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Analysis of courtship behavior in closely related species of *Drosophila virilis* group: a new approach arises new questions

Varvara Y. Vedenina · Tatyana I. Ivanova · Oleg E. Lazebny

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Abstract Courtship behavior of males was studied in four closely related *Drosophila* species: *D. virilis, D. lummei, D. a. americana* and *D. littoralis*. Using a video-computing approach, we compared behavior in males courting conspecific and heterospecific females. In males of all species studied, touching and licking were found to be the most prolonged courtship elements. Touching and licking were typically proceeding together; wing vibration was usually produced against a background of touching and licking. We found only minor interspecific variations in courtship rituals. Heterospecific courtships in *D. virilis* and *D. lummei* were almost as active as conspecific ones; however, isolation between *D. a. americana* and *D. littoralis* appeared to be much stronger than between *D. virilis* and *D. lummei*. Analysis of prolonged touching and licking raises a question about chemical and tactile sensory stimuli exchanged between sexes in the developed courtship of *D. virilis* group.

Keywords *Drosophila* · courtship behavior · sensory stimuli · sexual isolation

Introduction

Sexual isolation is known to be one of the important reproductive isolating mechanism preventing hybridization and gene exchange between closely related species of *Drosophila* flies in their natural habitats. The courtship rituals of *Drosophila* include

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an exchange of several signals with different modalities, chemical, visual, acoustical and tactile stimuli, between sexes. Courtship behavior of *Drosophila* males was usually described as sequential stereotypical elements such as orienting towards a female, touching her with their foreleg tarsi, wing vibration, circling around the female, and licking her genitalia. When touching and licking the females, both sexes receive from each other chemical and tactile cues. In particular, when touching with his fore legs, which tarsi have contact chemical receptors, the male is suggested to recognize the female epicuticular hydrocarbons. In the course of wing vibration, the males produce acoustic and sometimes visual cues; when circling around the female, the males produce visual stimuli (see e.g. Spieth 1951, 1974; Shorey 1962; Jallon and Hotta 1979; Ewing 1983; Markow and O'Grady 2005). Signals of same modalities may crucially differ between closely related species. For example, in the species of *D. melanogaster* group, the most studied group of *Drosophila*, the profiles of epicuticular hydrocarbons vary significantly not only between the species, but also within the species and sexes (see e.g. Jallon and David 1987; Coyne et al. 1994; Ferveur 2005; Foley et al. 2007; Everaerts et al. 2010; Grillet et al. 2012). The male courtship songs are known to differ in the time interval between pulses (interpulse interval) among different species, and this parameter was shown to be crucial during mate choice (see e.g. Shorey 1962; Ewing 1983; Kyriacou and Hall 1982; Ritchie and Kyriacou 1996; Popov et al. 2000).

Courtship rituals in closely related species of *D. virilis* group differ substantially from the rituals in *D. melanogaster* group. For example, touching usually precedes wing vibration in *D. melanogaster*; after wing vibration, the male licks genitalia of the female, and then he mounts her to "attempt to copulate" (reviewed in Sawamura and Tomaru 2002). In *D. virilis*, the male licks the female after touching her, and only after this, he starts wing vibration (Saarikettu et al. 2005a). One of the most prolonged courtship elements demonstrated by *D. melanogaster* males was shown to be wing vibration (Lasbleiz et al. 2006). In *D. virilis* males, the most prolonged element was shown to be licking (Saarikettu et al. 2005a).

In *D. virilis* group, similarly to *D. melanogaster* group, the most attention was paid to the studies of the role of two signal types: acoustic signals produced by both sexes and chemical signals obtained by the males during touching the female. The male songs were shown to differ in both temporal (pulse length, interpulse interval, pause between pulses), and frequency parameters between the species (Hoikkala and Lumme 1987; Hoikkala and Aspi 1993; Hoikkala et al. 1998; Päällysaho et al. 2003). Some sibling species may be reliably identified on the only basis of songs and genital characters (Kulikov et al. 2004). Numerous experiments with mate choice and playback of the songs showed that specific traits of the courtship song play an important role in inter-and intraspecific mate choice (see e.g. Hoikkala and Aspi 1993; Aspi and Hoikkala 1995; Ritchie et al. 1998; Saarikettu et al. 2005b; Klappert et al. 2007). The profiles of epicuticular hydrocarbons were shown to differ among the species and among sexes (Bartelt et al. 1986; Oguma et al. 1992; Liimatainen and Jallon 2007). Contact chemoreceptors were shown to localize on the foreleg tarsi and proboscis, and some of these sensilla appeared to be male specific, occurring in twice the number on male fore legs as on those of females (Nayak and Singh 1983). When the females of *D. virilis* and *D. montana* were placed with the males whose foreleg tarsi have been amputated, the mating percentage of the flies lowered significantly (Hoikkala 1988). In addition, the males stopped courting after touching the heterospecific females, which indicated that the hydrocarbon profiles play an important role in species recognition (Liimatainen and Hoikkala 1998).

Until now, it remains unclear what signals are received by both sexes during prolonged licking. In *D. melanogaster*, male licking is stimulated by ovipositor extrusion in the female, which is sometimes accompanied by emission of a tiny droplet of volatile compounds at the tip of the ovipositor (Cobb and Ferveur 1996; Lasbleiz et al. 2006). Contrasting to *D. melanogaster*, licking in *D. virilis* was shown to be the most prolonged courtship signal that did not necessarily result in copulation attempt. It remains unknown whether *D. virilis* females also secrete volatile compounds due to ovipositor extrusion.

To describe courtship behavior in *Drosophila*, most authors used kinetographs (kinematic flow charts) with transitions between different courtship elements of a male and corresponding behavior of a female (Manning 1959; Brown 1965; Cobb et al. 1985, 1989; Liimatainen and Hoikkala 1998; Hoikkala and Crossley 2000; Saarikettu et al. 2005a; Dankert et al. 2009). This method of describing courtship allows expressing relative frequencies of each courtship element and transitions between the elements. This method, however, implies that the elements follow sequentially. Meanwhile, Lasbleiz et al. (2006) showed that even in *D. melanogaster,* which is known to demonstrate highly stereotypic sequences of the courtship elements, some elements may occur simultaneously. Spieth (1951) indicated that in *D. virilis* group, licking and touching typically occur together and extend over relatively long periods. However, he did not use a video-computing approach and therefore, this statement requires verification.

In the current study, we analyzed the courtship behavior of the males in four closely related species: *D. virilis, D. lummei, D. a. americana* and *D. littoralis. D. virilis* is a domestic species being found with man around the world, *D. lummei* and *D. littoralis* are from the Palearctic region where they may occur sympatrically, while *D. a. americana* is from the eastern United States (Throckmorton 1982). We recorded behavior in males courting conspecific and heterospecific females by the method of videotaping. We analyzed latency and total duration of each courtship element, and studied whether different elements were produced simultaneously or consecutively. Comparison of the courtship rituals among four species allowed us to see whether these rituals can be regarded as species specific. We analyzed the particular courtship elements to clarify which chemical and tactile sensory stimuli are exchanged between sexes in the developed courtship.

Materials and methods

All sibling species used were from the stock of the Koltzov Institute of Developmental Biology: *D. virilis* (strains 1 and 102 originated from Erevan, Armenia and Berlin, Germany, respectively), *D. lummei* (strain 1100 originated from Kuopio, Finland), *D. a. americana* (strain FP 99.28 originated from Arkansas, USA) и *D. littoralis* (strain DmO 06.3 originated from Moscow region, Russia). The flies were cultured in semolina-yeast medium shell vials at 24–26 °C and exposed under a normal 12 h light/12 h dark cycle. The two-day old virgin flies were collected and the sexes were separated under ether anesthesia. They were subsequently kept in the glass vials (10×2.5 cm) and used in the experiments at the age of 10–20 days.

We videotaped single-pair courtships in a circular glass vial (10×2.5 cm). Each fly was used only once in experiments. Courtships were recorded using a Sony TRV355E and Sony HDR-SR12E video cameras. If the male didn't show any interest toward the female within 30 min after starting the experiment, the sexes were separated. In case of successful courtship, the behavior was recorded until the flies copulated or 30 min had elapsed. The courtships were analyzed with the Adobe Premiere and Virtual Dub programs. For each pair we calculated the total duration of each behavior element and the length of the active courtship time measured from the first male behavior element to beginning of copulation, excluding the long breaks. We distinguished seven male behavioral elements (Table 1) and the latency and duration of each element was measured independently. This means that after measuring one element, the processing was repeated for the second element, etc., and the corresponding data, differently labeled, were recorded in the same file according to the time. Five minutes of recorded behavior required about 30 min for analysis. The analysis of 155 trials was conducted by the two authors. To ensure against errors, 15 trials were analyzed by both authors and the comparison of the results showed no significant differences. Besides, each author analyzed about one third of the trials two times, and comparison of the results showed no significant differences. Statistical analysis was made with the MS Excel and Statistica v6.0 programs. All studied behavioral traits were analyzed using non-parametric tests, since they were not normally distributed. We used Bonferroni correction to account for multiple testing.

Table 1 The number and percentage of trials containing a given male courtship element in conspecific and heterospecific courtships

Combination of species	The total number of trials	The number of trials with the occurrence of courtship elements:						
		following	touching	licking	singing	circling	copulation attempt	copulation
♀+♂ *virilis*	21	14 (67 %)	21 (100 %)	21 (100 %)	16 (76 %)	7 (33 %)	10 (48 %)	5 (24 %)
♀+♂ *lummei*	13	2 (15 %)	13 (100 %)	13 (100 %)	12 (92 %)	1 (8 %)	4 (31 %)	3 (23 %)
♀+♂ *americana*	24	9 (37 %)	24 (100 %)	24 (100 %)	22 (92 %)	16 (67 %)	11 (46 %)	9 (37 %)
♀+♂ *littoralis*	23	5 (22 %)	23 (100 %)	23 (100 %)	22 (96 %)	15 (65 %)	10 (44 %)	9 (39 %)
♀ *virilis*+♂*lummei*	18	18 (100 %)	14 (78 %)	14 (78 %)	8 (44 %)	7 (39 %)	10 (56 %)	7 (39 %)
♀ *lummei*+♂*virilis*	26	23 (88 %)	26 (100 %)	26 (100 %)	23 (88 %)	10 (38 %)	18 (69 %)	11 (42 %)
♀ *americana* +♂ *littoralis*	20	5 (25 %)	19 (95 %)	11 (55 %)	10 (50 %)	6 (30 %)	2 (10 %)	1 (5 %)
♀ *littoralis*+♂ *americana*	10	7 (70 %)	10 (100 %)	10 (100 %)	8 (80 %)	1 (10 %)	3 (30 %)	1 (10 %)

Results

Courtship behavior in conspecific pairs

In conspecific trials, the male began the courtship by orienting toward the female and by touching her with his fore legs. Almost immediately, he extended his proboscis and started to lick her abdomen without stopping touching her abdomen. Touching and licking typically occurred together and were the most prolonged courtship elements (Figs. 1, 2, and 3, Online Resource 1–2). In all four species, touching and licking highly correlated with each other in both latencies from the beginning of courtship (Spearman rank correlation, $r=0.74$–0.81, $p<0.0009$, Bonferroni corrected) and overall duration ($r=0.92$–0.95, $p<0.00025$, Bonferroni corrected). Significant differences between latencies of touching and licking were only found in *D. virilis* (Sign Test, $Z=3.18$, $p=0.002$). Later on, the male vibrated his wings, producing the song, usually without stopping to touch and lick (Fig. 1, Online Resource 1–2). In three species except for *D. lummei*, latency of singing was significantly higher than latency of licking (Mann–Whitney U Test, $U=46$–149, $0.0001<p<0.02$). Next, the male circled around the female. In *D. virilis* and *D. littoralis*, the males started circling significantly later than singing ($U=24$–91, $0.02<p<0.03$). During circling, the male could continue singing but he did not touch or lick. We did not find significant correlations between three courtship elements (touching-singing-circling or licking-singing-circling) in all species studied. When the female started to walk, the male followed her. During following, the male could continue touching, licking or even singing (Fig. 1), however, it depended on the walking speed of female. If the female walked relatively fast, the male was only able to follow her and didn't show other behavior elements.

In courtships of all four species, different behavior elements occurred with almost equal probability (Table 1), with the exception of following and circling. The males followed the walking females in higher number of courtships in *D. virilis,* than in *D. littoralis* and *D. lummei* (Fisher's Exact Test, two-tailed, $p=0.005$), whereas circling was recorded in a higher number of courtships in *D. a. americana* and *D. littoralis,* than in *D. lummei* and *D. virilis* ($p<0.04$).

Significant differences between different species were found in latencies of licking (Median Test, $\chi^2=13.2$, df=3, $p=0.004$) and latencies of singing ($\chi^2=10.4$, $p=0.015$). The shortest latency of licking was found in *D. a. americana* (1 s), the longest latency—in *D. virilis* (12 s). The shortest latency of singing was shown by *D. littoralis* (7 s), the longest latency—in *D. virilis* (34 s) (Fig. 2). The species also differed in relative duration of following ($\chi^2=12.8$, df=3, $p=0.005$), singing ($\chi^2=18.5$, $p=0.0003$) and circling ($\chi^2=16.3$, $p=0.001$) (Fig. 3). The longest following was found in *D. virilis* (23 % of the active courtship time), the shortest—in *D. lummei* (1.3 %). The males of *D. littoralis* demonstrated the most prolonged singing (36 %), whereas *D. virilis* males—the shortest singing (12 %). The highest duration of circling was found in *D. a. americana* (10 %).

Courtship behavior in heterospecific pairs

Heterospecific males demonstrated some behavior elements more rarely than conspecific males (Table 1). For example, the males of *D. littoralis* licked heterospecific

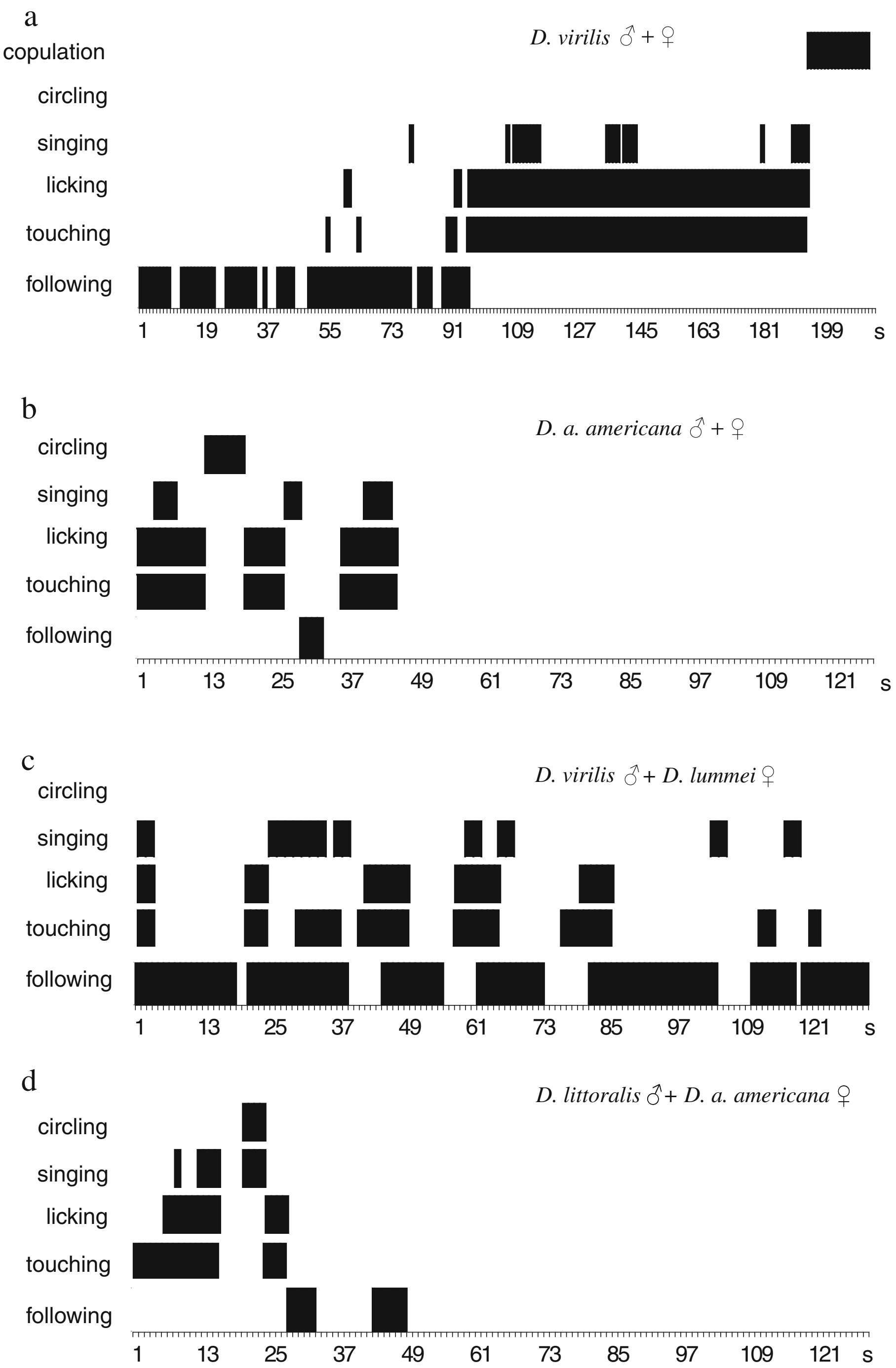


Fig. 1 Timing and duration of the behavioral elements in individual conspecific courtships of *D. virilis* male (**a**) and *D. a. americana* male (**b**), and heterospecific courtships of *D. virilis* male courting *D. lummei* female (**c**) and *D. littoralis* male courting *D. a. americana* female (**d**)

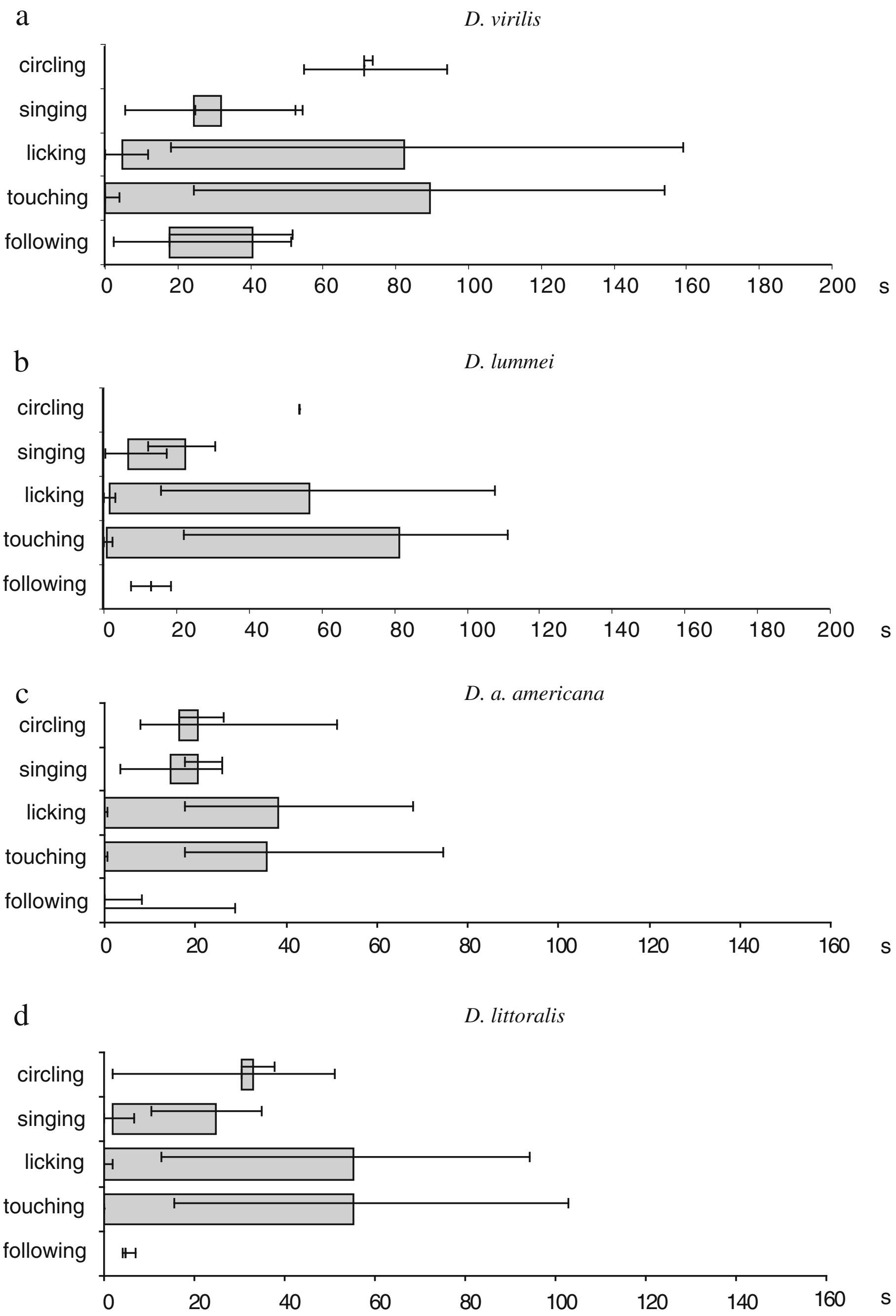


Fig. 2 Latencies and duration of male behavioral elements in conspecific courtships of *D. virilis* males (**a**), *D. lummei* males (**b**), *D. a. americana* males (**c**) and *D. littoralis* males (**d**). Length of boxes corresponds to the median duration of the male behavioral elements, whiskers show lower and upper quartiles for beginning and duration of each element

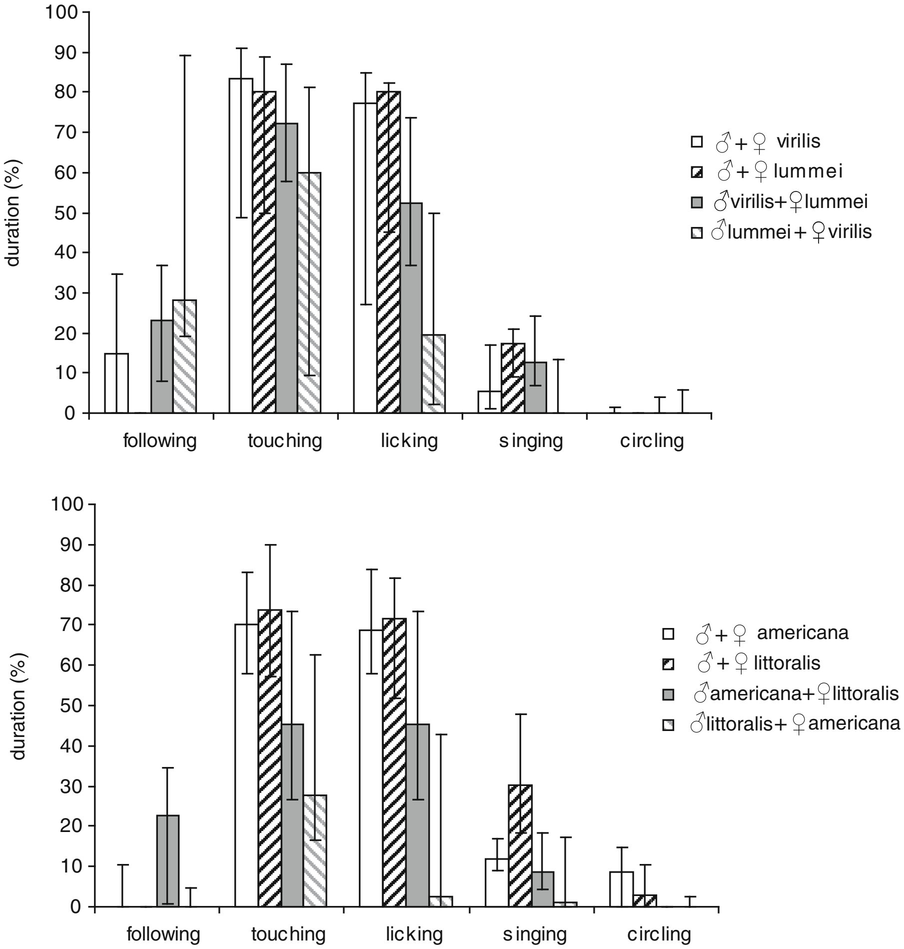


Fig. 3 The median duration of the male behavioral elements as percentages of the active courtship. Whiskers show lower and upper quartiles

females in only 55 % of trials, whereas in conspecific courtships they always demonstrated this behavior. The males of *D. lummei* and *D. littoralis* were singing in about half of heterospecific trials, which was significantly less than in conspecific trials (Fisher's Exact Test, two-tailed, $0.001<p<0.008$). The males of *D. a. americana* и *D. littoralis* demonstrated circling in less number of heterospecific courtships in comparison to conspecific trials ($0.007<p<0.03$). The males of *D. littoralis* made copulation attempts and mated more rarely in heterospecific than conspecific courtships ($0.01<p<0.02$). On the other hand, the males followed heterospecific females more often than the conspecific ones (Table 1).

In all heterospecific courtships, touching duration correlated with licking duration (Spearman rank correlation, $r=0.73–0.86$, $p<0.0025$, Bonferroni corrected), except for courtships of *D. a. americana* males. However, in contrast to conspecific courtships, licking duration was significantly shorter than duration of touching (Sign Test, $Z=2.4–3.7$, $0.0002<p<0.016$) (Figs. 3 and 4). The latencies of these two elements did not correlate in all courtships. The males of *D. littoralis, D. virilis* and *D. lummei* started licking heterospecific females significantly later than touching (Mann–Whitney *U* Test, $U=46$–165.5, $0.001<p<0.01$).

Comparison of latencies of the same courtship elements in con-and heterospecific trials showed significant differences between them. In *D. virilis* and *D. lummei*, following started earlier (Median Test, $\chi^2=19$, df=3, $p=0.0003$), whereas licking started later ($\chi^2=19.6$, $p=0.0002$) in heterospecific than conspecific courtships (Fig. 4). The differences were also found between durations of the same elements in con-and heterospecific trials. The males of *D. virilis* and *D. lummei* followed heterospecific females longer than conspecific females ($\chi^2=19.2$, $p=0.0002$) (Figs. 3 and 4). The males of *D. a. americana* and *D. littoralis* demonstrated longer duration of following ($\chi^2=8.2$, $p=0.04$) and shorter duration of licking ($\chi^2=11.2$, $p=0.01$), singing ($\chi^2=23.4$, $p<0.0001$) and circling ($\chi^2=14.4$, $p=0.002$) in heterospecific than conspecific courtships.

Discussion

Can courtship rituals be regarded as species specific?

The three species studied, *D. virilis*, *D. lummei* and *D. a. americana*, belong to the *virilis* phylad, whereas *D. littoralis* is a member of *montana* phylad (Throckmorton 1982). Despite this fact, we found many similarities in the behavior elements of conspecific courtships in all four species. The most prolonged courtship elements were touching and licking that occurred together and were highly correlated with each other. This finding is in concordance with the data of Spieth (1951). Licking usually started very shortly after touching, with the exception of *D. virilis*, in which licking started significantly later than touching. In all four species, singing was the much shorter element than touching or licking. This result is partly similar to the data of Saarikettu et al. (2005a) who found singing to be much shorter than licking in the species they studied. In the current study, singing started later than touching and licking in all four species. It is notable that singing usually occurred without stopping of touching and licking. Finally, in all four species, circling and following were the shortest elements. Circling, being more expressed in *D. a. americana* and *D. littoralis* than in *D. lummei* and *D. virilis*, typically started after the start of singing. The only significant difference between *D. littoralis,* the member of one phylad, and other three species belonging to another phylad, was found in latency and duration of singing: *D. littoralis* males started to sing with the shortest latency and sang for the longest time.

Among the species studied, only *D. lummei* and *D. littoralis* are known to occur sympatrically and even syntopically (Aspi et al. 1993; Liimatainen and Hoikkala 1998). Thus, one could expect that the differences between the courtships of these two species would be the largest compared to the courtship differences between the other species. We found, however, a significant difference in only latency and duration of singing, as noted above. Liimatainen and Hoikkala (1998) showed that *D. lummei* males licked the female after touching her, while *D. littoralis* males first circled around her and licked her only after that. The differences between the courtships of *D. littoralis* described by Liimatainen and Hoikkala (1998) and shown in the

Fig. 4 Latencies and duration of male behavioral elements in heterospecific courtships of *D. virilis* males courting *D. lummei* females (**a**), *D. lummei* males courting *D.virilis* females (**b**), *D. a. americana* males courting *D. littoralis* females (**c**) and *D. littoralis* males courting *D. a. americana* females (**d**). Length of boxes corresponds to the median duration of the male behavioral elements, whiskers show lower and upper quartiles for beginning and duration of each element ▶

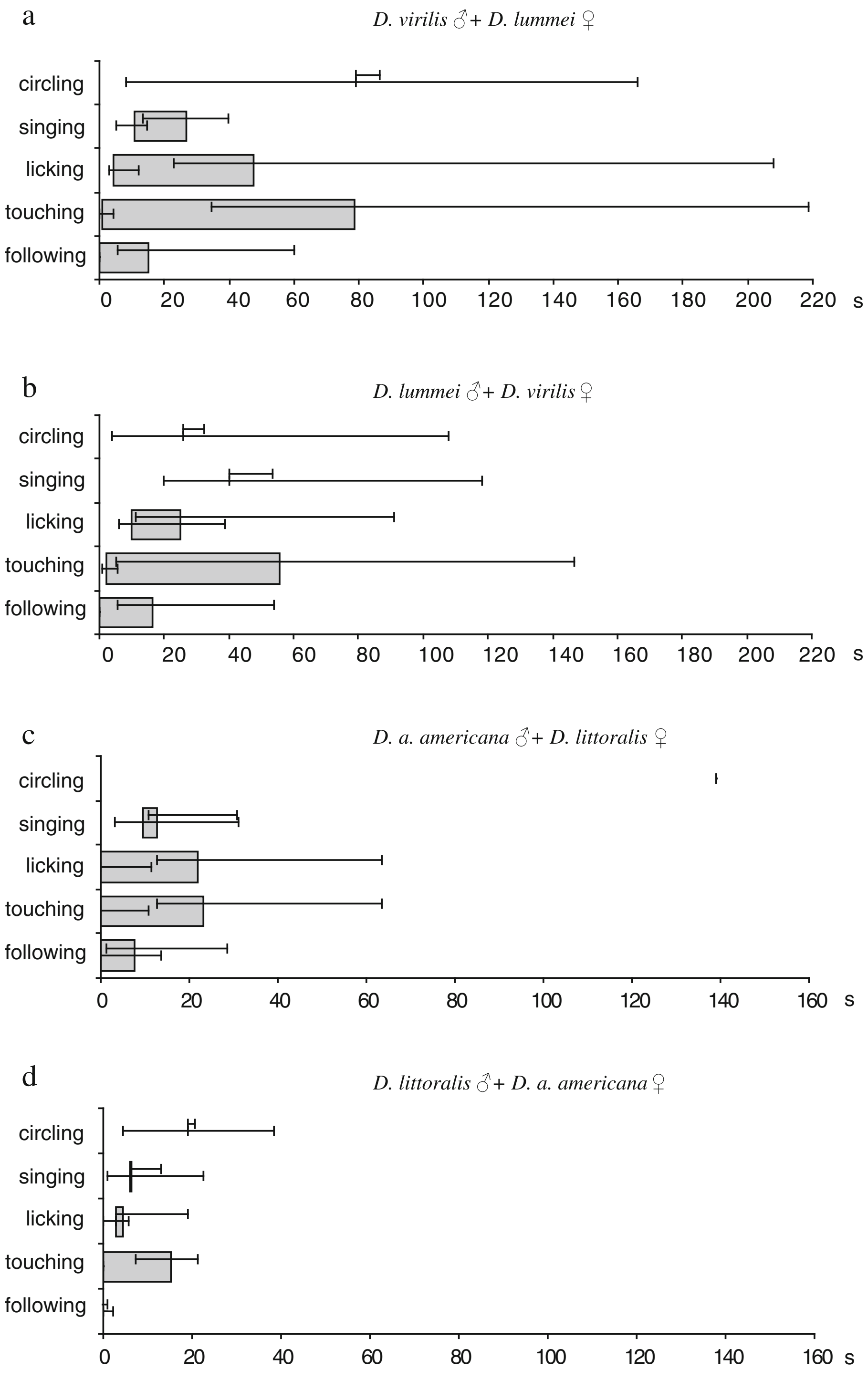
current paper could be due to the different strains used. Comparison of the courtship rituals between five *D. virilis* strains showed a relatively high intraspecific variation in these rituals (Saarikettu et al. 2005a): singing duration was rather high in courtships of some strains and very low in courtships of others; males of some strains demonstrated circling around the female, whereas males of other strains did not circle at all. We found some species specific differences between courtship rituals in three species of the *virilis* phylad, however, these differences are not reliable to distinguish between the species.

Sexual isolation between the species

In heterospecific courtships, we found that licking started significantly later and lasted shorter than touching. Besides, the males of *D. a. americana* and *D. littoralis* demonstrated shorter singing and circling in heterospecific than in conspecific courtships. Our results partly coincide with the data of Liimatainen and Hoikkala (1998) who showed that heterospecific courtships usually broke off when the male touched the female or when the male or the female produced the song. They, however, used other combinations of species: *D. littoralis* males were found to court heterospecific females (*D. montana* or *D. lummei*) more often than *D. lummei* males did. In our study, heterospecific interactions between *D. virilis* and *D. lummei* were almost as active as the conspecific ones, and the number of copulation attempts and copulations was almost equal in con-and heterospecific trials in these two species (Table 1). Very weak isolation barriers between these species were also shown by Throckmorton (1982) and Hoikkala (1988). To the contrary, isolation between *D. a. americana* and *D. littoralis* appeared to be much stronger: the males made copulation attempts and mated more rarely in hetero-than in conspecific courtships (Table 1). This is also in concordance with the previous data about crosses between these species (Throckmorton 1982): crosses between *D. littoralis* females and *D. a. americana* males resulted in sterile F_1 hybrids, and reciprocal crosses did not produce any F_1 generation. In our study, *D. a. americana* males courted *D. littoralis* females more actively than *D. littoralis* males courted *D. a. americana* females.

What is the function of prolonged licking and touching?

It was shown by Saarikettu et al. (2005a) that the median duration of touching is much shorter than the duration of licking in the courtships of five *D. virilis* strains. It was not the case in the conspecific courtships studied in the current paper, when touching and licking were of a similar duration and highly correlated with each other. It is possible to explain this discrepancy by different methods of the courtship analysis. Touching was shown to be crucial for conspecific recognition (Liimatainen and Hoikkala 1998); in this case, this element should occur at the beginning of


a
D. virilis ♂+ D. lummei ♀
circling
singing
licking
touching
following
0 20 40 60 80 100 120 140 160 180 200 220 s
b
D. lummei ♂+ D. virilis ♀
circling
singing
licking
touching
following
0 20 40 60 80 100 120 140 160 180 200 220 s
c
D. a. americana ♂+ D. littoralis ♀
circling
singing
licking
touching
following
0 20 40 60 80 100 120 140 160 s
d
D. littoralis ♂+ D. a. americana ♀
circling
singing
licking
touching
following
0 20 40 60 80 100 120 140 160 s

courtship. However, we suggest that in the developed courtship, touching may stimulate the female and facilitate her acceptance response. According to Spieth (1951) and our data, the male when touching, usually rubs the female abdomen with alternate back and forth movements of the fore legs. It is known that numerous mechanosensory bristles cover the abdominal segments in *Drosophila* (Fabre et al. 2008); these bristles are good candidates for perception of tactile stimulation during touching.

Function of licking is more obscure. Spieth (1951) showed that the *D. virilis* male never mounted the female unless he subsequently reached her genitalia with his proboscis. Thus, during licking, the male may receive the acceptance response from the female, and this certainly should occur at the final steps of courtship. In *D. melanogaster*, emission of the tiny droplet at the tip of the ovipositor was rapidly followed by strongly increased male sexual activity. It was suggested by Lasbleiz et al. (2006) that chemical components of the droplet could spread on the female abdomen by surface tension, or female abdominal preening may actively disperse compounds on the abdomen, stimulating the male courtship. It was shown by Spieth (1951) that the *D. virilis* male often licks not only genitalia but also the abdominal sclerites of the female. Therefore, it is possible that *D. virilis* males also lick the volatile compounds synthesized by a yet unknown glandular structure located beneath the ovipositor of the female, similarly to that suggested in *D. melanogaster*.

In all our heterospecific trials, except for those with *D. littoralis* males, a male was usually sufficiently motivated when courting heterospecific female. High motivation was indicated by the fact that males followed heterospecific females more actively than the conspecific females. Why did licking start significantly later and last shorter than touching in heterospecific courtships? It is likely that the male receives appropriate signals (e.g., volatile compounds) from the female, and these signals may or may not stimulate him to lick. On the other hand, a temporal change in behavior during *Drosophila* courtship suggests a mutually increasing recognition between mates (Lasbleiz et al. 2006). Prolonged touching without licking in heterospecific courtship may indicate that the male needs more time to recognize the female. As soon as he recognizes her, he starts licking that may be also a stimulation signal for the female. As it was shown by Spieth (1951), the male usually exerts considerable force by licking so that the posterior end of the female abdomen is often pushed upward. Thus, licking may be more important for the female than for the male.

Up to now, almost nothing is known what chemical and tactile signals are perceived by both sexes in *D. virilis* group in the developed courtship. It seems reasonable to further study the importance of chemical and tactile courtship stimuli in mate choice in *D. virilis* group using more delicate methods (e.g., selective disabling of the female abdominal receptors or male chemoreceptors on the proboscis) and considering the behavior of both sexes.

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