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**Author(s):** Poikela, Noora; Kinnunen, Johanna; Wurdack, Mareike; Kauranen, Hannele; Schmitt, Thomas; Kankare, Maaria; Snook, Rhonda R.; Hoikkala, Anneli

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

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**Running title:** Reinforcement of reproductive barriers

### Authors

Noora Poikela<sup>1</sup>, Johanna Kinnunen<sup>1</sup>, Mareike Wurdack<sup>2</sup>, Hannele Kauranen<sup>1</sup>, Thomas Schmitt<sup>2</sup>, Maaria Kankare<sup>1</sup>, Rhonda R. Snook<sup>3</sup> and Anneli Hoikkala<sup>1</sup>

<sup>1</sup>Department of Biological and Environmental Science, University of Jyväskylä, Finland

<sup>2</sup>Department of Animal Ecology and Tropical Biology, University of Würzburg, Germany

<sup>3</sup>Department of Zoology, Stockholm University, Sweden

### Corresponding author:

Noora Poikela, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

E-mail: noora.p.poikela@student.jyu.fi

Phone: +358 40 5383527

### Author contributions

Poikela, N.: study planning, experiments, data analyses, writing

Kinnunen, J.: participation in study planning and experiments

Wurdack, M.: pheromone analyses

Kauranen, H.: pheromone analyses

Schmitt, T.: pheromone analyses

Kankare, M: Wolbachia studies

Snook, R. R.: supervision in PMPZ isolation studies, writing

Hoikkala, A.: study planning, participation in experiments, writing

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### 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 (Sun 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 pre-mating 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 coexistence 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  $19 \pm 1^\circ\text{C}$  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  $\text{CO}_2$  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<sub>1</sub> 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 3<sup>rd</sup> 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> hybrid females differed from the expected 0.50 among crosses and population types, and whether fertility of F<sub>1</sub> 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<sup>3</sup> 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 arista 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<sub>2</sub>-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<sub>2</sub>-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  $3.34 \pm 0.02$   $\mu\text{m}$  and of *D. flavomontana* is  $5.53 \pm 0.01$   $\mu\text{m}$  (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<sub>1</sub> HYBRIDS

The costs of interspecific matings at postzygotic level were defined by measuring the viability (from the 3<sup>rd</sup> instar larvae to adults), sex ratio and fertility of F<sub>1</sub> progeny. Crosses between *D. montana* females and *D. flavomontana* males produced a lower number of 3<sup>rd</sup> 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> females were fertile. In the reciprocal crosses, where 101 of 106 F<sub>1</sub> hybrids mated with one of the parental species, fertility of F<sub>1</sub> females deviated significantly from the expected 1 in Allopatry and Sympatry M, while in Sympatry F all 5 F<sub>1</sub> females were fertile (Fig. A3B; Table S5). All F<sub>1</sub> 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 ( $0.52 \pm 0.11$ ) and in Sympatry M ( $0.51 \pm 0.13$ ) than in Allopatry ( $0.36 \pm 0.10$ ; 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 ( $0.39 \pm 0.14$ ) than in Allopatry ( $0.30 \pm 0.12$ , LMM,  $t_{1,910} = 2.52$ ,  $P = 0.016$ ), but of similar level as in Sympatry F ( $0.31 \pm 0.13$ , 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 ( $0.51 \pm 0.13$ ) and Sympatry F ( $0.41 \pm 0.10$ ) than in Allopatry ( $0.37 \pm 0.10$ ; 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-bond 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 <i>D. montana</i> and <i>D. flavomontana</i>	2methylC24-alkane	C27-alkene-1	C27-alkene-2
Between sexes of <i>D. montana</i>	C27-alkene-3	C27-alkene-3	2methylC24-alkane
Between sexes of <i>D. flavomontana</i>	C25-alkene-2	C25-alkene-4	C27-alkene-2
Between sexes of <i>D. montana</i>	C29-alkene-1	C29-alkadiene-2	C29-alkene-2
Between sexes of <i>D. flavomontana</i>	C27-alkene-5	2methylC28-alkane/C29-alkadiene-5	2methylC28-alkane/C29-alkadiene-5
Between sexes of <i>D. flavomontana</i>	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.

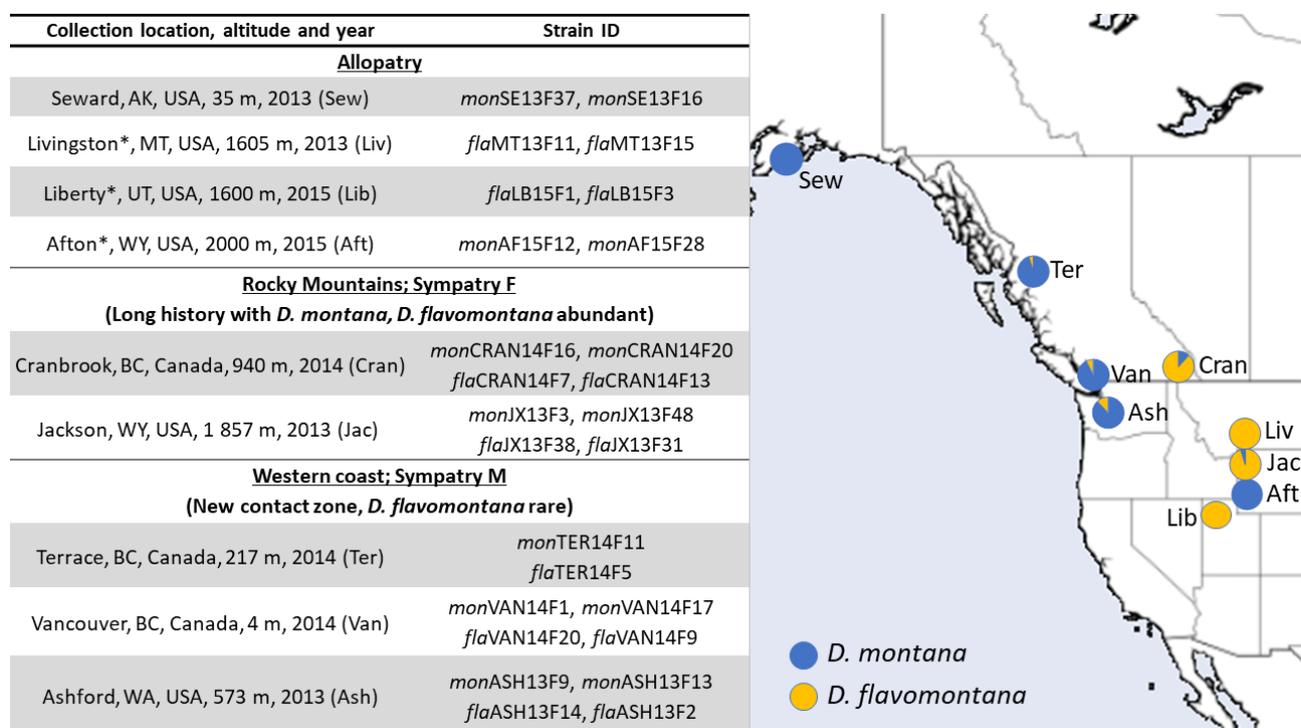
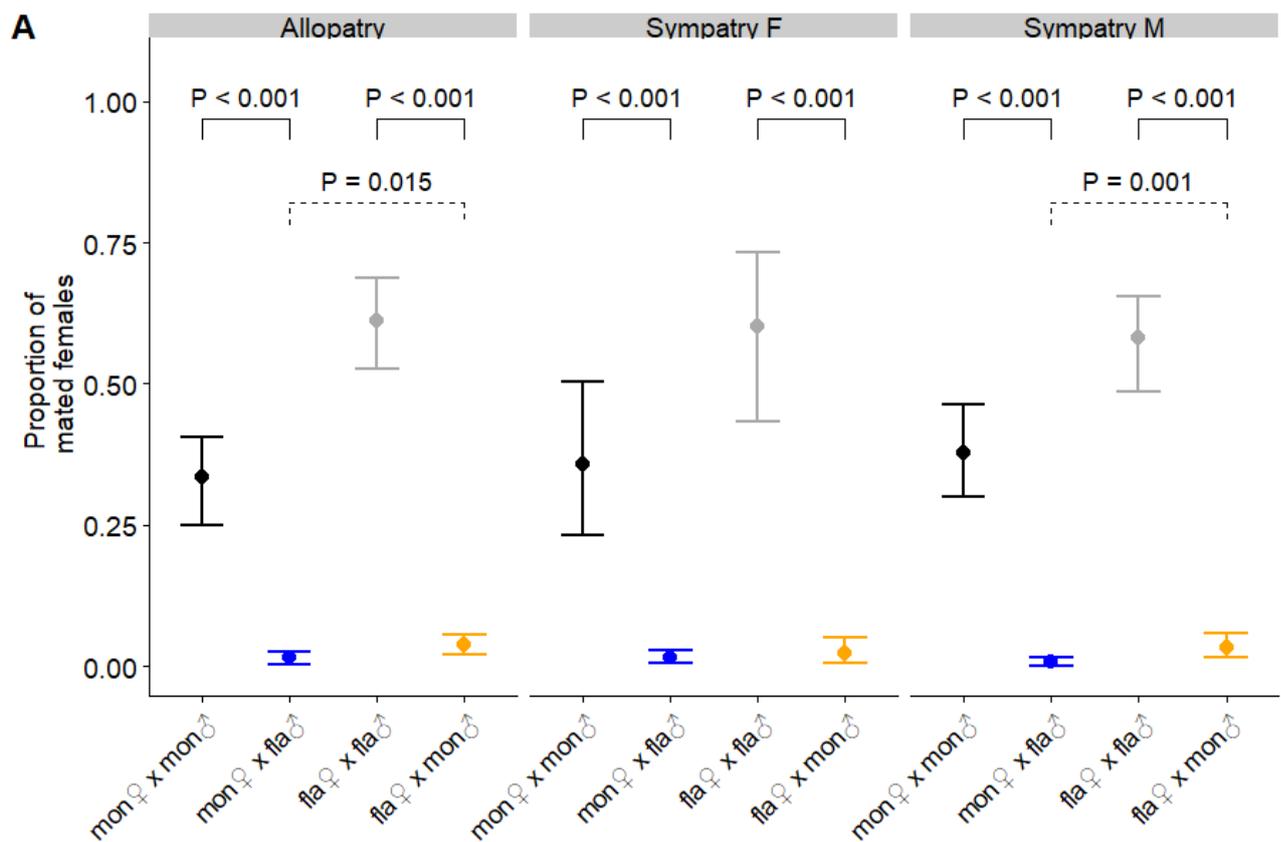


Figure 2. The strength of sexual isolation between *D. montana* and *D. flavomontana* in (A) multiple-choice tests and (B) no-choice tests in different population types. (C) The strength of sexual isolation between *D. flavomontana* from the same and different population types in multiple-choice tests. 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*.



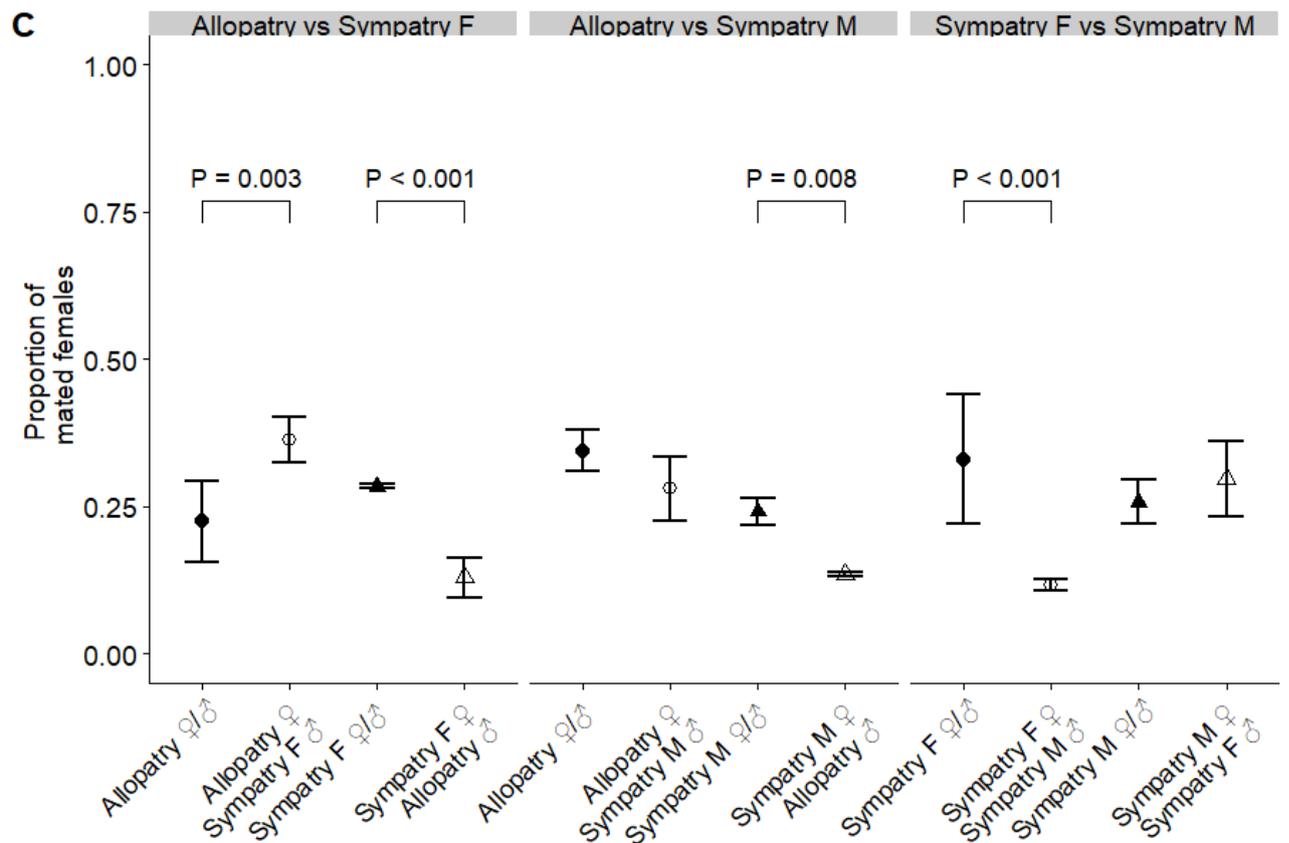
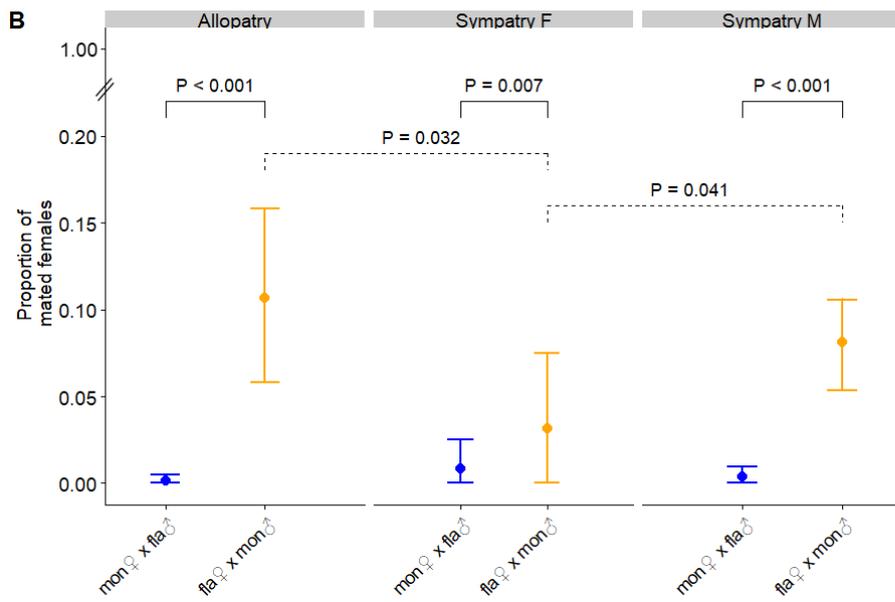


Figure 3. The impact of blocking the transfer of sensory cues on the proportion of mated females in crosses between (A) *D. montana* flies, (B) *D. montana* females and *D. flavomontana* males, (C) *D. flavomontana* flies and (D) *D. flavomontana* females and *D. montana* males. Error bars represent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

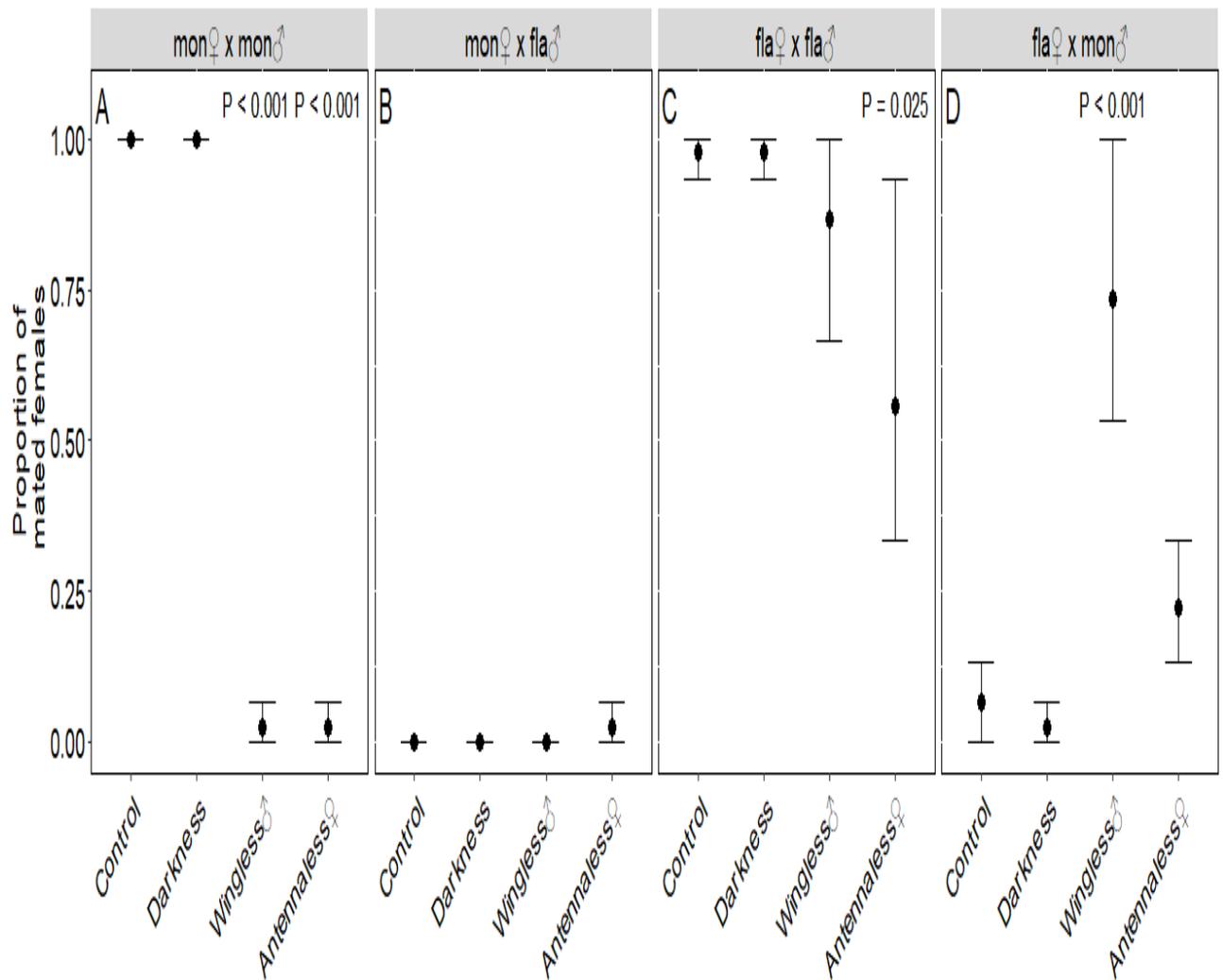


Figure 4. Variation between species and population types in (A) male song traits based on principal component analysis (PCA) and (B) cuticular hydrocarbons (CHCs) of both sexes based on multivariate Linear Discriminant Analysis (LDA). Song traits: PN = number of pulses in a pulse train, PL = length of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

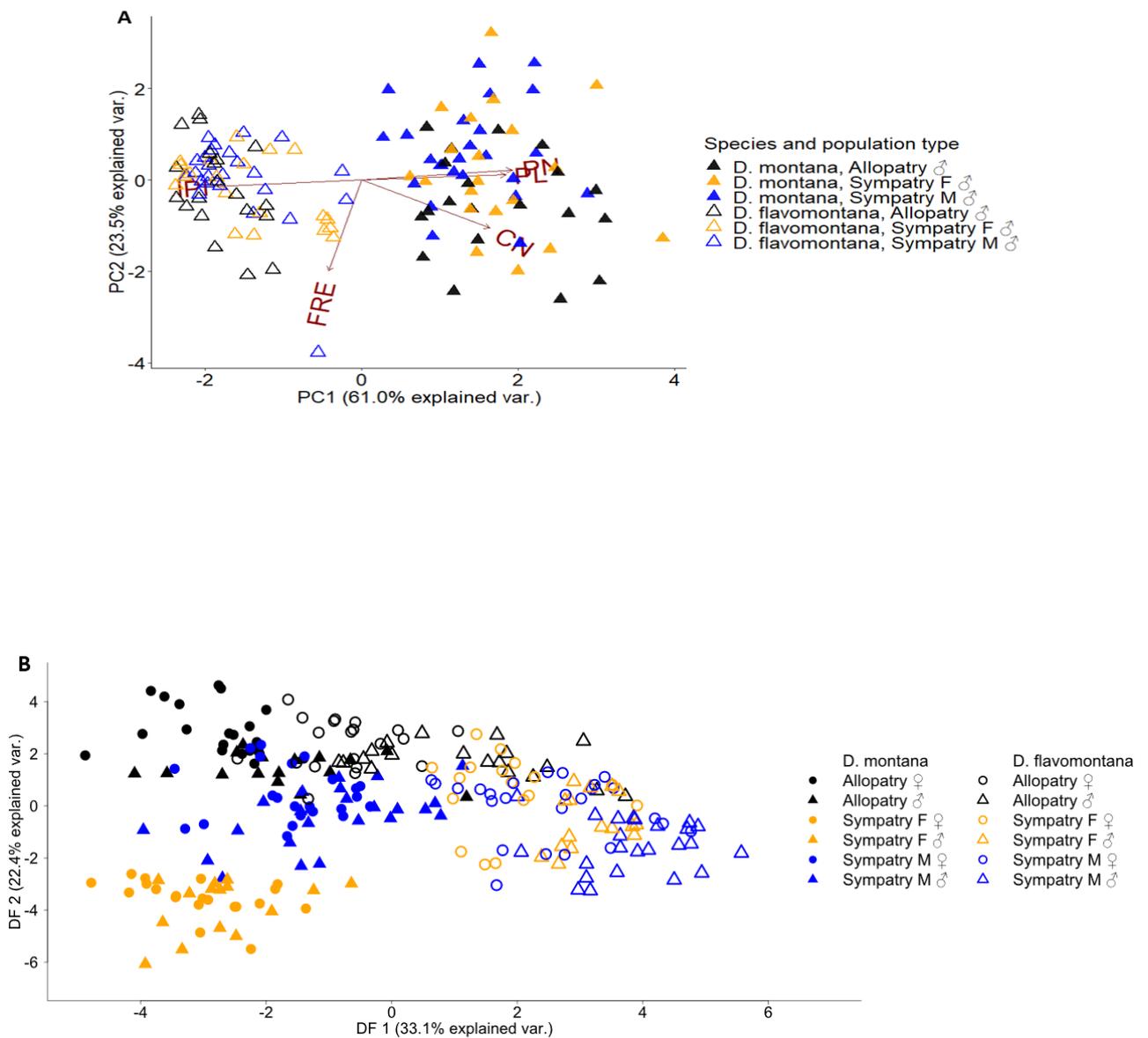


Figure 5. The quantity of stored sperm in interspecific crosses compared to intraspecific ones (P-values above solid lines) and between reciprocal interspecific crosses (P-values above dashed lines). P-values are obtained from CLMMs. Numbers above x-axis refer to the number of studied females in each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

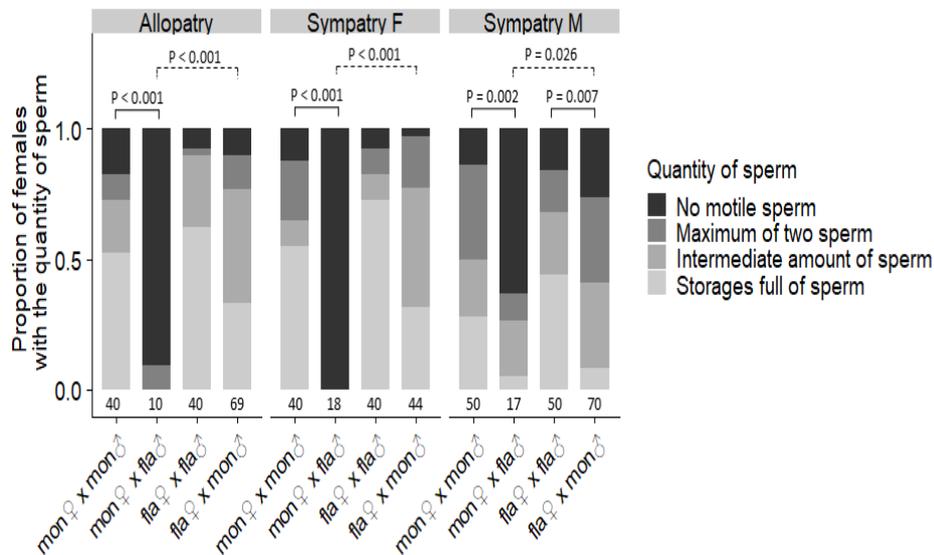


Figure 6. Proportion of fertilized eggs (A) in crosses between *D. flavomontana* females and *D. montana* males in different population types and (B) in the ones between *D. flavomontana* females and males from the same or different population type. Error bars represent bootstrapped 95% confidence intervals. 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.

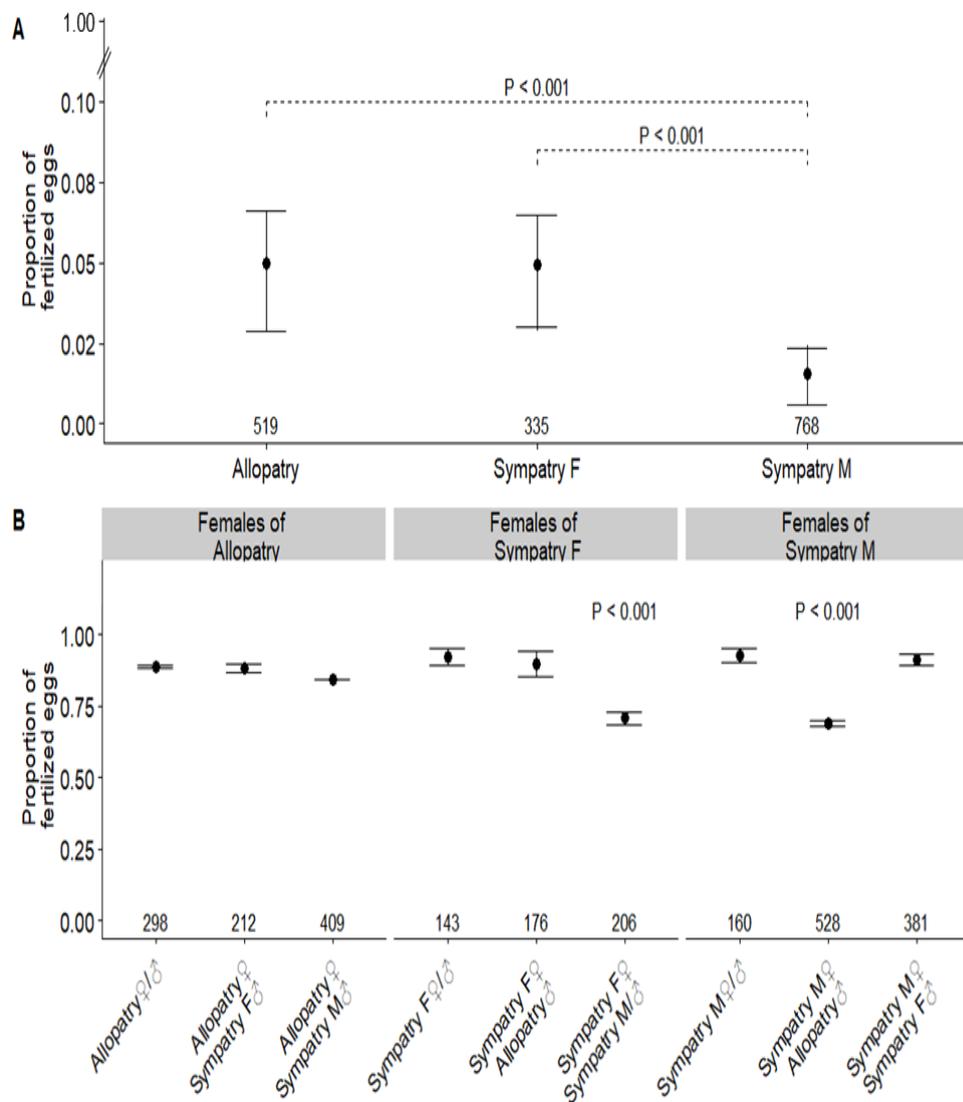
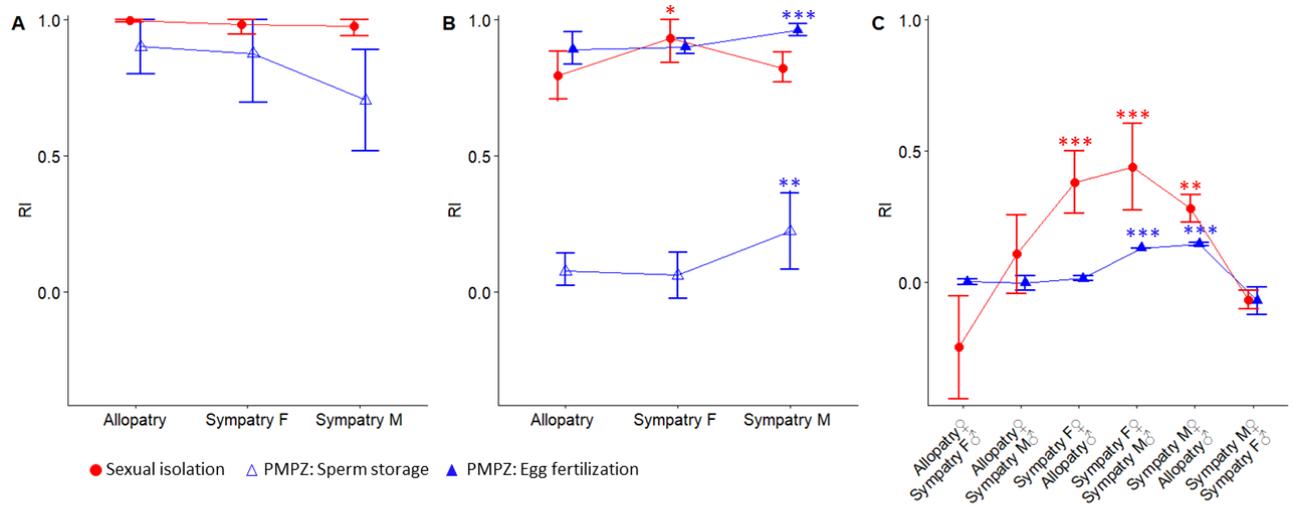


Figure 7. Reproductive isolation indices (RIs) calculated for sexual isolation and PMPZ barriers in sperm storage and / or fertilization in (A) interspecific crosses between *D. montana* females and *D. flavomontana* males and (B) interspecific crosses between *D. flavomontana* females and *D. montana* 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

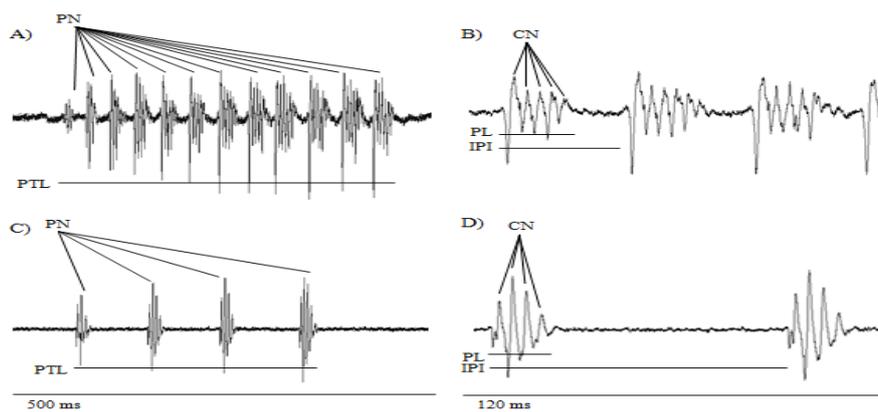


Figure A1. Oscillograms of the courtship songs of *D. montana* (A, B) and *D. flavomontana* (C, D) males and the traits measured from them. PN = number of pulses in a pulse train, PTL

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

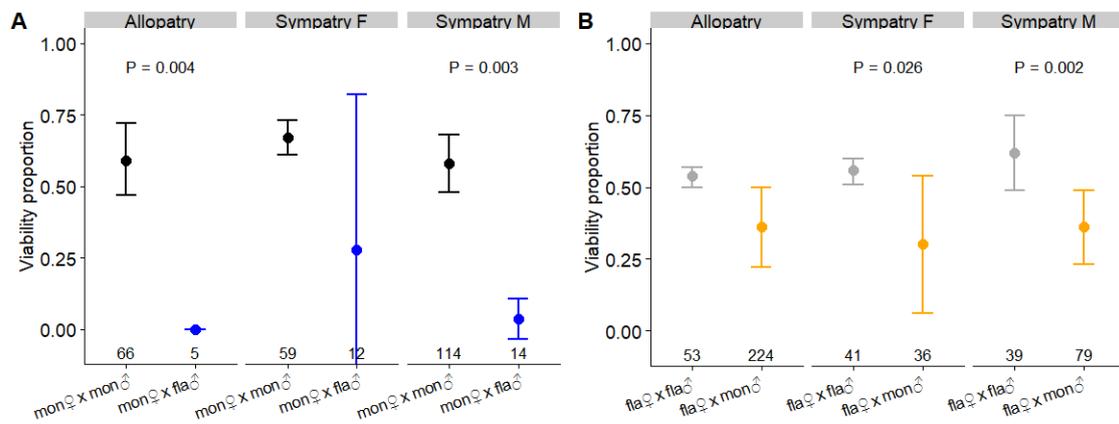


Figure A2. F<sub>1</sub> hybrid viability in intra- and inter-specific crosses involving (A) *D. montana* females and (B) *D. flavomontana* females. Error bars represent 95% confidence intervals. P-values from GLMMs indicate significant differences between intra- and interspecific crosses in different population types, and the numbers above x-axis refer to the total number of studied larvae per cross.

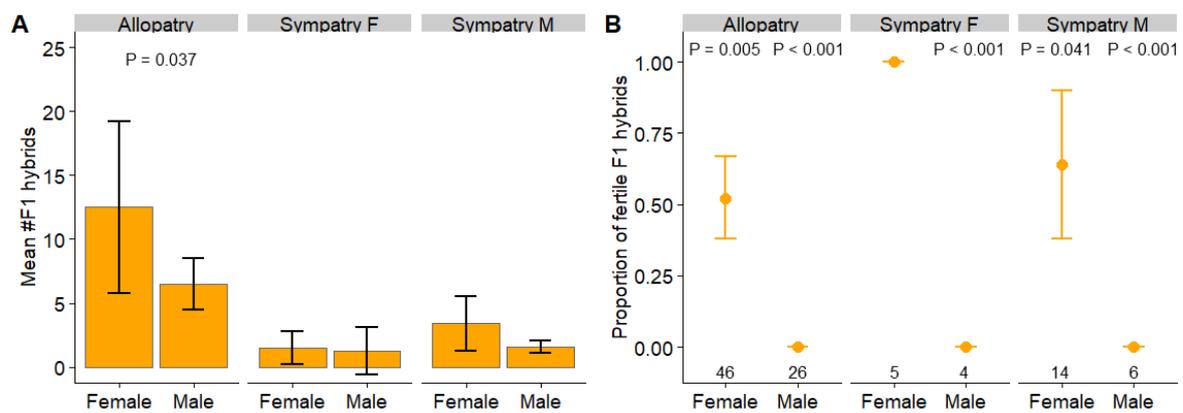


Figure A3. (A) Sex ratio and (B) fertility of F<sub>1</sub> hybrids produced by *D. flavomontana* females and *D. montana* males. Error bars represent 95% confidence intervals. P-values from student's t tests refer to significant deviation from the expected 0.5 in sex ratio and 1 in fertility. The numbers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

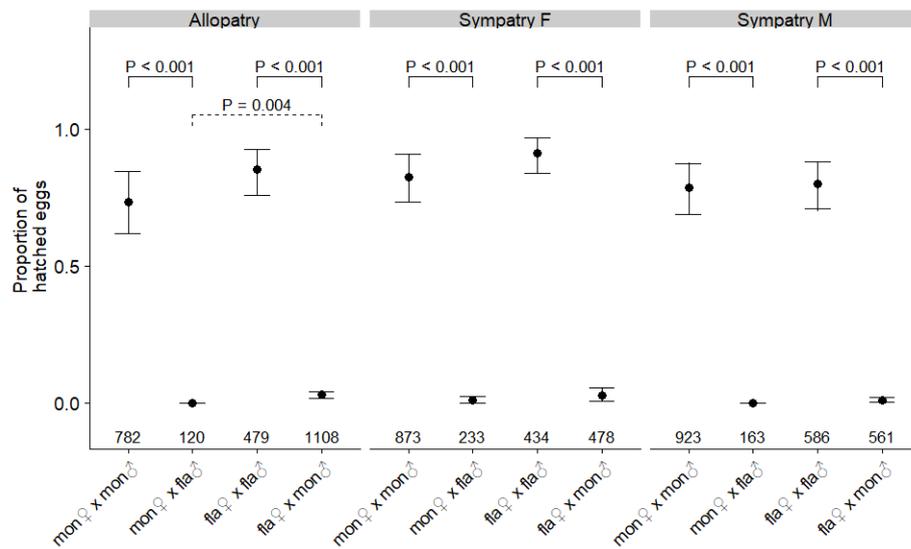


Figure A4. Proportion of hatched eggs in interspecific and in intraspecific crosses (P-values above solid lines) and in crosses between interspecific reciprocal crosses (P-value above dashed line). Error bars represent bootstrapped 95% confidence intervals. P-values are obtained from GLMMs. Numbers above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