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 reliance of insect cold tolerance traits on latitudinally and locally varying environmental conditions, and their associations with different morphological traits and molecular mechanisms, is essential for understanding the processes involved in adaptation. We explored these issues in non-diapausing females of two closely-related species, *Drosophila montana* and *Drosophila flavomontana*, originating from diverse climatic locations across several latitudes on the western coast and the Rocky Mountains of North America. Moreover, we traced the impact of one of the key regulators of circadian behavioural rhythms, *vrille*, on fly cold tolerance and cold acclimation ability by silencing this gene with RNA interference in *D. montana*.

Results

We performed principal component analysis (PCA) on variables representing bioclimatic conditions on 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 in North-South direction based on summer and annual mean temperatures and growing season length. Cold tolerance tests showed *D. montana* to be more cold-tolerant than *D. flavomontana*, as could be expected by species distributions, and chill coma resistance (CT_{min}) of this species showed association with latitudinally varying temperatures (PC2). Chill coma recovery time (CCRT) showed cold tolerance of both species to increase towards northern latitudes, and in *D. flavomontana* this trait was also associated with PC1. *D. flavomontana* flies were darkest in coastal populations and in the northern parts of the Rocky Mountains, but colouration showed no linkage with fly cold tolerance. Body size (measured as weight) decreased towards cold environments in both species, but large size correlated with fast CCRT among *D. montana* individuals. Finally, silencing of *vrille* suggested this gene to play an essential role in CT_{min} and cold acclimation, but not in CCRT.

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 functional circadian clock gene *vrille* is essential both for short- and long-term cold acclimation elucidating connection between circadian clock 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 and plants, 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' adaptation in these regions, it is essential to identify latitudinal and bioclimatic selection pressures driving these adaptations [2, 9–11]. It would also be important to discover molecular mechanisms underlying cold tolerance, but a functional link between candidate genes and cold tolerance has rarely been established (but see e.g. [12, 13]).

Both a high resistance to chill coma and fast recovery from it offer fitness advantages [14–16]. Insects' chill coma resistance can be assessed by measuring their critical thermal minimum (CT_{min}), where they lose neuromuscular coordination and fall into chill coma during a gradual decrease in temperature [17]. The second widely-used cold tolerance measurement, chill coma recovery time (CCRT), is based on time required for an individual to recover from the coma after removal of the chill coma inducing temperature [14, 18]. 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. [16, 19]). The onset of chill coma is associated with depolarisation of muscle resting membrane potential due to low temperature (e.g. [20, 21]), while the recovery process involves a complete restoration of ion gradients and upregulation of genes involved in repairing cold injuries [22–26]. The recovery process involves high energetic costs but has also several implications on insect fitness in cold environments (e.g. territory defence, forage, escape from predators, and mating). Thus, this trait is likely to be under strong divergent selection.

Latitudinal variation in cold tolerance has been detected in a variety of species, including *Drosophila* flies [18, 27, 28], *Myrmica* ants [29], *Porcellio laevis* woodlouse [30] and *Arabidopsis thaliana* plant [31, 32]. 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 that the populations or species experience [33]. 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 [34]. Thus, it is important to investigate cold tolerance in temperature and light conditions, where insects' reproductive status is controlled.

Also, morphological traits, such as body colour (the degree of melanism) and size, have been found to show latitudinal variation, potentially being associated with cold tolerance. In insects, an increase in melanism towards higher latitudes has been detected both between and within species [35–37]. 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 [38]). However, body colour is likely to be affected also by other selection pressures, including protection against UV-radiation [39, 40], desiccation [41, 42] or pathogens [43]. The body size of endothermic species increases towards higher latitudes and cooler climates (Bergman's rule), but ectothermic species have also commonly been found to obey converse Bergman's rule or U-shaped clines as a result of interactions between several factors covarying with latitude (e.g. [44–46]). This is true

especially for insect species with a long generation time, for which the short growing season on high latitudes limits the time available for development, growth and foraging [46–48]. Overall, correlations between latitudinal clines in cold tolerance and morphological traits give only indirect evidence on functional linkages between these traits. One way to obtain more direct evidence on these linkages would be to measure cold tolerance and morphological traits for the same individuals. For example, it has been shown that melanistic wood tiger moths (*Parasemia plantaginis*) warm up more quickly than the less melanistic ones [49] and *Drosophila montana* males' overwinter survival to increase along with an increase in body size in nature [50].

Insects can mitigate cold stress also through plastic responses, long-term cold acclimation and rapid cold hardening (RCH, [51, 52]). In nature, they can anticipate the forthcoming cold season from a decreasing day length and/or temperature and adapt to these changes through a gradual increase in cold tolerance [53, 54]. Insects can also be cold-acclimated by maintaining them in relatively low temperature or short day conditions for a few days to weeks prior to cold shock [55, 56]. Short-term cold acclimation of minutes to hours (rapid cold hardening, RCH) has been suggested to share mechanisms with longer-term acclimation [57] and allow insects to cope with sudden cold snaps and to optimize their performance during diurnal cooling cycles [25, 54]. Here the circadian clock system, which monitors daily and seasonal light and temperature cycles and entrains behavioural and physiological rhythms to match with them [58], could play a central role. In plants, the linkage between circadian clock and cold acclimation has already been established [59, 60], but in insects direct evidence on this link is still missing. However, the expression level of circadian clock genes has been found to change during long-term cold acclimation in several insect species, including *Drosophila* species [61–64] and *Gryllus pennsylvanicus* cricket [65]. For example *vrille*, which is one of the core genes in the central circadian clock system in *D. melanogaster* and a key regulator of circadian behavioural rhythms [66–68], is highly upregulated during cold acclimation in northern *Drosophila virilis* group species [63].

Drosophila virilis group species possess a very high cold tolerance compared to most other species of the genus [2]. Our study species, *Drosophila montana* and *Drosophila flavomontana* (both belonging to *virilis* group), have populations in diverse climatic conditions on the western coast and in the Rocky Mountains of North America across several latitudes (Fig. 1; Table S1). *D. montana* lives generally at higher latitudes and altitudes than *D. flavomontana*, but in some sites the species occur sympatrically [69–71]. Moreover, the body colour of *D. montana* is almost black, while that of *D. flavomontana* varies from light to dark brown [69]. Thus, these species offer a good opportunity to complement latitudinal studies performed on less cold-tolerant southern species, and to add new insights in clinal studies by exploring associations of insect cold tolerance with latitudinally and locally varying photoperiods and macroclimatic factors, as well as with body colour and size. We studied these genetic adaptations in non-diapausing *D. montana* and *D. flavomontana* females, which are susceptible to sudden temperature decreases in spring and autumn when the snow cover is missing, and measured all traits in a common environment to eliminate plastic responses. (i) We quantified environmental variation between fly collecting sites by performing a principal component analysis (PCA) on several bioclimatic variables, and (ii) investigated whether latitude, as a proxy of photoperiod, and latitudinally or locally varying climatic conditions have shaped female cold tolerance (CT_{min} and CCRT). We predicted that variation in fly cold tolerance, especially in CCRT given its high benefits and costs, shows association with latitudinally varying photoperiods which serve as the most reliable cue for seasonal temperature changes at given localities [7]. (iii) We tested whether fly body colour and size show association with the environmental cues and

whether cold tolerance traits and morphological traits are correlated with each other when measured for the same individuals. Body colour could be expected to show a latitudinal cline and get darker towards higher latitudes, if dark body colour increases fly cold tolerance in North (thermal melanin hypothesis, [38]) or if light body colour increases their resistance against UV radiation in South (UV protection hypothesis, [39, 40]). Fly size could be expected to decrease towards high latitudes like in other ectothermic species whose development relies on the length of growing season (converse Bergman's rule, [48, 72]). Collecting cold tolerance and morphological data for the same individuals gave us a chance to trace the effects of the morphological traits on fly cold tolerance and obtain more explicit information on selection pressures underlying the clines. Finally, (iv) we traced the role of the circadian clock gene *vrille* in regulating females' cold tolerance and cold acclimation ability by silencing it with RNAi in more cold-tolerant *D. montana*. We expected *vrille* inactivation to decrease at least females' ability to get cold-acclimated, as this gene shows expression changes during cold acclimation in this species [63]. This kind of finding could then suggest a link between cold acclimation and the circadian clock system in insects.

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

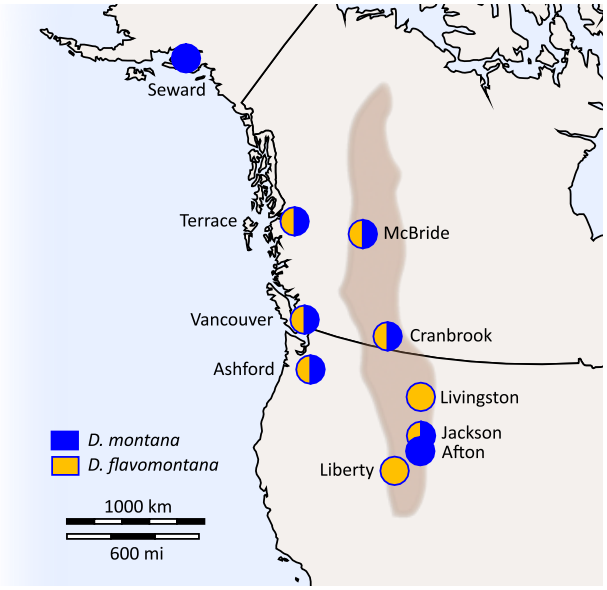

Figure 1. Sample locations. Table shows fly collecting sites and years, and the coordinates for each site. Map contains information on whether we have samples from one or both species in each site on the western coast and in the Rocky Mountains (brown area on the map) in North America (detailed information 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, were *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 populations 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.

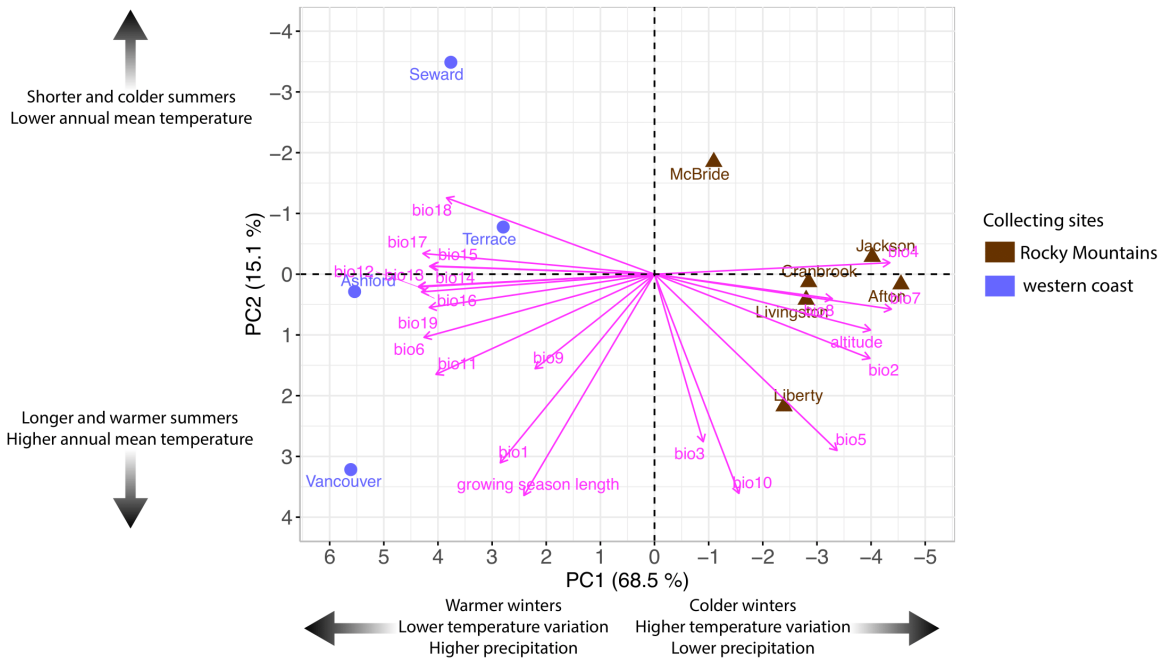

Figure 2. Principal component analysis (PCA) on 21 variables describing environmental conditions in fly collecting sites (Table S2, S3). Big black arrows show the change in given conditions.

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. All traits were measured for summer-acclimated non-diapausing *D. flavomontana* and *D. montana* females, reared in constant light at 19 ˚C. Contrary to other traits, weight is shown only for the flies used in CCRT tests (see Methods). The simplest model, which enabled us to distinguish between latitudinally varying photoperiods and temperatures, included latitude and PC2 as explanatory factors (see Fig. 2; Table S6). The more complicated models included macroclimatic conditions varying between the western coast and the Rocky Mountains (PC1), different interaction terms and fly body colour and size (Table S6).

The best-fit models explaining the cold tolerance (CT_{min} and CCRT), body colour and body size of *D. flavomontana* and *D. montana* females are presented in Fig. 3A-D (model comparisons are given in Table S6). The models show that the selection pressures driving the evolution of these traits vary between the species. Most of the pairwise correlations between fly cold tolerance traits, colour and weight were non-significant, and hence not included in the best-fit models; Fig. 3, 4; Table S6).

CT_{min} of *D. flavomontana* showed only low variation and was not significantly explained by any of the variables (Fig. 3A; Table S6). However, in *D. montana* this trait showed significant association with PC2 (Fig. 3A; Table S6), which suggests that the chill coma resistance of *D. montana* flies is highest in northern populations with a short growing season and cold summer and low annual mean temperatures (Fig. 2). CCRT tests showed *D. flavomontana* flies' cold tolerance to be significantly associated with latitude and to improve towards North (Fig. 2, 3B; Table S7). Moreover, this trait was affected by PC1, especially on latitudes around 50-55 ˚N, suggesting that fly cold tolerance is higher in the humid, low-altitude western coast populations 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 like CT_{min} (Fig. 3B; Table S7). Moreover, in this species large flies recovered from chill coma faster 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).

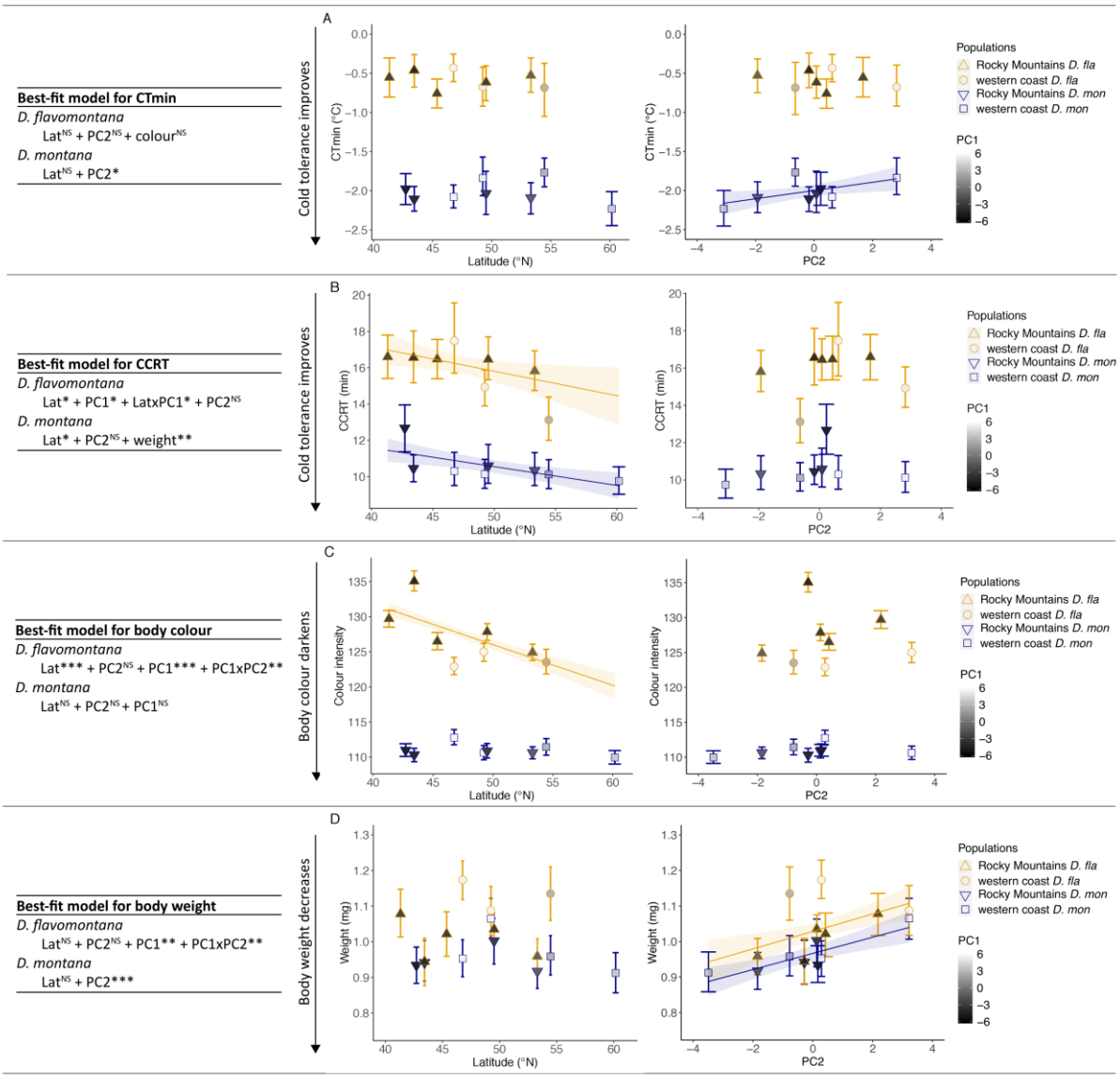

Figure 3. Relationship between latitude (as a proxy of photoperiod) and PC2 (as a proxy of latitudinally varying temperature) and (A) chill coma temperature (CT_{min}), (B) chill coma recovery time (CCRT), (C) fly body colour (measured as colour intensity) and (D) fly body size (measured as weight) in *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. Significance levels were obtained from GLMMs or LMM: ^{NS} non-significant, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

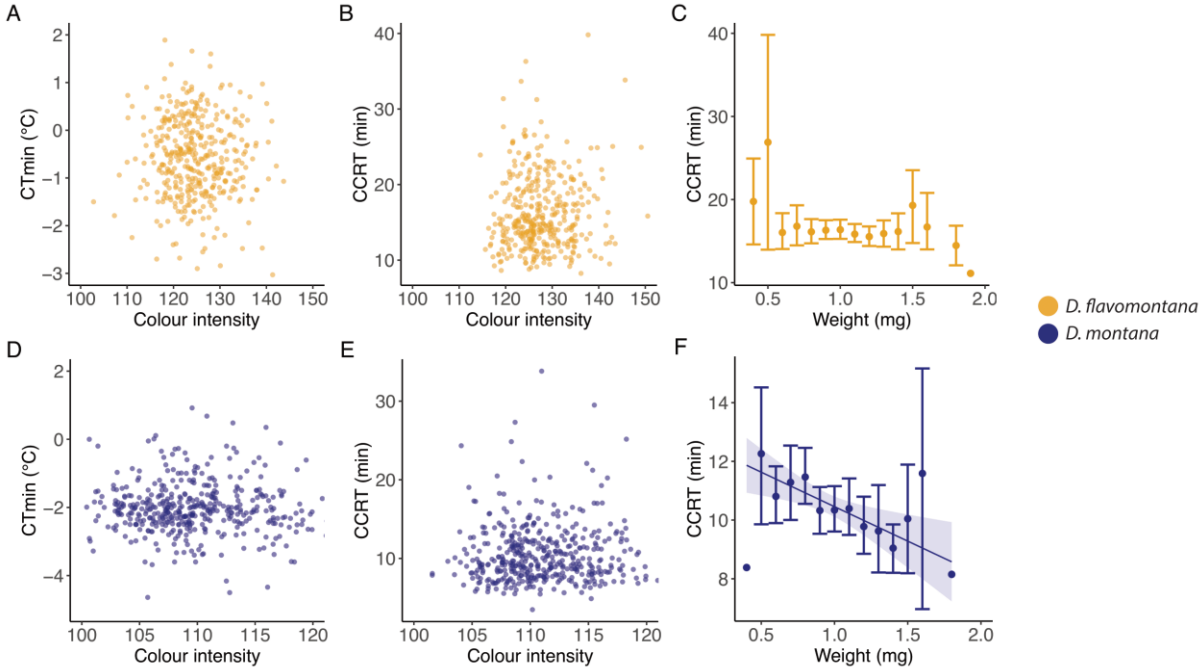

Figure 4. Correlations between fly cold tolerance traits (CT_{min}, CCRT) and morphological traits (body colour (low color intensity indicates dark colour) and body size (measured as weight) in *D. flavomontana* (A-C) and in *D. montana* (D-F). Error bars represent bootstrapped 95% confidence intervals. Significant regression lines are shown with standard errors (best-fit models are presented in Fig. 3 and Table S6).

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

Since the expression levels of circadian clock genes are known to fluctuate during the day, we first investigated daily rhythm in *vrille* expression in LD (light:dark cycle) 18:6, where the females of the study population (Seward, Canada) can be expected to be non-diapausing (and which was also verified, see methods). Our results showed that *vrille* has a clear daily rhythm and that its highest expression corresponds to ZT16 and ZT20 (ZT = Zeitgeber Time which refers to the number of hours after the lights are switched on; see Fig. 5A). From these two time points we chose to perform RNAi-injections at ZT16, when the lights were still on.

We next compared *vrille* expression levels in *LacZ*-injected control females to those of *vrille*-injected and no-injection females 12, 24 and 48 hours after the injections. This enables us to measure the effects of *vrille* RNAi on the expression level of this gene controlling possible effects of immune responses and physical damage. Differences between *LacZ*- and *vrille*-injected females were most pronounced 48h after the RNAi-injections (Fig. 5B; Fig. S1), where *vrille*-injected females had approximately 56% lower *vrille* expression compared to *LacZ* controls (Fig. 5B; Table S8). Accordingly, all experiments were performed 48h after the injections at ZT16 in LD 18:6.

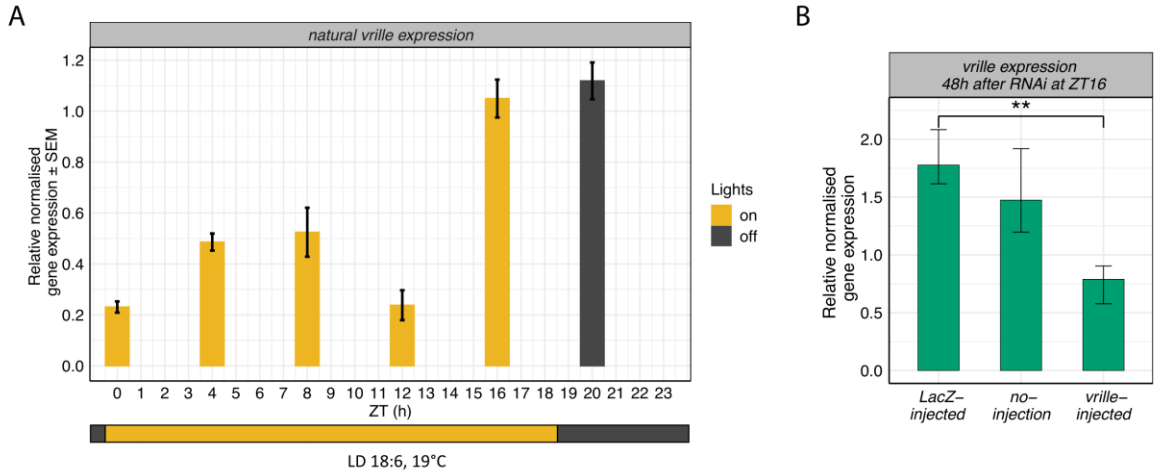

Figure 5. (A) Natural relative normalised expression of *vrille* at six time points, starting at ZT0 (Zeitgeber Time = sampling time every 4 hours over a 24 hour period), in LD 18:6 and 19 ˚C. Yellow represents the time of the day when the lights were on and dark grey when the lights were off. (B) Relative normalised expression levels of *vrille* in *LacZ*-injected and no-injection control females, and in females injected with dsRNA targeting on *vrille* 48h after the injections at ZT16. 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 *** $P < 0.001$.

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

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

We first investigated whether acclimation in 6 ˚C (cold-acclimated females) compared to the flies kept in 19 ˚C (non-acclimated females) had improved female cold tolerance within the three experimental groups (*LacZ*-injected females, no-injection females and *vrille*-injected females). CT_{min} tests showed that cold acclimation had improved the chill coma resistance of *LacZ*-injected and no-injection females by decreasing their CT_{min} on average by 0.7 ˚C and 0.6 ˚C, respectively, while acclimation had no significant effect on the chill coma resistance of *vrille*-injected females (Fig. 6A; Table S9). In CCRT tests, cold-acclimated no-injection females recovered from chill coma on average 1.5 minutes faster than the non-acclimated ones (Fig. 6B; Table S9), i.e. their ability to recover from coma was faster, as could be expected. However, cold acclimation had no significant effect on the recovery time of *LacZ*-injected

females, which suggests that either immune responses or physical damage had overridden the positive effects of the acclimation (Fig. 6B; Table S9). Finally, CCRT tests for *vrille*-injected females showed that cold acclimation had slowed down their recovery time by ~3.5 minutes instead of fastening it (Fig. 6B; Table S9). Such a significant effect in females' ability to recover from chill coma cannot be explained solely by immune responses or physical damage, which means that a functional *vrille* gene is essential for females' cold acclimation ability.

Next, we compared CT_{min} and CCRT of *LacZ*-injected females to those of the no-injection and *vrille*-injected females separately among the non-acclimated and cold-acclimated females. CT_{min} tests showed no differences between the chill coma tolerance of *LacZ*-injected and no-injection females in either of these groups (Fig. 6A; Table S10). On the other hand, *vrille*-injected females entered chill coma in 1 ˚C (non-acclimated females) and 2.5 ˚C (cold-acclimated females) higher temperature than *LacZ*-injected females of the same groups (Fig. 6A; Table S10). These results show that low expression levels of *vrille* significantly decreased females' ability to resist low temperatures. In CCRT tests, both the non-acclimated and cold-acclimated *LacZ*-injected females recovered from chill coma ~3 minutes more slowly than respective no-injection females (Fig. 6B; Table S10), 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. 6B; Table S10). However, cold-acclimated *vrille*-injected females recovered from chill coma ~3.5 minutes more slowly than *LacZ*-injected females (Fig. 6B; Table S10), which again brings up the poor acclimation ability of *vrille*-injected females.

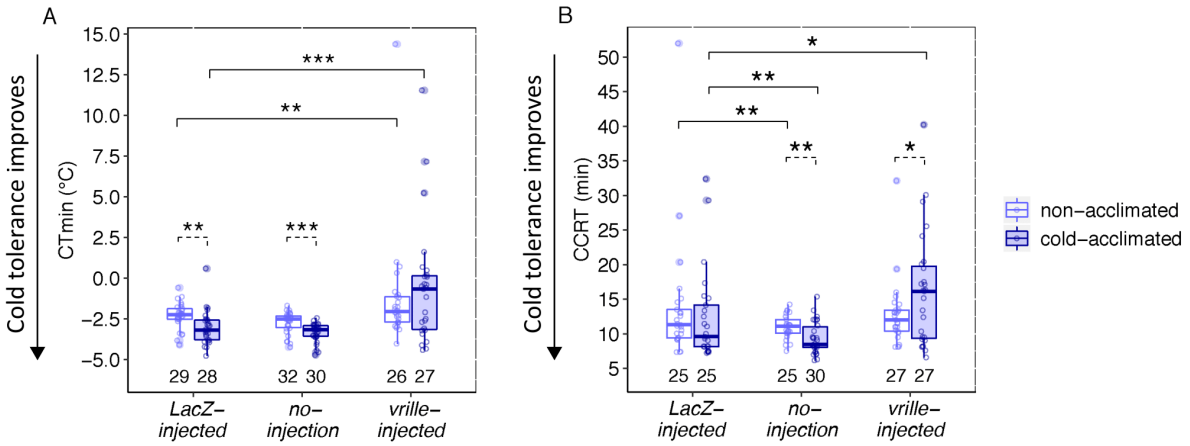

Figure 6. (A) CT_{min} and (B) CCRT tests for *LacZ*-RNAi, no-injection, and *vrille*-RNAi females that were kept at +19 ˚C for the whole time, or at first at +19 ˚C and the last 5 days at +6 ˚C (cold acclimation period). Dashed lines indicate significant effects of the acclimation in each treatment, 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 evidenced inter- and intraspecific latitudinal variation in insect cold tolerance (e.g. [1, 2, 30, 3–6, 18, 27–29]) and identified candidate genes for it (e.g. [61–63, 65]). Understanding the factors involved in species adaption into harsh environmental conditions requires information on the effects of latitudinal and macroclimatic factors and morphological traits on different aspects of insect cold tolerance, as well as on molecular mechanism underlying it. Moreover, to build a convincing case for clinal adaptation, it is important to gather several independent sources of evidence, including sibling species, multiple populations or geographic regions and environmental correlations that account for population structure [73]. We explored these questions in non-diapausing females of *D. montana* and *D. flavomontana* strains originating from diverse climatic environments across different latitudes on the western coast and the Rocky Mountains of North America. Also, performing RNAi experiments on one of the key regulators of the circadian behavioural rhythms, *vrille*, in *D. montana* enabled us to investigate the role of this gene on female cold tolerance and cold acclimation ability.

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

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

We predicted that cold tolerance traits have evolved in response to photoperiod, because it serves as a more reliable cue for seasonal temperature changes than environmental temperature itself [7]. However, females' chill coma resistance (CT_{min}) showed a significant variation only in *D. montana*, where it was associated with latitudinally varying temperature (PC2) instead of photoperiod (latitude). Also this association is logical since non-diapausing *D. montana* females may face quite cold temperatures during the late spring and early autumn in the northern parts of the species distribution [72, 74]. Chill coma recovery time (CCRT) of both species was associated with photoperiod (latitude), as hypothesized. In *D. montana*, northern females recovered faster from chill coma than the southern ones independent of local climatic conditions (PC1 or PC2), while in *D. flavomontana* CCRT was also associated with macroclimatic conditions varying between the coastal and mountain sites (PC1). Surprisingly, the *D. flavomontana* females from the colder high-altitude mountainous populations recovered more slowly from chill coma than the ones from the humid low-altitude coastal populations. This could be due to opposing selection pressures on fly stress tolerances, induced by high daily and seasonal temperature variation in the mountains, and/or mountain population flies could compensate their low inherent cold tolerance with high cold acclimation ability [15, 32, 57, 75, 76]. Rocky Mountains *D. flavomontana* females could also enhance their cold tolerance by entering reproductive diapause at an earlier time of the year than the females from the coastal populations on the same latitude, as *D. montana* females do [74], and/or they could occupy lower mountain slopes during the cold season.

Our second prediction was that CCRT is more prone to selection than CT_{min}, since it involves high fitness advantages and energetically costly processes [14, 16, 22–26]. Latitudinal variation in CCRT, but not in CT_{min}, has been found e.g. in two widespread ant species, *Myrmica rubra* and *Myrmica ruginodis* [29]. Our study gave some support to this hypothesis, as CCRT showed significant variation in both species, while CT_{min} showed it only in *D. montana*. Overall, the high heritability of CCRT [27, 57, 77–79] and strong selection pressures directed on the costs and benefits of short recovery time, make this trait a good indicator of climatic adaptation. CT_{min} tests may not be as fit as CCRT tests for studying insects' inherent cold tolerance, as rapid cold hardening (RCH) occurring during the gradual cooling period in CT_{min} tests can change the composition of membrane phospholipids, improving the chill coma resistance [20, 21, 80, 81]. A trade-off between inherent cold tolerance and RCH, detected in several studies [15, 57, 75, 76], could lead to skewed species- or population -level variation in CT_{min}.

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

Dark cuticle pigmentation (high melanism) can be expected to offer an advantage in cold environments, as it increases insects' ability to absorb solar radiation and enables them to warm up faster in cold environments (thermal melanin hypothesis, [38]). This assumption has received support e.g. from latitudinal variation in the degree of melanism in 473 European butterfly and dragonfly species [35]. However, clinal variation in body colour can be induced also by selection favouring light individuals in the south due to their higher resistance against UV radiation (UV protection hypothesis, [39, 40]). Overall, body colour genes are highly pleiotropic and can play a role also in a variety of other processes, including immunity, camouflage and mate choice [82, 83]. In our study, *D. montana* flies were darker and more cold-tolerant than *D. flavomontana* flies, which could be expected as *D. montana* is found on higher latitudes and altitudes than *D. flavomontana* [69, 70]. Body colour of *D. montana* showed no significant variation between populations, while that of *D. flavomontana* showed two trends. In the Rocky Mountains, *D. flavomontana* flies became darker towards North, as predicted by thermal melanin hypothesis [38], but the lack of association between fly colour and cold tolerance reduced support to this theory (see [38]). Rocky Mountains cline could also be explained by UV protection hypothesis [39, 40], if the light flies collected from the southern populations proved to have higher UV resistance. The second trend, detected in *D. flavomontana* body colour, was increased melanism in the coastal populations. Also this trend could be explained by UV protection hypothesis, as the flies of the misty coastal populations receive less UV radiation than the ones living on high mountains. Desiccation resistance, linked with high melanism in many systems [41, 42, 84], is not likely to explain the formation of either trend, as dark *D. flavomontana* individuals inhabit humid rather than arid regions. Sexual selection could play a role in *D. flavomontana* colour variation, as cuticular hydrocarbons (CHCs), which can be regulated by pigmentation genes [85], are under sexual selection in this species [71]. Moreover, the darkness of *D. flavomontana* flies throughout the western coast could be explained by a founder effect, since this species has relatively recently distributed to the coastal area likely through British Columbia in Canada, where its body colour is dark [69, 71]. Finally, *D. flavomontana* may hybridise with *D. montana* to some degree [69–71], which could potentially have led to introgression of dark body colour from *D. montana* to *D. flavomontana*.

Body size is one of the most important quantitative traits of individuals, being associated e.g. with metabolic rate, fecundity and mating success, and is potentially under multiple selection pressures in nature [45]. *D. montana* and *D. flavomontana* flies' body size (measured as weight) was largest in sites with warm summers and winters, likely due to a longer growing season in

these locations, which gives support for the converse Bergmann's rule [46–48]. In *D. montana*, body size is clearly affected by multiple selection pressures. While the population-level variation showed body size to decrease towards cold environments, the largest individuals of the populations recovered faster from chill coma than the smaller ones. The latter finding is consistent with previous observations, where the overwinter survival of *D. montana* males increases along with an increase in body size in nature [50]. In *D. flavomontana*, the largest flies 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 [86–89].

Circadian clock gene *vrille* plays an essential role in *D. montana* females' cold acclimation

Insects' circadian clock system monitors changes in daily light and temperature cycles and entrains behavioural and physiological rhythms to match with them [58], and hence it could be expected to be involved in cold acclimation. Here, we used RNAi to silence the expression of one of the key regulators of circadian behavioural rhythms, *vrille*, and investigated its effects on *D. montana* females' cold tolerance traits and cold acclimation ability. We found *vrille* to play an important role in CT_{min} and in females' cold acclimation ability, but not in CCRT. The effect of non-functional *vrille* in cold-acclimated females was of the same magnitude as the differences between *D. montana* and *D. flavomontana* cold tolerance. This highlights the importance of *vrille*, and possibly the whole circadian clock system, in enhancing females' cold tolerance both during the rapid cold hardening occurring in CT_{min} test [81] and during the longer-term cold acclimation. Both traits, short- and long-term cold acclimation, have important ecological implications in cold environments with high daily and seasonal temperature changes. Previous RNAi experiments have verified e.g. *Hsp22 and Hsp23* genes to contribute to CCRT in *D. melanogaster* [12], and *myo-inositol-1-sphosphate synthase* (*Inos*) to contribute to *D. montana* flies' survival during cold stress (5 ˚C), but not to affect their CCRT [13]. *vrille* is an interesting addition to this list, and it also gives new insights on molecular mechanisms underlying insect cold tolerance and cold acclimation.

Conclusions

Studying the mechanisms that generate and maintain variation in species stress tolerances is essential for understanding adaptation processes and predicting the likely outcomes of climate change or invasion biology. Species, whose cold or heat tolerance is tightly linked with photoperiod, may encounter more difficulties in adapting to changing temperature conditions than the ones whose tolerances are regulated by local climatic conditions. We show that insect cold tolerance may rely on different environmental cues and morphological traits even in closely-related species, and that insects' chill coma recovery time is likely affected by stronger selection pressures in nature than chill coma resistance. We also propose that *vrille* gene, and possibly the whole circadian clock system, play an essential role in molecular mechanisms underlying short- and long-term cold acclimation, both of which are ecologically important traits on high latitudes with high daily and seasonal temperature variation. In future, it would be interesting to study the relationship of reproductive stage and cold tolerance traits in individuals originating from diverse climatic conditions to deepen our understanding on adaptation to seasonally varying environments. Also, investigating the effects of silencing the other circadian clock genes under cold environment would give valuable insights on the role of the whole circadian clock system on cold acclimation in insects.

Methods

Variation in cold tolerance and body colour in *D. montana* and in *D. flavomontana* populations

Study species and populations

D. montana and *D. flavomontana* belong to the *montana* phylad of the *virilis* group, and our recent whole genome analyses have shown their divergence time to be ~1 mya (Poikela et al., in preparation). *D. montana* is distributed around the northern hemisphere across North America, Asia and Europe [70], while the distribution of *D. flavomontana* is restricted to North America [69, 70]. 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 [69, 71]).

We investigated cold tolerance traits and measures of body colour and body weight using 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 eliminate plastic responses and 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 [90].

Cold tolerance traits

We investigated variation in female cold tolerance using two well-defined and ecologically relevant methods: chill coma temperature (CT_{min}; also called critical thermal minimum) and chill coma recovery time (CCRT). CT_{min} corresponds to the temperature, at which the fly resists cold until it loses all neurophysiological activity and coordination and falls into a chill coma during a gradual decrease in temperature (reviewed in [17]). In this test, we placed the females individually in glass vials, which were submerged into a 30 % glycol bath. We then decreased the bath temperature from the starting temperature of 19 °C at the rate of 0.5 °C/min and scored the CT_{min} for each fly. The second method, CCRT, measures the time taken from a fly to recover from a standardized exposure time at a chill-coma-inducing temperature (reviewed in [17]). In this test, we submerged the females individually in glass vials into a 30 % glycol bath for 16 hours at -6 °C [64]. After returning the vials into 19 ± 1 °C in fly laboratory, we measured the time required for each female to recover from the chill coma and stand on its legs. CT_{min} tests were replicated 21 times and CCRT tests 20 times with Julabo F32-HL Refrigerated/Heating Circulator and Haake k35 DC50 Refrig Circulating Bath Chiller, respectively. To account for possible variation between replicates, each test included 1-3 females from each strain.

Fly body colour and body weight

We analysed variation in the body colour and body weight (as a proxy of body size) of the same females that had been phenotyped in CT_{min} or CCRT tests. Immediately after the cold tolerance tests, females were put individually into tightly sealed PCR plates and kept in -20 ˚C freezer until measuring their body colour and weight. For body colour measurements, the females were

532 photographed under Leica L2 microscope with 5x magnification, using Leica DFC320 R2
533 camera together with the Leica Application Suite software v4.3.0. Exposure time, zoom factors
534 and illumination level were kept constant, and the photographing arena was surrounded by a
535 plastic cylinder to eliminate glares on the chitinous surface of the fly. All photographs were
536 taken within 3 months after the cold tolerance tests. Images were saved in 24-bit (RGB) format
537 and the colour intensity was measured using grayscale image analysis (ImageJ, [91]); linearly
538 scaling from 0 to 255 (0 = black, 255 = white). We took colour measurements from part of
539 thorax (scutum; see Fig. S2), as our preliminary tests showed that it best incorporates the colour
540 variation among flies (Fig. S2). Body weight was measured by weighting them with Mettler
541 Toledo™ NewClassic Balance (model ME). The weight of the females used in CT_{min} test was
542 measured after females had been frozen for 2 to 4 months, which appeared to be problematic
543 as the females had started to dry and their weight correlated with the freezing time (Fig.
544 S3). The weight of the females used in CCRT tests was measured after 6 to 17 months in
545 freezer. All these females had lost most of their body liquids so that their weight was close to
546 dry weight and freezing time had no effect on it (Fig. S3). Accordingly, only the latter dataset
547 was used in the analyses.

548 *Statistical analyses*

549 We used different statistical models to investigate whether variation in fly cold tolerance, body
550 colour and body weight was associated with latitude, as a proxy of photoperiod, and/or local
551 climatic variables, and whether cold tolerance traits and morphological traits showed a
552 correlation with each other. In these models, we used either CT_{min} data (chill coma temperatures
553 in Celsius degrees + 10 ˚C to prevent negative values from affecting the analysis), CCRT data
554 (in minutes), body colour (measured from CCRT flies) or body weight (mg; measured from
555 CCRT flies) as response variables. *D. flavomontana* CT_{min} data were normally distributed (Fig.
556 S4) and they were analysed with generalized linear mixed model (GLMM) with gaussian
557 distribution (equivalent of linear mixed model). Other datasets showed deviation from the
558 normality (Fig. S4), and they were analysed using generalized linear mixed model (GLMM)
559 with gamma distribution, using *glmmTMB* function from glmmTMB package [92]. Technical
560 replicates and isofemale strains were handled as crossed random effects.

561 In our dataset latitude and altitude were negatively correlated (Pearson correlation coefficient
562 = -0.82) and to prevent this multicollinearity from affecting the analysis, we used only latitude
563 as an explanatory factor. However, we considered the effect of altitude and climatic variables
564 through a principal component analysis (PCA). We downloaded climatic information from
565 WorldClim database v2.1 (2.5 min spatial resolution; current data 1970-2000; [93];
566 http://www.worldclim.org) and extracted 19 bioclimatic variables for each site using their
567 latitudinal and longitudinal coordinates (Table S3; Fig. 1) with raster package v. 2.8-19 [94]. In
568 addition, we obtained growing season length (days) for each site from article by [74] and
569 www.weatherbase.com. Growing season is defined as the average number of days per year
570 when the average daily temperature is at least 5 °C. We performed the PCA on the 19
571 bioclimatic variables, altitude and growing season length (Table S3) to summarize climatic
572 differences on temperature and precipitation in each site using "FactoMineR" package [95].

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

578 scale the variables) and body weight were included in the model selection (Table S6). The best-
579 fit model for CT_{min}, CCRT, body colour and body weight of both species was chosen for further
580 analysis based on Akaike information criterion (AIC) value and Akaike weight (Table S6) using
581 *aictab* function from AICcmodavg package [96]. All the analyses were conducted in R
582 (v1.2.1335-1) and R studio (v3.6.1).

583 **RNA interference (RNAi) on circadian clock gene *vrille* and its effects on fly cold tolerance**

584 *Candidate gene and study material*

585 We used RNAi to silence the expression of one of the key regulators of the circadian
586 behavioural rhythms, *vrille*, and investigated its effects on cold tolerance traits. We performed
587 RNAi studies using only the more cold-tolerant *D. montana*, which has become a model species
588 for studying cold adaption at phenotypic and genetic level (e.g. [63, 74, 97]). Here, we used
589 females of a mass-bred cage population originating from Seward (Alaska, North America; see
590 Fig. 1) to increase genetic diversity. The population cage has been established from the 4th
591 generation progenies of 20 isofemale strains (total of 400 flies) in 2013 and maintained in
592 continuous light at 19 ± 1 °C since the establishment (30 generations). Malt bottles with freshly
593 laid eggs were transferred from the population cage into a climate chamber in LD 18:6
594 (light:dark cycle) at 19 ± 1 °C to reinforce flies' circadian rhythmicity prior to the experiments
595 and ensure they are non-diapausing. Critical day length for the induction of reproductive
596 diapause (CDL; 50 % of the females of given population enter diapause) in 19 °C is LD 17:7
597 in Seward population [74], and thus the females emerging in LD 18:6 can be expected to
598 develop ovaries. After ~4 weeks, we collected newly emerged females (≤ 1 day old) using light
599 CO_2 anaesthesia and placed them back in malt-vials into the above-mentioned conditions.
600 Females were changed into fresh vials once a week until they were used in the experiments at
601 the age of 21 days.

602 *Defining daily expression rhythm of vrille*

603 Expression levels of circadian clock genes are known to show daily fluctuations, and in order
604 to perform RNAi experiments at a right time of the day, we defined the time when the
605 expression of *vrille* is highest at LD 18:6. To do this, we collected females every four hours
606 (i.e. at six time points / day), starting at 6 am when lights were switched on. At each time point,
607 we stored samples of females into -80˚C through liquid N_2 and transferred them later on into
608 RNAlater®-ICE frozen tissue transition solution (Thermo Fisher Scientific). We then checked
609 the size of female ovaries (see [90]) and used only the females with fully developed ovaries
610 (>95 % of the females). For each time point, RNA was extracted from three pools, each of
611 which consisted of three whole females, using ZR Tissue & Insect RNA MicroPrep kit with
612 DNAse treatment (Zymo Research®). RNA purity was analysed with NanoDrop® ND-1000
613 spectrophotometer (Thermo Fisher Scientific) and concentration with Qubit® 2.0 Fluorometer
614 and RNA BR kit (Thermo Fisher Scientific). cDNA was synthesized using equal quantities of
615 RNA (200 ng) with iScript Reverse Transcription kit (Bio-Rad Laboratories®).

616 We measured the expression levels of *vrille* with quantitative real time PCR (qPCR). qPCR
617 primers for *vrille* and reference genes were designed based on *D. montana* genomic sequences
618 under NCBI accession number LUVX00000000 [98], together with information from *D. virilis*
619 exons using Primer3 (primer3.ut.ee) and NetPrimer (www.premierbiosoft.com/netprimer)
620 programs (gene accession numbers and primer sequences in Table S11). qPCR mix contained
621 10 µl of 2x Power SYBR Green PCR Master Mix (Bio-Rad Laboratories), 0.3 µM of each gene-
622 specific primer and 1 µl of cDNA solution. Cycling conditions in Bio-Rad CFX96 instrument

623 were: 3 min at 95 °C, 10 sec at 95 °C, 10 sec at annealing temperature of 53 °C (for reference
624 genes 56 °C) and 30 sec at 72 °C (repeated 40x), followed by melting curve analysis (65-95 °C)
625 for amplification specificity checking. Each run included three technical replicates for each
626 sample and the final threshold value (Cq) was defined as a mean of the technical replicates that
627 produced good quality data. The relative qPCR data was normalised with ΔΔ(Ct) normalisation
628 method [99] using two reference genes, *Tubulin beta chain* (*Tub2*) and *Ribosomal protein L32*
629 (*RpL32*), that showed equal expression levels in all samples (data not shown). Real efficiency
630 values of the genes used in the qPCR are given in Table S11.

631 *Synthesis of double-stranded RNA for RNAi*

632 *LacZ,* which codes a part of a bacterial gene, was used as a control for dsRNA injections. We
633 generated fragments of *vrille* and *LacZ* genes, with the length of 347 and 529 bp, respectively,
634 with PCR (primer information given in Table S11). PCR products were purified with GeneJET
635 Gel Extraction kit (Thermo Fisher Scientific) and cloned using CloneJET PCR Cloning kit
636 (Thermo Fisher Scientific). PCR products were ligated into the vector (pJET1.2/blunt Cloning
637 vector), transformed into *E. coli* Zymo JM109 (Zymo Research) cells, which were grown on
638 Luria Broth (LB) ampicillin plates. Individual colonies were picked up after 16 hours and
639 cultivated overnight in LB solution with ampicillin using a Unimax 1010 shaker with incubator
640 (Heidolph Instruments). The samples from the extracted bacterial solutions were analysed for
641 the size of the products in the second PCR, which was carried out with pJET primers from the
642 cloning kit, followed by agarose gel runs. We then selected the colonies with the right size
643 products for the third PCR using pJET primers, where the R primer contained T7 promoter
644 sequence at the 5' end of the primer (primer sequences in Table S11). PCR products were first
645 purified with GeneJet Gel Extraction kit and then used in transcription synthesis of the double-
646 stranded RNA (dsRNA), using the TranscriptAid T7 High Yield Transcription kit (Thermo
647 Fisher Scientific). Finally, we purified and precipitated the synthesized products with ethanol,
648 suspended them in salt buffer and quantified them using NanoDrop and agarose gel.

649 *RNAi microinjection procedure, response time screening and vrille expression at the chosen*
650 *response time*

651 Injecting dsRNA targeting *vrille* gene is expected to cause gene-specific effects, but it may also
652 cause immune responses and physical damage (injection) in the flies. Accordingly, we used
653 *LacZ* (encoding for bacterial gene) injections as control for both immune response to dsRNA
654 and to physical damage of injections, and no-injection as a baseline control. We checked the
655 effectiveness of RNAi treatment on *vrille* expression 12h, 24h and 48h after injections, at a time
656 of the day when it shows highest expression (see Results). The females were injected into thorax
657 with 138 nl of ~20 µM dsRNA targeting *vrille* or *LacZ* using Nanoject II Auto-nanoliter Injector
658 with 3.5" Drummond glass capillaries (Drummond Scientific) pulled with P-97 Flaming/Brown
659 Micropipette Puller (Sutter Instrument). No-injection control females were not injected, but
660 were otherwise handled in the same way as the injected females. To prevent CO_2 anaesthesia
661 from inducing variation between these groups, we injected six females at a time. For each
662 response time (12h, 24h, 48h) all three treatments (*vrille*, *LacZ*, no-injection) were performed.
663 Each treatment consisted of three pools, each pool containing 10 whole females. After reaching
664 the response time, the females were transferred into -80 ºC through liquid N_2. Then, the females
665 were transferred into RNAlater®-ICE solution and their ovaries were checked as explained
666 above. RNA was extracted from the pools using TRIzol® Reagent with the PureLink® RNA
667 Mini kit (Thermo Fisher Scientific). RNA purity was analysed with NanoDrop and
668 concentration with Qubit and RNA BR kit. cDNA was synthesized using equal quantities of

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

673 *Experimental design for studying the functional role of vrille in cold tolerance*

674 We investigated the role of *vrille* gene in resisting (CT_{min}) and recovering from cold stress
675 (CCRT) in summer-acclimated, non-diapausing *D. montana* females originating from Seward,
676 Alaska. Moreover, we considered the plastic effects of *vrille* during cold acclimation. Prior to
677 performing cold tolerance tests, females were maintained for 16 days in LD 18:6 at 19 ˚C,
678 corresponding to summer conditions at their home site (LD 18:6 was used throughout the
679 experiment). Then, half of these females were subjected to cold acclimation treatment at 6 °C
680 for 3 days (cold-acclimated females; [64]), while the other half was maintained at 19 °C. At the
681 age of 19 days, both cold-acclimated and non-acclimated females were collected from the
682 chambers, anesthetized with CO_2 and injected as described above. They were then placed back
683 to either cold acclimation (6 ˚C) and non-acclimation (19 ˚C) conditions for two more days, as
684 the expression levels of target genes had been found to be lowest 48h after RNAi treatment
685 (Fig. S1). At the age of 21 days, females' cold tolerance was quantified by measuring their chill
686 coma temperature (CT_{min}) or chill coma recovery time (CCRT) using Julabo F32-HL
687 Refrigerated/Heating Circulator. Sample sizes for CT_{min} and CCRT tests were 26-32 and 25-30
688 females per treatment, respectively.

689 *Statistical analyses*

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

694 To test the cold acclimation effect within each treatment group, we compared the cold tolerance
695 of the females that had or had not been cold-acclimated. We then investigated the gene specific
696 effects on cold tolerance by comparing *LacZ*-injected females to *vrille*-injected females and
697 traced possible immune and physical effects of the injections by comparing *LacZ*-injected
698 females to no-injection females. These analyses were performed separately for the cold-
699 acclimated and non-acclimated females. All data showed deviation from normality (Fig. S5)
700 and were analysed with generalized linear mixed model using gamma distribution (GLMM;
701 *glmmTMB* function from glmmTMB package [92]. In the models, response variables were
702 either CT_{min} (Celsius degrees + 10 to prevent negative values from affecting the results) or
703 CCRT (minutes) data, and the test replicates were used as a random effect. All the analyses
704 were conducted in R (v1.2.1335-1) and R studio (v3.6.1).

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712 **Data accessibility**

713 The data will be made available in Dryad after acceptance of the paper.

714 **Author contribution**

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

717 **Conflict of interest**

718 The authors have declared no conflicting interests.

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