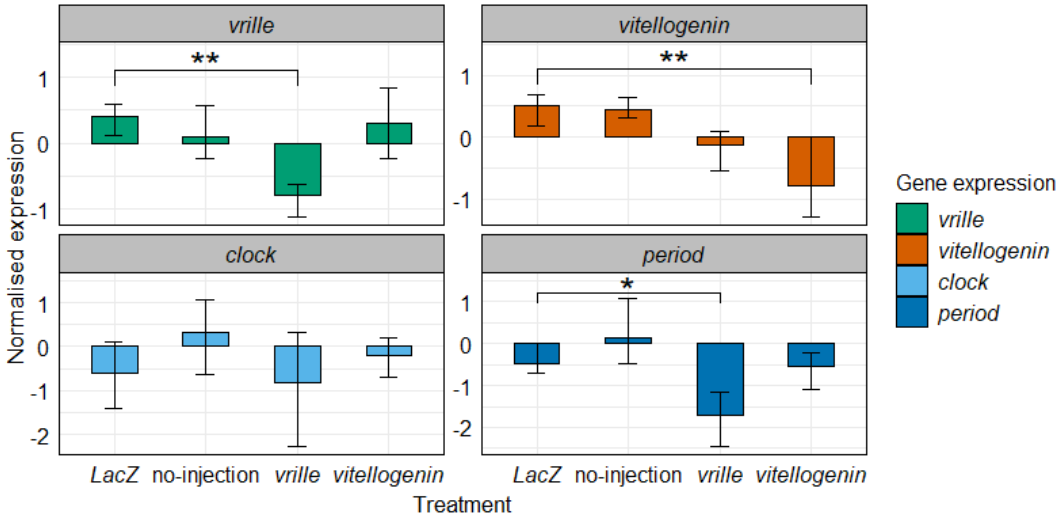


Figure 6. Expression levels of *vrille*, *vitellogenin, clock* and *period* genes in *LacZ*-injected and no-injection control females, and in females injected with dsRNA targeting on *vrille* or *vitellogenin*. Error bars represent bootstrapped 95% confidence intervals. Significance levels were obtained 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 *vitellogenin* silencing with RNAi on female basal cold tolerance and cold acclimation ability were studied with CT_{min} 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_{min} and CCRT data between *LacZ* and *vrille* and *vitellogenin* 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_{min} test.

Firstly, we compared the cold tolerances of non-acclimated (basal) and cold-acclimated females within each group. In CT_{min} 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

488 tolerance of *vrille*-injected females, and it decreased that of *vitellogenin*-injected females (Fig.
489 7A; Table S11). In CCRT tests, cold acclimation improved the cold tolerance of no-injection
490 females, had no effect on the cold tolerance of *LacZ*- or *vitellogenin*-injected females, and
491 decreased that of *vrille*-injected females (Fig. 7B; Table S11).

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

499 Silencing of *vrille* and *vitellogenin* genes decreased the cold tolerance of cold-acclimated
500 females compared to *LacZ* controls in CT_{min} tests, even though variation among *vitellogenin*-
501 injected females was quite high (Fig. 7A; Table S12). The same phenomenon was also detected
502 in the CCRT test, but only for *vrille* (Fig. 7B; Table S12). In the CCRT test, a significant
503 difference between *LacZ* and no-injection control implicates physical damage or immune
504 response induced by injection (Fig. 7B; Table S12).

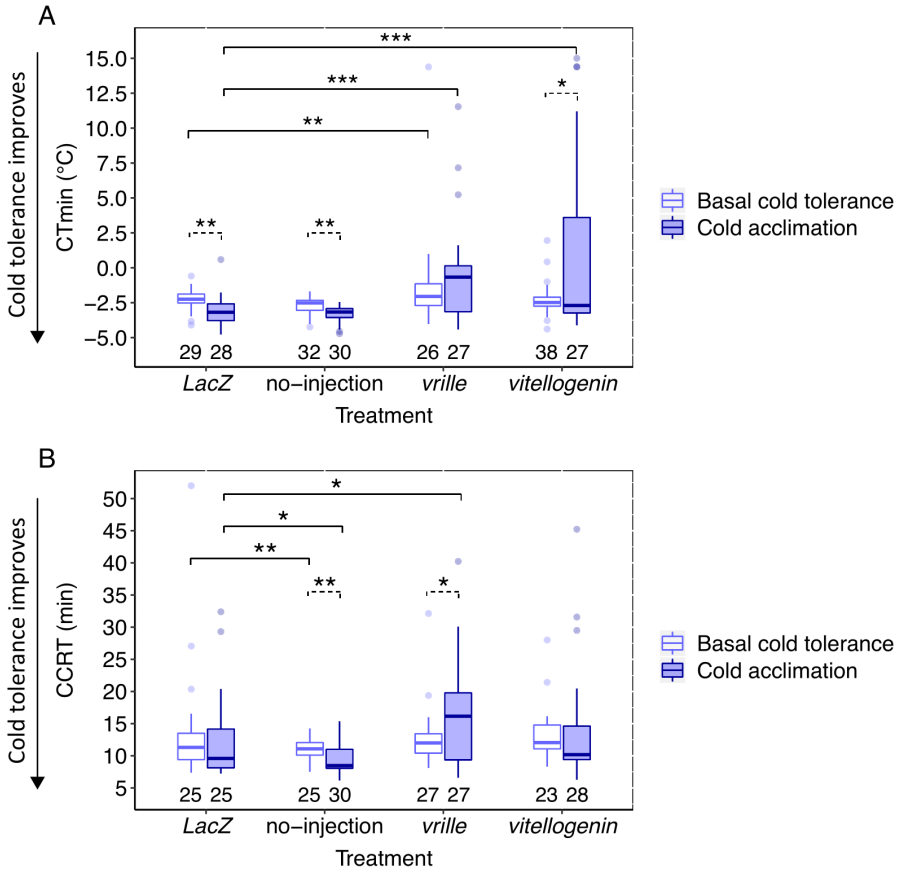


Figure 7. Female cold tolerance before (basal cold tolerance) and after cold acclimation in *LacZ* and no-injection control groups, and in RNAi treated *vrille* or *vitellogenin* groups measured with (A) CT_{min} and (B) CCRT tests. Dashed lines indicate significant differences between females' basal 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 *vitellogenin* 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.

541 Latitudinal variation in the basal cold tolerance (as measured with CCRT) of *D. montana*
542 females showed no association with local climatic conditions, which emphasizes its
543 dependency on photoperiodic and temperature conditions varying according to the latitude in a
544 wider scale. This is interesting, as latitudinal variation in another photoperiodically regulated
545 trait in this species, the critical day length for reproductive diapause (CDL), has been found to
546 be affected also by the climatic conditions prevailing at different altitudes (Tyukmaeva et al.,
547 2020). In *D. flavomontana*, latitudinal variation in basal cold tolerance showed an association
548 with local climatic conditions and was, surprisingly, higher in the humid low-altitude western
549 coast populations than in the high-altitude Rocky Mountains populations. However, *D.*
550 *flavomontana* flies of the mountain populations could use at least three strategies to enhance
551 their cold tolerance. Firstly, females could enter reproductive diapause at an earlier time of year
552 than the females from the low-altitude populations from the same latitude, as Tyukmaeva et al.
553 (2020) have shown *D. montana* females to do. In *D. montana* diapause improves female cold
554 tolerance considerably (Kauranen et al., 2019; Vesala & Hoikkala, 2011). Secondly, the low
555 basal cold tolerance of the mountain flies could also be compensated by a higher cold
556 acclimation ability, as several studies have detected a linkage between low cold tolerance and
557 high cold acclimation ability (Gerken et al., 2015; Hoffmann et al., 2003; Isobe et al., 2013;
558 Noh et al., 2017; Nyamukondiwa et al., 2011; Stillman, 2003; Zuther et al., 2012). Finally, the
559 flies could avoid freezing injuries by migrating towards warmer habitats and thermally buffered
560 microclimates (Stevenson, 1985). This kind of behaviour has been detected in the Colorado
561 population of *D. flavomontana*, where the flies occupy lower mountain slopes during early
562 spring than in summer (Hoikkala, pers. com.).

563 Insects' dark body colour has been suggested to absorb solar radiation, heat up faster and
564 maintain the optimum body temperature better than a light body colour (Clusella-Trullas et al.,

565 2007; Rajpurohit et al., 2008). For example in *Drosophila americana,* darker body colour of
566 the coastal populations, compared to that of the inland populations, (Wittkopp et al., 2011) has
567 been suggested to be an adaptation to lower solar radiation and smaller temperature variation
568 on the coast (Clusella-Trullas & Terblanche, 2011). On the other hand, several studies have
569 shown the dark body colour to play more important role in desiccation tolerance than in
570 thermoregulation (Parkash, Sharma, & Kalra, 2009; Ramniwas, Kajla, Dev, & Parkash, 2013).
571 *D. montana* flies were equally dark in all populations, while in *D. flavomontana* body colour
572 was dark brown in the western coast populations and turned from light to dark brown towards
573 North in the Rocky Mountains. Despite its association with climatic conditions, the body colour
574 of *D. flavomontana* showed no correlation with CT_{min} or CCRT. However, its variation could
575 reflect species demographic history, as *D. flavomontana* may have distributed from the Rocky
576 Mountains into the coastal area during the last decades (Patterson, 1952; Poikela et al. 2019),
577 most probably through British Columbia in Canada where *D. flavomontana* body colour is dark.
578 *D. flavomontana* may also hybridize with *D. montana* to some degree (Patterson, 1952;
579 Throckmorton, 1982; Poikela et al. 2019), which could potentially have led to introgression of
580 dark body colour from *D. montana* to *D. flavomontana*.

581 4.2 | The role of the circadian clock system and *vitellogenin* in cold 582 adaptation in *D. montana*

583 Cold acclimation has been found to induce shifts e.g. in insect metabolic profile and in the
584 production of cryoprotectants, which helps to maintain osmotic balance and stabilise cell
585 membrane structure during cold stress (Enriquez & Colinet, 2019; Lee, 2010; Storey & Storey,
586 2012). In addition, cold acclimation has been shown to be accompanied by expression changes
587 in genes involved in stress response, protein folding, repair, transport, signalling, neuronal

588 activity, carbohydrate metabolism and circadian rhythmicity (Des Marteaux, McKinnon,
589 Udaka, Toxopeus, & Sinclair, 2017; Enriquez & Colinet, 2019). Parker et al. (2015) found the
590 biological processes associated with cold acclimation of *D. montana* flies to cover largely the
591 same classes of genes as in above-mentioned studies, excluding stress response. Furthermore,
592 Parker et al. (2018) found evidence for selection for higher cold tolerance acting on neuronal,
593 membrane-transport and ion-transport related genes in genome-wide comparisons between *D.*
594 *montana* and several less cold-tolerant *Drosophila* species, as well as between *D. montana*
595 populations. These two studies brought up several candidate genes for cold tolerance and cold
596 acclimation in *D. montana*. Vigoder et al., (2016) used RNAi to silence one of these candidate
597 genes, *myo-inositol-1-phosphate synthase* (*Inos*), coding the major metabolite of overwintering
598 *D. montana* flies, *myo*-inositol (Vesala, Salminen, Kostál, Zahradníĉková, & Hoikkala, 2012),
599 and found this treatment to increase *D. montana* flies' mortality during cold exposure (5 ˚C)
600 but not to affect CCRT. We chose for RNAi studies two of the candidate genes, *vrille* and
601 *vitellogenin*, representing the circadian clock system and functioning in the insulin signalling
602 pathway, respectively.

603 *Drosophila* flies can monitor light and temperature changes e.g. through their thermosensory
604 organs located in aristae and legs, and to entrain behavioural and physiological changes to fit
605 with environmental conditions, with the aid of the circadian clock system (Sehadova et al.,
606 2009; Yadlapalli et al., 2018). For example in *Gryllus pennsylvanicus* crickets cold acclimation
607 increases the expression levels of two core circadian clock genes, *period* and *clock*, and
608 decreases that of *timeless* (Des Marteaux et al., 2017), while in *D. montana* and *D. virilis* it
609 increases the expression of *vrille* and *period* (Parker et al., 2015; Vesala, Salminen, Laiho, et
610 al., 2012). Blau & Young (1999) have shown that suppressing *vrille* expression decreases the
611 expression levels of *period* and *timeless*, even though a simulation performed by Smolen,

612 Hardin, Lo, Baxter, & Byrne (2004) suggests it to lead to a higher expression of *clock* and
613 *period*. Our study gives support to the finding of Blau & Young (1999), as silencing *vrille*
614 expression with RNAi decreased the expression of *period*, but not that of *clock*. Moreover,
615 *vrille*-injected acclimated and non-acclimated females had significantly decreased cold
616 tolerance in CT_{min} tests, and the same was true for *vrille*-injected acclimated females in CCRT
617 tests. Thus, we propose that circadian clock system is essential both in maintaining insect basal
618 cold tolerance and in increasing it during short cold acclimation.

619 Neuroendocrine signalling coordinates several processes involved in metabolism, development,
620 reproduction and cold acclimation (Des Marteaux et al., 2017; Hahn & Denlinger, 2011;
621 MacMillan et al., 2016; Torson, Yocum, Rinehart, Kemp, & Bowsher, 2015), and *vitellogenin*
622 genes play an important part in this system (Elgendy et al., 2019; Gonçalves Santos, Humann,
623 & Hartfelder, 2019). In insects, vitellogenin is primarily an egg-yolk protein, which is
624 synthesized in the fat body cells, released into the haemolymph and taken up by targets via
625 receptor-mediated endocytosis (reviewed in Tufail & Takeda, 2008), but it has also been
626 proposed to act as an antifreeze protein in the haemolymph (Crosthwaite et al., 2011; Qin et al.,
627 2019). Studies on the function of *vitellogenin* genes during cold acclimation have given
628 contradictory results in different species. For example in *D. melanogaster* (Colinet, Overgaard,
629 Com, & Sørensen, 2013) and *D. suzukii* (Enriquez & Colinet, 2019) flies and *G. pennsylvanicus*
630 crickets (Des Marteaux et al., 2017), cold acclimation decreases the expression level of
631 *vitellogenin* genes. On the other hand, in *Apis cerana cerana* honeybees the expression of
632 *vitellogenin* genes increases during the cold stress (Zhang et al., 2017), and in *Apis mellifera*
633 honeybees the level of vitellogenin protein in haemolymph increases towards the winter and is
634 higher in northern than in southern populations (Amdam et al., 2005; Qin et al., 2019).
635 Accordingly, vitellogenin has been suggested to act as an antifreeze protein in both bee species

636 (Crosthwaite et al., 2011; Qin et al., 2019). In the present study, silencing *vitellogenin* with
637 RNAi did not affect *D. montana* females' basal cold tolerance in CT_{min} or CCRT test, but
638 decreased their cold acclimation ability in CT_{min} test, which suggests that *vitellogenin* could act
639 as an antifreeze protein or affect fly cold resistance through its extensive regulatory and
640 signalling roles in the insulin-signalling pathway during cold acclimation (Crosthwaite et al.,
641 2011; Flatt et al., 2005; Qin et al., 2019).

642 5 | Conclusions

643 Understanding the mechanisms that generate variation in species stress tolerance is a key
644 component for predicting their adaptation ability in the face of global warming. Species, whose
645 cold or heat tolerance is tightly linked with latitude, may encounter more difficulties in adapting
646 to changing environmental conditions than species whose tolerances are also affected by local
647 climatic conditions. This study deepens our understanding on how latitudinally varying factors
648 and local climatic conditions shape the evolution of cold tolerance. We show that cold tolerance
649 of *D. montana* and *D. flavomontana* flies relies partly on different environmental cues. While
650 the basal cold tolerance of *D. montana* is tightly linked with latitudinal variation in photoperiod
651 and temperature, that of *D. flavomontana* is also affected by local climatic conditions. While
652 variation in the body color of *D. flavomontana* shows no correlation with cold tolerance *per se*,
653 its association with latitude and climatic conditions indicates that it may have thermoregulatory
654 functions in terms of solar radiation. However, possible effects of species and population
655 phylogenies and interspecific gene flow from *D. montana* cannot be ruled out. Our study also
656 propose that a functional circadian clock system is essential both in insect basal cold tolerance
657 and cold acclimation ability. Moreover, *vitellogenin*, and possibly the whole insulin-signalling
658 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

Amdam, G. V, Norberg, K., Omholt, S. W., Kryger, P., Lourenço, A. P., Bitondi, M. M. G., & Simões, Z. L. P. (2005). Higher vitellogenin concentrations in honey bee workers may be an adaptation to life in temperate climates. *Insectes Sociaux*, *52*(4), 316–319. doi: 10.1007/s00040-005-0812-2

Amdam, G. V, & Omholt, S. W. (2003). The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. *Journal of Theoretical Biology*, *223*, 451–464. doi: 10.1016/S0022-5193(03)00121-8

Andersen, J. L., Manenti, T., Sørensen, J. G., Macmillan, H. A., Loeschcke, 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

Baker, D. A., & Russell, S. (2009). Gene expression during *Drosophila melanogaster* egg development before and after reproductive diapause. *BMC Genomics*, *16*, 1–16. doi: 10.1186/1471-2164-10-242

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.cub.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

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. *Heredity*, *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

Crosthwaite, J. C., Sobek, S., Lyons, D. B., Bernards, M. A., & Sinclair, B. J. (2011). The overwintering physiology of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). *Journal of Insect Physiology*, *57*, 166–173. doi: 10.1016/j.jinsphys.2010.11.003

David, J. R., Gibert, P., Moreteau, B., Gilchrist, G. W., & Huey, R. B. (2003). The fly that came in from the cold: geographic variation of recovery time from low-temperature exposure in *Drosophila subobscura*. *Functional Ecology*, *17*, 425–430.

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

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

Elgendy, A. M., Tufail, M., Mohamed, A. A., & Takeda, M. (2019). A putative direct repeat element plays a dual role in the induction and repression of insect *vitellogenin-1* gene expression. *Comparative Biochemistry and Physiology, Part B*, *234*, 1–8. doi: 10.1016/j.cbpb.2019.04.003

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

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

Findsen, A., Pedersen, T. H., Petersen, A. G., Nielsen, O. B., & Overgaard, J. (2014). Why do insects enter and recover from chill coma? Low temperature and high extracellular potassium compromise muscle function in *Locusta migratoria*. *The Journal of Experimental Biology*, *217*, 1297–1306. doi: 10.1242/jeb.098442

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

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

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

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

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

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

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

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.

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

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.

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

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

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

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

Isobe, K., Takahashi, A., & Tamura, K. (2013). Cold tolerance and metabolic rate increased by cold acclimation in *Drosophila albomicans* from natural populations. *Genes & Genetic Systems*, *88*, 289–300.

Kankare, M., Parker, D. J., Merisalo, M., Salminen, T. S., & Hoikkala, A. (2016). Transcriptional differences between diapausing and non-diapausing *D. montana* females reared under the same photoperiod and temperature. *PLoS ONE*, *11*(8), 1–18. doi: 10.1371/journal.pone.0161852

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: 10.1177/0748730412455916

Kellermann, V., Loeschcke, V., Hoffmann, A. A., Kristensen, T. N., Fløjgaard, 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 halticella* 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 $2^{-\Delta\Delta C}$T 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.jinsphys.2010.10.004

Magnone, M. C., Jacobmeier, B., Bertolucci, C., Foa, A., & Albrecht, U. (2005). Circadian expression of the clock gene *Per2* 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.cub.2017.01.036

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/ece3.3112

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 Univerisity 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.1748-5967.2008.00129.x

Ramirez, L., Negri, P., Sturla, L., Guida, L., Vigliarolo, T., Maggi, M., … Lamattina, L. (2017). Abscisic acid enhances cold tolerance in honeybee larvae. *Proceedings of the Royal Society B: Biological Sciences*, *284*, 20162140.

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.076166

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

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. doi: 10.1038/nmeth.2089

Sehadova, H., Glaser, F. T., Gentile, C., Simoni, A., Giesecke, A., Albert, J. T., & Stanewsky, R. (2009). Temperature entrainment of *Drosophila*'s circadian clock involves the gene nocte 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

Sheng, Z., Xu, J., Bai, H., Zhu, F., & Palli, S. R. (2011). Juvenile hormone regulates *vitellogenin* gene expression through insulin-like peptide signaling pathway in the red flour beetle, *Tribolium castaneum*. *The Journal of Biological Chemistry*, *286*(49), 41924–41936. doi: 10.1074/jbc.M111.269845

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., Asplen, 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 relative importance of behavioral and physiological adjustments controlling body temperature in terrestrial ectotherms. *American Society of Naturalists*, *126*(3), 362–386.

Stillman, J. H. (2003). Acclimation capacity underlies susceptibility to climate change. *Science*, *301*(5629), 65–65. doi: 10.1126/science.1083073

Storey, K. B., & Storey, J. M. (2012). Insect cold hardiness: Metabolic, gene, and protein adaptation. *Canadian Journal of Zoology*, *90*(4), 456–475. doi: 10.1139/Z2012-011

Sunday, J. M., Bates, A. E., & Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proceedings of the Royal Society B*, *278*, 1823–1830. doi: 10.1098/rspb.2010.1295

Teets, N. M., & Denlinger, D. L. (2013). Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiological Entomology*, *38*(2), 105–116. doi: 10.1111/phen.12019

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

Tufail, 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 ofphotoperiodically 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

Zhang, W., Liu, Z., Zhu, M., Ma, L., Wang, Y., Wang, H., … Xu, B. (2017). Molecular cloning, expression and oxidative stress response of the *vitellogenin* gene (AccVg) from *Apis cerana cerana*. *Apidologie*, *48*(5), 599–611. doi: 10.1007/s13592-017-0503-9

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 used in the study are in Supplementary Information and 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.