



Variation and consistency of female preferences for simulated courtship songs in *Drosophila virilis*

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To study the variation and consistency of song preferences in *Drosophila virilis* females, we played them species-specific song and songs with modified sound pulses and/or interpulse intervals on 3 consecutive days. Species-specific song was played again on the fourth day. All playbacks were done without the presence of males. About 62% of the females indicated their readiness to mate by spreading their wings in at least one of the trials. The proportion of the females responding to species-specific song was about twice that of the females responding to modified songs. The majority of females responded to only one song type, which suggests that the females varied in their preferences and that their preference windows were rather narrow. The females were consistent in their responsiveness to species-specific song played on 2 days. If the female responded to normal song during the first trial, the probability of her responding to the same song during the second trial increased by about 32%. The number of songs required by the females before responding in the two subsequent trials was also correlated within the females (repeatability 0.328). Repeatability of female preferences for male sexual traits is expected both in the viability and Fisherian models of sexual selection.

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The courtship of *Drosophila* species consists of complicated signal-response chains between the courting flies. This enables the females to exercise choice and to indicate their refusal or readiness to mate at any courtship stage. According to Spieth (1974), *Drosophila* females can indicate their readiness to mate by spreading their vaginal plates, by spreading their wings, and/or by extruding their ovipositor. In *Drosophila virilis* group species, females spread their wings when ready to mate (Vuoristo et al. 1996).

Females may have specific preferences for the quality of signals produced by the courting male. The width of the preference window of a given female is determined by the probability that she will accept the courtship of a male with a particular trait value. The intensity of intersexual selection on a male character is mainly based on the width of the preference window of individual females and on the variation between the females in this character (Butlin 1993). In addition to genetic factors, her age, nutritional state and reproductive stage may also influence the female's receptivity.

Studies on female preferences in *Drosophila* have dealt mostly with differences between species (e.g. Bennet-Clark & Ewing 1969; Kyriacou & Hall 1982; Tomaru et al.

1995). It is also important, however, to study variation in female preferences within species (see e.g. Aspi & Hoikkala 1995; Boake & Poulsen 1997; Ritchie et al. 1998). Characterizing the phenotypic distribution of female mating preferences within populations, particularly in terms of how variable the preference windows are within and between females, is essential to understanding the evolution of these preferences. It is also important to know how consistent the female preferences are, that is whether females behave the same way on different days. Consistency of females' preferences for male signals is one of the primary assumptions of theories of sexual selection (e.g. Bradbury & Andersson 1987).

In the *D. virilis* species group the importance of the courtship songs varies between the species. In *D. montana*, females hardly ever accept the courtship of a 'mute' (wingless) male or that of a male producing abnormal song (Hoikkala 1988; Hoikkala & Aspi 1993; Liimatainen et al. 1993). In contrast, female *D. virilis* may accept the courtship of mute males (Hoikkala 1988). These females are, however, very sensitive to auditory signals and they have been observed to spread their wings as a sign of acceptance even when a male directs courtship song at another nearby female (Spieth 1951; Ewing 1983). This sensitivity makes them especially useful for song simulation studies.

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Table 1. Male song characters ($\bar{X} \pm \text{SD}$; $N=10$ males) of the *D. virilis* strain A used in playback experiments, together with the characters of the simulated songs

Song character	Strain A	Simulated songs		
		Normal	Mod 1	Mod 2
Pulse train length (ms)	218.1±22.4	218	218	218
Interpulse interval (ms)	16.7±3.5	17	52	52
Pulse length (ms)	15.5±3.2	16	16	48

We investigated how readily *D. virilis* females respond to simulated song if the song resembles the song of conspecific males, or if some characters of the song have been modified. We also studied whether females vary in their responsiveness to simulated songs and how consistent their preferences are.

METHODS

Flies and their Songs

We used flies from the wild-type laboratory stock A, obtained from the Moscow Institute of Developmental Biology in 1988. Flies were sexed under light carbon dioxide anaesthesia within 2 days of eclosion. Females were kept individually in vials containing *Drosophila* medium at 18°C, on a 16:8 h light:dark cycle. They were used in experiments when sexually mature, at the age of 10–18 days.

We analysed the courtship songs of the males of *D. virilis* strain A to adjust the song parameters of normal and modified songs used in our playback experiments (Table 1). We recorded songs of 10 sexually mature males of this strain when they were courting a female in a single pair courtship in a net-covered petri dish. Song recordings were made with a unidirectional JVC-150 electric microphone and Technics RS-BX404 cassette player at a temperature of $19 \pm 1^\circ\text{C}$. Songs were analysed from oscillograms made with a Gould 1425 oscilloscope and a Hitachi 672 Graph Plotter. For each male we analysed one randomly chosen pulse train by measuring the length of the pulse train and by counting the number of pulses in a train. We analysed the fourth pulse of these trains by counting the number of cycles in a pulse and by measuring the length of the pulse (PL) and the distance from the beginning of the pulse to the beginning of the next pulse (interpulse interval, IPI).

Song Simulator

Our objective was to determine whether *D. virilis* females respond selectively to simulated songs if some characters of these songs have been modified. Where female preference for a male secondary sexual trait has been experimentally demonstrated in other studies, manipulation of the male character has been quite extreme. For example, in Andersson's (1982) study, the tails of male widowbirds, *Euplectes progne*, were lengthened by about 5.4 SD. Female *D. virilis* seem to have a

very wide preference window with respect to male song, since they even accept the songs of two sibling species, *D. montana* and *D. littoralis* (Hoikkala 1988). The mean IPI of *D. montana* song is about 8.7 SD longer than that of *D. virilis* (estimated from data presented in Hoikkala et al. 1982), and the mean PL of *D. littoralis* is about 10.9 SD longer than that of *D. virilis* (estimated from data presented in Hoikkala et al. 1982). Accordingly, we decided to use modified male songs in which the song characters were modified by about 10 SD.

We played a normal simulated song and two kinds of modified simulated songs to each female. In normal song the IPIs and PLs were as long as those of average song in strain A. In modified song 1 (henceforth mod 1), the IPIs were about 10 SD longer than those of the average song in strain A and in modified song 2 (henceforth mod 2) both the PLs and the IPIs were about 10 SD longer than those in strain A (Table 1). The modified IPI and PL values are outside the range of *D. virilis*. The songs of 90 males belonging to six *D. virilis* strains originating from different localities varied from 15.3 to 25.3 ms in IPI and from 10.4 to 23.0 ms in PL (A. Hoikkala, unpublished data).

The simulator used to produce the simulated songs was based on a programmable sound generator connected to a personal computer (Aspi et al. 1992). The simulator was based on a fully programmable sound generator chip (Intel 6581), which contained three sound generators used in conjunction. Specifically designed software controlled the songs produced. With this software, we could control independently all the species-specific song characters of the *D. virilis* group species (see Aspi et al. 1992 for more details). The audio input of the sound generator chip was fed through the low-pass (1200 Hz) and high-pass (200 Hz) filters to a bridge-connected amplifier (designed around a stereo audio amplifier circuit TDA 2009) and finally to a loudspeaker.

The intensity of the simulated song was adjusted by two methods. First, we watched the flies during song simulation: they clearly became more active when the sound intensity increased over a certain level, whereas they stood in one place and began to tremble along with the song when the song intensity exceeded another level. More accurate calibration within this window was made with a particle velocity microphone. This kind of microphone should receive the male songs the same way as the particle velocity receptors in the female's antennae (Bennet-Clark 1971). The microphone was kept at the same distance from the loudspeaker as the females during the playback experiments. The amplitudes of the

oscillograms of these recordings were similar to those of the oscillograms of the male song recorded near the courting pair.

We measured the intensity of the simulated songs with a Bruel & Kjaer type 2203 microphone. The intensity of normal song was 128 dB at the level of the floor of the observation chamber and 124 dB at the level of the top of the chamber. The respective values for mod 1 song were 120 and 116 dB and for mod 2 song 124 and 120 dB. These values are very similar than those used by Tomaru et al. (1995) for *D. biauvaria*. They are considerably higher than those used in playback experiments with *D. melanogaster* (e.g. Crossley et al. 1995), but the songs of *D. virilis* males are much louder than those of *D. melanogaster* males.

Playback Experiment

To observe responses to simulated songs, we introduced females singly into a special chamber prepared from a petri dish (1.0 cm high and 3.4 cm in diameter). The floor of the chamber was made of a nylon net, and transparent thin plastic folded in the shape of a cone formed the roof of the chamber. The chamber was placed on top of a loudspeaker through which simulated courtship songs were played.

We used 204 females, half as controls. We observed the 102 females used in the song experiment in four sessions on 4 successive days, and the control females (102) only during the first three sessions. Females that heard simulated songs were divided into three groups, which differed from each other in the order in which the three song types were played. Groups A, B and C included 44, 29 and 29 females, respectively. This kind of grouping enabled us to test whether the females' responsiveness to courtship songs is influenced by their earlier experience (the order in which the songs were played) and/or by their 1–3-day age differences. Since the sexual activity of the flies may vary from day to day (A. Hoikkala, unpublished data), we also included the experimental day in our analysis.

After playing the females all the song types (normal, mod 1 and mod 2), we played them the normal song for a second time. Two of the females, which had heard the normal song in the first session, had died before they could be tested with this song for a second time. Consequently, we studied the repeatability of responses to normal song with 100 females.

The songs were played to the females between 0800 and 1200 hours. After the female was introduced into the chamber, we allowed her to settle down for 1 min. We then presented each female with at most 100 pulse trains of courtship song (henceforth referred to as songs) for 2 min. The consecutive number of simulated songs (i.e. pulse trains) after which the female clearly spread her wings (keeping them up for at least 2 s; Vuoristo et al. 1996) was recorded.

After each session, we introduced a control female into the chamber and observed her for 2 min without playing any songs. We observed her in the same way as that of the experimental female. Some authors have played white noise to control females; however, since this can halt all

movement (Crossley et al. 1995), we decided to keep our control females in silence.

Females appeared to respond to simulated courtship songs by spreading their wings only when standing or walking on the floor of the chamber. This is where they spent most of their time. In a few trials the female spent more than half of the observation time upside down on the roof of the chamber. In those trials the female's responses could not be measured reliably, and the trials were discarded. The final data set consisted of 102 (first session) and 100 (second session) trials with normal song, 99 trials with mod 1 song and 100 trials with mod 2 song.

Statistical Analysis

We analysed the effects of the experimental group (differing from each other in the order in which the songs were played to the females), the day when the experiment was done and the type of song played (normal, mod 1 and mod 2) on female responses by subjecting the female response data to a logit analysis (Christensen 1990). In this analysis the proportion of the females responding to the song was regarded as a response variable, and the experimental group (A, B and C), the experimental day and the type of song as explanatory variables. The GLIM statistical package (Aitkin et al. 1990) was used to fit and determine the parameters of the logit models. We also estimated Akaike's information criterion (AIC) for each logit model to select the model with the highest information content. For logit models this means choosing a model that minimizes Akaike's information criterion AIC:

$$AIC = G^2(X) - [q - 2r] \quad (1)$$

where $G^2(X)$ is the likelihood ratio test statistic for testing the X model against the saturated model, r is the number of degrees of freedom for the X model and q is the degrees of freedom for the saturated model. Because q does not depend on the model, we used a slight modification of AIC:

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

where $[q - r]$ is the test degrees of freedom (Christensen 1990). Minimizing $AIC - q$ is equivalent to minimizing AIC.

Repeatability of the number of songs required by the females before responding in the two trials with normal songs was estimated as a ratio of the between-individual variance to the total phenotypic variance (Falconer & Mackay 1996). The standard error for repeatability was estimated as a square root of the sampling variance of this intraclass correlation (Becker 1984).

Each female could respond (1) or not respond (0) to normal, mod 1 and mod 2 song. Categorization of the females on the basis of their responses to different song types resulted in eight response types ranging from 000 (in which category females responded to none of the song types) to 111 (in which females responded to all song types). For example, if a female did not respond to normal

Table 2. Logit main factor models of the factors influencing the responses of *D. virilis* females in playback experiments

Model	G^2	df	P	$AIC-q$
{S}{G}{D}	3.12	3	0.373	-2.88
{S}{D}	3.55	5	0.616	-6.45
{S}{G}	3.12	4	0.537	-4.88
{G}{D}	24.81	5	0.000	14.81
{S}	3.55	6	0.737	-8.45
{D}	25.21	7	0.001	11.21
{G}	25.65	6	0.000	13.65

G: the experimental group (A, B, C); D: the day when the experiment was performed; S: the type of song (normal, mod 1, mod 2) played during the experiment. $AIC-q$ is the Akaike's information criterion and G^2 is the log-likelihood test value

song, but responded to both modified songs, she would be categorized as type 011.

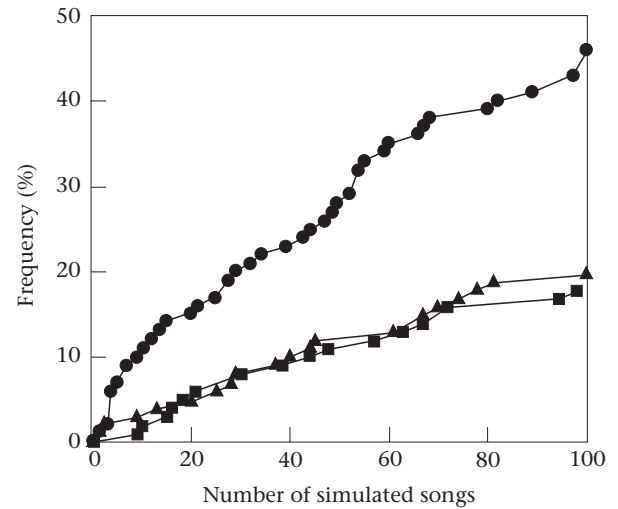
RESULTS

Responses to Normal and Modified Simulated Songs

Of the females that heard simulated songs, 61.6% spread their wings in at least one of the experiments. The results of the logit analysis (Table 2) suggest that only the type of playback song (normal, mod 1, mod 2) had an effect on female responses in the experiments. The model including only song type ({S}) as a main effect was the simplest model that fitted the female response data adequately (no significant deviation, $P=0.737$), and it also had the largest information content (i.e. smallest $AIC-q$). Adding the main effects of day or group into this model did not significantly improve the model. None of the models including two-factor interactions was significantly better than this model ($P>0.10$ in each case).

Since there were no differences between the females of different groups or between the days when the songs were played, the data for different groups and days were combined. The relationship between the number of simulated songs played to the females and the number of females responding to songs appeared to be linear for each song type (Fig. 1). Linear regression explained 96.4% of the total variation in the number of females responding to normal song. The respective percentages for the females responding to mod 1 and mod 2 songs were 98.3 and 99.2%. The mean number of songs (i.e. pulse trains) required to evoke a female response was not significantly different (Kruskal-Wallis test: $\chi^2_2=0.453$, $P=0.797$) between the song types (normal song: $\bar{X} \pm SD=41.6 \pm 31.3$, $N=46$; mod 1: 44.6 ± 28.4 , $N=18$; mod 2: 44.6 ± 28.9 , $N=20$), that is, females responding to modified songs did not require more (or fewer) songs than those responding to normal songs.

The cumulative percentage of females responding to normal song was 45.1% ($P=102$), to mod 1 song 18.2% ($N=99$) and to mod 2 song 20.0% ($N=100$). A higher percentage of females responded to normal song than to

**Figure 1.** Cumulative percentage of females responding to simulated normal (●), mod 1 (■) and mod 2 (▲) songs.

songs with modified characters (chi-square test between normal and mod 1 song: $\chi^2_1=16.77$, $P<0.001$; between normal and mod 2 song: $\chi^2_1=14.46$, $P<0.001$). The respective difference between the experiments with modified songs was nonsignificant (chi-square test: $\chi^2_1=0.11$, $P=0.744$). In control experiments, the females spread their wings during the 2-min silent observation period in only 3.6% of the trials ($N=306$). Consequently, the frequency of the females giving a wing-spreading signal was significantly higher in experiments with normal (chi-square test: $\chi^2_1=37.69$, $P<0.001$), mod 1 (chi-square test: $\chi^2_1=8.35$, $P=0.004$) and mod 2 (chi-square test: $\chi^2_1=9.27$, $P=0.001$) songs than in controls.

The observed and expected distributions of female responses (Fig. 2) were quite similar to each other (chi-square test: $\chi^2_4=1.51$, $P=0.824$). If the female responded to one of the modified songs, she did not respond to the other one more often than expected (chi-square test with continuity correction: $\chi^2_1=0.189$, $P=0.664$). Accordingly, the females who responded to mod 1 song were not the same ones that responded to mod 2 song. This result suggests that the females differ in their preferences and that both IPI and PL might affect female mate choice.

The mean number (\pm variance) of song types accepted by the females that gave a positive response in at least one trial was 1.38 ± 0.37 . The frequency distribution of the number of song types accepted by these females was not random (i.e. did not fit a Poisson distribution, $\chi^2_2=16.21$, $P<0.001$). The variance in the number of accepted song types was less than its mean, suggesting that the female preference windows were more narrow than expected. In fact, 43% of the receptive females accepted only one song type, whereas 15% accepted two and 4% all song types. The narrowness of the females' preference windows was not due to only the most receptive females responding to modified songs. The mean number (\pm variance) of song types accepted by the females responding to at least one of the modified songs was even lower than that for the females in general (1.15 ± 0.129).

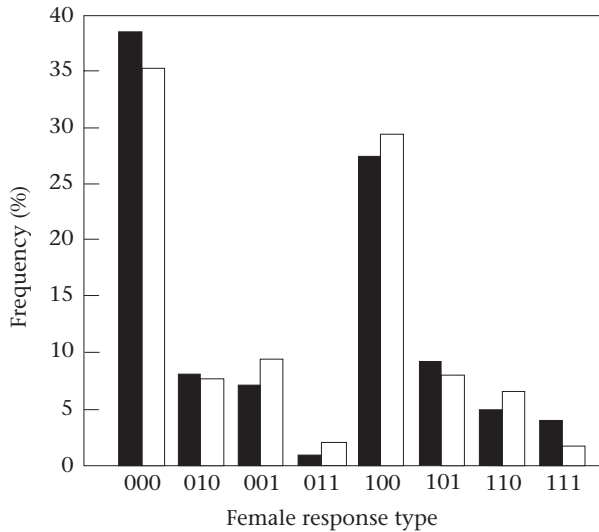


Figure 2. The frequency distribution of females by their response type for simulated normal, mod 1 and mod 2 song types (■), together with the distribution expected if the females' responses to different song types are totally independent (□). See text for an explanation of female response categories.

Consistency of Female Response to Normal Songs

The percentage of females responding to normal song in the first trial (45.1%; $N=102$) was not significantly different from that responding to normal song in the second trial (38.0%; $N=100$; chi-square test: $\chi^2_1=1.05$, $P=0.306$). Females' responsiveness to simulated songs was significantly correlated in the two trials (chi-square test: $\chi^2_1=10.70$, $P=0.001$), suggesting that the females' tendency to spread their wings during the song simulation was consistent within individuals. If the female had responded to normal simulated song in the first trial, the probability of her responding to this song in the second trial was as high as 0.56 ($N=45$). On the other hand, if she did not respond in the first trial, the probability of responding in the second trial was 0.24 ($N=55$). Goodman and Kruskal's predictive ability (Everitt 1977) computed from these probabilities was $\lambda=0.316$ indicating that if a female responded to a normal simulated song during the first trial, the probability that she responded to this song in the second trial increased by about 32%.

More simulated songs were required for a female to respond in the first trial ($\bar{X} \pm \text{SD}=41.6 \pm 31.3$ songs) than in the second trial ($\bar{X} \pm \text{SD}=35.9 \pm 27.9$ songs; Wilcoxon signed-ranks test: $Z=2.503$, $N=100$, $P=0.012$). The number of songs required to evoke a female response appeared to be consistent within the females between the two trials (Fig. 3). Because of the difference in the mean number of songs between the two trials, we added trial as a covariate in the ANOVA when estimating variance components of required song numbers for the repeatability estimate. The repeatability of the number of songs required for a response (square root-transformed data) \pm SE was estimated to be $r=0.328 \pm 0.236$ ($F_{24,49}=1.98$, $P<0.05$), suggesting that individual females were consistent in their responses in this respect.

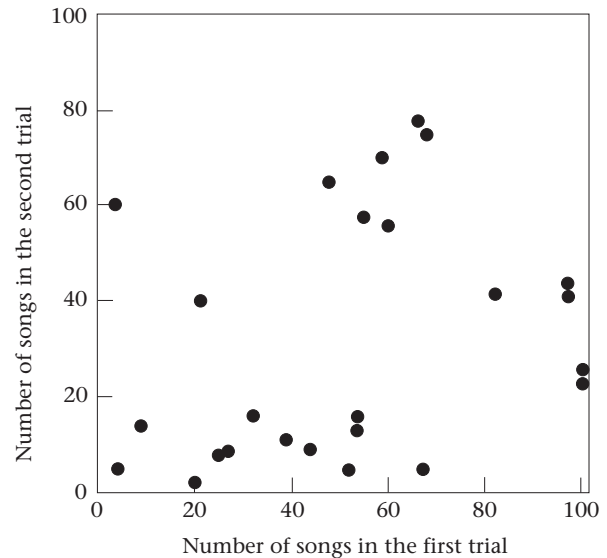


Figure 3. Relationship between the number of songs required for a female to respond in the first and second trial with normal simulated songs.

DISCUSSION

Drosophila virilis females responded readily to simulated songs. Even though the playback experiments were conducted without the presence of males, more than 60% of the females indicated their readiness to mate by spreading their wings in at least one of the experiments. The females varied in their preferences and they also appeared to be consistent in their responsiveness to species-specific song played on 2 days.

The majority of females responded to courtship song resembling that of the conspecific males. Some females, however, responded only to songs in which the IPI and PL were 10 SD longer than in the species-specific song of *D. virilis* males. Consistency of the female preferences for the last-mentioned songs was not studied. The modified IPI and PL used in our experiment were not within the range of variation (15.3–25.3 ms for IPI and 10.4–23.0 ms for PL) previously found in the songs of *D. virilis* males (A. Hoikkala, unpublished data). They were, however, within the range of variation found in other species of the *D. virilis* group. *Drosophila virilis* females will hybridize in laboratory conditions with the males of several *D. virilis* group species differing by as much as 10 SD in IPI and PL from the songs of conspecific males (Hoikkala 1988; Hoikkala & Lumme 1987). This suggests that song differences do not prevent these females from mating. Our study, however, shows that songs exceeding the normal range of the species are not as stimulatory for most *D. virilis* females as normal songs.

Hoikkala & Aspi (1993) have shown that the females of several species of the *D. virilis* group use relative criteria ('best-of- n ') in their mate choice. They can accept atypical males in the absence of other males, even though they would not accept these males in choice tests. Our experimental design did not allow the presentation of an alternative song for females (Godin & Dugatkin 1995).

However, some females responded only to modified and not to normal song. Moreover, the preference window of these females appeared to be narrow. Thus, it is unlikely that females in our experiments were accepting songs typical of drab males only because of a lack of better ones. It does not seem either that the females responding to modified songs were more receptive than the other females. These females accepted fewer song types than females in general, and they required the same number of songs as the females responding to normal song.

Different females responded to modified songs with long IPIs and with long IPIs and PLs. This suggests that both of these traits may affect female choice. The importance of these male song characters to female mate choice has been demonstrated in other *Drosophila* species. For example, in some of the species of the *D. melanogaster* group IPI is an important determinant of choice of males (Bennet-Clark & Ewing 1969; von Schilcher 1976; Kyriacou & Hall 1982; Greenacre et al. 1993). PL seems also to be one of the preferred song characters in wild populations of two *D. virilis* group species, *D. montana* and *D. littoralis* (Aspi & Hoikkala 1995). Ritchie et al. (1998) have shown that *D. montana* females respond most readily in playback experiments to song consisting of short sound pulses and a high carrier frequency.

Even though the male songs with exceptionally long IPIs and PLs appeared not to be so stimulatory for *D. virilis* females as the songs with species-specific characters, they evoked responses in some females. It is possible that *D. virilis* females vary in their preferences, and this causes some of them to mate with a heterospecific male. In *D. bauraria*, females also respond to courtship songs with nonspecies-specific characters. Tomaru et al. (1995) have shown that females of this species can accept male songs in which the IPI is about 3.1 SD longer than that of an average male of the species, even though the female rejections increased by 50% when females heard this kind of song.

Theoretical models explaining the evolution of male secondary sexual characters have several assumptions about the nature of the male character and the female preference. Both viability indicator and Fisherian models of sexual selection require that the preferred male character and female preference are inherited (e.g. Bradbury & Andersson 1987). However, there is only limited evidence for genetic variation in female mating preference (Bakker & Pomiankowski 1995). To obtain heritability estimates of female mating preferences one must test the females of different generations under standard conditions, and this is often very difficult. Many researchers have estimated repeatabilities instead of heritabilities (Møller 1994; Godin & Dugatkin 1995; Jennions et al. 1995; Johnsen & Zuk 1996). Repeatability is informative as it sets an upper limit for heritability (Boake 1989).

Our repeated trials with normal song showed that *D. virilis* females are consistent in their responses to male songs. If they responded to normal songs in the first trial, they usually also responded to it in the second one. Some of the females required only a few, and others considerably more songs. The repeatability of female requirement

in this respect appeared to be high (0.328). Our experiments also revealed variation in the width of the female preference window, and showed that the preference windows of individual females might be narrow. We used females from a stock maintained in the laboratory for a long period. Variation between the females in their receptivity and in the width of their preference windows might be even larger if one were to use the F₁ progenies of wild-caught females.

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