Latitudinal variation in cold tolerance and the role of *vrille* and *Yolk protein 3* 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 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 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

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

97 Several studies have suggested species cold tolerance and cold acclimation ability to be
98 associated with genes involved in the circadian clock system (Fowler & Thomashow, 2002;
99 Magnone, Jacobmeier, Bertolucci, Foa, & Albrecht, 2005; Vesala, Salminen, Laiho, Hoikkala,
100 & Kankare, 2012) and/or metabolic pathways, such as the insulin-signalling pathway (e.g. Sim
101 & Denlinger, 2013; Teets et al., 2012). The role of the circadian clock system in insect cold
102 adaptation, especially in cold acclimation, is logical, as this clocks monitors light and
103 temperature cycles and entrains behavioural and physiological rhythms to match with them
104 (Barber & Sehgal, 2018). In the central circadian clock, described most thoroughly in *D.*
105 *melanogaster*, daily rhythms are driven via one or several transcriptional feedback loops
106 involving changes in the expression of core circadian clock genes (studies reviewed e.g.
107 Gunawardhana & Hardin, 2017; Hardin, 2011; Williams & Sehgal, 2001). Briefly, the first core
108 timekeeping loop includes *clock* (*clk*) – *cycle* (*cyc*) and *period* (*per*) – *timeless* (*tim*) complexes
109 that activate and repress each other. *clock* and *cycle* further activate an interlocked feedback
110 loop that includes *vrille* (*vri*) and *Par domein protein 1* (*Pdp1*), which repress and activate *clock*
111 transcription, respectively. *vrille* also regulates neuropeptide Pigment-dispersing factor (PDF)
112 expression in clock neurons, which makes it a key regulator of circadian behavioural rhythms
113 (Gunawardhana & Hardin, 2017). Interestingly, *vrille* expression has been found to increase
114 during cold acclimation e.g. in *Drosophila montana* (Parker et al., 2015; Vesala, Salminen,

115 Laiho, et al., 2012). The insulin-signalling pathway, on the other hand, is known to have
116 widespread effects e.g. on insect development, aging, immune function and stress resistance
117 (reviewed in Flatt, Tu, & Tatar, 2005). This pathway involves major hormonal regulators,
118 juvenile hormone (JH) and 20-hydroxy-ecdysone (20E), for which effects are mediated, among
119 other genes, by *Yolk proteins* (reviewed in Flatt et al., 2005). *Yolk protein* genes code for a
120 phospholipoglycoprotein vitellogenin, which is primarily an egg-yolk protein synthesized in
121 insect ovarian follicle and fat body cells (reviewed in Flatt et al., 2005), but these genes play a
122 role also in neurogenesis (Neumüller et al., 2011). Under diapause inducing temperature and
123 light conditions, the *Yolk protein* genes *Yp1*, *Yp2* and *Yp3* are dozens of times higher in *D.*
124 *melanogaster* head than in ovaries (Zhao et al., 2015), which suggests that the genes have
125 important regulatory functions at neuronal level during diapause. Parker, Ritchie, & Kankare
126 (2016) have suggested that the same could be true during cold acclimation. Moreover, Davis &
127 Moyle (2020) suggest the function of *Yolk proteins* to extend beyond female reproductive
128 functions and to be associated broadly with stress response.

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

139 sympatrically and may hybridize to some degree (Patterson, 1952; Poikela et al., 2019;
140 Throckmorton, 1982). Body colour of *D. montana* is almost black, while that of *D.*
141 *flavomontana* varies from light to dark brown (Patterson, 1952).

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

152 2 | MATERIALS AND METHODS

153 2.1 | Variation in the basal cold tolerance and body colour in *D. montana* and
154 in *D. flavomontana* populations

155 2.1.1. | Study species and populations

156 *D. montana* and *D. flavomontana* belong to the *montana* phylad of the *virilis* group, and our
157 recent whole-genome analyses have shown their divergence time to be ~1 mya (Poikela et al.,
158 in preparation). *D. montana* is distributed around the northern hemisphere across North
159 America, Asia and Europe (Throckmorton, 1982), while the distribution of *D. flavomontana* is
160 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 CO_2 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

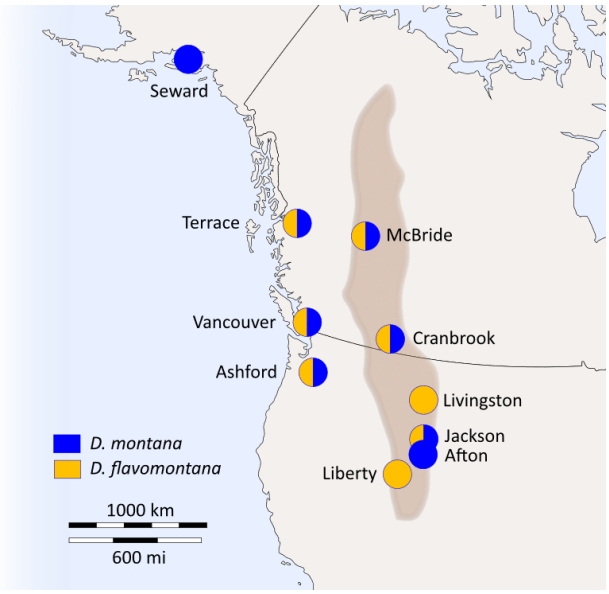

Figure 1. Table shows fly collecting sites and years, and the coordinates for each site. Map contains information on whether we have samples from one or both species in each site in the western coast and in the Rocky Mountains (brown area on the map) in North America (detailed information in Table S1).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

179 ### 2.1.2 | Cold tolerance tests

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

196 ### 2.1.3 | Fly body colour

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

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

208 2.1.4 | Statistical analyses

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

220 We extracted climatic information for the fly collecting sites from WorldClim database v2.0
221 (~340 km^2 spatial resolution; current data 1970-2000; Fick & Hijmans, 2017;
222 http://www.worldclim.org) using the latitudinal and longitudinal coordinates of each site (Fig.
223 1). After extracting 19 bioclimatic variables using raster package v. 2.8-19 (Hijmans & Etten,
224 2020; Table S2), we performed a principle component analysis (PCA) to summarize climatic

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

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

238 2.2 | RNA interference (RNAi) and its effects on fly cold tolerance

239 2.2.1 | Study material

240 We performed RNAi studies in *D. montana*, which has become a model species for studying
241 adaption to seasonally varying environments at phenotypic (e.g. Tyukmaeva, Lankinen,
242 Kinnunen, Kauranen, & Hoikkala, 2020) and genetic (Parker et al., 2018) level. We traced the
243 role of *vrille* and *Yp3* genes in cold adaptation using females from a mass-bred *D. montana*
244 cage population from Seward (Alaska, North America; see Fig. 1). This population has been
245 established from the 4th generation progenies of 20 isofemale strains and maintained in
246 continuous light at 19 ± 1 °C. Malt bottles with freshly laid eggs were transferred from the
247 population cage into a climate chamber in LD 18:6 (Light:Dark cycle) at 19 ± 1 °C to reinforce

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

257 2.2.2 | Selecting candidate genes and defining their daily expression rhythm

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

264 To perform RNAi experiments at a right time of the day, we defined the time when the
265 expression of *vrille* and *Yp3* is highest at LD 18:6. To do this, we collected females every four
266 hours (i.e. at six time points / day), starting at 6 am when lights were switched on. At each time
267 point, we stored samples of females into -80˚C through liquid N_2, and transferred them later on
268 into RNAlater®-ICE frozen tissue transition solution (Thermo Fisher Scientific). We then
269 checked the size of female ovaries (see Salminen & Hoikkala, 2013) and used only the females
270 with fully developed ovaries (>95 % of the females). For each time point, RNA was extracted

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

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

282 2.2.3 | Synthesis of double-stranded RNA for RNAi

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

289 2.2.4 | RNAi microinjection procedure, response time screening and the 290 expression levels of *vrille*, *Yp3*, *clock* and *period* at the chosen response time

291 Injecting dsRNA targeting on *vrille* or *Yp3* genes is expected to cause gene-specific effects, but
292 it may also cause immune responses and physical damage (injection) in the flies. Accordingly,

293 we used *LacZ* (encoding for bacterial gene) injections as a control for both immune response to
294 dsRNA and to physical damage of injections and no-injection as a baseline cold tolerance
295 control.

296 We checked the effectiveness of RNAi treatment on *vrille* and *Yp3* expression 12h, 24h and
297 48h after injections, at a time of the day when both genes show highest expression (see Results).
298 The females were injected with 138 nl of ~20 µM dsRNA targeting *vrille* or *Yp3* (target genes)
299 or *LacZ* using Nanoject II Auto-nanoliter Injector with 3.5" Drummond glass capillaries
300 (Drummond Scientific) pulled with P-97 Flaming/Brown Micropipette Puller (Sutter
301 Instrument). No-injection control females were not injected, but were otherwise handled in the
302 same way as the injected females. To prevent CO_2 anaesthesia from inducing variation between
303 these groups, we injected six flies at a time. The samples consisting of three pools, 10 females
304 per pool, for each treatment and response time (12h, 24h or 48h), were transferred into -80 ºC
305 through liquid N_2. Then the females were transferred into RNAlater®-ICE solution and their
306 ovaries were checked as explained above. RNA was extracted from the pools using TRIzol®
307 Reagent with the PureLink® RNA Mini kit (Thermo Fisher Scientific). RNA purity was
308 analysed with NanoDrop and concentration with Qubit and RNA BR kit. cDNA was
309 synthesized using equal quantities of RNA (143 ng) using SuperScript IV First-Strand
310 Synthesis System (Thermo Fisher Scientific).

311 Expression levels of *vrille* and *Yp3* were quantified 12h, 24h and 48h after injections, as
312 described in Method S1. Normalised expression values of both genes, analysed with a linear
313 model (ANOVA) in base R, were lowest compared to controls 48h after injections (Fig. S3).
314 Thus, we used this time point to find out whether the silencing of *vrille* or *Yp3* had changed
315 also the expression of two circadian clock genes, *clock* and *period* (primer sequences in Table
316 S4). We synthesized cDNA using equal quantities of RNA (150 ng) and quantified the

317 expression levels of all four genes as described in Method S1, using ΔΔ(Ct) normalisation
318 method (Livak & Schmittgen, 2001) with *Tub2* as a reference gene.

319 2.2.5 | Measuring females' cold tolerance and cold acclimation ability after 320 RNAi treatment

321 Prior to performing cold tolerance tests, females were maintained for 16 days in LD 18:6 and
322 at 19 ˚C, corresponding to summer conditions at their home site (LD 18:6 was used throughout
323 the experiment). Half of the females were then subjected to cold acclimation treatment at 6 °C
324 for 3 days (cold-acclimated females; Vesala, Salminen, Laiho, et al., 2012), while the other half
325 was maintained at 19 °C (non-acclimated females, i.e. basal cold tolerance). At the age of 19
326 days, both non-acclimated and cold-acclimated females were collected from the chambers,
327 anesthetized with CO_2 and injected as described above. They were then placed back to either
328 non-acclimation (19 ˚C) or cold acclimation (6 ˚C) conditions for two more days, as the
329 expression levels of target genes had been found to be lowest 48h after RNAi treatment (Fig.
330 S3). At the age of 21 days, females' cold tolerance was quantified by measuring their chill coma
331 temperature (CT_{min}) or chill coma recovery time (CCRT) using Julabo F32-HL
332 Refrigerated/Heating Circulator. Sample sizes for CT_{min} and CCRT tests were 26-38 and 23-30
333 females per treatment, respectively.

334 2.2.6 | Statistical analyses

335 Expression levels of *vrille, Yp3, clock* and *period* in *LacZ*-injected females were compared to
336 three other groups (no-injection control and females injected with dsRNA targeting on either
337 *vrille* or *Yp3*) 48h after injections. Log_2-transformed normalised expression values were
338 analysed using a linear model (ANOVA) in base R.

339 To test the effect of cold acclimation on female cold tolerance, we first compared the cold
340 tolerance of non-acclimated and cold-acclimated females with each other within different
341 groups (*LacZ* injection, no-injection, and *vrille* and *Yp3* injections). We then investigated the
342 effect *vrille* and *Yp3* RNAi on female cold tolerance by comparing these groups to *LacZ* control.
343 *LacZ* was also compared to no-injection control to trace possible immune and physical effects
344 of the injections. These analyses were performed separately for non-acclimated and cold-
345 acclimated females. All data showed deviation from normality (Fig. S4) and were analysed with
346 generalized linear mixed model using gamma distribution (GLMM; *glmmTMB* function from
347 glmmTMB package (Brooks et al., 2017). In the models, response variables were either CT_{min}
348 (Celsius degrees + 10 to prevent negative values from affecting the results) or CCRT (minutes)
349 data, and the test replicates were used as a random effect. All the analyses were conducted in R
350 (v1.2.1335-1) and R studio (v3.6.1).

351 **3 | RESULTS**

352 **3.1 | Variation in the climatic conditions at fly collecting sites and the factors**
353 **affecting fly cold tolerance and body colour**

354 **3.1.1 | Variation in the climatic conditions on fly collecting sites**

355 We performed principal component analysis (PCA) on 19 bioclimatic variables specific for
356 each fly collecting site to describe the patterns of climatic variation along these sites (Table S2,
357 S3). PCA revealed three principal components (PCs) with eigenvalues > 1 (Table S5). The first
358 PC (PC1) explained 63.5 % of the total variation (Fig. 2; Table S5), and it clearly separated the
359 Rocky Mountains populations from the ones on the western coast. The variables with a highest
360 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).

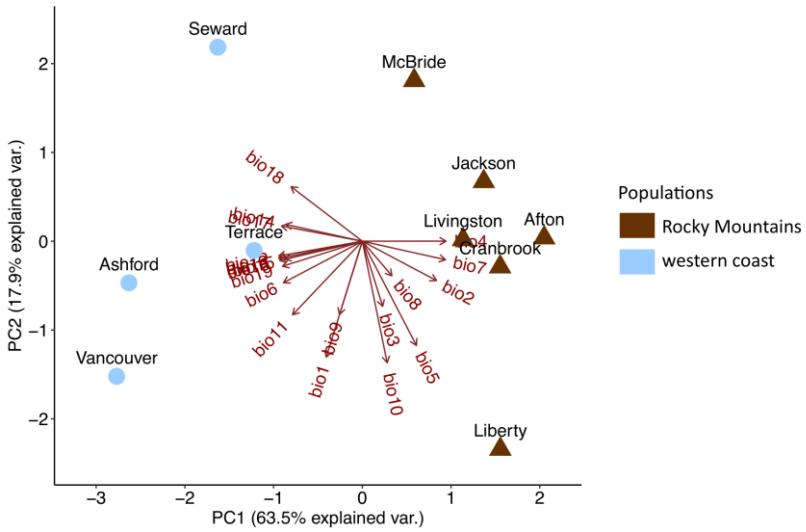

Figure 2. Principal component analysis (PCA) on 19 bioclimatic variables on fly collecting sites (Table S2). Direction of the arrows indicate which bioclimatic variables contribute to PC1 and which to PC2. The longer the arrow is, the greater its contribution is.

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

378 than *D. flavomontana* flies, and that the CCRT tests discover variation in this trait better than
379 the CT_{min} tests. Fly body colour showed significant variation only in *D. flavomontana*.

380 To choose the best-fit linear model explaining the effects of latitude and climatic variation
381 (PCA) on variation in fly cold tolerance, we performed model selection for different
382 combinations of latitude and PC1, which explained most variation in PCA (Fig. 2; Table S7).
383 The best-fit model for CT_{min}, CCRT and body colour of *D. montana*, judged from their Akaike
384 information criterion (AIC) and Akaike weight values, included only latitude (Table S7). CT_{min}
385 and body colour of this species showed only minor variation, which was not significantly
386 associated with latitude (Fig. 3A, 3C; Table S8), while CCRT showed the fly cold tolerance to
387 improve towards northern latitudes (Fig. 3B; Table S8). In *D. flavomontana*, best-fit model for
388 CT_{min} included only latitude, that for CCRT latitude, PC1 and their interaction, and the one for
389 body colour latitude and PC1 (Table S7). CT_{min} of this species showed only minor variation
390 and no significant association with latitude (Fig. 3A; Table S8). However, CCRT values
391 showed the cold tolerance of *D. flavomontana* to improve towards North and to be strongly
392 affected by local climatic conditions (PC1) especially at latitudes around 50-55 ˚N (Fig. 3B;
393 Table S8). On these latitudes, fly cold tolerance was higher in the humid, low-altitude western
394 coast populations than in the high-altitude Rocky Mountains populations showing high
395 temperature variation (Fig. 2, 3B; Table S8). Body colour of *D. flavomontana* became darker
396 (i.e. its colour intensity decreased) towards North (Fig. 3C; Table S8). This trait was also
397 affected by climatic conditions (PC1), all western coast populations being equally dark and
398 darker than the Rocky Mountains populations from the similar latitudes (Fig. 3C; Table S8).

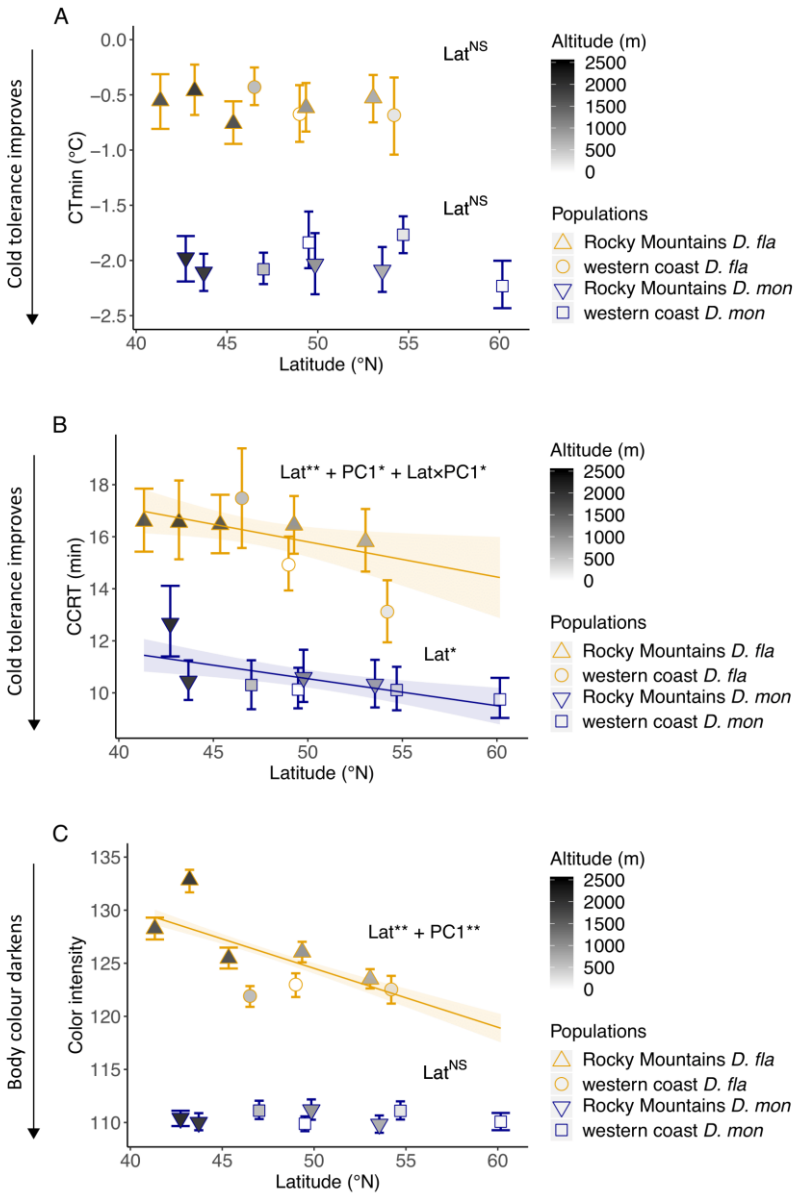

Figure 3. Relationship between latitude and (A) chill coma temperature (CT_{min}), (B) chill coma recovery time (CCRT) and (C) fly body colour in *D. montana* (*D. mon*) and *D. flavomontana* (*D. fla*) populations. Error bars represent bootstrapped 95% confidence intervals. Altitudes (m) are shown in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

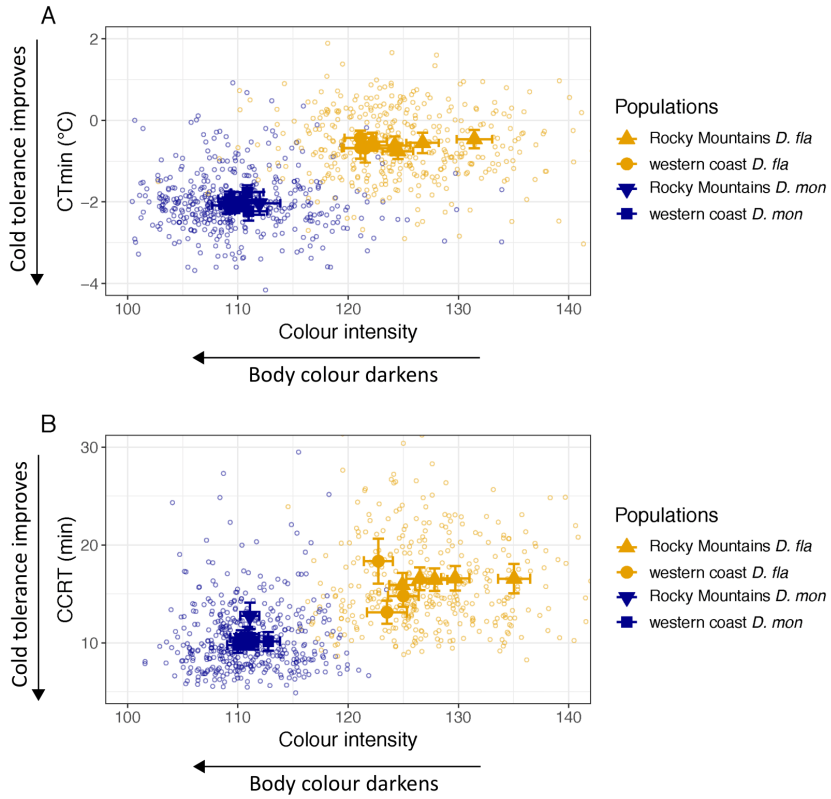

Figure 4. Association between body colour and (A) chill coma temperature (CT_{min}) and (B) chill coma recovery time (CCRT) in *D. montana* and *D. flavomontana* populations. Information on Rocky Mountains and western coast populations is given only for the purpose of illustration. Vertical error bars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

3.2. | The role of *vrille* and *Yp3* genes in the cold tolerance and cold acclimation of *D. montana*

3.2.1 | Daily rhythms of *vrille* and *Yp3* and effectiveness of RNAi

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

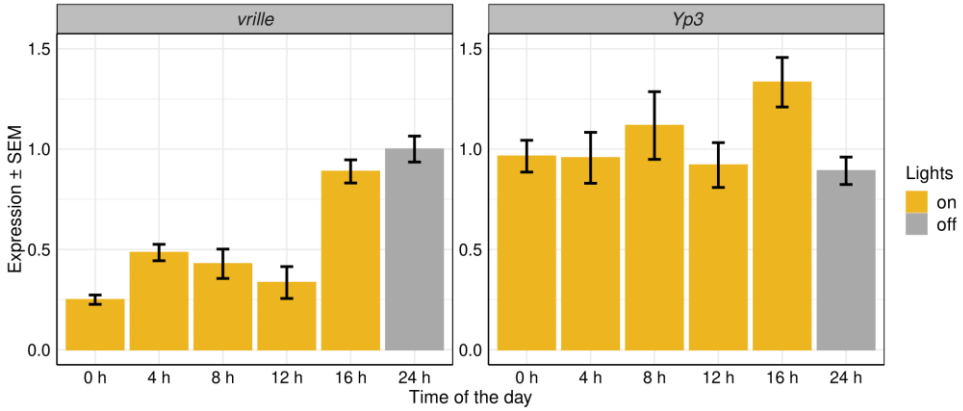

Figure 5. Baseline expression of *vrille* and *Yp3* at six time points, starting 0 h after lights were switched on, at LD 18:6. Orange represents the time of the day when the lights were on and grey when the lights were off.

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

Checking the expression levels of *vrille* 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 *vrille*, *Yp3*, *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 *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).

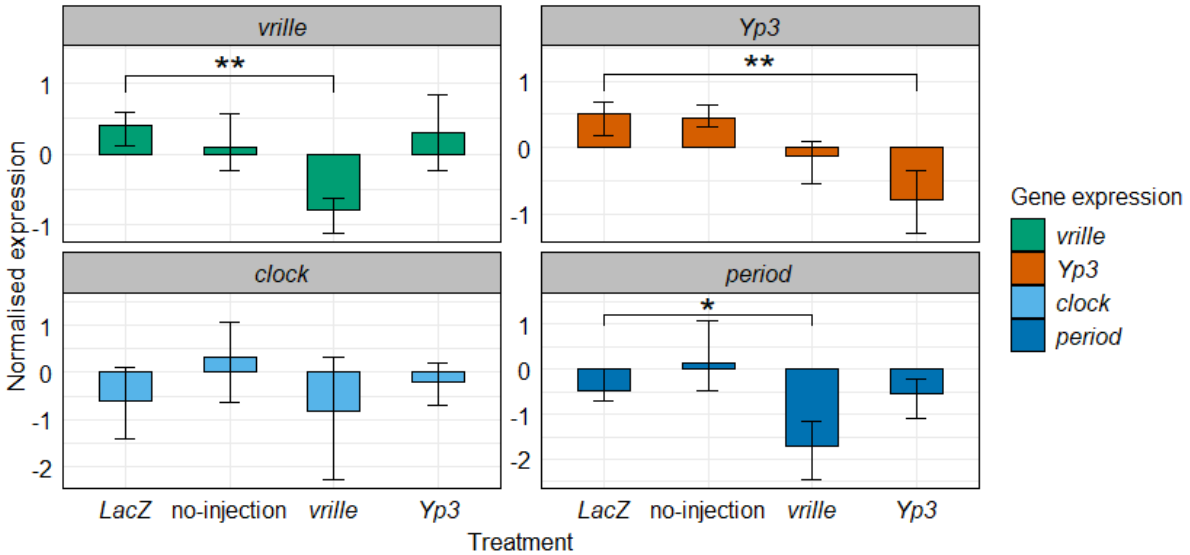

Figure 6. Expression levels of *vrille*, *Yp3, clock* and *period* genes in *LacZ*-injected and no-injection control females, and in females injected with dsRNA targeting on *vrille* or *Yp3*. Error bars represent bootstrapped 95% confidence intervals. Significance levels were obtained from linear model 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_{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 *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_{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

456 tolerance of *vrille*-injected females, and it decreased that of *Yp3*-injected females (Fig. 7A;
457 Table S11). In CCRT tests, cold acclimation improved the cold tolerance of no-injection
458 females, had no effect on the cold tolerance of *LacZ*- or *Yp3*-injected females, and decreased
459 that of *vrille*-injected females (Fig. 7B; Table S11).

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

467 Silencing of *vrille* and *Yp3* genes decreased the cold tolerance of cold-acclimated females
468 compared to *LacZ* controls in CT_{min} tests, even though variation among *Yp3*-injected females
469 was quite high (Fig. 7A; Table S12). The same phenomenon was also detected in the CCRT
470 test, but only for *vrille* (Fig. 7B; Table S12). In the CCRT test, a significant difference between
471 *LacZ* and no-injection control implicates physical damage or immune response induced by
472 injection (Fig. 7B; Table S12).

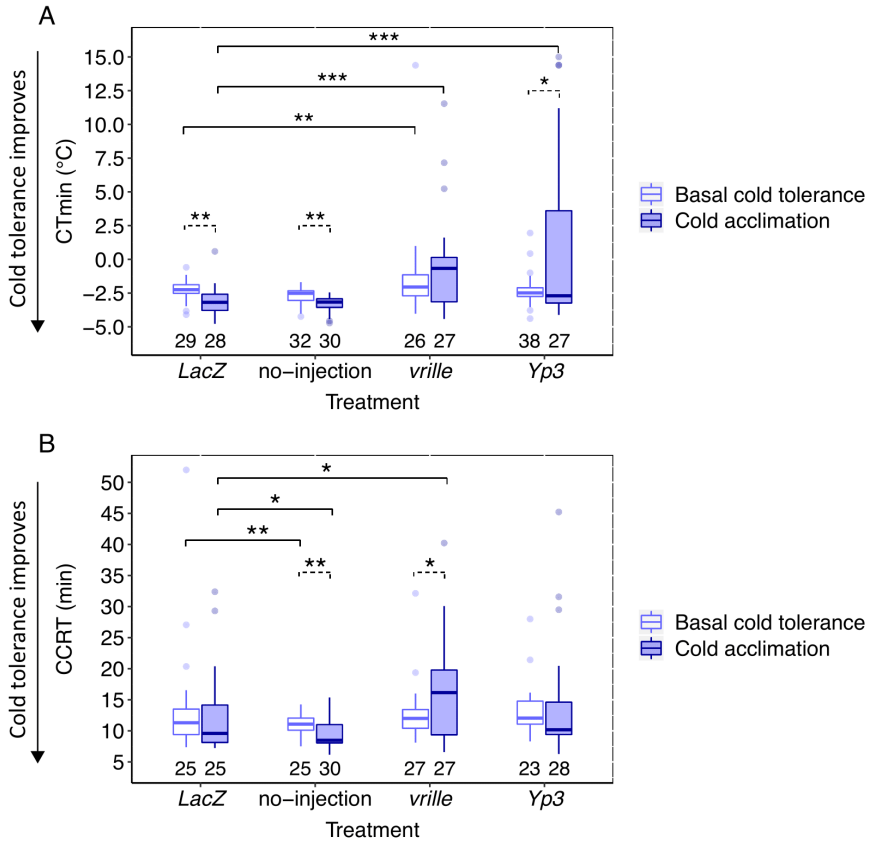

Figure 7. Female cold tolerance before (basal cold tolerance) and after cold acclimation in *LacZ* and no-injection control groups, and in RNAi treated *vrille* or *Yp3* groups measured with (A) CT_{min} and (B) CCRT tests. Dashed lines indicate significant differences between females' basal and acclimation 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*

488 *protein 3* (*Yp3*) enabled us to investigate the role of these genes on fly cold tolerance and cold
489 acclimation ability and get insight on the important molecular mechanisms underlying these
490 traits.

491 4.1 | Latitudinal variation in fly cold tolerance and its interaction with 492 climatic variables and body colour

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

509 Latitudinal variation in the basal cold tolerance (as measured with CCRT) of *D. montana*
510 females showed no association with local climatic conditions, which emphasizes its

511 dependency on photoperiodic and temperature conditions varying according to the latitude in a
512 wider scale. This is interesting, as latitudinal variation in another photoperiodically regulated
513 trait in this species, the critical day length for reproductive diapause (CDL), has been found to
514 be affected also by the climatic conditions prevailing at different altitudes (Tyukmaeva et al.,
515 2020). In *D. flavomontana*, latitudinal variation in basal cold tolerance showed an association
516 with local climatic conditions and was, surprisingly, higher in the humid low-altitude western
517 coast populations than in the high-altitude Rocky Mountains populations. However, *D.*
518 *flavomontana* flies of the mountain populations could use at least three strategies to enhance
519 their cold tolerance. Firstly, females could enter reproductive diapause at an earlier time of year
520 than the females from the low-altitude populations from the same latitude, as Tyukmaeva et al.
521 (2020) have shown *D. montana* females to do. In *D. montana* diapause improves female cold
522 tolerance considerably (Kauranen et al., 2019; Vesala & Hoikkala, 2011). Secondly, the low
523 basal cold tolerance of the mountain flies could also be compensated by a higher cold
524 acclimation ability, as several studies have detected a linkage between low cold tolerance and
525 high cold acclimation ability (Gerken et al., 2015; Hoffmann et al., 2003; Isobe et al., 2013;
526 Noh et al., 2017; Nyamukondiwa et al., 2011; Stillman, 2003; Zuther et al., 2012). Finally, the
527 flies could avoid freezing injuries by migrating towards warmer habitats and thermally buffered
528 microclimates (Stevenson, 1985). This kind of behaviour has been detected in the Colorado
529 population of *D. flavomontana*, where the flies occupy lower mountain slopes during early
530 spring than in summer (Hoikkala, pers. com.).

531 Insects' dark body colour has been suggested to absorb solar radiation, heat up faster and
532 maintain the optimum body temperature better than a light body colour (Clusella-Trullas et al.,
533 2007; Rajpurohit et al., 2008). For example in *Drosophila americana,* darker body colour of
534 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_{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

558 biological processes associated with cold acclimation of *D. montana* flies to cover largely the
559 same classes of genes as in above-mentioned studies, excluding stress response. Furthermore,
560 Parker et al. (2018) found evidence for selection for higher cold tolerance acting on neuronal,
561 membrane-transport and ion-transport related genes in genome-wide comparisons between *D.*
562 *montana* and several less cold-tolerant *Drosophila* species, as well as between *D. montana*
563 populations. These two studies brought up several candidate genes for cold tolerance and cold
564 acclimation in *D. montana*. Vigoder et al., (2016) used RNAi to silence one of these candidate
565 genes, *myo-inositol-1-phosphate synthase* (*Inos*), coding the major metabolite of overwintering
566 *D. montana* flies, *myo*-inositol (Vesala, Salminen, Kostál, Zahradníĉková, & Hoikkala, 2012),
567 and found this treatment to increase *D. montana* flies' mortality during cold exposure (5 ˚C)
568 but not to affect CCRT. We chose for RNAi studies two of the candidate genes, *vrille* and *Yp3*,
569 representing the circadian clock system and functioning in the insulin signalling pathway,
570 respectively.

571 *Drosophila* flies can monitor light and temperature changes e.g. through their thermosensory
572 organs located in aristae and legs, and to entrain behavioural and physiological changes to fit
573 with environmental conditions, with the aid of the circadian clock system (Sehadova et al.,
574 2009; Yadlapalli et al., 2018). For example in *Gryllus pennsylvanicus* crickets cold acclimation
575 increases the expression levels of two core circadian clock genes, *period* and *clock*, and
576 decreases that of *timeless* (Des Marteaux et al., 2017), while in *D. montana* and *D. virilis* it
577 increases the expression of *vrille* and *period* (Parker et al., 2015; Vesala, Salminen, Laiho, et
578 al., 2012). Blau & Young (1999) have shown that suppressing *vrille* expression decreases the
579 expression levels of *period* and *timeless*, even though a simulation performed by Smolen,
580 Hardin, Lo, Baxter, & Byrne (2004) suggests it to lead to a higher expression of *clock* and
581 *period*. Our study gives support to the finding of Blau & Young (1999), as silencing *vrille*

582 expression with RNAi decreased the expression of *period*, but not that of *clock*. Moreover,
583 *vrille*-injected acclimated and non-acclimated females had significantly decreased cold
584 tolerance in CT_{min} tests, and the same was true for *vrille*-injected acclimated females in CCRT
585 tests. Thus, we propose that circadian clock system is essential both in maintaining insect basal
586 cold tolerance and in increasing it during short cold acclimation.

587 Neuroendocrine signalling coordinates several processes involved in metabolism, development,
588 reproduction and cold acclimation (Des Marteaux et al., 2017; Hahn & Denlinger, 2011;
589 MacMillan et al., 2016; Torson, Yocum, Rinehart, Kemp, & Bowsher, 2015), and the insulin-
590 signalling pathway, including *Yolk protein* genes, play an important part in this system (Flatt et
591 al., 2005; Gonçalves Santos, Humann, & Hartfelder, 2019). Expression of *Yolk protein* genes
592 has been found to decrease during cold acclimation e.g. in *D. melanogaster* (Colinet,
593 Overgaard, Com, & Sørensen, 2013) and *D. suzukii* (Enriquez & Colinet, 2019). However, the
594 situation is quite opposite in *D. montana*, where these genes are upregulated during cold
595 acclimation (Parker et al., in preparation) and where the silencing of *Yp3* gene with RNAi was
596 found to decrease cold acclimation ability in CT_{min} test in the present study. *Yolk protein* genes
597 and other possible genes involved in vitellogenesis (often called *vitellogenins*) seem to affect a
598 wide range of traits involving insect development, immune function, endocytosis and stress
599 resistance through their regulatory and signalling functions in the insulin-signalling pathway
600 (Flatt et al., 2005; Schwerin et al., 2009; Tufail & Takeda, 2008). Moreover, e.g. in *Apis*
601 *mellifera* honey bees these genes have been shown to function as antifreeze proteins (Qin et al.,
602 2019) and in social hierarchies (Guidugli et al., 2005). However, comparisons of the function
603 of vitellogenins between species and species groups are difficult to be made at gene level,
604 because of the genes' different evolutionary histories and high sequence divergences (Chen,
605 Sappington, & Raikhel, 1997; Kaulenas, 2012).

606 ## 5 | Conclusions

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

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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.