JYU DISSERTATIONS 367

Noora Poikela

Mechanisms Underlying Speciation and Adaptation Processes in Two Closely Related *Drosophila virilis* Group Species

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi huhtikuun 9. päivänä 2021 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Science of the University of Jyväskylä,
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JYVÄSKYLÄN YLIOPISTO
UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2021

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Permanent link to this publication: http://urn.fi/URN:ISBN:978-951-39-8581-3

ISBN 978-951-39-8581-3 (PDF)
URN:ISBN:978-951-39-8581-3
ISSN 2489-9003

ABSTRACT

Poikela, Noora
Mechanisms underlying speciation and adaptation processes in two closely related *Drosophila virilis* group species
Jyväskylä: University of Jyväskylä, 2021, 51 p.
(JYU Dissertations
ISSN 2489-9003; 367)
ISBN 978-951-39-8581-3 (PDF)
Diss.

Speciation is a slow process that proceeds through populations' ecological divergence and the development of reproductive barriers. Populations living in the same area (sympatry) are susceptible to the disruptive effects of gene flow and recombination, which can slow down or prevent their divergence. Accordingly, natural selection may favour genetic mechanisms, like chromosomal inversions, which protect divergent loci from the homogenising effects of gene exchange and promote speciation. In this dissertation, I investigated the central aspects of speciation and adaptation using two closely related fly species, *Drosophila montana* and *D. flavomontana*. In the first chapter, I found the reproductive barriers between these species to be strong, but not complete. In *D. flavomontana*, the prezygotic barriers showed signs of reinforcement in sympatric populations, the type of barriers varying according to the length of species coexistence and/or species abundancies. The second chapter showed ecological isolation between *D. montana* and *D. flavomontana* to be enhanced by multiple environmental variables and to be largely based on species differences in cold tolerance. The third chapter suggested that chromosomal inversions had originated already before the species' split, where they may have played an important role in the development of early reproductive barriers and/or ecological differences between local populations of the ancestral form. The last chapter, where I performed repeated interspecific backcrosses, indicated that the X chromosomal inversions, together with an incompatibility locus residing within them, effectively prevent gene flow from *D. montana* to *D. flavomontana*. Overall, this dissertation gives a comprehensive overview of the mechanisms underlying speciation and adaptation processes in these two species. It supports the existing speciation theories, but also brings up new perspectives, and shows that finding the final answers in speciation research is extremely challenging.

Keywords: Chromosomal inversions; cold tolerance; *Drosophila*; genetic incompatibilities; reinforcement; reproductive barriers; speciation.

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TIIVISTELMÄ

Poikela, Noora
Lajiutumiseen ja sopeutumiseen liittyvät tekijät kahdella lähisukuisella *Drosophila virilis* –ryhmän lajilla
Jyväskylä: Jyväskylän yliopisto, 2021, 51 s.
(JYU Dissertations
ISSN 2489-9003; 367)
ISBN 978-951-39-8581-3 (PDF)
Diss.

Lajiutuminen on hidas prosessi, joka pohjautuu sekä populaatioiden sopeutumiseen erilaisiin elinympäristöihin että niiden välisten lisääntymisesteiden kehittymiseen. Samoilla alueilla elävät populaatiot (sympatria) ovat alttiita geenivirran ja rekombinaation vaikutuksille, jotka voivat hidastaa tai estää eriytymistä. Tällöin luonnonvalinta voi suosia mekanismeja, kuten kromosomin osien ja geenijärjestyksen kääntymistä (inversio), jotka vähentävät rekombinaatiota ja edesauttavat lajiutumista. Tutkin tässä väitöskirjassa lajiutumiseen ja sopeutumiseen liittyviä prosesseja kahdella lähisukuisella kärpäslajilla, *Drosophila montanalla* ja *D. flavomontanalla*. Ensimmäinen osatutkimus osoitti, että lajien väliset lisääntymisesteet ovat voimakkaita, mutta eivät täydellisiä. Lisäksi *D. flavomontanalla* tsygootin muodostumista edeltävät lisääntymisesteet osoittivat vahvistumisen merkkejä sympatrisissa populaatioissa, esteen tyypin vaihdellessa lajien välisen kontaktin pituuden ja/tai lajien runsaussuhteiden mukaan. Toisen osatutkimuksen tulokset osoittivat, että *D. montanan* ja *D. flavomontanan* ekologinen isolaatio perustuu useisiin ympäristömuuttujiin ja eroihin lajien kylmänkestävyydessä. Kolmas osatutkimus antoi viitteitä inversioiden merkityksestä varhaisten lisääntymisesteiden ja ekologisten erojen muodostumisessa jo ennen lajien eriytymistä. Viimeinen osatutkimus, jossa risteytin risteymäjälkeläisnaaraita kantalajien koiraiden kanssa, osoitti että X-kromosomissa sijaitseva geenilokus estää yhdessä inversioiden kanssa tehokkaasti geenien siirtymistä *D. montanalta D. flavomontanalle*. Väitöskirjani antaa kattavan kuvan näiden kahden lajin eriytymiseen vaikuttaneista tekijöistä. Sen tulokset tukevat olemassa olevia lajiutumisteorioita, mutta nostavat esille myös uusia näkökulmia. Toisaalta tutkimukseni osoittavat, että lopullisten vastausten löytäminen lajiutumistutkimuksessa on hyvin haasteellista.

Avainsanat: *Drosophila*; geneettiset yhteensopimattomuudet; kromosomin kääntymä (inversio); kylmänkestävyys; lajiutuminen; lisääntymisesteet; lisääntymisesteiden vahvistuminen.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–IV.

I Noora Poikela, Johanna Kinnunen, Mareike Wurdack, Hannele Kauranen, Thomas Schmitt, Maaria Kankare, Rhonda R. Snook and Anneli Hoikkala. 2019. Strength of sexual and postmating prezygotic barriers varies between sympatric populations with different histories and species abundances. *Evolution* 73(6): 1182-1199.

II Noora Poikela, Venera Tyukmaeva, Anneli Hoikkala, and Maaria Kankare 2021. Multiple paths to cold tolerance: the role of environmental cues, morphological traits and the circadian clock gene *vrille*. Submitted manuscript.

III Noora Poikela, Dominik R. Laetsch, Konrad Lohse and Maaria Kankare. Ancestrally polymorphic chromosomal inversions as potential drivers of speciation. Manuscript.

IV Noora Poikela, Dominik R. Laetsch, Maaria Kankare, Anneli Hoikkala[1] and Konrad Lohse[1]. Experimental introgression in *Drosophila* species: asymmetric postzygotic isolation associated with chromosomal inversions and an incompatibility locus on the X chromosome. Manuscript. [1] Shared last authorship

Responsibilities of the authors:

	I	II	III	IV
Study design	NP, AH	NP, MK, AH, VT	NP, MK	NP, AH, KL
Experiments	NP, AH, JK	NP		NP
Laboratory work	NP, MK, RS	NP, VT, MK	NP	lab assistant
Pheromone analysis	TS, MW, HK			
Statistical analysis	NP	NP	NP	NP
Bioinformatics			NP, DL	NP, DL
Demographic modelling			NP, DL, KL	
Gene analysis			NP, MK	NP, AH, MK
Mathematical simulations				KL
Drafting the manuscript	NP, AH, RS	NP	NP	NP, AH
Finalising	all authors	all authors	all authors	all authors

NP = Noora Poikela, MK = Maaria Kankare, AH = Anneli Hoikkala, VT = Venera Tyukmaeva
KL = Konrad Lohse, DL = Dominik Laetsch, RS = Rhonda Snook , JK = Johanna Kinnunen
TS = Thomas Schmitt, MW = Mareike Wurdack, HK = Hannele Kauranen

1 INTRODUCTION

1.1 A broad view of speciation

Speciation is a major evolutionary process that contributes to the patterns of biodiversity (e.g. Gavrilets and Losos 2009). Biological species concept defines species as a group of individuals that successfully interbreed and produce fertile hybrids, but are reproductively isolated from other such groups (Coyne *et al.* 2004 and references therein). In the past years, it has become increasingly clear that species are not entirely isolated from one another, since hybridisation between close relatives is common (e.g. Mallet 2005). The mixing of diverged genes and gene complexes and/or different chromosomal rearrangements in hybrids can have unpredictable consequences at the individual level. While some hybrid individuals may not survive a day, others may be superior compared to their parental species. For example, McQuillan *et al.* (2018) showed that the hybrids between two *Poecile* birds are less fit compared to their parents due to deficiencies in remembering where they had hidden food. On the other hand, Griebel *et al.* (2015) found the hybrids between two *Daphnia* water flea species to dominate a lake community for two years through their better overwintering survival and fecundity than either one of the parental species. Effects of hybridisation are not limited to the level of individuals, but they can have far-reaching, highly complex evolutionary consequences at the species level, and ultimately at the level of biodiversity (Abbott *et al.* 2013, Taylor and Larson 2019). Moreover, human-induced environmental changes, including global climate warming, intentional and unintentional invasion of species and habitat fragmentation and destruction, bring previously geographically and/or ecologically isolated closely related species in close contact. This, in turn, increases the likelihood for their hybridisation and may lead to major shifts and reductions in overall biodiversity (Abbott 1992, Perry *et al.* 2002, Rhymer and Simberloff 2007, Todesco *et al.* 2016, Taylor and Larson 2019). Thus,

understanding the forces that operate on species formation and maintenance is more crucial than ever.

1.2 Complexity of speciation process

For a long time one of the keystones of speciation research has been to understand whether speciation requires a geographical barrier that restricts gene flow between diverging populations (allopatry), or whether it can also occur with a limited amount of gene flow (parapatry) or with unrestricted gene flow (sympatry) (Butlin *et al.* 2012; Feder *et al.* 2012; Martin *et al.* 2013). In allopatric speciation, adequate time for random drift and indirect effects of selection inevitably lead to genetic differentiation between populations and facilitate a build-up of reproductive barriers (Coyne and Orr 1989, 2004, Seehausen *et al.* 2014). Speciation with gene flow, on the other hand, can proceed via direct effects of natural and sexual selection (Coyne and Orr 2004), but is prone to the homogenising effects recombination and gene flow (Felsenstein 1981, Coyne and Orr 2004). Accordingly, sympatric speciation requires simultaneous development of sufficient ecological differences and reproductive isolation, which is most likely to occur through the accumulation of mutations on chromosomal regions of low recombination, like chromosomal inversions (Felsenstein 1981, Wu 2001, Butlin 2005, Feder *et al.* 2012, Ortiz-Barrientos *et al.* 2016). However, the expansion of genome sequencing and effective computational approaches have allowed evolutionary biologists to move from the strict division of allopatric and sympatric speciation to evaluating the magnitude and the timing of interspecific gene flow, introgression (Anderson and Hubricht 1938), across species' divergence. Indeed, ancient and more recent introgression has been observed between lineages e.g. in insects, birds, mammals and plants (Marsden *et al.* 2011, Garrigan *et al.* 2012, Martin *et al.* 2013, Lamichhaney *et al.* 2015, Liu *et al.* 2015, Pease *et al.* 2016, Suvorov *et al.* 2020 and references therein). Measuring the magnitude of adaptive divergence and reproductive barriers, as well as tracing the evolutionary history of species and the occurrence of introgression, helps us take one step closer to understanding how species diverge in the face of gene flow and what kind of evolutionary consequences it can have.

1.3 Ecological differentiation and natural selection

Ecological divergence has been suggested to be the earliest stages of speciation in lineages that are in contact with each other, since no taxa can live in exactly the same ecological niche with another one (Coyne and Orr 2004). One of the clearest signs of natural selection acting on populations derives from inter- and intraspecific variation in adaptively important traits across climatic gradient

(cline) and emphasizes the genetic basis of these traits. Ectothermic species are particularly susceptible to changes in their external environment, since their locomotion, growth, survival and reproduction are greatly affected by environmental temperature (e.g. Deutsch *et al.* 2008), and both cold and hot temperatures can seriously damage cellular and molecular components of organisms (e.g. Denlinger and Yocum 1998, Findsen *et al.* 2014). Accordingly, species' distribution often correlates remarkably well with their physiological ability to tolerate local temperature variation, and especially the cold tolerance is a good predictor of species distribution (e.g. Addo-Bediako *et al.* 2000, Kimura 2004, Sunday *et al.* 2011). Inter- and intraspecific variation in cold tolerance across latitudinal or altitudinal clines have been detected e.g. in woodlice, flies and ants (Gibert *et al.* 2001, Hallas *et al.* 2002, Hoffmann *et al.* 2002, Castañeda *et al.* 2005, Overgaard *et al.* 2011a, b, Kellermann *et al.* 2012, Maysov 2014). Traits linked to thermal regulation, such as body colour or body size, often shows similar latitudinal clines as cold tolerance (reviewed in Clusella-Trullas *et al.* 2007, Chown and Gaston 2010), but it is less clear whether these traits are directly correlated. Cutter and Gray (2016) emphasize that harsh environmental conditions and related selection pressures in high latitudes can effectively drive local adaptation and ecological speciation.

Plastic responses to temperature changes are expected to be beneficial in environments with high variation in environmental temperature and when there is no superior phenotype that would be favoured by selection in all conditions (reviewed in Ghalambor *et al.* 2007). For example, Klok and Chown (2003) and Ayrinhac *et al.* (2004) have shown that inherent genetic clinal variation explains only a fraction of the variation in cold tolerance, while plastic responses (acclimation) account for most of it. Acclimation, which can last from days to weeks (seasonal acclimation), or from minutes to hours (rapid cold-hardening), helps organisms to survive over longer and shorter cold periods (Doucet *et al.* 2009, Teets *et al.* 2012, Teets and Denlinger 2013). Both long- and short-term acclimation involves adjustments e.g. to ion transport and membrane restructuring to increase membrane fluidity and maintenance of cell functioning at low temperatures and can be triggered by decreasing temperatures and/or shortening day lengths (Vesala *et al.* 2012a, Teets and Denlinger 2013, MacMillan *et al.* 2015). Moreover, cold acclimation has been found to be accompanied by expression changes in circadian clock genes e.g. in several *Drosophila* species and in *Gryllus pennsylvanicus* crickets (Vesala *et al.* 2012b, Parker *et al.* 2015, MacMillan *et al.* 2016, Des Marteaux *et al.* 2017, Enriquez and Colinet 2019), potentially indicating their involvement in plastic responses to cold. A functional link between circadian clock system and cold acclimation has been verified in plants (e.g. Espinoza *et al.* 2008), but in insects such link is still missing.

1.4 The diversity of reproductive barriers and the effects of selection and drift on their formation

1.4.1 Reproductive isolation is often a consequence of multiple barriers

Although ecological divergence is a prequisite for species living in contact with each other, the heart of speciation lies on the formation of reproductive barriers (or reproductive isolation mechanisms). Speciation is usually an extremely slow process, which occurs as multiple reproductive barriers develop and interact with each other (Coyne and Orr 2004, Butlin and Smadja 2018, Kulmuni *et al.* 2020). Reproductive barriers can be induced by any trait or mechanism that prevent or reduce species' hybridisation, and they can broadly be categorised into ecological and non-ecological (sexual) premating barriers, postmating-prezygotic (PMPZ) barriers and postzygotic barriers (Coyne and Orr 2004).

1.4.2 Ecological and sexual premating isolation

Premating barriers consist of a wide variety of factors that act before sperm or pollen transfer, and they can be based on ecological factors and/or mate preferences. Ecological isolation arises when the encounters and matings between individuals of two populations are reduced due to differences in their habitats and/or in the timing of reproduction (Coyne and Orr 2004). It is based on genetic preferences or tolerances and it arises as a direct by-product of adaptation to local environment (Nosil 2012). Two sibling species of *Rhagoletis pomonella* fruit flies offer an excellent example of the magnitude and variety of factors inducing and maintaining ecological isolation. Hybrids of these species are easy to obtain in laboratory conditions, but they are never found in nature, as one of the species inhabits apples and hawthorn, while the other one uses only blueberries (Feder and Bush 1989). The usage of specific host plants has minimised encounters between the flies of different species and host-races, and the differences in host plants' peak fruiting time has also induced partial temporal isolation in the timing of their pupal diapause and mating (Feder *et al.* 2003).

One of the most intensively studied forms of premating isolation is sexual (or behavioural) isolation, which is based on genetically determined species differences in male-female interactions, courtship signals and requirements for these signals (Chenoweth and Blows 2006). In insects, sexual selection is typically based on visual, auditory and chemosensory signals delivered during the courtship rituals to attract and recognise mates of the same species (Ewing 1983, Greenspan and Ferveur 2000). For example, courtship can be based on wing displays, songs, colour and/or pheromones (reviewed in Greenspan and Ferveur 2000). The courtships of even closely-related species may rely on different sensory modalities (production and reception of specific courtship cues) (Gleason *et al.* 2012, Giglio and Dyer 2013, Colyott *et al.* 2016).

Some sexual signals can evolve under both natural and sexual selection, which may fasten their evolution (Nosil 2012, Wang *et al.* 2021). For example, cuticular hydrocarbons (CHCs), a broad group of fatty-acid derived carbon-chain compounds on insect cuticle, can have various roles in insect communication, ecological divergence and stress tolerance. In *Drosophila*, CHCs are typically low volatile substances that are received at a relatively short distance by olfactory (smell) organs of the head and/or by gustatory (taste) organs mostly found on the legs (Stocker 1994, Ferveur 2005). CHCs are produced and received by both females and males, and thus they can affect mate choice and species' recognition in both sexes (Coyne and Oyama 1995, Dyer *et al.* 2014). CHCs are susceptible to changes in diet (Fedina *et al.* 2012), which can further enforce sexual isolation between ecologically diverged species (Wang *et al.* 2021). They show association with the properties of insect cuticula and play an important role e.g. in cold and desiccation tolerance (Foley and Telonis-Scott 2011, Chung and Carroll 2015, Dennis *et al.* 2015). Insects use their olfactory and gustatory organs also to localise food resources and suitable oviposition sites, as well as to avoid toxic substances and predators (Vosshall 2000, Wang *et al.* 2021). Perhaps due to their multifaceted role in mate choice and adaptation, odorant-binding proteins and olfactory and gustatory receptors are often among the most rapidly diverging systems across *Drosophila* species (Clark *et al.* 2007; McBride 2007; Smadja and Butlin 2009) and potentially one of the first steps towards reproductive isolation.

1.4.3 Postmating-prezygotic isolation

Postmating-prezygotic (PMPZ) isolation comprises a wide variety of factors that can reduce or prevent hybridisation between diverged lineages after mating, but before fertilisation (e.g. Price et al. 2001). PMPZ isolation arises from inherent problems in sperm or pollen transfer and storage, and/or in fertilisation between the individuals of different species. For example, sperm may be transferred in smaller amounts during interspecific copulations than in intraspecific ones or the females can remove it from their spermathecae afterwards (e.g. Price *et al.* 2001). If sperm transfer is successful, PMPZ isolation can result from sperm inviability, problems in storing or releasing the sperm or an inability of the sperm to pass the egg membrane and fertilise it (Howard 1999, Wirtz 1999, Howard *et al.* 2009). In some crosses, sperm may induce an insemination reaction where female reproductive tract swells and prevents sperm usage (Patterson 1946), or it may not stimulate female oviposition (Price *et al.* 2001). These problems typically involve protein incompatibilities between female reproductive tract and male seminal fluids, or between gametes (Howard *et al.* 2009, Garlovsky *et al.* 2020).

1.4.4 Postzygotic isolation

Postzygotic isolation manifests itself as low hybrid fitness and it can be based on extrinsic and intrinsic factors. Extrinsic barriers are environmental-

dependent factors, like challenges to find a suitable ecological niche or to attract the opposite sex and reproduce, and is potentially associated with similar genetic architecture and selection pressures as ecological or sexual isolation (Coyne and Orr 2004, Seehausen *et al.* 2014). Intrinsic postzygotic isolation, on the other hand, is independent of the external environment and evident as full or partial physiological inviability or sterility of hybrid progeny (Coyne and Orr 2004, Seehausen *et al.* 2014). Hybrid sterility or inviability often result from negative epistatic interactions between two or more loci, known as Bateson-Dobzhansky-Muller incompatibilities (BDMIs; see Orr 1995). BDMIs arise when alleles established in one of the species are functionally incompatible with alleles at interacting loci from another species (Orr 1995, Presgraves 2010a, b). BDMIs do not evolve by the direct effects of selection, but as a by-product of divergent selection operating on other traits (Coyne and Orr 2004, Seehausen *et al.* 2014, Kulmuni and Westram 2017). Accordingly, mutations accumulating in diverging species over time can generate enough divergence to lead to strong incompatibilities (Barbash *et al.* 2004, Presgraves 2010a).

Based on several independent evidence, the X chromosome (or bird and *Lepidoptera* equivalent, the Z) often plays a special role in the evolution of BDMIs in animals. First, the loci contributing to hybrid inviability and sterility have been found to map disproportionately on the X or Z chromosome e.g. in *Drosophila* and *Lepidoptera* species ("the large X-effect") (Coyne and Orr 1989, Jiggins *et al.* 2001, Tao *et al.* 2003, Masly and Presgraves 2007). Second, the heterogametic sex (XY, ZW) of essentially all taxa suffers more from hybrid inviability or sterility than the homogametic one ("Haldane's rule"; (Haldane 1922, Orr 1997, Presgraves 2010c, Schilthuizen *et al.* 2011). Third, the X chromosome often shows reduced levels of introgression like observed e.g. in Denisovans and Neanderthals (Sankararaman *et al.* 2016), *Heliconius* butterflies (Martin *et al.* 2013), *Drosophila* flies (Garrigan *et al.* 2012, Turissini and Matute 2017) and *Anopheles* malaria mosquitoes (Fontaine *et al.* 2015). Several competing theories have been proposed to explain these X-related patterns of postzygotic isolation (reviewed in Coyne 2018) and will be evaluated in the context of the Results in the Discussion.

1.5 Reinforcement of prezygotic barriers and other evolutionary consequences of barrier leakage

Hybridisation and introgression can have pervasive effects on species' divergence, depending on its costs and benefits (Abbott 1992, Burke and Arnold 2001, Servedio and Noor 2003, Currat *et al.* 2008, Abbott *et al.* 2013, Taylor and Larson 2019). First, if reproductive barriers between lineages are weak, they may break down and further diminish lineage differentiation and lead to species' fusion or extinction (Servedio and Noor 2003, Taylor *et al.* 2006). Second, lineages with stronger barriers may acquire adaptive genetic variation

from closely related species through introgression (Lewontin and Birch 1966, Barrett and Schluter 2008, Currat *et al.* 2008, Abbott *et al.* 2013, Hedrick 2013). For example, up to 8.8 % of Alaskan brown bear genomes contain polar bear ancestry (Cahill *et al.* 2015), which may have contributed to the transfer of salt tolerance allele and brought selective advantage for coastal brown bears (Miller *et al.* 2012). Third, if species produce unfit hybrids due to strong postzygotic isolation, natural selection is expected to strengthen earlier acting barriers in sympatric populations of the species (Dobzhansky 1940, Servedio and Noor 2003). Such reinforcement of prezygotic barriers has been identified as increased mate discrimination in several animal species (Noor 1995, 1999, Kronforst *et al.* 2007, Lemmon 2009, Dyer *et al.* 2014). However, basically any trait that prevents costly parental investment in unfit hybrid offspring can get reinforced in sympatric populations, including ecological divergence and PMPZ barriers (reviewed in Coughlan and Matute 2020), although these are less studied. Increased divergence of species-specific traits and their preferences in sympatric populations may also generate reproductive isolation between conspecific allopatric populations, namely cascading reinforcement, and initiate new speciation process (Ortiz-Barrientos *et al.* 2009, Comeault *et al.* 2016, Pfennig 2016).

Species differences in the cost of hybridisation, and the selection pressures to avoid it, are predicted to lead to the evolution of asymmetric prezygotic barriers (Jaenike *et al.* 2006, Cooley 2007). Accordingly, reinforcement may be influenced by the relative abundancies and population sizes of the interacting species. Females of the rarer species are expected to have higher hybridisation costs than those of the more abundant species, because they encounter more heterotypic mating attempts ("rarer-female hypothesis"; Noor 1995; Yukilevich 2012). Reinforcement could thus be expected to target on the species recognition ability of the rarer females (Yukilevich 2012). Alternatively, females' ability to distinguish heterospecific males from the conspecific ones may weaken when population density is small and the chances to encounter conspecific mating partners are low (reviewed in Wirtz 1999). In the presence of conspecific males, females usually reject heterospecific males, while in their absence females sometimes accept courtships by males of other species. Males rarely exercise such discrimination, and thus heterospecific matings occur mainly between the females of a rare species and the males of a common species (reviewed in Wirtz 1999).

1.6 The role of chromosomal inversions in the formation of ecological divergence and reproductive isolation

Chromosomal regions that are prone to breakage, like repetitive sequences, may contribute to the formation of chromosomal inversions, where gene order is reversed (reviewed in Kapun and Flatt 2018). Like mutations in general,

inversions evolve under selection and drift (reviewed in Kirkpatrick 2010). Inversions are expected to be highly beneficial if they protect locally adapted allele complexes from the homogenising effects of recombination and gene flow (Navarro and Barton 2003, Kirkpatrick and Barton 2006). Furthermore, inversion breakpoints may serve as hot spots for mutations creating new genetic variation, as alterations in their DNA sequences may change the reading frames or expression patterns of genes located on these regions (Matzkin *et al.* 2005, Kirkpatrick and Barton 2006, Castermans *et al.* 2007). Finally, neutral inversions may pick up a beneficial mutation by chance and spread them through population via the hitchhiking-effects of the positively selected allele (Kirkpatrick and Barton 2006, Charlesworth and Barton 2018).

Once established, inversions can generate speciation in several ways. They can directly act as barriers to gene flow by inducing problems in chromosome pairing during meiosis, which can further lead to reduced hybrid fertility and viability (Coyne & Orr, 2004; Hoffmann & Rieseberg, 2008; Rieseberg, 2001). These problems are partly avoided in *Drosophila* species, where malformed gametes become polar cells (Sturtevant and Beadle 1936, Hoffmann and Rieseberg 2008). Perhaps more importantly, after generations of limited recombination and gene flow, inversions tend to become hotspots for positively selected genetic differences and an incidental build-up of BDMIs (Kirkpatrick, 2010; Navarro & Barton, 2003; Noor et al., 2001; Rieseberg, 2001).

Demonstrating whether, and at which point of the speciation process, inversions may contribute to species divergence is not straightforward. First, increased genetic divergence between closely related is expected to be accumulated on inverted rather than collinear chromosomal regions, as observed e.g. in *Drosophila* flies (Noor *et al.* 2007, Kulathinal *et al.* 2009, Lohse *et al.* 2015), *Helianthus* sunflowers (Barb *et al.* 2014), *Sorex* shrews (Basset *et al.* 2006) and between humans and chimpanzees (Farré *et al.* 2013). Second, the loci that contribute to local adaptation, prezygotic barriers and hybrid sterility are expected to be found largely within inverted regions, as observed in some species of *Drosophila* (Noor *et al.* 2001, Brown *et al.* 2004, Khadem *et al.* 2011) and *Mimulus* monkeyflowers (Lowry and Willis 2010, Fishman *et al.* 2013). Experimental speciation studies may provide valuable sources for identifying barrier loci, specifically the ones involved in BDMIs (Ravinet *et al.* 2017, Moran *et al.* 2019, White *et al.* 2020). Third, demographic modelling is required for estimating the time of divergence of inversions compared to the species' split as well as the effects of introgression in the divergence process. For example, analysis of the likely evolutionary history of *Drosophila mojavensis* and *Drosophila arizonae* showed that introgression between the species had ceased around the time when several inversions had originated, suggesting the role of inversions in building up reproductive isolation and protecting divergent loci from the negative effects of hybridisation and introgression (Lohse *et al.* 2015). In contrast, Fuller *et al.* (2018) proposed that species-specific inversions of *Drosophila permilis* and *Drosophila pseudoobscura* had arisen already in the ancestral population of the species before the species' split, where they may have fuelled the build-up of BDMIs and reproductive isolation of the species.

1.7 Study species

My study species, *Drosophila montana* and *Drosophila flavomontana*, belong to *montana* subphyla of *Drosophila virilis* species group (Patterson 1952, Throckmorton 1982), which is one of the most cold-tolerant groups of *Drosophila* (Kellermann *et al.* 2012). *D. montana* has distributed around the northern hemisphere with southern extensions into the high-altitude Rocky Mountains and the low-altitude coastal regions of North America, while *D. flavomontana* is found only in North America (Throckmorton 1982). Population structure of both species is a mosaic due to the flies' dependence on suitable water bodies and species-specific host trees (Patterson, 1952, Throckmorton, 1982). Presently, these species have both sympatric and allopatric populations in North America (Fig. 1). Populations of *D. montana* and *D. flavomontana* in the Rocky Mountains are partly separated by altitude, as *D. montana* is found at altitudes from 1400 m to well over 3000 m, and *D. flavomontana* mainly below 2000 m (Patterson 1952, Throckmorton 1982). Some hybrids between the species have been found in the regions of overlap (usually from 1800 to 2100 m elevation; Patterson 1952; Throckmorton 1982). Both species has populations also on low-altitudes on the western coast of North America, where *D. flavomontana* has spread relatively recently and its population size is relatively small (Patterson 1952, Poikela *et al*. 2019, Chapter I). Hereafter, the sympatric populations in the Rocky Mountains are referred as "mountain sympatry" and the ones on the western coast as "coastal sympatry". Moreover, *D. montana* inhabits high latitudes in Alaska, where *D. flavomontana* has not spread. These populations are purely allopatric, while some of the Rocky Mountains sites can be called parapatric/allopatric. Previous studies have shown that reproductive isolation between these species is strong but not complete (Patterson 1952), and that the species have both fixed and polymorphic chromosomal inversions (Stone *et al.* 1960, Schäfer *et al.* 2010). These studies, as well as the studies on cold adaptation (Parker *et al.* 2015, 2018, Kauranen *et al.* 2019, Tyukmaeva *et al.* 2020, Wiberg *et al.* 2020) and sexual selection (Saarikettu *et al.* 2005, Klappert *et al.* 2007) of *D. montana* flies, give a good background for further studies on the genetics of speciation and adaptation. Overall, *D. montana* and *D. flavomontana* offer an ideal species-pair to find answers for ambitious questions involved in these fields, since they show incomplete reproductive isolation and latitudinal and altitudinal differences in their distribution ranges, and they possess several species-specific chromosomal inversions.

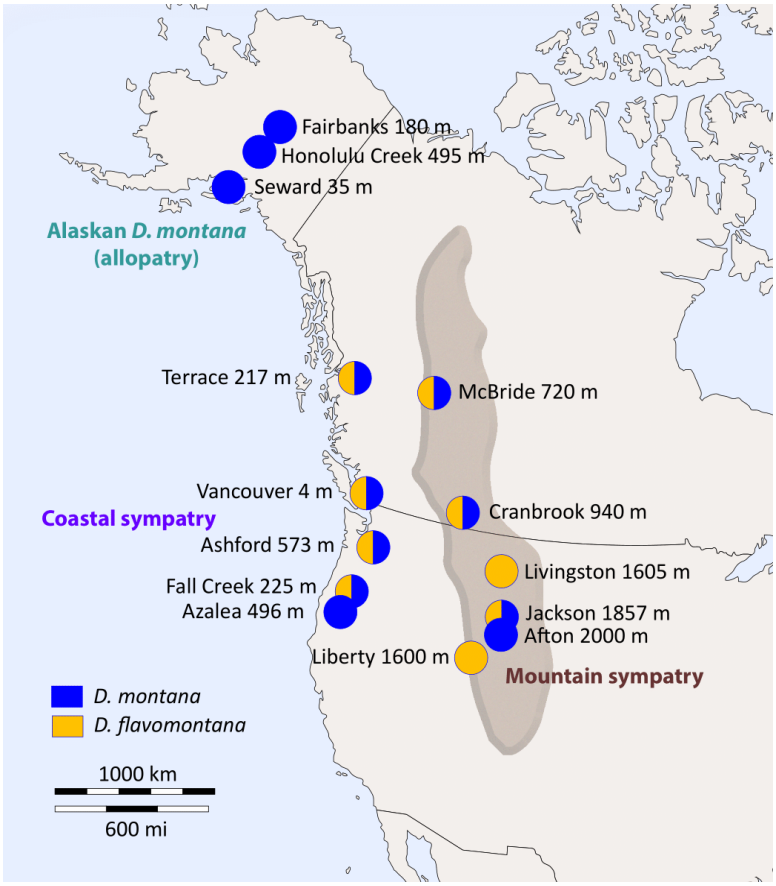

FIGURE 1 Collecting sites of the used *D. montana* and *D. flavomontana* populations in the Rocky Mountains (grey area), in the western coast and in Alaska. Pie charts of each collecting site refer to the presence of one or both species in each site. Detailed information e.g. on the used study populations is found in each of the chapters. The map template was obtained from https://d-maps.com/carte.php?num_car=5082&lang=en

1.8 Aims of the study

In this thesis, I used a variety of phenotypic, genetic, genomic and computational approaches to investigate the processes involved in speciation and adaptation and the links between them. My first aim was to study the evolution of reproductive barriers between sympatric and parapatric/allopatric *D. montana* and *D. flavomontana* populations in North America and to trace climatic factors and tolerances (especially cold tolerance) affecting the distribution of these species. My second aim was to find out whether species-specific chromosomal inversions have played a role in the development of reproductive barriers and ecological differences between diverging lineages before and/or after the species' split. Finally, I performed repeated interspecific backcrosses to recognize chromosomal regions restricting introgression between these species, and to identify the effects of inversions and BDMIs within these regions. The main questions of my thesis were:

1. What kind of reproductive barriers prevent hybridization between *D. montana* and *D. flavomontana*? Do prezygotic barriers show signs of reinforcement between and/or within the species, and does the occurrence of reinforcement depend on species' relative abundancies or evolutionary history?
2. Do *D. montana* and *D. flavomontana* populations show ecological isolation based on inter- and intraspecific genetic variation in their cold tolerance?
3. Have species-specific chromosomal inversions contributed to species' divergence before and/or after the species' split?
4. Is introgression between *D. montana* and *D. flavomontana* restricted in specific chromosomal regions and do these regions contain inversions and possible BDMIs? Does the X chromosome play a special role in the maintenance of species integrity?

2 MATERIALS AND METHODS

Detailed descriptions on materials and methods used in different studies are found in the respective chapters, and I give here only a brief overview. Phenotypic experiments were performed using isofemale strains, established from the progenies of fertilized wild-caught females, except the RNA interference (RNAi) experiment which was done using a mass-bred cage population. Short-read whole-genome Illumina data were obtained by sequencing the single wild-caught females that had been used to establish the isofemale strains and by sequencing the laboratory-produced backcross hybrids and their parents. Finally, some isofemale strains of both species were sequenced with long-read PacBio (Pacific Biosciences) sequencing to obtain enough genomic data for generating contiguous genome assemblies and characterising large species-specific inversions.

In the first chapter, I performed sensory deprivation experiments to study female requirements for different courtship signals, and analysed variation in courtship songs and CHCs both between and within the species. Then I made multiple- and no-choice mating experiments to measure the strength of sexual isolation between *D. montana* and *D. flavomontana* flies in the presence of both conspecific and heterospecific males, or only heterospecific males, respectively. I also performed similar tests between *D. flavomontana* flies originating from different populations. I studied the strength of PMPZ barriers between the species by investigating females' sperm storage ability and egg fertilisation after conspecific and heterospecific matings, as well as between *D. flavomontana* flies originating from different populations. Finally, I studied postzygotic isolation as species' ability to produce fertile interspecific hybrids and counted the hybrid fertility.

In the second chapter, I studied ecological isolation between *D. montana* and *D. flavomontana*. I first performed principal component analysis (PCA) on bioclimatic variables around the species distributions, and measured variation in fly cold tolerance, body colour and body size both between and within the species. Fly cold tolerance was measured using two ecologically relevant methods (CT_{min} = gradual decrease in temperature from 19 °C at the rate of

0.5 °C/min until chill coma; CCRT = time required for recovery after 16h at -6 °C). Moreover, I studied the role of a circadian clock gene *vrille* in the cold tolerance and cold acclimation ability of *D. montana* females by silencing its expression with RNAi.

In the third chapter, I investigated the role of chromosomal inversions in the divergence of *D. montana* and *D. flavomontana*. I first performed genome assemblies and annotations of the species to generate contiguous reference assemblies, and characterised large (>1Mb), high-frequency species-specific inversions between the species. Then I traced the role of inversions and postdivergence gene flow in the evolutionary history of *D. montana* and *D. flavomontana* using demographic modelling and gene enrichment analyses.

Finally, in the fourth chapter, I identified the chromosomal regions that prevented introgression in the sequenced 2nd generation backcross (BC_2) hybrids towards both species (experimental introgression). Only the BDMIs with dominant epistatic effects could be detected since only female hybrids were sequenced. Here the observed amount of introgression in BC_2 females was contrasted with the expected one in *in silico* replicates. I also traced diverged, non-introgressed X-chromosomal loci that could be sensitive to hybrid incompatibilities.

3 RESULTS AND DISCUSSION

3.1 Signatures of reinforcement at the levels of premating and postmating-prezygotic barriers (I)

The key prediction of the reinforcement theory is that accelerated evolution of prezygotic barriers during sympatry occurs to avoid the costs involved in the production of low fitness hybrids (Dobzhansky 1940, Servedio and Noor 2003). In addition to completing speciation, species' interactions and the reinforcement of their reproductive barriers may initiate new speciation through cascading reinforcement of barriers between conspecific populations (Ortiz-Barrientos *et al.* 2009, Comeault *et al.* 2016, Pfennig 2016). In this chapter, I studied the factors underlying reproductive isolation between *D. montana* and *D. flavomontana* and traced possible reinforcement of prezygotic barriers between the species at the levels of premating and postmating-prezygotic (PMPZ) barriers in two types of sympatries. Moreover, I studied whether the strengthening of species-specific barriers had induced reproductive isolation between conspecific populations of *D. flavomontana* that are or are not in contact with *D. montana*. Mountain sympatry represents here the old contact zone of *D. montana* and *D. flavomontana*, where both species have relatively large population sizes. Coastal sympatry, on the other hand, is a relatively new contact zone and the population size of *D. flavomontana* on this area is quite small.

Strong postzygotic isolation is a prerequisite for the reinforcement of reproductive barriers that function before zygote formation (Dobzhansky 1940, Servedio and Noor 2003), and thus I first studied the types and the strengths of these barriers between *D. montana* and *D. flavomontana*. The viability and fertility of interspecific hybrids were highly asymmetric between in the reciprocal crosses between these species. Hybrid production in crosses between *D. montana* females and *D. flavomontana* males was almost non-existent, which was partly explained by the low number of matings. Hybrid production

between *D. flavomontana* females and *D. montana* males, on the other hand, was more successful. Here, roughly half of the F_1 females and all F_1 males were sterile, which agrees well with Haldane's rule (Haldane 1922, Coyne and Orr 1989, Tao *et al.* 2003, Masly and Presgraves 2007). Given the strong postzygotic isolation in both reciprocal crosses, selection can be predicted to have strengthened prezygotic barriers in sympatric populations either at the level of premating (sexual) isolation or PMPZ barriers, or both.

I next studied the strength of sexual isolation between *D. montana* and *D. flavomontana*, and its possible reinforcement in sympatric populations. As courtship and mate choice have been found to rely on different signals in other closely related *Drosophila* species, including *D. nebulosa* and *D. willistoni* (Gleason *et al.* 2012) and *D. recens* and *D. subquinaria* (Giglio and Dyer 2013), I started these studies by identifying the courtship signals that are most influential in sexual selection and species-recognition in my study species. *D. montana* females required species-specific male courtship song for mating, while the mating of *D. flavomontana* females was more dependent on the reception of chemical cues, cuticular hydrocarbons (CHCs). This fits well with earlier studies on *D. montana*, which have shown that in this species male song plays an important role both in intraspecific sexual selection (Hoikkala *et al.* 1998, Klappert *et al.* 2007, Veltsos *et al.* 2011) and in species-recognition (Saarikettu *et al.* 2005). Also CHCs have been found to affect female attractiveness and male mating success in *D. montana*, even though are less important for females than the song (Jennings *et al.* 2014).

Previous studies have shown that the songs of *D. montana* and *D. flavomontana* differ clearly from each other, the songs of *D. flavomontana* males having much longer intervals between sound pulses than the ones of *D. montana* males (Päällysaho *et al.* 2003). Song analyses performed in the present study confirmed this, and they also showed variation in different song traits to be relatively small within the species. On the other hand, CHCs, quantified here for the first time in *D. flavomontana*, showed clear species differences, with large variation within the species. CHCs were more diverged between species and between females and males of the same species in both sympatries, particularly in mountain sympatry, than in allopatry. Sex differences were pronounced especially in *D. flavomontana*, where they showed an association with 2-methyl-branched alkanes and/or alkadienes in both sympatries, potentially indicating their role in female mate choice and/or species' recognition.

The occurrence of the reinforcement of sexual isolation, largely based on species differences in courtship songs and CHCs and females' preferences for them, was studied by comparing the strength of this barrier in sympatric vs. allopatric populations (Noor 1995, 1999, Kronforst *et al.* 2007, Lemmon 2009, Dyer *et al.* 2014). Sexual isolation was almost complete between *D. montana* females and *D. flavomontana* males and somewhat weaker between *D. flavomontana* females and *D. montana* males, which adds one more example of parallel asymmetries in pre- and postzygotic isolation (see Yukilevich 2012). Moreover, the strength of sexual isolation showed higher variation in crosses involving *D. flavomontana* females and *D. montana* males than in the reciprocal

crosses. Here, *D. flavomontana* females originating from mountain sympatry discriminated more effectively against heterospecific males than *D. flavomontana* females originating from allopatry or coastal sympatry. *D. flavomontana* females from the mountain sympatry also preferred the males of their own population over conspecific males from other regions. Overall, increased discrimination of the mountain *D. flavomontana* females towards both heterospecific males and conspecific males from other populations, combined with pronounced species and sex divergence in CHCs, suggested the reinforcement of sexual isolation in mountain sympatry *D. flavomontana* females.

Postmating-prezygotic (PMPZ) barriers involve discordant interactions between heterospecific gametes and/or between the female reproductive tract and male seminal fluids (Howard 1999, Wirtz 1999, Price *et al.* 2001, Howard *et al.* 2009), and the reinforcement of these barriers has been reported in some *Drosophila* species (Matute 2010, Castillo and Moyle 2017). *D. montana* and *D. flavomontana* females showed PMPZ barriers in terms of sperm storage and egg fertilisation, sympatric and allopatric *D. montana* females storing heterospecific sperm worse than *D. flavomontana* females. In *D. flavomontana*, coastal sympatry females stored lower number of sperm and showed lower egg fertilisation after heterospecific matings than females from mountain sympatry or allopatry, and these flies also showed lower fertilisation rates in matings with conspecific flies from the other regions. Thus, in contrast to the patterns observed in sexual isolation, *D. flavomontana* females from the coastal sympatry showed signs of reinforcement at PMPZ level.

In conclusion, all reproductive barriers between *D. montana* females and *D. flavomontana* males were practically complete, while in the reciprocal cross they were slightly weaker. *D. flavomontana* females showed signs of reinforcement at the levels of sexual and PMPZ barriers that may have facilitated reproductive barriers between conspecific *D. flavomontana* populations (cascading reinforcement; Ortiz-Barrientos *et al.* 2009; Comeault *et al.* 2016; Pfennig 2016). I did not observe increased species' recognition ability of the coastal *D. flavomontana* females with small population size, as would be expected in the "rarer female hypothesis" (Noor 1995; Yukilevich 2012). Instead, my results gave more support to a suggestion that it may sometimes be more beneficial for females of the rare species to mate with heterospecific males than remain unmated (reviewed in Wirtz 1999). Finally, even though these findings gave support for the reinforcement theory, reinforcement of prezygotic barriers can be very difficult to distinguish from other processes that lead to similar outcomes. For example, ecological adaptation and sexual selection within species may facilitate similar divergence in sexual traits as the reinforcement (Noor 1999, Ortiz-Barrientos *et al.* 2009, Nosil 2012). Templeton (1981) has offered an alternative explanation for the strong prezygotic barriers observed between sympatric species and proposed that effective prezygotic isolation is more likely to evolve before the secondary contact than after it. Accordingly, when the species meet in the secondary contact, individuals with the weakest reproductive barriers produce low-fitness hybrids and disappear, while the

remaining population has relatively stronger barriers, leading to an observational bias of reinforcement.

3.2 Species' ecological divergence and potential ecological isolation associated with cold adaptation (II)

Environmental stressors may be particularly effective drivers of adaptation in ectotherms whose fitness is highly dependent on the optimum temperature. Cutter and Gray (2016) propose that harsh environmental conditions at high latitudes accelerate both speciation and extinction. The aim of this chapter was to find out whether *D. montana* and *D. flavomontana* populations show ecological isolation based on inter- and intraspecific genetic variation in their cold tolerance. I found some environmental traits, including the photoperiod and temperature, to show latitudinal variation across the fly home sites, but majority of the climatic variation between the sites was captured by differences in the coastal and mountainous regions (Fig. 1). Populations originating from the western coast of the continent receive high amounts of precipitation and have relatively mild temperatures throughout the year, while the ones from the high-altitude Rocky Mountains are exposed to high daily and seasonal temperature variation. Variation in the climatic and geographic conditions on species' home site helped me to identify the potential selection pressures driving their cold adaptation. I also considered possible correlations between fly cold tolerance and morphological traits (body colour and body size) as drivers of ecological divergence and speciation. Finally, I examined the role of circadian clock gene *vrille* in *D. montana* females' ability to tolerate cold and get cold-acclimated.

I measured the cold tolerance of *D. montana* and *D. flavomontana* flies using two ecologically relevant methods, which measure slightly different aspects of this tolerance. Cold resistance (chill coma temperature, CT_{min}) represents the temperature where an individual can no longer resist cold and falls into chill coma, while chill coma recovery time (CCRT) indicates the time that an individual needs to recover from a chill coma inducing temperature (MacMillan and Sinclair 2011, Vesala and Hoikkala 2011). *D. montana* flies were more cold-resistant and recovered faster from chill coma than *D. flavomontana* flies, as could be expected on the basis of species distributions (e.g. Addo-Bediako *et al.* 2000; Kimura 2004). However, the mean CT_{min} of both species was below 0 °C, which is generally rare among *Drosophila*, and supports the earlier finding that *virilis* group species are among the most cold-tolerant species in genus *Drosophila* (Kellermann *et al.* 2012). *D. montana* and *D. flavomontana* showed differences in their body colour, but not in body size. The body colour of *D. montana* was almost black, while that of *D. flavomontana* varied from yellowish to brown, as noted earlier by Patterson (1952). The dark body colour of *D. montana* flies may have helped them to adapt to cold environments on higher

latitudes and altitudes, since increased melanism (darker colour) absorbs solar radiation and enables the insects to warm up faster in cold environments (Clusella-Trullas *et al.* 2007).

Comparisons of *D. montana* and *D. flavomontana* populations from different geographic areas demonstrated that fly cold tolerance, particularly CCRT, and its association with different environmental cues and morphological traits can be highly variable even among closely-related species. Genetic variation in CT_{min} was relatively lower than in CCRT in both species', which could be explained by higher fitness advantages and costs involved in CCRT (Gibert *et al.* 2001, Overgaard and Macmillan 2017). Early recovery from the cold, in the morning or in spring, could offer high fitness advantages in defending territories, escaping from predators, foraging and mating. Moreover, recovery from cold may be sensitive to selection, since it requires a complete re-establishment of normal trans-membrane ion gradients and is relatively costly (e.g. Gibert *et al.* 2001; Sinclair *et al.* 2007; MacMillan *et al.* 2012). Furthermore, CT_{min} method is susceptible to plastic short-term acclimation responses occurring during the gradual cooling period of the test, which induces changes in the composition of membrane phospholipids, and may thus not be a straightforward test for genetic adaptation of cold tolerance (Overgaard *et al.* 2005, 2006, Macmillan *et al.* 2014, Andersen *et al.* 2015).

CCRT of *D. montana* was associated with latitude and that of *D. flavomontana* with latitude and climatic conditions prevailing on the coastal vs. mountainous regions. Correlation between short CCRT (high cold tolerance) and large body size, detected in *D. montana*, refers to shared genetic mechanisms or linkage between the genes regulating these traits. On the other hand, variation in the body colour in *D. flavomontana* did not correlate directly with cold tolerance, which suggests that body colour may play a more important role in other processes than thermoregulation, like protection against UV radiation, immunity, camouflage or mate choice (True 2003, Wittkopp and Beldade 2009, Telonis-Scott *et al.* 2011, Bastide *et al.* 2014). Also desiccation tolerance has been found to be linked with high melanism in many systems (Rajpurohit *et al.* 2008, Ramniwas *et al.* 2013, Davis and Moyle 2019), but it is unlikely to explain our results since dark *D. flavomontana* flies originated from humid rather than arid regions.

Finally, I traced the role of *vrille* in the fly cold tolerance and cold acclimation ability of *D. montana* flies, using RNAi technique. *vrille* is one of the several core circadian clock genes and a key regulator of circadian behavioural rhythms (Gunawardhana and Hardin 2017, Helfrich-Förster *et al.* 2018), and it is also one of the most intriguing candidate genes linked to *D. montana* cold tolerance or cold acclimation (Vesala *et al.* 2012b, Parker *et al.* 2015). Silencing *vrille* expression in *D. montana* females showed that this gene plays an essential role in flies' short- and long-term cold acclimation ability. These findings suggest that *vrille*, and potentially the whole circadian clock system, may play a role in adaptation to changing environmental conditions. Direct link between the circadian clock system and cold acclimation has already been detected in *Arabidopsis thaliana* plant (e.g. Espinoza *et al.* 2008). However, determining the

role of this complicated system in the cold acclimation of ectothermic insect species in general requires further experiments.

Overall, species' differences in cold tolerance traits and their association with selection pressures and morphological traits suggest that *D. montana* and *D. flavomontana* have genetically adapted to somewhat different ecological niches, which may have helped them to develop and maintain reproductive barriers in partial isolation (see I).

3.3 Chromosomal inversions and their potential role in early reproductive isolation and local adaptation (III)

Chromosomal inversions are expected to be favoured by selection, when they protect locally adapted genes from the homogenising effects of gene exchange and recombination (Navarro and Barton 2003, Kirkpatrick and Barton 2006). Thus, they may become hotspots for positively selected genetic differences (Kirkpatrick and Barton 2006, Hoffmann and Rieseberg 2008), which can give rise to both pre- and postzygotic isolation as well as adaptation (Noor *et al.* 2001, Kirkpatrick and Barton 2006, Hoffmann and Rieseberg 2008). In this chapter, I investigated the role of introgression and chromosomal inversions in contributing to the patterns of divergence in *D. montana* and *D. flavomontana*. I identified several large species-specific inversions from both species in all chromosomes, except in the 3rd chromosome, and asked whether these inversions could have contributed to species' divergence before and/or after the species' split.

All species-specific inversions showed accumulated genetic divergence in non-coding sequences compared to collinear regions, which emphasizes their role in reducing the homogenising effects of gene exchange. Intriguingly, inversions on the X and 4th chromosomes showed elevated divergence also on coding sequences, which refers to an important role of these inversions in their divergence. Based on the demographic analyses and comparisons of allopatric and sympatric populations, *D. montana* and *D. flavomontana* have experienced no or very low levels of introgression after their split, and their species-specific inversions have started to diverge between the lineages already before the species' split. Consequently, these findings suggest that the inversions have reduced gene exchange between the local populations of the ancestral form, where they have potentially contributed to the formation of early reproductive isolation and/or ecological divergence between the lineages. These results resemble the likely evolutionary history of *D. permilis* and *D. pseudoobscura*. Majority of the genes contributing to pre- and postzygotic isolation between these two species have been found to reside within species-specific inversions (Noor *et al.* 2001, Brown *et al.* 2004), even though these inversions were already present in the ancestral population of the species well before the species split (Fuller *et al.* 2018). Fuller *et al.* (2018) suggest that the inversions had persisted in

the ancestral population of the species, where they underwent incomplete lineage sorting and started to slowly accumulate genetic divergence, even without strong postdivergence gene flow (although the species had also experienced postdivergence gene flow). Finally, accumulation of genetic differences between the diverging lineages is expected to facilitate an accrual of BDMIs over time, which can eventually lead to speciation.

While the increased genetic divergence associated with inversions, particularly on the X and 4th chromosome, could be a consequence of ancestrally polymorphic inversions contributing to species divergence, there are also other plausible reasons for their emergence that are unrelated to speciation. One possibility is that these inversions have arisen close to the species' split simply by chance. Moreover, Berdan *et al.* (2020) recently performed a simulation study, where they showed that non-adaptive, deleterious mutations could arise and be maintained within inversions, because purifying selection is less effective on inversion heterozygotes. Coding sequences, however, showed consistently lower genetic divergence in non-coding than coding sequences, which proposes that purifying selection can effectively remove deleterious mutation also within inversions. Also, other indirect evidence suggest that at least some of the inversions have contributed to the adaptation and/or early reproductive isolation between local populations of the ancestral form.

First, diverged regions between *D. montana* and *D. flavomontana* within inversions on the 4th chromosome were enriched for genes associated with membranes, transmembranes, lipid metabolism and immunoglobulins. The same clusters of genes have repeatedly been linked to *D. montana* flies' ability to withstand cold temperatures (Parker *et al.* 2018, 2020, Kauranen *et al.* 2019, Wiberg *et al.* 2020). Intriguingly, Wiberg *et al.* (2020) showed that clinal variation in *D. montana* cold resistance (CT_{min}) is associated with allelic variation in immunoglobulins and lipid metabolism, and that of the chill coma recovery time (CCRT) with membranes, transmembranes and immunoglobulins. The association between CCRT and membranes and transmembranes is logical, since recovery process requires a re-establishment of normal trans-membrane ion gradients (Gibert *et al.* 2001, Sinclair *et al.* 2007, MacMillan *et al.* 2012). Moreover, transmembrane ion balance and cell membranes are susceptible to damages by low temperatures (Koštál *et al.* 2004), and adjustments to ion transport and membrane restructuring are essential mechanisms to increase membrane fluidity and permeability in response to cold (Teets and Denlinger 2013). Inversions on the X and 5th chromosome were enriched e.g. for genes involved in olfaction, odorant binding and cuticle proteins. These genes could have contributed to the differences in the formation and use of CHCs in the mate choice of *D. montana* and *D. flavomontana* (see I). They could also be involved in identification of specific host trees of the species (Markow and O'Grady 2008), or in species' cold or desiccation adaptation (Foley and Telonis-Scott 2011, Chung and Carroll 2015, Dennis *et al.* 2015).

Second, about 3Mb long region, located exactly at the *D. flavomontana* inversion breakpoint on the 4th chromosome, showed several intriguing patterns. This region had relatively high similarity between mountain *D.*

montana and *D. flavomontana* and high dissimilarity between mountain *D. montana* and *D. montana* from the other regions. These observations could suggest that mountain *D. montana* has obtained genetic material from mountain *D. flavomontana* before the species' split, but after some divergence has occurred between the lineages (Cruickshank and Hahn 2014). Mountain *D. montana* has very high genetic diversity in this region, which may be highly beneficial. High genetic variation could e.g. allow mountain *D. montana* to use different habitats to better respond to large daily and seasonal temperature changes in these areas.

In conclusions, all species-specific chromosomal inversions were already present in the ancestral form of the species, where they may have contributed to the early reproductive isolation and ecological (cold) divergence by reducing recombination and gene flow between the lineages. Inversions on the X and 4th chromosome are particularly good candidates for the onset of speciation process, since multiple substitutions driven by selection are ultimately expected to lead to enough divergence to cause BDM incompatibilities (Barbash *et al.* 2004, Presgraves 2010a), and further reinforcement of prezygotic barriers (Dobzhansky 1940, Servedio and Noor 2003).

3.4 Restricted introgression associated with an incompatibility locus residing within the X chromosomal inversions (IV)

Inverted chromosomal regions with increased genetic divergence are particularly prone to the formation of genetic incompatibilities (Orr 1995, Kirkpatrick and Barton 2006, Hoffmann and Rieseberg 2008). If strong incompatibility locus appears within an inversion, gene exchange is expected to be reduced across the inverted region (Hoffmann and Rieseberg 2008). The goal of this study was to find out whether genomic regions showing restricted introgression between *D. montana* and *D. flavomontana* involve chromosomal inversions that contain incompatibility loci (BDMIs). In *D. montana* and *D. flavomontana*, the inversions on the X and 4th chromosome are particularly good candidates for the incidental build-up of BDMIs, since they show increased genetic divergence at intergenic, intronic and coding sequences compared to the collinear genomic regions (see III). The X chromosome is here of a special interest, as it has been associated with the formation of BDMIs and reduced introgression in several studies (e.g. Haldane 1922, Coyne and Orr 1989, Masly and Presgraves 2007, Presgraves 2010c, Garrigan *et al.* 2012).

I investigated these questions in an experimental introgression study, where the initial cross involved a single *D. flavomontana* female and *D. montana* male (the reciprocal cross is unsuccessful; Fig. 2, see I). Fertile F_1 females were backcrossed towards both parental males for two generations and the resulting second-generation backcross (BC_2) females were pool-sequenced to quantify the reductions in introgression. Since this experiment involved crosses between

hybrid females and pure males, and since only the female progeny were sequenced, we could detect only BDMIs with dominant epistatic interactions (any effects of recessive-recessive pairwise incompatibilities were masked; Coyne and Orr 2004). The significance of the results was evaluated with simulations, because random inheritance of gametes and recombination can induce considerable variation in the expected amount of introgression.

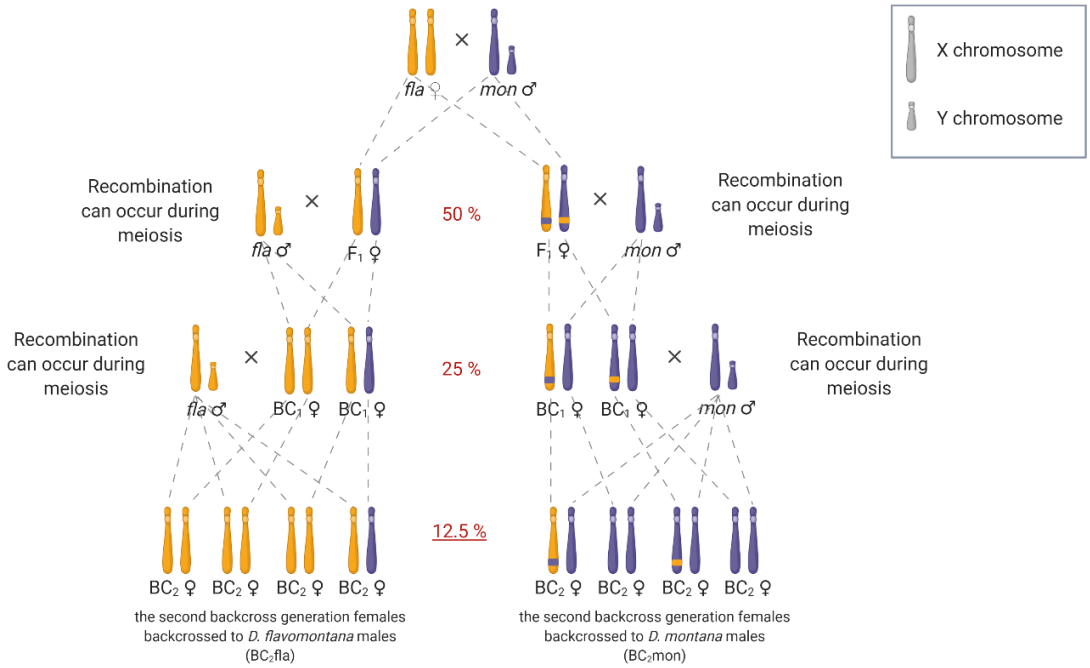

FIGURE 2 Illustration of the crossing experiment showing the inheritance of sex chromosomes (genetic combinations are similar for autosomes). Initial cross involved a single *D. flavomontana* (fla) female and *D. montana* (mon) male, and the resulting fertile F_1 and BC_1 females were backcrossed to either *D. flavomontana* or *D. montana* male. The expected amount of genetic material transferred between species (introgression) halves in every generation (red percentages). 12.5 % represents the expected amount of introgression in the BC_2 pools that were sequenced. Note that recombination can occur in F_1 and BC_1 generations and create substantial variation into the expected amount of introgression. For simplicity only one crossing-over occurring in the F_1 generation of BC_2mon pool is shown. The figure was created with BioRender.com. This figure is from Chapter IV.

I made three interesting observations in this study. First, backcrossing towards *D. montana* (BC_2mon) was more successful and showed weaker genetic incompatibilities than backcrossing towards *D. flavomontana* (BC_2fla). The potential for introgression from *D. flavomontana* to *D. montana* (via backcrossing towards *D. montana* (BC_2mon)) was surprising, because even though some interspecific hybrids have been found in nature (Patterson 1952), no significant introgression was observed between the species in nature in chapter III. The most obvious reason for this discrepancy is that laboratory experiments may not reveal all reproductive barriers, like extrinsic postzygotic barriers,

prevailing in wild populations. In the wild, hybrid flies may have difficulties in finding a suitable ecological niche and/or the female hybrids may fail to oviposit in the right host trees, which could induce disturbances in larvae development and host tree preferences, and also affect their CHCs. Furthermore, female requirement for species-specific song and female receptivity are determined by different genetic factors at least in *D. montana* (Isoherranen *et al.* 1999), and their mixing in species' hybrids may prevent matings. *D. flavomontana* flies also carry *Spiroplasma* sp. endosymbiont (III), which could prevent introgression, if reception of this endosymbiont is costly for *D. montana* flies.

The second finding was that the backcrosses towards *D. flavomontana* showed strong reduction in introgression on the X chromosome, which was associated with at least one dominant incompatibility locus residing within the X chromosomal inversions. While several other studies have shown the disproportionate role of the X chromosome (or Z) in backcross hybrid problems (Coyne and Orr 1989, Jiggins *et al.* 2001, Tao *et al.* 2003, Masly and Presgraves 2007), it was surprising that this incompatibility locus is a dominant one. "Dominance theory", which aims to explain the X-related patterns of postzygotic isolation, relies on the recessive incompatibility locus on the X chromosome (e.g. Turelli and Orr 1995, 2000), and thus it cannot explain the findings of the present study. However, the F_1 male sterility, detected in Chapter I could still be explained by the dominance theory, or by the "faster male evolution", where male sterility is suggested to result from the fast evolution of male traits and the highly sensitive spermatogenesis (Wu and Davis 1993). Meiotic drivers, which are expected to accumulate on the X chromosome, are largely involved in spermatogenesis (Patten 2018, Courret *et al.* 2019) and not relevant to female sterility. Meiotic drivers are also expected to increase their own transmission in both backcross directions, which was not observed in the present study. On the other hand, the findings could potentially be explained by "faster X evolution", based on the idea that selection increases the frequency of advantageous recessive alleles more effectively on the X chromosome than on autosomes, irrespectively of whether the incompatibilities themselves are recessive (Charlesworth *et al.* 1987, 2018). Moreover, hybrid problems could be induced by dosage compensation (females carry twice as many X-linked alleles than males, when the other X is silenced), and/or the X chromosome could contain more genes that are prone to creating hybrid problems than the autosomes (Coyne 2018).

The third finding concerned the highly diverged genes that showed low levels of introgression from *D. montana* to *D. flavomontana* on the X chromosome. These genes are potential candidate genes for postzygotic isolation and/or coadapted gene complexes that benefit from the tight linkage within inversions. Many of the identified genes were involved in embryo development, meiosis and/or gametogenesis (oogenesis and spermatogenesis). This agrees with Gompert et al. (2012) who have suggested that high levels of genetic divergence between species are typically enriched for genes associated with developmental and meiotic processes. Moreover, several interacting genes,

which likely benefit from the tight linkage, were associated with the circadian clock system and chemoreception, i.e. genes potentially contributing to adaptation and/or prezygotic barriers. Intriguingly, sexual selection and reinforcement has been suggested be favoured by selection, if the loci that contribute to postzygotic and prezygotic barriers reside within inversions (Trickett and Butlin 1994) or on the X chromosome (Lemmon and Kirkpatrick 2006). For example, loci associated with hybrid sterility and female sexual preferences are found within species-specific chromosomal inversions between *D. pseudoobscura* and *D. permilis* (Noor *et al.* 2001).

Overall, the results of this study suggest that the historical accumulation of genetic divergence within inversions, particularly on the X chromosome (see III, IV), has facilitated the formation of strong incompatibility locus or loci, preventing introgression from *D. montana* to *D. flavomontana.* Such strong BMDI could further induce the reinforcement of prezygotic barriers observed in chapter I.

3.5 Conclusions, caveats and future directions

Populations of closely related species that live in contact with each other are susceptible to the disruptive effects of gene flow and recombination if their reproductive barriers are not strong enough. In this kind of situation, species integrity can be enhanced by the reinforcement of prezygotic barriers, which can have far-reaching effects on population divergence also within species. The divergence of genes important in adaptation and reproductive isolation is often associated with chromosomal inversions, where the accumulation of genetic divergence may lead to the build-up of genetic incompatibilities through the actions of natural and/or sexual selection and drift. My aim in this thesis has been to give a comprehensive view on the central topics of speciation research and to obtain evidence on evolutionary forces that have enhanced the divergence of *D. montana* and *D. flavomontana*. This thesis also left several open questions, providing exciting avenues for the future research.

In chapter I, I identified a diverse group of reproductive barriers between *D. montana* and *D. flavomontana* that function together to form strong overall reproductive isolation (Butlin and Smadja 2018). Both pre- and postzygotic barriers were stronger, essentially complete, between *D. montana* females and *D. flavomontana* males than in the reciprocal cross (I). I also found signatures of the reinforcement of prezygotic barriers in the form of increased mating discrimination, sexual signal divergence and PMPZ isolation between *D. flavomontana* females and *D. montana* males and between *D. flavomontana* females and males from different populations (I). Prezygotic barriers were relatively stronger than the postzygotic ones, but the initial reproductive barriers that have functioned during the onset of species' divergence may differ from those that presently keep species apart (Butlin *et al.* 2012). However, the earliest reproductive barriers, detected between *D. flavomontana* populations,

involved sexual and PMPZ barriers (I), and Jennings *et al.* (2014b) have earlier found the same phenomenon among allopatric *D. montana* populations. Interestingly, *D. flavomontana* flies were found to carry *Spiroplasma* sp. endosymbiont (III), which could induce asymmetric isolation, if the reception of this endosymbiont is costly for *D. montana* flies. *Spiroplasma* has been detected e.g. in several *Drosophila* species and these endosymbionts can be divided into male-killing and non-male-killing ones (reviewed in Anbutsu and Fukatsu 2011). Investigating whether *D. flavomontana Spiroplasma* is a male-killing type would be interesting, since it could induce high costs and effectively strengthen prezygotic barriers (Jaenike *et al.* 2006, Cooley 2007), in this case between of *D. montana* females and *D. flavomontana* males.

Ecological premating isolation and extrinsic postzygotic isolation were not directly investigated in this thesis, but they are likely to be important and to considerably increase the total reproductive isolation between *D. montana* and *D. flavomontana*. In chapter II, I showed that *D. montana* and *D. flavomontana* have distinct abilities to withstand cold temperatures, which may have helped these species to develop and maintain reproductive barriers and adaptive divergence in partial isolation. One of the open questions in this thesis is the relative importance of natural and sexual selection driving the CHC divergence, detected between and within *D. montana* and *D. flavomontana* populations and between sexes. Natural selection could have induced divergence in the CHCs in populations living in different climatic conditions and affected by different environmental stressors, as several studies have shown CHCs to be linked with cold or desiccation tolerance (Foley and Telonis-Scott 2011, Chung and Carroll 2015, Dennis *et al.* 2015). In *D. flavomontana*, natural selection could also have affected CHCs through their association with body color. For example, among the *Drosophila* pigmentation genes, *tan*, *ebony* and *yellow* (Wittkopp *et al.* 2002), *tan* and *ebony* have been found to contribute also to CHC composition (Massey *et al.* 2019). Consequently, variation in *D. flavomontana* coloration, CHC composition and chill coma recovery time (CCRT) may be affected by a few highly pleiotropic genes (II). Ecological divergence cannot, however, explain higher sex differences in CHCs in sympatric than allopatric populations (I), since females and males of the same species and population live in the same ecological niche, which advocates the role of reinforcement in CHC divergence. Experimental speciation studies enable direct tests of speciation theory (White *et al.* 2020) and could be used to investigate the relative roles of natural and sexual selection in CHC divergence, as well as determine the first steps of speciation.

Even though interspecific hybrids between *D. montana* and *D. flavomontana* have been found in nature (Patterson 1952) and the reinforcement of prezygotic barriers indirectly suggests maladaptive hybridisation (I), I found no or very low levels of introgression between the species in nature (III). There are several plausible explanations for these observations that may feel somewhat contradictory. i) The species may have hybridised to some degree in nature, but strong selection against hybrids has not resulted in introgression (Cruickshank and Hahn 2014). This explanation is particularly fit since matings

between *D. flavomontana* females and *D. montana* males may sometimes produce fertile female hybrids (Patterson 1952, I), but strong incompatibility locus within inversions on the X chromosome effectively prevents introgression from *D. montana* to *D. flavomontana* (i.e. backcrossing towards *D. flavomontana*, IV). In theory, introgression could be more successful from *D. flavomontana* to *D. montana* (i.e. backcrossing towards *D. montana*, IV) or potentially through male hybrids, but investigating why this is not the case would require e.g. direct tests of ecological premating isolation and extrinsic postzygotic isolation, as well as more hybrid experimental designs. ii) Separate ecological niches of *D. montana* and *D. flavomontana* may have contributed to partial geographical isolation and restricted introgression between species (II). Finally, iii) prezygotic isolation could have been enhanced by other processes than reinforcement, like intraspecific sexual selection, ecological divergence and Templeton effect (Templeton 1981).

The age of the species-specific inversions and their association with increased genetic divergence and the presence of genes important in (cold) adaptation and reproductive isolation, suggest that inversions may have played an important role in the divergence of *D. montana* and *D. flavomontana* before the species' split (III, IV). The 4th and X chromosomal inversions provided good candidate inversions for the species' divergence due to their increased divergence both on coding and non-coding sequences. Loci of increased divergence within the 4th chromosomal inversions are particularly interesting in the context of cold adaptation (III), but it is difficult to know which of the genes have evolved under selection and which ones are hitchhiking (Cheng *et al.* 2012, Corbett-detig and Hartl 2012, Guerrero *et al.* 2012, Kirkpatrick 2017, Ravinet *et al.* 2017, Kapun and Flatt 2018). Examining the signs of positive selection (dn/ds ratios) of genes within the inverted and the collinear chromosomal regions could give more information on this. Ultimately, verifying the adaptive significance of the most prominent candidate loci associated with inversions could be done using gene editing techniques like RNA interference (RNAi) or CRISPR/Cas9 (Kapun and Flatt 2018). One interesting gene for further studies would be *vrille*, which is essential in *D. montana* females short- and long-term cold acclimation ability (II) and which is located only 200kb away from the breakpoint of *D. montana* inversion on the 4th chromosome. Moreover, tracing the genetic variation and inversion polymorphism within species across their distribution ranges could provide insights into the role of specific inversions in environmental adaptation and elucidate key genes associated e.g. with cold adaptation.

Finally, substitutions driven by selection and drift are expected to generate BDMIs (Barbash *et al.* 2004, Presgraves 2010a). Indeed, the X chromosomal inversions, containing high genetic divergence in non-coding and coding sequences (III), were associated with an incompatibility locus, which effectively restrict introgression from *D. montana* to *D. flavomontana* (IV). This regions is of a special interest also due to its potential to facilitate the reinforcement of prezygotic barriers (Trickett and Butlin 1994, Lemmon and Kirkpatrick 2006), as observed in *D. flavomontana* females (I). Identifying a specific BDMI locus from

the X chromosome would require other techniques, like quantitative trait loci (QTL) analyses, but BDMIs are often highly complex and difficult to localize (Satokangas *et al.* 2020). Also, it is difficult, if not impossible, to disentangle whether this incompatibility locus has played a part in species' split, or whether it has emerged much later.

Acknowledgements

I am thankful to so many people who have contributed to this thesis either directly by helping, advising, listening and encouraging, and/or indirectly by brining joy and experiences to my life. First, I would like to thank my awesome supervisors. You have been there for me during every step of this thesis. Maaria – you gave me the time I needed to learn cool analyses as well as opportunities to learn from my mistakes. There have been times when nothing worked and everything took longer than expected, but you were always encouraging and patient. Anneli – it has been amazing solving mysteries together since my master's thesis (sometimes also in an escape room). You have always been excited about all kinds of results, especially the weird ones that are contrary to our expectations. Venera – your passion for discovering new things and your dedication to students (including me) and scientific community have been highly motivating for me. Special thanks to Konrad and Dom – you have felt like a second set of supervisors and I am deeply grateful for your time, expertise and support. You taught me a great deal about population genetics, bioinformatics and genomics, and warmly welcomed me into your group. Many thanks also to the other people in Edinburgh (Gertjan, Sam, Derek, Mark, Lewis, Andrea, Rich, Pablo, Sujai, Samir, Ruben, Hannah, Brian, Deborah, Darren and the rest) for inspiring discussions, help and company. I experienced countless happy moments with you during my visits (DDH coffee times and happy hours, assembling jigsaws, pubs and slightly too spicy Tanjore, bird watching and butterfly hunt, watching movies at Domino or GOT and Daniel Sloss at Dom's and so on). You made me feel like home in Edinburgh. Many thanks to the other co-authors for your valuable input and special thanks to Rhonda for having me in Sheffield. Next, I would like to thank my follow-up group members Emily and Mike. Thank you for your kind support, enthusiasm, and valuable insights over the years. I would also like to express my appreciation to my department, and especially to the evolutionary genetics meetings. You have created a warm atmosphere for studying and starting out as a young scientist. I would like to thank the current and former staff at the fly room and DNA lab, and Sara and Andres for organising perhaps the best statistic/R course ever – I do not know how I would have survived without you. Thank you prof. Thomas Flatt and assistant prof. Daniel Matute for reviewing this thesis and giving valuable and constructive feedback. I am also very grateful for Jari for his kind suggestions during the thesis editing. I would like to express my gratitude to Emil Aaltonen Foundation, Finnish Cultural Foundation and the Academy of Finland for funding the research.

I'm extremely lucky to have so many friends in and outside the uni. Thank you for all the fellow PhD students, and special thanks to Annis, Elsi, Maiju, Anni and Mimmu for the shared therapeutic lunch and coffee breaks during the undergraduate and graduate studies. Thank you, Anne, for your constant support through the ups and downs over the past 17 years. You are irreplaceable. Thank you lovely "KF folks" for all the unforgettable moments

and experiences (kaverifestarit, Sherlock Holmes and other board game nights, “gang beasts” tournaments in Kuusamo, Lapland hiking, horror movie nights, beach volleyball -filled summer evenings, juhannus and New Year’s Eve festivities, trampoline park, Bättre Folk and so forth). Thank you Jenni, Taru, Aki, Jan, Juho, Ike, Topi, Jenna, Pätskä, Samuli, Henna, Kia, Jonna, Sanna, Eemi, Kata, Timo and Anna for your unique and fun company over the years. Thank you so much Anneli, Mikko, Tomppa, Timppa, Mari, Jatta, Hilma and Okke for relaxing family time in Sammallahti and Kaarikuja. I would like to express my deep gratitude to my mom and dad – I would not have done this without your help, support and encouragement during primary and high school as well as my undergraduate and graduate studies. Thank you so much. Jonna, Kai, Emil, Mihka, Veera and other family members – I am lucky to have you in my life and I look forward to spending more time together. Ville. I feel like I cannot thank you enough. You have been there for me this whole time, endured both good and bad moments, supporting, encouraging, listening, helping, celebrating and caring. Thank you for being you. Special thanks to my lovely little Aada – I got the best ideas and were the most relaxed while going for a walk with you. Every sunny and rainy day.

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ORIGINAL PAPERS

I

STRENGTH OF SEXUAL AND POSTMATING PREZYGOTIC BARRIERS VARIES BETWEEN SYMPATRIC POPULATIONS WITH DIFFERENT HISTORIES AND SPECIES ABUNDANCES

by

Noora Poikela, Johanna Kinnunen, Mareike Wurdack, Hannele Kauranen, Thomas Schmitt, Maaria Kankare, Rhonda R. Snook & Anneli Hoikkala 2019

Evolution, 73(6), 1182-1199

https://doi.org/10.1111/evo.13732

Strength of sexual and postmating prezygotic barriers varies between sympatric populations with different histories and species abundances

Keywords: speciation, sympatry, reinforcement, female discrimination, courtship cue, Drosophila

Running title: Reinforcement of reproductive barriers

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Acknowledgements

We would like to thank H. Järvinen for her help with the experiments, and people in the laboratory for the fly maintenance. We also thank E. Virtanen, A. Hiillos and E. Övermark for their contribution in Wolbachia studies, and V. Hoikkala for inspiring discussions. This work was supported by the grants from Academy of Finland (project 132619) and Ella and Georg Ehrnrooth Foundation to Anneli Hoikkala and Academy of Finland (projects 268214 and 272927) to Maaria Kankare.

Data archival

There is no data to be archived.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/evo.13732.

Conflict of Interest statement

We have no conflicts of interest with other researchers.

Abstract

The impact of different reproductive barriers on species or population isolation may vary in different stages of speciation depending on evolutionary forces acting within species and through species' interactions. Genetic incompatibilities between interacting species are expected to reinforce prezygotic barriers in sympatric populations and lead to cascade reinforcement between conspecific populations living within and outside the areas of sympatry. We tested these predictions and studied whether and how the strength and target of reinforcement between *Drosophila montana* and *Drosophila flavomontana* vary between sympatric populations with different histories and species abundances. All barriers between *D. montana* females and *D. flavomontana* males were nearly complete, while in the reciprocal cross strong postzygotic isolation was accompanied by prezygotic barriers whose strength varied according to population composition. Sexual isolation between *D. flavomontana* females and *D. montana* males was increased in long-established sympatric populations, where *D. flavomontana* is abundant, while postmating prezygotic (PMPZ) barriers were stronger in populations where this species is a new invader and still rare and where female discrimination against heterospecific males was lower. Strengthening of sexual and PMPZ barriers in this cross also induced cascade reinforcement of respective barriers between *D. flavomontana* populations, which is a classic signature of reinforcement process.

Introduction

Past and present climate change and human activity have induced shifts in species' distribution, which has had a strong impact on species interactions and speciation. When geographically or ecologically isolated populations or diverging species spread in the same area / habitat, their interaction may lead to different evolutionary outcomes depending on the strength of the reproductive barriers that they have evolved during isolation. If the barriers are weak to moderate, then the gene pools of the evolving species may be merged (Servedio and Noor 2003; Arnold and Martin 2009). Restricted gene flow between sympatric species may also promote adaptation into new environmental conditions, and it can even lead to the formation of new hybrid taxa (Abbott et al. 2013). If postzygotic barriers are strong enough, then the two species or populations may live in sympatry, and selection is predicted to reinforce barriers that function before zygote formation (Dobzhansky 1940; Howard 1993; Servedio and Noor 2003; Turissini et al. 2018). These barriers may occur at different stages of species interaction, from habitat and host choice to flowering or mating time and sexual and postmating prezygotic (PMPZ) isolation. Reinforcement of these barriers between the species can also induce divergence of respective traits between conspecific individuals from sympatric and allopatric and / or from different types of sympatric populations, this divergence potentially leading to some degree of reproductive isolation among these conspecific populations (reinforcement cascades or cascade reinforcement) (Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010; Abbott et al 2013; Comeault et al. 2016; Pfenning 2016) To understand how different reproductive barriers evolve during speciation, it is critical to elucidate the targets of reinforcement and to trace the role of reinforcement in completing and initiating speciation processes (Butlin et al. 2008; Nosil et al. 2009; The Marie Curie speciation network 2012).

Evolution of reproductive barriers through reinforcement has been studied in a variety of organisms from killifish (Kozak et al. 2015), frogs (Lemmon 2009) and plants (Suni and Hopkins 2018) to several insect species (Noor 1999; Kronforst et al. 2007). In *Drosophila*, sexual isolation has been shown to evolve faster than postzygotic isolation (Coyne and Orr 1997), and PMPZ isolation faster than hybrid inviability but more slowly than sexual isolation (Turissini et al. 2018). In this taxon, the male courtship cues, the acceptance threshold of females and / or the use of different sensory modalities in mate choice often vary between closely-related interacting species (Gleason et al. 2012; Giglio and Dyer 2013; Colyott et al. 2016). Reinforcement of female discrimination against heterospecific males can induce changes in any of the above-mentioned traits, and it may also increase female discrimination towards conspecific males from other populations (Noor 1999; Hoskin et al. 2005; Jaenike et al. 2006; Bewick and Dyer 2014; Comeault et al. 2016). PMPZ barriers, including incompatibilities in the transfer, storage and use of heterospecific sperm, involve discordant interactions between heterospecific gametes and / or between the female reproductive tract and male seminal fluids (Howard 1999; Wirtz 1999; Price et al. 2001; Howard et al. 2009). Reinforcement of these barriers has been reported so far only between *D. yakuba* and *D. santomea* (Matute 2010) and *D. pseudoobscura* and *D. persimilis* (Castillo and Moyle 2017) and between two nightingale species (*Luscinia megarhynchos* and *L. luscinia*; Albrect et al. 2019). Notably, strong PMPZ barriers can act as a driving force in the reinforcement of premating barriers, and selection pressure generated by them can be as strong or even stronger than that caused by low hybrid fitness (Servedio 2001).

Reinforcement is most likely to occur when species hybridization is common and its costs are high and when the opposing forces of gene flow and recombination are weak (e.g. Servedio and Noor 2003; Coyne and Orr 2004; Servedio 2009; Butlin and Smadja 2018). Accordingly, almost all sympatric *Drosophila* species have been found to show concordant pre- and

postzygotic isolation asymmetries, where the more costly reciprocal mating shows greater prezygotic isolation relative to the less costly mating, while no such patterns exist in allopatry (Yukilevich 2012). The outcome of reinforcement can also be affected by changes in species' distribution and abundance, the length of species coexistence, and the strength and targets of natural and sexual selection between and within species (Servedio 2001; Servedio and Noor 2003; Smadja and Butlin 2011; Nosil 2012). Whether and how species relative abundances affect female discrimination against heterospecific males is less clear. In the "rarer female hypothesis", reinforcement is expected to be targeted on the species recognition ability of females of the less abundant species, because these females encounter more heterotypic mating attempts in the wild and suffer from higher hybridization costs than those of the more abundant species (Noor 1995; Hoskin et al. 2005; Yukilevich 2012). On the other hand, females of the rarer species or genotype have been suggested to mate with heterospecific or heterotypic males because of the high costs involved in mate search and / or in the risk of remaining unmated (Wilson and Hedrick 1982; Wirtz 1999; Kokko and Mappes 2005; Matute 2014). In this scenario reduced mate choice of females of the rarer species may constrain the reinforcement of sexual isolation, and thus natural and sexual selection could be targeted on PMPZ barriers to limit the costs of maladaptive hybridization (Turissini et al. 2018).

Despite these predictions, only a few studies have examined whether the targets of reinforcement vary between species that have a long history of sympatry compared to a scenario in which one species has only recently invaded the area and is still rare, and whether this variation has an impact on the target and strength of cascade reinforcement (Matute 2010; Suni and Hopkins 2018). We study these outstanding speciation questions using two *virilis* group species, *Drosophila montana* and *Drosophila flavomontana*. Morales-Hojas et al. (2011) have estimated that the species have diverged from each other 4.9 million years

ago, but our recent genome-level studies show the divergence time to be considerably shorter (Poikela and Lohse, unpublished). Species divergence has probably occurred in the Great Basin / Rocky Mountains area in North America (Stone et al. 1960; Throckmorton 1982). *D. montana* has distributed from this region around the northern hemisphere, including the western coast of North America, long time ago (Throckmorton 1982), while *D. flavomontana* has spread to the western coast only after the extensive collections carried out on this area in 1950's (see Patterson 1952), and is still rare. Both species have a patchy population structure, as they live only on watersides, and as their distribution and abundance depend on climatic factors and the presence of species-specific host trees (Patterson 1952). Sympatric populations of the species are found in the Rocky Mountains area, where the species have a long history of sympatry and where *D. flavomontana* is abundant, and on the western coast of North America, where this species is rare. Reproductive barriers between *D. montana* females and *D. flavomontana* males are nearly complete, while the leakage of these barriers in the reciprocal cross occasionally leads to species hybridization at least in the Rocky Mountains region (Patterson 1952).

To study the reinforcement of prezygotic barriers in sympatric *D. montana* and *D. flavomontana* populations, we first determined the strength of postzygotic barriers between the species. We then studied whether and how the length of species coexistence, combined with their relative abundances, has affected the reinforcement of sexual and / or PMPZ barriers between species in sympatric populations compared to allopatry. Finally, we studied whether reinforcement of these barriers between *D. flavomontana* and *D. montana* in sympatry has induced cascade reinforcement between allopatric and sympatric populations of *D. flavomontana*. We predicted that the high abundance of *D. flavomontana* and its long co-existence with *D. montana* in sympatric Rocky Mountains populations would select for reinforcement of sexual barriers preventing mating. In contrast, in sympatric western coast

populations, where the rareness of *D. flavomontana* could constrain the reinforcement of sexual isolation, reinforcement was expected to be targeted on PMPZ barriers. Finally, we expected that reinforcement of sexual and / or PMPZ barriers between species has induced cascade reinforcement between *D. flavomontana* populations in the same barriers.

Material and Methods

STUDY SPECIES

D. montana and D. flavomontana populations

D. montana and *D. flavomontana* belong to the *montana* subphylad of the *virilis* group (Morales-Hojas et al. 2011). *D. montana* is distributed on different continents around the northern hemisphere. In North America, it is found in high latitudes in Canada and Alaska, in high altitudes (from 1400 to above 3000 m) in the southern Rocky Mountains and in low altitudes along the western coast of the United States (US) and Canada (Patterson 1952; Stone et al. 1960; Throckmorton 1982). In the 1950s distribution of *D. flavomontana* was restricted to the Rocky Mountains region, where it is typically found in lower altitudes than *D. montana* (usually below 2000 m; Patterson 1952; Stone et al. 1960). However, our fly collecting trips in North America in 2010 – 2015 showed that the distribution of both species has shifted northwards and towards higher altitudes and that *D. flavomontana* has invaded also the western coast. Population structure of both species is mosaic because of their dependence on waterways, suitable climatic conditions and specific host trees (Patterson 1952; Throckmorton 1982). *D. montana* is associated with aspen (e.g. *Populus tremuloides*) and alder (e.g. *Alder rubra*) and *D. flavomontana* with narrowleaf cotton-wood (*Populus angustifolia*; Throckmorton 1982).

D. montana and *D. flavomontana* strains used here were collected 2013 – 2015 (Fig. 1). *D. montana* strains from Seward (Alaska, USA) and Afton (Wyoming, USA) are allopatric to *D.*

flavomontana, as Alaska is too cold and the collecting site in Afton lacks suitable host trees for the latter species. In contrast, *D. flavomontana* strains collected at an altitude of about 1600 m from Livingston (Montana, USA) and Liberty (Utah, USA) are allopatric to *D. montana*, because these sites lack higher altitudes necessary for the latter species to migrate during hot summers (typical behavior of Rocky Mountains *D. montana*; A. Hoikkala, unpublished observations). Sympatric flies were collected from the lower slopes of the Rocky Mountains (altitude up to 2000 m) and from the western coast of North America. In the Rocky Mountains, collections were made in Cranbrook (British Columbia, Canada) and Jackson (Wyoming, USA), where *D. flavomontana* is more abundant than *D. montana*, and thus the populations are hereafter referred to as "Sympatry F". In the western coast, flies were collected from Terrace (British Columbia, Canada), Vancouver (British Columbia, Canada) and Ashford (Washington, USA). In these sites *D. montana* is more abundant than *D. flavomontana*, and thus these populations are referred to as "Sympatry M". We also refer to the origin of the strains (Allopatry, Sympatry F and M) as "population type".

All flies were collected over several days during local spring and represented the overwintered generation. Sample sizes varied between ~40 and 100 individuals per site. In Liberty we succeeded to collect only six flies (all *D. flavomontana*; see Fig. 1 and Table S1), but according to Patterson (1952) the area between this site and the closest mountain peaks is almost solely occupied by *D. flavomontana* (only 1 of more than 200 flies was *D. montana*). Our earlier fly collecting trips on different times of spring and summer on the Rocky Mountains (3 trips) and the western coast (3 trips) 2003 - 2009 confirm the high and low abundance of *D. flavomontana* on the Rocky Mountains and the western coast, respectively, and thus the proportions presented here can be regarded as good representation of the relative abundances of *D. montana* and *D. flavomontana* on above-mentioned areas.

Isofemale strains

The present study was performed using 2 isofemale strains per species per location, established from the progenies of fertilized wild-caught females (in Terrace we succeeded to collect only 1 *D. flavomontana* female). Species identification was performed by sequencing part of the mtDNA *COI* region of one progeny per isofemale line as described in Simon et al. (1994; see Table S2 for primer information). As *Wolbachia* has been found to induce reproductive isolation between some *Drosophila* species (Clark et al. 2006), we also tested for its presence in our study strains by performing PCR on 2 females and males per strain (see Method S1; Table S2; Fig. S1) and by scanning whole-genome sequences of 5 *D. montana* and *D. flavomontana* strains (Kankare et al. unpublished data). Neither method found evidence of *Wolbachia* genomic products in our study strains, and thus the detected reproductive incompatibilities are not explained by this endosymbiont.

Fly strains were maintained on malt medium (Lakovaara 1969) under conditions that prevented variation in flies' circadian rhythm and / or diapause susceptibility from affecting the results (continuous light at 19 ± 1°C and 60-70% humidity). Fly strains were maintained in laboratory for 7 to 14 generations before using them in experiments involving both species, and 14 to 28 generations in the ones involving only *D. flavomontana*. For all assays, flies were separated by sex under light CO_2 anesthesia within three days after emergence and maintained in plastic malt vials (15-20 virgin females or males per vial). Cuticular hydrocarbons (CHCs) were extracted at the age of 14 days when the females' ovaries have reached full size (Salminen and Hoikkala 2013). Other assays, including reproductive isolation experiments, were conducted when the flies were 18-22 d old, as normally done in *D. montana* (e.g. Jennings et al. 2014b). Information on strain pairs used in studies on reproductive barriers is given in Table S1.

POSTZYGOTIC BARRIERS

Postzygotic barriers between *D. montana* and *D. flavomontana* were studied by quantifying the viability, sex ratio and fertility of hybrid offspring from reciprocal interspecific crosses. F_1 hybrids were obtained by placing 10 females of one and 10 males of the other species in malt vials (20 replicates for each reciprocal cross) and transferring them into a fresh vial once a week for about one month. Intraspecific controls were obtained by placing 5 conspecific females and males in a malt vial (one replicate for two strain pairs per population type) and transferring them into a new vial every day for a week to prevent overcrowding. In both crosses, progeny viability and sex ratio were determined by counting the number of 3rd instar larvae and adult females and males that were viable at least 24 hours after emergence (numbers of earlier stage larvae could not be counted reliably).

Interspecific F_1 hybrids were collected from the vials within three days after eclosion, and females and males were transferred into different malt vials. Fertility of sexually mature hybrids was measured as the ability to produce progeny (at least one larva), when backcrossed to *D. montana* or *D. flavomontana* (each hybrid was given up to 3 possibilities to mate with a fly of either species).

All statistical analyses were conducted in R (Version 3.4.3; R Core Team 2017) and R studio (Version 1.1.383). Variation in the viability of intra- and interspecific F_1 progeny among crosses or among population types within a cross was tested using generalized linear mixed model (GLMM), with viability as response variable and cross or population type as an explanatory variable. These analyses were done using *glmer* function of nlme package (Pinheiro et al. 2018) with binomial distribution. Strains were treated as a random effect (nested within population type and cross). In one mon♀×fla♂ cross, variation in viability was low (excess of zeroes), and here the significance was tested using a chi squared likelihood

ratio test instead of a z-test. We also used one-sample student's t test (*t test* function of the stats package) to test whether the proportion of F_1 hybrid females differed from the expected 0.50 among crosses and population types, and whether fertility of F_1 hybrid females and males deviated from the expected 1. Detailed statistics (degrees of freedoms, test statistics, P-values) and additional information on results of different experiments are reported in Supporting Material.

PREMATING SEXUAL ISOLATION AND IMPORTANCE OF COURTSHIP CUES

Multiple-choice and no-choice tests

The magnitude of sexual isolation between *D. montana* and *D. flavomontana* was quantified using both multiple-choice and no-choice tests performed between 9 am – 11 am. For multiple-choice tests, 30 flies of each sex of both species were introduced into a 6 cm^3 Plexiglas mating chamber without anesthesia (see Jennings et al. 2014b). Mating pairs were removed by aspiration through holes in the mating chamber walls and their species was identified by body color (*D. montana* is darker than *D. flavomontana*). In Terrace population, where the color differences were small, different strains were marked by mixing either red or blue food coloring in malt medium 24 h before each test, altering the colors between tests (see Wu et al. 1995; Jennings et al. 2014b). Multiple-choice tests were replicated 5 times, and the data for the first 30 matings (50% of possible matings) in each test were used for calculating the strength of sexual isolation. No-choice tests involved reciprocal tests with 30 females of one and 30 males of the other species (5 replicates per cross), and here the mating pairs were collected for 2 h from the beginning of experiment. Controls for these tests were obtained by performing reciprocal crosses between 2 conspecific strains per population type, with 1 replicate per cross (see Table S1). Variation in the proportion of females mated with con- or heterospecific males in multiple-choice and no-choice tests was analyzed using

generalized linear mixed model (GLMM) with binomial distribution, using cross and population type within a cross as an explanatory variable as described in "Postzygotic barriers" section.

Sexual isolation was also studied between *D. flavomontana* strains (see Table S1), both between and within population types, using similar multiple-choice tests as for interspecific crosses. All tests were replicated 3 times and the flies were always marked with a different food color. To prevent strain differences in fly mating activity from affecting measures of sexual isolation between *D. flavomontana* population types, the results were normalized by taking into account the mating activity of flies of each strain (see Method S2 in Supporting Information). After normalization the data were analyzed the same way as those of the interspecific tests.

Species differences in the importance of potential sexual cues for mate discrimination

Contribution of visual, auditory (courtship song) and olfactory (cuticular hydrocarbons) cues in mate choice and species recognition of *D. montana* and *D. flavomontana* was determined by performing four sets of experiments with partially sensory-deprived individuals within and between the species. Mating success was measured in the following treatments: (1) control - both females and males were unmanipulated and the experiments were performed in light, (2) visual - both females and males were unmanipulated, but experiments were performed in darkness, (3) auditory – females were unmanipulated but males were muted by removing their wings with micro-scissors, and (4) olfactory and auditory - the entire antennae of females, including the third segment and aristae that act as olfactory and auditory cue receivers (Carlson 1996; Tauber and Eberl 2003), were removed with tweezers. Sense organ removals were done under CO_2-anesthetization, and anesthetized flies were given 1 d to recover from treatment before being used in mating assays.

Experiments were performed on 1 strain per species from each population type, and different experiments involving the females of the same strain were run on the same day. In each treatment and experiment, 15 females and 15 males (either conspecific or heterospecific) were placed in a food vial for 24 h. After this the females were CO_2-anesthetized, and their mating status was determined by dissecting their reproductive tracts in a drop of PBS on a microscope slide, covered with a cover slip, and by examining presence of sperm under light microscopy. Differences between treatments in the proportion of mated females was analyzed with generalized linear mixed model (GLMM) with binomial distribution (other details described in the "Postzygotic barriers" section above).

Male courtship song analysis

The songs of *D. montana* and *D. flavomontana*, produced by male wing vibration, are species-specific (Hoikkala and Lumme 1987). We studied variation in courtship song parameters by recording the songs of five males of each study strain (Method S3 in Supporting Material). Song traits analyzed from oscillograms included number of pulses in a pulse train (PN), length of a pulse train (PTL), length of a sound pulse (PL), interpulse interval (IPI) and number of cycles in a sound pulse (CN; see Fig. A1). PN and PTL were analyzed for three pulse trains per male, and PL, IPI and CN for the third or fourth pulse of each train. In addition, song carrier frequency (FRE) was measured from the frequency spectrum of the same pulse trains. Mean values of song traits were averaged over three pulse trains of each male.

We applied principal component analysis (PCA) for the song data using the *prcomp* function in R (Version 3.4.3) and R studio (Version 1.1.383). As PTL and PN were strongly correlated in both species (>0.80; Table S3), we removed PTL from the PCA. PCA scores for each study strain were centered and scaled. Variation in each song trait between population types

of both species were also analyzed with linear mixed model (LMM) using study strains as a random effect. These analyses were done using *lmer* function of nlme package (Pinheiro et al. 2018).

Cuticular hydrocarbon (CHC) profiles

CHCs may serve as contact pheromones and function in mate discrimination (Ferveur 2005; Jennings et al. 2014a). CHCs were extracted in hexane and analyzed with a gas chromatograph/mass spectrometer for both sexes of all study strains (usually 5 individuals/sex/strain; Table S4). CHC profile similarity was assessed by means of multivariate Linear Discriminant Analysis (LDA) and Random forest classification using the functions *lda* (from the MASS package) and *randomForest* (from the randomForest package) in R (Version 3.4.3) and R studio (Version 1.1.383). In addition, Bray-Curtis dissimilarities were analyzed for species and sex differences for each population type. For methodological details see Method S4 in Supporting Material.

POSTMATING-PREZYGOTIC (PMPZ) BARRIERS

PMPZ barriers were quantified by assessing sperm transfer and storage (hereafter referred as sperm storage) and egg hatch rate in all interspecific crosses and their controls. In these tests we used females that had mated with a heterospecific male in no-choice experiments for at least 3 minutes (ensures sperm transfer; Mazzi et al. 2009; see section "*Multiple-choice and no-choice tests*" above). As the number of matings between *D. montana* females and *D. flavomontana* males was low, we generated more matings in this direction by playing females conspecific song (see Saarikettu et al. 2005) while being exposed to muted *D. flavomontana* males (Method S5 in Supporting Material).

Mated females were placed individually into a set of 20 vials ("manifold") with 1 cm of malt medium at the bottom. Females were removed after 48 h and dissected to check for the

presence of sperm in their seminal receptacle and spermathecae (see section "*Species differences in the importance of potential sexual cues for mate discrimination*"). The amount of sperm was estimated and categorized into four levels: 0 = no motile sperm, 1 = maximum of two sperm cells, 2 = intermediate amount of sperm, and 3 = seminal receptacles and / or spermatheca full of sperm. The number of eggs oviposited by each female was counted immediately after her removal, and again after 3 d, to calculate the proportion of eggs that had hatched and proceeded to larval stage during this period (Jennings et al. 2014b).

Reduction in the proportion of hatched eggs may result from either fertilization failure (PMPZ barrier) or from problems in embryo development due to genetic incompatibilities (postzygotic barriers). To distinguish between these alternatives, we determined the fertilization status of eggs oviposited by *D. flavomontana* females that had mated with *D. montana* males (reciprocal cross was not studied because *D. montana* females did not store *D. flavomontana* sperm), and between flies from *D. flavomontana* population types. Freshly laid eggs of 17-33 mated females per cross were collected each day for 3 d, then fixed and processed for fluorescence microscopy (DAPI; Snook and Karr 1998; Jennings et al. 2014b). Eggs were classified as fertilized if either clear mitotic division or cellular differentiation was evident (Fig. S2). Eggs that did not meet these criteria (Fig. S2) were examined for the presence of sperm inside the egg to determine whether they were fertilized but karyogamy had not yet occurred or whether they were unfertilized (i.e. sperm were absent). The presence of sperm inside eggs was scored using differential interference contrast (DIC) light microscopy (Jennings et al. 2014b). Sperm length of *D. montana* is 3.34 ± 0.02 mm and of *D. flavomontana* is 5.53 ± 0.01 mm (Pitnick et al. 1999), thus the sperm flagellum can easily be seen as a coiled structure near the anterior end of the egg (see Fig. S2).

Variation in sperm storage ability among females from intra- and interspecific crosses, and between population types within a cross, was tested treating this trait as an ordinal variable in

a cumulative link mixed model (CLMM). These analyses were conducted using *clmm* function of ordinal package (Christensen 2018). Proportion of hatched / fertilized eggs were analyzed as in the "Postzygotic barriers" section, using generalized linear mixed model (GLMM) with binomial distribution. In mon♀×fla♂ crosses, where the variation in the proportion of hatched eggs was low (excess of zeroes), we used a chi squared likelihood ratio test instead of a z-test was used to test the significance.

REPRODUCTIVE ISOLATION INDEX

To determine whether the costs involved in interspecific matings had potentially reinforced prezygotic reproductive barriers in sympatric populations and promoted cascade reinforcement between *D. flavomontana* populations, we calculated reproductive isolation index (RI; Sobel and Chen 2014) separately for sexual isolation and 2 PMPZ barriers (sperm storage and fertilization):

$$RI = 1 - 2 \times (H / (H + C)),$$

where H = heterospecific / heteropopulation and C = conspecific / conpopulation.

RI for sexual isolation in interspecific matings was calculated from no-choice results, as species differences in mating activity decreased the reliability of multiple-choice experiments (see Table S6). RI for sexual isolation between *D. flavomontana* populations was calculated from multiple-choice results, where minor variation in the mating activity of the flies of different strains was normalized with parallel tests within populations. Among PMPZ barriers, RI was calculated for sperm storage for reciprocal interspecific crosses, and for

fertilization in crosses between *D. flavomontana* females and *D. montana* males and between *D. flavomontana* from different population types.

Results

POSTZYGOTIC BARRIERS – FITNESS OF F_1 HYBRIDS

The costs of interspecific matings at postzygotic level were defined by measuring the viability (from the 3rd instar larvae to adults), sex ratio and fertility of F_1 progeny. Crosses between *D. montana* females and *D. flavomontana* males produced a lower number of 3rd instar larvae than the reciprocal cross (31 vs. 339 larvae), which could be due to problems before or after zygote formation. Viability was significantly higher in intra- than in interspecific crosses involving *D. montana* females in Allopatry and Sympatry M (Fig. A2A), and the ones involving *D. flavomontana* females in Sympatry F and Sympatry M (Fig. A2B; Table S5). Crosses between *D. montana* females and *D. flavomontana* males produced only a few F_1 hybrids (4 females and 2 males). In the reciprocal crosses hybrid sex-ratio was female-biased and deviated significantly from the expected 0.5 in Allopatry (Fig; A3A, Table S5). Backcrossing the F_1 hybrids to their parental species showed no effect of cross direction on hybrid fertility (GLMM, $z_{1,99} = 1.21$, $P = 0.228$), so subsequent statistics were performed on combined data within the reciprocal crosses. In crosses between *D. montana* females and *D. flavomontana* males, 2 of the 3 mated F_1 females were fertile. In the reciprocal crosses, where 101 of 106 F_1 hybrids mated with one of the parental species, fertility of F_1 females deviated significantly from the expected 1 in Allopatry and Sympatry M, while in Sympatry F all 5 F_1 females were fertile (Fig. A3B; Table S5). All F_1 males from these crosses were sterile (Fig. A3B; Table S5).

SEXUAL ISOLATION AND THE COURTSHIP CUES

The strength and asymmetry of sexual isolation between the species and between D. flavomontana populations

In interspecific multiple-choice tests between *D. montana* and *D. flavomontana*, matings occurred mainly within the species in all population types (Fig. 2A; Table S6). Matings between *D. flavomontana* females and *D. montana* males were significantly more common than the reciprocal ones in Allopatry and Sympatry M, but not in Sympatry F (Fig. 2A; Table S6; data for individual strain pairs shown in Table S7).

In no-choice tests, the proportions of mated females remained very low in both interspecific crosses (*D. montana* females with *D. flavomontana* males: 0.00-0.01; *D. flavomontana* females with *D. montana* males: 0.03-0.11), compared to intraspecific ones (*D. montana*: 0.90-0.97; *D. flavomontana:* 0.77-0.93). In all population types *D. flavomontana* females mated significantly more often with heterospecific males than *D. montana* females (Fig. 2B; Table S6). *D. montana* females were equally reluctant to mate with heterospecific males in all population types, while *D. flavomontana* females from Allopatry and Sympatry M mated more frequently with heterospecific males than the females from Sympatry F (Fig. 2B; Table S6).

In multiple-choice tests between *D. flavomontana* from different population types, Allopatry females preferred Sympatry F males over their own males, whereas both Sympatry F and Sympatry M females discriminated against Allopatry males (Fig. 2C; Table S6). Additionally, Sympatry F females discriminated against Sympatry M males. Detailed information on individual strain pairs is shown in Table S8.

Importance of sexual cues in species recognition / sexual selection

The importance of visual, auditory and olfactory cues in species recognition and / or sexual selection was studied by comparing fly mating propensity between control trials and the test trials in which transmission of one or more cues was prevented. Visual cues did not play an essential role in mating success in either species, as flies' mating frequency did not differ between light (control) and dark conditions (Fig. 3A and C; Table S9). However, the species differed in the impact of auditory and olfactory signals on mating success. In *D. montana* both removal of male wings, which prevented the passage of auditory cues, and removal of female antennae, which silenced both auditory and volatile olfactory cues, prevented mating (Fig. 3A; Table S9). In contrast, in *D. flavomontana* only removal of female antennae significantly reduced fly mating (Fig. 3C; Table S9). These results suggest that *D. montana* require male song (and perhaps CHCs) for mating, whereas the courtship of *D. flavomontana* relies more on CHCs. The outcome of interspecific sense-deprivation experiments confirms this conclusion. Here *D. montana* females did not mate with *D. flavomontana* females in any experiment (Fig. 3B; Table S9). On the other hand, *D. flavomontana* females mated significantly more often with wingless than with normal *D. montana* males, which means that hearing a heterospecific song decreased their mating willingness more than hearing no song (Fig. 3D; Table S9).

Divergence in song traits and CHCs within and between species

Variation in song traits (Table S10) within and between species is illustrated with a principal component (PC) analysis plot (Fig. 4A). The first two components accounted for 84.5% of the total variance (Fig. S3; Table S11). The first PC explained 61.0% of variation and separated PN, PL and CN from IPI (Fig. 4A; see Fig. A1). The second PC explained 23.5% of variation; here CN varied both within and between species, while FRE varied only within *D. montana*. In *D. montana* CN and FRE were slightly higher in males from Allopatry than in

the ones from Sympatry M (LMM, CN: $t_{1,36} = -3.04$, P = 0.019; FRE: $t_{1,36} = -2.45$, P = 0.040), while none of the *D. flavomontana* song parameters varied significantly between population types (Table S12).

CHCs of sympatric *D. montana* and *D. flavomontana* populations diverged from each other more than those of allopatric ones (Fig. 4B). Species differences, measured as Bray-Curtis dissimilarities, were significantly higher in Sympatry F (0.52 ± 0.11) and in Sympatry M (0.51 ± 13) than in Allopatry (0.36 ± 0.10; LMM, $t_{1,2670} = 6.60$, P < 0.001 and $t_{1,3783} = 5.81$, P < 0.001, respectively), while Sympatry F and Sympatry M showed no significant difference (LMM, $t_{1,3491} = -0.39$, P = 0.697).

In *D. montana* CHC differences between sexes, measured as Bray-Curtis dissimilarities, were significantly higher in Sympatry M (0.39 ± 0.14) than in Allopatry (0.30 ± 0.12, LMM, $t_{1,910}$ = 2.52, P = 0.016), but of similar level as in Sympatry F (0.31 ± 0.13, LMM, $t_{1,850} = 1.05$, P = 0.299). Also, CHC differences between sexes showed no significant difference between Allopatry and Sympatry F (LMM, $t_{1,658} = 1.09$, P = 0.284). In *D. flavomontana*, CHC differences between sexes were more pronounced in both Sympatry M (0.51 ± 0.13) and Sympatry F (0.41 ± 0.10) than in Allopatry (0.37 ± 0.10; Sympatry M: LMM, $t_{1,978} = 4.13$, P < 0.001; Sympatry F: LMM, $t_{1,887} = 2.30$, P = 0.027), but did not differ between sympatric population types (LMM, $t_{1,667} = 1.63$, P = 0.113). Overall, sex differences were higher in *D. flavomontana* than in *D. montana* (LMM, $t_{1,2479} = -4.55$, P < 0.001) and the sexes were misidentified slightly more often in the latter species (Table S13). Together, these results indicate that CHCs are relatively more important in sexual selection and / or species-recognition of *D. flavomontana* than *D. montana*.

The most influential CHC substances for the chemical dissimilarities between species and between sexes within species in each population type were defined using random forest

analysis (Table 1; Fig. S4). Most of the substances were alkenes with varying numbers of carbons in a chain and with different double-pond positions. Interestingly, in both sympatric *D. flavomontana* population types, 2-methyl-branched alkanes and / or alkadienes had a large contribution to sex differences, which indicates a signal function of these compound classes. The relative amounts of these compounds were higher in males than in females (Table S14).

POSTMATING PREZYGOTIC (PMPZ) BARRIERS

D. montana females from all population types had fewer sperm after mating with a heterospecific than with a conspecific male (Fig. 5; Table S15). *D. flavomontana* females from Allopatry and Sympatry F, but not the ones from Sympatry M, stored sperm equally well regardless of whether it was received from conspecific or heterospecific males (Fig. 5; Table S15). In interspecific crosses, sperm was more successfully stored in *D. flavomontana* than in *D. montana* females in all population types (Fig. 5; Table S15).

The proportion of hatched eggs was significantly lower in all interspecific crosses (*D. montana* females and *D. flavomontana* males: 0.00-0.01; *D. flavomontana* females and *D. montana* males: 0.01-0.03) than in intraspecific ones (*D. montana* = 0.73-0.83; *D. flavomontana* = 0.80-0.91; Fig. A4; Table S15). In interspecific crosses the proportion of hatched eggs was higher in *D. flavomontana* females than in *D. montana* females in Allopatry, but not in either of the sympatric populations (Fig. A4; Table S15).

In crosses between *D. flavomontana* females and *D. montana* males, the low proportion of hatched eggs was found to be due to fertilization failure as only 1.3–5.1% of the eggs had started to develop, on average, and the non-developing ones lacked sperm (Fig. 6A). This PMPZ barrier significantly stronger in Sympatry M than in Allopatry or Sympatry F, respectively, but did not differ between Allopatry and Sympatry F (Fig. 6A; Table S16).

PMPZ barriers were also detected in crosses between *D. flavomontana* populations. Proportion of fertilized eggs was significantly reduced in crosses between Sympatry F females and Sympatry M males, and the ones between Sympatry M females and Allopatry males, compared to controls (Fig. 6B; Table S17).

PREZYGOTIC REPRODUCTIVE ISOLATION INDICES

Reproductive isolation indices (RIs) were calculated for sexual isolation and PMPZ barriers in sperm storage (only for interspecific crosses) and fertilization (for crosses between *D. flavomontana* females and *D. montana* males and between *D. flavomontana* populations).

Crosses between *D. montana* females and *D. flavomontana* males showed no variation between population types in RI for sexual isolation or for PMPZ barrier in sperm storage (Fig. 7A), while the reciprocal cross showed variation between population types in all measured barriers (Fig. 7B). RI for sexual isolation was highest in Sympatry F, whereas RIs for both PMPZ barriers were highest in Sympatry M. Thus, in sympatric populations where sexual isolation is less effective, PMPZ barriers could block interspecific gene flow.

Among *D. flavomontana* crosses, RI for sexual isolation was increased in crosses between Sympatry F females and Allopatry and Sympatry M males, as well as between Sympatry M females and Allopatry males (Fig. 7C). RI for PMPZ in egg fertilization was highest in crosses between Sympatry F females and Sympatry M males and between Sympatry M females and Allopatry males (Fig. 7C).

Discussion

Reinforcement can enhance speciation both by strengthening prezygotic reproductive barriers between sympatric species and by creating new barriers between conspecific populations that live within and outside the area of sympatry (Howard 1993; Ortiz-Barrientos et al. 2009). In

our study, both post- and prezygotic barriers between *D. montana* females and *D. flavomontana* males were nearly complete in all population types. However, in crosses between *D. flavomontana* females and *D. montana* males, strong postzygotic isolation was accompanied by sexual and PMPZ barriers whose strength varied between population types. In these crosses sexual isolation was 27% stronger in sympatric Rocky Mountains populations, and PMPZ barriers 25% stronger in sympatric western coast populations, compared to allopatric populations. These percentages are of the same level as the ones detected for reinforcement of prezygotic barriers in sympatric populations of *Drosophila* species with partly overlapping distributions (18–26% on average; Yukilevich 2012). Strengthening of prezygotic barriers in sympatric populations of several species, including mammals, frogs, fishes, insects, birds and plants, gives strong support for speciation via reinforcement (see e.g. Smadja and Ganem 2005; Ortiz-Barrientos et al. 2009; Bímová et al. 2011). However, distinguishing the effects of reinforcement on prezygotic barriers from the those of other selection pressures acting within the species, like ecological adaptation and / or sexual selection, is challenging (Noor 1999; Nosil 2007; Ortiz-Barrientos et al. 2009).

D. montana and *D. flavomontana* females differ in their receptivity and requirement of courtship cues. *D. montana* females mate only after hearing male song (Liimatainen et al. 1992), and in this species certain song characters and female preferences for them vary between populations, suggesting a strong role in sexual selection (Ritchie et al. 1998; Klappert et al. 2007). Thus, variation that we detected in these characters between *D. montana* populations is likely to be due to sexual selection within the species. In *D. flavomontana*, on the other hand, mate choice appeared to rely mainly on CHCs, which showed higher divergence between the species in sympatric populations than in allopatric ones. Furthermore, sympatric males had greater relative amounts of 2-methyl-branched

alkanes than females, suggesting a signal function of these compound classes in mate choice and / or species recognition.

PMPZ barriers have only recently received attention as important suppressors of interspecific gene flow, even though their reinforcement may be a common and rapid process (Castillo and Moyle 2014; Comeault et al. 2016; Turissini et al. 2018). For example, Matute (2010) detected an increase in PMPZ barriers between sympatric *D. yakuba* and *D. santomea*, where *D. yakuba* females depleted the sperm of *D. santomea* males faster than that of conspecific males. Also, sympatric populations of two nightingale species (*Luscinia*) showed greater divergence in sperm morphology than the allopatric ones, with evidence for character displacement in sperm head length in one species (Albrecht et al. 2018). In our study, species differences in the length of female seminal receptacle (*D. montana*: 3.43 mm; *D. flavomontana*: 10.54 mm) and male sperm (*D. montana*: 3.34 mm; *D. flavomontana*: 5.53 mm; Pitnick et al. 1999) could induce problems in sperm transfer and storage especially in matings between *D. montana* females and *D. flavomontana* males. In addition, the male ejaculate may also induce an insemination reaction in females, in which a mass formed in female vagina inhibits sperm storage (Patterson 1946; Knowles and Markow 2001). This reaction has been detected in *D. montana* females after intraspecific matings (Wheeler 1947) and they could be even more pronounced after mating with *D. flavomontana* males. Reduced egg hatch rate detected in both reciprocal crosses, on the other hand, could be due to problems in sperm release from storage and / or to an inability of sperm to penetrate the egg membrane arising from incompatibilities between proteins of either male seminal fluid and female reproductive tract and / or between sperm and egg (Howard 1999; Wirtz 1999; Lawniczak and Begun 2007; Howard et al. 2009; Kelleher et al. 2009). In our study the reasons for decreased egg hatch rate were traced in crosses between *D. flavomontana* females and *D. montana* males, and between *D. flavomontana* flies from different population types,

and in both cases failure in egg development appeared to be due to an inability of sperm to enter the egg. Similar barriers have been detected also between other *virilis* group species (Sweigart 2010; Sagga and Civetta 2011; Ahmed-Braimah and McAllister 2012) and previously between *D. montana* populations (Jennings et al. 2014b; Garlovsky and Snook 2018).

Crosses between *D. flavomontana* females and *D. montana* males enabled us to trace the strength and possible reinforcement of sexual and PMPZ barriers in sympatric populations with different histories and species abundancies. Sexual isolation was strongest in sympatric Rocky Mountains populations (Sympatry F), as expected if reinforcement targets barriers functioning at early stages of species interaction in populations with a long history of co-existence and high abundance of *D. flavomontana*. Reinforcement of sexual isolation in Sympatry F is likely to be driven by strong postzygotic barriers, but it could also be affected by strong PMPZ barrier in egg fertilization (see Servedio 2001). On the other hand, both PMPZ barriers, sperm storage and fertilization, were strongest in sympatric western coast populations (Sympatry M), as expected if reduced choosiness of rare females has restrained reinforcement of sexual isolation (Turissini et al. 2018). Crosses between *D. flavomontana* from different population types also gave support for cascade reinforcement, as Sympatry F females showed highest discrimination against males of other populations, while PMPZ barriers were strongest in crosses involving flies from Sympatry M. The strength of sexual isolation in inter- and intraspecific crosses involving *D. flavomontana* females could, at least partly, be due to variation in CHCs, which showed highest species divergence in Sympatry F and lowest in Allopatry. Divergence of male CHCs in Sympatry M vs. Allopatry could also explain why Sympatry M females discriminated against Allopatry males. Species and sex differences in CHC could also be driven by natural selection, e.g. through insect desiccation tolerance (Gibbs 2002). However, divergence of CHCs of *D. flavomontana* Rocky Mountains

populations (Sympatry F and Allopatry) was higher than Sympatry F and Sympatry M (Fig. 4B), suggesting that the climatic conditions on the Rocky Mountains and western coast may not have played a major role in CHC divergence.

In conclusion, reinforcement has been shown to play a key role in both strengthening species boundaries and enhancing new barriers, and the field of speciation is beginning to evaluate its broader evolutionary and ecological consequences (Pfennig 2016). Speciation research also needs to consider the origin of barrier effects and the ways in which they are coupled, as strong barriers to gene flow will evolve only if multiple barrier effects coincide (Butlin and Smadja 2018). Our results show that reinforcement may target either sexual or PMPZ barriers depending on the length of species coexistence and / or species abundancies, and we also demonstrate that the consequences of such reinforcement can be detected between conspecific populations. Accordingly, we argue that the reliance of reproductive isolation on multiple barriers is beneficial because different barriers can compensate each other in situations where reinforcement of some barriers is restricted (Seehausen 2004; Currat et al. 2008; Abbott et al. 2013).

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Table 1. The most influential CHC substances based on random forest analysis (see Fig. S4).

Random forest analysis	Allopatry	Sympatry F	Sympatry M
Between *D. montana*	2methylC24-alkane	C27-alkene-1	C27-alkene-2
and *D. flavomontana*	C27-alkene-3	C27-alkene-3	2methylC24-alkane
Between sexes of	C25-alkene-2	C25-alkene-4	C27-alkene-2
D. montana	C29-alkene-1	C29-alkadiene-2	C29-alkene-2
Between sexes of	C27-alkene-5	2methylC28-alkane/C29-alkadiene-5	2methylC28-alkane/C29-alkadiene-5
D. flavomontana	C25-alkene-4	C27-alkene-2	2methylC30-alkane/C31-alkadiene-4

Figure 1. North American fly collection sites. Pie charts show the proportion of *D. montana* (blue) and *D. flavomontana* (yellow) at each site 2010-2015; in the present study we used strains collected 2013-2015.

Collection location, altitude and year	Strain ID
Allopatry	
Seward, AK, USA, 35 m, 2013 (Sew)	*mon*SE13F37, *mon*SE13F16
Livingston*, MT, USA, 1605 m, 2013 (Liv)	*fla*MT13F11, *fla*MT13F15
Liberty*, UT, USA, 1600 m, 2015 (Lib)	*fla*LB15F1, *fla*LB15F3
Afton*, WY, USA, 2000 m, 2015 (Aft)	*mon*AF15F12, *mon*AF15F28
Rocky Mountains; Sympatry F (Long history with *D. montana, D. flavomontana* abundant)	
Cranbrook, BC, Canada, 940 m, 2014 (Cran)	*mon*CRAN14F16, *mon*CRAN14F20 *fla*CRAN14F7, *fla*CRAN14F13
Jackson, WY, USA, 1 857 m, 2013 (Jac)	*mon*JX13F3, *mon*JX13F48 *fla*JX13F38, *fla*JX13F31
Western coast; Sympatry M (New contact zone, *D. flavomontana* rare)	
Terrace, BC, Canada, 217 m, 2014 (Ter)	*mon*TER14F11 *fla*TER14F5
Vancouver, BC, Canada, 4 m, 2014 (Van)	*mon*VAN14F1, *mon*VAN14F17 *fla*VAN14F20, *fla*VAN14F9
Ashford, WA, USA, 573 m, 2013 (Ash)	*mon*ASH13F9, *mon*ASH13F13 *fla*ASH13F14, *fla*ASH13F2

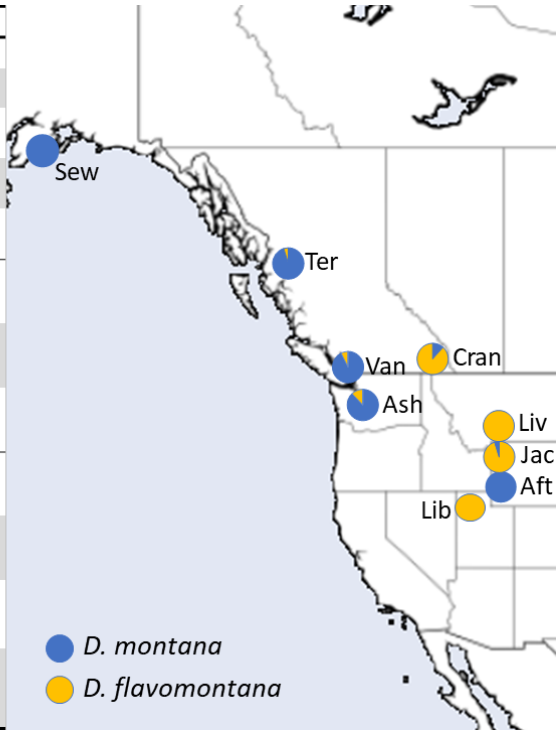
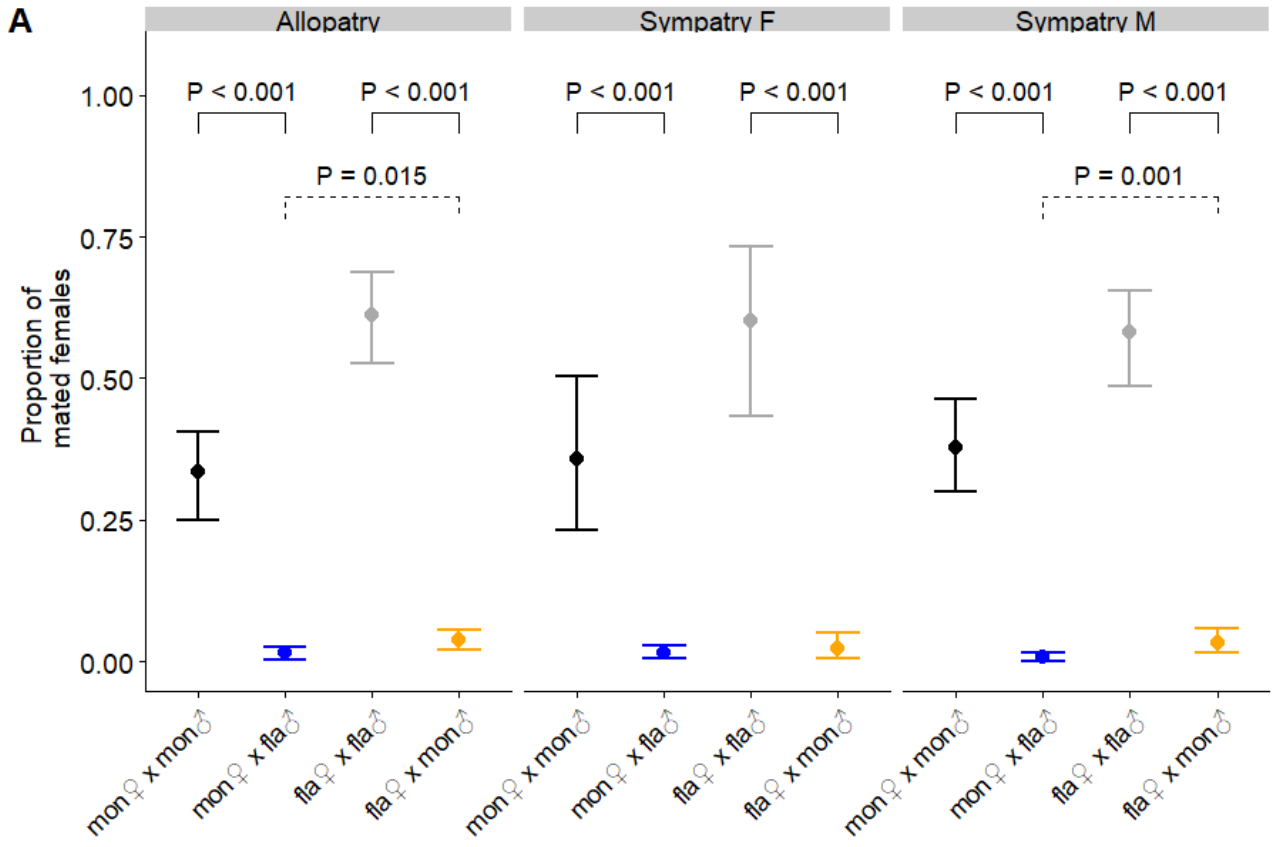
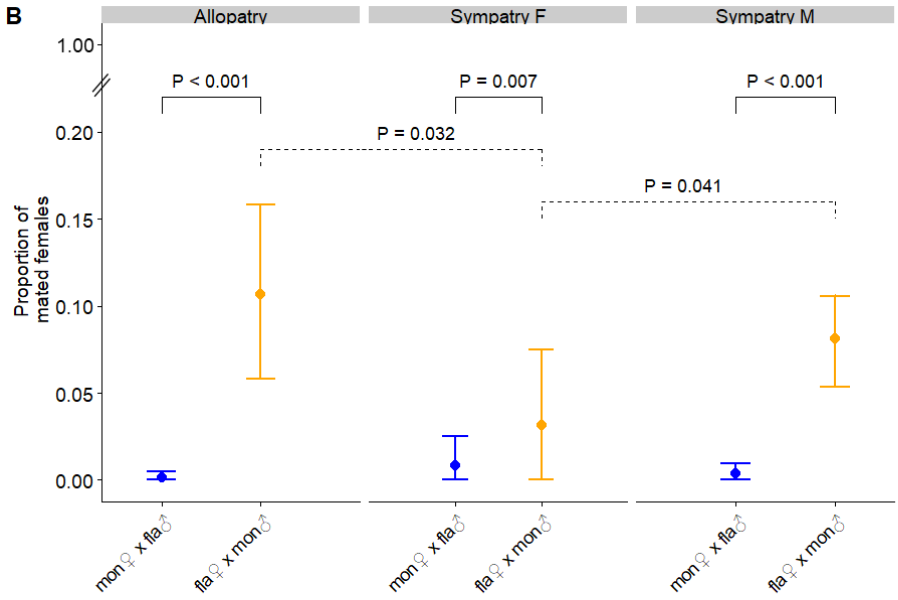


Figure 2. The strength of sexual isolation between *D. montana* and *D. flavomontana* in (A) multiple-choice tests and (B) no-choice tests in different population types. (C) The strength of sexual isolation between *D. flavomontana* from the same and different population types in multiple-choice tests. Error bars represent bootstrapped 95% confidence intervals. P-values are obtained from GLMMs. (A) P-values above solid lines refer to differences between inter- and intraspecific crosses, and the ones above dashed lines to differences between reciprocal crosses. (B) P-values above solid lines refer to differences between reciprocal crosses, and the ones above dashed lines to differences between similar crosses. (C) P-values show statistically significant differences between intra- and interpopulation crosses in *D. flavomontana*.





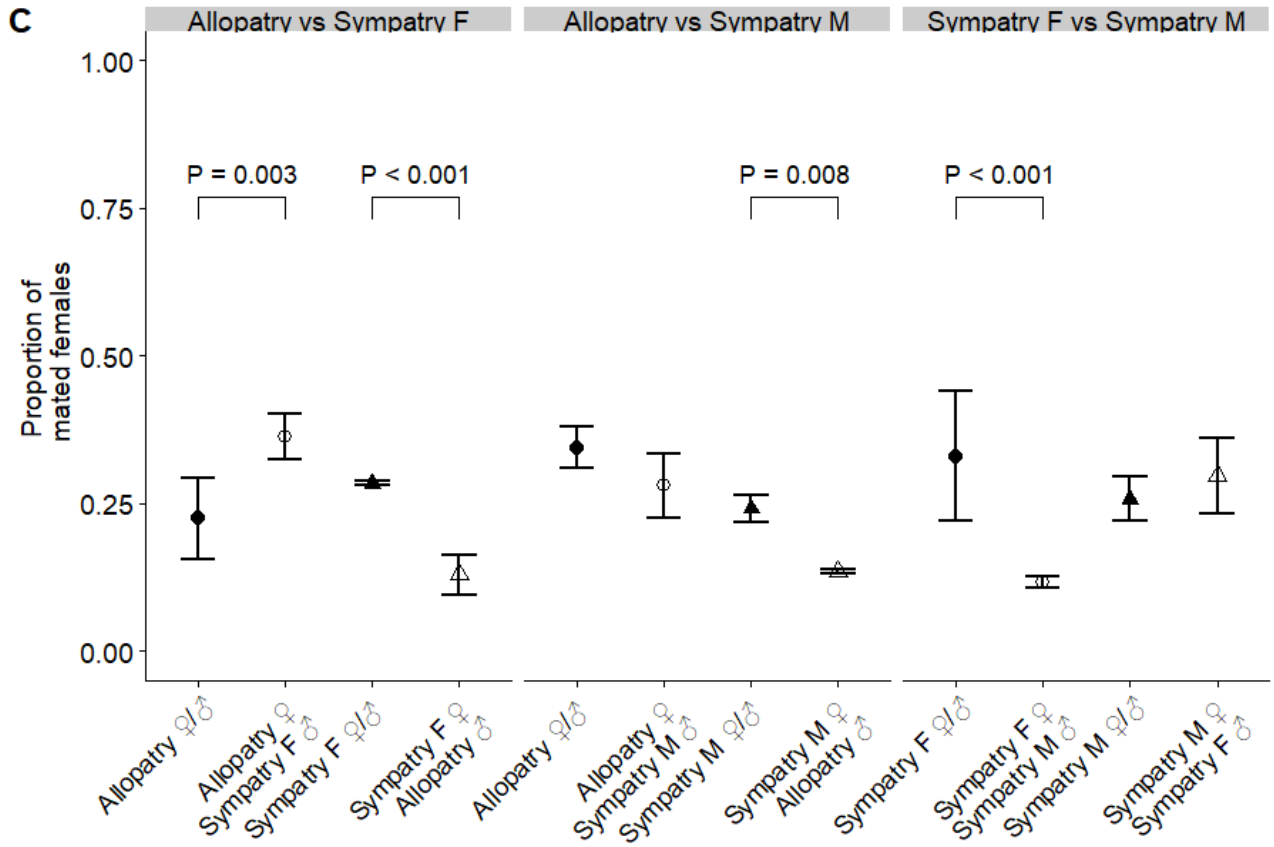


Figure 3. The impact of blocking the transfer of sensory cues on the proportion of mated females in crosses between (A) *D. montana* flies, (B) *D. montana* females and *D. flavomontana* males, (C) *D. flavomontana* flies and (D) *D. flavomontana* females and *D. montana* males. Error bars represent bootstrapped 95% confidence intervals. P-values are obtained from GLMMs and show significant differences between the control and sense-deprivation experiments.

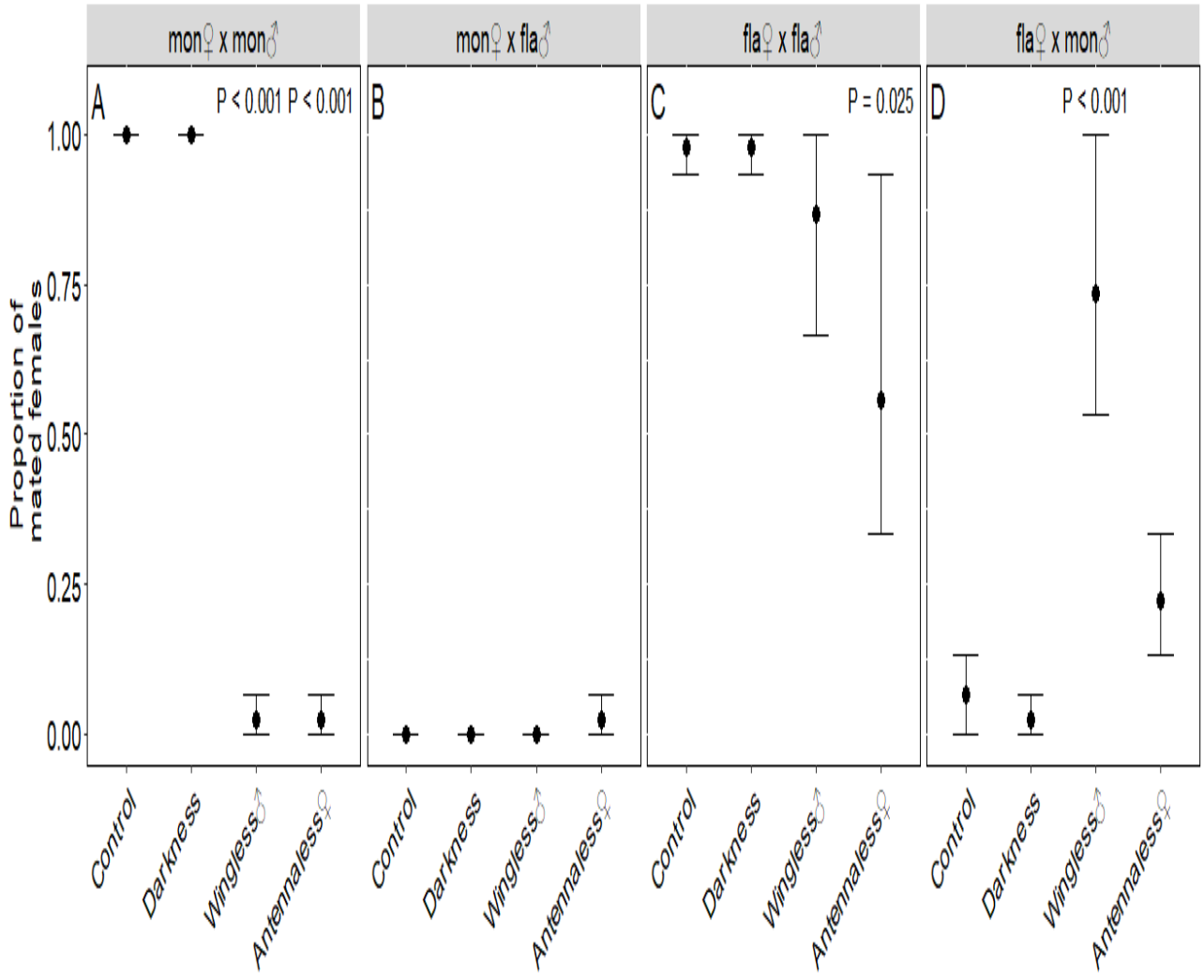


Figure 4. Variation between species and population types in (A) male song traits based on principal component analysis (PCA) and (B) cuticular hydrocarbons (CHCs) of both sexes based on multivariate Linear Discriminant Analysis (LDA). Song traits: PN = number of pulses in a pulse train, PL = length of a sound pulse, IPI = interpulse interval, CN = number of cycles in a sound pulse and FRE = song carrier frequency.

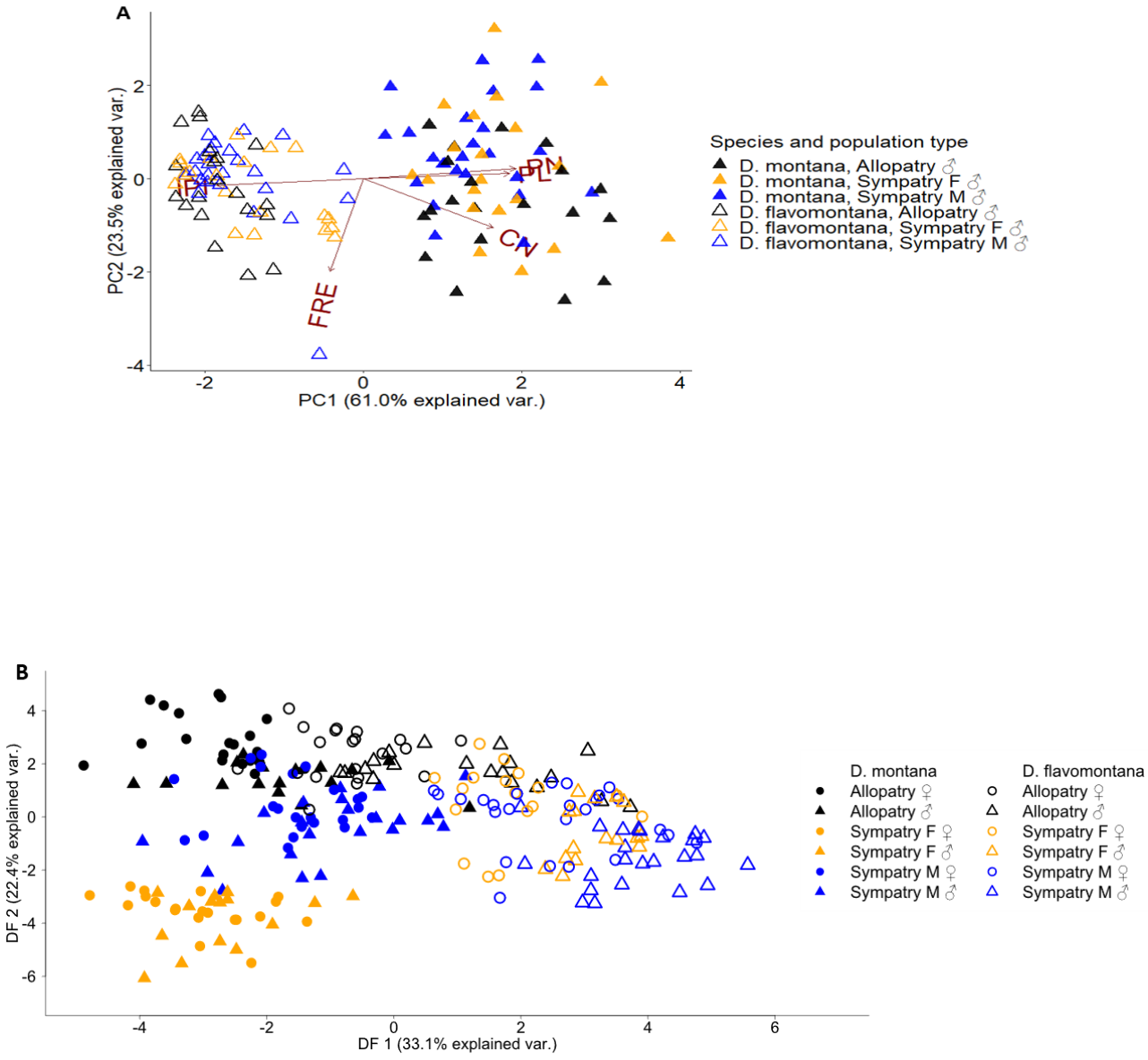


Figure 5. The quantity of stored sperm in interspecific crosses compared to intraspecific ones (P-values above solid lines) and between reciprocal interspecific crosses (P-values above dashed lines). P-values are obtained from CLMMs. Numbers above x-axis refer to the number of studied females in each cross.

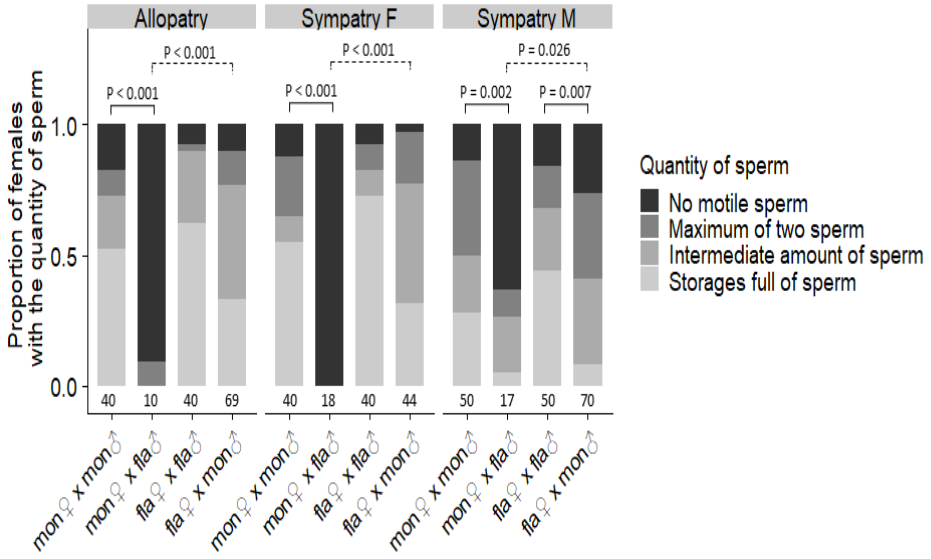


Figure 6. Proportion of fertilized eggs (A) in crosses between *D. flavomontana* females and *D. montana* males in different population types and (B) in the ones between *D. flavomontana* females and males from the same or different population type. Error bars represent bootstrapped 95% confidence intervals. P-values are obtained from GLMMs, and in (B) they show significant differences in matings between females and males from different population

types compared to intrapopulation controls. Numbers above x-axis refer to the number of eggs examined.

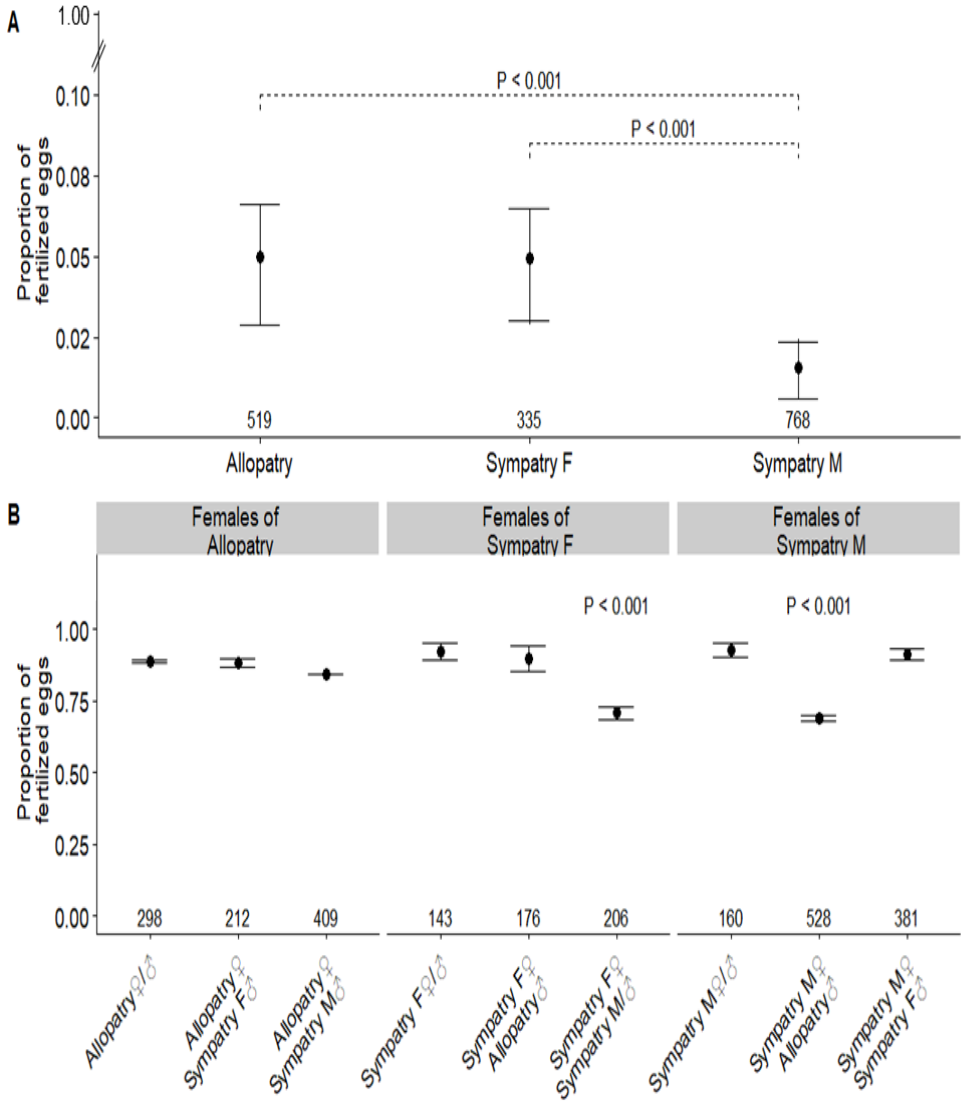
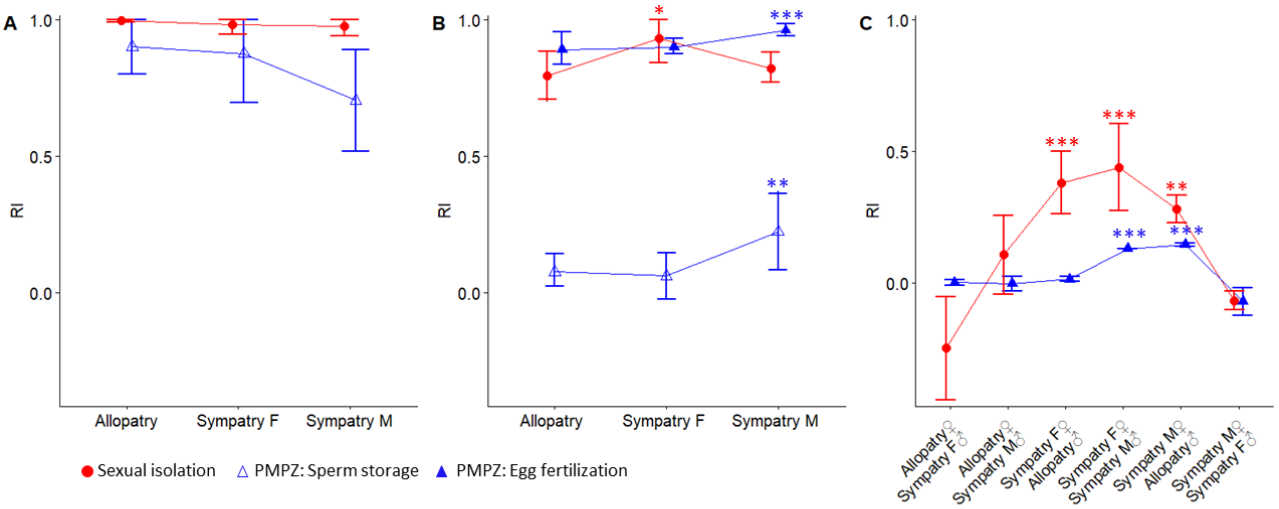


Figure 7. Reproductive isolation indices (RIs) calculated for sexual isolation and PMPZ barriers in sperm storage and / or fertilization in (A) interspecific crosses between *D. montana* females and *D. flavomontana* males and (B) interspecific crosses between *D. flavomontana* females and *D. montana* males, and (C) in crosses between *D. flavomontana* flies from different population types (C). Error bars represent bootstrapped 95% confidence intervals. Significance levels are obtained from the analyses (GLMMs and CLMMs)

performed on respective barriers (see Fig. 2B-C, 5 and 6): * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.



Appendix

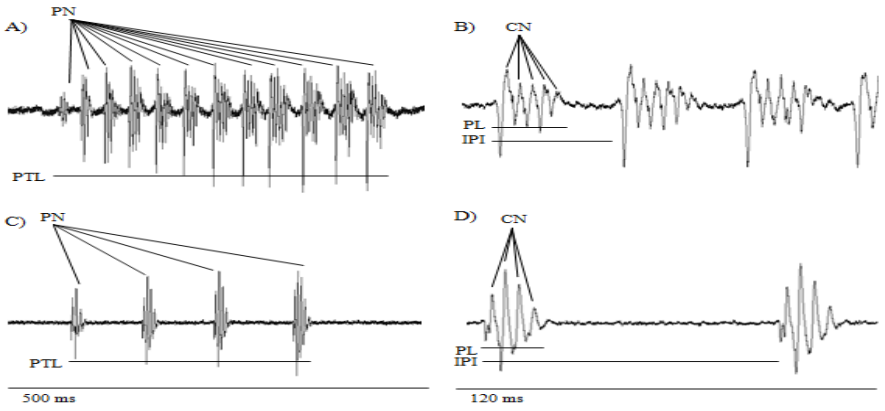


Figure A1. Oscillograms of the courtship songs of *D. montana* (A, B) and *D. flavomontana* (C, D) males and the traits measured from them. PN = number of pulses in a pulse train, PTL

= length of a pulse train, CN = number of cycles in a sound pulse, PL = length of a sound pulse, IPI = interpulse interval.

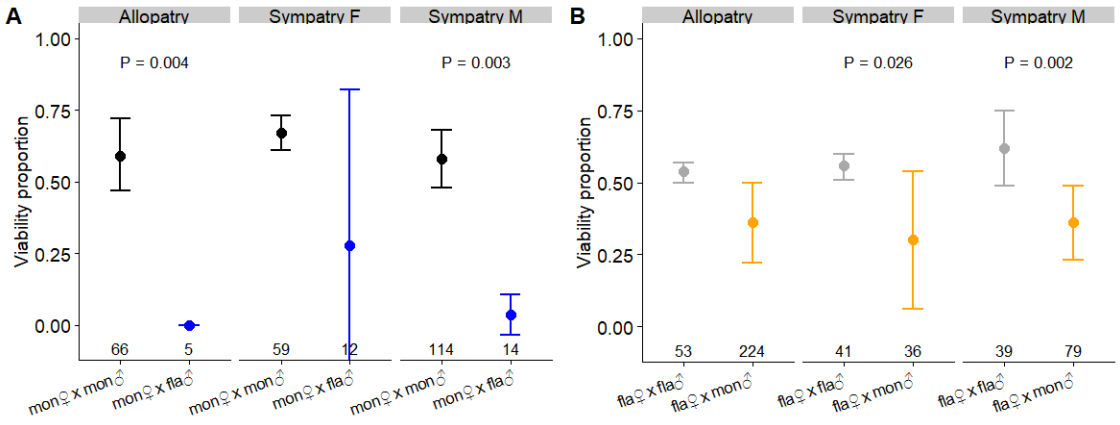


Figure A2. F_1 hybrid viability in intra- and inter- specific crosses involving (A) *D. montana* females and (B) *D. flavomontana* females. Error bars represent 95% confidence intervals. P-values from GLMMs indicate significant differences between intra- and interspecific crosses in different population types, and the numbers above x-axis refer to the total number of studied larvae per cross.

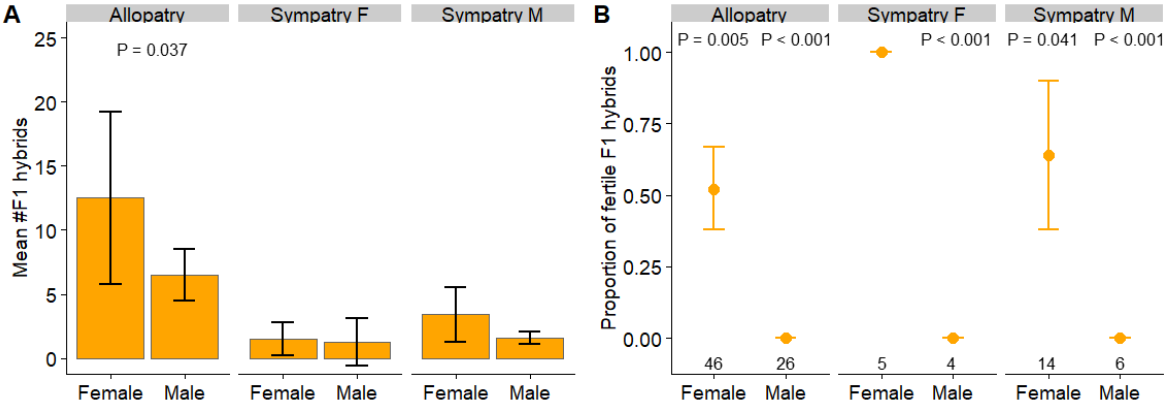


Figure A3. (A) Sex ratio and (B) fertility of F_1 hybrids produced by *D. flavomontana* females and *D. montana* males. Error bars represent 95% confidence intervals. P-values from student's t tests refer to significant deviation from the expected 0.5 in sex ratio and 1 in fertility. The numbers above x-axis refer to the total number of studied adult flies.

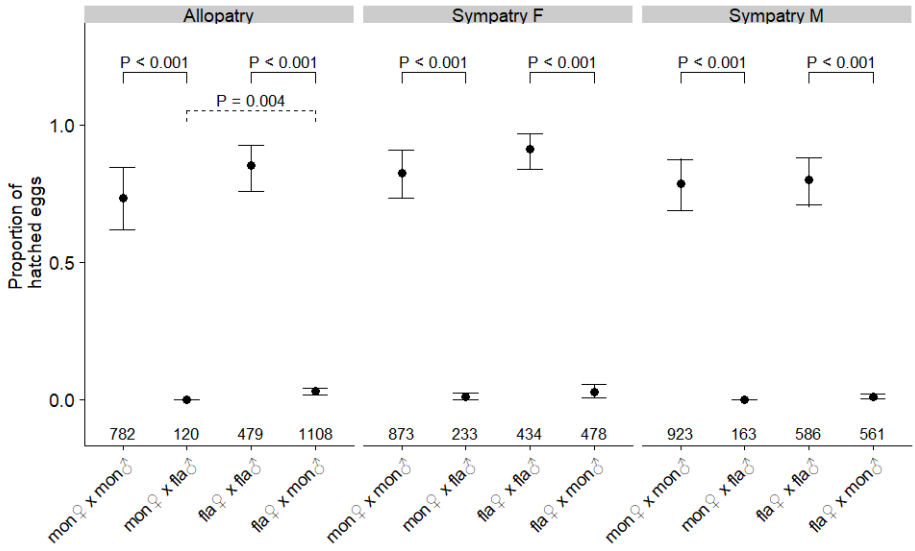


Figure A4. Proportion of hatched eggs in interspecific and in intraspecific crosses (P-values above solid lines) and in crosses between interspecific reciprocal crosses (P-value above dashed line). Error bars represent bootstrapped 95% confidence intervals. P-values are obtained from GLMMs. Numbers above x-axis refer to the number of studied eggs in each cross.

Reinforcement of sexual and postmating prezygotic barriers varies between sympatric populations with different histories and species abundances

S*upporting Material*

Content

Methods

Method S1. *Wolbachia* detection

Presence of possible *Wolbachia* infection in *D. montana* and *D. flavomontana* was checked with PCR from DNA extractions from two 21 d old females and males of each study strain, using Wolbachia_1-3FR and wsp_1F+R primers (Osborne et al. 2009). Primer sequences are given in Table S2. Wolbachia_1-3FR primers were designed using *Wolbachia* sequences collected from the *D. montana* genome (old inbred Vancouver population; Parker et al. 2018) with Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and Netprimer (http://www.premierbiosoft. com/netprimer/) programs. PCR was carried out with Bio-Rad's C1000 Touch™ thermal cycler using the following program: 95°C for 3 min, 95°C for 10 s, 52°C for 10 s, 72°C for 30 s, and finally 72°C for 10 min. *Wolbachia* infected flies of *Drosophila melanogaster* and *Drosophila borealis* (a species of *D. virilis* group) were used as positive controls. PCR products were run on a 1.5% agarose gel for 30 minutes with Sybr safe™ dye to check for the presence of *Wolbachia* infections in different samples.

Methods S2. Normalization of sexual isolation data between *D. flavomontana* population types.

To control for differences in mating activity across strains in tests between population types, the data from these experiments were normalized using the following procedure.

Tests within population types were first used to establish intrinsic mating activities (matings per minute) for both sexes of each strain. For example, the intrinsic mating activity of strain A females consisted of the number of matings with A and B males (strain B originating from the same population type).

Using the intrinsic mating activities of both sexes of each strain, expected mating activities for each cross in tests between population types were calculated by taking the mean of the mating activities of the participating strains. For example, the expected mating activity for cross A x C (A and C originating from different population types) is the mean of the mating activities of A and C derived from tests within population types. This is a theoretical value that shows the expected mating activities, if females and males of different strains were not constrained by reproductive barriers.

The observed mating activity in each cross in tests between population types was then calculated by taking the mean of the numbers of matings both sexes participated in. For example, the observed mating

activity in cross A x C was calculated from the mean of the overall number of matings between A females and C males. Note that the observed mating activity does not only reflect the number of matings in this cross, but also the number of matings both sexes had in other crosses.

The ratio between expected and observed mating activities for each cross reveals the deviation of the observed activities from the expected ones. Finally, the absolute number of matings in each cross was multiplied by this ratio to yield normalized values that take into account differences in fly mating activities.

Method S3. Courtship song recording methods

For song recording, a sexually mature virgin female and male of the same strain were transferred into a small petri dish, which had a moistened filter paper on the bottom and a nylon net roof. Courting males walked upside down on the roof of the chamber, which allowed song recording by holding the microphone (JVC) directly above the male. Songs were recorded using a digital Handy Recorder H4n at a temperature of 20 ± 1˚C and analyzed with the Signal 4.0 sound analysis system (Engineering Design, Belmont, MA, USA).

Method S4. Methods used in analyzing cuticular hydrocarbon (CHC) profiles.

CHC extractions were performed in the morning by immersing individuals in 200 µl n-hexane in glass vials (Micro Liter Analytical Supplies; 1.8 ml) for 10 min, after which individuals were removed. Open vials were maintained in a sterile fume hood at room temperature until the hexane had evaporated, then vials were sealed and stored at -20°C. Control vials with pure solvent (n-hexane) were prepared in the same way.

CHC extracts were analysed with an Agilent 7890 gas chromatograph (GC) coupled with an Agilent 5975C Mass Selective (MS) Detector (Agilent, Waldbronn, Germany) at the University of Würzburg (Germany). The GC (split/splitless injector in splitless mode for 1 min, injected volume: 1 µl at 300°C) was equipped with a DB-5 Fused Silica capillary column (30m x 0.25 mm ID, df = 0.25 µm; J&W Scientific, Folsom, USA). Helium served as a carrier gas at a constant flow of 1 ml/min. The temperature program consisted of the start temperature 60°C, temperature increase by 5°C/min up to 300°C and maintenance at 300°C for 10 min. The electron ionization mass spectra (EI-MS) were acquired at an ionization voltage of 70 eV (source temperature: 230°C). Chromatograms and mass spectra were recorded and quantified with the software Agilent Enhanced Chem Station G1701AA (version A.03.00).

Individual CHC compounds were chemically identified using the MS data base Wiley275 (John Wiley & Sons, New York, USA), retention indices, and the detected diagnostic ions (Bernier et al. 1998). Some substances could not be accurately separated and, in these cases, the combined quantity was calculated by integrating over all substances within a peak.

CHC profile similarity was assessed by means of multivariate Linear Discriminant Analysis (LDA) and Random forest classification (Liaw and Wiener 2002) using the functions *lda* (from the MASS package) and *randomForest* (from the randomForest package) in R (Version 3.4.3) and R studio (Version 1.1.383). In addition, Bray-Curtis dissimilarities were analyzed for differences between species in each population type and differences between sexes within a population type for both species. Values range from 0 to 1, where 0 means the same composition and 1 means complete dissimilarity. Significance levels were tested with linear mixed model (LMM) using study strains as a random effect.

Method S5. Methods used in song play-back experiments.

D. flavomontana males were muted by removing their wings with micro-scissors 1d before the mating experiment. *D. montana* females and muted *D. flavomontana* males (n=10-15 per trial) were placed in a mating arena (small petri dish and a nylon net roof) placed above a subwoofer (Harman Kardon JBL Platinum Series Speakers) connected to a computer. Recorded *D. montana* song was played for the flies throughout the courtship, and mating pairs were collected once copulation had ended.

Tables

Table S1. Strains used in reproductive isolation experiments and phenotypic assays (Strain ID prefix *mon = D. montana*, *fla = D. flavomontana*) consisting of 4 strain pairs from Allopatry, 4 strain pairs from Sympatry F (*D. flavomontana* abundant, long history with *D. montana*) and 4 strain pairs from Sympatry M (*D. montana* abundant, new contact zone). *D. montana* and *D. flavomontana* strains that were used as pairs in interspecific experiments are marked with the same alphabet in superscript above the strain ID. The strains collected from the same location were used as controls, except studies of postzygotic barriers and interspecific no-choice tests, where strains from Seward, Livingston, Jackson and Vancouver were used as intraspecific controls. Strains used for studying reproductive isolation between *D. flavomontana* populations are marked in the last column with strains used as pairs marked with the same alphabet in superscript above the strain ID. The strains collected from the same location were used as controls.

Collecting site and year	Strain ID; Interspecific experiments	Strain ID; Experiments between *D. flavomontana*
Allopatry		
Seward, AK, USA (2013) 60°10'N; 149°27'W Altitude 35 m	*mon* SE13F37[A] *mon* SE13F16[B]	
Livingston, MT, USA (2013) 45°21'N; 110°36'W Altitude 1 605 m	*fla* MT13F11[A] *fla* MT13F15[B]	*fla* MT13F11[A, B] *fla* MT13F15[C, D]
Liberty, UT, USA (2015) 41°20'N; 111°51'W Altitude 1 600 m	*fla* LB15F1[C] *fla* LB15F3[D]	
Afton, WY, USA (2015) 42°43'N; 110°55'W Altitude 2 000 m	*mon* AF15F12[C] *mon* AF15F28[D]	
Sympatry F		
Cranbrook, BC, Canada (2014) 49°36'N; 115°46'W Altitude 940 m	*mon* CRAN14F16[J] *mon* CRAN14F20[K] *fla* CRAN14F7[J] *fla* CRAN14F13[K]	
Jackson, WY, USA (2013) 43°26'N; 110°50'W Altitude 1 857 m	*mon* JX13F3[L] *mon* JX13F48[M] *fla* JX13F38[L] *fla* JX13F31[M]	 *fla* JX13F38[C, E] *fla* JX13F31[A, H]
Sympatry M		
Terrace, BC, Canada (2014) 54°27'N; 128°34'W Altitude 217 m	*mon* TER14F11[E] *fla* TER14F5[E]	
Vancouver, BC, Canada (2014) 49°15'N; 123°10'W Altitude 4 m	*mon* VAN14F1[F] *mon* VAN14F17[G] *fla* VAN14F20[F] *fla* VAN14F9[G]	 *fla* VAN14F20[D, H] *fla* VAN14F9[B, E]
Ashford, WA, USA (2013) 46°45'N; 121°57'W Altitude 573 m	*mon* ASH13F9[H] *mon* ASH13F13[I] *fla* ASH13F14[H] flaASH13F2[I]	
AK = Alaska, BC = British Columbia, MT = Montana, UT = Utah, WA = Washington, WY = Wyoming		

Table S2. Primer information for mitochondrial *COI*, Wolbachia and wsp primers.

Region/primers	Primer sequences
COI_1F	5'-ATCTATCGCCTAAACTTCAGCC-3'
COI_1R	5'-ACTTCAGGGTGACCAAAAAATC-3'
Wolbachia_1F	5' – GGTGTCCAAGGTCCAGAAAA – 3'
Wolbachia_1R	5' – AGTGCTCTTCTAGCCGTCCA – 3'
Wolbachia_2F	5' – CGGTTGACGGAGTCGTAAAT – 3'
Wolbachia_2R	5' – AGGAAGTGCCGATTGAAAAC – 3'
Wolbachia_3F	5' – CCGTCCTTTCACAGGAAAAC – 3'
Wolbachia_3R	5' – TGGTTGATGGTCTGTTTGGA – 3'
wsp_1F	5'-GCATTTGGTTAYAAAATGGACGA-3'
wsp_1R	5'-GGAGTGATAGGCATATCTTCAAT-3'

Table S3. Correlations between male courtship song parameters in *D. montana* (values above grey fields) and in *D. flavomontana* (values below grey fields).

	PN	PTL	PL	IPI	CN	FRE
PN		0.844	0.019	0.159	0.052	-0.008
PTL	0.855		0.277	0.556	0.110	-0.163
PL	0.205	0.366		0.681	0.600	-0.286
IPI	-0.063	0.418	0.267		0.244	-0.386
CN	0.262	0.310	0.739	0.029		0.434
FRE	-0.038	-0.174	-0.038	-0.280	0.453	

Table S4. Number of females (f) and males (m) per study strain used in CHC analyses. Prefix in the Strain ID: *mon* = *D. montana* and *fla* = *D. flavomontana.*

Population type	Strain ID	N
Allopatry	*mon* SE13F37	5 f, 4 m
	mon SE13F16	5 f, 5 m
	mon AF15F12	5 f, 4 m
	mon AF15F28	5 f, 5 m
	fla MT13F11	5 f, 4 m
	fla MT13F15	5 f, 5 m
	fla LB15F1	5 f, 5 m
	fla LB15F3	5 f, 5 m
Sympatry F	*mon* CRAN14F16	5 f, 1 m
	mon CRAN14F20	5 f, 4 m
	mon JX13F3	5 f, 5 m
	mon JX13F48	5 f, 5 m
	fla CRAN14F7	4 f, 2 m
	fla CRAN14F13	4 f, 5 m
	fla JX13F38	5 f, 5 m
	fla JX13F31	4 f, 5 m
Sympatry M	*mon* TER14F11	4 f, 4 m
	mon VAN14F1	4 f, 5 m
	mon VAN14F17	5 f, 5 m
	mon ASH13F9	5 f, 5 m
	mon ASH13F13	5 f, 5 m
	fla TER14F5	5 f, 4 m
	fla VAN14F20	5 f, 5 m
	fla VAN14F9	5 f, 5 m
	fla ASH13F14	5 f, 5 m
	fla ASH13F2	5 f, 5 m

Table S5. Statistical tests on the viability (from 3rd instar larvae to adult) of intra- and interspecific F_1 progeny in different population types performed using generalized linear mixed model (GLMM) with binomial distribution. Sex ratio and fertility (mean and 95% confidence interval) were analyzed with one-sample student's t tests (expected sex ratio 0.5 and fertility 1). Significant values are shown in bold.

Viability of offspring produced by	**Df**	**z**	**P-value**
mon♀xmon♂ vs mon♀xfla♂			
Allopatry	1, 4	-	**0.004** (χ2 test)
Sympatry F	1, 4	0.90	0.370
Sympatry M	1, 5	2.98	**0.003**
fla♀xfla♂ vs fla♀xmon♂			
Allopatry	1, 4	-1.87	0.061
Sympatry F	1, 4	-2.22	**0.026**
Sympatry M	1, 5	-3.04	**0.002**

Sex ratio	**N♀**	**N♂**	**Proportion of females**	**P-value**
Allopatry				
mon♀xfla♂	0	0	-	-
fla♀xmon♂	50	26	0.64 (0.52-0.76)	**0.037**
Sympatry F				
mon♀xfla♂	4	1	-	-
fla♀xmon♂	7	4	0.62 (-0.37-1.62)	0.652
Sympatry M				
mon♀xfla♂	0	1	-	-
fla♀xmon♂	15	7	0.63 (0.42-0.85)	0.160

F1 female fertility	**N**	**Proportion fertile females**	**P-value**
Allopatry			
mon♀xfla♂	0	-	
fla♀xmon♂	46	0.45 (0.22-0.68)	**0.005**
Sympatry F			
mon♀xfla♂	3	-	
fla♀xmon♂	5	1.00	-
Sympatry M			
mon♀xfla♂	0	-	
fla♀xmon♂	14	0.33 (-0.42-0.85)	**0.041**

F1 male fertility	**N**	**Proportion fertile males**	**P-value**
Allopatry			
mon♀xfla♂	0	-	
fla♀xmon♂	26	0	**> 0.001**
Sympatry F			
mon♀xfla♂	0	-	
fla♀xmon♂	4	0	**> 0.001**
Sympatry M			
mon♀xfla♂	0	-	
fla♀xmon♂	6	0	**> 0.001**

Table S6. Statistical analyses of multiple-choice and no-choice tests performed using generalized linear mixed model (GLMM) with binomial distribution. Significant values are shown in bold.

Sexual isolation			
Multiple-choice tests between *D. montana* and *D. flavomontana*	**Df**	**Z**	**P-value**
Allopatry			
mon♀ x mon♂ vs mon♀ x fla♂	1,7	10.13	**< 0.001**
fla♀ x fla♂ vs fla♀ x mon♂	1,7	-16.1	**< 0.001**
mon♀ x fla♂ vs fla♀ x mon♂	1,7	-2.44	**0.015**
Sympatry F			
mon♀ x mon♂ vs mon♀ x fla♂	1,7	10.82	**< 0.001**
fla♀ x fla♂ vs fla♀ x mon♂	1,7	-14.88	**< 0.001**
mon♀ x fla♂ vs fla♀ x mon♂	1,7	-0.84	0.403
Sympatry M			
mon♀ x mon♂ vs mon♀ x fla♂	1,9	10.4	**< 0.001**
fla♀ x fla♂ vs fla♀ x mon♂	1,9	-17.17	**< 0.001**
mon♀ x fla♂ vs fla♀ x mon♂	1,9	-3.21	**0.001**
No-choice tests between *D. montana* and *D. flavomontana*	**Df**	**Z**	**P-value**
Allopatry			
mon♀ x fla♂ vs fla♀ x mon♂	1,63	-4.28	**< 0.001**
Sympatry F			
mon♀ x fla♂ vs fla♀ x mon♂	1,22	-2.71	**0.007**
Sympatry M			
mon♀ x fla♂ vs fla♀ x mon♂	1,62	-5.24	**< 0.001**
Allopatry vs. Sympatry F			
mon♀ x fla♂	1, 4	0.48	0.629
fla♀ x mon♂	1, 81	-2.15	**0.032**
Allopatry vs. Sympatry M			
mon♀ x fla♂	1, 2	0.76	0.448
fla♀ x mon♂	1, 123	-0.84	0.401
Sympatry F vs. Sympatry M			
mon♀ x fla♂	1, 6	-0.16	0.871
fla♀ x mon♂	1, 78	2.05	**0.041**
Multiple-choice tests between *D. flavomontana* from different population types	**Df**	**Z**	**P-value**
Allopatry (A) vs Sympatry F (F)			
A♀ x A♂ vs A♀ x F♂	1,3	2.95	**0.003**
F♀ x F♂ vs F♀ x A♂	1,3	3.74	**< 0.001**
Allopatry (A) vs Sympatry M (M)			
A♀ x A♂ vs A♀ x M♂	1,3	-1.4	0.161
M♀ x M♂ vs M♀ x A♂	1,3	2.66	**0.008**
Sympatry F (F) vs Sympatry M (M)			
F♀ x F♂ vs F♀ x M♂	1,3	-4.88	**< 0.001**
M♀ x M♂ vs M♀ x F♂	1,3	-0.9	0.369

Table S7. Results of multiple-choice tests for each *D. montana* and *D. flavomontana* strain pair from different population types. Total number of flies from the five replicates of each cross was 150 females and 150 males (150 copulating pairs). Values refer to the number of copulating pairs that occurred when half of the flies had mated. Numbers in parentheses refer to the number of matings observed after 50% of the flies had mated.

		Copulating pairs / strain pair			
Population type	**Strain pair**	**fla♀× fla♂**	**mon♀× mon♂**	**fla♀× mon♂**	**mon♀× fla♂**
Allopatry	monSE13F37 x flaMT13F11	109 (+8)	32 (+28)	4 (+3)	5 (+1)
	monSE13F16 x flaMT13F15	77 (+4)	64 (+10)	8 (+0)	1 (+1)
	monAF15F12 x flaLB15F1	97 (+15)	51 (+31)	2 (+0)	0 (+0)
	monAF15F28 x flaLB15F3	84 (+23)	54 (+40)	9 (+1)	3 (+0)
Sympatry F	monCRAN14F16 x flaCRAN14F7	89 (+13)	58 (+44)	3 (+1)	0 (+1)
	monCRAN14F20 x flaCRAN14F13	52 (+19)	86 (+17)	9 (+4)	3 (+2)
	monJX13F3 x flaJX13F38	116 (+9)	27 (+34)	2 (+0)	5 (+3)
	monJX13F48 x flaJX13F31	104 (+13)	44 (+51)	0 (+1)	2 (+1)
Sympatry M	monVAN14F1 x flaVAN14F20	62 (+48)	80 (+26)	5 (+1)	3 (+0)
	monVAN14F17 x flaVAN14F9	83 (+28)	64 (+31)	1 (+0)	2 (+1)
	monASH13F9 x flaASH13F14	104 (+21)	43 (+45)	2 (+3)	1 (+0)
	monASH13F13 x flaASH13F2	95 (+22)	43 (+37)	12 (+0)	0 (+0)
	monTER14F11 x flaTER14F5	92 (+15)	53 (+41)	5 (+0)	0 (+1)

Table S8. Results of multiple-choice tests for each *D. flavomontana* strain pair within and between different population types. Total number of matings from the three replicates of each cross was 90 females and 90 males (90 copulating pairs). Values refer to the number of observed matings. Values in parentheses are matings normalized by the activity of the respective strains within and between population types to prevent the fly activity from affecting the results. Statistical tests were performed using the normalized values.

		Copulating pairs / strain pair			
Population type	**Strains**	**A♀xA♂**	**B♀xB♂**	**A♀xB♂**	**B♀xA♂**
Allopatry	flaMT13F11 (A) x flaMT13F15 (B)	28	22	17	23
Sympatry F	flaJX13F31 (A) x flaJX13F38 (B)	16	32	12	30
Sympatry M	flaVAN14F9 (A) x flaVAN14F20 (B)	30	21	13	25
Allopatry vs Sympatry F	flaMT13F11 (A) x flaJX13F31 (B)	19 (11)	19 (21)	34 (30)	18 (12)
	flaMT13F15 (A) x flaJX13F38 (B)	28 (33)	26 (33)	25 (37)	11 (11)
Allopatry vs Sympatry M	flaMT13F11 (A) x flaVAN14F9 (B)	35 (42)	13 (29)	27 (25)	15 (14)
	flaMT13F15 (A) x flaVAN14F20 (B)	35 (19)	16 (13)	24 (21)	15 (8)
Sympatry F vs Sympatry M	flaVAN14F20 (A) x flaJX13F31 (B)	19 (30)	26 (23)	33 (37)	12 (13)
	flaVAN14F9 (A) x flaJX13F38 (B)	17 (18)	44 (35)	17 (19)	12 (9)

Table S9. The effects of blocking the transfer of potential courtship cues on flies' mating success in intra- and interspecific crosses. The tests were performed using generalized linear mixed models (GLMM) with binomial distribution, and significant values are shown in bold.

Females' requirement	Df	z-statistic	P-value
mon♀ x mon♂			
Control – darkness	3, 88	0.00	1.000
Control – wingless♂	3, 88	-44	**< 0.001**
Control – antennaless♀	3, 88	-44	**< 0.001**
mon♀ x fla♂			
Control – darkness	3, 88	0.00	1.000
Control – wingless♂	3, 88	0.00	1.000
Control – antennaless♀	3, 88	1.41	0.195
fla♀ x fla♂			
Control – darkness	3, 88	0.00	1.000
Control – wingless♂	3, 88	-0.72	0.491
Control – antennaless♀	3, 88	-2.74	**0.025**
fla♀ x mon♂			
Control – darkness	3, 88	-0.4	0.700
Control – wingless♂	3, 88	6	**< 0.001**
Control – antennaless♀	3, 88	1.4	0.200

Table S10. Male courtship song traits for each strain from different population types. Means and standard errors calculated for the songs of 5 males per strain. PN = pulse number; PTL =pulse train length; PL = pulse length; IPI = interpulse interval; CN = cycle number and FRE = carrier frequency of the song.

	Strain	PN	PTL	PL	IPI	CN	FRE
D. montana							
Allopatry	monSE13F37	10.4 ± 0.60	354.6 ± 26.83	22.3 ± 1.37	38.6 ± 1.45	5.8 ± 0.25	266.8 ± 14.93
	monSE13F16	10.5 ± 0.17	358.3 ± 14.84	21.4 ± 1.38	34.9 ± 1.76	5.9 ± 0.46	282.4 ± 15.37
	monAF15F12	10.1 ± 0.37	330.0 ± 19.54	21.3 ± 1.49	35.0 ± 1.63	5.3 ± 0.24	250.6 ± 9.49
	monAF15F28	11.0 ± 0.49	395.2 ± 25.93	26.2 ± 1.57	40.6 ± 2.67	6.4 ± 0.19	269.8 ± 20.51
Sympatry F	monCRAN14F16	11.1 ± 0.59	382.2 ± 32.17	26.2 ± 1.39	38.4 ± 1.60	6.3 ± 0.51	247.3 ± 25.30
	monCRAN14F20	10.9 ± 0.40	332.7 ± 13.41	21.3 ± 1.23	34.6 ± 0.67	5.5 ± 0.25	267.4 ± 10.48
	monJX13F3	9.7 ± 0.61	336.0 ± 19.63	22.9 ± 1.51	39.5 ± 1.55	5.4 ± 0.19	233.0 ± 22.63
	monJX13F48	11.3 ± 0.32	418.0 ± 17.16	21.6 ± 0.85	37.1 ± 0.57	5.1 ± 0.32	233.7 ± 9.88
Sympatry M	monTER14F11	10.1 ± 0.37	362.9 ± 14.82	25.1 ± 1.04	42.3 ± 1.33	5.4 ± 0.19	212.6 ± 15.28
	monVAN14F1	11.9 ± 0.46	408.2 ± 28.67	21.6 ± 1.13	36.9 ± 1.65	4.9 ± 0.48	229.4 ± 18.40
	monVAN14F17	9.5 ± 0.27	329.8 ± 21.44	23.4 ± 1.89	37.9 ± 2.61	5.5 ± 0.40	233.9 ± 5.68
	monASH13F9	11.8 ± 0.56	409.7 ± 22.41	20.3 ± 0.31	36.2 ± 0.45	4.9 ± 0.07	252.7 ± 2.88
	monASH13F13	9.2 ± 0.56	278.0 ± 19.39	19.4 ± 0.26	30.0 ± 1.14	5.1 ± 0.12	264.5 ± 11.62
D. flavomontana							
Allopatry	flaMT13F11	4.5 ± 0.23	362.5 ± 36.36	18.2 ± 1.41	98.5 ± 6.37	4.3 ± 0.19	260.2 ± 8.76
	flaMT13F15	4.5 ± 0.13	330.7 ± 18.83	15.4 ± 0.30	88.5 ± 3.56	3.7 ± 0.07	240.4 ± 8.44
	flaLB15F1	4.6 ± 0.29	348.9 ± 29.76	16.9 ± 0.55	91.0 ± 0.90	4.5 ± 0.17	287.6 ± 6.38
	flaLB15F3	4.0 ± 0.00	282.4 ± 8.80	17.1 ± 0.57	85.4 ± 2.74	4.7 ± 0.30	292.8 ± 10.89
Sympatry F	flaCRAN14F7	4.7 ± 0.19	358.3 ± 20.76	19.0 ± 1.21	90.6 ± 1.06	4.9 ± 0.39	262.6 ± 9.29
	flaCRAN14F13	5.3 ± 0.11	387.5 ± 17.78	17.5 ± 1.25	85.2 ± 2.27	4.7 ± 0.46	262.5 ± 8.00
	flaJX13F31	4.5 ± 0.17	376.2 ± 12.92	17.3 ± 0.76	99.0 ± 1.55	4.7 ± 0.24	262.7 ± 11.68
	flaJX13F38	4.0 ± 0.00	335.0 ± 9.22	18.2 ± 1.20	100.6 ± 4.21	4.8 ± 0.34	265.7 ± 4.77
Sympatry M	flaTER14F5	4.5 ± 0.20	329.9 ± 22.32	16.2 ± 0.40	88.0 ± 1.95	4.3 ± 0.18	256.8 ± 6.34
	flaVAN14F9	4.9 ± 0.34	355.0 ± 28.75	17.3 ± 0.55	88.5 ± 2.75	4.7 ± 0.43	285.0 ± 20.72
	flaVAN14F20	4.6 ± 0.19	366.2 ± 20.63	17.3 ± 0.46	96.0 ± 2.87	4.3 ± 0.11	246.8 ± 7.07
	flaASH13F2	4.0 ± 0.28	306.4 ± 31.40	16.8 ± 1.02	94.8 ± 3.25	4.5 ± 0.29	260.2 ± 2.99
	flaASH13F14	5.1 ± 0.17	388.4 ± 23.28	18.6 ± 1.51	87.5 ± 2.01	4.8 ± 0.37	242.8 ± 6.53

Table S11. Importance of 5 principal components (PCs) for song analysis, and correlations between PC loadings and courtship song traits. PN = pulse number; PL = pulse length; IPI = interpulse interval; CN = cycle number and FRE = carrier frequency of the song.

Importance of components	PC1	PC2	PC3	PC4	PC5
Standard deviation	1.746	1.083	0.794	0.276	0.265
Proportion of variance	0.61	0.235	0.126	0.015	0.014
Cumulative proportion	0.61	0.845	0.971	0.986	1
Loadings					
PN	0.917	0.108	-0.329	0.089	0.175
PL	0.88	0.053	0.436	-0.172	0.063
IPI	-0.884	-0.064	0.427	0.081	0.16
CN	0.783	-0.501	0.324	0.157	-0.081
FRE	-0.199	-0.951	-0.212	-0.088	0.056

Table S12. Comparison of song traits between population types in *D. montana* and *D. flavomontana*, performed using linear mixed model. Significant values are shown in bold.

	Song parameter	Df	t	P-value
D. montana				
Allopatry vs Sympatry F	CN	32	-0.82	0.445
	PN	32	0.63	0.549
	PTL	32	0.32	0.763
	PL	32	0.11	0.920
	IPI	32	0.08	0.937
	FRE	32	-1.87	0.111
Allopatry vs Sympatry M	CN	36	-3.04	**0.019**
	PN	36	0.02	0.989
	PTL	36	-0.04	0.969
	PL	36	-0.55	0.598
	IPI	36	-0.24	0.816
	FRE	36	-2.45	**0.044**
Sympatry F vs Sympatry M	CN	36	-1.57	0.160
	PN	36	-0.35	0.737
	PTL	36	-0.27	0.798
	PL	36	-0.67	0.523
	IPI	36	-0.32	0.762
	FRE	36	-0.55	0.601
D. flavomontana				
Allopatry vs Sympatry F	CN	32	2.21	0.069
	PN	32	0.76	0.479
	PTL	32	1.59	0.164
	PL	32	1.53	0.176
	IPI	32	0.65	0.540
	FRE	32	-0.56	0.596
Allopatry vs Sympatry M	CN	36	1.12	0.301
	PN	36	0.85	0.424
	PTL	36	0.81	0.445
	PL	36	0.47	0.653
	IPI	36	0.03	0.978
	FRE	36	-0.88	0.411
Sympatry F vs Sympatry M	CN	36	-1.12	0.302
	PN	36	-0.07	0.945
	PTL	36	-0.79	0.454
	PL	36	-1.14	0.293
	IPI	36	-0.76	0.473
	FRE	36	-0.6	0.567

Table S13. Confusion matrix for Random forest analysis. Grey fields describe CHC consistency in both sexes of one species within the same population type, and black-edged boxes describe CHC consistency of both sexes of both species within the same population type.

	D. fla Allopatry females	D. fla Allopatry males	D. mon Allopatry females	D. mon Allopatry males	D. fla Sympatry F females	D. fla Sympatry F males	D. mon Sympatry F females	D. mon Sympatry F males	D. fla Sympatry M females	D. fla Sympatry M males	D. mon Sympatry M females	D. mon Sympatry M males	class.error	class.error with other sex of the same pop	class.error beyond the other sex
D. fla Allopatry females	18	1	0	0	0	0	0	0	0	0	0	1	0.10	0.05	0.05
D. fla Allopatry males	1	17	0	0	0	0	0	0	0	1	0	0	0.11	0.05	0.05
D. mon Allopatry females	0	0	18	1	0	0	0	0	0	0	1	0	0.10	0.05	0.05
D. mon Allopatry males	1	1	3	11	0	0	0	0	0	0	2	0	0.39	0.17	0.22
D. fla Sympatry F females	1	0	0	0	14	1	0	0	0	1	0	0	0.18	0.06	0.12
D. fla Sympatry F males	0	0	0	0	0	17	0	0	0	0	0	0	0.00	0.00	0.00
D. mon Sympatry F females	0	0	0	0	0	0	19	1	0	0	0	0	0.05	0.05	0.00
D. mon Sympatry F males	0	0	0	0	0	0	2	12	0	1	0	0	0.20	0.13	0.07
D. fla Sympatry M females	0	0	0	0	0	0	0	0	25	0	0	0	0.00	0.00	0.00
D. fla Sympatry M males	0	0	0	0	0	0	0	0	1	23	0	0	0.04	0.04	0.00
D. mon Sympatry M females	0	0	1	0	0	0	0	0	0	0	20	2	0.13	0.09	0.04
D. mon Sympatry M males	0	0	0	1	0	0	0	0	1	0	3	19	0.21	0.13	0.08

Table S14. Chemical compounds (mean ± SD) on the cuticle of *D. montana* (*D. mon*) and *D. flavomontana* (*D. fla*) females and males in different population types.

	Retention Index	*D. mon* Allopatry females	*D. mon* Allopatry males	*D. fla* Allopatry females	*D. fla* Allopatry males	*D. mon* Sympatry F females	*D. mon* Sympatry F males	*D. fla* Sympatry F females	*D. fla* Sympatry F males	*D. mon* Sympatry M females	*D. mon* Sympatry M males	*D. fla* Sympatry M females	*D. fla* Sympatry M males
2MeC24-Alkane	2464	1.10 ± 0.64	1.14 ± 0.99	**0.33 ± 0.34**		1.07 ± 0.69	0.94 ± 1.13	0.25 ± 0.29	0.07 ± 0.11	1.12 ± 0.72	1.19 ± 0.94	0.38 ± 0.27	0.16 ± 0.16
C25-Alkene 1	2470		0.05 ± 0.11			0.09 ± 0.16	0.09 ± 0.15	0.03 ± 0.06		0.13 ± 0.22	0.07 ± 0.19		
C25-Alkene 2	2473	0.88 ± 0.85	1.09 ± 1.63	0.04 ± 0.08		0.28 ± 0.31	0.58 ± 0.71	0.08 ± 0.15	0.04 ± 0.06	0.68 ± 0.53	0.54 ± 0.49	0.09 ± 0.12	0.01 ± 0.04
C25-Alkene 3	2481	0.86 ± 1.38	1.12 ± 2.18	0.13 ± 0.20		2.27 ± 1.47	1.75 ± 1.04	2.27 ± 1.63	1.32 ± 0.95	2.71 ± 1.84	1.80 ± 1.14	1.92 ± 1.31	1.50 ± 1.04
C25-Alkene 4	2491	4.09 ± 1.67	2.42 ± 1.21	3.60 ± 1.17	1.63 ± 0.66	1.15 ± 1.17	0.16 ± 0.23	1.19 ± 2.28	0.60 ± 1.03	1.68 ± 2.01	1.62 ± 1.82	1.27 ± 2.10	0.47 ± 1.00
C25-Alkane	2500	0.20 ± 0.20	0.20 ± 0.37	0.04 ± 0.09		0.13 ± 0.31				0.33 ± 0.35	0.29 ± 0.28		
C27-Alkadiene 1	2646		0.01 ± 0.03										
C27-Alkadiene 2	2651		0.06 ± 0.18										
C27-Alkadiene 3	2655	0.25 ± 0.24	0.26 ± 0.37	9.04 ± 9.50	4.63 ± 6.85						0.13 ± 0.35		
C27-Alkadiene 4	2660	8.40 ± 6.39	3.83 ± 4.56	5.24 ± 9.53	1.82 ± 4.21	1.38 ± 4.26	1.23 ± 4.75		1.86 ± 3.21	1.82 ± 4.48	4.43 ± 8.64	11.22 ± 12.50	1.87 ± 4.76
2MeC26-Alkane/C27-Alkadiene 5	2664	9.51 ± 2.60	11.31 ± 6.94	11.90 ± 2.96	10.48 ± 4.56	11.57 ± 3.75	10.48 ± 5.20	23.80 ± 9.06	14.97 ± 5.15	13.12 ± 8.21	11.83 ± 8.39	19.21 ± 9.83	18.82 ± 6.83
C27-Alkene 1	2673					20.79 ± 6.22	18.51 ± 7.81	1.48 ± 2.89	3.45 ± 4.99	2.43 ± 4.74	3.20 ± 6.45	3.47 ± 7.33	3.82 ± 6.98
C27-Alkene 2	2678	16.76 ± 2.98	13.81 ± 6.07	12.13 ± 4.78	7.50 ± 8.59	7.73 ± 5.36	7.82 ± 6.14	13.80 ± 9.28	2.61 ± 2.88	23.26 ± 3.42	15.59 ± 5.93	7.46 ± 8.74	2.92 ± 4.65
C27-Alkene 3	2683	8.79 ± 2.53	10.41 ± 3.36	7.17 ± 4.04	4.52 ± 1.63	18.41 ± 6.50	18.20 ± 9.41	6.10 ± 6.15	5.31 ± 3.49	8.98 ± 2.90	11.51 ± 4.49	4.27 ± 4.54	7.61 ± 6.69
C27-Alkene 4	2691	15.15 ± 3.08	16.20 ± 6.33	18.14 ± 7.74	19.17 ± 2.06	6.92 ± 7.97	9.48 ± 8.73	16.50 ± 7.46	17.05 ± 6.75	11.84 ± 6.84	9.01 ± 7.08	9.10 ± 7.6	12.32 ± 9.08
C27-Alkene 5	2695	1.40 ± 1.04	1.50 ± 1.53	1.37 ± 1.00	4.18 ± 1.61	2.35 ± 1.63	2.58 ± 2.30	3.01 ± 3.25	6.10 ± 2.80	7.70 ± 12.4	7.92 ± 11.78	11.83 ± 10.98	5.26 ± 6.59
C27-Alkane	2700	1.09 ± 0.88	1.16 ± 1.16	0.29 ± 0.34	1.04 ± 1.95	0.48 ± 0.91		1.12 ± 1.99		0.94 ± 0.89	0.50 ± 0.70	0.40 ± 0.71	0.30 ± 0.75
C29-Alkadiene 1	2831	1.21 ± 1.19	0.69 ± 0.67	0.38 ± 0.39	0.21 ± 0.39	0.15 ± 0.20	0.45 ± 0.27	0.09 ± 0.28		0.60 ± 0.66	0.47 ± 0.51	0.28 ± 0.59	0.01 ± 0.04
C29-Alkadiene 2	2840	3.31 ± 1.46	3.11 ± 1.24	2.27 ± 1.02	2.44 ± 1.84	0.93 ± 0.57	1.46 ± 0.80	0.83 ± 0.81	1.09 ± 0.66	1.72 ± 1.25	1.88 ± 1.23	1.04 ± 1.79	1.00 ± 1.42
C29-Alkadiene 3	2851	1.26 ± 0.95	2.54 ± 4.79	1.20 ± 0.96	3.91 ± 2.67	0.16 ± 0.40		0.23 ± 0.55	1.11 ± 1.56	0.60 ± 0.84	1.10 ± 1.25	1.70 ± 1.56	3.60 ± 2.91
C29-Alkadiene 4	2860							0.18 ± 0.46	1.53 ± 2.48	0.15 ± 0.34	0.43 ± 1.07	0.66 ± 1.28	0.83 ± 1.98
2MeC28-Alkane/C29-Alkadiene 5	2863	13.40 ± 4.24	19.58 ± 8.72	17.01 ± 5.74	26.93 ± 7.18	12.98 ± 6.49	14.33 ± 10.22	18.78 ± 6.44	31.69 ± 6.65	11.41 ± 4.55	16.34 ± 5.51	15.60 ± 8.68	30.85 ± 11.22
C29-Alkene 1	2879	8.56 ± 3.91	4.53 ± 4.12	5.04 ± 4.15	4.83 ± 7.45	10.43 ± 3.40	10.24 ± 4.84	4.59 ± 4.64		6.87 ± 4.03	5.10 ± 3.19	5.74 ± 6.14	1.42 ± 4.29
C29-Alkene 2	2884	1.14 ± 1.09	2.12 ± 2.78	2.38 ± 2.15	2.23 ± 1.67	0.36 ± 1.11	1.14 ± 2.07	2.90 ± 3.83	3.32 ± 2.97	1.20 ± 2.27	3.35 ± 2.53	2.93 ± 3.05	1.94 ± 2.20
C29-Alkene 3	2892	0.51 ± 0.46	1.12 ± 1.43	0.78 ± 0.56	1.64 ± 1.44		0.58 ± 1.21	1.57 ± 1.97	2.56 ± 1.77	0.07 ± 0.22	0.73 ± 0.88	0.43 ± 0.64	1.23 ± 1.32
C29-Alkene 4	2895			0.16 ± 0.35	0.52 ± 1.10			0.05 ± 0.15	0.58 ± 0.83				0.18 ± 0.48
C31-Alkadiene 1	3030	0.40 ± 0.51	0.23 ± 0.24	0.15 ± 0.19	0.10 ± 0.19							0.09 ± 0.19	
C31-Alkadiene 2	3045	0.24 ± 0.23	0.23 ± 0.28	0.24 ± 0.24	0.39 ± 0.38			0.07 ± 0.19	0.10 ± 0.19		0.07 ± 0.19	0.11 ± 0.18	0.34 ± 0.42
C31-Alkadiene 3	3055			0.05 ± 0.14	0.20 ± 0.42			0.05 ± 0.14	0.38 ± 0.48		0.11 ± 0.14	0.02 ± 0.05	0.36 ± 0.53
2MeC30-Alkane/C31-Alkadiene 4	3063	1.49 ± 0.77	1.06 ± 0.72	0.92 ± 0.72	1.62 ± 0.97	0.37 ± 0.39		1.01 ± 0.73	2.05 ± 1.71	0.62 ± 0.57	0.80 ± 0.76	0.78 ± 0.39	2.24 ± 1.31
C31-Alkene	3080		0.04 ± 0.09						0.08 ± 0.23				
C33-Alkadiene 1	3238		0.05 ± 0.16						0.32 ± 0.64				0.05 ± 0.10
C33-Alkadiene 2	3246		0.04 ± 0.13						0.41 ± 0.87				0.15 ± 0.28
C33-Alkadiene 3	3257		0.01 ± 0.06						0.20 ± 0.61				0.28 ± 0.49
C33-Alkene 1	3266		0.01 ± 0.03										0.24 ± 0.45
C33-Alkene 2	3276								0.69 ± 1.69				0.13 ± 0.27
C33-Alkene 3	3284		0.07 ± 0.22						0.53 ± 1.34				0.08 ± 0.22

Table S15. The transfer / storage of sperm in females and the proportion of hatched eggs in intra- and interspecific crosses within different population types. Significant values are shown in bold.

		Transfer and/or storage of sperm (CLMM)			Proportion of hatched eggs (GLMM)		
		Df	χ2	P-value	Df	z	P-value
Allopatry							
	mon♀ x mon♂ vs mon♀ x fla♂	1,48	3.44	**< 0.001**	1,40	-	**< 0.001** (χ2 test)
	fla♀ x fla♂ vs fla♀ x mon♂	1,107	-1.83	0.067	1,91	-9.5	**< 0.001**
	mon♀ x fla♂ vs fla♀ x mon♂	1,77	-4.32	**< 0.001**	1,65	-	**0.004** (χ2 test)
Sympatry F							
	mon♀ x mon♂ vs mon♀ x fla♂	1,56	4.08	**< 0.001**	1, 56	9.92	**< 0.001**
	fla♀ x fla♂ vs fla♀ x mon♂	1,82	-1.59	0.111	1,68	-12.1	**< 0.001**
	mon♀ x fla♂ vs fla♀ x mon♂	1,60	-5.1	**< 0.001**	1,47	-0.04	0.967
Sympatry M							
	mon♀ x mon♂ vs mon♀ x fla♂	1,66	3.05	**0.002**	1,54	-	**< 0.001** (χ2 test)
	fla♀ x fla♂ vs fla♀ x mon♂	1,118	-2.68	**0.007**	1,90	-9.32	**< 0.001**
	mon♀ x fla♂ vs fla♀ x mon♂	1,86	-2.22	**0.026**	1,55	-	0.110 (χ2 test)

Table S16. Generalized linear mixed model (GLMM) with binomial distribution for PMPZ barrier in egg fertilization between *D. flavomontana* females and *D. montana* males from different population types. Significant values are shown in bold.

	Df	z-statistic	P-value
Allopatry vs Sympatry F	1, 6	-0.19	0.846
Allopatry vs Sympatry M	1, 7	-3.94	**< 0.001**
Sympatry F vs Sympatry M	1, 7	-3.51	**< 0.001**

Table S17. Generalized linear mixed model (GLMM) with binomial distribution for PMPZ barrier in egg fertilization in crosses between *D. flavomontana* strains from the same vs different population types. Significant values are shown in bold.

	Df	z-statistic	P-value
Allopatry ♀/♂ vs Allopatry♀ x Sympatry F♂	2,3	-0.57	0.571
Allopatry ♀/♂ vs Allopatry♀ x Sympatry M♂	2,3	-1.48	0.138
Sympatry F♀/♂ vs Sympatry F♀ x Allopatry♂	2,3	-1.55	0.122
Sympatry F♀/♂ vs Sympatry F♀ x Sympatry M♂	2,3	-4.94	**< 0.001**
Sympatry M♀/♂ vs Sympatry M♀ x Allopatry♂	2,3	-5.48	**< 0.001**
Sympatry M♀/♂ vs Sympatry M♀ x Sympatry F♂	2,3	-0.64	0.526

Figures

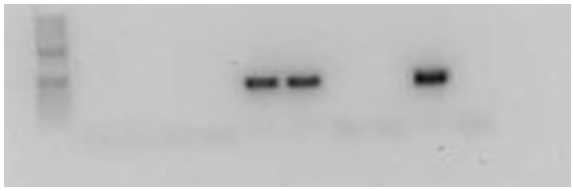


Figure S1. Example of an agarose gel run with wsp_1FR primers and the following samples: 1. DNA size ladder, 2. and 3. *D. flavomontana* males from Liberty, 4. *D. montana* female from Ashford, 5. *D. montana* male from Cranbrook, 6. and 7. *D. borealis*, *Wolbachia* +, 8. and 9. *D. borealis*, *Wolbachia* -, 10. *D. melanogaster*, *Wolbachia* +, 11. negative control. All our *D. montana* and *D. flavomontana* samples were negative while *Wolbachia* infected *D. borealis* and *D. melanogaster* flies showed clear bands.

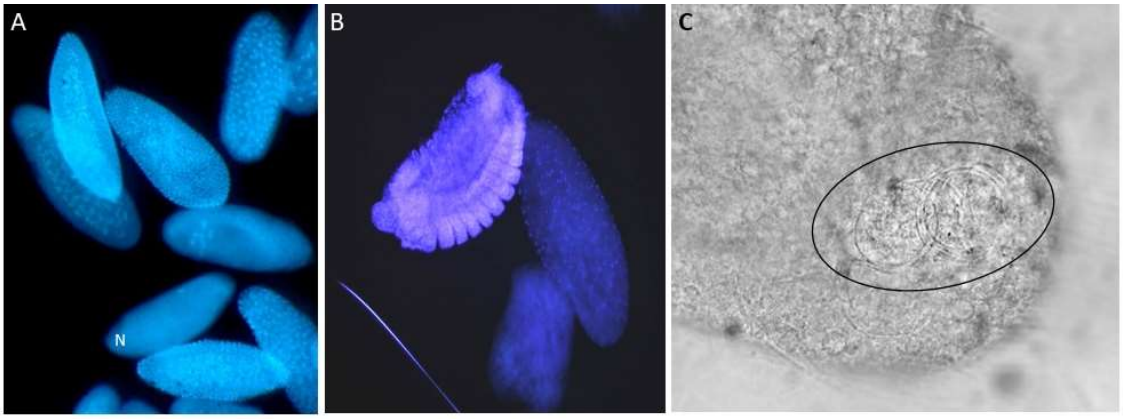


Figure S2. (A) Developing eggs with either clear mitotic division or (B) with cellular differentiation. Non-developing eggs had fewer than four nuclei visible within the egg (marked with N) (C) Sperm is visible as a spiral structure near the anterior end of the egg.

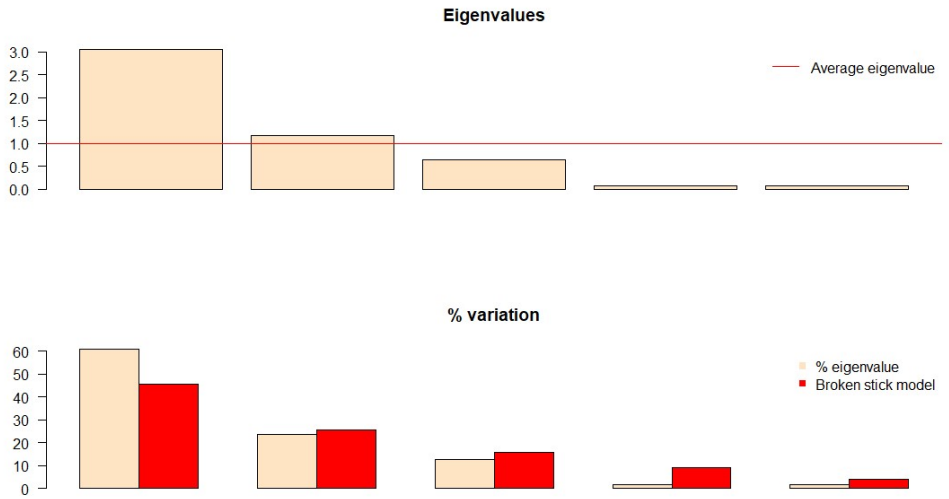
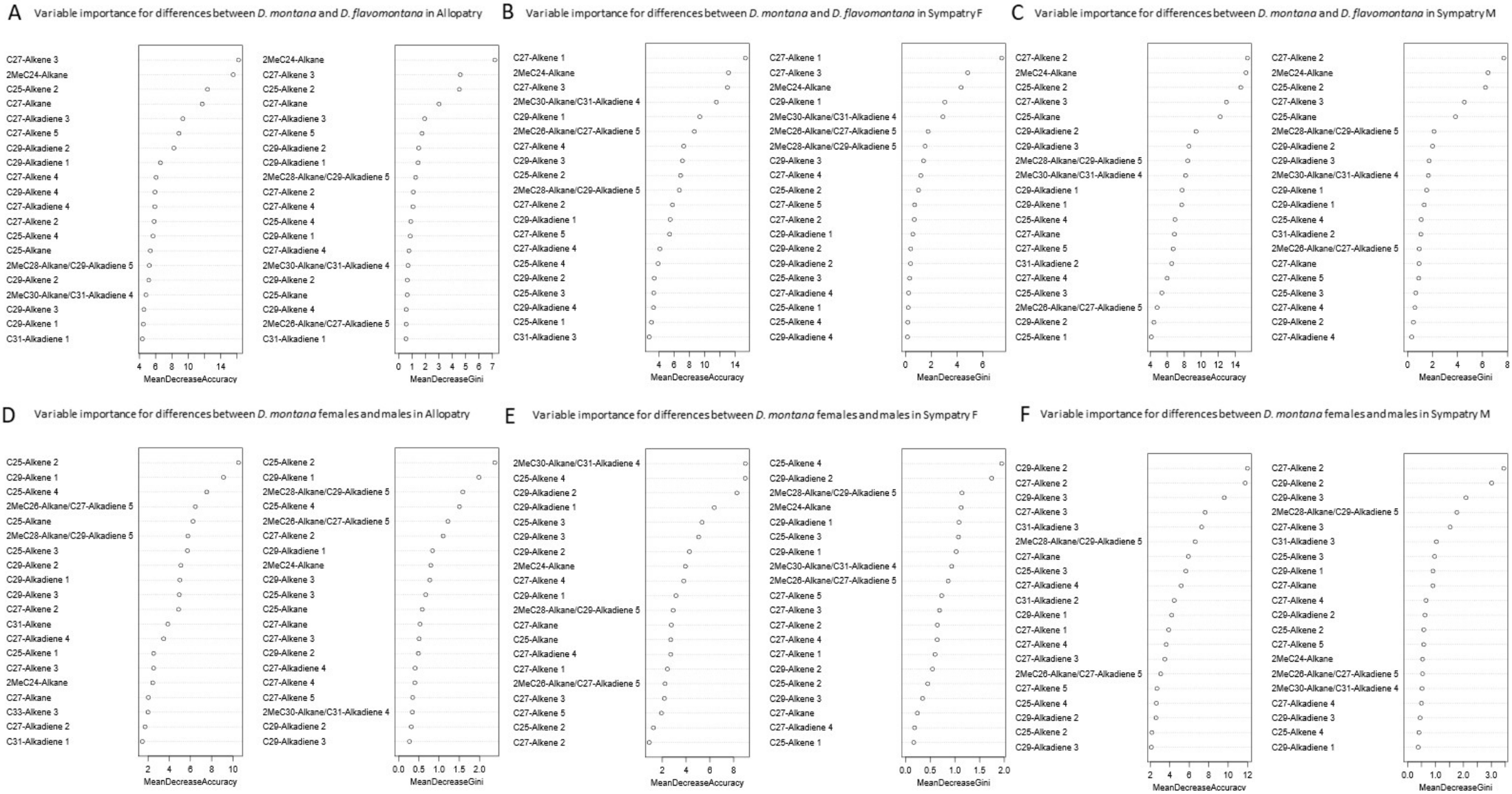


Figure S3. Eigenvalues and broken stick model for principal component analysis (PTL removed).


A Variable importance for differences between D. montana and D. flavomontana in Allopatry
C27-Alkene 3
2MeC24-Alkane
C25-Alkene 2
C27-Alkane
C27-Alkadiene 3
C27-Alkene 5
C29-Alkadiene 2
C29-Alkadiene 1
C27-Alkene 4
C29-Alkene 4
C27-Alkadiene 4
C27-Alkene 2
C25-Alkene 4
C25-Alkane
2MeC28-Alkane/C29-Alkadiene 5
C29-Alkene 2
2MeC30-Alkane/C31-Alkadiene 4
C29-Alkene 3
C29-Alkene 1
C31-Alkadiene 1
4 6 8 10 14
MeanDecreaseAccuracy
2MeC24-Alkane
C27-Alkene 3
C25-Alkene 2
C27-Alkane
C27-Alkadiene 3
C27-Alkene 5
C29-Alkadiene 2
C29-Alkadiene 1
2MeC28-Alkane/C29-Alkadiene 5
C27-Alkene 2
C27-Alkene 4
C25-Alkene 4
C29-Alkene 1
C27-Alkadiene 4
2MeC30-Alkane/C31-Alkadiene 4
C29-Alkene 2
C25-Alkane
C29-Alkene 4
2MeC26-Alkane/C27-Alkadiene 5
C31-Alkadiene 1
0 1 2 3 4 5 6 7
MeanDecreaseGini
B Variable importance for differences between D. montana and D. flavomontana in Sympatry F
C27-Alkene 1
2MeC24-Alkane
C27-Alkene 3
2MeC30-Alkane/C31-Alkadiene 4
C29-Alkene 1
2MeC26-Alkane/C27-Alkadiene 5
C27-Alkene 4
C29-Alkene 3
C25-Alkene 2
2MeC28-Alkane/C29-Alkadiene 5
C27-Alkene 2
C29-Alkadiene 1
C27-Alkene 5
C27-Alkadiene 4
C25-Alkene 4
C29-Alkene 2
C25-Alkene 3
C29-Alkadiene 4
C25-Alkene 1
C31-Alkadiene 3
4 6 8 10 14
MeanDecreaseAccuracy
C27-Alkene 1
C27-Alkene 3
2MeC24-Alkane
C29-Alkene 1
2MeC30-Alkane/C31-Alkadiene 4
2MeC26-Alkane/C27-Alkadiene 5
2MeC28-Alkane/C29-Alkadiene 5
C29-Alkene 3
C27-Alkene 4
C25-Alkene 2
C27-Alkene 5
C27-Alkene 2
C29-Alkadiene 1
C29-Alkene 2
C29-Alkadiene 2
C25-Alkene 3
C27-Alkadiene 4
C25-Alkene 1
C25-Alkene 4
C29-Alkadiene 4
0 2 4 6
MeanDecreaseGini
C Variable importance for differences between D. montana and D. flavomontana in Sympatry M
C27-Alkene 2
2MeC24-Alkane
C25-Alkene 2
C27-Alkene 3
C25-Alkane
C29-Alkadiene 2
C29-Alkadiene 3
2MeC28-Alkane/C29-Alkadiene 5
2MeC30-Alkane/C31-Alkadiene 4
C29-Alkadiene 1
C29-Alkene 1
C25-Alkene 4
C27-Alkane
C27-Alkene 5
C31-Alkadiene 2
C27-Alkene 4
C25-Alkene 3
2MeC26-Alkane/C27-Alkadiene 5
C29-Alkene 2
C25-Alkene 1
4 6 8 10 12 14
MeanDecreaseAccuracy
C27-Alkene 2
2MeC24-Alkane
C25-Alkene 2
C27-Alkene 3
C25-Alkane
2MeC28-Alkane/C29-Alkadiene 5
C29-Alkadiene 2
C29-Alkadiene 3
2MeC30-Alkane/C31-Alkadiene 4
C29-Alkene 1
C29-Alkadiene 1
C25-Alkene 4
C31-Alkadiene 2
2MeC26-Alkane/C27-Alkadiene 5
C27-Alkane
C27-Alkene 5
C25-Alkene 3
C27-Alkene 4
C29-Alkene 2
C27-Alkadiene 4
0 2 4 6 8
MeanDecreaseGini
D Variable importance for differences between D. montana females and males in Allopatry
C25-Alkene 2
C29-Alkene 1
C25-Alkene 4
2MeC26-Alkane/C27-Alkadiene 5
C25-Alkane
2MeC28-Alkane/C29-Alkadiene 5
C25-Alkene 3
C29-Alkene 2
C29-Alkadiene 1
C29-Alkene 3
C27-Alkene 2
C31-Alkene
C27-Alkadiene 4
C25-Alkene 1
C27-Alkene 3
2MeC24-Alkane
C27-Alkane
C33-Alkene 3
C27-Alkadiene 2
C31-Alkadiene 1
2 4 6 8 10
MeanDecreaseAccuracy
C25-Alkene 2
C29-Alkene 1
2MeC28-Alkane/C29-Alkadiene 5
C25-Alkene 4
2MeC26-Alkane/C27-Alkadiene 5
C27-Alkene 2
C29-Alkadiene 1
2MeC24-Alkane
C29-Alkene 3
C25-Alkene 3
C25-Alkane
C27-Alkane
C27-Alkene 3
C29-Alkene 2
C27-Alkadiene 4
C27-Alkene 4
C27-Alkene 5
2MeC30-Alkane/C31-Alkadiene 4
C29-Alkadiene 2
C29-Alkadiene 3
0.0 0.5 1.0 1.5 2.0
MeanDecreaseGini
E Variable importance for differences between D. montana females and males in Sympatry F
2MeC30-Alkane/C31-Alkadiene 4
C25-Alkene 4
C29-Alkadiene 2
C29-Alkadiene 1
C25-Alkene 3
C29-Alkene 3
C29-Alkene 2
2MeC24-Alkane
C27-Alkene 4
C29-Alkene 1
2MeC28-Alkane/C29-Alkadiene 5
C27-Alkane
C25-Alkane
C27-Alkadiene 4
C27-Alkene 1
2MeC26-Alkane/C27-Alkadiene 5
C27-Alkene 3
C27-Alkene 5
C25-Alkene 2
C27-Alkene 2
2 4 6 8
MeanDecreaseAccuracy
C25-Alkene 4
C29-Alkadiene 2
2MeC28-Alkane/C29-Alkadiene 5
2MeC24-Alkane
C29-Alkadiene 1
C25-Alkene 3
C29-Alkene 1
2MeC30-Alkane/C31-Alkadiene 4
2MeC26-Alkane/C27-Alkadiene 5
C27-Alkene 5
C27-Alkene 3
C27-Alkene 2
C27-Alkene 4
C27-Alkene 1
C29-Alkene 2
C25-Alkene 2
C29-Alkene 3
C27-Alkane
C27-Alkadiene 4
C25-Alkene 1
0.0 0.5 1.0 1.5 2.0
MeanDecreaseGini
F Variable importance for differences between D. montana females and males in Sympatry M
C29-Alkene 2
C27-Alkene 2
C29-Alkene 3
C27-Alkene 3
C31-Alkadiene 3
2MeC28-Alkane/C29-Alkadiene 5
C27-Alkane
C25-Alkene 3
C27-Alkadiene 4
C31-Alkadiene 2
C29-Alkene 1
C27-Alkene 1
C27-Alkene 4
C27-Alkadiene 3
2MeC26-Alkane/C27-Alkadiene 5
C27-Alkene 5
C25-Alkene 4
C29-Alkadiene 2
C25-Alkene 2
C29-Alkadiene 3
2 4 6 8 10 12
MeanDecreaseAccuracy
C27-Alkene 2
C29-Alkene 2
C29-Alkene 3
2MeC28-Alkane/C29-Alkadiene 5
C27-Alkene 3
C31-Alkadiene 3
C25-Alkene 3
C29-Alkene 1
C27-Alkane
C27-Alkene 4
C29-Alkadiene 2
C25-Alkene 2
C27-Alkene 5
2MeC24-Alkane
2MeC26-Alkane/C27-Alkadiene 5
2MeC30-Alkane/C31-Alkadiene 4
C27-Alkadiene 4
C29-Alkadiene 3
C25-Alkene 4
C29-Alkadiene 1
0.0 1.0 2.0 3.0
MeanDecreaseGini

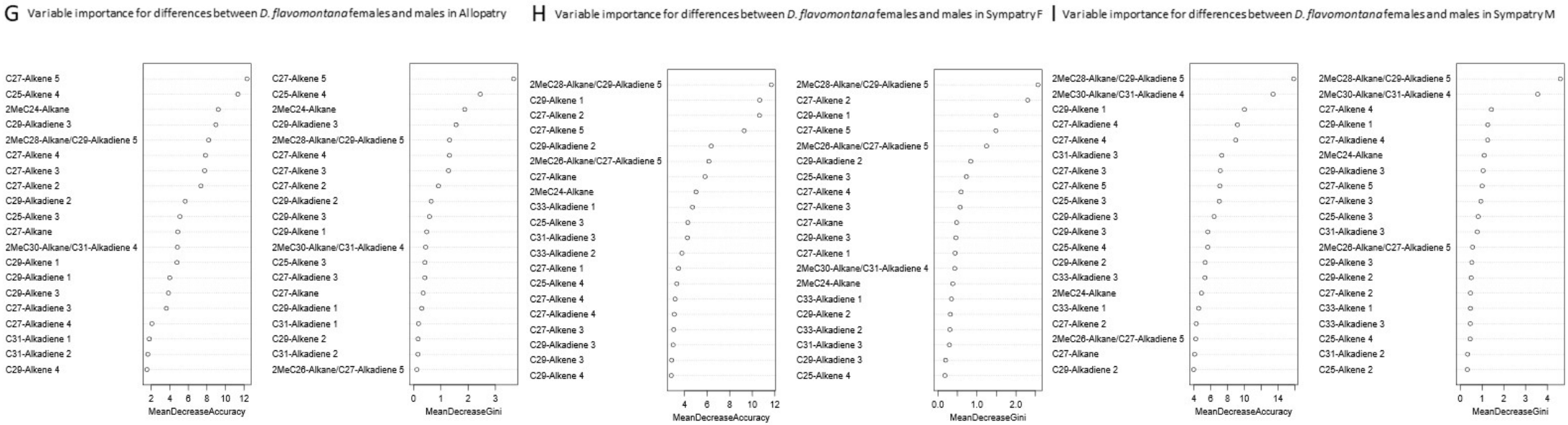


Figure S4. Importance of CHC variables between species (A-C), between sexes in each population type for *D. montana* (D-F) and between sexes in each population type for *D. flavomontana* (G-I) in Random forest analysis.

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II

MULTIPLE PATHS TO COLD TOLERANCE: THE ROLE OF ENVIRONMENTAL CUES, MORPHOLOGICAL TRAITS AND THE CIRCADIAN CLOCK GENE *VRILLE*

by

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Manuscript.

bioRxiv https://doi.org/10.1101/2020.04.20.050351

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). *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

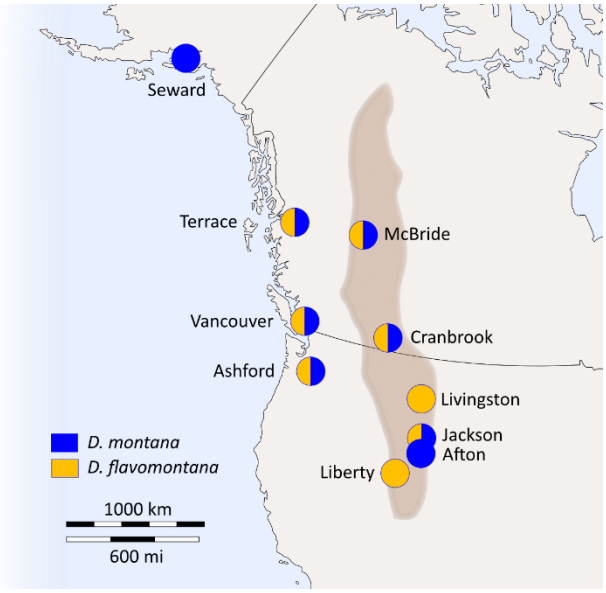

Figure 1. Sample locations. Table shows fly collecting sites and years, and the coordinates for each site. Map contains information on whether we have samples from one or both species in each site on the western coast and in the Rocky Mountains (brown area on the map) in North America (detailed information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

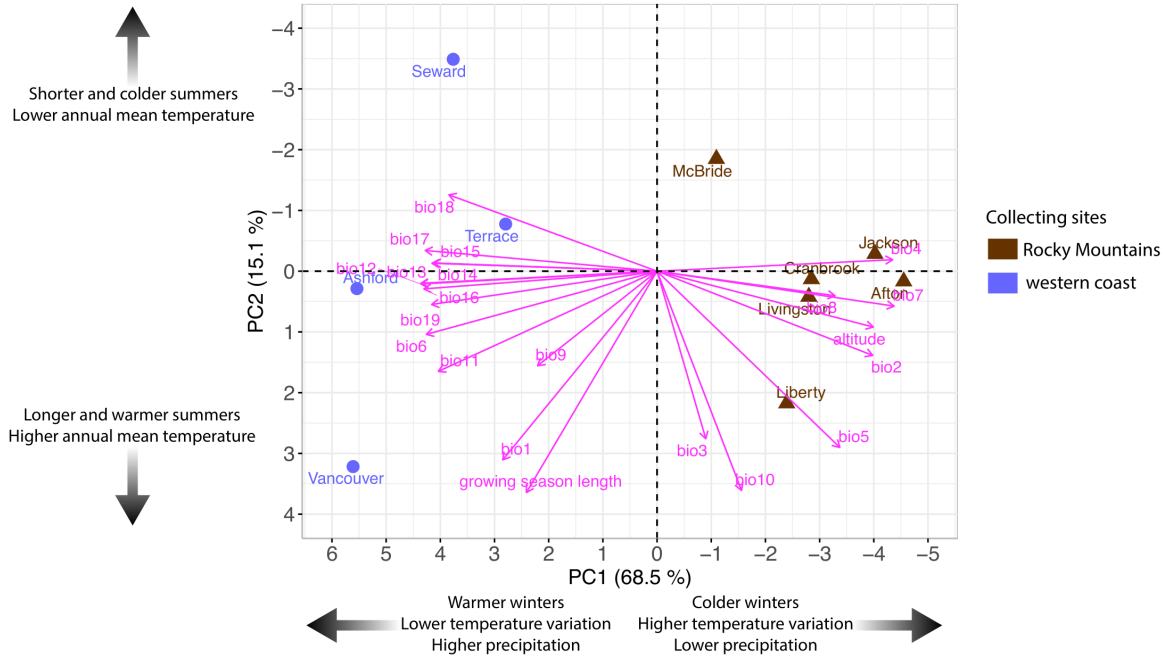

Figure 2. Principal component analysis (PCA) on 21 variables describing environmental conditions in fly collecting sites (Table S2, S3). Big black arrows show the change in given conditions.

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).

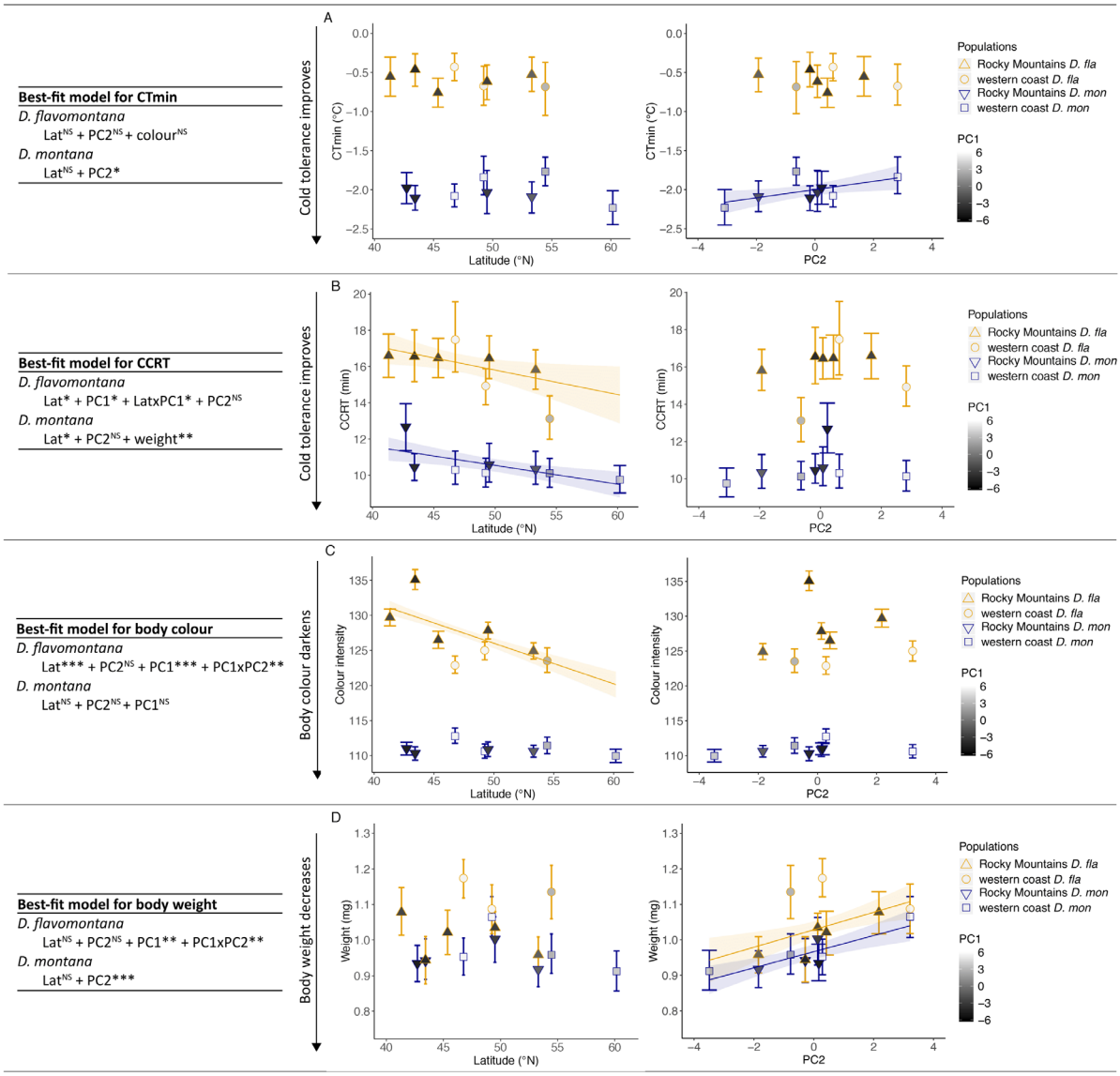

Figure 3. Relationship between latitude (as a proxy of photoperiod) and PC2 (as a proxy of latitudinally varying temperature) and (A) chill coma temperature (CT_{min}), (B) chill coma recovery time (CCRT), (C) fly body colour (measured as colour intensity) and (D) fly body size (measured as weight) in *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 *** $P < 0.001$.

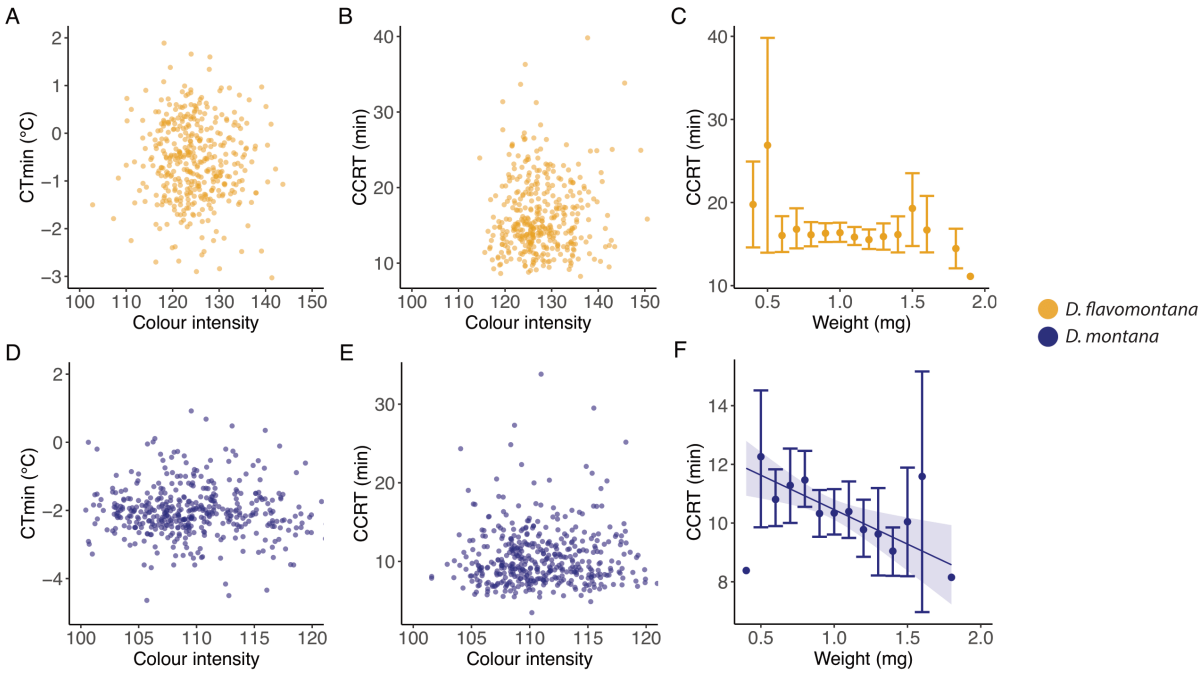

Figure 4. Correlations between fly cold tolerance traits (CT_{min}, CCRT) and morphological traits (body colour (low color intensity indicates dark colour) and body size (measured as weight) in *D. flavomontana* (A-C) and in *D. montana* (D-F). Error bars represent bootstrapped 95% confidence intervals. 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.

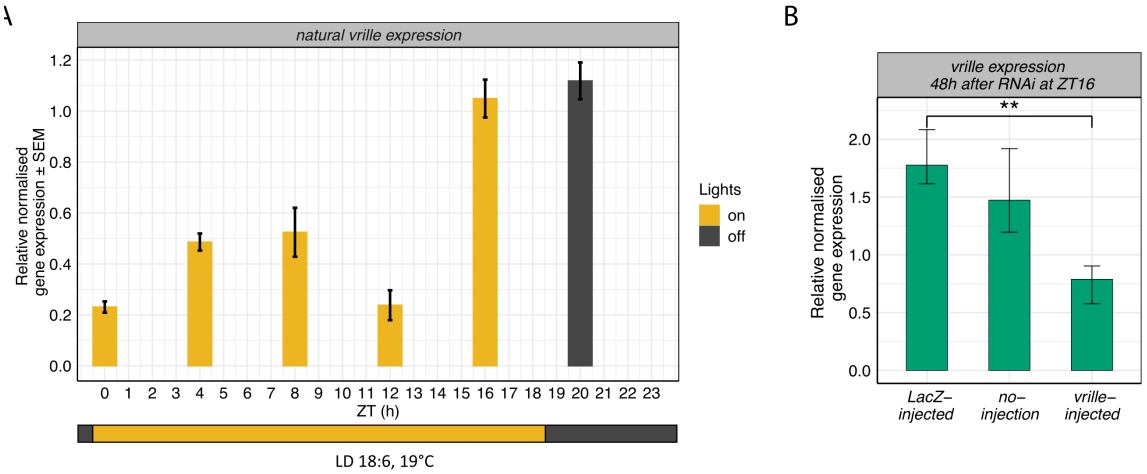

Figure 5. (A) Natural relative normalised expression of *vrille* at six time points, starting at ZT0 (Zeitgeber Time = sampling time every 4 hours over a 24 hour period), in LD 18:6 and 19 ˚C. Yellow represents the time of the day when the lights were on and dark grey when the lights were off. (B) Relative 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.

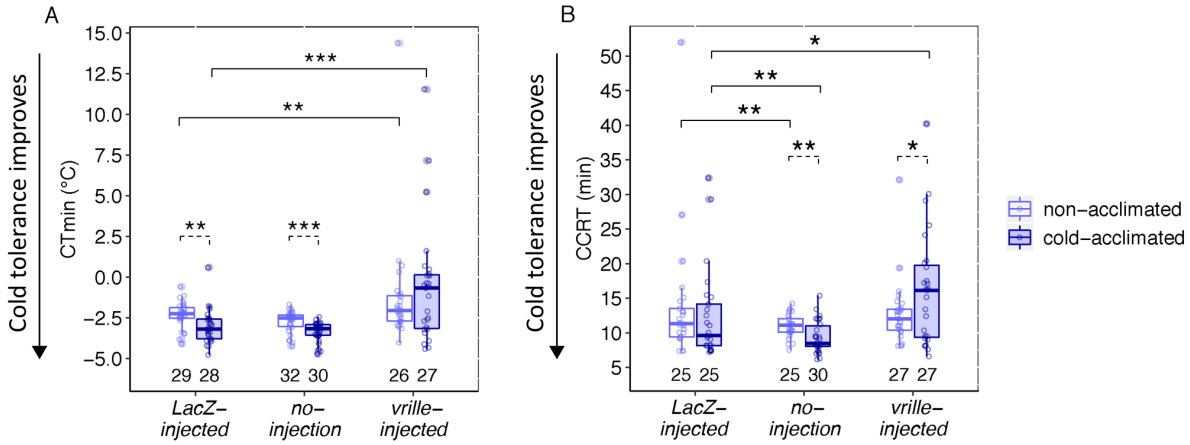

Figure 6. (A) CT_{min} and (B) CCRT tests for *LacZ*-RNAi, no-injection, and *vrille*-RNAi females that were kept at +19 ˚C for the whole time, or at first at +19 ˚C and the last 5 days at +6 ˚C (cold acclimation period). Dashed lines indicate significant effects of the acclimation in each treatment, 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 processes 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. Thus, in our dataset latitude represents photoperiodic differences (day length) between the sites, while PC2 corresponds to latitudinally varying temperature 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 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 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) 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 thus 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 the 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 play an important role in 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 only recently distributed to the coastal area 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 thus is likely under several 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 shows 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 also 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 fly cold acclimation.

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

Statistical analyses

We used different statistical models to investigate whether variation in fly cold tolerance, body colour and body weight was associated with latitude, as a proxy of photoperiod, and/or local climatic variables, and whether cold tolerance traits and morphological traits showed a correlation with each other. In these models, we used either CT_{min} data (chill coma temperatures in Celsius degrees + 10 ˚C to prevent negative values from affecting the analysis), CCRT data (in minutes), body colour (measured from CCRT flies) or body weight (mg; measured from CCRT flies) as response variables. *D. flavomontana* CT_{min} data were normally distributed (Fig. S4) and they were analysed with generalized linear mixed model (GLMM) with gaussian distribution (equivalent of linear mixed model). Other datasets showed deviation from the normality (Fig. S4), and they were analysed

using generalized linear mixed model (GLMM) with gamma distribution, using *glmmTMB* function from glmmTMB package [92]. Technical replicates and isofemale strains were handled as crossed random effects.

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

We included the first two PCs in the model comparison of cold tolerance, body colour and body weight (see Results). The simplest model included latitude and PC2, which enabled us to distinguish between the latitudinally varying photoperiods and temperatures. Moreover, the effect of macroclimatic conditions varying between the western coast and the Rocky Mountains (PC1), interaction terms between latitude/PC2 and PC1, and body colour (divided by 100 to scale the variables) and body weight were included in the model selection (Table S6). The best-fit model for CT_{min}, CCRT, body colour and body weight of both species was chosen for further analysis based on Akaike information criterion (AIC) value and Akaike weight (Table S6) using *aictab* function from AICcmodavg package [96]. All the analyses were conducted in R (v1.2.1335-1) and R studio (v3.6.1).

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

Candidate gene and study material

We used RNAi to silence the expression of one of the key regulators of the circadian behavioural rhythms, *vrille*, and investigated its effects on cold tolerance traits. We performed RNAi studies using only the more cold-tolerant *D. montana*, which has become a model species for studying cold adaption at phenotypic and genetic level (e.g. [63, 74, 97]). Here, we used females of a mass-bred cage population originating from Seward (Alaska, North America; see Fig. 1) to increase genetic diversity. The population cage has been established from the 4th generation progenies of 20 isofemale strains (total of 400 flies) in 2013 and maintained in continuous light at 19 ± 1 °C since the establishment (30 generations). Malt bottles with freshly laid eggs were transferred from the

population cage into a climate chamber in LD 18:6 (light:dark cycle) at 19 ± 1 °C to reinforce flies' circadian rhythmicity prior to the experiments and ensure they are non-diapausing. Critical day length for the induction of reproductive diapause (CDL; 50 % of the females of given population enter diapause) in 19 °C is LD 17:7 in Seward population [74], and thus the females emerging in LD 18:6 can be expected to develop ovaries. After ~4 weeks, we collected newly emerged females (≤ 1 day old) using light CO_2 anaesthesia and placed them back in malt-vials into the above-mentioned conditions. Females were changed into fresh vials once a week until they were used in the experiments at the age of 21 days.

Defining daily expression rhythm of vrille

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

We measured the expression levels of *vrille* with quantitative real time PCR (qPCR). qPCR primers for *vrille* and reference genes were designed based on *D. montana* genomic sequences under NCBI accession number LUVX00000000 [98], together with information from *D. virilis* exons using Primer3 (primer3.ut.ee) and NetPrimer (www.premierbiosoft.com/netprimer) programs (gene accession numbers and primer sequences in Table S11). qPCR mix contained 10 µl of 2x Power SYBR Green PCR Master Mix (Bio-Rad Laboratories), 0.3 µM of each gene-specific primer and 1 µl of cDNA solution. Cycling conditions in Bio-Rad CFX96 instrument were: 3 min at 95 °C, 10 sec at 95 °C, 10 sec at annealing temperature of 53 °C (for reference genes 56 °C) and 30 sec at 72 °C (repeated 40x), followed by melting curve analysis (65-95 °C) for amplification specificity checking. Each run included three technical replicates for each sample and the final threshold value (Cq) was defined as a mean of the technical replicates that produced good quality data. The relative qPCR data was normalised with ΔΔ(Ct) normalisation method [99] using two reference genes, *Tubulin beta chain* (*Tub2*) and *Ribosomal protein L32* (*RpL32*), that showed equal expression levels in all samples (data not shown). Real efficiency values of the genes used in the qPCR are given in Table S11.

Synthesis of double-stranded RNA for RNAi

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

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

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

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

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

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

Statistical analyses

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

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

Acknowledgements

This work was supported by Academy of Finland projects 268214 and 322980 to MK, and grants from Emil Aaltonen to NP and Finnish Cultural Foundation to NP and MK. We thank Anna-Lotta Hiillos and Johanna Kinnunen for help in analysing fly photographs and performing cold tolerance experiments and Ville Hoikkala for help in finalizing the figures.

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Data accessibility

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

Author contribution

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

Conflict of interest

The authors have declared no conflicting interests.

Supplementary information

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

Content

Supplementary tables

Table S1. Information on fly collecting sites and years, and the exact coordinates (latitude, longitude) and altitudes for each collecting site. Table also shows isofemale strains of both species (*mon* = *D. montana*, *fla* = *D. flavomontana*) used in the study.

Site	Year	Latitude	Longitude	Altitude (m)	Species prefix and Strain ID
Seward, AK, USA	2013	60˚10'N	149˚27'W	35	monSE13F14, -F16, -F37
Terrace, BC, Canada	2014	54˚27'N	128˚34'W	217	monTER14F4, -F11, -F13 flaTER14F5
McBride, BC, Canada	2014	53˚07'N	120˚18'W	720	monMB14F1, -F2, -F3 flaMB14F10, -F20, -F27
Cranbrook, BC, Canada	2014	49˚36'N	115˚46'W	940	monCRAN14F16, -F20 flaCRAN14F7, -F10, -F13
Vancouver, BC, Canada	2014	49˚15'N	123˚10'W	4	monVAN14F1, -F17, -F24 flaVAN14F9, -F20
Ashford, WA, USA	2013	46˚45'N	121˚57'W	573	monASH13F9, -F11, -F13 flaASH13F2, -F14
Livingston, MT, USA	2013	45˚21'N	110˚36'W	1605	flaMT13F8, -F11, -F15
Jackson, WY, USA	2013	43˚26'N	110˚50'W	1857	monJX13F3, -F41, -F48 flaJX13F31, -F37, -F38
Afton, WY, USA	2015	42˚43'N	110˚55'W	2000	monAF15F12, -F19, -F28
Liberty, UT, USA	2015	41˚20'N	111˚51'W	1600	flaLB15F1, -F2, -F3

Table S2. A List of 19 bioclimatic variables used in the PCA (WorldClim database v2.1, 2.5 min spatial resolutions; current data 1970-2000; Fick and Hijmans 2017; www.worldclim.org).

Variable	Description
bio1	Annual mean temperature
bio2	Mean diurnal range (mean of monthly (max - min temperature))
bio3	Isothermality (bio2/bio7*100)
bio4	Temperature seasonality (standard deviation * 100)
bio5	Max temperature of the warmest month
bio6	Min temperature of the coldest month
bio7	Annual temperature range (bio5-bio6)
bio8	Mean temperature of the wettest quarter
bio9	Mean temperature of the driest quarter
bio10	Mean temperature of the warmest quarter
bio11	Mean temperature of the coldest quarter
bio12	Annual precipitation
bio13	Precipitation of the wettest month
bio14	Precipitation of the driest month
bio15	Precipitation seasonality (coefficient of variation)
bio16	Precipitation of the wettest quarter
bio17	Precipitation of the driest quarter
bio18	Precipitation of the warmest quarter
bio19	Precipitation of the coldest quarter

Table S3. 19 bioclimatic variables for each site were extracted from WorldClim database v2.1 using latitudinal and longitudinal coordinates (2.5 min spatial resolutions; current data 1970-2000; (Fick & Hijmans, 2017); www.worldclim.org) and growing season lengths for each site were obtained from Tyukmaeva et al., (2020) and www.weatherbase.com.

Population	longitude	latitude	altitude	bio1	bio2	bio3	bio4	bio5	bio6	bio7	bio8	bio9	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio18	bio19	growing season length (days)
Seward	-149.45	60.16	35	3.84	6.65	28.52	643.90	16.80	-6.54	23.34	3.56	10.33	12.13	-3.44	1551	245	53	47	620	204	228	432	169
Terrace	-128.57	54.45	217	6.41	7.72	27.57	730.96	21.38	-6.63	28.01	1.75	13.43	15.32	-2.71	1379	210	50	52	578	157	170	487	200
McBride	-120.16	53.30	720	4.07	11.31	32.24	850.10	22.13	-12.94	35.07	4.04	0.10	14.23	-6.66	706	75	39	21	210	127	201	163	184
Cranbrook	-115.74	49.52	940	5.55	12.24	32.16	899.03	25.95	-12.10	38.06	14.11	0.93	16.50	-5.85	461	61	26	27	151	85	133	113	188
Vancouver	-122.60	49.24	4	10.04	9.15	36.73	577.06	24.20	-0.70	24.90	3.52	17.02	17.09	3.06	1972	308	64	49	818	221	226	691	289
Ashford	-121.96	46.76	573	6.25	9.40	39.81	512.91	20.72	-2.88	23.60	1.02	12.65	13.00	0.69	2333	361	48	58	1057	199	213	964	227
Livingston	-110.61	45.36	1605	5.13	14.06	38.05	836.35	25.90	-11.05	36.95	12.93	-4.85	15.66	-4.85	500	72	24	35	185	83	154	83	193
Jackson	-110.84	43.43	1857	3.51	15.73	38.05	924.38	26.55	-14.80	41.35	8.07	-1.79	14.87	-8.04	456	52	28	20	132	94	110	120	173
Afton	-110.92	42.72	2000	3.14	16.64	36.87	1030.70	27.49	-17.63	45.12	8.47	15.39	15.39	-10.24	466	53	30	16	140	104	104	112	176
Liberty	-111.89	41.33	1600	5.95	13.73	36.51	866.64	27.02	-10.59	37.61	4.65	16.83	16.83	-4.49	515	55	27	21	162	89	89	129	239

Table S4. Principal components with their variance, cumulative variance and Eigenvalues.

PC	Eigenvalue	Variance (%)	Cumulative variance (%)
PC1	14.39	68.52	68.52
PC2	3.16	15.07	83.59
PC3	1.39	6.60	90.18
PC4	1.15	5.48	95.67
PC5	0.39	1.86	97.53
PC6	0.28	1.34	98.87
PC7	0.14	0.66	99.53
PC8	0.06	0.30	99.83
PC9	0.04	0.17	100.00

Table S5. Contributions (loadings) of the altitude and 19 bioclimatic variables on the Principal Component (PC).

Variable	PC1	PC2	PC3	PC4	PC5
altitude	5.5	1.3	10.5	0.2	1.6
bio1	2.8	15.1	7.0	1.4	0.1
bio2	5.5	3.0	7.4	0.0	1.8
bio3	0.3	11.9	36.9	4.4	4.2
bio4	6.5	0.1	0.8	2.7	0.3
bio5	3.9	13.2	0.1	0.0	0.0
bio6	6.3	1.7	0.4	1.8	1.4
bio7	6.6	0.5	0.3	0.8	0.6
bio8	3.7	0.3	1.9	23.8	5.4
bio9	1.7	3.8	0.0	49.3	5.8
bio10	0.8	20.4	16.1	0.0	0.0
bio11	5.6	4.3	0.3	3.1	0.3
bio12	6.6	0.1	3.2	0.0	0.2
bio13	6.6	0.1	2.7	0.0	1.2
bio14	6.0	0.0	1.0	2.6	13.1
bio15	5.9	0.0	0.0	3.0	23.0
bio16	6.4	0.1	4.1	0.0	2.0
bio17	6.3	0.2	0.4	1.2	7.2
bio18	5.1	2.5	0.1	5.5	24.8
bio19	6.0	0.5	6.0	0.0	2.2
growing season length	2.0	20.8	0.7	0.1	4.7

Table S6. The best-fit model for CCRT, CT_{min}, body colour and size of *D. montana* and *D. flavomontana* was defined based on Akaike Information Criterion (AIC). The model selection included Latitude and PC2, as well as PC1, different interaction terms and body colour and size. The model with the highest Akaike weight (AICcWt), i.e. probability of being the best model, was chosen for further analysis. AICcWt of the best-fit model is shown in grey.

Model selection	**CT_{min}**				**CT_{min}**				**CCRT**				**CCRT**				**Body colour**				**Body colour**				**Body weight**				**Body weight**			
	D. montana				***D. flavomontana***				***D. montana***				***D. flavomontana***				***D. montana***				***D. flavomontana***				***D. montana***				***D. flavomontana***			
Model	**df**	**AICc**	**ΔAICc**	**AICcWt**	**df**	**AICc**	**ΔAICc**	**AICcWt**	**df**	**AICc**	**ΔAICc**	**AICcWt**	**df**	**AICc**	**ΔAICc**	**AICcWt**	**df**	**AICc**	**ΔAICc**	**AICcWt**	**df**	**AICc**	**ΔAICc**	**AICcWt**	**df**	**AICc**	**ΔAICc**	**AICcWt**	**df**	**AICc**	**ΔAICc**	**AICcWt**
latitude + PC2	6	1046.1	0.0	1.0	6	979.8	0.4	0.8	6	2248.4	7.8	0.0	6	2238.7	0.7	0.7	6	2370.3	1.0	0.2	6	2301.7	9.4	0.0	6	-129.7	0.0	1.0	6	-118.0	5.3	0.1
latitude + PC2 + PC1	7	1047.6	1.5	0.5	7	981.2	1.9	0.4	7	2249.0	8.4	0.0	7	2240.7	2.6	0.3	7	2369.2	0.0	0.3	7	2297.4	5.1	0.1	7	-127.7	2.0	0.4	7	-119.9	3.4	0.2
latitude * PC1 + PC2	NA	NA	NA	NA	NA	NA	NA	NA	8	2250.4	9.8	0.0	8	2238.0	0.0	1.0	8	2369.9	0.7	0.2	8	2295.2	2.9	0.2	8	-125.6	4.1	0.1	8	-117.8	5.5	0.1
latitude + PC2 * PC1	8	1049.3	3.2	0.2	8	983.3	3.9	0.1	8	2250.3	9.7	0.0	8	2242.7	4.7	0.1	8	2371.1	1.8	0.1	8	2292.3	0.0	0.8	8	-126.0	3.7	0.2	8	-123.3	0.0	1.0
latitude * PC1 + PC2 * PC1	NA	NA	NA	NA	NA	NA	NA	NA	9	2252.2	11.6	0.0	9	2240.0	2.0	0.4	9	2370.3	1.0	0.2	9	NA	NA	NA	9	-123.9	5.8	0.1	9	-121.2	2.1	0.4
latitude + PC2 + weight	8	1049.0	2.9	0.2	8	983.3	3.9	0.1	7	2240.6	0.0	1.0	7	2239.8	1.8	0.4																
latitude + PC2 + PC1 + weight	9	1050.4	4.2	0.1	9	984.8	5.4	0.1	8	2241.1	0.5	0.8	8	2241.7	3.7	0.2																
latitude * PC1 + PC2 + weight	NA	NA	NA	NA	NA	NA	NA	NA	9	2242.2	1.6	0.4	9	2238.9	0.8	0.7																
latitude + PC2 * PC1 + weight	10	1052.2	6.1	0.0	10	986.8	7.4	0.0	9	2242.0	1.5	0.5	9	2243.8	5.7	0.1																
latitude * PC1 + PC2 * PC1 + weight	NA	NA	NA	NA	NA	NA	NA	NA	10	2243.9	3.3	0.2	10	2241.0	2.9	0.2																
latitude + PC2 + colour	7	1047.6	1.4	0.5	7	979.4	0.0	1.0	7	2249.2	8.6	0.0	7	2240.7	2.7	0.3																
latitude + PC2 + PC1 + colour	8	1049.0	2.9	0.2	8	981.1	1.8	0.4	8	2249.5	8.9	0.0	8	2242.6	4.6	0.1																
latitude * PC1 + PC2 + colour	NA	NA	NA	NA	NA	NA	NA	NA	9	2250.7	10.1	0.0	9	2239.6	1.6	0.5																
latitude + PC2 * PC1 + colour	9	1050.6	4.5	0.1	9	983.1	3.7	0.2	9	2250.8	10.2	0.0	9	2244.7	6.7	0.0																
latitude * PC1 + PC2 * PC1 + colour	NA	NA	NA	NA	NA	NA	NA	NA	10	2252.6	12.0	0.0	10	2241.7	3.7	0.2																
latitude + PC2 + weight + colour	9	1049.5	3.4	0.2	9	981.6	2.2	0.3	8	2242.3	1.7	0.4	8	2241.9	3.8	0.1																
latitude + PC2 + PC1 + weight + colour	10	1050.7	4.5	0.1	10	983.4	4.0	0.1	9	2242.6	2.0	0.4	9	2243.7	5.7	0.1																
latitude * PC1 + PC2 + weight + colour	NA	NA	NA	NA	NA	NA	NA	NA	10	2243.6	3.0	0.2	10	2240.6	2.6	0.3																
latitude + PC2 * PC1 + weight + colour	11	1052.4	6.3	0.0	11	985.3	6.0	0.1	10	2243.6	3.0	0.2	10	2245.8	7.8	0.0																
latitude * PC1 + PC2 * PC1 + weight + colour	NA	NA	NA	NA	NA	NA	NA	NA	11	2245.4	4.8	0.1	11	2242.7	4.7	0.1																

df = degrees of freedom
AICc = Akaike Information Criterion with a correction for small sample sizes
ΔAICc = difference between the best and other models
NA = model overparameterized given the data

Table S7. Summary of the best-fit model results on the effects of latitude and/or climatic factors (PC1) on cold tolerance and body colour of *D. flavomontana*. Model selection was based on Akaike Information Criterion (AIC) results (shown in Table S6). Significant P-values are shown in bold. df = degrees of freedom

Species	Test	Fixed effect	Df	Estimate	SE	Statistic	P-value
D. montana	**CT_{min}**	Intercept	7,443	2.023	0.059	34.42	< 0.001
		Latitude		0.001	0.001	0.98	0.329
		PC2		0.008	0.004	2.36	**0.018**
D. flavomontana	**CT_{min}**	Intercept	7,360	2.495	0.156	15.95	< 0.001
		Latitude		-0.002	0.002	-1.00	0.316
		PC2		-0.005	0.006	-0.95	0.341
		colour		-0.123	0.078	-1.58	0.114
D. montana	**CCRT**	Intercept	7,443	3.123	0.236	13.22	< 0.001
		Latitude		-0.011	0.005	-2.48	**0.013**
		PC2		-0.009	0.014	-0.61	0.540
		weight		-0.210	0.069	-3.07	**0.002**
D. flavomontana	**CCRT**	Intercept	7,385	3.729	0.357	10.44	< 0.001
		Latitude		-0.019	0.007	-2.65	**0.008**
		PC1		0.267	0.128	2.09	**0.036**
		Latitude × PC1		-0.006	0.003	-2.07	**0.038**
		PC2		-0.013	0.019	-0.70	0.484
D. montana	**Body colour**	Intercept	7,440	4.771	0.039	122.08	< 0.001
		Latitude		-0.001	0.001	-1.60	0.109
		PC2		-0.002	0.002	-0.96	0.339
		PC1		0.002	0.001	1.83	0.067
D. flavomontana	**Body colour**	Intercept	7,384	5.080	0.069	73.79	< 0.001
		Latitude		-0.005	0.001	-3.51	**< 0.001**
		PC2		-0.005	0.004	-1.33	0.183
		PC1		-0.006	0.001	-4.00	**< 0.001**
		PC1 × PC2		0.002	0.001	2.94	**0.003**
D. montana	**Body weight**	Intercept	7,440	-0.299	0.141	-2.13	0.034
		Latitude		0.005	0.003	1.86	0.063
		PC2		0.033	0.009	3.83	**< 0.001**
D. flavomontana	**Body weight**	Intercept	7,384	-0.224	0.311	-0.72	0.471
		Latitude		0.006	0.006	0.89	0.373
		PC2		0.031	0.018	1.71	0.087
		PC1		0.020	0.007	3.05	**0.002**
		PC1 × PC2		-0.010	0.004	-2.60	**0.009**

Table S8. Summary of the effect of treatment (*LacZ* and no-injection controls and RNAi with *vrille*) on the expression levels of *vrille*. Significant P-values are in bold.

Gene	Treatment	Coefficient	SE	t	P
vrille	*LacZ* (intercept)	1.777	0.169	10.538	< 0.001
	No-injection	-0.303	0.239	-1.272	0.251
	vrille	-0.988	0.239	-4.143	**0.006**

Table S9. Summary of the effects of cold acclimation treatment on cold tolerance, measured with CT_{min} or CCRT, in *D. montana* females in different treatments (*LacZ* and no-injection controls and RNAi with *vrille*) using generalized linear mixed model (GLMM) with gamma distribution. Significant P-values are in bold.

Test	Acclimation effect	Treatment	Coefficient	SE	z-statistic	P-value	DF
CT_{min}	*LacZ*-injected	Intercept	2.041	0.023	90.040	< 0.001	1,53
		Acclimation: yes	-0.100	0.032	-3.090	**0.002**	
	No-injection	Intercept	1.982	0.018	110.030	< 0.001	1,58
		Acclimation: yes	-0.084	0.020	-4.140	**< 0.001**	
	vrille-injected	Intercept	2.184	0.072	30.456	< 0.001	1,49
		Acclimation: yes	0.043	0.079	0.545	0.586	
CCRT	*LacZ*-injected	Intercept	2.611	0.115	22.756	< 0.001	1,46
		Acclimation: yes	-0.089	0.116	-0.764	0.445	
	No-injection	Intercept	2.386	0.046	51.480	< 0.001	1,51
		Acclimation: yes	-0.154	0.054	-2.880	**0.004**	
	vrille-injected	Intercept	2.547	0.076	33.520	< 0.001	1,50
		Acclimation: yes	0.237	0.107	2.200	**0.028**	

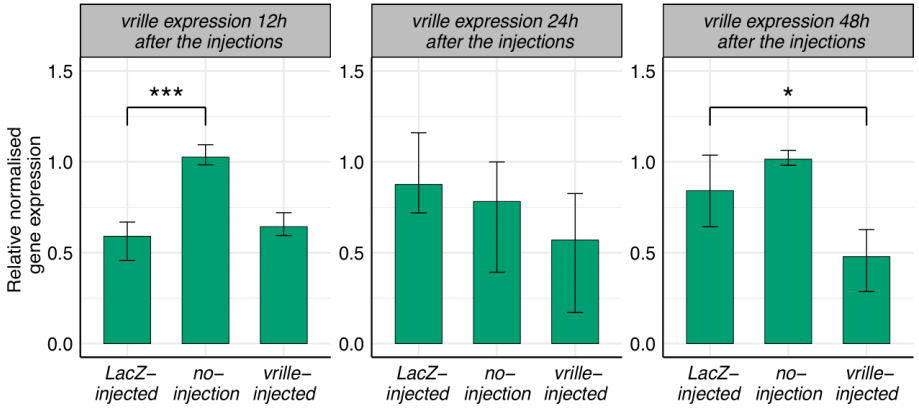
Table S10. Summary of the effects of silencing *vrille* gene on cold tolerance, measured with CT_{min} or CCRT, in non-acclimated and cold-acclimated *D. montana* females using generalized linear mixed model (GLMM) with gamma distribution.

Test	Gene effect	Treatment	Coefficient	SE	z	P	Df
CT_{min}	Non-acclimated	*LacZ*-injected (intercept)	2.042	0.036	57.510	< 0.001	2,80
		No-injection	-0.059	0.044	-1.330	0.185	
		vrille-injected	0.123	0.046	2.670	**0.008**	
	Cold-acclimated	*LacZ*-injected (intercept)	1.941	0.040	48.640	< 0.001	2,82
		No-injection	-0.043	0.055	-0.780	0.436	
		vrille-injected	0.300	0.058	5.170	**< 0.001**	
CCRT	Non-acclimated	*LacZ*-injected (intercept)	2.597	0.087	29.940	< 0.001	2,72
		No-injection	-0.248	0.085	-2.918	**0.004**	
		vrille-injected	-0.054	0.083	-0.652	0.514	
	Cold-acclimated	*LacZ*-injected (intercept)	2.513	0.084	29.842	< 0.001	2,77
		No-injection	-0.275	0.103	-2.667	**0.008**	
		vrille-injected	0.261	0.106	2.454	**0.014**	

Table S11. Quantitative real time PCR (qPCR) primers and their efficiencies (%) for *vrille* gene and reference genes (*Tub2*, *Rpl32*), and primers designed for dsRNA used in RNA interference (RNAi). Primers were designed based on *D. montana* genomic sequences under NCBI accession number LUVX00000000 (Parker et al. 2018) together with information from *D. virilis* exons (Flybase) using Primer3 (primer3.ut.ee) and NetPrimer (www.premierbiosoft.com/ netprimer) programs.

Primers	Gene	Source	Forward primer sequence	Reverse primer sequence	Efficiency value (E%) for qPCR primers
qPCR	*vrille*	Flybase (FBgn0204709)	5′ – CTTTTTTCAAGAGACTGGAACTACT – 3′	5′ – GCACTGCGTATGTAGAATGTTG – 3′	98.1
	Tubulin beta chain (*Tub2*)	Flybase (FBgn0208711)	5′ – CGTGCTGTGTTTGTCGATCT– 3′	5′ –GATCTCCTTGCCAATGGTGT– 3′	96.0
	Ribosomal Protein L32 (*RpL32*)	Flybase (FBgn0016459)	5′ – CATCAGCAGCACCTCCAGTTC – 3′	5′ – GATATGCCAAGCTGTCGCACAA – 3′	97.8
dsRNA synthesis	*vrille*	Flybase (FBgn0204709)	5′ GCGATATACATATGGTGAATGAAGTG 3′	5′ GCTCCAACGGGCTTTCTATC 3′	-
	LacZ	Vigoder et al., 2016	5′ AGAATCCGACGGGTTGTTACT 3′	5′ CACCACGCTCATCGATAATTT 3′	-
	pJET 1.2 F / pJET 1.2. T7 R	CloneJET PCR Cloning Kit (Thermo Fisher Scientific)	5′ –CGACTCACTATAGGGAGAGCGGCC – 3′	5′ – TCTCCTATAGTGAGTCGTATTACGGTACCTTTTAGCTACAAGAA – 3′	-

Supplementary figures



Response time	Treatment	Coefficient	SE	t	P
12h	*LacZ*- injected (intercept)	0.742	0.062	12.045	< 0.001
	No-injection	0.547	0.087	6.277	**< 0.001**
	vrille -injected	0.067	0.087	0.766	0.473
24h	*LacZ*- injected (intercept)	4.031	0.837	4.817	0.003
	No-injection	-0.431	1.184	-0.364	0.728
	vrille- injected	-1.409	1.184	-1.191	0.279
48h	*LacZ*- injected (intercept)	1.208	0.128	9.467	< 0.001
	No-injection	0.249	0.180	1.378	0.218
	vrille- injected	-0.521	0.180	-2.888	**0.028**

Figure S1. The effectiveness of RNAi was investigated 12, 24 and 48 hours after injections. Expression levels of *vrille* were compared between *LacZ*-injected females and no-injection and *vrille*-injected females. Error bars represent bootstrapped 95% confidence intervals. Significance levels were obtained from a linear model (ANOVA) and only significant differences are shown: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

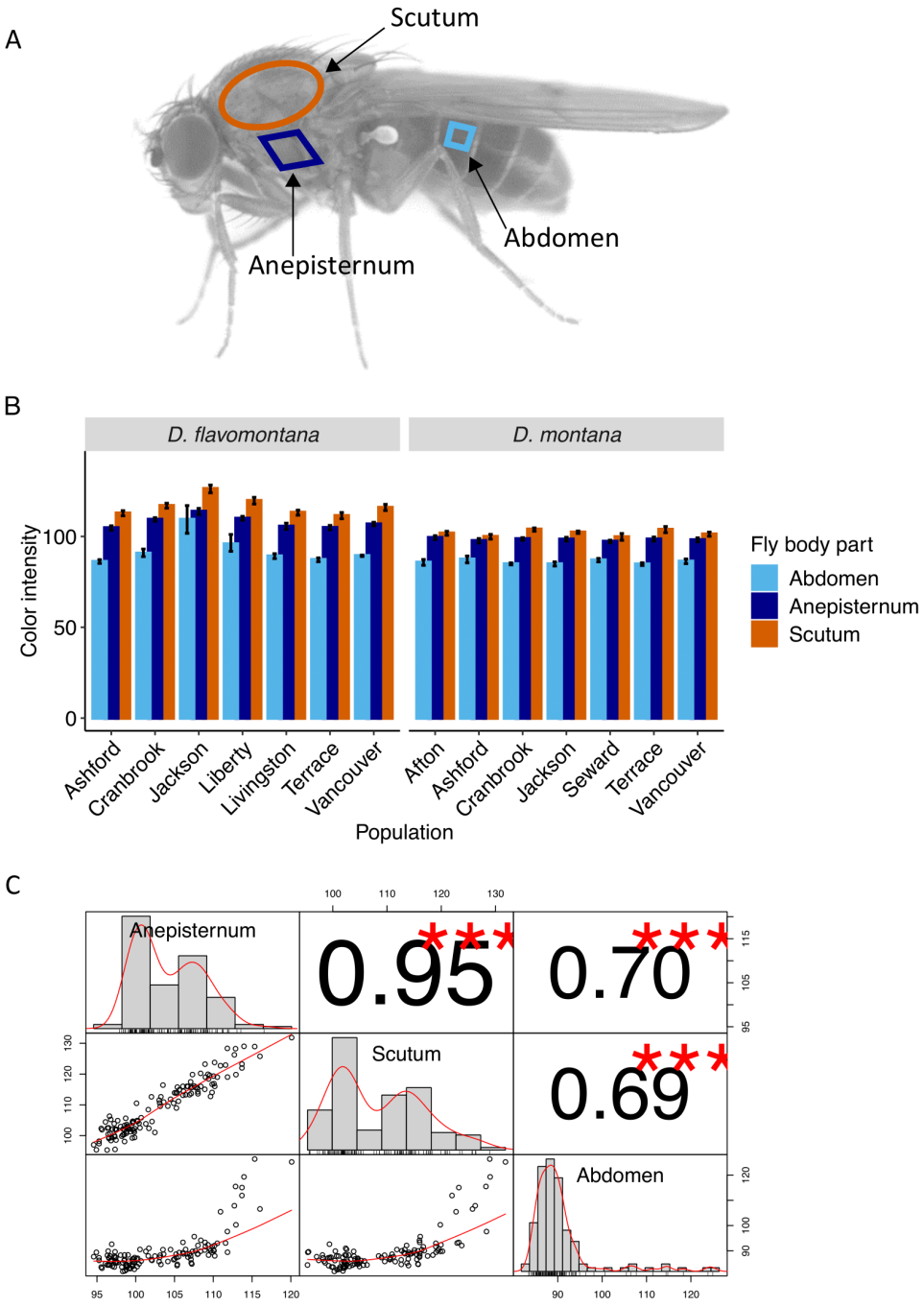


Figure S2. Preliminary colour intensity measurements of different *D. montana* and *D. flavomontana* populations. (A) Colour measurements were taken from two parts of the thorax, scutum and anepisternum, and from A3 segment of the abdomen. (B) Measurements were taken from 5 females per strain, and 1-2 strains per population in each species (McBride populations were not included), and linearly scaled from 0 to 255 (0 = black, 255 = white). Scutum and anepisternum incorporated most of the colour intensity variation among *D. montana* and *D. flavomontana* flies, while abdomen was equally dark among them, except in *D. flavomontana* from Jackson and Liberty populations, which showed slightly more variation. Error bars represent bootstrapped 95% confidence intervals. (C) Scutum and anepisternum were highly correlated with each other (Pearson correlation coefficient = 0.95), enabling us to use only the former in our colour analysis.

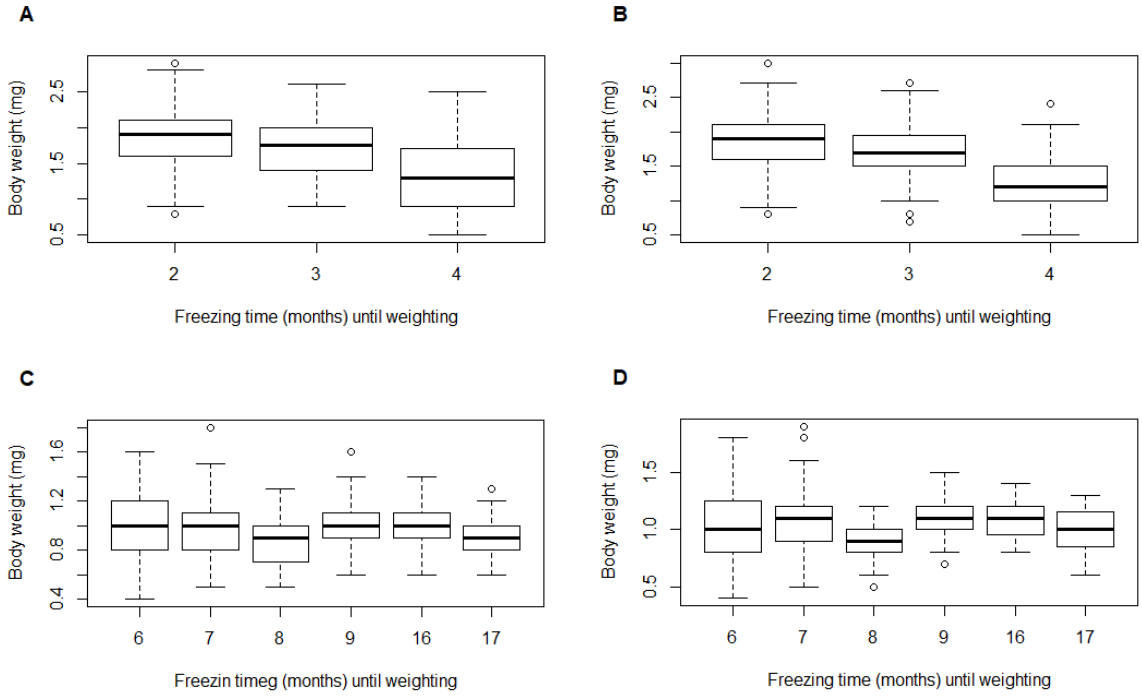


Fig. S3. The effect of the freezing time (in months) on body weight in (A) CT_{min} *D. montana* flies (GLMM, $z_{2,443}$=-5.556, P<0.001), (B) CT_{min} *D. flavomontana* flies (GLMM, $z_{2,334}$= -6.437, P<0.001), (C) CCRT *D. montana* flies (GLMM, $z_{2,437}$=-0.900, P=0.368) and (D) CCRT *D. flavomontana* flies (GLMM, $z_{2,437}$=-0.645, P=0.519).

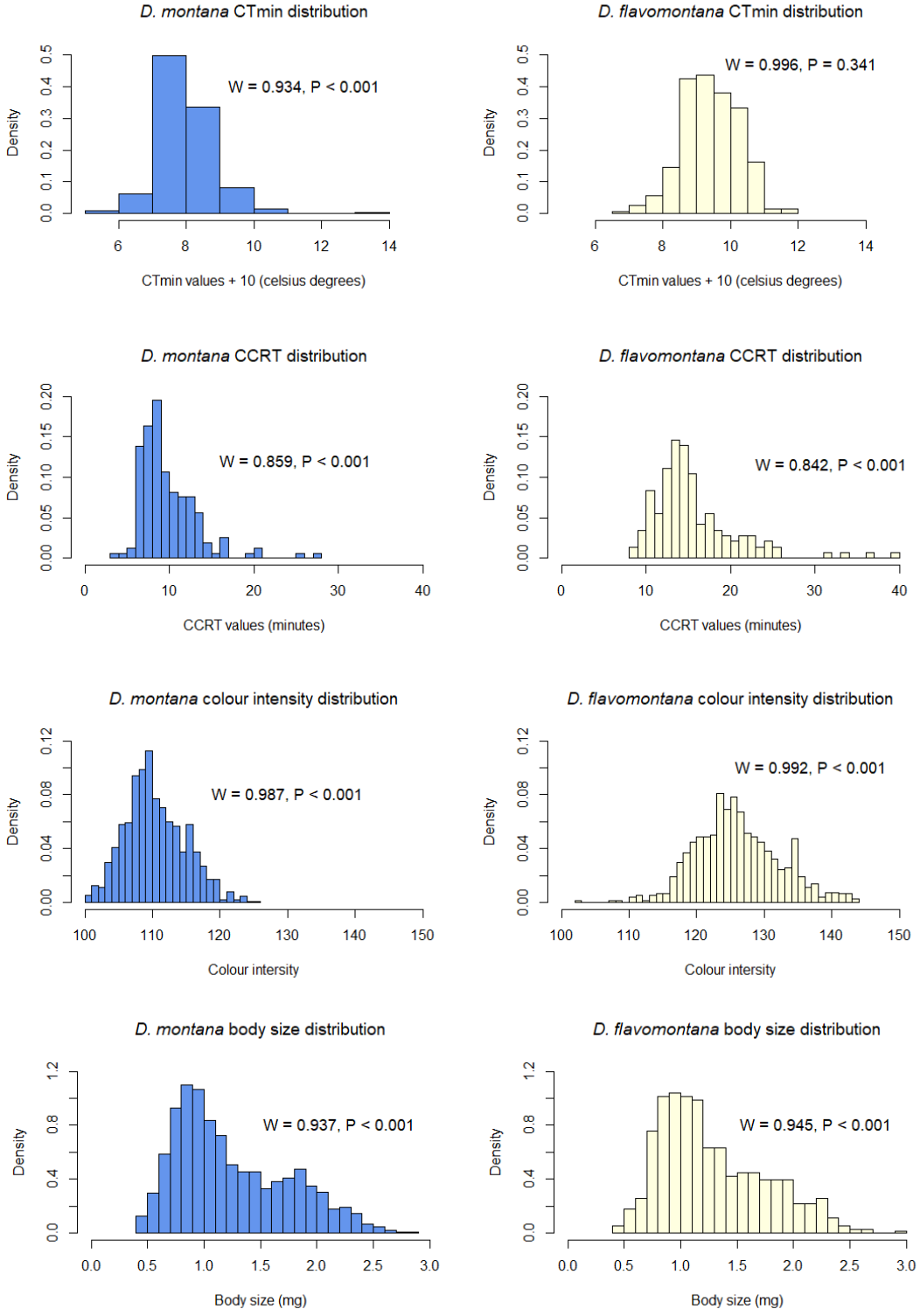


Figure S4. Distributions and Shapiro-Wilk test statistics and P-values for testing normality of CT_{min}, CCRT, body colour and body size (measured as weight) data of *D. montana* and *D. flavomontana*

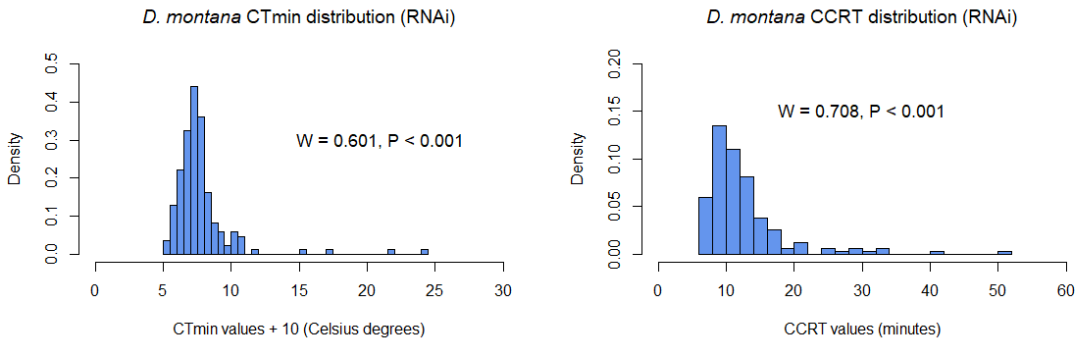


Figure S5. Distributions and Shapiro-Wilk test statistics and P-values for testing normality of CT_{min} and CCRT data of *D. montana* in RNAi studies.

Supplementary references

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III

ANCESTRALLY POLYMORPHIC CHROMOSOMAL INVERSIONS AS POTENTIAL DRIVERS OF SPECIATION

by

Noora Poikela, Dominik R. Laetsch, Konrad Lohse & Maaria Kankare

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IV

EXPERIMENTAL INTROGRESSION IN *DROSOPHILA* SPECIES: ASYMMETRIC POSTZYGOTIC ISOLATION ASSOCIATED WITH CHROMOSOMAL INVERSIONS AND AN INCOMPATIBILITY LOCUS ON THE X CHROMOSOME

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