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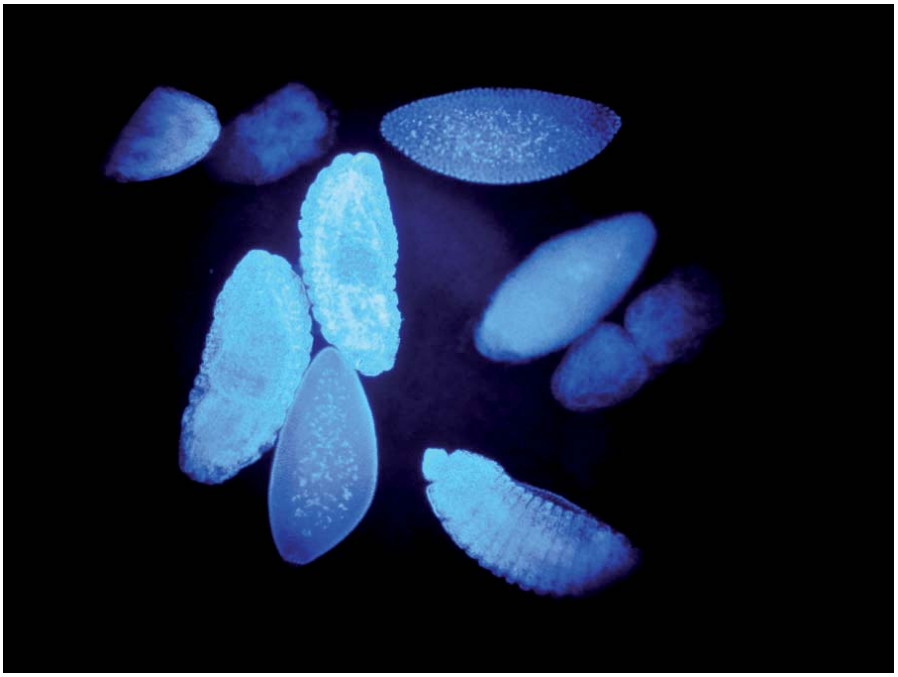
236

Jackson Hubbard Jennings

Barriers Evolving

Reproductive Isolation and the Early Stages of Biological Speciation



JYVÄSKYLÄN YLIOPISTO

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UNIVERSITY OF JYVÄSKYLÄ

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ABSTRACT

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The process of speciation can be complex and represents the ultimate basis for biodiversity on the planet Earth. The contribution of various intrinsic reproductive barriers and their underlying phenotypic mechanisms were studied using two *Drosophila* model systems: the cactophilic sister species *Drosophila arizonae* and *D. mojavensis*, from the deserts of Mexico and the Southwestern USA, and populations of the circumboreal, hydrophilic fly, *Drosophila montana*, from North America and Northern Europe. Levels of premating isolation between *D. arizonae* and *D. mojavensis* as well as between populations of *D. montana* were significant and sensitive to experimental design. Further investigations of intrinsic barriers to gene flow among populations of *D. montana* from Canada, Finland and the USA showed that different mechanisms (premating vs. postmating) act with different strengths depending on the populations. Premating isolation was significant between all populations and postmating isolation was strongest in crosses between American (Colorado) females and Canadian (Vancouver) males. This was found to be due to a postmating, prezygotic barrier; while sperm from Canadian males were successfully transferred and stored after matings with American females, the majority of these eggs were not fertilized. The last study in this thesis aimed to determine whether cuticular hydrocarbons might play a role in sexual selection in *D. montana*. The study revealed significant variation in cuticular hydrocarbons among populations and between the sexes, as well as correlations between particular principal components or individual hydrocarbon peaks and behavioural measurements relevant to sexual selection. These effects appeared to be strongest in the Canadian population of the species. Thus, cuticular hydrocarbons may be involved in sexual selection within and sexual isolation between populations, although more direct tests using manipulation of CHCs are still needed.

Keywords: *Drosophila*; cuticular hydrocarbons; reproductive isolation; sexual isolation; sexual selection; speciation.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following four articles, which are referred to throughout the text with Roman numerals I-IV. I fully participated in the planning, data collection, analysis and writing of all of these articles, as indicated by first authorship.

W. J. Etges contributed to the planning of experiments and to the text in study I and helped in gas chromatography and data analysis in study IV. D. Mazzi carried out some experiments and contributed to the text and M.G. Ritchie helped in data analysis and contributed to the text in study II. R. Snook contributed greatly to the text, carried out dissections and helped with microscope work in study III. T. Schmitt carried out gas chromatography–mass spectrometry work and helped in interpreting the data in study IV. A. Hoikkala contributed to the text and helped to provide the general framework for studies II, III and IV.

I Jennings, J.H. & Etges, W.J. 2010. Species hybrids in the laboratory but not in nature: A reanalysis of premating isolation between *Drosophila arizonae* and *D. mojavensis*. *Evolution* 64: 587-598.

II Jennings, J.H., Mazzi, D., Ritchie, M.G. & Hoikkala, A. 2011. Sexual and postmating reproductive isolation between allopatric *Drosophila montana* populations suggest speciation potential. *BMC Evolutionary Biology* 11: 68.

III Jennings, J.H., Snook, R.R. & Hoikkala, A. 2012. The relative strengths of different reproductive barriers depend on the populations considered: lessons from a circumboreal Drosophilid. Manuscript.

IV Jennings, J.H., Etges, W.J., Schmitt, T. & Hoikkala, A. 2012. Variation and sexual dimorphism in cuticular hydrocarbon profiles of *Drosophila montana* populations and population-specific evidence for their role in mating behaviour. Manuscript.

1 INTRODUCTION

1.1 Speciation

Evolutionary biologists generally seek to explain two major phenomena of the living world: adaptation of organisms to their environment and the origin of biodiversity (The Marie Curie SPECIATION Network 2012). While biological diversity may be manifest at many levels, speciation research focuses particularly on the discontinuous distribution of phenotypes and genotypes into units called 'species', classically defined by Mayr (1942) as "groups of actually or potentially interbreeding populations, which are reproductively isolated from other such groups". Since biodiversity is explained by the balance between extinction and speciation, speciation has remained a central focus of evolutionary science. Research on the patterns and processes underlying speciation has undergone tremendous advances since *Origin of Species* was first published in 1859 (which had little to say about the process itself), particularly in the past two to three decades (Darwin 1859, Otte & Endler 1989, Coyne & Orr 2004). New techniques and model systems have emerged that will allow this fundamental biological process to be more fully evaluated (The Marie Curie SPECIATION Network 2012).

Among speciation models, allopatric speciation is the most basic and well-understood (Mayr 1942). In this model, intrinsic reproductive isolation can arise through genetic drift as well as ecological and/or nonecological processes under natural and/or sexual selection when gene flow is halted between divergent populations (Schluter 2009). In ecological speciation, reproductive barriers between populations evolve through pleiotropic effects of local adaptation (Sobel 2010). In a non-ecological speciation scenario, genetic divergence of populations occurs through the fixation of different advantageous mutations in each population, even though the populations are adapting to similar environmental conditions. In the latter case (mutation-order model; Mani & Clarke 1990), diverging populations may not exhibit the same mutations or the mutations may not be fixed in the same order in each

population. Consequently, during secondary contact, incompatible alleles may interact negatively in hybrids creating pre- and/or postmating reproductive barriers.

Sexual selection also may be important in speciation. Kirkpatrick and Ravigné (2002) suggest that it is even more effective than natural selection in generating disequilibria (i.e., non-random association of alleles at two or more loci) and hence new species. Sexual selection can contribute to reproductive isolation by driving the divergence of important male mating signals and corresponding female preferences in particular populations (e.g. Lande 1981) and/or through sexual conflict (Pizzari & Snook 2003). Natural and sexual selection may also work in concert by favoring the evolution of female sexual preferences for male ornaments that signal local adaptation, potentially creating reproductive barriers even in the face of substantial gene flow (van Doorn et al. 2009). However, the actual mechanisms contributing to speciation may differ from the mechanisms maintaining isolation between already diverged species (The Marie Curie SPECIATION Network 2012). Studying the mechanisms of speciation, therefore, requires not only a consideration of all potential mechanisms of reproductive isolation, but also a study system where speciation has not reached completion.

As Dobzhansky (1935) and Mayr (1942) noted long ago, the evolution of reproductive isolation between divergent conspecific populations is a key requirement for speciation to occur. Barriers maintaining reproductive isolation have generally been categorized as occurring prior to mating (premating), after mating but before zygote formation (postmating, prezygotic [PMPZ]) or after zygote formation (postzygotic). Understanding the order of appearance of the particular mechanisms and their relative strengths during speciation has been described as the "holy grail" of speciation research by Sobel et al. (2010). However, this is made difficult by disagreement over which barriers to include and how to measure total reproductive isolation (for review see Sobel et al. 2010). For example, the two most commonly measured reproductive isolating barriers, premating (or "sexual") isolation and postzygotic isolation (hybrid inviability or sterility) provide valid estimates of reproductive isolation, but fail to take into account other potential barriers, such as those that occur after mating but before the fusion of gametes (e.g. cryptic female choice or sperm-egg incompatibilities).

Determining whether particular demes exhibit some form of reproductive isolation is, of course, an important first step in investigating the speciation process, but it is also important to identify the specific mechanisms that underlie different barriers to gene flow. For example, premating isolation may be determined by one or many incompatibilities, e.g. in courtship song and/or pheromones, and the same can be said for postmating, prezygotic barriers and the genetics of postzygotic isolation. Thus, all potential mechanisms at each stage of reproduction should be assessed in order to gain a better understanding of the causes and consequences of particular reproductive isolating mechanisms and to determine in which order the mechanisms develop in the early stages of species formation.

1.2 Premating reproductive barriers

While demes in nature may be isolated by geographical, temporal or ecological barriers, all of which may contribute to premating isolation, the focus of the present work is on intrinsic barriers to gene flow, which occur upon contact between members of divergent demes and thereafter. Thus, premating isolation in this context is limited to sexual isolation. In *Drosophila* spp., the degree to which species or intraspecific populations are sexually isolated from one another is often investigated by carrying out mate choice experiments in the laboratory. However, as Spieth and Ringo (1983) noted, the "normal rearing techniques and protocols used [in the laboratory] perturb the normal ontogeny of the flies." They state that "in the absence of prior knowledge about the effects of experimental design on mating behavior, the best design is the one that imitates nature most closely" (Spieth & Ringo 1983). Understanding how laboratory conditions affect mating behavior can thus help to elucidate mechanisms responsible for maintaining reproductive isolation between nascent species in nature (Noor & Ortiz-Barrientos 2006). For example, rearing techniques and mating chamber designs may cause changes in fly mating behavior that could affect measurements of sexual isolation, sexual selection, and mating propensity in the laboratory. If realistic estimates of the strength of sexual isolation occurring in nature are to be obtained, the effects of such conditions need to be disentangled.

For *Drosophila*, the element of mate-choice opportunity is one example of how experimental design may affect mate-choice behaviour. Coyne et al. (2005) found that multiple-choice mating experiments yielded significantly higher estimates of sexual isolation between *Drosophila santomea* and *D. yakuba* than no-choice, male-choice, or female- choice experiments. Hoikkala and Aspi (1993) provided similar evidence using a different experimental design. In their study, providing females with the ability to choose between two males of differential fitness – one with normal wings and one with shortened wings – significantly increased the mating success of the fitter, unmanipulated male. In the three species used in their study (*Drosophila littoralis, D. montana,* and *D. ezoana),* discrimination between conspecific normal and wing-manipulated males by females increased when both males were present, as opposed to no-choice situations, and was strongest when the females were courted by both types of male during the trial rather than just one of them (Hoikkala & Aspi 1993).

Mating behaviour may also be influenced by differences in diet. In crosses between populations of the cactophilic *D. mojavensis,* reduction in sexual isolation and time to copulation due to different cactus rearing substrates was first discovered by Brazner (1983). Flies reared on either agria or organ pipe cactus tissue had a fourfold decrease in copulation latency (or time to copulation) when compared to flies reared on synthetic laboratory media. Further, levels of premating isolation between populations were significant when flies were reared on laboratory food, but not on cactus tissue (Etges 1992,

Brazner & Etges 1993). In *D. mojavensis* and its sister species, *D. arizonae*, rearing substrate type has been shown also to affect the composition of epicuticular hydrocarbons (Stennett & Etges 1997), which serve as contact pheromones, mediating sexual isolation between them (Etges & Ahrens 2001, Etges & Tripodi 2008, Etges et al. 2009).

1.3 Postmating reproductive barriers

Postmating isolation between populations is generally assessed by quantifying egg and progeny production or by testing for sterility or inviability of the hybrids produced from interpopulation crosses. Reductions in egg or progeny number after heterotypic matings represent a true postmating barrier, which may or may not be postzygotic in origin, while sterility or death of developing hybrids represents a genetic, postzygotic incompatibility. For example, reduction in F_1 hybrid progeny may be due to failure of sperm to successfully fertilize heterotypic eggs (PMPZ) or to developmental problems occurring after the zygote is formed (postzygotic). Postmating barriers thus include postzygotic ones, but not all postmating barriers are postzygotic. Incompatibilities that occur between the act of copulation and zygote formation include incomplete sperm transfer, sperm death or depletion after mating, improper egg-sperm nuclear fusion and general incompatibilities between sperm or seminal fluids and the female reproductive tract. Because of the complex nature of these interactions, progeny production may break down at any of these stages and may be governed by different genetic factors. For this reason, it is necessary to study all potential areas of breakdown in order to understand the mechanisms underlying reproductive isolation between particular demes.

1.3.1 Postmating, prezygotic isolation

There has been a bias in the accumulation of knowledge on different categories of reproductive isolating barriers; while both premating and postzygotic barriers have been studied extensively in a number of taxa (Coyne & Orr 2004), PMPZ barriers have received less attention until more recently. Thus detecting barriers that occur between copulation and zygote development and understanding how they may influence total reproductive isolation is comparatively depauperate. Perhaps the most well understood PMPZ barriers are in externally fertilizing species, where male-female interactions are limited to their gametes. Here, chemical incompatibilities between egg and sperm cells result in reproductive isolation (Shaw et al. 1994, Metz et al. 1994, Metz & Palumbi 1996, Palumbi & Lessios 2005, Palumbi 2008). In animals with internal fertilization however, a suite of complex processes and interactions between male and female reproductive elements (e.g., ejaculate-female reproductive tract interactions, sperm storage and release and egg–sperm interactions) occur after copulation. These interactions provide ample opportunity for selection to

drive sufficient evolutionary change and cause reproductive isolation, however they are difficult to detect and measure since these processes occur within the female reproductive tract (Howard et al. 1998, 2009, Knowles & Markow 2001, Snook et al. 2009).

Recently, more research in *Drosophila* has sought to identify PMPZ barriers at the level of egg production, sperm transfer, storage and usage, sperm viability and motility, and sperm-egg interactions (either extracellular or intracellular; Snook et al. 2009). For example, between some members of the *D. melanogaster* and *D. simulans* species groups, reduction in sperm transfer, depletion of transferred sperm and/or inefficient sperm storage in heterospecific matings contribute to PMPZ isolation (Matute & Coyne 2010, Fuyama 1983, Price et al. 2001). In the *D. virilis* group, cases of PMPZ isolation between species have been found to involve incompatibility between the male ejaculate or sperm and the female reproductive tract, which results in the incapacitation, death or loss of sperm after heterospecific matings (Sweigart et al. 2010, Sagga & Civetta 2011, Ahmed-Braimah & McAllister 2012). While these studies have gone some way in elucidating the occurrence of PMPZ barriers, they involve crosses between species and thus cannot distinguish whether these mechanisms have contributed to the speciation process or arose thereafter.

1.3.2 Postzygotic isolation

Postzygotic isolation can occur when one or more alleles that are fixed in one population or species are no longer compatible with the genetic background of another (Dobzhansky-Muller incompatibilities; Turelli & Orr 2000). The genetic basis of intrinsic postzygotic isolation differs profoundly from that of ordinary species differences by involving strong epistasis between the loci involved. Postzygotic isolation can be detected as a decline in the viability and/or fertility of the progeny in crosses between individuals from different species or populations. Here, sterility of the heterogametic sex (usually males; Haldane's Rule) is commonly found to be one of the first barriers to gene flow detectable between diverging animal populations (Coyne & Orr 1989, Unckless & Orr 2009). Hybrid male sterility can be tested easily and intuitively, yet separating Dobzhansky-Muller incompatibilities in the F_1 or subsequent generations from other postmating mechanisms can be more difficult. Disentangling the potential mechanisms involved in apparent hybrid inviability thus requires investigating the process of reproduction from the onset of mating, through PMPZ processes, and finally, postzygotic incompatibilities.

1.4 Cuticular hydrocarbons as mating signals

Insects have evolved complex cuticular chemistry, which has allowed them to cope with life in terrestrial habitats. Cuticular hydrocarbons (CHCs) found on the body surface can play important roles in waterproofing, desiccation or

disease resistance and/or mate choice (Edney 1977, Wagner et al. 2001, Howard & Blomquist 2005, Ferveur 2005, Blomquist & Bagnères 2010). The evolution of these compounds can be correlated with abiotic environmental factors (Wagner et al. 2001), but they may also be under sexual selection if particular components confer a mating advantage or increase the fitness of resulting offspring. In sexual selection, the "quality" of a male signal depends partly on the preference of the female, which can vary both within and between species. Thus, signal-preference coevolution can have a major effect on speciation in the early stages of population divergence by providing mechanisms for both sexual selection and species recognition.

1.5 Study species

Drosophila mojavensis and *D. arizonae* belong to the *mulleri* complex of the *D. repleta* group. They exhibit incomplete, yet strong, pre- and postzygotic isolation in the laboratory (Baker 1947, Wasserman & Koepfer 1977, Reed & Markow 2004) but are not known to produce hybrids in nature (Ruiz et al. 1990, Etges et al. 1999, Counterman & Noor 2006, Machado et al. 2007). Both species complete their life cycle in the necrotic tissues of various cactus species and are endemic to the arid lands of the southwestern United States and Mexico. *Drosophila arizonae* is widespread with a range that extends from southern New Mexico and Arizona to Guatemala; it is sympatric with *D. mojavensis* in Sonora and northern Sinaloa on the Mexican mainland and parts of southern Arizona. *Drosophila mojavensis* is found in southern Arizona, Baja California, northwestern mainland Mexico, and southern California (see Fig. 1 in Jennings & Etges 2011). Cytological evidence suggests that *D. mojavensis* originated in Baja California and was derived from an ancestral population of a *D. arizonae*-like ancestor on the mainland (Ruiz et al. 1990). The current estimate of the date of divergence between *D. arizonae* and *D. mojavensis* is 2.4 ± 0.7 mya (Matzkin & Eanes 2003).

Drosophila montana is a *D. virilis* group species with a circumpolar distribution. The *D. virilis* group originated in continental Asia about 20 Mya and gave rise to 12 species which have spread throughout the northern hemisphere, west to Fennoscandia and east to North America by way of Beringia (Throckmorton 1982). North American and Scandinavian clades of *D. montana* have been isolated for 450,000 to 900,000 years and mtDNA data suggest that there has been no recent gene exchange (Mirol et al. 2007). Adaptation to annual changes in light and temperature conditions at high latitudes and altitudes include strong photoperiodic reproductive diapause of overwintering females (Lumme 1978), which shows latitudinal variation (Tyukmaeva et al. 2011), and extreme cold tolerance of both sexes (Vesala & Hoikkala 2011). Both northern and high altitude populations of this species are practically univoltine (i.e., one generation per year; Baker 1975), while more southern populations on the west coast of North America are bivoltine

(Moorhead 1954). Divergent populations of *D. montana* provide an excellent model system for tracing the onset of reproductive barriers in the early stages of speciation, as a wealth of information concerning this species' ecology, mating system, life history, genetics and phylogeography is available. Reproductive isolating barriers between these populations, however, have not been investigated before this project.

1.6 Aims of the thesis

The main objectives of the thesis were to investigate the impact of mate-choice experimental design on levels of sexual isolation in the laboratory between the sister species, *Drosophila arizonae* and *D. mojavensis,* and to investigate the strengths and mechanisms of reproductive isolation, spanning across premating, postmating-prezygotic (PMPZ) and postzygotic barriers, among three focal populations of *Drosophila montana.* I also tested for the role of cuticular hydrocarbons in mate choice in *D. montana* in order to better understand the mechanisms of behavioural isolation among populations, once it was established that the populations were indeed sexually isolated. This was done by analyzing the cuticular hydrocarbon profiles of flies from three focal populations (two from North America and one from northern Europe) and testing whether these hydrocarbons played any role in within-population mate choice in this species.

In the first part of the study (I), I aimed to obtain more realistic estimates of sexual isolation between *D. arizonae* and *D. mojavensis* (which is complete in nature) in the laboratory by rearing flies on fermenting cactus tissue, presenting them with a simulated cactus rot and/or altering the mating chamber size. Using four different experimental designs, I not only developed good methodology for measuring sexual isolation between different *Drosophila* types, but also found that more natural experimental designs yielded results more in line with what occurs in nature, laying some groundwork for the subsequent studies on *D. montana.* Container size and host plant use both affected levels of premating isolation in the laboratory, and isolation was stronger in sympatric population crosses than allopatric ones, consistent with reproductive character displacement in these species.

Then, using the knowledge I had gained from the first part of the study and some of the methods therein, I studied more deeply the onset of reproductive barriers between allo- and parapatric *D. montana* populations from different parts of the species distribution (II and III). I first aimed to establish whether or not allopatric populations of *D. montana* representing the European and North American clades exhibit significant sexual isolation (II). This set the stage for deeper investigation into other potential mechanisms of reproductive isolation in which I incorporated a third, high-altitude population from Colorado, USA. In this work (III), I investigated barriers to gene flow occurring not only at the premating level, but also the PMPZ and postzygotic stages. I

then assessed the relative contributions of pre- and postmating isolation to total reproductive isolation for each pairwise population cross.

In the last part of this thesis (IV), I aimed to assess variation in cuticular hydrocarbon profiles among flies from the three *D. montana* populations and to determine whether the flies of these populations use these compounds as a chemical cue in mating, since understanding premating isolation requires the consideration of all potential mating cues. Earlier studies on this species have concentrated mainly on acoustic signalling (studies reviewed in Hoikkala *et al*. 2005).

2 MATERIALS AND METHODS

2.1 Study species and populations

In the first study we used two sympatric and two allopatric populations of both *D. arizonae* and *D. mojavensis*. The sympatric populations were collected in 1996 and 2003 from Las Bocas and Punta Onah, respectively, both in the Mexican state of Sonora. The allopatric *D. mojavensis* populations were collected in 2002 from Organ Pipe National Monument (Arizona, USA) and in 2003 from San Quintín (Baja California, Mexico). Both allopatric populations of *D. arizonae* were collected in 1999 from the Mexican state of Hidalgo (Metztitlán and Vaquerías). The number of founders ranged from 7 to 2,559 individuals (see Table 1 in Jennings & Etges 2010). Since their collection from banana bait buckets or by aspirating flies directly from cactus rots in the field, all stocks were mass cultured on banana agar food (Brazner and Etges 1993) in 8-dram shell vials at room temperature at the University of Arkansas (Fayetteville, AR, USA).

The *D. montana* populations used in study II were collected from riparian habitats in Oulanka (Finland) and Vancouver (Canada) in the summer of 2008. Studies III and IV used these same populations along with a third population collected in Colorado (USA) in summer of 2009. All founders were collected using malt bait buckets and aspirators. Isofemale lines were established from the progenies of wild-caught females and these lines were maintained in half-pint bottles on malt medium until a large number of F_3 flies were available. 20 F_3 males and 20 F_3 females from each isofemale line (≈ 20 lines, 800 total flies per population) were then combined in a population cage and bred in overlapping generations. Each representative population was maintained in a 25×25×60 cm wooden cage with a Plexiglas top and eight available food bottles for feeding, oviposition and larval rearing. Study II also included matings with flies from isofemale lines (four per population) to study potential postmating isolation.

2.2 Mating experiments

Measurements of premating isolation were obtained by carrying out mating trials with various experimental designs. All mating trials between *D. arizonae* and *D. mojavensis* were multiple-choice and were carried out in mating chambers of various sizes with 120 mature, virgin flies (30 males and 30 females of each species). Flies were anesthetized with CO_2 and placed gently into the chamber. Copulating pairs were aspirated out of the chamber as they occurred and stored in individual vials for identification. Adults of both species were placed on laboratory food colored with one drop of either red or blue food coloring 12–24 h before each trial began so that species identification could be verified.

Study I involved four population crosses (two allopatric-allopatric and two sympatric-sympatric), which were carried out under four experimental conditions to test for the effects of mating chamber size, larval rearing substrate, and the presence of a simulated cactus rot on levels of sexual isolation between populations. Here our attempt was to mimic circumstances in nature and create conditions where no hybridization would occur. The first treatment was designed to crowd flies into a small space, thus increasing the number of interactions with potential mates. The mating chamber was a 20 mL cylindrical glass specimen jar fitted with a perforated latex lid. Flies in this treatment were reared on standard banana laboratory food. In the next treatment, flies were also reared on lab food, but the size of the mating chamber was increased to 28.4 L. In the last two treatments, the mating chamber was 28.4 L in volume but flies were either reared on lab food and provided with a simulated cactus rot or reared on fermenting cactus tissue instead of lab food. The effects of geographic origin (allopatry vs. sympatry) were also examined for evidence of character displacement in these species.

Studies II - IV were performed on *D. montana* flies. Study II included no-choice, female-choice and multiple choice mating trials using *D. montana* populations from Vancouver and Oulanka. For each no-choice trial, one female and one male were transferred into a gauze-covered plastic dish (diameter 5 cm, height 0.7 cm) with a piece of moistened filter paper covering the floor. This was done for all possible combinations of flies. The behaviour of the flies was observed until the end of copulation or until two hours had elapsed and the frequencies of each type of pair mating were recorded. For each individual pair of flies, we also recorded the lengths of courtship latency, courtship duration and copulation duration to test for differences in mating behaviour across the different combinations of flies. Female-choice trials were carried out in the same way as no-choice trials, except that for each trial the female was combined with two males (one from each population). Multiple-choice trials were carried out as in study I, with 120 total flies, however flies were not anesthetized before being introduced into the mating chamber and the mating chamber was a 6×6×6 cm Plexiglas box. Males in female-choice trials and the flies of both sexes in multiple-choice trials were marked for identification as in study I, but flies

were dissected to verify their identity, since the color of the food in the gut was rarely visible through the abdomen in *D. montana*. Study III involved only multiple-choice trials, carried out the same way as in study II, but it also included a third population of *D. montana* (Colorado, USA). For all mating experiments, the frequencies of each type of pair mating were used to generate measurements of premating isolation and other mating statistics in the program JMating (Rolán-Alvarez & Caballero 2000).

In study IV, I used population-specific, female-choice mating trials to test for the potential role of CHCs in mating behaviour. Each trial was carried out in a small plastic dish (diameter 5 cm, height 0.7 cm) covered with netting, using one female and two males from the same population. When one male was accepted by the female, indicated by the spreading of her wings (Vuoristo et al. 1996) and a mounting attempt by the male, the mating was interrupted and this "winning" male was removed from the chamber. If a female was courted by only the winning male before accepting him as a mate, the mating trial was allowed to continue until the losing male also initiated a courtship bout. This was done to control for the possible effects of courtship activity on the male CHC profile, since CHCs have been shown to be sensitive to social experience and mating activity (Kent et al. 2008, Etges et al. 2009, Everaerts et al. 2010). All flies were then immediately frozen at -20°C and stored for CHC extractions.

2.3 Postmating isolation in *D. montana*

2.3.1 Egg and progeny production

Postmating isolation among *D. montana* populations was assessed first by simply counting the number of eggs laid and progeny produced by singly mated females in all population cross combinations. For each mating, we combined a single male and a single female in a food vial (II) or small plastic dish covered with netting (III). In study II, matings were made using both mass-bred populations and isofemale lines. Crosses within and between isofemale lines were carried out using a diallel design with all 64 possible crosses. After mating, males were discarded and females were transferred singly to fresh food vials and allowed 7 days of oviposition, changing them into a new vial after the first 3 days. Eggs were counted under a dissecting microscope and resulting progeny (males and females) were counted after their emergence.

2.3.2 Postmating, prezygotic isolation

While general postmating isolation was assessed in all possible cross types using the three focal populations, investigations of PMPZ isolation were limited to crosses between the flies of the Vancouver and Colorado populations, since crosses between Colorado females and Vancouver males showed the strongest reduction in F_1 progeny production of any interpopulation cross carried out. To

determine whether this reduction was due to PMPZ mechanisms or postzygotic incompatibilities, I measured traits that contribute to PMPZ isolation including sperm transfer, storage and usage. Sperm usage was measured by observing the hatchability, development and fertilization rates of eggs laid by females from the four possible cross combinations of flies from the two populations.

To qualitatively determine whether differences in progeny production could be due to inefficient sperm transfer or storage in heterotypic crosses, we mated virgin females to virgin males and dissected the females either one or three days after mating. We dissected females' reproductive tracts under a dissecting microscope and separated the uterus, spermathecae and ventral (seminal) receptacle. These organs were then scored for the presence or absence of motile sperm.

Since sperm transfer and storage appeared to be normal in heterotypic crosses, we then investigated whether the observed decrease in progeny number was due to a decrease in egg hatch rate or whether the breakdown occurred after the eggs had hatched. Again using the two North American populations, we carried out single pair matings for all possible cross combinations in plastic shell vials. We then transferred mated females to an oviposition manifold (as in Crudgington et al. 2005, Snook et al. 2000) for egg laying. Manifolds consisted of 20 replicate chambers connected to a plate with corresponding oviposition dishes containing molasses-yeast-agar food sprinkled with dried yeast. Females were left individually in these chambers for two days and then transferred to new food plates for another two days of oviposition. Laid eggs were counted immediately after the plates were removed from the manifold and the number of unhatched eggs on each plate was counted 2 days later. From these data we calculated the proportion of eggs that hatched for each mated female of each cross type.

Since the egg hatch rate in crosses between Colorado females and Vancouver males was found to be significantly reduced compared to pure parental crosses, we used fluorescent and compound light microscopy to score eggs for development and/or sperm presence, respectively. Eggs from all four cross types between the Colorado and Vancouver populations were observed. This allowed us to determine whether the decreased egg hatch rate was due to either fertilization failure (a PMPZ mechanism) or abnormal development after fertilization (a postzygotic mechanism). To obtain eggs, we combined large numbers of males and females (30-40 per sex) in bottles, each covered by an oviposition plate. Oviposition plates were removed within 24 hours and eggs were collected, dechorionated, fixed and stained with the nuclear stain, DAPI, for microscopy (as in Snook & Karr 1998). For each of the four cross types we observed all eggs collected from the 24 hour oviposition period to determine if they were developing or not. We classified eggs as "developing" if clear mitotic division or cellular differentiation was evident (Fig. 1) and "non-developing" if fewer than four apparent nuclei were visible within the egg. In general, the two classes of eggs were easily distinguished, since developing eggs fluoresced brightly and showed clear signs of mitotic division or cellular differentiation, while "non-developing" eggs were dark, lacked clear nuclei and were thus conspicuous.

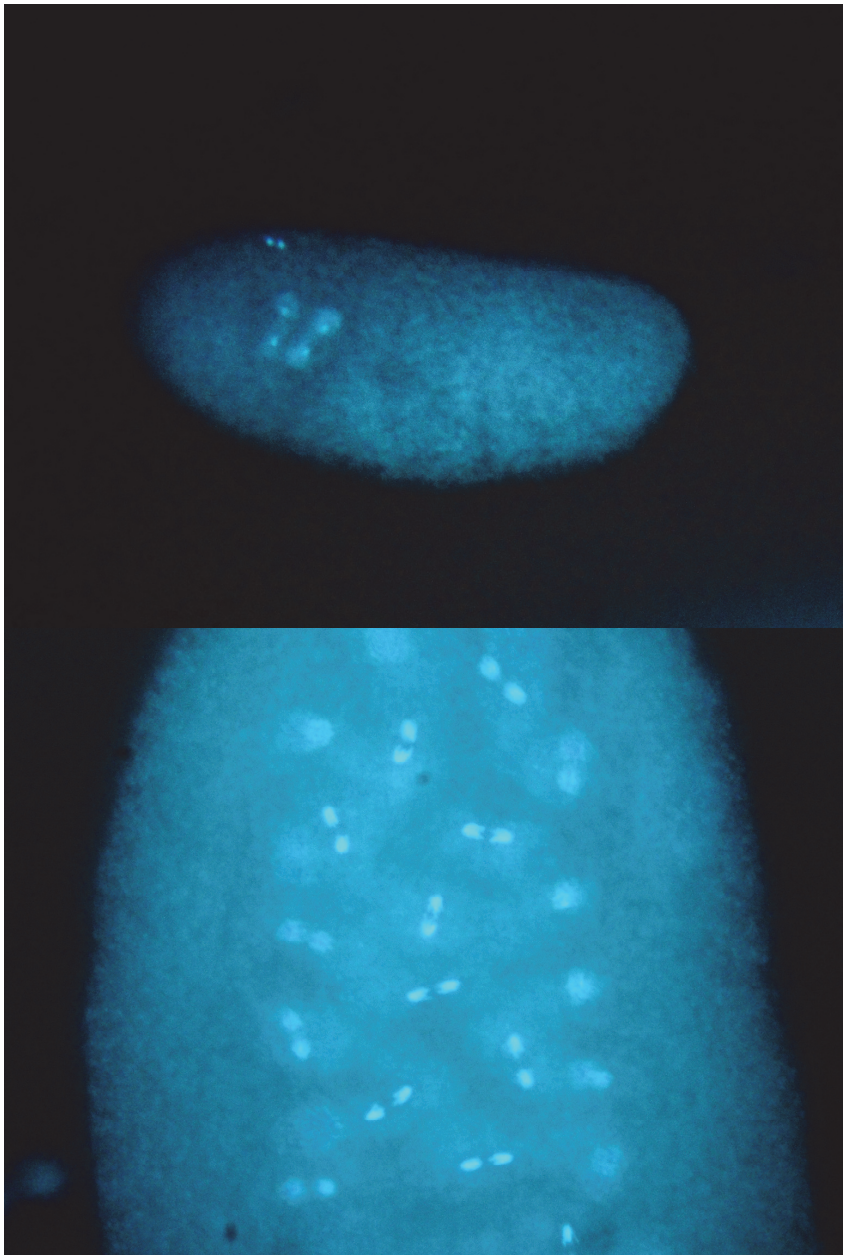

FIGURE 1 Examples of mitotic division visible in DAPI-stained eggs of *D. montana* under fluorescent microscopy. Eggs containing fewer than 4 apparent nuclei were categorized as "non-developing" and scored later for the presence or absence of sperm.

Finally, to determine whether the "non-developing" eggs were unfertilized or had failed to develop due to incompatibilities arising after fertilization, we scored them for the presence or absence of sperm using differential interference contrast (DIC) light microscopy. Since sperm length of *D. montana* is 3.34 ± 0.02 mm (Pitnick et al. 1995), the tail was easily seen as a coiled structure near the anterior end of the egg under 20-40X magnification (Fig. 2).

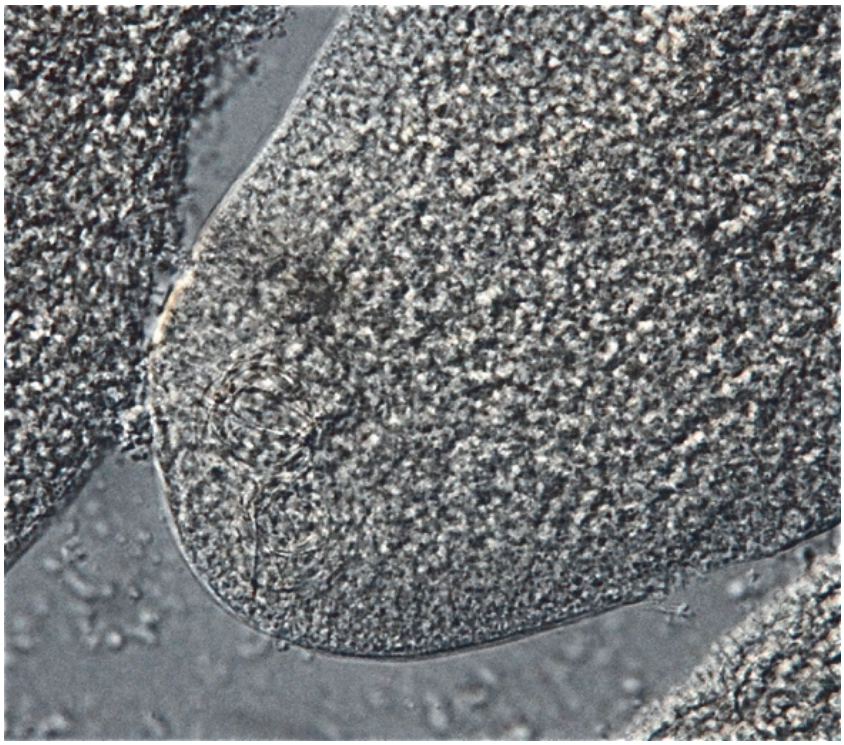

FIGURE 2 Sperm tail visible in a successfully fertilized *D. montana* egg. The tail is visible under DIC light microscopy as a coiled structure near the anterior portion of the egg.

2.3.3 Postzygotic isolation

To search for evidence of intrinsic postzygotic isolation in the form of sterility (at the F_1 level) or inviability (at the F_2 level), we carried out single pair matings within and between the reciprocal F_1 "hybrids" resulting from crosses between Colorado and Vancouver flies. We also backcrossed Vancouver males to both F_1 "hybrid" type females. These matings were carried out in the same way as the single pair matings used to assess general postmating isolation, except that the mated females were allowed eight days of oviposition instead of seven and eggs were not counted.

2.4 Cuticular hydrocarbon analysis

CHCs were extracted from *D. montana* flies by washing single individuals with N-hexane in 300µL conical microvial inserts (Microliter Inc.) for ten minutes, agitating them twice on a vortexer. After CHC extraction, flies were removed from the solvent and the vials were placed in a sterile fume hood at room temperature until dry. Extracts were then sealed, labeled and stored at -20°C until they were shipped together on ice to the University of Arkansas (Fayetteville, AR, USA) to be analyzed by gas chromatography.

Once in the laboratory at the University of Arkansas, each hydrocarbon sample was redissolved in hexane containing docosane as an internal standard. Samples were analyzed by capillary gas liquid chromatography using a Shimadzu G14 (Shimadzu Scientific, Columbia, MO, USA) and peak integration was carried out using the program Class VP 4.2 provided by Shimadzu.

Subsequent identification of peak constituents was carried out by mass spectrometry at the University of Freiburg (Germany) using a separate set of representative samples from the same source populations. A gas chromatograph coupled with a mass spectrometer (GC-MS; Agilent Technologies 6890N gas chromatograph, Agilent Technologies 5975 inert mass selective detector) was used to analyse the components of the polar and non-polar fractions. The software MSD ChemStation (Version A.03.00) for Windows was used for data acquisition and constituents were identified using diagnostic ions and retention indices calculated using Kovats' method (Carlson et al. 1998). The positions of the double bonds in n-alkenes was determined using DMDS derivatization following Dunkelblum et al. (1985).

2.5 Statistical Analyses

2.5.1 Premating isolation

I_{PSI} was the index of sexual isolation used in all parts of this study. I_{PSI} ranges from -1 to 1, where 0 represents random mating and 1 is complete sexual isolation. Negative values reflect disassortative mating, which is rare in crosses between different populations or species. This index (along with other response variables; see Study I) is calculated based on the frequencies of each type of pair mating observed in the mating trials. All calculations were performed in the program JMating (Rolán-Alvarez & Caballero 2000, Carvajal-Rodriguez & Rolán-Alvarez 2006) and significance of sexual isolation was determined by bootstrapping 10,000 times in the program. In study I, we first compared sexual isolation indices from mating trials across treatments and by geography (sympatric-sympatric vs. allopatric-allopatric) using analysis of variance (ANOVA) in SAS (SAS Institute 2004). We then performed nonparametric Kruskal–Wallis tests to assess pairwise differences due to mating chamber type,

rearing substrates and geography. In all cases, when multiple comparisons were made using single datasets, probability levels were adjusted using step-down sequential Bonferroni correction (Rice 1989).

In Study II, we measured some aspects of fly behaviour in the no-choice trials to look for differences among cross types. We used Kruskal-Wallis tests to analyze differences in courtship latency and courtship duration among cross types, since these data were non-normally distributed. Copulation duration was normally distributed and thus analyzed with ANOVA. All statistical analyses in Studies II-IV were performed in SPSS vs. 16.0 (SPSS Inc., Chicago Illinois, USA).

2.5.2 Postmating isolation

All analyses of factors potentially contributing to postmating isolation (egg and progeny production and egg hatchability, development and fertilization rates) were performed in SPSS. We used analysis of variance ANOVA and post-hoc Tukey's HSD tests to assess variation among different cross types when these responses were normally distributed, and nonparametric Kruskal-Wallis tests when they were not. In cases where proportional data was used (egg hatchability, development and fertilization) data were arcsine transformed then analyzed with ANOVA. The absolute and relative contributions of pre- and postmating barriers to total reproductive isolation for the three population crosses in study III were calculated according to Ramsey et al. (2003).

2.5.3 Cuticular hydrocarbon analysis

We found 17 hydrocarbon peaks that were consistent and measurable across all samples. We first calculated peak areas in nanograms/fly based on the docosane internal standard. We then $\log_{10}$ transformed the data to improve normality. We used a multivariate analysis of variance (MANOVA) on the $\log_{10}$ transformed data to assess overall differences in CHC profiles among populations and between the sexes and then ran a principal component analysis (PCA) to characterize variation in the entire dataset.

We then ran PCAs on the male and female data separately for each population and subsequently tested for the effects of PCs explaining at least 1% of the variance in the dataset on male mating success (male data) and courtship latency (female data). For the male data, we used binary logistic regression to determine whether variation in CHC profiles influenced male mating success with male status (winner or loser) as the dependent variable and PCs as covariates. For the female data, we tested for the effects of the PCs on courtship latency with linear regression. Subsequent analyses were carried out on individual $\log_{10}$ transformed data for each peak (linear or binary logistic regression) to aid in biological interpretation of the PCA results.

3 RESULTS AND DISCUSSION

3.1 Effects of experimental design on sexual isolation between *D. arizonae* and *D. mojavensis*

D. mojavensis and *D. arizonae* are sister species that have been studied extensively in a speciation context, however, there has remained a discrepancy between natural and laboratory estimates of the strength of sexual isolation between them. Decreasing the physical space available to flies during mating trials in study I significantly increased sexual isolation (I_{PSI}) between *D. mojavensis* and *D. arizonae*. This indicates the importance of physical space in mate choice experimental design. Sexual isolation also increased when flies were reared on fermenting cactus tissue and when they were exposed to it during the mating experiments. Also, consistent with reproductive character displacement in these species, sympatric-sympatric crosses showed higher levels of sexual isolation than allopatric-allopatric crosses.

The finding that sexual isolation is strongest in the smallest container could be due to the increased possibility for females to choose between con- and heterospecific males in this treatment. We initially predicted that confining large numbers of flies in close quarters would result in more interspecific mating, due perhaps to interference of male mating signals (e.g., courtship songs or epicuticular hydrocarbons), increased interaction of individuals with flies of a different species or simply a lack of space for females to evade undesirable males. During mating trials in the small (20 mL) container, most females were courted by multiple males simultaneously, increasing the frequency of interaction among potential mates of each species. On the other hand, in the large container, flies generally explored the floor of the chamber after recovering from the anesthesia, before dispersing throughout the chamber. Females often walked up the container walls where they stopped and remained motionless, sometimes for the entire duration of the experiment. Males appeared to roam about the container until a lone female was encountered and courtship began. Therefore, many of the females in the large container were

courted by only a single male and many of the males courted only a single female, a situation that more closely resembles a no-choice experimental design.

Coyne et al. (2005) concluded that "space itself. . . appears to be an unimportant factor in sexual isolation," although in the same study, they found significantly higher estimates of sexual isolation between the sister species *D. yakuba* and *D. santomea* in multiple choice mating experiments compared to no-choice, male-choice, or female-choice trials. Hoikkala and Aspi (1993) provided similar evidence using a different experimental design. In the three *D. virilis* group species studied (D. *littoralis, D. montana,* and *D. ezoana),* discrimination between conspecific normal and wing-manipulated males by females increased when both males were present, as opposed to no-choice situations. Further, their discrimination was strongest when they were courted by both types of male during the trial rather than just one (Hoikkala &Aspi 1993).

Both rearing flies on fermenting cactus and providing them fermenting cactus tissue during the experiment also increased sexual isolation, demonstrating the sensitivity of mate choice to exposure to host plant tissue. Sexual isolation in the sympatric crosses was stronger than in the allopatric ones, but only when flies were reared on lab food; this difference was reduced to nonsignificant levels when fermenting cactus tissue was used either as larval rearing medium or during the mating trial.

Brazner (1983) first showed that in crosses between populations of *Drosophila mojavensis*, flies reared on either agria or organ pipe cactus tissue had a four-fold decrease in copulation latency when compared to flies reared on synthetic laboratory media. Also, substrate type has also been shown to affect the composition of cuticular hydrocarbons in *D. mojavensis* and *D. arizonae* (Stennett & Etges 1997), which act as pheromones and are known to mediate sexual isolation between populations (Etges & Ahrens 2001, Etges & Tripodi 2008, Etges et al. 2009). While we did not determine the mechanism underlying the changes in the level of sexual isolation due to cactus tissue, it is clear that more realistic estimates of sexual isolation were obtained when the native host plant tissue was used.

FIGURE 3 *Drosophila mojavensis* copulation on agria cactus rot in the Sonoran Desert, Punta Onah, Mexico. Photo credit: Jackson Jennings.

3.2 Reproductive isolation among allopatric *D. montana* populations

3.2.1 Premating isolation

In the second study, we first used no-choice, female-choice and multiple choice mating trials to establish whether sexual isolation could be found in crosses between flies representing the North American and European clades of *D. montana,* since reproductive isolation among these divergent populations had not been investigated previously. This study showed that there was no sexual isolation between populations (Oulanka and Vancouver) in the no-choice experimental design, but female- and multiple-choice trials did yield significant sexual isolation indices. This again illustrates the importance of experimental design, and specifically the opportunity for choice, when testing for possible sexual isolation between closely related taxa. Also, in the female-choice trials, females were choosier when they were courted by both males instead of just one of them, consistent with previous work in the *D. virilis* group (Hoikkala & Aspi 1993). In nature, *D. montana* flies aggregate on feeding sites, such as sap fluxes, where females are usually courted simultaneously by several males (Liimatainen & Hoikkala 1998, Hoikkala, pers. comm.). However, individuals may occasionally encounter problems finding mates when population densities are low, so females may exercise choice when they have a possibility to do so and accept less-favoured males when there are no "better" ones available (Hoikkala & Aspi 1993).

In the third study, all pairwise crosses between the three *D. montana* populations showed significant premating isolation at least in one direction in multiple-choice situations. The strength of sexual isolation did not differ among the cross types, however the frequencies of pair matings for different combinations of flies showed that this isolation was largely asymmetric. Females from Colorado and Oulanka accepted Vancouver males nearly as frequently as their own males (61 vs. 63 matings for Colorado females, 73 vs. 78 matings for Oulanka females) while Vancouver females showed clear preference for their own males versus males from Colorado or Oulanka (80 vs. 33 and 59 vs. 30 matings, respectively; see Table 1). This seems to suggest that either Vancouver females are more discriminatory or that Vancouver males are more attractive to heterotypic females than the males from their own population.

FIGURE 4 *Drosophila montana;* copulating pair. Photo credit: Anne Lehtovaara.

3.2.2 Postmating isolation

Females' egg production showed no significant variation among the different cross types in either study II or III. However, both studies showed differences in the number of progeny produced in crosses involving the Vancouver population. The lowest number of progeny was produced by Colorado females mated to Vancouver males. The reciprocal interpopulation cross (Vancouver females mated to Colorado males) showed a similar, but less drastic, reduction in progeny, producing significantly fewer progeny than pure Colorado crosses. There was no reduction in progeny production in crosses between the Colorado and Oulanka populations and we found no bias in offspring sex ratio in any experimental cross performed in these studies.

Since "hybrid" progeny production was most severely compromised in crosses between Colorado females and Vancouver males, we focused on the crosses within and between these populations to determine whether the breakdown in progeny number involves PMPZ or strictly postzygotic mechanisms. Dissections of singly mated females revealed that Vancouver males successfully transferred sperm to both Vancouver and Colorado females and that both types of females successfully stored sperm in both of their storage organs (spermathecae and seminal receptacle). Sperm were motile both one and three days after mating, suggesting that transfer, storage and motility of sperm were normal. This suggested that the mechanism responsible for the low progeny production occurs either at the sperm-egg level (PMPZ) or after fertilization (postzygotic). Mean egg hatchability was found to be significantly lower in crosses between Colorado females and Vancouver males than in any other cross type, even though the females successfully received and stored motile sperm. Egg hatchability followed the same trend as total progeny produced, indicating that the reduction in progeny was indeed due to fewer eggs hatching rather than larval or pupal developmental problems.

To further determine whether the decrease in egg hatch rate was due to developmental problems in the egg (i.e., early intrinsic genetic incompatibilities) or lack of fertilization by foreign sperm, we observed eggs laid by females in all cross types with flies from Colorado and Vancouver using fluorescent and compound light microscopy. The proportion of developing eggs in the cross between Colorado females and Vancouver males was lower than that of any other cross, again mirroring the trends seen in overall progeny production and egg hatchability. We then examined "non-developing" eggs for the presence or absence of sperm, and found that these eggs were mostly unfertilized in all crosses. This showed that the reduction in progeny observed in the C×V cross was due to the lack of fertilization and not the result of incompatibilities arising thereafter. Furthermore, we found no evidence of "hybrid" sterility or inviability in any test of postzygotic isolation.

Previous work on PMPZ isolation in *Drosophila* has mostly been carried out using demes that are already "good" species, instead of focusing on conspecific populations as we have done here. Between some members of the *D. melanogaster* and *D. simulans* species groups, reduction in sperm transfer, depletion of transferred sperm and/or inefficient sperm storage in heterospecific matings contribute to PMPZ isolation (Matute & Coyne 2010, Fuyama 1983, Price et al. 2001). In the *D. virilis* group, cases of PMPZ isolation between species have been found to involve incompatibility between the male ejaculate or sperm and the female reproductive tract, which results in the incapacitation, death or loss of sperm after heterospecific matings (Sweigart et al. 2010, Sagga and Civetta 2011, Ahmed-Braimah & McAllister 2012). Ahmed-Braimah and McAllister (2012) showed that in crosses between *D. americana* and *D. novamexicana*, two sister species in the *D. virilis* group found east and west of the Rocky Mountains, low progeny production in heterospecific crosses is due to a decrease in fertilization rate by heterospecific sperm. While *D. americana* sperm was successfully transferred to *D. novamexicana* females, this sperm was incapacitated or lost during storage, such that fewer eggs were fertilized. Sagga and Civetta (2011) found a similar phenomenon in crosses between *D. virilis* females and *D. novamexicana* males in which the sperm was successfully transferred, but depleted rapidly, leading to a low hatch rate of eggs. A similar phenomenon has also been found in crosses between the two subspecies *D. pseudoobscura pseudoobscura* and *D. p. bogotana,* where "consubspecific" sperm precedence occurs in both reciprocal crosses (Dixon et al. 2003).

3.2.3 Postzygotic isolation

We found no evidence for "hybrid" sterility and the number of F_2 progeny resulting from matings within and between Colorado × Vancouver F_1 "hybrids" showed no variation among the crosses. Backcrosses involving Vancouver males and "hybrid" F_1 females also showed no decrease in progeny production and there was no bias in offspring sex ratio in any of the crosses in this study. This suggests no evidence of downstream postzygotic incompatibilities and further supports the idea that premating and PMPZ isolation have both evolved

earlier on in divergence than postzygotic isolation. Indeed, PMPZ incompatibilities in this system may be the strongest barrier to gene flow between the intracontinental (Colorado and Vancouver) populations, although premating isolation has also evolved to some degree.

3.2.4 Relative contributions of pre- and postmating barriers

I used criteria proposed by Ramsey et al. (2003) to calculate the absolute and relative contributions of pre- and postmating isolation for each pairwise population comparison. These comparisons clearly showed that premating and postmating isolation are effectively independent from one another. In the cross where the measurement of premating isolation was highest (Colorado × Oulanka) postmating isolation played little to no role, while the lower level of premating isolation found in the Colorado × Vancouver cross was accompanied by substantial postmating isolation, in the form of a PMPZ barrier (see Fig. 7 in study III).

3.3 Cuticular hydrocarbon variation in *D. montana* and evidence for its role in mating behaviour

There were no qualitative differences in CHC profiles across populations or between the sexes, i.e. all hydrocarbon peaks used in the analysis were identifiable and present in each sample. The CHCs of *D. montana* consisted of 25, 27, 29 and 31 carbon *n*- and methylalkanes, alkenes and alkadienes with varying branch positions, as well as low amounts of alcohols with 27, 29 and 31 carbons. There were, however, significant quantitative differences in CHCs among the populations and to a lesser extent, between the sexes. There was also a significant population*sex interaction effect on CHC profiles, indicating that differences between males and females were not consistent across populations.

We first tested whether male mating success (i.e., winner/loser status) was influenced by any male CHC PCs in each study population. We found no significant effects in the Oulanka population, but both North American populations showed significant effects of particular PCs, indicating that these PCs were somehow involved in the mating success of males in the female-choice trials. Subsequent analyses failed to reveal significant effects of individual CHC peaks on mating success in the Colorado or Oulanka populations, however three individual peaks (representing 4-methyloctacosane, hentriacontanol and 4-methylhentriacontane) were significantly correlated with the mating success of Vancouver males. The data from analyses on PCs and individual peaks were in agreement and suggested stronger effects of CHCs on male mating success in Vancouver than in either the Colorado or the Oulanka population.

To test whether female hydrocarbons influenced male mating behaviour and thus, potentially female attractiveness, we looked for correlations between

courtship latency and female hydrocarbons for each population separately. While none of the PCs showed significant relationships with courtship latency in females from Colorado or Oulanka, PC2 showed a significant correlation between these traits in Vancouver females. Post-hoc analyses of individual peak data revealed that three peaks (representing 7-pentacosene, heptacosanol and Δ-nonacosadiene) were significantly correlated with courtship latency. Data from PCs and individual peak data were again in agreement and suggested that elevated amounts of these compounds correspond to shorter latencies to courtship in the Vancouver population, but not in Oulanka or Colorado.

Bartelt et al. (1986) analyzed the CHCs of 11 species in the *D. virilis* group, including *D. montana*, and showed that species clusters based on CHC profiles agreed with Throckmorton's (1982) previous phylogeny of the group. They found *D. montana* to exhibit the least sexual dimorphism of all the species studied and the authors concluded that sexual dimorphism was essentially lacking in this species. Our results show that sexual dimorphism is indeed significant, although not as strong as the differences among populations. Evidence for geographic variation in *D. montana* CHCs has also been found previously; Suvanto et al. (2000) traced divergence in the CHCs of five inbred *D. montana* isofemale lines from different parts of world and found significant geographical variation as well as strong sexual dimorphism some of the strains used. However, their study did not allow conclusions to be drawn concerning natural populations since the strains used had been inbred for 20 generations in the laboratory before being analyzed. The same study (Suvanto et al. 2000) also showed that male latency to copulation was influenced by the female, suggesting that some females were more attractive to particular males than others. The role of courtship song in mate choice in *D. montana* has been studied more extensively than that of CHCs (reviewed in Hoikkala et al. 2005). These studies have shown that male courtship song is important for both within-population mate choice and species recognition, and that both the song and female preferences for different traits may vary among populations (Routtu et al. 2007, Klappert et al. 2007). Klappert et al. (2007) showed that females from the Oulanka and Vancouver populations prefer high frequency male songs, while those from Colorado, where males actually sing at a higher frequency than males from the other two populations, females show a preference for low frequency songs. Within population sexual selection has likely driven male signal traits and female preferences along different evolutionary trajectories in the different populations, resulting in partial sexual isolation. Cuticular hydrocarbons (CHCs), which can act as pheromones and influence mate choice many *Drosophila* species (Ferveur 2005), may also play some role. Recently, Veltsos et al. (2011) studied both male courtship song and CHCs for their role in sexual selection in *D. montana* and concluded that song is a better indicator of male mating success than CHCs. Similar to study IV of this thesis, they found population differences in CHCs to be considerably greater than differences between the sexes. While variation in male and female CHC PCs was limited, it indicated strong linear selection towards opposite directions in Oulanka and

Vancouver populations, a form of selection most likely to contribute to population divergence and reproductive isolation (Veltsos et al. 2011).

One explanation for geographical variation in CHCs is sympatry with closely-related species, which can potentially drive the evolution of pheromone blends. A good example of this is *D. serrata*, which has different CHCs in the areas of sympatry with *D. birchii* than in allopatric populations (Higgie et al. 2000) as a result of reproductive character displacement. Environmental factors may also play role in CHC variation, as rates of water loss have been found to be correlated with some structural features of CHCs including chain length and the number and position of double bonds and of methyl groups (Ferveur 2005). *D. montana* flies from near the western USA-Canadian border have previously been termed "giant montana" as opposed to simply "montana" which has been used to describe more inland populations (Moorhead 1954). Flies from the Vancouver population have not only adapted to warmer climates than flies from the other focal populations, but also persist in the near absence of other *D. virilis* group species; *D. flavomontana* may be sympatric, but if so, is extremely rare (M. Ritchie, personal observation). In the Colorado and Oulanka populations, however, *D. montana* lekking and breeding sites are often visited by other closely related *D. virilis* group species (*D. borealis* and *D. flavomontana* in Colorado and *D. ezoana, D. littoralis* and historically *D. lummei* in Oulanka). In Oulanka *D. montana* females have been shown to experience frequent courtship from different types of heterospecific males (Liimatainen and Hoikkala 1998). Thus, sexual selection in Colorado or Oulanka may rely more heavily on traits that discriminate between con- and heterospecifics, while in Vancouver this selection may have been relaxed and shifted more towards traits that discriminate between conspecific males of varying fitness, as there is no pressure from closely related species.

Drosophila species show a great diversity of CHCs with regard to chain lengths and the position and number of double bonds and in the role of these compounds in mate choice. None of the three CHCs found to affect the mating success of Vancouver males (4-methyloctacosane, hentriacontanol and 4-methylhentriacontane) have been found to play a role in mating behavior or mate choice in any other *Drosophila* species, but 4-methyloctacosane has been shown to elicit precopulatory behaviours in longicorn beetles (Yasui 2009). The three CHCs that were found to be significantly correlated with male courtship latency in Vancouver population (7-pentacosene, heptacosanol and Δ-nonacosadiene) are more familiar in *Drosophila* literature. 7-pentacosene is structurally similar to 7-tricosene (the former contains two more carbon atoms than the latter), which has been shown to act as an aphrodisiac for *D. simulans* and *D. melanogaster* males (Jallon 1984) and to be the most efficient pheromone in preventing or reducing male homosexual courtship in *D. melanogaster* (Ferveur & Sureau 1996). Heptacosanol has been shown to elicit a chemoreceptive response in the antennae of the cabbage butterflies *Pieris rapae* and *Pieris brassicae* (Yildizhan et al. 2009) and nonacosadienes are known to play a role both in sex recognition (Antony et al. 1985) and courtship stimulation

(Ferveur & Sureau 1996) in *D. melanogaster*. More information on the role of these compounds in *Drosophila* and other species can be found in Study IV.

3.4 Population divergence in *D. montana*: historical events and adaptation to biotic and abiotic environmental factors

Drosophila montana was first found in the Rocky Mountains of North America in 1941 (Stone et al. 1942, Throckmorton 1982). It occurs there partly sympatrically with *D. flavomontana*, with which it has occasionally been found to hybridize in nature (Patterson 1952). The basic form of *D. montana* has a circumpolar distribution and it is found in the northern parts of North America as well as in Japan, northern Scandinavia and high altitude sites (up to 3,000 meters) in the Rocky Mountains (Throckmorton 1982). Another form of this species, 'giant *D. montana*', is found at low altitudes in the Pacific Coastal Northwest area of USA (Moorhead, 1954). Moorhead (1954) has suggested that on the basis of pupae and adult size and inversion polymorphism, giant *montana* could be classified as a distinct race or subspecies of *D. montana* (he found 14 inversions in giant *montana* which have not been detected in the basic *montana* form). The fact that the giant forms are able to exploit habitats at much lower altitudes (and relatively lower latitudes) than flies in other parts of the species range suggests that basic and giant *montana* populations occupy quite different niches at least in terms of the length of warm season and the harshness and duration of the winter period. Lakovaara and Hackman (1972) originally described Finnish *D. montana* as "*D. ovivororum*", but later studies have shown that there is no reason to give this population a species status (Routtu et al. 2007, Jennings et al. 2011). However, Morales-Hojas et al. (2007) detected 14 polymorphic inversions in Finnish *D. montana*, nine of which had not been described in North American populations, showing that this population also has evolved in its own direction.

D. montana is heterozygous for at least 40 chromosomal inversions (Stone et al. 1960, Morales-Hojas et al. 2007) and the species shows high variation in the number and location of fixed and polymorphic inversions between populations. Interestingly five of the 14 inversions unique to Finnish *D. montana* population are located on the 5th chromosome and only two are on the 4th chromosome (Morales-Hojas et al. 2007), while in giant *montana* seven of the 14 new rearrangements have occurred on the 4th chromosome and only one is found on the 5th (Moorhead 1954). The role of these inversion differences in adaptation to local environmental conditions and in generating reproductive isolation among *D. montana* populations deserves further attention.

A study by Mirol et al. (2007) on microsatellite and mtDNA variation in *D. montana* populations showed clear genetic differentiation between North American and Scandinavian populations, with estimated divergence time of 450,000 to 900,000 years. Two *D. montana* strains included in their study represented giant *montana* and these were found to differ from all other North

American populations. The Vancouver population used in the present study is located at the border of an area where giant *montana* has been suggested to be (Moorhead 1954), and it could be classified as giant *montana* on the basis of its geographic location or it may exchange genes with the more southern population on the Western Coast of USA. Interestingly, microsatellite data show no signs of admixture between Colorado and Vancouver populations, supporting their genetic distinctiveness (Mirol et al. 2007). Also, Routtu et al. (2007) studied the extent of variation in male courtship song as well as wing and genital morphology among Vancouver, Colorado and Oulanka populations and found the divergence to be especially high between the Colorado and Vancouver populations, implying the role of natural and/or sexual selection in their divergence. In the present study, these two populations showed the strongest reproductive isolation, which was mostly governed by a PMPZ barrier.

PMPZ isolation was strongest in crosses between the two populations from North America (Colorado and Vancouver), which show the lowest divergence in mtDNA (Mirol et al. 2007). This low divergence could be due to common ancestry, such that *D. montana* essentially invaded North America through Beringia only once, after which the populations split, subsequently adapting to different kinds of environments. Another possibility is that there have been two or more invasions, leading directly to the establishment of the giant and basic forms, but that there has been gene exchange between the two forms after their establishment.

At present, the arid lands extending from the Canadian border and eastern Washington southward through the center of Nevada towards the Rocky Mountains constitute an effective barrier to gene flow between the basic and giant *montana* populations, but gene flow in the past cannot be discounted. Undoubtedly, the glacial history of North America has had an effect on the population dynamics of *D. montana,* as the Cordilleran ice sheet has repeatedly advanced south into the western United States, and characteristically retreated over the past several hundred thousand years. This likely created glacial refugia that fluctuated in space and time, potentially resulting in periods of allopatry and secondary contact among populations as this species has adapted to the changing topography and climate (Hewitt 2001, 2004). Currently, it is possible that the giant *montana* may exchange genes or gene regions (inversions) with basic *montana* in Canada and northern Washington and Idaho via the Rocky Mountains, which connect their habitats through a high altitude corridor. Basic *montana* from these areas have been found share some inversions common in giant *montana* (Moorhead 1954), which may suggest a role of reinforcement of isolation mechanisms in keeping the two forms separate. Since these populations show both pre- and postmating isolation, reinforcement remains a possibility, although more collections from this area and further testing are needed.

4 CONCLUSIONS

The studies in this thesis have provided valuable information concerning the importance of experimental design in sexual isolation studies along with characterizing the strengths and mechanisms involved in barriers that could prevent gene flow between conspecific populations that appear to be in the early throes of speciation. The finding that divergence between *D. montana* populations has occurred to the extent that significant pre- and postmating reproductive barriers have evolved, and the fact that these barriers contribute differently to total reproductive isolation in the different population crosses provides a good opportunity to trace mode and tempo of the onset of these barriers and the mechanisms that govern them. Whether these populations are diverging by ecological or mutation-order processes, and whether the reproductive barriers reported here would be sufficient to prevent fusion in sympatry, remain to be explored.

Experimental design of mating trials can clearly influence the intensity of sexual isolation within and between species. Thus, failure to take into account ecologically relevant aspects of the natural mating environment (rearing substrates, chemical cues, etc.) in the laboratory may lead to inaccurate measurements of sexual isolation. Determining which factors affect sexual isolation between *D. mojavensis* and *D. arizonae* has yielded information about the possible mechanisms responsible for maintaining reproductive isolation between these species in nature. Attempts to create a more natural setting in the laboratory, in terms of both biotic and abiotic factors, may yield more realistic approximations of sexual isolation in natural populations. Along characterizing the frequency and nature of interspecific courtship and copulation in the wild (as in Liimatainen & Hoikkala 1998) and measuring the fitness of hybrid larvae in natural host plant tissues and hybrid adults when exposed to natural abiotic factors (as in Bono & Markow 2009), more laboratory studies should be carried out to better understand the effects of early sexual experience, temperature and rearing conditions (e.g. larval or adult crowding) on reproductive isolation between these and other sibling species.

Studies I and II support the idea that providing flies (particularly females) with more opportunity to compare multiple potential mates before choosing one can lead to stronger discrimination by females, and thus stronger sexual isolation between types. It is therefore necessary to consider what type of choice situation most closely resembles circumstances in nature when designing experiments to test for sexual isolation in the laboratory. In studies II and III we directly measured the barriers that could potentially play a role at the early stages of speciation, rather than those that may have evolved after the speciation event has occurred, by focusing on *D. montana* populations between which barriers to gene flow are incomplete. Our data show that in the early stages of speciation pre- and postmating isolation can evolve independently from one another and that they may arise in complete absence of any postzygotic isolation. Mate choice tests between the Colorado, Oulanka and Vancouver *D. montana* populations revealed significant premating isolation in all population crosses. The mechanisms of this barrier are likely based on the differential evolution of male mating signals and female preference in the different populations, as both of these traits show geographic variation (Klappert et al. 2007). Our investigation of CHCs in *D. montana* suggests that some of these compounds may be used in communication during courtship and mating, but our data is only correlational. Verification of such a role will require rub-off or perfuming experiments (as in Blows & Allen 1998 or Etges & Ahrens 2001) to show that CHCs actually cause changes in fly mate choice decisions.

Data from study IV showed that differences in CHCs among populations are stronger than the differences between sexes, although there is still significant quantitative sexual dimorphism in *D. montana*. We found no correlations between CHCs and any mating behavior in the Oulanka population, limited effects in the Colorado population and stronger effects in the Vancouver flies, suggesting that sexual selection on CHC properties may be more prominent in North American *D. montana*. Further, the CHCs involved in male mating success differed from those involved in female attractiveness. Thus, the role of CHCs in mating behaviour may not be uniform across populations and different compounds may be used by each sex in chemical communication.

Postmating barriers were strongest and had the greatest relative contribution to total reproductive isolation in crosses between the two *D. montana* populations with the least geographic distance between them (i.e., Colorado and Vancouver). Interestingly, postmating barriers between these populations proved to be caused by the lack of ability of sperm from Vancouver males to successfully penetrate and fertilize the eggs of Colorado females. While postmating isolation was studied in the other crosses only at a more general level, it appeared to be effectively independent of premating isolation in all three crosses. Future work on reproductive barriers between giant *montana* populations on the West Coast of North America and the Vancouver and Colorado populations would help to determine whether this putative speciation is ecological and whether the barriers have become stronger in areas of increased gene flow, consistent with secondary contact and potential

reinforcement. This work would be most fruitful if combined with adaptation studies and estimates of gene flow between populations using a large set of freshly collected population samples from North America.

The rapid evolution and divergence of seminal fluid proteins between closely related species is emerging as a common trend in animals including *Drosophila* (Swanson et al. 2001, Mueller et al. 2005). Singh and colleagues have shown that reproductive proteins are more divergent between closely related *Drosophila* species than non-reproductive proteins (Coulthart & Singh 1988, Civetta & Singh 1995, 1998) among species pairs that have diverged 1-16 million years ago. Given the topography and glacial history of the western U.S., it seems plausible that changing population dynamics and distributions may have contributed to the evolution of PMPZ between *D. montana* populations from the central Rocky Mountains and those from more coastal, Northwestern populations. We did not determine here if the PMPZ isolation between the Colorado and Vancouver populations is due to the failure of sperm to successfully reach unfertilized eggs or rather their inability to penetrate heterotypic eggs after contact is made, although the latter would suggest a mismatch in gamete chemistry (e.g., sperm/egg surface proteins). Further work should aim to identify the specific mechanisms responsible for this phenomenon in *D. montana* and to determine if similar systems, particularly species with recently diverged populations in which female remating occurs frequently and where sexual selection is known to play a role, exhibit incompatibility at the PMPZ level and how this isolation compares to other forms of intrinsic reproductive isolation. Proteomic studies using multiple, freshly collected populations of *D. montana* may help to elucidate the specific molecular mechanisms involved in the PMPZ isolation we have found here and would address this phenomenon within a single species that appears to be in the early throes of speciation.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Raja-aitojen kehittyminen: Lisääntymisisolaatio ja biologisen lajiutumisen ensimmäiset vaiheet

Eriytymisvaiheessa olevien lajien ja populaatioiden välisten lisääntymisesteiden evoluution tutkiminen auttaa ymmärtämään luonnon monimuotoisuuden, biodiversiteetin, syntyä ja säilymistä maapallolla. Uusien lajien kehittyminen on jo pitkään ollut yksi evoluutiotutkimuksen pääkohteista, mutta lajien ja populaatioiden risteytymistä estävien tekijöiden evoluutiosta, ja varsinkin sen ensimmäisistä askelista, on vielä verrattain vähän tietoa. Lajiutumisprosessien ymmärtämiseksi on tärkeää selvittää kuinka nopeasti ja missä järjestyksessä lajien ja populaatioiden väliset lisääntymisesteet syntyvät ja millaiset tekijät ylläpitävät niitä luonnossa. Tässä väitöskirjatyössä kehitettiin kolmen *Drosophila*-lajin, *D. arizonae*, *D. mojavensis* ja *D. montana,* pariutumiskumppanin valinnan ja siihen perustuvan seksuaalisen isolaation tutkimisessa käytettäviä menetelmiä mahdollisimman hyvin luonnossa tapahtuvaa valintaa vastaaviksi ja tutkittiin erilaisten isolaatiomekanismien voimakkuutta ympäri pohjoista pallonpuoliskoa levinneiden *D. montana* -populaatioiden välillä.

Väitöskirjatutkimuksen ensimmäisenä kohteena oli hiljattain eriytyneet sisarlajit, *D. arizonae* ja *D. mojavensis*. Nämä lajit elävät osittain samoilla alueilla Sonoran autiomaassa Meksikossa, missä molempien lajien kärpäset kerääntyvät parittelemaan ja munimaan samalle kaktuslajille. Kaktuksen mädäntyessä sen solukoissa alkaa kasvaa monenlaisia hiivoja ja bakteereita, joita kärpästoukat käyttävät ravinnokseen kaktuksen solukkonesteiden lisäksi. Ei tiedetä varmasti parittelevatko eri lajien kärpäset keskenään kaktuksilla, mutta lajien välisiä risteymiä ei ole koskaan tavattu luonnossa ja geneettisten tutkimusten perusteella on voitu päätellä, ettei lajien välillä ole geenivirtaa. Kyseisten lajien kärpäset kuitenkin pariutuvat ja tuottavat keskenään elinkykyisiä jälkeläisiä laboratorio-oloissa, vaikka risteymäkoiraat ovatkin usein steriilejä. Selvittääkseni mahdollisia syitä luonnossa ja laboratoriossa tapahtuvan risteytymisen välisille eroille mittasin lajien välisen seksuaalisen isolaation voimakkuutta laboratoriossa erilaisissa oloissa antaen sekä koiraille että naaraille mahdollisuuden valita pariutumiskumppaninsa. Testasin näillä kokeilla kärpästen tiheyden (pariutumiskammion koko), toukkien kasvatusalustan laadun (kaktuksen solukosta vs. banaanista tehty elatusalusta) ja mätänevän kaktuksen vaikutusta lajien väliseen isolaatioon. Kaikki edellä mainitut tekijät vaikuttivat lajien välisen seksuaalisen isolaation tasoon, mikä kertoo kärpästen parinvalinnan ja/tai lajintunnistuksen olevan hyvin herkkä käytettäville menetelmille. Lajin välinen seksuaalinen isolaatio oli korkeampi, kun kokeet tehtiin pienessä tilassa, kun kärpäsiä kasvatettiin kaktuksen solukosta tehdyllä elatusalustalla ja kun pariutumiskammioon laitettiin mätänevää kaktusta. Samalta alueelta peräisin olevien kärpäskantojen välisen seksuaalisen isolaation havaittiin olevan voimakkaampi kuin eri alueilta peräisin olevien kantojen, mikä tukee ko. lajeilla aiemmin tehtyjä havaintoja.

Työn seuraavassa vaiheessa tutkin erilaisten isolaatiomekanismien voimakkuutta *D. montana* -lajilla, jonka populaatioiden välisten lisääntymisestei-

den synty on vielä alkuvaiheessa. Risteytin näissä töissä pareittain Coloradon (USA), Oulangan (Suomi) ja Vancouverin (Kanada) populaatioista peräisin olevia kärpäsiä. Pariutumiskokeet osoittivat seksuaalisen isolaation olevan lähes yhtä voimakasta kaikkien populaatioiden kärpästen välillä, vaikka Vancouverin populaation naaraat olivatkin muiden populaatioiden naaraita valikoivampia ja parittelivat mieluiten oman populaationsa koiraiden kanssa. Joidenkin populaatioiden välille oli kehittynyt myös pariutumisen jälkeen, mutta ennen tsygoottien syntymistä toimivia lisääntymisesteitä. Coloradosta peräisin olevien naaraiden ja Vancouverista peräisin olevien koiraiden välisissä risteytyksissä naaraat vastaanottivat koirailta siittiöitä ja myös säilyttivät niitä elimistössään, mutta naaraiden munimien munien hedelmöitysaste oli huomattavan alhainen. Oulangan naaraiden ja Vancouverin koiraiden välisessä risteytyksessä löytyi sama ilmiö lievempänä, mutta Coloradon ja Oulangan kärpästen välisissä risteytyksissä sitä ei havaittu lainkaan. Tämä oli yllättävää, sillä Pohjois-Amerikan populaatiot ovat toisilleen läheisempää sukua kuin Suomen populaatiolle, josta ne ovat eronneet puolesta yhteen miljoonaa vuotta sitten. Risteytyksissä ei myöskään löytynyt viitteitä tsygootin syntymisen jälkeen toimivista isolaatiomekanismeista (hybridien heikentynyt elinkyky tai hedelmällisyys), vaikka koiraiden ja naaraiden hedelmällisyyteen vaikuttavien tekijöiden yhteisevoluutio eri populaatioissa voi johtaa lisääntymiseen liittyvien tekijöiden yhteensopimattomuuteen populaatioiden ollessa eristyksissä pitkiä aikoja. Ei tiedetä milloin Pohjois-Amerikan *D. montana* –lajin populaatiot ovat olleet viimeksi kosketuksissa toistensa kanssa, mutta mahdollinen kontakti on voinut vaikuttaa populaatioiden välisten, munien hedelmöitysvaiheessa toimivien, isolaatiomekanismien voimakkuuteen. *D. montanalla* tehty tutkimus osoittaa, että lisääntymisesteet voivat kehittyä eri teitä eri populaatioiden välille, ja että pariutumista edeltävät ja pariutumisen jälkeen, mutta ennen tsygootin muodostumista toimivat lisääntymisesteet ovat kehittyneet ko. lajin populaatioiden välille ennen tsygootin syntymisen jälkeen toimivia mekanismeja.

Väitöskirjani viimeisessä tutkimuksessa selvitin käyttävätkö *D. montana* kärpäset parinvalinnassaan hyödyksi kutikulan pinnassa olevia hiilivetyjä (CHC). Kaikki hiilivetymittaukset tehtiin yksittäisistä koiraista ja naaraista kaasukromatografin ja massaspektromerin avulla, mikä mahdollisti sukupuolten ja populaatioiden välisten erojen jäljittämisen. Aikaisemmat tutkimukset ovat osoittaneet, että tällä lajilla on kyseisissä hiilivedyissä jonkin verran muuntelua sekä sukupuolten että populaatioiden välillä, mutta hiilivetyjen laadun ja määrän vaikutusta kärpästen pariutumiskäyttäytymiseen ja parinvalintaan on tutkittu hyvin vähän ja tulokset ovat myös olleet osittain ristiriitaisia. Pariutumiskokeissa kolmen edellä mainitun *D. montana* populaation naaraiden annettiin valita parittelukumppaninsa kahden oman populaationsa koiraan joukosta. Voittaja- ja häviäjäkoiraiden kutikulan hiilivetyjen määrittäminen antoi mahdollisuuden selvittää korreloiko joku niistä koiraiden pariutumismenestyksen kanssa. Kokeissa mitattiin myös koiraiden kosinnan aloittamiseen kuluvan aika, jota voidaan pitää naaraiden ”puoleensa vetävyyden” mittana, ja tutkittiin sen korrelaatiota naaraiden kutikulan hiilivetyjen laadun ja määrän kanssa. Tulok-

set osoittivat, että populaatioiden väliset erot ovat sukupuolten välisiä eroja suurempia, ja että koiraiden ja naaraiden väliset erot eivät ole yhdenmukaisia populaatioiden välillä. Koiraiden pariutumismenestyksen havaittiin korreloivan yhden hiilivedyistä lasketun pääkomponentin kanssa molemmissa pohjoisamerikkalaisissa populaatioissa, Vancouverin populaatiossa myös kolmen yksittäisen hiilivedyn määrän kanssa. Naaraiden kohdalla yksi pääkomponenteista ja kolmen yksittäisen hiilivedyn määrä korreloivat koiraiden kosinnan aloituksen kanssa, mutta jälleen vain Vancouverin populaatiossa. Nämä tulokset viittaavat siihen, että kärpästen kutikulan hiilivedyillä voi olla suurempi merkitys kärpästen kosinnassa ja parittelukumppanin valinnassa pohjoisamerikkalaisissa populaatioissa (erityisesti Vancouverissa) kuin Suomen populaatiossa. Tulokset perustuvat kuitenkin vain korrelaatioihin ja vaativat tulevaisuudessa tuekseen erilaisia manipulaatioita, kuten hajustuskokeita.

Tämä väitöskirjatyö osoittaa kuinka tärkeää on valita sopivat menetelmät ja hyvät tutkimuskohteet lajien välisten isolaatiomekanismien tutkimiseen ja kuinka samankin lajin eri populaatioiden välille voi kehittyä erilaisia lisääntymisesteitä. Työ antaa hyvän pohjan jatkotutkimuksille, joilla voidaan tutkia seksuaalivalinnan vs. luonnonvalinnan ja neutraalin geneettisen eriytymisen vaikutusta lisääntymisisolaation syntyyn lajiutumisen ensimmäisten vaiheiden aikana sekä selvittää populaatioiden välisten isolaatiomekanismien geneettistä taustaa.

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ORIGINAL PAPERS

I

SPECIES HYBRIDS IN THE LABORATORY BUT NOT IN NATURE: A REANALYSIS OF PREMATING ISOLATION BETWEEN *DROSOPHILA ARIZONAE* AND *D. MOJAVENSIS*

by

Jennings, J.H. & Etges, W.J. 2010

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II

SEXUAL AND POSTMATING REPRODUCTIVE ISOLATION BETWEEN ALLOPATRIC DROSOPHILA MONTANA POPULATIONS SUGGEST SPECIATION POTENTIAL

by

Jennings, J.H., Mazzi, D., Ritchie, M.G. & Hoikkala, A. 2011

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III

THE RELATIVE STRENGTHS OF DIFFERENT REPRODUCTIVE BARRIERS DEPEND ON THE POPULATIONS CONSIDERED: LESSONS FROM A CIRCUMBOREAL DROSOPHILID

by

Jackson H. Jennings, Rhonda R. Snook & Anneli Hoikkala

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IV

VARIATION AND SEXUAL DIMORPHISM IN CUTICULAR HYDROCARBON PROFILES OF *DROSOPHILA MONTANA* AND POPULATION-SPECIFIC EVIDENCE FOR THEIR ROLE IN MATING BEHAVIOUR

by

Jackson H. Jennings, William J. Etges, Thomas Schmitt & Anneli Hoikkala

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