

# The Role of Male Courtship Song in Species Recognition in *Drosophila montana*

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In many *Drosophila* species the male courtship song is an essential part of a successful courtship, playing a role in species-recognition and/or in sexual selection exercised by the females on conspecific males. In *Drosophila montana*, specific traits of the courtship song (the length and the carrier frequency of sound pulses) have earlier been shown to play an important role in intraspecific mate choice. Here we show that the male song (especially the interpulse interval) also plays a role in species-recognition, maintaining sexual isolation between sympatric species. We succeeded in breaking down sexual isolation between *D. montana* females and *D. lummei* males by playing the females simulated courtship song with a species-specific interpulse interval while they were courted by a mute (wingless) male. Involvement of different song traits in intra- and interspecific mate choice suggests that the song may be affected by both directional and stabilizing selection.

**KEY WORDS:** acoustic cues; mate choice; sexual isolation; song simulations.

## INTRODUCTION

During courtship, the males of most *Drosophila* species vibrate their wings, producing species-specific courtship song. This song may play a role in species recognition or in intraspecific mate choice, or in both. In many species, including *D. melanogaster*, 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 song typical to their own species, but heterospecific song still enhances mating speed over the silent control (Ritchie *et al.*, 1999; Tomaru and Oguma, 2000).

Ritchie *et al.* (1999) have stated that species-specific mating signals may not make a significant contribution to sexual isolation, if differences between species are not matched by narrowly tuned

differences in female preferences. In *D. melanogaster* complex and in *D. biauraria* the females have a rather broad preference window, the songs with a species-specific mean interpulse interval (IPI) being most stimulatory for the females (Tomaru *et al.*, 1995; Tomaru and Oguma, 2000). *D. melanogaster* exhibits smaller variation in song IPI than *D. simulans* (Kawanishi and Watanabe, 1980), and the females of this species are also more likely to discriminate against heterospecific song than the females of the latter species (Ritchie *et al.*, 1999). In *D. montana* (a species of *D. virilis* group), the female preferences are more narrowly tuned than in any of the species mentioned above, and the females do not accept the courting male before hearing his song (Liimatainen *et al.*, 1992). In intraspecific courtship the females of this species prefer males producing a song with short sound pulses and a high carrier frequency (e.g., Ritchie *et al.*, 1998) and they also have been found to get indirect benefits (better surviving progeny), when mating with these males (Hoikkala *et al.*, 1998). The song also seems to play an important role in

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species-recognition (Liimatainen and Hoikkala, 1998), and it is not possible to produce hybrids between *D. montana* females and *D. lummei* males in laboratory, largely because of high discrimination exercised by the females (Hoikkala, 1988).

In northern Scandinavia, *D. montana* occurs partly sympatrically with three other species of the *D. virilis* group. Three of the sympatric species, *D. montana*, *D. ezoana* and *D. littoralis*, belong to the montana subgroup and one, *D. lummei*, to the virilis subgroup, the estimated divergence time between the two subgroups being over 10 million years (Spicer and Bell, 2002). The flies of all four species 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 the species have some differences in the timing of their mating period (Aspi *et al.*, 1993), interspecific courtships are quite common in the wild (Liimatainen and Hoikkala, 1998). These courtships, however, break down, when the male begins to vibrate his wings producing courtship song (e.g., 75% of the courtships between *D. montana* females and *D. lummei* males are interrupted at this stage; Liimatainen and Hoikkala, 1998). The courtship songs of sympatric *D. virilis* group species differ clearly from each other (Hoikkala *et al.*, 1982).

Sexual isolation wherein potential mates meet but do not mate is a most efficient isolating mechanism as it prevents the wastage of gametes, food and space for developing hybrids (Ehrman, 1965). Cobb and Jallon (1990) have shown that males of the melanogaster subgroup species can be induced to court other flies, regardless of sex or species, if the hydrocarbons of the courted fly correspond to the main female cuticular hydrocarbons of the species of courting male. In addition to chemical cues, visual, auditory, tactile and olfactory cues may also play roles in species-recognition. Here we show that the male song, especially the interpulse interval (IPI) of the song, plays an important role in maintaining sexual isolation between *D. montana* females and *D. lummei* males, and that it is possible to break the isolation by playing to the females a simulated song of *D. montana* or a *D. lummei* song with *D. montana* IPI during interspecific courtship. The study also shows that the main characters of the song have to be species-specific before the female preference for short sound pulses (see e.g., Ritchie *et al.*, 1998) can be revealed.

## MATERIALS AND METHODS

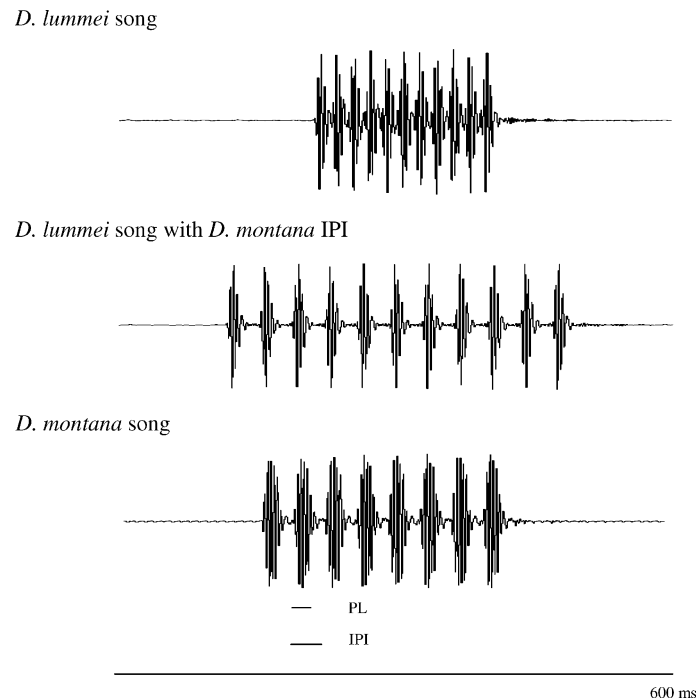
### Flies

*D. montana* females used in our song simulation experiments were from the strain 1251/20 (Oulanka, Finland, established 1984 and inbred for 20 generations) and *D. lummei* males from the strain 1143 (Hokkaido, Japan, established 1974). *D. montana* strain is the same strain that has been used in our earlier song simulation studies (e.g., Ritchie *et al.*, 1998), and the progenies of wild-caught females have been found to show similar preferences as did the females of this strain (Ritchie *et al.*, in press). The flies were kept in malt medium vials in continuous light in a culture room (19 °C). Males and females were collected in separate vials at the age of 2 days and used in experiments when they were sexually mature (*D. montana* females at the age of 21–28 days and *D. lummei* males at the age of 14–21 days). Wings of the males were removed with a scalpel under light CO<sub>2</sub> anesthesia one day before the males were used in playback experiments.

### Simulated songs

The songs of *D. montana* and *D. lummei* strains used in the present study were recorded (seven males per strain, three pulse trains per male) at 20 ± 1 °C. For song recording, a sexually mature virgin male and a female were transferred into a mating chamber (diameter 5.5 cm, height 1.3 cm) with a nylon net roof. Male song was recorded with a JVC condenser microphone placed above the chamber and a Sony TC-FX33 cassette recorder.

The songs were analyzed with the SIGNAL Sound Analysis System, and the simulated songs used in the playback experiments were constructed according to the parameters measured for these songs (Fig. 1, Table I), using the same program. The song analysis involved counting or measuring the following parameters from the oscillograms of the male song: the interpulse interval (IPI), i.e., the distance from the beginning of one pulse to the beginning of the next pulse, the length of the sound pulse (PL), the number of sound pulses in a pulse train (PN) and the intertrain interval (ITI), i.e., the distance from the beginning of one pulse train to the beginning of the next one. Carrier frequency (FRE) was set in all simulated songs at 300 Hz, which is a higher frequency than that measured from Fourier spectra of the songs of the studied strains (fulfills female criteria in intraspecific mate choice; Ritchie *et al.*, 1998). All



**Fig. 1.** Oscillograms of the simulated songs: the *D. lummei* song, the *D. lummei* song with the *D. montana* song IPI and *D. montana* song.

**Table I.** Parameters of the Simulated Songs (Means of the Song Characters of *D. montana* and *D. lummei* Strains Used in this Study are Given in Parenthesis)

|  | IPI (ms)  | PN        | PL (ms)   | ITI (ms)    | FRE (Hz)  |
|--|-----------|-----------|-----------|-------------|-----------|
| <i>D. lummei</i> song                            | 18 (18.0) | 11 (10.6) | 12 (13.4) | 1100 (1102) | 300 (297) |
| <i>D. lummei</i> song with <i>D. montana</i> IPI | 35        | 11        | 12        | 1100        | 300       |
| <i>D. montana</i> song                           | 35 (35.4) | 8 (8.3)   | 21 (20.6) | 1600 (1631) | 300 (237) |

See Materials and Methods for the explanation of the abbreviations.

songs were played at 105 dB, measured with a TECPEL DSL-330 Sound Level Meter at the level of the floor of the observation chambers.

### Female song preferences

In song simulation experiments *D. montana* females were placed individually in small chambers (diameter 25 mm, height 10 mm) with a mute (wingless) *D. lummei* male. These chambers were laid above a subwoofer (Boston Acoustics, Inc. Micro-Media PC Sound system), through which the simulated song was played. Copulations starting within the 10-minute observation period were counted and the copulating flies were immediately separated to prevent sperm transfer. Observations were made between 8:00 a.m. and noon at  $20 \pm 1$  °C.

Each experimental group consisted of 45 females observed for three successive days. The behavior of the flies was observed in sets of five pairs of flies at the same time, each pair in its own chamber. The three simulated songs were played for the females in a random order and all the females were tested three times during the three-day observation period (once per day and once with each song type). The procedure was repeated for the females of the four similar experimental groups during a 4-week period (altogether 180 females). The males were used in the trials only once.

### Statistical analysis

We analyzed the effects of the song type (*D. montana* song, *D. lummei* song with *D. montana* song

IPI, and *D. lummei* song), the experimental group (the females observed during the same three days), the day when the song was played (1st, 2nd or 3rd day) and the set where the females belonged (sets of five females) on the number of copulations, using logit analysis (Christensen, 1990). In this analysis the number of copulating females in the sets of five females was regarded as a response variable, and the song, the experimental group, the day and the set of five females as explanatory variables. The GLIM statistical package (Aitkin *et al.*, 1990) was used to fit and determine the parameters of the logit models. We tested each model [X] against the full model using a log-likelihood test, and we obtained the test statistic  $G^2(X)$  and degrees of freedom  $r$  for each model [X]. For the full model the degrees of freedom is  $q$ . Akaike's information criterion (AIC) was estimated for each logit model [X] to find the model with the highest information content, that is, the model that minimizes Akaike's information criterion AIC:

$$AIC = G^2(X) - [q - 2r].$$

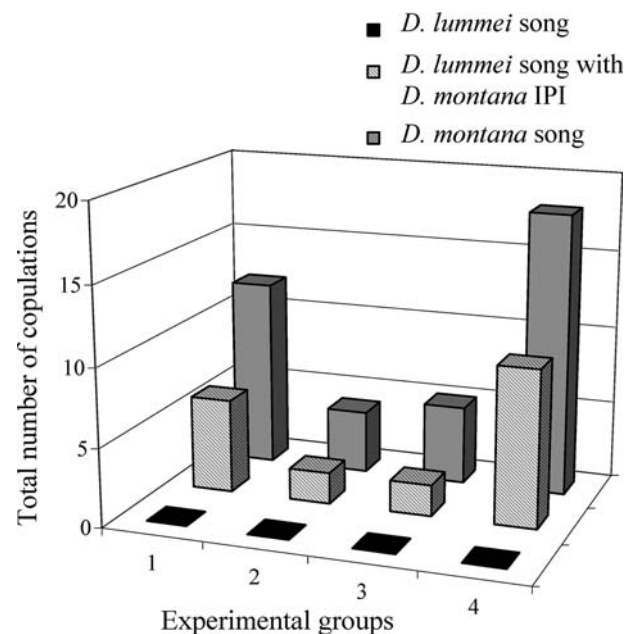
Because  $q$  (the degrees of freedom for the full model) does not depend on the model [X] tested against the full model, we used a modification of AIC:

$$AIC - q = G^2(X) - 2[q - r],$$

where  $[q-r]$  is the test degrees of freedom for the model [X] (Christensen, 1990). Minimizing  $AIC-q$  is equivalent to minimizing AIC. The best model to describe the data was obtained by comparisons of the models with the highest information contents (i.e., smallest  $AIC-q$ -values) beginning with the simplest ones. The test statistic is calculated as  $G^2(X_1) - G^2(X_2)$ , and the degrees of freedom is  $r(X_1) - r(X_2)$ , where  $[X_1]$  and  $[X_2]$  are the models compared.

## RESULTS

*D. montana* females were played three kinds of simulated songs while in the chamber with a mute (wingless) *D. lummei* male (see Fig. 1 and Table I). None of the females copulated when hearing the *D. lummei* song, 20/180 (11%) of the females copulated when hearing the *D. lummei* song with the *D. montana* song IPI, and 39/180 (22%) of the females copulated when hearing the *D. montana* song (see Fig. 2). The differences in the females' willingness to mate confirm our earlier suggestion that *D. montana* females need to hear a song with species-specific characters to mate (Liimatainen and Hoikkala, 1998),



**Fig. 2.** The total number of copulations during ten minute observation periods in playback experiments (three types of simulated songs, four experimental groups). The simulated song types are the *D. lummei* song, the *D. lummei* song with the *D. montana* IPI, and the *D. montana* song. The number of copulations in experiments with the *D. lummei* song type was always zero.

and they also show that IPI plays a major role in song specificity. The percentage of females responding to the *D. lummei* song with the *D. montana* IPI was only half of that responding to the *D. montana* song. This fact suggests that while a species-specific interpulse interval is a critical parameter for the females to recognize male song and to accept the courting male, other traits by which *D. montana* and *D. lummei* songs differ from each other (PL, PN, ITI) also play a role in song recognition.

Logit analysis revealed significant variation in the number of copulations among the sets of five females ( $G^2 = 138.4$ , d.f. = 107,  $0.01 < p < 0.05$ ). All tested models, except the one having only day as main factor, were adequate models to explain the data (i.e., did not differ statistically significantly from the full model, Table II). The models [S][E][D], [S][E], [S][D] and [S] had the highest information contents (i.e., smallest  $AIC-q$ -values). The model [S][E] was significantly better than [S] ( $G^2 = 26.8$ , d.f. = 3,  $p < 0.001$ ), but [S][D] was not better than [S] ( $G^2 = 4.5$ , d.f. = 2,  $p > 0.1$ ), and [S][E][D] was not better than [S][E] ( $G^2 = 4.88$ , d.f. = 2,  $p > 0.05$ ). None of the models including two factor interactions was

**Table II.** Logit Main Factor Models of the Factors Affecting the Number of Copulating *D. montana* Females

| Model        | $G^2(X)$ | df  | $p$               | $AIC-q$  |
|--------------|----------|-----|-------------------|----------|
| [S]          | 79.579   | 105 | NS                | -130.421 |
| [E]          | 113.85   | 104 | NS                | -94.15   |
| [D]          | 132.74   | 105 | $0.01 < p < 0.05$ | -77.26   |
| [F]          | 87.259   | 72  | NS                | -56.741  |
| [S][E]       | 52.779   | 102 | NS                | -151.221 |
| [S][D]       | 75.079   | 103 | NS                | -130.921 |
| [S][F]       | 24.202   | 70  | NS                | -115.798 |
| [E][D]       | 107.92   | 102 | NS                | -96.08   |
| [E][F]       | 87.259   | 72  | NS                | -56.741  |
| [D][F]       | 81.097   | 70  | NS                | -58.903  |
| [S][E][D]    | 47.895   | 100 | NS                | -152.105 |
| [S][E][F]    | 24.202   | 70  | NS                | -115.798 |
| [S][D][F]    | 17.113   | 68  | NS                | -118.887 |
| [E][D][F]    | 81.097   | 70  | NS                | -58.903  |
| [S][E][D][F] | 17.113   | 68  | NS                | -118.887 |

S: song type; E: experimental group; D: day of playback; F: set of the five females;  $AIC-q$  is the Akaike's information criterion and  $G^2(X)$  is the log-likelihood test value for model [X].

significantly better than the [S][E] model ( $p > 0.10$  in each case).

The fact that the activity of the females varied depending on the experimental group in which the females' behavior was observed (i.e., the calendar days), was not a surprise, as daily or weekly changes in the courtship activity of the flies have been detected in many studies on *Drosophila* mating behavior. What is new here is the finding that the strength of selection exercised by the females did not depend on the height of their acceptance threshold. The females of all experimental groups preferred the *D. montana* song, mated occasionally when hearing the *D. lummei* song with the *D. montana* IPI, and refused mating when hearing the *D. lummei* song.

## DISCUSSION

Our song simulation experiments show that the male song is an important species-recognition signal in *D. montana*. The females of this species can be tricked into mating with a male of a foreign species by playing them a simulated song with species-specific characters, even though the females exercise strong sexual isolation against nonconspecific males (Hoikkala, 1988). The fact that none of the *D. montana* females copulated when hearing the *D. lummei* song shows that specific song characters (especially the IPI) have to be species-specific before *D. montana* females will accept the courting male.

The songs of *D. montana* and *D. lummei* males differ clearly from each other. The *D. montana* song has long sound pulses (PLs) with clear pauses between the pulses (leading to long interpulse intervals, IPIs), while there are no pauses between the pulses in the *D. lummei* song (PLs and IPIs short and equal to each other). Göpfert and Robert (2002) have shown that in *D. melanogaster*, antennal hearing organs mediate the detection of the male courtship song, but it is not known how thoroughly the females can recognize different song traits. The present study gives indirect evidence that females can recognize species-specific IPI as well as some other traits differing between the species. However, even though *D. montana* females are known to prefer short PLs in courtship with conspecific males (e.g., Ritchie *et al.*, 1998), they did not show any preference for short sound pulses of the *D. lummei* song in the present study. The song clearly has to be species-specific before this kind of preference can be revealed, and the *D. lummei* song with the *D. montana* IPI did not fill that criteria.

Species differences in the male courtship song are an effective mechanism preventing interspecific matings in the wild in *D. virilis* group species. All four species of this group living sympatrically in northern Europe (*D. montana*, *D. lummei*, *D. littoralis* and *D. ezoana*) have species-specific songs with clear differences in IPI (Hoikkala *et al.*, 1982) and, as Liimatainen and Hoikkala (1998) have shown, interspecific courtships in the wild break most frequently when the male begins to sing. Species-specificity of the song does not, however, automatically lead to strong sexual isolation, and in some species song evolution has been found to be more rapid than the evolution of sexual isolation (Gleason and Ritchie, 1998). Sexual isolation may be largely affected by species differences in behavior and pheromones (Cobb and Jallon, 1990). The courtship rituals of *D. montana* and *D. lummei* are basically similar to each other (Liimatainen, 1993). The species differ in their cuticular hydrocarbons (Bartelt *et al.*, 1986), but this does not prevent *D. lummei* males from courting *D. montana* females, or *D. montana* females from mating with *D. lummei* males when hearing an acceptable song.

Previous song simulation experiments with *D. montana* (e.g., Ritchie *et al.*, 1998) have focused on female preferences within the species. In these studies females' willingness to mate has been studied by observing whether the females spread their wings as a sign of acceptance response (Vuoristo *et al.*, 1996) when hearing the song. In Ritchie *et al.*, (1998), the

song simulation experiments were done both for solitary females and for females placed in the observation chamber with a mute (wingless) male. Around 20% of the females responded to the songs in the absence of males and 40% in the presence of males. In the present study the females' willingness to mate was studied by observing whether the females copulated with a nonconspecific male when hearing an acceptable song. Female acceptance was, on average, lower than in the experiments with conspecific males, except in one experimental group where 40% of the females mated when hearing the *D. montana* song. Percentages of responding females in different experiments are not, however, strictly comparable because of the temporal changes in female receptivity.

Many studies (e.g., Tomaru *et al.*, 1995; Ritchie *et al.*, 1999) have shown that the male song is likely to influence sexual isolation between *Drosophila* species, but direct evidence on this is not easy to find. The IPI of the male song has been suggested to be an important species recognition signal in several *Drosophila* species, showing little geographic variation (Ritchie *et al.*, 1994, but see Colegrave *et al.*, 2000). The present study shows undisputedly that this song trait plays an important role in species recognition also in *D. montana*, while our earlier studies have shown the pulse characters of the song (PL, FRE) to be important in intraspecific sexual selection in this species (Ritchie *et al.*, 1998). The fact that the male song, or different traits of the song, plays a role both in species recognition and in sexual selection may restrict song evolution, as signals important in species-recognition should not vary too much to retain species-specificity (Lambert and Henderson, 1986). On the other hand, directional sexual selection may enhance the evolution of species-recognition signals and strengthen sexual isolation, if the female preferences vary between the species. Studies on the interaction and conflicts between species recognition and sexual selection in signal evolution is critical for understanding the rise of sexual isolation between diverging species.

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