Multiple paths to cold tolerance: the role of environmental cues, morphological traits and the circadian clock gene *vrille*

Running title: Multiple paths to cold tolerance

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Abstract

Background

Tracing the association between insect cold tolerance and latitudinally and locally varying environmental conditions, as well as key morphological traits and molecular mechanisms, is essential for understanding the processes involved in adaptation. We explored these issues in two closely-related species, *Drosophila montana* and *Drosophila flavomontana*, originating from diverse climatic locations across several latitudes on the coastal and mountainous regions of North America. We also investigated the association between sequence variation in one of the key circadian clock genes, *vrille*, and cold tolerance in both species. Finally, we studied the impact of *vrille* on fly cold tolerance and cold acclimation ability by silencing it with RNA interference in *D. montana*.

Results

We performed a principal component analysis (PCA) on variables representing bioclimatic conditions on the study sites and used latitude as a proxy of photoperiod. PC1 separated the mountainous continental sites from the coastal ones based on temperature variability and precipitation, while PC2 arranged the sites based on summer and annual mean temperatures. Cold tolerance tests showed *D. montana* to be more cold-tolerant than *D. flavomontana* and chill coma resistance (CT_{min}) of this species showed an association with PC2. Chill coma recovery time (CCRT) of both species improved towards northern latitudes, and in *D. flavomontana* this trait was also associated with PC1. *D. flavomontana* flies were darkest in the coast and in the northern mountainous populations, but coloration showed no linkage with cold tolerance. Body size decreased towards cold environments in both species, but only within *D. montana* populations largest flies showed fastest recovery from cold. Finally, both the sequence

analysis and RNAi study on *vrille* suggested this gene to play an essential role in *D. montana* cold resistance and acclimation, but not in recovery time.

Conclusions

Our study demonstrates the complexity of insect cold tolerance and emphasizes the need to trace its association with multiple environmental variables and morphological traits to identify potential agents of natural selection. It also shows that a circadian clock gene *vrille* is essential both for short- and long-term cold acclimation, potentially elucidating the connection between circadian clock system and cold tolerance.

Keywords: CT_{min}, CCRT, body colour, body weight, latitude, bioclimatic variables, RNA interference (RNAi), *Drosophila montana*, *Drosophila flavomontana*

Background

Species geographical distribution is largely defined by their ability to tolerate stressful conditions and to respond to daily and seasonal temperature changes (e.g. [1–6]). Accordingly, ectothermic species, especially the ones living at high latitudes, may use a variety of physiological and behavioural strategies to increase their cold tolerance [7, 8]. To understand species' cold adaptation in these regions, it is essential to identify latitudinal and bioclimatic selection pressures driving these adaptations [2, 9–11] and to trace associations between cold tolerance and adaptationally important morphological traits. Also, it is also important to discover molecular mechanisms underlying cold tolerance, but a functional link between candidate genes and cold tolerance has relatively rarely been established (but see e.g. [12, 13]).

Insect cold tolerance is often measured with two ecologically relevant methods. Insects' chill coma resistance can be assessed by measuring their critical thermal minimum (or chill coma temperature, CT_{min}), where they lose neuromuscular coordination and fall into chill coma during a gradual decrease in temperature [14]. Chill coma recovery time (CCRT), on the other hand, is based on time required for an individual to recover from the coma after removal of the chill coma inducing temperature [15, 16]. Even though the ability to resist chill coma and recover from it are thought to be somewhat of a continuum, they are affected by different physiological and molecular mechanisms (e.g. [17, 18]). The onset of chill coma is associated with depolarisation of muscle resting membrane potential due to low temperature (e.g. [19, 20]), while the recovery process involves energetically costly restoration of ion gradients and upregulation of genes involved in repairing cold injuries [21–25]. Both the high resistance to chill coma and the fast recovery from it have several implications on insect fitness in cold environments [15, 18, 26]. For example, fast recovery from chill coma after winter, night or otherwise sudden temperature drops ensures finding high-quality mates on feeding and

breeding sites, as well as fast escape from predators. High chill coma resistance covers largely the same advantages, but it may also save insects from falling into energetically costly chill coma. Thus, these traits are likely to be under strong selection.

Latitudinal clines in cold tolerance traits, detected in a variety of species, including *Drosophila* flies [16, 27, 28], *Myrmica* ants [29], *Porcellio laevis* woodlouse [30], highlight adaptive genetic variation in these traits. However, it is challenging to distinguish whether such variation has evolved in response to changes in photoperiod (day length), temperature or their combination [7]. One possible approach is to use GIS (geographic information system) -based environmental data to trace correlations between evolutionary changes in the studied traits and the environments the populations or species experience [31]. Studies on clinal variation in insect cold tolerance can also be complicated by the fact that many species spend the coldest time of the year in reproductive diapause, which induces various kinds of changes in their physiology, metabolism and behaviour in addition to increasing their cold tolerance [32]. Thus, it is important to investigate genetic variation in cold tolerance in temperature and light conditions, where insects' reproductive status is controlled.

In addition to cold tolerance, morphological traits, like body colour (the degree of melanism) and body size, often show latitudinal variation. Thus, to understand insect adaptation to cold environments, it would be important to investigate cold tolerance traits together with other adaptationally important traits and consider correlations between them. In insects, an increase in melanism towards higher latitudes has been detected both between and within species [33–35]. According to thermal melanin hypothesis, this can be explained by an increased ability of dark individuals to absorb solar radiation and warm up fast in cold environments with low solar radiation (reviewed in [36]). However, body colour may also be affected by other selection pressures, including protection against UV-radiation [37, 38], desiccation [39, 40] and

111 pathogens [41]. Body size is one of the most important quantitative traits associated with insect
112 metabolism, fecundity, mating success and stress tolerance, and thus it is likely under several
113 selection pressures in nature (e.g. [42, 43]). Body size of ectothermic species has been either
114 shown to increase (Bergmann's rule) or decrease (converse Bergmann's rule or U-shaped cline)
115 towards higher latitudes and cooler climates (e.g. [42, 44, 45]). Converse Bergmann's rule
116 applies especially for insects with a long generation time, for which the short growing season
117 on high latitudes limits the time available for development, growth and foraging [45–47].
118 Overall, parallel clines in cold tolerance and morphological traits give only indirect evidence
119 on the functional linkages between these traits. One way to obtain more direct evidence on their
120 association would be to measure cold tolerance and morphological traits for the same
121 individuals. For example, it has been shown that melanistic wood tiger moths (*Parasemia*
122 *plantaginis*) warm up more quickly than the less melanistic ones [48] and *Drosophila montana*
123 males' overwinter survival increases along with an increase in body size in nature [49].

124 At high latitudes with clear seasonal and diurnal temperature variation, insects can enhance
125 their cold tolerance through long- and short-term cold acclimation [50, 51]. In nature, insects
126 can anticipate the forthcoming cold season from a decreasing temperature and/or day length
127 and adapt to these changes through a gradual increase in cold tolerance [52, 53]. Insects can
128 also be experimentally cold-acclimated by maintaining them in relatively low temperature or
129 short day conditions for a few days to weeks prior to cold shock [54, 55]. Short-term cold
130 acclimation of minutes to hours (rapid cold hardening, RCH) has been suggested to share
131 mechanisms with longer-term acclimation [56] and allow organisms to cope with sudden cold
132 snaps and to optimize their performance during diurnal cooling cycles [24, 53]. Here the
133 circadian clock system, which monitors daily and seasonal light and temperature cycles and
134 entrains behavioural and physiological rhythms to match with them [57], could play a vital role.

135 In the central circadian clock, described most thoroughly in *D. melanogaster*, daily rhythms are
136 driven via one or several transcriptional feedback loops involving changes in the expression of
137 core circadian clock genes (reviewed in [58–60]). Intriguingly, in *Drosophila montana* flies
138 cold tolerance traits (CT_{min} and CCRT) are under photoperiodic regulation [55, 61], and hence
139 could be associated with circadian clock system. Also, the expression levels of circadian clock
140 genes have been found to change during long-term cold acclimation e.g. in several *Drosophila*
141 species [62–65] and *Gryllus pennsylvanicus* cricket [66]. In plants, the linkage between
142 circadian clock and cold acclimation has been established [67, 68], but in insects direct evidence
143 on this link and its extent is still missing.

144 Northern *Drosophila virilis* group species possess a very high cold tolerance compared to most
145 other species of the genus [1, 2]. Our study species, *D. montana* and *D. flavomontana*, belong
146 to this group, and they live in diverse climatic conditions in the low-altitude western coast and
147 in the high-altitude Rocky Mountains of North America across several latitudes (Fig. 1). *D.*
148 *montana* lives generally at higher latitudes and altitudes than *D. flavomontana*, but in some sites
149 the species occur sympatrically [69–71]. Moreover, the body colour of *D. montana* is almost
150 black, while that of *D. flavomontana* varies from light to dark brown [69]. These features make
151 *D. montana* and *D. flavomontana* an ideal species pair to investigate genetic adaptation to cold
152 environments, complementing latitudinal studies performed on less cold-tolerant southern
153 species. They also enable us to study connections between fly cold tolerance and morphological
154 traits and to trace selection pressures underlying cold adaptation. Another interesting point is
155 that the circadian clock system of *D. virilis* group species differs from that of *D. melanogaster*,
156 and shows features that have helped them to adapt to high latitudes [72–76]. Here *vrille*, which
157 is one of the core genes in the central circadian clock system and a key regulator of circadian

158 behavioural rhythms in *D. melanogaster* [58–60], is of special interest, as it has been found to
159 be highly upregulated during cold acclimation in *D. montana* [64, 65, 77].

160 In this study, we addressed two main objectives. 1) We traced genetic variation in cold tolerance
161 traits (CT_{min} and CCRT) across distribution ranges of *D. montana* and *D. flavomontana*, and
162 identified environmental selection pressures and morphological (body colour and size)
163 correlations driving these adaptations. 2) We compared *vrille* sequence variation to cold
164 tolerance traits among clinal populations of *D. montana* and *D. flavomontana,* and tested the
165 role of this gene in cold tolerance and cold acclimation ability in *D. montana* using RNA
166 interference (RNAi). In the first part of the study, we quantified environmental variation across
167 fly collecting sites by performing a principal component analysis (PCA) on several bioclimatic
168 variables, and investigated whether latitude, as a proxy of photoperiod, and latitudinally or
169 locally varying climatic conditions have shaped fly cold tolerance (CT_{min} and CCRT). Here we
170 predicted that genetic variation in cold tolerance traits shows association with latitudinally
171 varying photoperiods, which serves as the most reliable cue for seasonal temperature changes
172 at given localities [7]. We also quantified genetic variation in fly body colour and body size
173 across species' distribution range, identified the likely selection pressures affecting these traits
174 and measured direct correlations between the morphological and cold tolerance traits from the
175 same individuals. Body colour could be associated with cold adaptation if it shows latitudinal
176 cline, becoming darker towards northern latitudes, and if it correlates directly with cold
177 tolerance measures (thermal melanin hypothesis, [36]). If large body size and better cold
178 tolerance are associated with each other (e.g [49]), the size could be expected to increase
179 towards cold environments (Bergmann's rule, [47, 78]) and the traits should be directly
180 correlated. We studied these adaptations in non-diapausing individuals in a common
181 environment to eliminate plastic responses. In the second part of the study, we evaluated the

adaptive role of *vrille* in fly cold tolerance by comparing its nucleotide and amino acid variation with variation in two cold tolerance traits (CT_{min} and CCRT) among *D. montana* and *D. flavomontana* cline populations. We also investigated the role of this gene in regulating females' cold tolerance traits and cold acclimation ability by silencing it with RNAi in *D. montana*. We expected *vrille* to be involved at least in females' ability to become cold-acclimated, as it shows expression changes during cold acclimation in *D. montana* [64, 77].

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

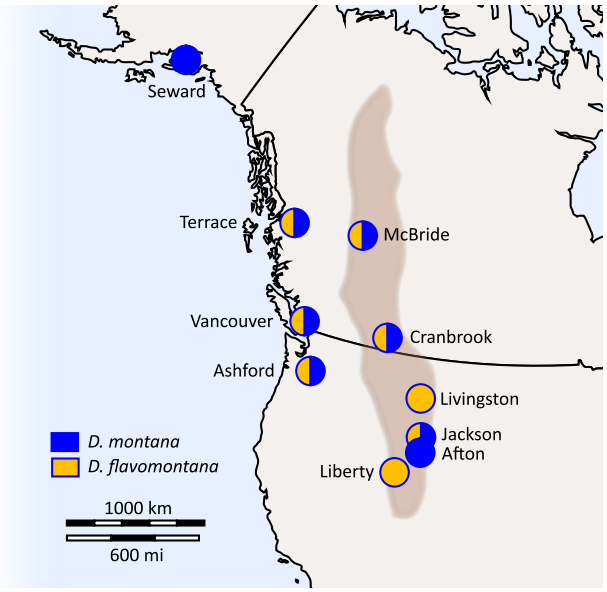


Figure 1. Fly collection sites. Table shows fly collecting sites and years, and the coordinates for each site. Map contains information on whether we have samples from one or both species in each site on the western coast and in the Rocky Mountains (brown area on the map) in North America (detailed information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

Results

Variation in the climatic conditions at fly collecting sites

We investigated macroclimatic variability among the sites, where *D. montana* and *D. flavomontana* strains originated from, by performing a principal component analysis (PCA) on 19 bioclimatic variables, growing season length (days) and altitude (Table S2, S3). PCA revealed four principal components (PCs) with eigenvalues > 1 (Table S4). The first two PCs explained more than 83% of the total variation (Fig. 2; Table S4) and were included in further analyses.

PC1 clearly separated the Rocky Mountains sites from the ones on the western coast. Variables with the highest contribution on this separation included altitude and the ones describing daily and seasonal temperature variation (bio2, bio4, bio7), the minimum temperature of the coldest month (bio6), and the mean temperature of the coldest quarter (bio11) and precipitation (Fig. 2; Table S5). Together they showed the high-altitude Rocky Mountains sites to have higher temperature variation and colder winters than the ones on the western coast sites. On the other hand, the western coast sites had higher precipitation throughout the year than the Rocky Mountains sites.

PC2 arranged the sites on the basis of the growing season length, annual mean temperature (bio1), the mean temperature of the warmest quarter (bio10), the maximum temperature of the warmest month (bio5) and isothermality (bio3, i.e. how large day-to-night temperatures oscillate relative to the summer-to-winter oscillations; Fig. 2; Table S5). They showed that while some fly collecting sites differ in latitude (photoperiod), they resemble each other in

growing season length and summer temperatures due to their variability in altitude and closeness to sea.

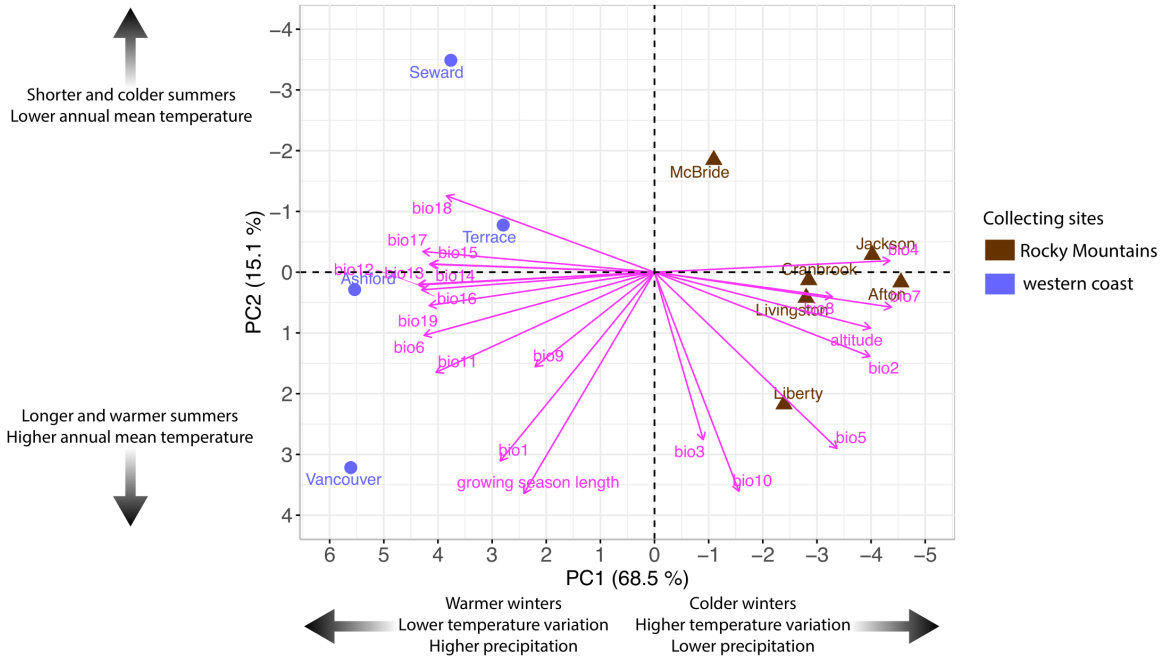


Figure 2. Climatic conditions in the fly collecting sites. Principal component analysis (PCA) was performed on 21 variables describing environmental conditions in fly collecting sites (Table S2, S3). Big black arrows show the change in given conditions.

The effects of latitude, climatic conditions and morphological traits on fly cold tolerance

Studying the effects of photoperiod, temperature-related factors (PC1, PC2), body colour and size (measured as weight) on fly cold tolerance enabled us to identify selection pressures affecting this trait. In these experiments, we used three isofemale strains for majority of the populations, but for four populations we succeeded to establish only one or two strains due to the rarity of the species at the collection sites (Table S1). All traits were measured for non-

233 diapausing *D. flavomontana* and *D. montana* females, reared in constant light at 19 ˚C, to
234 minimize plastic responses. Contrary to other traits, weight was analysed only for the flies used
235 in CCRT tests (see Methods). The simplest model, which enabled us to distinguish between
236 latitudinally varying photoperiods and temperatures, included latitude and PC2 as explanatory
237 factors (see Fig. 2; Table S6). The more complicated models included macroclimatic conditions
238 varying between the western coast and the Rocky Mountains (PC1), different interaction terms
239 and fly body colour and size (Table S6).
240
241 The best-fit models explaining the cold tolerance (CT_{min} = chill coma temperature, CCRT =
242 chill coma recovery time), body colour and body size of *D. flavomontana* and *D. montana*
243 females are presented in Fig. 3A-D (model comparisons are given in Table S6). The models
244 show that the selection pressures driving the evolution of these traits vary between the species.
245 Most of the pairwise correlations between fly cold tolerance traits, colour and weight were non-
246 significant, and were not included in the best-fit models; Fig. 3, 4; Table S6).
247
248 CT_{min} of *D. flavomontana* showed only low variation and was not significantly explained by
249 any of the variables (Fig. 3A; Table S7). However, in *D. montana* this trait showed significant
250 association with PC2 (Fig. 3A; Table S7), which suggests that the chill coma resistance of *D.*
251 *montana* flies is highest in northern populations with a short growing season and cold summer
252 and low annual mean temperatures (Fig. 2). CCRT tests showed *D. flavomontana* flies' cold
253 tolerance to be significantly associated with latitude and to improve towards North (Fig. 2, 3B;
254 Table S7). Moreover, this trait was affected by PC1, especially on latitudes around 50-55 ˚N,
255 suggesting that fly cold tolerance is higher in the humid, low-altitude western coast populations
256 than in the high-altitude Rocky Mountains populations with colder temperatures and higher

temperature variation (Fig. 2, 3B; Table S7). CCRT of *D. montana* was significantly associated with latitude, improving towards North (Fig. 3B; Table S7). Moreover, in this species large flies recovered faster from chill coma than the small ones (Fig. 3B, 4G; Table S7).

D. flavomontana flies' body colour was significantly affected by latitude, PC1 and an interaction between PC2 and PC1 (Fig. 3C; Table S7). In the Rocky Mountains, the flies became darker (their colour intensity decreased) towards North, while in the western coast populations flies were equally dark and darker than the ones from the Rocky Mountains populations on similar latitudes. *D. montana* body colour showed only minor population-level variation and no significant association with latitude, PC2 or PC1. The body size of *D. flavomontana* was significantly associated with PC1 and an interaction between PC1 and PC2 (Fig. 3D; Table S7), increasing towards warmer winters and summers and longer growing season. The body size of *D. montana* was significantly associated with PC2, being highest in sites with high summer temperatures and a long growing season (Fig. 3D; Table S7).

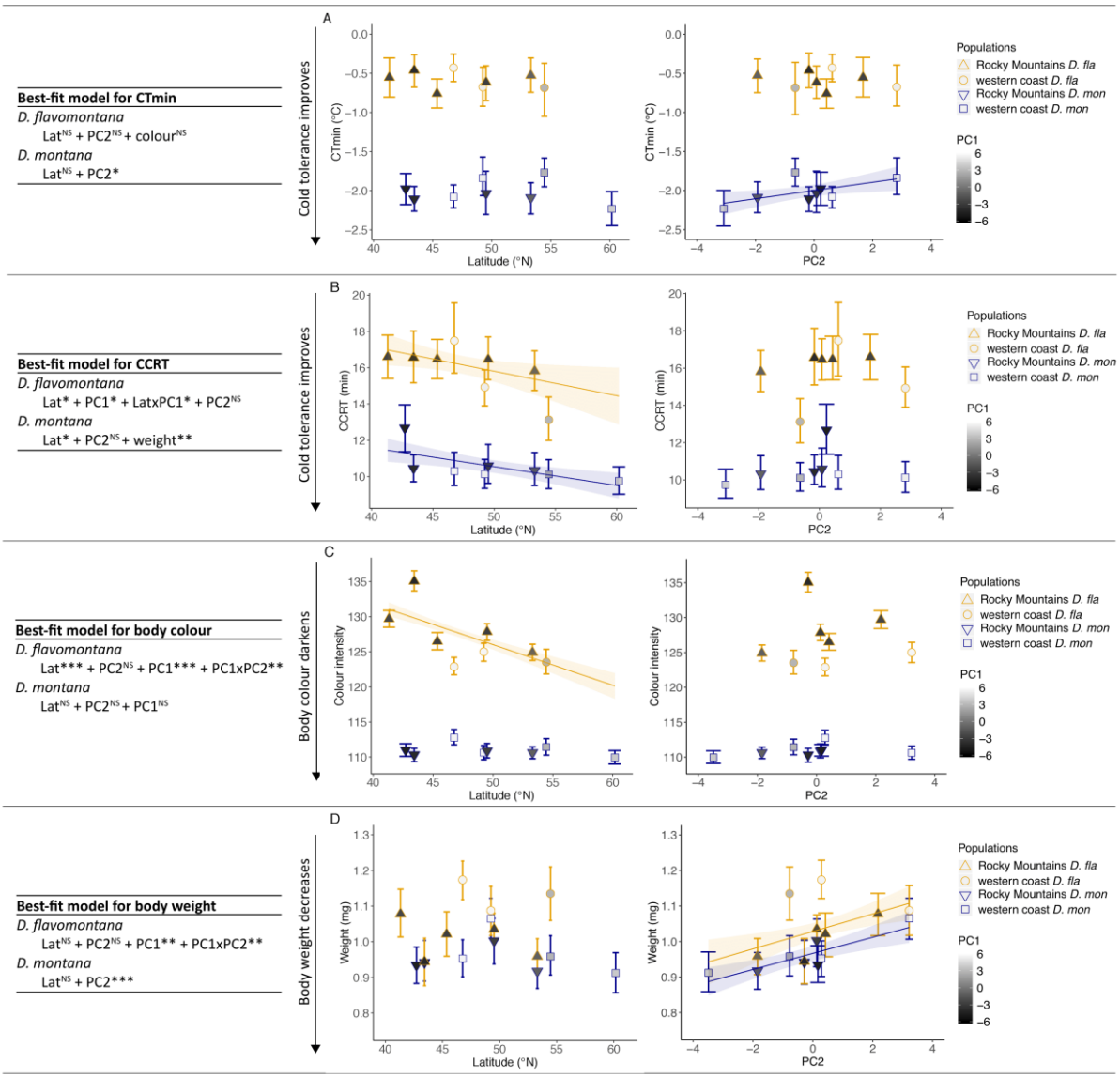
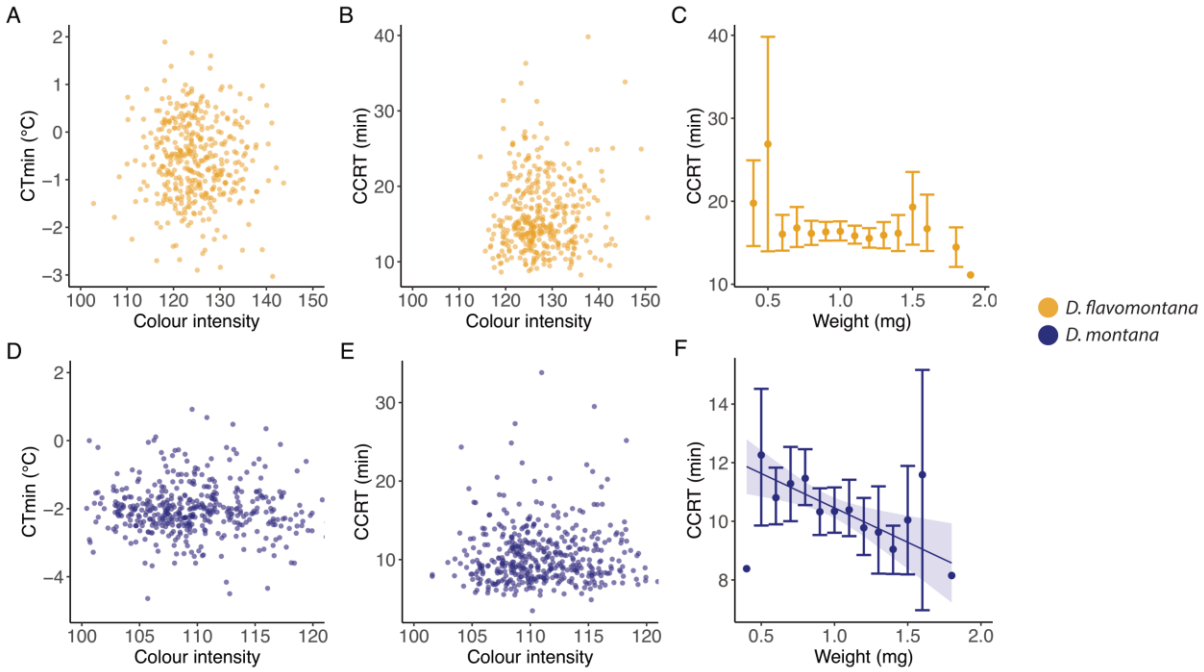


Figure 3. Cold tolerance, morphological traits (body colour and body size) and environmental conditions. The best-fit models are presented in tables. Relationship between latitude (as a proxy of photoperiod) and PC2 (as a proxy of latitudinally varying temperature) and (A) chill coma temperature (CT_{min}), (B) chill coma recovery time (CCRT), (C) fly body colour (measured as colour intensity) and (D) fly body size (measured as weight) in *D. flavomontana* (*D. fla*) and *D. montana* (*D. mon*) populations. The effects of local climatic conditions (PC1; see Fig. 2) on the western coast and in the Rocky Mountains are illustrated in grey scale (lighter colours represent the western coast populations and darker ones the Rocky Mountains populations). Error bars represent bootstrapped 95% confidence intervals (Mean ± CI). Significant regression lines for latitude or PC2 with standard errors are shown.

281 Significance levels were obtained from GLMMs or LMM: ^{NS} non-significant, * P < 0.05, ** P < 0.01
282 and *** P < 0.001.



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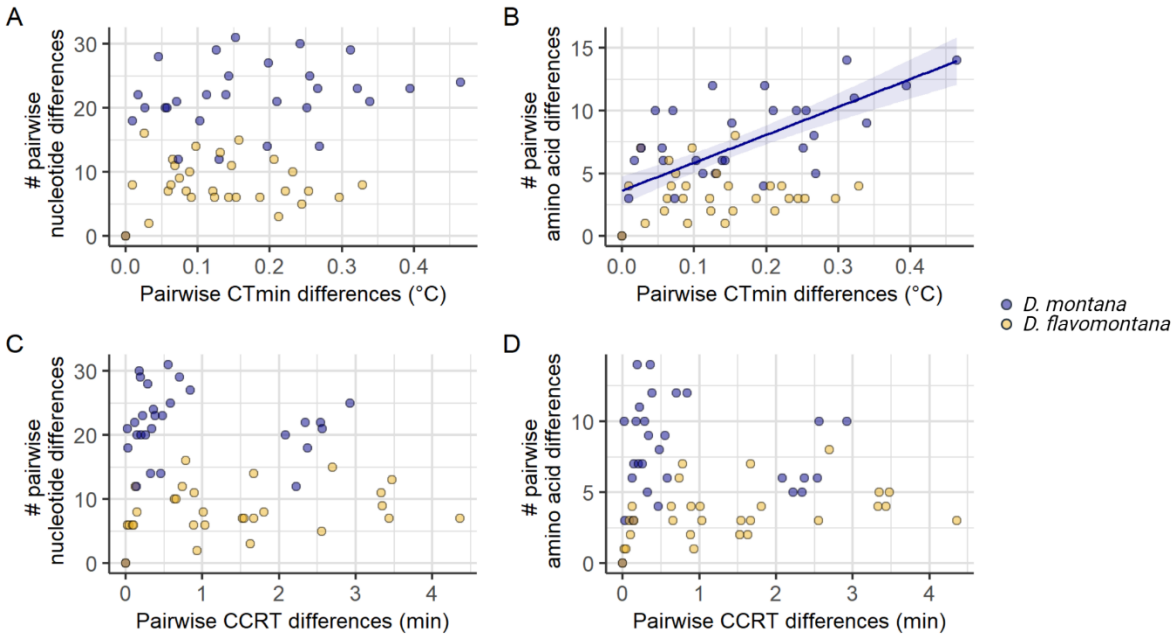
284 Figure 4. Correlations between fly cold tolerance and morphological traits (body colour or body size).
285 Correlation between (A) *D. flavomontana* CT_{min} and body colour, (B) *D. flavomontana* CCRT and body
286 colour, (C) *D. flavomontana* CCRT and body size (measured as weight), (D) *D. montana* CT_{min} and
287 body colour, (E) *D. montana* CCRT and body colour, (F) *D. montana* CCRT and body size (measured
288 as weight). Error bars represent bootstrapped 95% confidence intervals. Significant regression lines are
289 shown with standard errors (best-fit models are presented in Fig. 3 and Table S6).

290 **Association between the nucleotide and amino acid variation in *vrille* and variation in fly**
291 **cold tolerance traits among clinal *D. montana* and *D. flavomontana* populations**

292 To elucidate the adaptive potential of *vrille* in cold tolerance among *D. montana* and *D.*
293 *flavomontana* populations, we traced the association between pairwise differences in *vrille*
294 sequence variation (the number of nucleotide and amino acid differences) and differences in fly
295 cold tolerance traits among the studied populations. The number of amino acid differences and

296 the differences in mean CT_{min} in *D. montana* populations were significantly correlated (Mantel
297 test: r=0.60, P=0.007; Fig. 5B; Table S8), i.e. the greater the amino acid differences were
298 between *D. montana* populations, the greater were the differences in their chill coma resistance.
299 Other measured correlations were non-significant (Fig. 5; Table S8).



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301 Figure 5. The association between *vrille* sequence variation and cold tolerance among *D. montana* and
302 *D. flavomontana* populations. (A) Pairwise mean CT_{min} differences and the number of pairwise
303 nucleotide differences among *D. montana* and *D. flavomontana* populations. (B) Pairwise mean CT_{min}
304 differences and the number of pairwise amino acid differences among *D. montana* and *D. flavomontana*
305 populations. (C) Pairwise mean CCRT differences and the number of pairwise nucleotide differences
306 among *D. montana* and *D. flavomontana* populations. (D) Pairwise mean CCRT differences and the
307 number of pairwise amino acid differences among *D. montana* and *D. flavomontana* populations.

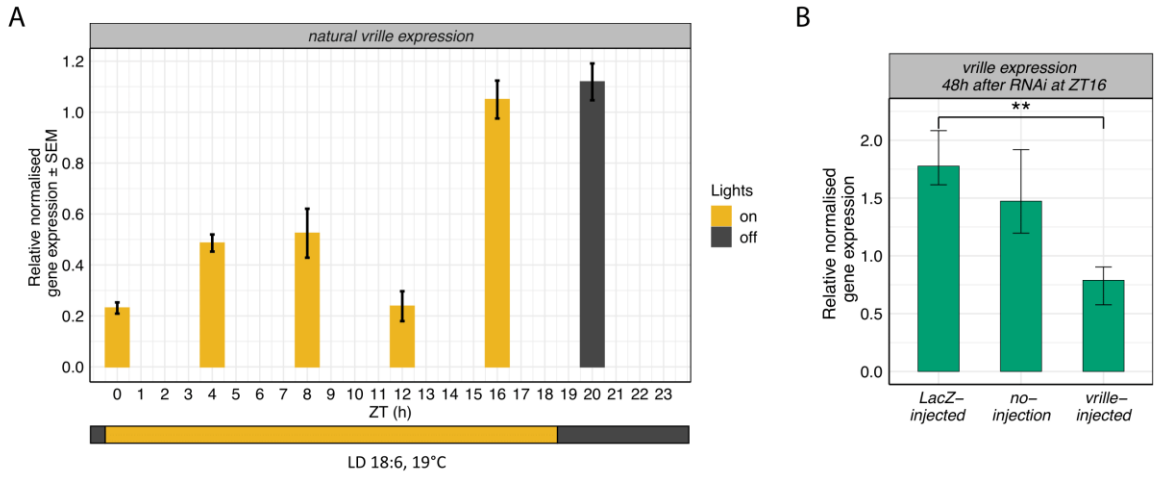
308

309 **Daily rhythm of *vrille* and the expression levels of *vrille* after RNAi in *D. montana***

310 Since the expression levels of circadian clock genes are known to fluctuate during the day, we
311 first investigated daily rhythm in *vrille* expression in LD (light:dark cycle) 18:6, where the

312 females of the study population of *D. montana* (Seward, Alaska, USA) can be expected to be
313 non-diapausing (and which was also verified, see methods). Our results showed that *vrille* has
314 a clear daily rhythm and that its highest expression corresponds to ZT16 and ZT20 (ZT =
315 Zeitgeber Time which refers to the number of hours after the lights are switched on; see Fig.
316 6A). From these two time points we chose to perform RNAi-injections at ZT16, when the lights
317 were still on.

318 We next compared *vrille* expression levels in *LacZ*-injected control females to those of *vrille*-
319 injected and no-injection females 12, 24 and 48 hours after the injections (Fig. S1, Fig. 6B).
320 This enabled us to measure the effects of *vrille*-RNAi on the expression level of this gene
321 controlling possible effects of immune responses and physical damage. Differences between
322 *LacZ*- and *vrille*- injected females were most pronounced 48h after the RNAi-injections (Fig.
323 6B; Fig. S1), where *vrille*-injected females had approximately 56% lower *vrille* expression
324 compared to *LacZ* controls (Fig. 6B; Table S9). Accordingly, all RNAi experiments were
325 performed 48h after the injections at ZT16 in LD 18:6.



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327 Figure 6. *vrille* expression during the day and after RNA interference (RNAi). (A) Relative normalised
328 expression of naturally cycling *vrille* at six time points, starting at ZT0 (Zeitgeber Time = sampling time
329 every 4 hours over a 24 hour period), in LD 18:6 and 19 ˚C. Yellow represents the time of the day when

330 the lights were on and dark grey when the lights were off. (B) Relative normalised expression levels of
331 *vrille* in *LacZ*-injected and no-injection control females, and in females injected with dsRNA targeting
332 on *vrille* 48h after the injections at ZT16. Error bars represent bootstrapped 95% confidence intervals.
333 Significance levels were obtained from linear model and only significant observations are shown: * P
334 < 0.05, ** P < 0.01 and *** P < 0.001.

335 **The effects of *vrille*-RNAi on *D. montana* females' cold tolerance and cold acclimation**
336 **ability**

337 The effects of *vrille*-RNAi on female cold tolerance and cold acclimation ability were studied
338 by quantifying these traits in *vrille*-injected, *LacZ*-injected and no-injection females.
339 Comparisons between *LacZ*- and *vrille*-injected females enabled us to determine whether a
340 expression of *vrille* increases *D. montana* females' ability to resist chill coma (CT_{min}), to
341 recover from it (CCRT) and/or to achieve higher cold-tolerance after cold acclimation.
342 Comparisons between *LacZ*-injected and no-injection females, on the other hand, revealed
343 possible immune responses to dsRNA and/or physical damage caused by the injection itself.
344 All cold tolerance tests were started 48h after the injections at ZT16, when the effects of *vrille*-
345 RNAi were at a highest level (Fig. 6, Fig. S1). Cold tolerance tests (CT_{min} and CCRT) were
346 conducted the same way as for the females of cline populations, except that now the females
347 were maintained in LD 18:6 instead of LL throughout the experiments (also during the
348 acclimation treatment). Moreover, to measure females' cold acclimation ability, half of the
349 females (cold acclimation group) were maintained in 6 ˚C and the other half (non-acclimation
350 group) in 19 ˚C for 5 days prior to cold tolerance tests. RNAi-injections were performed two
351 days (48 h) before finishing acclimation treatment and performing the tests.

352 We first investigated whether cold acclimation in 6 ˚C (cold-acclimated females) had improved
353 female cold tolerance compared to the flies kept in 19 ˚C (non-acclimated females) within the

354 three experimental groups (*LacZ*-injected females, no-injection females and *vrille*-injected
355 females). In CT_{min} tests, cold acclimation improved the chill coma resistance in *LacZ*-injected
356 and no-injection females by decreasing their CT_{min} on average by 0.7 ˚C and 0.6 ˚C,
357 respectively, while acclimation had no significant effect on the chill coma resistance of *vrille*-
358 injected females (Fig. 7A; Table S10). In CCRT tests, cold-acclimated no-injection females
359 recovered from chill coma on average 1.5 minutes faster than the non-acclimated ones (Fig. 7B;
360 Table S10), i.e. their ability to recover from coma was faster, as could be expected. However,
361 cold acclimation had no significant effect on the recovery time of *LacZ*-injected females, which
362 suggests that either immune responses or physical damage had overridden the positive effects
363 of the acclimation (Fig. 7B; Table S10). Finally, CCRT tests for *vrille*-injected females showed
364 that cold acclimation had slowed down their recovery time by ~3.5 minutes instead of fastening
365 it (Fig. 7B; Table S10). Such a significant effect in females' ability to recover from chill coma
366 cannot be explained solely by immune responses or physical damage, which means that
367 expression of *vrille* is essential for females' cold acclimation ability.

368 Next, we compared CT_{min} and CCRT of *LacZ*-injected females to those of the no-injection and
369 *vrille*-injected females separately among the non-acclimated and cold-acclimated females. In
370 CT_{min} tests, chill coma resistance did not differ significantly between *LacZ*-injected and no-
371 injection females in either acclimation groups (Fig. 7A; Table S11). On the other hand, *vrille*-
372 injected females entered chill coma in 1 ˚C (non-acclimated females) and 2.5 ˚C (cold-
373 acclimated females) higher temperature than *LacZ*-injected females of the same groups (Fig.
374 7A; Table S11). These results show that low expression levels of *vrille* significantly decreased
375 females' ability to resist low temperatures. In CCRT tests, both the non-acclimated and cold-
376 acclimated *LacZ*-injected females recovered from chill coma ~3 minutes more slowly than the
377 respective no-injection females (Fig. 7B; Table S11), which suggests that immune and/or

injection effects might have played a role in chill coma recovery in both groups. The recovery time of non-acclimated *vrille*-injected females was on the same level as that of the *LacZ*-injected females (Fig. 7B; Table S11). However, cold-acclimated *vrille*-injected females recovered from chill coma ~3.5 minutes more slowly than *LacZ*-injected females (Fig. 7B; Table S11), which again brings up the poor acclimation ability of *vrille*-injected females.

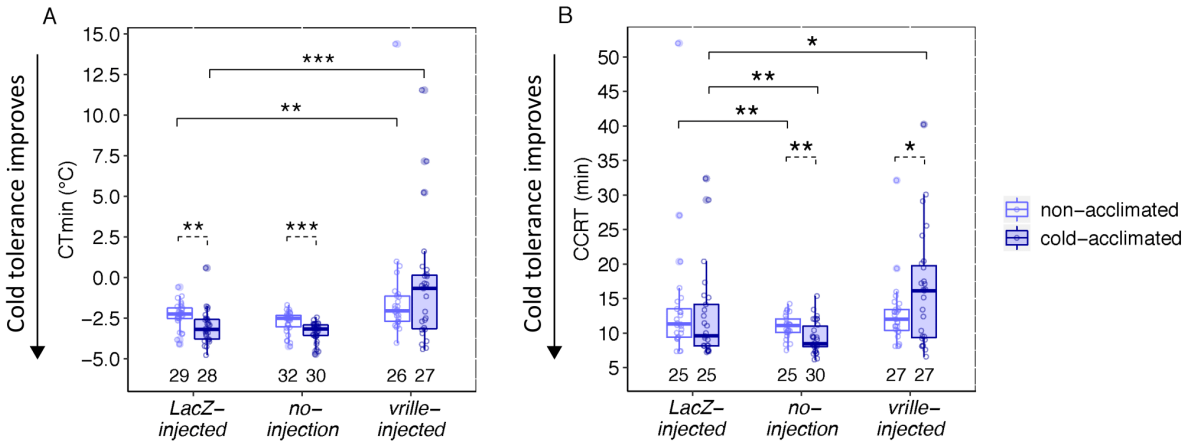

Figure 7. *D. montana* cold tolerance after RNA interference (RNAi). (A) Chill coma resistance (CT_{min}) and (B) chill coma recovery time (CCRT) for *LacZ*-RNAi, no-injection, and *vrille*-RNAi females that were kept at +19 ˚C for the whole time, or at first at +19 ˚C and the last 5 days at +6 ˚C (cold acclimation period). Dashed lines indicate significant effects of the acclimation in each treatment, and solid lines significant differences between the *LacZ* control and the other treatments in females that were or were not acclimated. Significance levels were obtained from GLMMs and only significant observations are shown: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Numbers below boxplots refer to sample sizes. Whiskers represent ±1.5×IQR.

Discussion

Numerous studies have found inter- and intraspecific latitudinal variation in insect cold tolerance (e.g. [1–4, 6, 16, 27–30]) and identified candidate genes for it (e.g. [62–64, 66]).

395 Spatially varying selection on insect fitness along latitudinal clines can be based on photoperiod
396 and/or climatic factors, and sometimes it is difficult to detect the actual selection pressures
397 driving the evolution of cold tolerance. To deepen our understanding on clinal adaptation, it is
398 important to gather several independent sources of evidence, including sibling species, multiple
399 populations or geographic regions and environmental correlations that account for population
400 structure [79], as well as to investigate associations between cold tolerance and morphological
401 traits (e.g. body colour and size) that often show parallel clines. Here, we explored factors
402 affecting cold tolerance of non-diapausing females of *D. montana* and *D. flavomontana*
403 originating from diverse climatic environments across different latitudes on the western coast
404 and the Rocky Mountains of North America. Moreover, tracing sequence variation in both
405 species and performing RNAi experiments in *D. montana* on one of the key regulators of the
406 circadian behavioural rhythms, *vrille*, enabled us to investigate the role of this gene on female
407 cold tolerance and cold acclimation ability.

408 **Effects of latitude and bioclimatic variables on fly cold tolerance vary even between**
409 **closely-related species**

410 The principal component analysis (PCA), which we performed on macroclimatic variables on
411 fly collection sites, showed PC1 to separate the low-altitude coastal sites from the high-altitude
412 mountainous ones based on differences in winter temperatures, temperature variability and
413 precipitation. PC2 further arranged the clinal populations according to their summer and annual
414 mean temperatures. Thus, in our dataset latitude represents photoperiodic differences (day
415 length) between the sites, while PC2 corresponds to latitudinally varying mean temperatures
416 and PC1 to macroclimatic variation between the coast and mountains.

417 Overall, genetic variation was lower in chill coma resistance (CT_{min}) than in chill coma recovery
418 time (CCRT), and CT_{min} showed significant variation only in *D. montana*. There are some

419 plausible explanations for this pattern. CT_{min} may not be as suitable as CCRT for studying
420 insects' inherent cold tolerance, as rapid cold hardening (RCH) during the gradual cooling
421 period in CT_{min} tests can change the composition of membrane phospholipids, improving the
422 chill coma resistance [19, 20, 80, 81]. A trade-off between inherent cold tolerance and RCH,
423 detected in several studies [26, 56, 82, 83], could lead to skewed or unapparent population-level
424 variation in CT_{min}. For example, sub-Antarctic *Ectemnorhinus* weevils and *Glossina pallidipes*
425 tsetse flies show low inherent genetic variation and greater plastic responses in CT_{min} between
426 populations [84, 85]. In contrast, CCRT has high heritability [27, 56, 86–88], and fast recovery
427 from chill coma may be under strong selection due to energetically costly repair of cold injury
428 and high fitness advantages involved in it (e.g. earlier recovery ensures high-quality territories,
429 mates, feeding and breeding sites and escape from predators) [15, 18, 21–25]. These
430 characteristics make CCRT a good indicator of climatic adaptation. Latitudinal variation in
431 CCRT, but not in CT_{min}, has also been observed in two widespread ant species, *Myrmica rubra*
432 and *Myrmica ruginodis* [29]. Although it is possible that the low variation in cold tolerance in
433 our study is a consequence of relatively low number of studied strains or populations, a recent
434 study found similar trends when using mass-bred *D. montana* populations for a limited number
435 of places from North America [89].

436 We predicted that cold tolerance traits have evolved in response to photoperiod, because it
437 serves as a more reliable cue for seasonal temperature changes than environmental temperature
438 itself [7]. In contrast to our expectations, *D. montana* CT_{min} was associated with latitudinally
439 varying summer and annual mean temperatures (PC2) instead of photoperiod (latitude). This
440 finding could indicate that the non-diapausing *D. montana* flies have adapted to cope with
441 sudden temperature drops and diurnal temperature cycles at their home sites during the late
442 spring, summer and early autumn [53]. CCRT of both species, on the other hand, was associated

443 with photoperiod (latitude), as hypothesized. In *D. montana*, northern females recovered faster
444 from chill coma than the southern ones independent of local climatic conditions (PC1 or PC2),
445 while in *D. flavomontana* CCRT was also associated with macroclimatic conditions varying
446 between the coastal and mountain sites (PC1). Surprisingly, *D. flavomontana* females from the
447 colder high-altitude mountain populations recovered more slowly from chill coma than the ones
448 from the humid low-altitude coastal populations. The most logical explanation for this is that
449 high daily and seasonal temperature variation in the mountains create opposing selection
450 pressures, which select for plastic responses. Accordingly, the flies could compensate their low
451 inherent cold tolerance with high cold acclimation ability, as observed in several studies [26,
452 56, 82, 83, 90, 91]. For example, non-diapausing *D. montana* females from the coast have been
453 shown to recover faster from chill coma than those from the mountains, but diapausing females
454 from both regions recover at similar rates [91]. Mountain *D. flavomontana* females could also
455 enhance their cold tolerance by entering reproductive diapause at earlier time of the year than
456 females from the coastal populations on the same latitude, like *D. montana* females do [92].
457 Finally, the mountain flies could occupy lower mountain slopes, or thick snow cover could
458 protect them during the cold season.

459 **Fly body colour and size and the effects of these traits on fly cold tolerance**

460 Dark cuticle pigmentation (high melanism) can be expected to offer an advantage in cold
461 environments, as it increases insects' ability to absorb solar radiation and enables them to warm
462 up faster in cold environments (thermal melanin hypothesis, [36]). This assumption has
463 received support e.g. from latitudinal variation in the degree of melanism in 473 European
464 butterfly and dragonfly species [33]. However, clinal variation in body colour can be induced
465 also by selection favouring light individuals in the south due to their higher avoidance of UV
466 radiation (UV protection hypothesis, [37, 38]). Overall, body colour genes are highly

467 pleiotropic and can play a role also in a variety of other processes, including immunity,
468 camouflage and mate choice [93, 94]. In our study, *D. montana* flies were darker and more
469 cold-tolerant than *D. flavomontana* flies, which could be expected as *D. montana* is found on
470 higher latitudes and altitudes than *D. flavomontana* [69, 70]. Body colour of *D. montana*
471 showed no significant variation between populations, while that of *D. flavomontana* showed
472 two trends. In the Rocky Mountains, *D. flavomontana* flies became darker towards North, as
473 predicted by thermal melanin hypothesis [36], but the lack of association between fly colour
474 and cold tolerance reduced the support to this theory (see [36]). Rocky Mountains cline could
475 also be explained by UV protection hypothesis [37, 38], if the light flies collected from the
476 southern populations proved to have higher UV resistance. The second trend, detected in *D.*
477 *flavomontana* body colour, was increased melanism in the coastal populations. Also this trend
478 could be explained by UV protection hypothesis, as the flies of the misty coastal populations
479 receive less UV radiation than the ones living on high mountains. Desiccation resistance, linked
480 with high melanism in many systems [39, 40, 95], is not likely to play an important role in the
481 formation of either trend, as dark *D. flavomontana* individuals inhabit humid rather than arid
482 regions. Sexual selection could play part in *D. flavomontana* colour variation, as cuticular
483 hydrocarbons (CHCs), which can be regulated by pigmentation genes [96], are under sexual
484 selection in this species [71]. Moreover, the darkness of *D. flavomontana* flies throughout the
485 western coast could be explained by a founder effect, since this species has only recently
486 distributed to the coastal area through British Columbia in Canada, where its body colour is
487 dark [69, 71]. Finally, *D. flavomontana* may hybridise with *D. montana* to some degree [69–
488 71], which could potentially have led to introgression of dark body colour from *D. montana* to
489 *D. flavomontana*.

We hypothesized that body size (measured as weight) of our study species increases towards cold environments like e.g. in *Drosophila serrata* [27], consistent with Bergmann's rule [45–47], and that the body size is correlated with cold tolerance traits. Contrary to our expectations, the body size of both species was largest in sites with warm summers and winters, which gives support for the converse Bergmann's rule [45–47]. This likely results from the short growing season in cold regions and long generation time of *D. montana* and *D. flavomontana* (~7 weeks) when the time available for development, growth and foraging is limited, resulting in smaller individuals [45–47]. However, *D. montana* body size is clearly affected by multiple selection pressures. While the body size of these flies decreased towards cold environments at population level, the large individuals recovered faster from chill coma than the smaller ones within populations. The latter finding is consistent with a previous study, where the overwinter survival of *D. montana* males was found to increase along with an increase in body size in nature [49]. In *D. flavomontana*, the largest individuals came from the southern Rocky Mountains and from the coastal region with warm summers and mild winters. Lack of correlation between body size and cold tolerance, detected in this species, resembles the situation in several other insect species [97–100].

Circadian clock gene *vrille* plays an essential role in *D. montana* females' short- and long-term cold acclimation

Insects' circadian clock system monitors changes in daily light and temperature cycles and entrains behavioural and physiological rhythms to match with them [57]. This clock system shows adaptive divergence between northern and southern species [72–75], and several clock genes have also been found to be differentially expressed during cold acclimation in studied species [62, 63, 66], suggesting an association between the circadian clock system and cold adaptation. Here, we traced correlation between the number of nucleotide or amino acid

514 differences in *vrille* and variation in the mean chill coma resistance (CT_{min}) or chill coma
515 recovery time (CCRT) in *D. montana* and *D. flavomontana* populations, and found a significant
516 correlation only between amino acid variation and CT_{min} in *D. montana.* We then used RNAi
517 to silence *vrille* and to investigate its effects on *D. montana* females' cold tolerance traits and
518 cold acclimation ability, and found this gene to play an important role in CT_{min} and in females'
519 cold acclimation ability, but not in CCRT. These findings together highlight the importance of
520 *vrille* in enhancing females' cold tolerance both during the rapid cold hardening occurring in
521 CT_{min} test [81] and during the longer-term cold acclimation. Both traits, short- and long-term
522 cold acclimation, have important ecological implications in cold environments with high daily
523 and seasonal temperature changes.

524 Although we found a link between *vrille* and short- and long-term cold acclimation, it is not
525 clear what are the genetic mechanisms underlying this link and to what extent the link exists.
526 First, *vrille* might contribute to cold acclimation ability via circadian clock system, which may
527 either directly contribute to cold acclimation, or regulate other pathways contributing to it. Here,
528 any disruptions in the clock genes could cause similar effects. Alternatively, *vrille* might have
529 more direct role on cold acclimation ability. For example, circadian clock system has been
530 linked to Na^+/K^+ ion channel activity in *D. melanogaster* [101], which is also involved in
531 avoiding cold injury, as observed in *D. montana* [102]. The second open question is whether
532 the direct or indirect effects of *vrille* are limited to *D. montana*, *D. virilis* group species, or
533 insects or organisms in general. *D. virilis* group species are among the most cold-tolerant
534 *Drosophila* species [1, 2], their circadian clock system is adapted to northern habitats [72–75]
535 and *vrille* expression is highly upregulated during cold-acclimation [64, 65], which gives
536 indirect support for the role of *vrille* or the circadian clock system in cold adaptation in this
537 group.

538 Although becoming more feasible, pinpointing the functions of genes affecting ecologically
539 relevant traits is still rare. Previous RNAi experiments have verified e.g. *Hsp22 and Hsp23*
540 genes to contribute to CCRT in *D. melanogaster* [12], *myo-inositol-1-sphosphate synthase*
541 (*Inos*) to contribute to *D. montana* flies' survival during cold stress (5 ˚C), but not to affect their
542 CCRT [13], and *Gs1-like* (*Gs1l*) gene to be involved in *D. montana* cold acclimation [103].
543 *vrille* is an interesting addition to this list, and while the details on the specific molecular
544 mechanisms remain unclear, it gives new insights on the genetics underlying insect cold
545 tolerance and cold acclimation.

546 Conclusions

547 Studying the mechanisms that generate and maintain variation in species stress tolerances is
548 essential for understanding adaptation processes. We show that insect cold tolerance may rely
549 on different environmental cues and morphological traits even in closely-related species, and
550 that insects' chill coma recovery time may be affected by stronger selection pressures in nature
551 than chill coma resistance. These kinds of studies may have applications e.g. in predicting the
552 likely outcomes of climate change or invasion biology. Species, whose cold tolerance is tightly
553 linked with photoperiod, may encounter more difficulties in adapting to changing temperature
554 conditions than the ones whose tolerances are driven by local climatic conditions. We also
555 propose that *vrille*, and possibly the whole circadian clock system, play an essential role in
556 molecular mechanisms underlying short- and long-term cold acclimation, both of which are
557 ecologically important traits on high latitudes with high daily and seasonal temperature
558 variation. In the future, studies on the relationship of reproductive stage and cold tolerance traits
559 in individuals originating from diverse climatic conditions could deepen our understanding on
560 adaptation to seasonally varying environments. Also, investigating the effects of silencing the

561 other core circadian clock genes under cold environment and in other organisms would give
562 valuable insights on the role of the circadian clock system on cold acclimation in general.

563 Methods

564 Genetic variation in cold tolerance, body colour and body size in *D. montana* and in *D.* 565 *flavomontana* populations

566 *Study species and populations*

567 *D. montana* and *D. flavomontana* belong to the *montana* phylad of the *Drosophila virilis* group,
568 and our recent whole genome analyses have shown their divergence time to be ~1 mya (Poikela
569 et al., in preparation). *D. montana* is distributed around the northern hemisphere across North
570 America, Asia and Europe [70], while the distribution of *D. flavomontana* is restricted to North
571 America [69, 70]. In the central Rocky Mountains, *D. montana* is found at altitudes from 1400
572 m to well over 3000 m, while *D. flavomontana* is found mainly below 2000 m. In the western
573 coast, where *D. flavomontana* has probably invaded only during the last decades, both species
574 live at much lower altitudes (see [69, 71]).

575 We investigated cold tolerance traits and measures of body colour and body weight using
576 females from 23 *D. montana* and 20 *D. flavomontana* isofemale strains, which were established
577 from the progenies of fertilized females collected from several sites in North America between
578 2013 and 2015 (Fig. 1). Each site was represented by three isofemale strains per population per
579 species, when possible (Fig. 1; Table S1). *D. flavomontana* is particularly rare in the western
580 coast of North America [71] and we succeeded to collect only 1-2 strains from the study
581 populations. All the strains were maintained in continuous light at 19 ± 1 °C since their
582 establishment (15-30 generations) to eliminate plastic responses and prevent females from

583 entering reproductive diapause. For the experiments, we sexed emerging flies under light CO_2
584 anaesthesia within three days after emergence. Females were changed into fresh malt-vials once
585 a week and used in experiments at the age of 20 ± 2 days, when they all had fully developed
586 ovaries [104].

587 ***Cold tolerance traits***

588 We investigated variation in female cold tolerance using two well-defined and ecologically
589 relevant methods: chill coma temperature (CT_{min}; also called critical thermal minimum) and
590 chill coma recovery time (CCRT). CT_{min} corresponds to the temperature, at which the fly resists
591 cold until it loses all neurophysiological activity and coordination and falls into a chill coma
592 during a gradual decrease in temperature (reviewed in [14]). In this test, we placed the females
593 individually in glass vials, which were submerged into a 30 % glycol bath. We then decreased
594 the bath temperature from the starting temperature of 19 °C at the rate of 0.5 °C/min and scored
595 the CT_{min} for each fly. The second method, CCRT, measures the time taken from a fly to recover
596 from a standardized exposure time at a chill-coma-inducing temperature (reviewed in [14]). In
597 this test, we submerged the females individually in glass vials into a 30 % glycol bath for 16
598 hours at -6 °C [65]. After returning the vials into 19 ± 1 °C in fly laboratory, we measured the
599 time required for each female to recover from the chill coma and stand on its legs. CT_{min} tests
600 were replicated 21 times and CCRT tests 20 times with Julabo F32-HL Refrigerated/Heating
601 Circulator and Haake k35 DC50 Refrig Circulating Bath Chiller, respectively. To account for
602 possible variation between replicates, each test included 1-3 females from each strain.

603 ***Fly body colour and body weight***

604 We analysed variation in the body colour and body weight (as a proxy of body size) of the same
605 females that had been phenotyped in CT_{min} or CCRT tests. Immediately after the cold tolerance

606 tests, the females were put individually into tightly sealed PCR plates and kept in -20 ˚C freezer
607 until measuring their body colour and weight. For body colour measurements, the females were
608 photographed under Leica L2 microscope with 5x magnification, using Leica DFC320 R2
609 camera together with the Leica Application Suite software v4.3.0. Exposure time, zoom factors
610 and illumination level were kept constant, and the photographing arena was surrounded by a
611 plastic cylinder to eliminate glares on the chitinous surface of the fly. All photographs were
612 taken within 3 months after the cold tolerance tests. Images were saved in 24-bit (RGB) format
613 and the colour intensity was measured using grayscale image analysis (ImageJ, [105]); linearly
614 scaling from 0 to 255 (0 = black, 255 = white). We took colour measurements from part of
615 thorax (scutum), as our preliminary tests showed that it best incorporates the colour variation
616 among flies (Fig. S2). Body weight was measured by weighting the females with Mettler
617 Toledo™ NewClassic Balance (model ME). The weight of the females used in CT_{min} tests was
618 measured after females had been frozen for 2 to 4 months, which appeared to be problematic
619 as the females had started to dry and their weight correlated with the freezing time (Fig.
620 S3). The weight of the females used in CCRT tests was measured after 6 to 17 months in
621 freezer. These females had lost most of their body liquids so that their weight was close to dry
622 weight and freezing time had no effect on it (Fig. S3). Accordingly, only the latter dataset was
623 used in the analyses.

624 ***Statistical analyses***

625 We used different statistical models to investigate whether variation in fly cold tolerance, body
626 colour and body weight was associated with latitude, as a proxy of photoperiod, and/or local
627 climatic variables, and whether cold tolerance traits and morphological traits showed a
628 correlation with each other. In these models, we used either CT_{min} data (chill coma temperatures
629 in Celsius degrees + 10 ˚C to prevent negative values from affecting the analysis), CCRT data

630 (in minutes), body colour (measured from CCRT flies) or body weight (mg; measured from
631 CCRT flies) as response variables. *D. flavomontana* CT_{min} data were normally distributed (Fig.
632 S4) and they were analysed with generalized linear mixed model (GLMM) with gaussian
633 distribution (equivalent of linear mixed model). Other datasets showed deviation from the
634 normality (Fig. S4), and they were analysed using generalized linear mixed model (GLMM)
635 with gamma distribution, using *glmmTMB* function from glmmTMB package [106]. Technical
636 replicates and isofemale strains were handled as crossed random effects.

637 In our dataset latitude and altitude were negatively correlated (Pearson correlation coefficient
638 = -0.82) and to prevent this multicollinearity from affecting the analysis, we used only latitude
639 as an explanatory factor. However, we considered the effect of altitude and climatic variables
640 through a principal component analysis (PCA). We downloaded climatic information from
641 WorldClim database v2.1 (2.5 min spatial resolution; current data 1970-2000; [107];
642 http://www.worldclim.org) and extracted 19 bioclimatic variables for each site using their
643 latitudinal and longitudinal coordinates (Table S3; Fig. 1) with raster package v. 2.8-19 [108].
644 Moreover, we obtained growing season length (days) for each site from article by [92] and
645 www.weatherbase.com. Growing season is defined as the average number of days per year
646 when the average daily temperature is at least 5 °C. We performed the PCA on the 19
647 bioclimatic variables, altitude and growing season length (Table S3) to summarize climatic
648 differences on temperature and precipitation in each site using "FactoMineR" package [109].

649 We included the first two PCs in the model comparison of cold tolerance, body colour and body
650 weight (see Results). The simplest model included latitude and PC2, which enabled us to
651 distinguish between the latitudinally varying photoperiods and temperatures. Moreover, the
652 effect of macroclimatic conditions varying between the western coast and the Rocky Mountains
653 (PC1), interaction terms between latitude/PC2 and PC1, and body colour (divided by 100 to

654 scale the variables) and body weight were included in the model selection (Table S6). The best-
655 fit model for CT_{min}, CCRT, body colour and body weight of both species was chosen for further
656 analysis based on Akaike information criterion (AIC) value and Akaike weight (Table S6) using
657 *aictab* function from AICcmodavg package [110]. All the analyses were conducted in R
658 (v1.2.1335-1) and R studio (v3.6.1).

659 **Investigating circadian clock gene *vrille* and its effects on fly cold tolerance using sequence**
660 **variation and RNA interference (RNAi)**

661 ***The candidate gene***

662 *D. virilis* species group shows several distinct features in their circadian clock system compared
663 to *D. melanogaster*, which has enabled their adaptation in high-latitudes [72–74]. Interestingly,
664 *vrille* was the only core circadian clock gene that was found to show both population-level
665 differentiation in clinal cold tolerance study [89] as well as expression level differences when
666 cold-acclimated and non-acclimated *D. montana* flies were compared [77]. This gene was also
667 one of the differentially expressed genes in our earlier microarray study investigating cold
668 tolerance of *D. montana* and *D. virilis* [65]. Accordingly, we investigated the sequence variation
669 in *vrille* and traced its association with cold tolerance traits (CT_{min} and CCRT) in *D. montana*
670 and *D. flavomontana* populations. We also used RNAi to silence the expression of *vrille* and
671 investigated its effects on cold tolerance traits (CT_{min} and CCRT) and cold acclimation ability
672 in *D. montana*, where this gene showed correlation with CT_{min} at the amino acid level (see Fig.
673 5B).

674 ***Association between the number of nucleotide or amino acid differences and differences in***
675 ***cold tolerance traits in D. montana and D. flavomontana populations***

676 To evaluate the role of *vrille* in cold adaptation, we investigated the association between *vrille*
677 sequence variation and variation in cold tolerance traits (CT_{min} and CCRT) in *D. montana* and
678 *D. flavomontana* populations. We obtained published *D. montana* genomic sequences from
679 NCBI under accession number LUVX00000000 and the respective genome annotation from
680 Dryad: doi:10.5061/dryad.s813p55 [111]. Based on *D. virilis* and *D. melanogaster* orthologs in
681 the annotation file, *vrille* sequence is found in scaffold2327-size32305 at position 13933-17558.
682 We used Illumina 150 bp paired-end data from single wild-caught females or their F_1 female
683 progeny, i.e. the founder females of the studied isofemale strains, from all study populations of
684 both species (one individual per population; Table S1; Poikela et al., in prep.).

685 Before mapping, Illumina paired-end reads were quality-checked with FastQC v0.11.8
686 (Andrews 2010: FastQC: a quality control tool for high throughput sequence data. Available
687 online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimmed for adapter
688 contamination and low-quality bases using fastp v0.20.0 [112]. Each trimmed Illumina sample
689 was mapped against *vrille D. montana* reference *vrille* gene using BWA mem v0.7.17 [113].
690 The alignments were sorted with SAMtools v1.10 [114] and PCR duplicates marked with
691 sambamba v0.7.0 [115]. The resulting BAM-files were used for variant calling with freebayes
692 v1.3.1-dirty [116], and the variant calling (VCF) file was filtered for minimum depth and
693 quality of 20 using bcftools view v1.8 (SAMtools. 2018.
694 https://github.com/samtools/bcftools/releases/tag/1.8). Finally, *vrille* FASTA sequences for
695 each sample were obtained using bcftools consensus.

696 The *vrille* sequences together with information on exons from the annotation file (see Fig. S5,
697 S6) were imported to Geneious v9.1.8. (Biomatters ltd). For exons, *D. montana* and *D.*
698 *flavomontana* nucleotide sequences and translated amino acid sequences were aligned using
699 muscle algorithm. The number of pairwise nucleotide and amino acid differences were then

700 calculated for *D. montana* and *D. flavomontana* populations (Table S12, S13). Similarly,
701 pairwise differences in mean CT_{min} (in Celsius degrees) and CCRT (in minutes) were calculated
702 for *D. montana* and *D. flavomontana* populations (Table S14, S15). The correlations between
703 distance matrices were statistically tested with a Mantel test with 1000 permutations using
704 mantel.rtest function from ade4 package [117]. The correlations were tested between i) the
705 number of nucleotide differences and differences in mean CT_{min} ii) the number of amino acid
706 differences and differences in mean CT_{min}, iii) the number of nucleotide differences and
707 differences in mean CCRT, and iv) the number of amino acid differences and differences in
708 mean CCRT among *D. montana* and *D. flavomontana* populations (only within-species
709 correlations).

710 ***Study material for RNAi***

711 We performed RNAi study using only the more cold-tolerant *D. montana*, where *vrille* showed
712 correlation with CT_{min} at the amino acid level (see Fig. 5B) and which has become a model
713 species for studying cold adaption at phenotypic and genetic level (e.g. [61, 64, 92]). Here, we
714 used females of a mass-bred cage population originating from Seward (Alaska, USA; see Fig.
715 1) to increase genetic diversity. The population cage has been established from the 4th
716 generation progenies of 20 isofemale strains (total of 400 flies) in 2013 and maintained in
717 continuous light at 19 ± 1 °C since its establishment (30 generations). Malt bottles with freshly
718 laid eggs were transferred from the population cage into a climate chamber in LD 18:6
719 (light:dark cycle) at 19 ± 1 °C to reinforce flies' circadian rhythmicity prior to the experiments
720 and ensure they are non-diapausing. Critical day length for the induction of reproductive
721 diapause (CDL; 50 % of the females of given population enter diapause) in 19 °C is LD 17:7
722 in Seward population [92], and thus the females emerging in LD 18:6 can be expected to
723 develop ovaries. After ~4 weeks, we collected newly emerged females (≤ 1 day old) using light

724 CO_2 anaesthesia and placed them back into the above-mentioned conditions in malt-vials.
725 Females were changed into fresh vials once a week until they were used in the experiments at
726 the age of 21 days.

727 ***Defining daily expression rhythm of vrille for RNAi study***

728 Expression levels of circadian clock genes are known to show daily fluctuations, and in order
729 to perform RNAi experiments at a right time of the day, we defined the time when the
730 expression of *vrille* is highest at LD 18:6. To do this, we collected females every four hours
731 (i.e. at six time points / day), starting at 6 am (ZT0) when lights were switched on. At each time
732 point, we stored samples of females into -80˚C through liquid N_2 and transferred them later on
733 into RNAlater®-ICE frozen tissue transition solution (Thermo Fisher Scientific). We then
734 checked the size of female ovaries (see [104]) and used only the females with fully developed
735 ovaries (>95 % of the females). For each time point, RNA was extracted from three pools, each
736 of which consisted of three whole females, using ZR Tissue & Insect RNA MicroPrep kit with
737 DNAse treatment (Zymo Research®). RNA purity was analysed with NanoDrop® ND-1000
738 spectrophotometer (Thermo Fisher Scientific) and concentration with Qubit® 2.0 Fluorometer
739 and RNA BR kit (Thermo Fisher Scientific). cDNA was synthesized using equal quantities of
740 RNA (200 ng) with iScript Reverse Transcription kit (Bio-Rad Laboratories®).

741 We measured the expression levels of *vrille* with quantitative real time PCR (qPCR). qPCR
742 primers for *vrille* and reference genes were designed based on *D. montana* genomic sequences
743 under NCBI accession number LUVX00000000 [111], together with information from *D.*
744 *virilis* exons using Primer3 (primer3.ut.ee) and NetPrimer
745 (www.premierbiosoft.com/netprimer) programs (gene accession numbers and primer
746 sequences in Table S16). qPCR mix contained 10 µl of 2x Power SYBR Green PCR Master

747 Mix (Bio-Rad Laboratories), 0.3 µM of each gene-specific primer and 1 µl of cDNA solution.
748 Cycling conditions in Bio-Rad CFX96 instrument were: 3 min at 95 °C, 10 sec at 95 °C, 10 sec
749 at annealing temperature of 53 °C (for reference genes 56 °C) and 30 sec at 72 °C (repeated
750 40x), followed by melting curve analysis (65-95 °C) for amplification specificity checking.
751 Each run included three technical replicates for each sample and the final threshold value (Cq)
752 was defined as a mean of the technical replicates that produced good quality data. The relative
753 qPCR data was normalised with ΔΔ(Ct) normalisation method [118] using two reference genes,
754 *Tubulin beta chain* (*Tub2*) and *Ribosomal protein L32* (*RpL32*), that showed equal expression
755 levels in all samples (data not shown). Real efficiency values of the genes used in the qPCR are
756 given in Table S16.

757 ***Synthesis of double-stranded RNA for RNAi***

758 *LacZ,* which codes a part of a bacterial gene, was used as a control for dsRNA injections. We
759 generated fragments of *vrille* and *LacZ* genes, with the length of 347 and 529 bp, respectively,
760 with PCR (primer information given in Table S16). PCR products were purified with GeneJET
761 Gel Extraction kit (Thermo Fisher Scientific) and cloned using CloneJET PCR Cloning kit
762 (Thermo Fisher Scientific). PCR products were ligated into the vector (pJET1.2/blunt Cloning
763 vector), transformed into *E. coli* Zymo JM109 (Zymo Research) cells, which were grown on
764 Luria Broth (LB) ampicillin plates. Individual colonies were picked up after 16 hours and
765 cultivated overnight in LB solution with ampicillin using a Unimax 1010 shaker with incubator
766 (Heidolph Instruments). The samples from the extracted bacterial solutions were analysed for
767 the size of the products in the second PCR, which was carried out with pJET primers from the
768 cloning kit, followed by agarose gel runs. We then selected the colonies with the right size
769 products for the third PCR using pJET primers, where the R primer contained T7 promoter
770 sequence at the 5' end of the primer (primer sequences in Table S16). PCR products were first

771 purified with GeneJet Gel Extraction kit and then used in transcription synthesis of the double-
772 stranded RNA (dsRNA), using the TranscriptAid T7 High Yield Transcription kit (Thermo
773 Fisher Scientific). Finally, we purified and precipitated the synthesized products with ethanol,
774 suspended them in salt buffer and quantified them using NanoDrop and agarose gel.

775 ***RNAi microinjection procedure, response time screening and vrille expression at the chosen***
776 ***response time***

777 Injecting dsRNA targeting *vrille* gene is expected to cause gene-specific effects, but it may also
778 cause immune responses and physical damage (injection) in the flies. Accordingly, we used
779 *LacZ* (encoding for bacterial gene) injections as a control for both immune response to dsRNA
780 and to physical damage of injections, and no-injection as a baseline control. We checked the
781 effectiveness of RNAi treatment on *vrille* expression 12h, 24h and 48h after injections, at a time
782 of the day when it shows highest expression (ZT16, see Results). The females were injected
783 into thorax with 138 nl of ~20 μM dsRNA targeting *vrille* or *LacZ* using Nanoject II Auto-
784 nanoliter Injector with 3.5" Drummond glass capillaries (Drummond Scientific) pulled with P-
785 97 Flaming/Brown Micropipette Puller (Sutter Instrument). No-injection control females were
786 not injected, but were otherwise handled in the same way as the injected females. To prevent
787 CO_2 anaesthesia from inducing variation between these groups, we injected six females at a
788 time. For each response time (12h, 24h, 48h) all three treatments (*vrille*, *LacZ*, no-injection)
789 were performed. Each treatment consisted of three pools, each pool containing 10 whole
790 females. After reaching the response time, the females were transferred into -80 ^{o}C through
791 liquid N_2. Then, the females were transferred into RNAlater®-ICE solution and their ovaries
792 were checked as explained above. RNA was extracted from the pools using TRIzol® Reagent
793 with the PureLink® RNA Mini kit (Thermo Fisher Scientific). RNA purity was analysed with
794 NanoDrop and concentration with Qubit and RNA BR kit. cDNA was synthesized using equal

795 quantities of RNA (143 ng) using SuperScript IV First-Strand Synthesis System (Thermo Fisher
796 Scientific). Expression levels of *vrille* 12, 24 and 48 hours after the injections were quantified
797 with qPCR, as described above. The response time of 48 hours was the most effective (Fig. S1),
798 and its effect was double-checked with another round of cDNA synthesis (200 ng) and qPCR
799 (Fig. 6B).

800 ***Experimental design for studying the functional role of vrille in cold tolerance using RNAi***

801 We investigated the role of *vrille* gene in resisting chill coma (CT_{min}) and recovering from it
802 (CCRT) in non-diapausing *D. montana* females originating from Seward, Alaska, USA.
803 Moreover, we considered the plastic effects of *vrille* during cold acclimation. Prior to
804 performing cold tolerance tests, females were maintained for 16 days in LD 18:6 at 19 ˚C,
805 corresponding to summer conditions at their home site (LD 18:6 was used throughout the
806 experiment). Then, half of these females were subjected to cold acclimation treatment at 6 °C
807 for 3 days (cold-acclimated females; [65]), while the other half was maintained at 19 °C. At the
808 age of 19 days, both cold-acclimated and non-acclimated females were collected from the
809 chambers, anesthetized with CO_2 and injected as described above. They were then placed back
810 to either cold acclimation (6 ˚C) and non-acclimation (19 ˚C) conditions for two more days, as
811 the expression levels of target genes had been found to be lowest 48h after RNAi treatment
812 (Fig. S1). At the age of 21 days, females' cold tolerance was quantified by measuring their chill
813 coma temperature (CT_{min}) or chill coma recovery time (CCRT) using Julabo F32-HL
814 Refrigerated/Heating Circulator. Sample sizes for CT_{min} and CCRT tests were 26-32 and 25-30
815 females per treatment, respectively.

816 ***Statistical analyses of RNAi experiment***

817 For investigating the effects of RNAi, expression levels of *vrille* in *LacZ*-injected females were
818 compared to no-injection control females and females injected with dsRNA targeting on *vrille*.
819 The relative normalised expression values were analysed using a linear model (ANOVA) in
820 base R.

821 To test the cold acclimation effect within each treatment group (*vrille*-RNAi, *LacZ*-RNAi, no-
822 injection control), we compared the cold tolerance of the females that had or had not been cold-
823 acclimated. We then investigated the gene specific effects on cold tolerance by comparing
824 *LacZ*-injected females to *vrille*-injected females and traced possible immune and physical
825 effects of the injections by comparing *LacZ*-injected females to no-injection females. These
826 analyses were performed separately for the cold-acclimated and non-acclimated females. All
827 data showed deviation from normality (Fig. S7) and were analysed with generalized linear
828 mixed model using gamma distribution (GLMM; *glmmTMB* function from glmmTMB package
829 [106]. In the models, response variables were either CT_{min} (Celsius degrees + 10 to prevent
830 negative values from affecting the results) or CCRT (minutes) data, and the test replicates were
831 used as a random effect. All the analyses were conducted in R (v1.2.1335-1) and R studio
832 (v3.6.1).

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Abbreviations

CT_{min} = critical thermal minimum or chill coma temperature; cold tolerance method that measures the ability of an individual to resist cold and chill coma

CCRT = chill coma recovery time; cold tolerance method that measures the ability of an individual to recover from chill coma

RNAi = RNA interference; technique for gene silencing

Declarations

Ethics approval and consent to participate

Neither of the species is endangered, and the flies were collected along watersides on public lands outside National and State parks, where insect collecting does not require permits in the USA or Canada (The Wilderness Act of 1964, section 6302.15).

Consent to publish

Not applicable.

Availability of data and materials

The climatic data were obtained from WorldClim database v2.1, http://www.worldclim.org. The phenotypic data generated and analysed during the current study are available in the Dryad repository, https://doi.org/10.5061/dryad.98sf7m0fv. *D. montana* genome sequences were obtained from NCBI repository, under accession number LUVX00000000, and the respective genome annotation was obtained from the Dryad repository, https://doi.org/10.5061/dryad.s813p55. *vrille* sequence data (BAM files) analysed during the

current study are available in the EMBL Nucleotide Sequence Database (ENA) repository, under accession number PRJEB45370.

Competing interests

The authors declare no competing interests.

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Authors' Contributions

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.

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