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The importance of different courtship stimuli in the mating behaviour of European species of the *Drosophila virilis* group

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Four species of the *D. virilis* group, *D. lummei*, *D. littoralis*, *D. ezoana* and *D. montana*, occur sympatrically, at least in Scandinavia. These species are sexually isolated and prohibiting the passage of visual, auditory or chemical cues between the males and the females does not break this isolation. On the other hand *D. virilis* (cosmopolitan domestic species) flies mate with *D. lummei* males and females and *D. montana* males in a no-choice situation. Removing the aristae or the antennae of the females or the wings or tarsal sense organs of the males reduces the mating success of the males of all of these species except *D. lummei*. Differences between species suggest that the species-recognition and sexual stimulation of the flies relies on different stimuli depending on the species. In *D. montana* and *D. ezoana* the females, whose aristae or antennae have been removed, or who are courted by wingless males, persistently refuse to copulate. Cessation of mating in these species is probably due to their courtship relying mainly on auditory cues i.e. on the sounds produced by the wing vibration of the males.

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1. Introduction

The courtship rituals of *Drosophila* flies are very complicated and differences exist between species, as well as in such intraspecific behaviour as the stimuli delivered by both males and females during courtship. These differences help the flies to recognize individuals of their own species and thus they may play a part in maintaining sexual isolation between species.

The males of most *Drosophila* species begin their courtship by orienting towards the female and touching her with their foreleg tarsi. They may then attempt to get under the wings of the female and lick the tip of her abdomen, and/or they may circle around the female touching and licking her in a species-specific way. In most *Drosophila* species the males vibrate their wings during some phases of the courtship, producing acoustic cues, so called male courtship sounds, first described by Shorey (1962). The movements of the flies may stimulate the individuals of both sexes sexually and, in some species, showy patterns in the head and the wings of the males are important visual mating stimuli for the females (Fuyama 1979, Spieth 1984). When touching and licking the females the males (and the females) receive from each other tactile and chemical cues (Manning 1959, Jallon & Hotta 1979) and, according to Shorey & Bartell (1970), pheromones acting over a long distance also play an important role in the initiation of the courtship or in the mating success of the males.

Bastock & Manning (1955) have suggested that each behavioural trait of the males has its own threshold for firing. The inhibitatory and excitatory stimuli which the male receives from the female during the courtship interact with the internal factors of the male to produce a "common excitation" and lead to the output of certain types of behaviour. On the other hand the stimuli delivered by the males raise the receptivity of the females until a certain threshold is attained and the female accepts the courtship (Manning 1967a). The females not only summate these stimuli, but the effects of the stimuli also persevere for some minutes in the absence of continuous stimulation (Bennet-Clark et al. 1973, Kyriacou &

Hall 1984). Courtship stimuli may also act in a trigger-like fashion (von Schilcher 1976b) being important for either sex in the initiation of the courtship or in species recognition and further in maintaining the sexual isolation between species.

The importance of different courtship stimuli has been studied in several *Drosophila* species by removing the sense organs (or wings) of the flies, playing simulated courtship sounds to the females and observing the behaviour of mutant or normal flies in the light or in darkness. The results of these studies (which have been reviewed by Ewing, 1983) indicate that the importance of different mating stimuli varies between and even within the species. In some *Drosophila* species courtship relies mainly on one or two kinds of courtship stimuli, the lack of these stimuli blocking the entire courtship. The flies of several *Drosophila* species do not, for example, mate at all, if they are unable to receive visual cues during courtship (Grossfield 1971). The lack of acoustic or olfactory cues has not been found to be so critical in any species studied so far, even though Ikeda et al. (1981) have reported that antennaless *D. mercatorum* females are "unreceptive to courting males".

In the present paper I have studied the importance of visual, acoustic and chemical cues in the mating behaviour of the flies of five European species of the *Drosophila virilis* group. Four of these species, *D. lummei*, *D. littoralis*, *D. ezoana* and *D. montana*, occur sympatrically in Scandinavia (Lumme et al. 1978) and *D. lummei*, *D. ezoana* and *D. montana* have been found, together with *D. virilis,* also in Japan (Watabe & Higuchi 1979). The first four species share same kinds of biotypes near lakes, rivers or ponds, while D. *virilis* is a domestic species, being associated with man around the world. *D. virilis* has, however, been collected wild in China (Okada 1956) and in Japan (Watabe & Higuchi, 1979). According to Throckmorton (1982) *D. virilis* and *D. lummei,* have also been collected wild in the southern USSR. The nature and extent of ecological isolation among the five species studied here is, however, as yet unknown.

2. Material and methods

2.1. Flies

All the strains used in the present study are isofemale lines cultured in the laboratory for at least five years prior to the experiments. The codes of the strains refer to the stock codes of the Department of Genetics, University of Oulu, Finland. *D. virilis* strain1416 comes from Caucasus, USSR and *D. lummei* strain 1134 from Oulu, Finland. *D. littoralis* strain 1056 originates from Strängnäs, Sweden, *D. ezoana* strain 1317 from Padasjoki, Finland and *D. montana* strain 1219 from Kiuruvesi, Finland.

During and prior to the experiments the flies were maintained in culture bottles or vials containing malt medium, in continuous light at 19°C. Freshly emerged males and females were collected in separate vials. The flies were used in the experiments at the age of two to three weeks.

2.2. Surgical operations

All the flies were etherized at the age of ten to twelve days, when the aristae or the antennae of some females, or the wings or the tarsal sense organs of some males, were ablated using a fine forceps. Scanning electron microscope photographs of these organs have been published by Hodgkin & Bryant (1978).

Auditory and olfactory receptors are located in different segments of the antennae of the flies. The main olfactory receptors are located in the large third antennal segment, the funiculus (Hertweck 1931, Kellogg et al. 1962). Auditory stimuli are perceived by Johnston's organ, which is in the second antennal segment, the pedicellus, and which is stimulated by the movements of featherly aristae (Manning 1967b, Bennet-Clark 1971, Ewing 1978). Thus, removing the aristae of the females should deafen these flies and removing the whole antennae should prevent them from receiving acoustic and olfactory cues. Von Schilcher (1976a) has, however, found that aristaless *D. melanogaster* flies react to a simulated sound, if the intensity of the sound is extremely high (at least 106 dB), while Ikeda et al. (1981) have demonstrated that while the aristae may be very important for *D. mercatorum* females to efficiently detect the courtship sounds, they may not always be necessary, if the stimulus is strong enough.

The passage of auditory cues was also prevented by cutting off the wings of the males, i.e. by "mutening" the males. Wingless males (or the males having small wing stumps) may produce low-amplitude sounds (Waldron 1964). These sounds cannot, however, be recorded with a ribbon microphone, which is a particle velocity receptor similar to the female's aristae (Bennet-Clark 1971, von Schilcher 1976a). Thus, it is probable that the females do not hear these sounds, at least not properly.

The flies have chemical sense organs in the tip of their foreleg tarsi (Hertweck 1931). These organs were removed by cutting off the tips of the foreleg tarsi of some males to prevent the males from receiving chemical cues when touching the females.

2.3. Experimental procedures

To study the importance of different courtship stimuli in the mating behaviour of the flies of the five *D. virilis* group species and the effectiveness of the sexual isolation between these species I made six sets of mating combinations within and between the species (25 combinations and three to five replicates per set). In each replicate 15 males and 15 females (conspecific or of different species) were put in a vial (length 9 cm, diameter 1.5 cm) containing malt medium. The flies were left together for

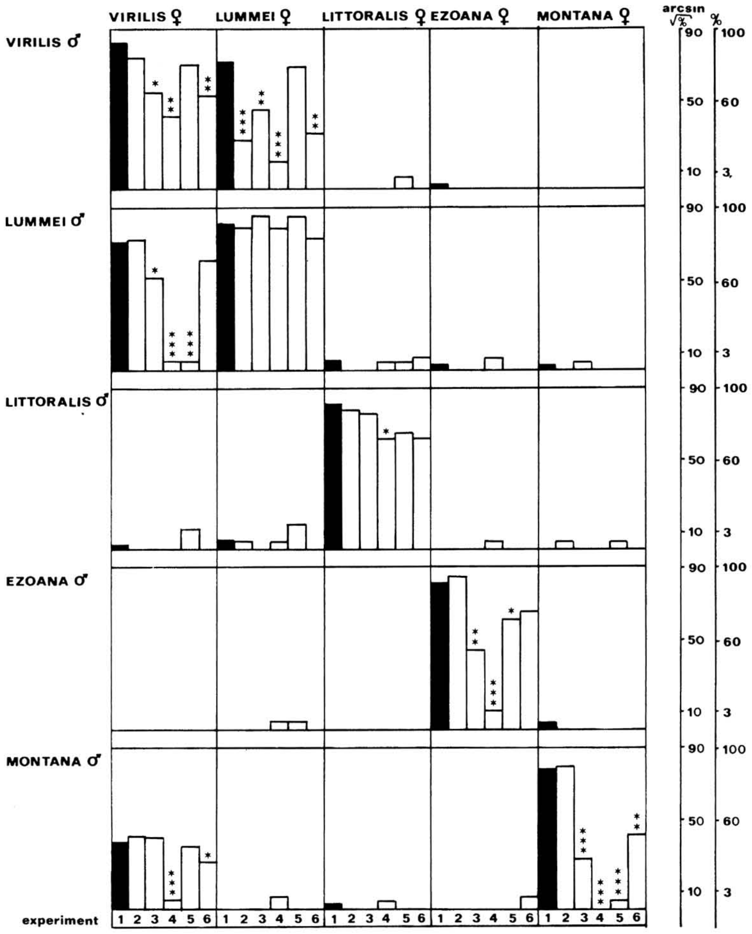

Fig. 1. Percentage of females inseminated in experiments 1, 2, 3, 4, 5 and 6. Statistically significant differences between experiment 1 (control) and the rest of the experiments have been indicated with stars. (1) normal females with normal males (control), (2) normal females with normal males in dark, (3) aristaless females with normal males, (4) antennaless females with normal males, (5) normal females with wingless males, (6) normal females with males whose foreleg tarsi have been amputated.

24 hours. The experiments were began at 9.00 a.m. and the vials were kept continuously in a lighted room at 19°C. After 24 hours the females were etherized and their receptaculum seminis and spermathecae were prepared on an object glass in a drop of 0.67% NaCl and covered with a cover slip. These slides were studied under a microscope to detect the presence of sperm.

In experiment 1 (control) I defined the portion of females which would be inseminated by conspecific males or by the males of foreign species, if the flies were able to receive all the stimuli delivered during courtship. This experiment was repeated five times on different days.

In experiment 2 the vials containing the flies were kept in a dark box, so that the flies would not be able to receive visual cues. In other experiments I used aristaless females (experiment 3), antennaless females (experiment 4), wingless males (experiment 5) or males, whose foreleg tarsi had been amputated (experiment 6). All these experiments were repeated three times.

The differences on one hand between the experiments and on the other hand between the species (in intraspecific combinations) were tested using an analysis of variance (two-way classification; Snedecor & Cochran 1976). In all intraspecific combinations and also in some interspecific ones (*D. virilis* × *D. lummei*, *D. lummei* × *D. virilis* and *D. virilis* × *D. montana*) the percentages of the females inseminated in experiments 2 to 6 were also compared with the percentages found in experiment 1 (control). Statistical analysis was conducted on arc sin $\sqrt{p}$ transformed data.

3. Results

3.1. Intraspecific combinations

Two to three week old virgin females of the *D. virilis* group species appeared to copulate with conspecific males quite readily, if they were able to receive all the stimuli delivered during the courtship. In Fig. 1 the average portion of the females inseminated in experiments 1 to 6 has been given for each of the 25 intra- and interspecific combinations.

In control experiments (normal females with normal males in light) and in experiment 2 (normal females with normal males in darkness) more than 90% of the females were inseminated in each of the intraspecific combinations. In experiments 3 to 6 the mating percentage of the females of most species was significantly lower than this. The analysis of variance made for the intraspecific combinations confirmed that the percentage of inseminated females was affected by the operations made for the flies and by the species in question, as well as by the interaction of these two factors, i.e. removing the sense organs or the wings of the flies affected the mating percentage of the females in different ways in different species.

In *D. lummei* more than 70% of the females were inseminated in each experiment irrespective of whether or not the sense organs or wings of the flies had been removed (Fig. 1). In *D. littoralis* the mating percentage of the females was significantly reduced (i.e. lower than in control experiments) in experiments with antennaless females and in *D. virilis* also in experiments with aristaless females and with males whose tarsal sense organs had been removed. In *D. ezoana* and *D. montana* manipulation of the flies had affected the mating success of the males most dramatically blocking, in some cases, the entire courtship. Only 2/45 antennaless *D. ezoana* females had accepted the courting male during the 24 hour period; the mating percentage of the flies was also reduced in experiments with aristaless females and with wingless males. In *D. montana* none of the antennaless females, and only one female courted by a wingless male, had accepted the courtship. In this species the mating of the flies was also reduced in experiments with aristaless females and with males lacking tarsal sense organs.

3.2. Interspecific combinations

In interspecific combinations the mating percentage of the flies was on average much lower than in the intraspecific ones. The number of inseminated females in different vials did not, however, approach the Poisson distribution, i.e. insemination was more common in some combinations than in others. Most of the interspecific matings occurred between *D. virilis* and *D. lummei* (more than 80% of the females were inseminated in control experiments in both combinations between these species) and between *D. virilis* females and *D. montana* males (more than 40% of the females inseminated in control experiments). In all other interspecific combinations the percentage of inseminated females fell between 0 and 5%. In *D. lummei* only 11/900 males and 12/900 females had copulated with the flies of a species other than *D. lummei* or *D. virilis*. In *D. virilis* 4/600 females had mated with the males of some species other than *D. virilis*, *D. lummei* or *D. montana*, and 4/900 males had mated with the females of some species other than *D. virilis* or *D. lummei*. In *D. littoralis* 12/1200 males and 10/1200 females and in *D. ezoana* 4/1200 males and 7/1200 females had copulated with alien flies. In *D. montana* 92/300 males had copulated with *D. virilis* females and 6/900 males and 6/1200 females had copulated with the flies of other foreign species.

The lack of visual, auditory or chemical cues did not break the sexual isolation between species. On the contrary, it reduced the percentage of inseminated females in some interspecific combinations even if the lack of these stimuli had not disturbed the mating of the flies in intraspecific combinations of these species. For example, antennaless *D. virilis* females refused to copulate with *D. lummei* or *D. montana* males. Also wingless *D. lummei* males were unsuccessful with *D. virilis* females. *D. lummei* females, which had accepted the courtship of conspecific males in all experiments discriminated against *D. virilis* males in experiments 2,3,4 and 6. In the rest of the interspecific combinations it was not possible to test the effects of different operations on the mating willingness of the flies due to the small number of inseminations. However, on average 11.7% of the flies had copulated with the flies of foreign species in control experiments, 7.7% in dark, 8.1% in experiments with aristaless females, 1.8% in experiments with antennaless females and 7.4% and 6.3% in experiments with males whose wings or foreleg tarsi had been amputated.

4. Discussion

The twelve species of the *D. virilis* group can be divided into two subgroups: the "conservative" *virilis* subgroup and the "more evolved" *montana* subgroup (Throckmorton 1982). *Virilis* subgroup consists of *D. americana americana*, *D. a. texana*, *D. novamexicana*, *D. lummei* and *D. virilis* and *montana* subgroup of *D. kanekoi*, *D. littoralis*, *D. borealis*, *D. ezoana*, *D. lacicola*, *D. flavomontana* and *D. montana*. Most of the studies on sexual isolation between the *D. virilis* group species were made in the 1950s, when many of the presently known species of the group had not yet been described. According to Patterson et al. (1947) and Patterson & Stone (1952) the role of the males and the females in maintaining the sexual isolation between the *D. virilis* group species varies according to the species pair in question. In many species pairs the sexual isolation is one-sided, i.e. the females of species A mate with the males of species B, while the females of species B do not mate with the males of species A.

Watanabe & Kawanishi (1981) and Watabe & Higuchi (1984) have recently studied the effectiveness of sexual isolation between some species of the *D. virilis* group in a no-choice situation. Watanabe and Kawanishi put ten five day old males and females (conspecific or of different species) in a vial containing standard medium. The flies were left in these vials for five days, after which the females were dissected to detect whether they had been inseminated or not. The percentage of inseminated females in vials in *D. virilis* was 100%, in *D. littoralis* 92% and in the combinations *D. virilis* × *D. littoralis* and *D. littoralis* × *D. virilis* 52% and 9% respectively. Watabe and Higuchi put five one to two day old males and females of two different species (*D. virilis*, *D. lummei*, *D. littoralis*, *D. ezoana*, *D. montana* or *D. kanekoi*) in a vial for 30 days, after which the females were dissected. In most of the vials the percentage of inseminated females appeared to be very high (from 0 to 90.3%, $\bar{x}$ 35.0%). Only *D. ezoana* males and females discriminated persistently against the flies of foreign species in most combinations. Thus it seems that the sexual isolation between most of the species studied here will be broken, if the flies are left together for several days in a no-choice situation. In fact, many of these species can be crossed with each other in the laboratory to obtain F_1 and even F_2 or backcross hybrids (Throckmorton 1982). In both studies mentioned above the males and the females were mixed together before the females were sexually mature, which could have effected the discrimination ability of the females. Watabe & Higuchi (1984) state that at least *D. virilis* and *D. lummei* females begin to mate before their ovaries are fully developed.

In the present study the importance of visual, auditory and chemical mating stimuli appeared to vary a lot between the species of the *D. virilis* group. *D. lummei* females were quite willing to copulate in an intraspecific combination irrespective of whether they (or the males) were capable of receiving the stimuli delivered during the courtship, or not. This indicates that even though removing the aristae or antennae of the females, or the wings or tarsal sense organs of the males are rather crude operations, they had not injured the flies seriously. In experimental vials the females were probably courted by several males and the males and the females also received courtship stimuli from other courting pairs. The fact that the quantitity of stimuli did not compensate their quality in most species indicates that the effects of the stimuli are not merely additive. Furthermore, prohibiting the passage of certain courtship stimuli between the sexes lowered the mating percentage of the females in interspecific combinations more than in the intraspecific ones, which indicates that courtships in which the male and the female belong to different species are more sensitive to disturbances than courtships between conspecific flies.

Visual cues were the only stimul the lack of which did not significantly lower the percentage of inseminated females in vials in any species. Even though such cues do not seem to be necessary for the flies of these species in regard to species- recognition, they surely help the flies in locating other individuals and in keeping in contact with them. Spieth (1952) has stated that moving insects activate the males of the *D. virilis* group species into approaching and investigating them and that "courting males apparently keep a sharp lookout for approaching intruders". The lack of visual cues might also affect the behaviour pattern of the flies without affecting the 24 hour insemination percentages.

Bartelt & Jackson (1984) and Bartelt et al. (1985) have characterized some aggregation pheromones of *D. virilis*. Other pheromones and their effects on the behaviour of the flies of this group have not yet been studied. According to Spieth (1952) *D. virilis* males, when encountering the females of foreign species, merely tap the female and then turn away from her. If, however, the males pass the tapping stage they will court alien female vigorously and persistently. In the present study removing the tarsal sense organs of the males reduced the percentage of matings in *D. virilis* × *D. virilis*, *D. lummei* × *D. virilis*, *D. montana* × *D. montana* and *D. virilis* × *D. montana*, which implies that chemical cues received through tarsal sense organs would be of special importance to *D. virilis* and *D. montana* males. It should, however, be kept in mind that only one or two active males are able to inseminate practically all the females in a vial within 24 hours. Thus, the experimental procedure of the present study is not a good way of testing the mating propensity of the males. Again, the stimuli which the males receive when licking the females may substitute the stimuli normally received through tarsal sense organs.

Surgical operations carried out on the flies reduced the percentage of inseminated females most radically in *D. ezoana* and *D. montana.* In these species removing the antennae of the females practically blocked the whole courtship and removing the aristae of the females or the wings of the males lowered the mating percentage of the flies significantly. It should be noted that removing the wings of *D. montana* males did not lower the success of these males with *D. virilis* females, i.e. the reduction in the percentage of females inseminated by wingless males in *D. montana* was not due the to incapability of the males to court. Reduction in the mating percentage of the antennaless and aristaless females, as well as of the females courted by wingless males, was probably to a large extent due to the females being unable to receive auditory cues delivered by the wing vibration of the males. The former females were more unwilling to mate than the latter two, which could be due to the passage of auditory stimuli being most effectively prohibited by removing the whole of the antennae of the females (see Materials and Methods). Antennaless females have lost, in addition to their hearing organs, the olfactory sense organs located in the antennae. Thus, the difference in the receptivity of the antennaless and aristaless females may also reflect the importance of olfactory cues in the mating behaviour of the flies.

Cessation of mating due to a lack of visual stimuli has been found to occur in several species (Grossfield 1971), but cessation of mating due to a lack of auditory and/or olfactory stimuli (found in *D. ezoana* and *D. montana*) has not been reported earlier. This cessation may either be due to the females being unable to recognize the males of their own species when they cannot hear their sounds, or to sounds being more important in sexual stimulation of the females. I have found that the males of the *D. virilis* group species occurring sympatrically in northern Finland also produce sounds when courting the females of a foreign species in the wild (Hoikkala 1986) and so the sounds may play a part in maintaining sexual isolation between these species.

The sounds of the *virilis* subgroup species (including *D. virilis* and *D. lummei*) consist of dense pulse trains with very short interpulse intervals, while the sounds of the *montana* subgroup species differ from these sounds and from each other in several sound traits (Hoikkala et al. 1982). For example, the sounds of *D. littoralis*, *D. ezoana* and *D. montana* can be distinguished from each other by the length of an interpulse interval, by the number of cycles in a pulse and by the number of pulses in a train. It is interesting to note that the lack of auditory cues does not block the courtship in *D. littoralis* even though the males of this species have a highly specific courtship sound. Thus, the importance of the sound in the mating behaviour of the flies does not correlate with the complexity of the sound.

In *D. montana* there is quite a lot of variation between the sounds of the males of different "laboratory" strains (Hoikkala 1986). It will be interesting to study whether these differences have evolved during maintenance of the strains in the laboratory (due to genetic drift or sexual selection) or whether there is detectable variation in wild populations of this species as well. Again, the behaviour of the females of different *D. ezoana* and *D. montana* strains should be studied further using more delicate methods (courtship sequencing, simulated courtship sounds, etc.), paying special attention to the quality of the sounds that stimulate the females sexually and to the variation in the receptivity of the females within the species.

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