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COEVOLUTION OF MALE SIGNALS AND FEMALE PREFERENCES IN DROSOPHILA MONTANA AND D. VIRILIS
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**Abstract**

Divergence of behavioral traits (e.g. courtship rituals, habitat choice) has had a major impact on species formation and isolation. Species-specific courtship rituals preventing species hybridization may arise as a by-product of natural selection during spatial isolation or through direct action of natural selection to prevent species hybridization after a secondary contact. Coordination leading to the assumption of coevolution of signals and preferences is a prerequisite for effective courtship signaling between a male and a female of the same species.

We found a reasonable amount of variation in the mate traits within the *Drosophila montana* and *D. virilis* species, but our findings did not reveal evidence of the coevolution between the male courtship signals and female preference for these signals. Variation also did not cause isolation within species. The form of female preference for carrier frequency of male song was found to be stabilizing even in changing environments, when female preference for the carrier frequency and pulse length of the male song was measured at ambient temperature. *D. montana* females always preferred males with high frequency songs, which probably advertises the male condition. The frequency of male song decreased due to male aging, although males with larger body size were able to maintain frequencies better. Ageing also had a deterioration effect on male reproductive success. The importance of male courtship song was not only highlighted in mate choice, but also in species-recognition. We were able to persuade *D. montana* females to copulate with alien *D. lummei* species by playing them simulated song resembling the song of conspecific males. Simulated courtship songs were demonstrated to be a practical tool in studies of preference between sexually isolated *Drosophila* species.

**Keywords:** courtship, *Drosophila montana*, *Drosophila virilis*, mate choice, reproductive behavior, sexual selection
Saarikettu-Känsälä, Mari, Koiraan signaalien ja naaraan vasteiden yhteisevoluutio *Drosophila montana*lla ja *D. virilissellä*.

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**Tiivistelmä**

Kosintapiirteiden (esim. kosintarituaalit, habit aatin valinta) vaihtelevuudella on ollut suuri vaikutus lajien muodostumisessa ja eriytymissä. Lajien välisiä risteytymisiä estäviä lajispesifisiä kosintarituaaleja voi syntyä luonnonvalinnan sivutuotteena spaatialisen eristäytymisen aikana tai luonnonvalinnan suoralla vaikutuksella estämään lajiristeymät lajien uudelleen kohdatessa. Tehokkaan koiraan ja naaraan välisen kosintalautan edellytyksenä on koordinointi, mikä vuoksi on oletettava, että signaalien ja vasteiden välillä on yhteisevoluutiota.


**Asiasanat:** Drosophila montana, Drosophila virilis, kosinta, lisääntymiskäyttäytyminen, parinvalinta, sukupuolivalinta
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Oulu, October 2011

Mari Saarikettu-Känsälä
List of original papers

This thesis is based on the following papers, which are referred in the text by their Roman numerals:


The author’s contribution: Mari Saarikettu-Känsälä (MS) was responsible for the experimental works in II, III and V, and participated in the experimental work in IV. MS was also responsible for the experimental work of Experiment 2 in I. Statistical analyzes were performed by MS and Jaana O. Liimatainen in III and V, by MS, Jaana O. Liimatainen and Janne S. Kotiaho in IV, and by Michael G. Ritchie in I and II. MS was responsible for writing and interpretation of the results in III and V and participated in writing and interpretation in I, II and IV.
1 Introduction

1.1 Sexual selection by female choice

Charles Darwin introduced the theory of natural selection (1859), where he explained the continual development process of species. Some individuals will have more progeny than others or some individuals may not have any progeny at all. Heritable traits owned by individuals that reproduce more often will become more frequent in a population and traits owned by those reproducing little or not at all will become infrequent in a population. These more reproductive individuals have traits that give them advantages in survival. In this almost inclusive theory there was still an open question: Why do the males tend to invest in extreme male traits even though the traits seem to reduce their survival? Darwin’s solution was to distinguish two selection regimes, natural and sexual selection, from each other. Sexual selection is a special case of natural selection, which refers specifically to competition over mates and to mate choice and is a result of variance in mating success, whereas natural selection arises from variance in individual survival and fecundity (Andersson 1994; Lande 1981).

There are two forms of sexual selection: competition between individuals of the same sex (‘intrasexual selection’) and choice of the mating partner (‘intersexual selection’). This thesis is focused on the latter one, which is also known as mate choice or female/male choice. In many species the females are the ones in the position to choose their mating partner, while the males compete against each other to be one of those who will get a chance to reproduce. This is quite understandable taking into account the high investment of the females in their progeny production. Females usually have few large gametes compared to the quantity of gametes that males can spread. Also factors like the time and energy used to select the best possible mate (Milinski & Bakker 1992), increased exposure to predation during mate choice (Hedrick & Dill 1993) or toxic compounds in male seminal fluids (Chapman et al. 1995) are costly for females. Therefore they may choose their mating partner on the basis of advantages that they can get from their choice. These advantages may include direct benefits from a male to a female, like parental care (Qvarnström et al. 2000), territories (Alatalo et al. 1986) or nutrients (Wagner & Harper 2003), or indirect benefits in the form of better offspring fitness. In most fly species, the females only get sperm from their mating partner, so the benefits they get are likely to be of an indirect nature.
Females have three alternative strategies to find the best possible mate in the time and space they are given. They may prefer a male trait that exceeds some internally determinate and genetically fixed threshold value. With this strategy any male having a trait above the threshold value is a potentially acceptable mate for a female. More limited is absolute choice, where females require a male trait to exceed a threshold value, but also to not exceed it too far. So the female preference is within a narrow preference window. Both the threshold and absolute choice strategies may make the female unable to find a mating partner, if none of the available males fit within required trait values that females prefer. A third mate choice strategy, relative choice, is more flexible, and gives females the opportunity to choose the best mate from the available males ("best-of-n" rules, Jennions & Petrie 1997). Hoikkala & Aspi (1993) have shown that Drosophila montana females choose their mates on the basis of relative criteria if the signals emitted by the courting males are within the range of acceptable cues and the results of this thesis support this view (Paper I).

1.2 Coevolution of female and male mating behavior

Several theories have been developed to explain how females choose their mating partner on the basis of indirect benefits. One of the earliest theories is a runaway process originally introduced by Fisher (1930) and further framed by Lande (1981). It suggests that if some attractive male trait has a selective advantage due to arbitrary female preference, the proportion of individuals having that trait will increase. At the first phase the males have a diverged trait that may in some form slightly (but not significantly) improve their survival and the females possess genetic variation in their preference on this trait. Females mating with the males with the preferred trait will have male offspring with both the preference allele and the allele of the preferred trait. That will create a genetic correlation between female preference and the male trait (Bakker 1993; Wilkinson & Reillo 1994). Females indirectly benefit from better reproductive success of their attractive male offspring (Møller & Alatalo 1999). The process continues until the male trait becomes too costly for the bearer.

Indicator models of sexual selection, also referred to as good genes models, assume that the males are able to advertise their fitness to females through their secondary sexual traits. Zahavi’s (1975; Zahavi 1977) handicap principle predicts that a preferred male trait gives information on a male’s viability to a female, but the trait is costly for its owner and so only males in the best shape are able to bear
it. The cost is higher to males with lower fitness than to males with better fitness. Therefore the trait is an honest indicator of males’ genetic quality, because males with low fitness are not able to afford it (Andersson 1982; Møller 1989). As in the Fisherian model the females benefit indirectly through their offspring, because along the preferred handicap trait they indirectly inherit the better fitness genes. That idea has been developed further by Hamilton & Zuk (1982) in their parasite model. According to this model, the males’ secondary sexual trait exposes the male’s genetic resistance to parasites and other pathogens. Offspring of females choosing to mate with males with exaggerated ornaments also receive resistance genes (Milinski & Bakker 1990; Møller 1990). Also fluctuating asymmetry (FA), which estimates the effects of minor developmental failures (Van Valen 1962) and is a popular measure of developmental stability (Palmer & Strobeck 1986), has been found to be an indicator of the individual’s capacity to tolerate parasites (Møller 1996; Polak 1993). Individuals having minor deviations from perfect symmetry are less capable to buffer environmental and genomic stress during development.

If the good genes models are a reality in nature, this gives rise to another question. Females choosing mates without direct benefits should lead to directional selection on strongly preferred traits. That constant preference for particular male traits may eventually lead to loss of variance in these traits, which would finally prevent females from getting any benefit from their choice. This dilemma is called the lek paradox (Pomiankowski & Møller 1995; Rowe & Houle 1996). Solutions to the problem of maintaining variation in mate choice traits have included fluctuating selection, mutation-selection and most recently condition-dependence (Tomkins et al. 2004). According to the idea of fluctuating selection, the best phenotype is not fixed. It changes depending on environmental conditions. For example, parasites are constantly changing to resist their host’s attempts to defend against them. Mutation-selection thus argues that new mutations arise so often that it is enough to maintain the genetic variance of a trait. Currently most of the debate has focused on the idea of condition-dependence of a preferred trait and especially on the genic capture hypothesis. It states that costly male mate traits are condition dependent and show additive genetic variance. Male condition depends on genes at many loci and there are positive genetic correlations between sexually selected traits and condition (Birkhead et al. 2006). An example of this was showed by Kotiaho et al. (2001) in the mating behavior of the dung beetle (Onthophagus taurus). Condition-
dependent male courtship rate (male residual body mass) was found to have high
genetic variance and was genetically correlated with courtship rate.

Unlike the runaway and good genes processes, the sensory exploitation
hypothesis (also called sensory drive, sensory trap, receiver bias) (e.g. Ryan &
Rand 1990; 1995; Smith et al. 2004), suggests that male sexual traits do not
coevolve with female preferences. Female preference for the trait exists
beforehand and has evolved without the effect of sexual selection. When the male
trait appears the females start to favor the males with this trait. Originally a
sensory system of female preference may be evolved because of other important
factors like foraging or avoiding predators.

Females and males of the same species usually have different mate choice
strategies to achieve optimal fitness for themselves and/or for their offspring.
Therefore mating strategies of opposite sexes might compete against each other,
because enhanced fitness of one sex may reduce that of another sex. In sexual
conflict genetic interests of the male and female are diverged (Chapman et al.
2003). The chase-away hypothesis proposes that coevolution of male and female
mating traits are antagonistic rather than mutualistic and reproductive strategies of
opposite sexes manipulate and counteract each other (Pizzari & Snook 2003).

1.3 Reproductive isolation

According to the biological species concept (Mayr 1942) a species is a group of
populations which are capable to form viable and fertile offspring. New species
are born when isolation mechanisms prevent or limit gene exchange between
populations (Dobzhansky 1951). Breeding between the individuals of different
species is prevented by isolation barriers. The reproductive barriers may be either
prezygotic or postzogotic and they may arise by chance in allopatry or through
selection to prevent species hybridization in sympatry. Prezygotic barriers prevent
copulation between the individuals of different species/populations helping to
avoid the wastage of time and recourses to ‘bad’ hybrids. It may only require a
few loci in a genetic interaction among maternal and paternal alleles to prevent
fertilization of hybrid offspring (Sweigart 2010). These barriers may be caused,
e.g., by species differences in the timing of reproduction, by mechanical
incompatibility of sex organs and/or by divergence of courtship rituals. Ritchie et
al. (1999) have stated that species-specific mating signals may not make a
significant contribution to sexual isolation unless differences between species are
matched by narrowly tuned differences in female preferences. Postzygotic
barriers decrease the hybrid viability or cause sterility in hybrid offspring, either in the first or later generations.

Reinforcement is the evolution of mating signals and preferences in order to increase reproductive isolation as a result of selection against hybrids (Servedio & Noor 2003). Its role in speciation is still under debate, but recent studies have highlighted the validity of reinforcement (Hoskin & Higie 2010; Matute & Coyne 2010). According to Howard (1993) it can give rise to reproductive character displacement (RCD) and greater divergence of mating traits. Butlin’s (1987) definition is more limited and expects fertile hybrids to actually be formed. In this view reproductive character displacement can increase difference of mate traits when there is no gene flow between sympatric populations of closely related species. If there is even minor gene flow, differentiation of mate traits can still develop as a result of selection against less fit hybrids (i.e. reinforcement; Butlin 1987).

Reproductive isolation does not have to be a product of selection. It may as well develop as a by-product of random changes in genetic variance, though genetic drift alone is rarely enough for speciation to happen (Sobel et al. 2010), because of gene flow. Theoretical approaches have suggested drift and sexual selection to act in concert in the speciation process (Uyeda et al. 2009; Tazzyman & Iwasa 2010). During colonization of a new habitat founder effect speciation is expected, because a population is usually established only by a few individuals. There could even be repeatable bottlenecks called ‘flush and crash’ cycles. Genetic drift is stronger in small populations making it presumably to be an important process in founder effect speciation (Carson 1975). In his genetic transilience model Templeton (1980) has suggested that a genetically variable founder population can respond to drift and the altered selective forces by a rapid shift to a new adaptive peak.

1.4 Courtship behavior of flies

The male fly intuitively knows how to court the female even when it has been isolated from egg to adulthood (Greenspan & Ferveur 2000), whereas for example birds learn to sing through experience (see e.g. Leonardo & Konishi 1999, Soha & Marler 2000). The male and the female flies perform several courtship elements in a certain sequence to ensue copulation (Welbergen et al. 1987, Liimatainen et al. 1992). During different stages of the courtship the flies also send each other acoustic, pheromone, touch and/or visual stimuli (Spiess 1987).
Acoustic signals are an important part of courtship in many species (e.g. birds, frogs and many insects). For example, male mice have been found to emit ultrasonic sound, inaudible to humans, when they smell female urine (Holy & Guo 2005). In most Drosophila species courtship includes song produced by the male vibrating his wings. The male fly vibrates one of his wings up and down (Spieth 1974) and the female senses movement of air particles through their antennal hearing organs (Göpfert & Robert 2001). In many species the male song is not necessary for the flies to copulate, but hearing the song lowers the amount of courtship required by the female (Ewing, 1964). For example D. melanogaster, D. simulans and D. sechellia females mate most quickly when stimulated by species-specific song, but heterospecific song still improves mating speed more than silence (Ritchie et al. 1999; Tomaru and Oguma 2000). There are also a few species, where the females are more demanding in their courtship requirements: D. montana (Hoikkala 1988, Liimatainen et al. 1992), D. ezoana (Hoikkala 1988) and D. busckii (Bixler et al. 1992) females do not copulate at all without hearing the courtship song.

Blows & Allan (1998) demonstrated that species-specific hydrocarbons may be used in mate choice. For a few species like D. clavisetaella the pheromones are very important. The males of this species spray odors straight over the female during the courtship (Spieth 1982). D. serrata females prefer extreme male cuticular hydrocarbon blends and this female preference is positively and genetically correlated with their offspring fitness (Hine et al. 2002). Also D. virilis group species have male-specific hydrocarbons, which in some cases may act like pheromones (Bartelt et al. 1986; Liimatainen & Jallon 2007). Most of the studies investigating genetic bases of pheromones acting in mate choice have focused on the D. melanogaster group (Greenspan & Ferveur 2000).

In some species groups, like the D. nasuta group (Lambert 1982) Hawaiian picture-winged Drosophila (Spieth 1982), visual signals play an important role in courtship. D. nasuta males exhibit a species-specific behavior of circling to the front of the female they are courting. This way they show their wing patterns so that the female can recognize the males of her own species (Lambert 1982). The flies of the D. virilis group species do not need light to mate (Hoikkala 1988) and so the visual signals are probably not very important factors in their courtship.
1.5 Study species: *Drosophila montana* and *D. virilis*

The *Drosophila virilis* group (Diptera, Drosophilidae) is one of the major species groups in the subgenus *Drosophila*. It consists of twelve holarctic species (Throckmorton 1982, Spicer 1992) and has been further divided into the montana and virilis subgroups; the divergence time between subgroups being about 10 mya (Spicer & Bell 2002). The courtship songs of sympatric *D. virilis* group species clearly differ from each other (Hoikkala et al. 1982) and so the females can use them in species-recognition. *D. virilis* females also have an easily observable acceptance signal for the male courtship song. They lift their wings when ready to copulate (Vuoristo et al. 1996).

*Drosophila montana* (montana subgroup) is distributed around the northern hemisphere, having known populations in North America, Europe and Asia. The origin of the species is probably from east Asia (Throckmorton 1982), but European *D. montana* populations are known to be separated from the North American ones (Mirol et al. 2007), differing from each other, e.g., in male courtship song and genitalia structure and fly wing size and shape (Routtu et al. 2007) The first signs of pre- and postzygotic isolation have been detected among these populations (Jennings et al. 2011).

In Finland, *D. montana* flies occur partly sympatrically with *D. lummei* (virilis subgroup), *D. littoralis* and *D. ezoana*. The flies have a short mating period in spring, during which they gather on food patches of rotting plant material where courtship and mating take place (Aspi et al. 1993). Even though there are some differences in timing of the mating period of the species (Aspi et al. 1993), interspecific courtships are quite common in nature (Liimatainen and Hoikkala, 1998). *D. montana* females exercise strong selection towards courting males, accepting the male as their mating partner only if he produces a species-specific courtship song. When courted by two conspecific males, the females usually choose the male singing with a higher carrier frequency. The strong song preferences of the females allow the study of variation in male songs and female preferences within and between populations and to trace factors affecting possible coevolution of these traits.

*Drosophila virilis* (virilis subgroup) has spread world-wide with humans and as *D. montana*, it has its ancestral population in Asia (Throckmorton 1982). It is a convenient model species, because the whole genome of the species has been sequenced (*Drosophila* 12 Genome Consortium 2007). This species has a high rate of reproduction and the females of this species have a very low mate
acceptance threshold. They often accept a conspecific male as their mating partner even without hearing the male’s courtship song (Hoikkala 1988, Isoherranen et al. 1999). The females of this species have also been found to mate with non-conspecific males in laboratory conditions (Stalker 1942). The males are more selective and usually quit courting if they touch a female of an alien species (Spieth 1951).

1.6 Goals of this study

The goal of this thesis was to determine how variation in the mating rituals and signals within the species affects female mate choice and whether the male signals and the female preferences for these signals have evolved in connection with each other. In the first and third paper the female preference functions have been studied using computer-simulated male songs. In paper I we characterized female preference functions for the carrier frequency and pulse length of male song in *D. montana*. We also tested the temperature coupling hypothesis by studying the effect of environmental temperature on the carrier frequency of male song and female preference for it. In paper II we estimated variation in female preference functions and male songs and the covariance between these traits in natural populations of *D. montana*. In paper III we demonstrated the importance of interpulse interval of male song in species recognition in *D. montana*. We also showed how sexual isolation (in this case between *D. montana* and *D. lummei*) can be broken by playing the females a simulated song of their own species. In paper IV we studied mate choice by observing how ageing affects male mating success and song quality in *D. montana*. In the last paper (paper V) we studied the variation of mating rituals and acoustic signals and the effects of variation in these traits on mate choice in *D. virilis*, a close relative to *D. montana*. 
2 Materials and methods

Detailed descriptions of the used materials and methods are presented in the original papers and only a brief summary is given here.

2.1 Flies

In studies described in papers I and III we used females from the D. montana strain 1251/20 (Oulanka, Finland, established 1984), inbred for 20 generations. In paper I the males were from the same strain, while in paper III we used D. lummei males from strain 1143 (Hokkaido, Japan, established 1974).

Data in paper II consisted of the F1 progenies of 27 D. montana females collected in summer 2000 in Oulu (Finland). In studies involved in paper IV we used overwintered D. montana males collected in spring 2001 in Oulanka (Finland), and females from D. montana multi-female strain mo1 (Kemi, Finland, established 1990).

In paper V we studied the behavior of the flies of five D. virilis laboratory strains: 1051.51 (Santiago, Chile 1956), 1431 (Leeds, UK 1981), B22 (Matsuyama, Ehime, Japan 1973), Hunan (Hunan, China) and B15 (Matsuyama, Ehime, Japan 1973).

All the flies, except the overwintered D. montana males (paper IV), were cultured in malt medium vials in continuous light in a culture room (19°C). The overwintered males were maintained in sugar-agar vials in continuous darkness in a cold room (4°C). For all behavioral experiments the males and the females were collected into separate vials at the age of one to two days and used in the experiments, when sexually mature (D. virilis and D. lummei flies at the age of 10–21 days and D. montana flies at the age of 18–28 days).

2.2 Morphological traits

Body and wing size of the overwintered males was measured in paper IV to determine whether the size of the male correlates with the male’s mating drive and offspring production. For body size we measured thorax length, face width and head width and for wing size we measured wing length, wing width and the distance between the inner and outer wing vein for both wings. All these measures were derived to one parameter, body size, by a principal component analysis.
2.3 Playback experiments

In playback experiments (papers I-III), the females were placed individually in small chambers with a mute (wingless) male. Males’ wings were removed with a scalpel under a light CO₂ anesthesia one day before the males were used in experiments. The chambers were laid above a loudspeaker (Boston Acoustics, Inc. MicroMedia PC Sound System) through which the simulated male courtship songs were played. The songs were produced using the SIGNAL Sound Analysis System (© Engineering Design). Female wing lifting, which is an acceptance signal in the *D. virilis* group (Vuoristo *et al.* 1996), was used in papers I and II, and the fly copulations in paper III, to measure the female preferences for the songs of different quality.

In paper I the female preference functions were analyzed both by model fitting and nonparametrically and in paper II by generalized linear model with a logit link function. The data sets (male song type, experimental group, day of playback and the set of females studied at the same time) in paper III were analyzed by logit analysis (Christensen 1990). The GLIM statistical package (Aitkin *et al.* 1990) was used to fit and determine the parameters of the logit models.

2.4 Song recording and analysis

Recording and analyzing the male courtship songs formed an important part in all five studies. A customized Insectavox microphone (Gorczyga and Hall 1987) and a Marantz (Eindhoven, The Netherlands) CP430 cassette recorder were used for song recording in paper I and a JVC condenser microphone and a Sony TC-FX33 cassette player in papers II-V. The recorded songs were analyzed with the SIGNAL Sound Analysis System (© Engineering Design).

In paper I covariance between mean song carrier frequency and environmental temperature was observed using a generalized linear model. In paper II the male courtship song traits were normally distributed and therefore analyzed using a one-way ANOVA, while in paper V some song traits showed deviation from normal distribution and were accordingly tested using a Kruskal-Wallis analysis of variance followed by multiple comparisons after Dunn (Zar 1984). In paper IV we used a repeated measures ANOVA to analyze the change in male song characters during male ageing. To study whether the age of the flies could have an effect on the relationship between male body size and song quality,
we extracted Pearson’s correlation coefficients between male body size and each of the song traits separately for each age category and then correlated the extracted correlation coefficients with the age category of the flies. Finally we used a meta-analysis (Rosenthal 1991, Cooper and Hedges 1994, Kotiaho and Tomkins 2002) to study whether there was an overall positive trend between male body size - song parameter correlation coefficient and the age category of the fly.

2.5 Mating studies

In paper IV we studied how many copulations the males were able to achieve during the two-hour testing period and whether the male mating success changed during aging. We also counted the number of progeny produced by the females after these copulations. The effect of age on male mating success was studied by repeated measures ANOVA. In order to partial out the effect of male age and the ordinal of the copulation on the number of offspring per mating, we ran a mixed model nested analysis of covariance. We also analyzed the effect of male identity on the number of offspring separately for each age category. From each of these analyses of variance we extracted the probability level and regressed these levels on the age category of the flies.

In paper V we observed courtships of *D. virilis* males and females belonging to the same or different fly strain. The no-choice tests were performed to find out, if variation in the courtship behavior of the studied strains caused sexual isolation between them. We first analyzed the effects of the identity of the males and the females and the number of pairs that began to court using a logit analysis (Christensen 1990) with the GLIM statistical package (Aitkin et al. 1990). As these analyses did not provide an explanation for observed variation in fly mating, we continued the analysis by comparing the number of copulations achieved by the males of different strains within each female strain with a chi-square test. The adjusted residuals (Everitt 1977) were used to find strain combinations causing the significant chi-square values.

2.6 Courtship interactions

We videotaped single-pair courtships of *D. virilis* flies belonging to the same fly strain (paper V) and recorded the courtship of each pair with a Sony DCR-TRV510E video camera until the flies copulated. The courtships were registered and analyzed using the ETHOSOFT program (see e.g. Welbergen et al. 1987;
Liimatainen & Hoikkala 1998). From the resulting transition frequency matrices we drew pictograms showing the progress of courtship. The duration and/or the percentages of the active courtship time that males used for different behavior elements were measured and compared by using a Kruskal-Wallis analysis of variance followed by multiple comparisons after Dunn (Zar 1984).

We also compared data (courtships of five D. virilis phylad species) from an earlier study (Liimatainen 1993) with our new data set. To enable comparisons within and between the strains/species we compressed each courtship sequence into one variable that provides information about the overall variation of courtship interaction. Here we used Shannon’s second-order entropy, based on information theory (Shannon 1948), where conditional probabilities are structured as the stream of events (McCowan et al. 1999). In this case the events were behavioral elements, which follow each other with different probabilities. The variance in entropy indices was studied using Levene’s test of homogeneity of variances.
3 Results and discussion

3.1 The shape of female preference functions and the preference for the high carrier frequency courtship song

Ritchie et al. (1998) have previously found that D. montana females are attracted by a high-frequency male song with a short pulse length. Papers I and II describe the shape of the independent preference functions for these two song traits in D. montana. In paper I the females were found to strongly prefer intermediate/high carrier frequency and a short pulse length. The shape of the female preference function for the carrier frequency was quadratic and stabilizing while for the pulse length it was linear (Paper I, Figure 1). The direct favoritism toward short pulses was unexpected, because pulse length and offspring quality do not correlate (Hoikkala et al. 1998) and short pulses are not more attractive in D. melanogaster (Bennet-Clark & Ewing 1969). D. montana males also have longer pulses than many other Drosophila species (Hoikkala et al. 1982; Suvanto et al. 1999). Because the interpulse interval was held constant in our synthetic songs, the female preference for a short pulse length also means that females favor song stimuli with the lowest duty cycle, which is rather unusual for acoustic insects (Hedrick 1986; Jang & Greenfield 1996; Shaw & Herlihy 2000) and animal communication in general (Bradbury & Vehrencamp 1998).

The highest peak in the female preference functions for the carrier frequency was around 250–350 Hz depending on the temperature during the experiment (Paper I). The normal range of the male song variation in this trait is usually below 350 Hz like found among the F1 progeny of the females from a natural population in Paper II. Therefore, despite the quadratic component of female preference for male song frequency, the female preference functions are in practice linear and directional toward higher frequencies (Papers I and II). Sexual selection will then favor males with high carrier frequency, but some females are choosier than others (Paper II).

Offspring of the D. montana males with a high-frequency song have been shown to enjoy increased larval survival (Hoikkala et al. 1998). It seems likely that a high-frequency song is more costly to produce and may therefore be a reliable indicator trait (Andersson 1994). The overwintered D. montana flies have a short mating period in spring (Aspi et al. 1993) probably after the stressful winter dormancy. Repeatability analysis indicated that the trait is highly
repeatable only among overwintered males with a high variation in male condition (Hoikkala & Isoherranen 1997). In Paper IV, sexually selected trait pulse length and song frequency (Aspi & Hoikkala 1995; Ritchie et al. 1998) expectedly became a less preferred state due to males ageing, while non-sexually selected traits did not show linear changes. The produced songs of 32 males surviving to the last age category revealed a decrease of frequency of courtship song. (Table II and Figure 4), but large males were able to maintain high song frequency better than small males (Table 3). This implies the condition-dependence nature on song frequency, because traits under intense directional selection should be more sensitive to changes in condition than the ones not selected (Cotton et al. 2004; Tomkins et al. 2004).

3.2 Covariance and variance of mating traits

In paper I we studied the effects of environmental conditions on the carrier frequency of male song and the female song preference in D. montana species. The male songs and the female preferences were studied at three different room temperatures (at 15°C, 20°C and 25°C) and compared with each other to determine possible correspondence between them. Temperature coupling is described as maintaining coordination between signals and preferences among a range sexes of Species (Walker 1957; Carlson et al. 1976; Gerhardt 1978; Doherty 1985; Pires & Hoy 1992), although in some species the covariance is relatively low (Skovmand & Pederson 1983; Shimizu & Barth 1996). The carrier frequency strongly covaried with the ambient temperature ($F_{1.15} = 128.2$, $P < 0.001$), but the peaks of the female preference functions did not match with those of the male song traits at the same temperature (Paper I, Figure 3). The variance in male songs and female preferences among the F1 progeny of the females collected from a natural population of D. montana were analyzed in Paper II. All song traits showed considerable variation among families (Paper II, Table I and II). The variation was greatest in the carrier frequency of male song (65% of the variance was among families), but no covariance was found between the female preferences and the carrier frequency of male song (Pearson correlation: $r = 0.148$, $P = 0.47$). Average variation among families of the other traits was 39%. A likely explanation for the higher variance in song frequency is its condition dependence, because it correlates with overwinter survival and offspring quality (Hoikkala et al. 1998; Hoikkala & Isoherranen 1999). Genic capture is assumed to happen for these kinds of traits, because mutational input to
heritability will be greater for a trait influenced by condition genes (Rowe & Houle 1996; Kotiaho et al. 2001).

There was no evidence of coevolution between the studied male song traits and the female preference for them in *D. montana* (Papers I, Table 2 and Paper II, Figure 2). The lack of correlation between these traits has later been confirmed also in a study by Klappert et al. (2007) on male songs and female preferences in allopatric *D. montana* populations. Still other studies have demonstrated strong genetic correlation between song and preference on different species. Wiley and Shaw (2010) found that the coevolution of male and female components of sexual communication is facilitated by physical linkage between genes underlying signals and preferences in Hawaiian crickets. Also, covariance between the female preferences and male mating signals were found in the *Enchenopa binotata* species complex of treehoppers (Rodríguez 2006).

### 3.3 Mating interactions of *Drosophila virilis*

In paper V we demonstrated that courtship rituals may vary greatly within a species. In Papers I-IV our model species was *D. montana*, but here we observed courtships of *D. virilis*, which is a close relative to *D. montana*, but differs in that the courtship song is not as important to this species. *D. montana* females would not copulate with a male that is not able to sing the courtship song (Liimatainen et al. 1992), but *D. virilis* females do not have strong criteria for their choice of mating partners (Isoherranen et al. 1999). One of the five studied *D. virilis* strains (strain B15) was not able to sing an audible song at all even though the wing vibration was still part of their courtship rituals (Fig. 2) and in courtship of the strain Hunan, males spent more time licking the female than wing vibrating (Fig. 1). But there was also one strain (1431) where male wing vibration was a crucial part of the female acceptance. The differences in their courtship interactions reflected geographical origins of the strains. According to Sobel et al. (2010) ecological adaption is the major driver for reproductive isolation, but we did not detect sexual isolation despite the found variances. B22, Hunan and B15 were all Asian-originated strains and had similar courtship interactions. European-originated 1431 had a simple courtship in comparison, whereas South American-originated 1051.51 had more complicated one. We could not, however, rule out the possible effects of 20–44 years maintenance in a laboratory, even if natural mate choice systems stay reasonably intact in laboratory lines (Carson 2003).
Two of the studied *D. virilis* strains (strains 1051.51 and 1431) were clearly more successful in their courtships than the other three (strains B22, Hunan and B15); almost all courtships of strains 1051.51 (91%) and 1431 (88%) led to copulations while a corresponding rate for strains B22 (29%), Hunan (45%) and B15 (23%) was less than half of that. Males of strains 1051.51 and B15 were also the most successful and least successful ones, respectively, in the no-choice mating experiments between strains. The successful males of strain 1051.51 were very active and persistent in their courtship and produced a lot of courtship song. Weakness of the B15 males was probably due to their inability to produce audible courtship song.

Paterson (1980) suggested mate recognition systems to be stable within species, because it would help individuals to find mating partners more easily and therefore reproduce more effectively. Earlier studies in *Drosophila melanogaster* supported this view and documented the flies having very stable mating systems (Lambert & Harper 1985; Henderson & Lambert 1982), but later there have been studies indicating variation in sexual behavior (Welbergen *et al.* 1992; Capy *et al.* 2000; Colegrave *et al.* 2000). Paterson’s idea does not take into account the effect of hybrid progeny between sympatric species, which can be a waste of resources, if a hybrid is sterile or less viable. Populations may diverge in their mating rituals as a result of selection against unfit hybrid progeny (reinforcement; Butlin 1987).
Fig. 1. The courtship pictogram of strain Hunan.
Fig. 2. The courtship pictogram of strain B15.
3.4 External and internal effects on mate traits

In Paper I both the carrier frequency of male courtship song and female preference for it changed depending on the variant temperature where the measurements were carried out (Fig. 2 and Fig. 3). The temperature has earlier been found to affect the mating displays of many ectothermic animals. For example in field crickets (*Gryllus integer*) the temperature-based microhabitat choice affects the mating calls and the potential reproductive success of the males (Hedrick *et al.* 2002). Temperature-induced song variation may be largely irrelevant to mate choice, if the animals only court within a narrow temperature range under field conditions or if the females choose their mating partners using relative criteria (e.g. comparing the available males with each other). In *D. montana*, the females use relative criteria in their mate choice (Hoikkala & Aspi 1993), which enables them to find an acceptable mating partner even at a low temperature, where the frequency of the male song is quite low (Fig. 2).

Paper IV describes the influences of aging and body size on male reproductive success and song quality in field-collected *Drosophila montana* flies during their laboratory maintenance. Culturing the flies in sugar-agar vials in a cold room (4°C) enabled them to live beyond their natural breeding age. Mean life span of *D. montana* flies in laboratory conditions, being less than 45 days on corn meal medium (Durbin & Yoon 1987), can be lengthened by slowing metabolic rate in cold conditions and sugar-agar nutrition (Suvanto *et al.* 1999). The oldest fly in this study survived 300 days and reproductive success was studied five times at one-month intervals (age categories 1–5). In the first experiment (age category 1) the flies were still at their normal breeding age, but in the remaining four experiments exceeded that. Studies of reproductive success consisted of observing copulation rates and counting the number of progeny per copulation. Male courtship songs were recorded within three days after each mating test. The first experiment (age category 1) did not reveal a relationship between male mating success and longevity or body size. There was no correlation between number of copulations or the progeny produced from those copulations and male age or body size (Paper IV). This is not unexpected, because according the mutation accumulation theory, fitness components important in early life and those important in surviving to old age do not correlate (e.g. Hansen & Price 1995; Gavrilo & Gavriloava 2002; Patridge & Gems 2002). The copulation activity decreased linearly with male age, but progeny production started to decrease only after age category 2; the experiment showed a quadratic
decrease with male age and the trait was also affected by the ordinal of mating (Paper IV, Fig. 2). Mixed model nested analysis of covariance revealed the male age category and ordinal of mating having a negative effect on the number of progeny, but identity of male had no general effect on it (Paper IV, Table I). The decline in mating success and progeny reproduction could have been affected by a growing amount of deleterious mutations in older age groups and changes in male physical fitness. Differences in fecundity due to aging may be the result of damaging early life reproduction and/or weakening of sperm quality or its transfer ability (Jones et al. 2000; Prowse & Partridge 1997; Sgro & Partridge 1999). Mating can also reduce male immune function (McKean & Nunney 2001), so the activity in courtship and high mating rate is costly for males (e.g. Partridge & Farquhar 1981; Cordst & Partridge 1996; Kotiaho & Simmons 2003; Martin & Hosken 2004; Paukku & Kotiaho 2005).

The individual progeny production per mating for age categories was extracted to probability levels that were regressed on the age categories to study possible individual male variation compared to the declining amount of progeny production across male age categories. We found a quadratic relationship between the probability level and age category (Paper IV, Fig. 3) indicating that at younger ages there is no difference between individual males because every male is able to produce a number of progeny, but at older ages variation between males becomes significant. Results from a general model for male display in species with multiple reproductive bouts suggests that older males’ signaling is more reliable than that of the younger males (Proulx et al. 2002). At an early stage of their lifespan, high-quality males may not waste resources by advertising themselves to females, because they have a chance to reproduce in future. Closer to the end of their lifespan they will, however, start to invest more into signaling directed at females. On the other hand, Hansen & Price (1995) have suggested that for an overall greater lifetime reproductive success it may be more beneficial for the males to invest in survival to maturity and in traits increasing mating success than in longevity. Considering the short life span of flies, there is not time for them to delay investments in reproduction success.

3.5 Species recognition and reproductive isolation

The willingness of Drosophila montana females to mate depends on hearing a male courtship song with species-specific characters. Interspecific courtships in the wild break most frequently when the male begins to sing (Liimatainen &
Hoikkala 1998). Still studies of *D. montana* courtship have usually been focused on song characters that are important in discrimination within species. The role of the carrier frequency of male song in intraspecific mate choice of *D. montana* has been demonstrated in several studies (e.g. Ritchie *et al.* 1998; Klappert *et al.* 2007; paper I). In paper III we showed that while the song frequency is a discriminating factor within the species, the interpulse interval (IPI) plays a more important role in species recognition. We also demonstrated how *D. montana* females can be tricked into mating with a *D. lummei* male by playing the females a simulated song with species-specific characters, even though the females exercise strong sexual isolation against *D. lummei* males (Hoikkala, 1988).

*D. montana* females were played three kinds of computer-simulated songs in the presence of a mute (i.e. wingless) *D. lummei* male (Paper III: Figure 1 and Table I): two which had species-specific parameters of *D. montana* or *D. lummei* and one which had all other parameters as a species-specific *D. lummei* song, but the inter-pulse interval (IPI) was the same as in the *D. montana* song. None of the females copulated when hearing the *D. lummei* song, 11% of the females copulated when hearing the *D. lummei* song with *D. montana* IPI and 22% of the females copulated when hearing the *D. montana* song (Paper III, Figure 2). Logit analysis (Paper III, Table II) showed that results depended mostly on the song which was played and the experimental group to which the flies belonged. We had four experimental groups where the total amount of copulations varied, but all exhibited the same trend as the overall results (Paper III, Fig. 2).

The interpulse-interval of male song has been suggested to be an important species recognition signal in several *Drosophila* species (Ritchie *et al.* 1994). Results in Paper III show that it has a similar role in *D. montana* courtship. Many studies have shown evidence that male song is likely to create sexual isolation between *Drosophila* species (e.g. Tomaru *et al.* 1995; Ritchie *et al.* 1999), but in some species song evolution has been found to be more rapid than the evolution of sexual isolation (Gleason & Ritchie 1998). Signals important in species-recognition should not vary too much to retain species-specificity (Lambert & Henderson 1986), but directional sexual selection may enhance the evolution of species-recognition signals and strengthen sexual isolation, if the female preferences vary between species.

In paper V we demonstrated that within a species there can be great variation in courtship rituals. The mating experiments, however, did not reveal any favoritism towards the flies of their own strains, which suggests that there is no sexual isolation between these strains. Also, comparisons of the variation in
courtship interactions within *D. virilis* strains to variation between species (data from earlier studies of Liimatainen 1993) showed that variability among courtship behavior of *D. virilis* strains (Levene’s test statistic of homogeneity of variances: $F_{5,183} = 0.97, P = 0.437$) was less than that found at species level (Levene’s test statistic of homogeneity of variances: $F_{4,371} = 4.17, P = 0.003$).

Laboratory strains are not directly comparable to natural populations, but strains in the laboratory undergo selection for these conditions and are maintained in small population sizes. Therefore if a strain has been in culture for a long time, behavior may change through selection and/or drift. Variation of mating signals was likely to have evolved through adaptation to novel habitats or by random genetic drift rather than by direct sexual selection, because *D. virilis* females are not very selective in their choice of mating partners (Isoherranen *et al.* 1999). Through several founder events and bottlenecks, the genetic transilience (Templeton 1980) has shaped the behavioral trait of each population differently, and also occasional mutations may have affected or destroyed some characters of the courtships (Carson *et al.* 1982). Yet, stabilizing selection has maintained the species-specificity of traits.
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COEVOLUTION OF MALE SIGNALS AND FEMALE PREFERENCES IN *DROSOPHILA MONTANA* AND *D. VIRILIS*