

Courtship Behavior Analysis in Three Sibling Species of the *Drosophila virilis* Group

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Abstract—Courtship behavior was studied in three sibling species of the *Drosophila virilis* group: *D. virilis*, *D. lummei*, and *D. littoralis*. The latter species was represented by two strains the founders of which had been collected in the habitats of the southern and northern races of *D. littoralis* whose status is equivalent to subspecies. Con- and heterospecific tests were analyzed by video-typing. Analyses of conspecific tests of *D. virilis* and *D. lummei* revealed no differences in the duration of courtship elements and their latencies. By contrast, comparison of heterospecific tests of ♀ *D. virilis* + ♂ *D. lummei* and ♀ *D. lummei* + ♂ *D. virilis* showed a much lower duration of all the main courtship elements (touching, licking, and singing), as well as a significantly lower percentage of copulation. Comparison of con- and heterospecific tests of the northern and southern races of *D. littoralis* revealed some differences in the courtship structure, but no obstacles to successful mating were observed in heterospecific tests of the two races. By contrast, large differences in the structure of the courtship ritual were observed in heterospecific reciprocal tests of *D. littoralis* + *D. virilis* and *D. littoralis* + *D. lummei*. The males of the *D. virilis* phylad lost interest in *D. littoralis* females immediately after the beginning of touching. On the contrary, *D. littoralis* males demonstrated a complete courtship ritual towards both *D. lummei* and *D. virilis* females. At the same time, all the heterospecific tests of *D. lummei* + *D. littoralis* and *D. virilis* + *D. littoralis* were characterized by the lowest incidence of copulation.

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Mating behavior is one of the most important factors of reproductive isolation preventing hybridization and gene exchange among closely related species. The courtship behavior in *Drosophila* flies includes an exchange of signals with different modalities (chemical, acoustic, visual, and tactile) and thus provides a good model for studying the role of different signals in mate choice.

Sibling species of the *Drosophila virilis* group provide a popular model system for speciation studies. The group comprises 11 closely related species that are distributed in various regions of the globe and can cross under laboratory conditions (Throckmorton, 1982; Spicer, 1992). Complete genomes of two species, *D. virilis* and *D. americana*, have been sequenced (http://beta.flybase.org/static/sequenced_species; <http://cracs.fc.up.pt/~nf/dame/>). Besides, the species divergence pattern within this group has been determined based on polymorphism of chromosome mutations, proteins, and DNA fragments (Throckmorton, 1982; Spicer and Bell, 2002; Caletka and McAllister, 2004;

Wang et al., 2006). At the same time, comprehensive studies of reproductive behavior and especially the courtship rituals in species of the *D. virilis* group are scanty (Liimatainen and Hoikkala, 1998; Vedenina et al., 2013; LaRue et al., 2015). Most publications are devoted to two types of signals: the acoustic ones produced by the male (Hoikkala and Lumme, 1987; Hoikkala and Aspi, 1993; Suvanto et al., 1994; Aspi and Hoikkala, 1995; Päällysaho et al., 2003; Klappert et al., 2007) and the chemical ones received by the male when touching the female (Bartelt et al., 1986; Oguma et al., 1992; Liimatainen and Jallon, 2007).

The courtship ritual in species of the *D. virilis* group is considerably different from the well-studied ritual in *D. melanogaster*. The latter is characterized by a relatively stable sequence of rather short individual courtship elements; by contrast, in the *D. virilis* group several elements are usually observed concurrently while the whole courtship is longer and includes a less rigidly stereotypic sequence of elements (Spieth, 1951; Vedenina et al., 2013). Besides, the order of the

main courtship elements is substantially different in the two groups (Sawamura and Tomaru, 2002; Saarikettu et al., 2005; Lasbleiz et al., 2006). In particular, the *D. melanogaster* male first touches the female's abdomen with its forelegs, then emits an acoustic signal, and finally touches the female's genitalia with its proboscis (below, this element is referred to as "licking") (Sawamura and Tomaru, 2002). The male of *D. virilis* touches and licks the female's abdomen almost simultaneously and only then emits an acoustic signal (Spieth, 1951; Vedenina et al., 2013). Acoustic signals are produced only by males in *D. melanogaster* and by both sexes in the *D. virilis* group; the function of the female signals in the latter case remains obscure (Satokangas et al., 1994; LaRue et al., 2015).

Most researchers (Manning, 1959; Brown, 1965; Cobb et al., 1985, 1989; Liimatainen and Hoikkala, 1998; Hoikkala and Crossley, 2000; Saarikettu et al., 2005; Dankert et al., 2009) described the courtship behavior in *Drosophila* by means of kinematic flow charts showing transitions between the male's courtship elements and the corresponding behavior elements of the female. This method of data representation allows one to estimate the relative frequencies of individual courtship elements and transitions between them. However, this method is best suited to describing sequentially arranged elements that are typical of *D. melanogaster*, whereas in the *D. virilis* group at least three different elements may be concurrent. Therefore, we used a different method of visualizing courtship patterns (Vedenina et al., 2013; Belkina et al., 2016).

The goal of this work was to compare the courtship structure variability in three sibling species of the *D. virilis* group with regard to their phylogenetic relations, by analyzing the video records of courtship behavior in conspecific and heterospecific pairs (i.e., in the so-called con- and heterospecific tests). We used two phylogenetically close species *D. virilis* and *D. lummei* belonging to the *D. virilis* phylad, and also a relatively distantly related species *D. littoralis* included in the *D. montana* phylad (Spicer, 1992, 1993; Spicer and Bell, 2002). *Drosophila littoralis* was represented by two strains whose founders had been collected in the localities of the southern and northern races of the species. Based on allozyme variation and chromosome mutations, some authors earlier suggested that these geographic races should be regarded as subspecies but did not formally describe them (Mitrofanov and Poluektova, 1982; Goncharenko et al.,

1984, 2004; Kulikov et al., 2004; Temkina, 2005; Andrianov et al., 2008). In heterospecific tests, special attention was devoted to determining the key element of the courtship ritual after which courtship stopped. The following combinations were used in heterospecific tests: the southern and northern races of *D. littoralis* as the most closely related forms (for the purpose of our study this combination is regarded as heterospecific even though technically it is not); *D. virilis* and *D. lummei* as phylogenetically close forms not contacting in the nature; *D. lummei* and *D. littoralis* as phylogenetically distant forms co-occurring in the same natural biotopes; *D. virilis* and *D. littoralis* as phylogenetically distant forms not contacting in the nature. In this way, we expected to estimate the level of divergence between the tested species or races by variation in the structure of their courtship rituals and also to determine the role of courtship signals of different modalities in the evolution of this group.

MATERIALS AND METHODS

All the tested *Drosophila* strains were obtained from the collections of Koltzov Institute of Developmental Biology of the Russian Academy of Sciences: *D. virilis* strain 102 originating from flies collected in 1967 in Berlin, Germany; *D. lummei* strain 1109, collected in 1972 in Muonio, Finland; *D. littoralis* strains FP 12-01 and AB-58, collected in 2012 in Moscow, Russia and in 2013 in Pitsunda, Abkhazia, respectively. The flies were cultured on standard semolina-yeast medium in vials 100 mm high and 25 mm in diameter (5–10 ml of food in each), kept in a temperature-controlled room at a daily photoperiod of 12 h of light and 12 h of darkness. Adults were sorted by sex under cold anesthesia 1 day after emergence. Virgin females and unmated males were kept separately in vials with standard food. Testing of the courtship ritual was carried out in the same vials. Each individual was tested only once, after reaching maturation at the age of 14–21 days. Courtship behavior was studied by the video-typing method: all the interactions between the insects were recorded with a Sony HDR-SR12E camcorder (Japan) and analyzed using VirtualDub 1.10.3 software. If the male showed no interest in the female within 30 min after the beginning of test, the pair was separated. If courtship started, the behavior of the pair was recorded until copulation or for 30 min after the first courtship element.

For each pair, we measured the total duration of each behavior element and the total duration of court-

ship from the beginning of its first element to the beginning of copulation, not counting breaks longer than 1 min. Measurements were carried out independently by two researchers to reduce the possible errors. Eight courtship elements were distinguished: the male following the female, the male touching the female's abdomen, the male licking the female's genitalia, the male singing, the female singing, the male circling around the female, an attempt at copulation, and copulation. The category of "copulation attempts" included only those cases when the male mounted the female for less than a minute without the subsequent proper copulation. The term "singing" refers herein to the production of acoustic signals. The male sings by vibrating one of its wings abducted almost at the right angle to the body axis; the female sings while holding both its wings slightly sideways. Acoustic signals were not recorded in this study since simultaneous audio and video recording was impossible. However, the moments of wing abduction were always easily detectable in the video records, and these behavior elements were interpreted as singing.

The durations of the courtship elements and their latency periods (from the beginning of courtship to that of each individual element) were calculated in Microsoft Excel. Data were statistically processed using Microsoft Excel and Statistica software: the frequencies of courtship elements were compared by Fisher's exact test, and the mean durations of the elements and their latency periods, by Student's test after normalizing transformation. Species were compared by the overall structure of their courtship ritual using MANOVA with Wilks' statistic (λ_{Wilks}).

RESULTS

Courtship Behavior in Conspecific Tests

The main courtship elements in conspecific tests of the studied sibling species of the *D. virilis* group were touching and licking: these elements were the longest and were recorded in nearly 100% of the trials. Touching and licking occurred almost simultaneously and were accompanied by acoustic signals produced by both sexes. However, the duration of singing was shorter than that of touching or licking.

Comparison of courtship behavior in the sibling species *D. virilis* and *D. lummei* did not reveal any significant differences in the frequencies of individual elements and in the total courtship pattern ($\lambda_{\text{Wilks}} = 0.404$, d.f.1 = 2, d.f.2 = 1, $F = 0.74$, $p = 0.636$) (Table 1;

Figs. 1a, 1b). During prolonged touching (on average 83 s in *D. virilis* and 108 s in *D. lummei*) and licking (on average 66 and 95 s, respectively), the males occasionally emitted acoustic signals with average duration of 25 and 36 s, respectively. The circling started considerably later than the above elements and was brief: on average 12 and 10 s, respectively. The male followed the female also for a short time, on average 10 and 7 s, respectively. At the same time, the percentage of copulations was significantly lower in *D. lummei* than in *D. virilis* ($p = 0.00001$).

The courtship behavior of *D. littoralis* (Figs. 2a, 2b) was significantly different from that of *D. virilis* and *D. lummei* ($\lambda_{\text{Wilks}} = 0.237$, d.f.1 = 7, d.f.2 = 10, $F = 8.90$, $p = 0.015$): the four main elements (touching, licking, male and female singing) were significantly shorter in *D. littoralis* than in the two other species ($p < 0.003$). No differences in the duration of the courtship elements were observed between the two races of *D. littoralis* ($\lambda_{\text{Wilks}} = 0.043$, d.f.1 = 7, d.f.2 = 2, $F = 6.35$, $p = 0.143$) (Table 2; Figs. 2a, 2b); only the copulation proper was significantly longer in the northern (6 min 47 s) than in the southern race (4 min 46 s) ($t = 4.59$, d.f. = 47, $p = 0.00003$). Differences were also observed in the duration of the latency periods: licking, singing, and circling by males and singing by females started earlier in the southern race of *D. littoralis* than in the northern race (Table 3; Figs. 2a, 2b).

Significant correlations (0.65–0.84) in duration but not in latency were revealed between female singing and two elements of male courtship, namely licking and singing.

Courtship Behavior in Heterospecific Tests

Heterospecific Tests with Two Races of *D. littoralis*

In heterospecific tests with different races of *D. littoralis*, the courtship ritual almost invariably included three main elements: touching, licking, and singing. Most heterospecific tests resulted in copulation. In this respect, behavior was similar in con- and heterospecific tests. Significant differences between con- and heterospecific tests were observed in the duration and latencies of certain courtship elements. In particular, females of the northern and southern races paired with heterospecific males had a significantly greater duration of singing as compared with the conspecific test for the northern race ($p < 0.011$).

Table 1. Frequency of individual courtship elements in con- and heterospecific tests with three sibling species of the *Drosophila virilis* group

Combination	Number of trials	Number (in parentheses: percentage) of trials in which the given courtship element was recorded							
		following	touching	licking	male singing	circling	copulation attempts	copulation	female singing
♀ + ♂ <i>virilis</i>	30	17 (57)	30 (100)	30 (100)	30 (100)	12 (40)	9 (30)	26 (87)	29 (97)
♀ + ♂ <i>lummei</i>	30	19 (63)	30 (100)	30 (100)	29 (97)	17 (57)	12 (40)	10 (33)	28 (93)
♀ + ♂ <i>littoralis</i> (NR)	30	21 (70)	30 (100)	29 (97)	29 (97)	6 (20)	2 (7)	26 (87)	28 (93)
♀ + ♂ <i>littoralis</i> (SR)	30	18 (60)	30 (100)	30 (100)	29 (97)	12 (40)	3 (10)	23 (77)	30 (100)
♀ <i>virilis</i> + ♂ <i>lummei</i>	30	3 (10)	30 (100)	13 (43)	8 (27)	5 (17)	3 (10)	4 (13)	13 (43)
♀ <i>lummei</i> + ♂ <i>virilis</i>	30	4 (13)	30 (100)	12 (37)	10 (33)	0 (0)	2 (7)	1 (3)	12 (37)
♀ <i>littoralis</i> (NR) + ♂ <i>littoralis</i> (SR)	30	21 (70)	30 (100)	30 (100)	29 (97)	12 (40)	1 (3)	21 (70)	28 (93)
♀ <i>littoralis</i> (SR) + ♂ <i>littoralis</i> (NR)	30	23 (77)	30 (100)	30 (100)	30 (100)	15 (50)	7 (23)	19 (63)	29 (97)
♀ <i>lummei</i> + ♂ <i>littoralis</i> (NR)	30	27 (90)	30 (100)	26 (87)	22 (73)	13 (43)	11 (37)	0 (0)	22 (73)
♀ <i>littoralis</i> (NR) + ♂ <i>lummei</i>	30	0 (0)	30 (100)	1 (3)	1 (3)	0 (0)	0 (0)	1 (3)	1 (3)
♀ <i>lummei</i> + ♂ <i>littoralis</i> (SR)	30	23 (77)	30 (100)	22 (73)	21 (70)	10 (33)	7 (23)	0 (0)	25 (83)
♀ <i>littoralis</i> (SR) + ♂ <i>lummei</i>	30	1 (3)	30 (100)	4 (13)	2 (7)	0 (0)	0 (0)	0 (0)	1 (3)
♀ <i>virilis</i> + ♂ <i>littoralis</i> (NR)	30	16 (53)	30 (100)	26 (87)	26 (87)	14 (47)	9 (30)	2 (7)	26 (87)
♀ <i>littoralis</i> (NR) + ♂ <i>virilis</i>	30	2 (7)	30 (100)	2 (7)	3 (10)	0 (0)	0 (0)	0 (0)	4 (13)
♀ <i>virilis</i> + ♂ <i>littoralis</i> (SR)	30	16 (53)	30 (100)	27 (90)	27 (90)	17 (57)	13 (43)	1 (3)	28 (93)
♀ <i>littoralis</i> (SR) + ♂ <i>virilis</i>	30	16 (53)	29 (97)	9 (30)	7 (23)	1 (3)	0 (0)	0 (0)	12 (40)

NR, northern race of *D. littoralis*; SR, southern race of *D. littoralis*.

The activity patterns of the northern and southern males remained the same regardless of whether they were courting con- or heterospecific females. In particular, males of the northern race of *D. littoralis* on average started singing and circling later ($p < 0.011$), whereas males of the southern race started singing and licking earlier ($p < 0.0027$). Thus, the specific features of male behavior at least partly determined the structure of the courtship ritual in both con- and heterospecific tests. This was also true of heterospecific tests in which males of both races of *D. littoralis* were paired with females of *D. virilis* and *D. lummei*. In addition, females of the northern race started singing significantly earlier in the heterospecific test than in the conspecific test of the northern race ($p = 0.0487$) (Table 3; Fig. 2).

Comparison of the reciprocal variants revealed only one difference in the latency period: males of the northern race of *D. littoralis* courting females of the

southern race started singing later ($p = 0.0027$) than in the reciprocal variant (Table 3; Fig. 2). No differences in the element duration were observed between these variants.

Female singing was found to be significantly correlated with male licking and singing, both in latency (0.59–0.68) and in duration (0.50–0.88).

Heterospecific Tests with *D. virilis* and *D. lummei*

More than a half of heterospecific trials with *D. virilis* and *D. lummei* ended at the touching stage (Table 1). Males of *D. lummei* courting females of *D. virilis* showed significantly shorter durations of touching and licking ($p < 0.0016$) as compared with the conspecific tests of *D. virilis* and *D. lummei* (Table 2; Fig. 1c). In their turn, *D. virilis* females had a significantly shorter duration of singing when paired with *D. lummei* males ($t = -2.82$, d.f. = 40, $p = 0.0075$) than with conspecific

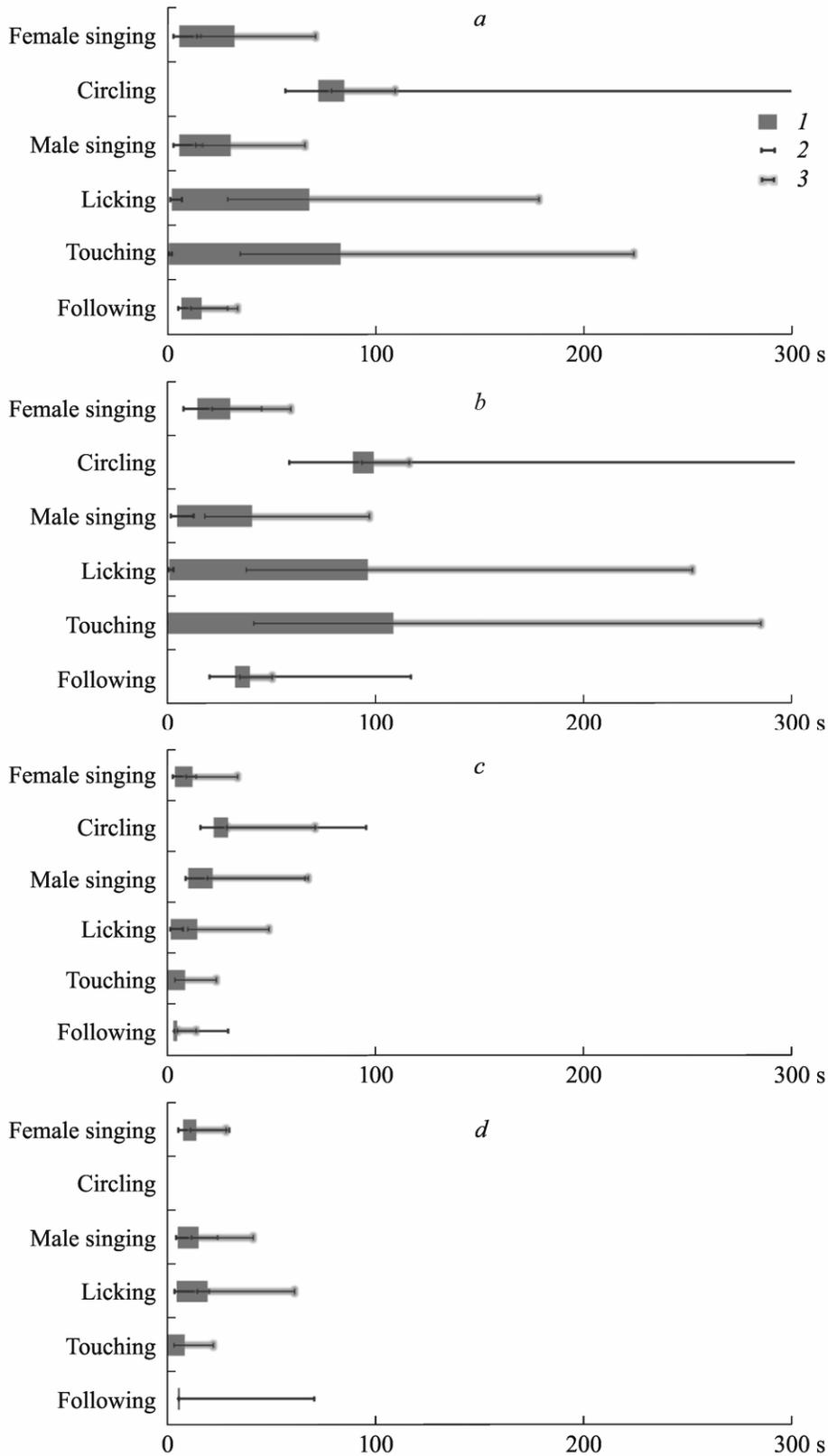


Fig. 1. Latency periods and duration of courtship elements in *D. virilis* and *D. lummei*: (a) ♀ *D. virilis* + ♂ *D. virilis*; (b) ♀ *D. lummei* + ♂ *D. lummei*; (c) ♀ *D. virilis* + ♂ *D. lummei*; (d) ♀ *D. lummei* + ♂ *D. virilis*: 1 (boxes), the duration of the given element (the mean timing of its beginning and end); 2 (bolder lines), 95% confidence interval for the mean latency; 3 (highlighted thin lines), 95% confidence interval for the mean duration.

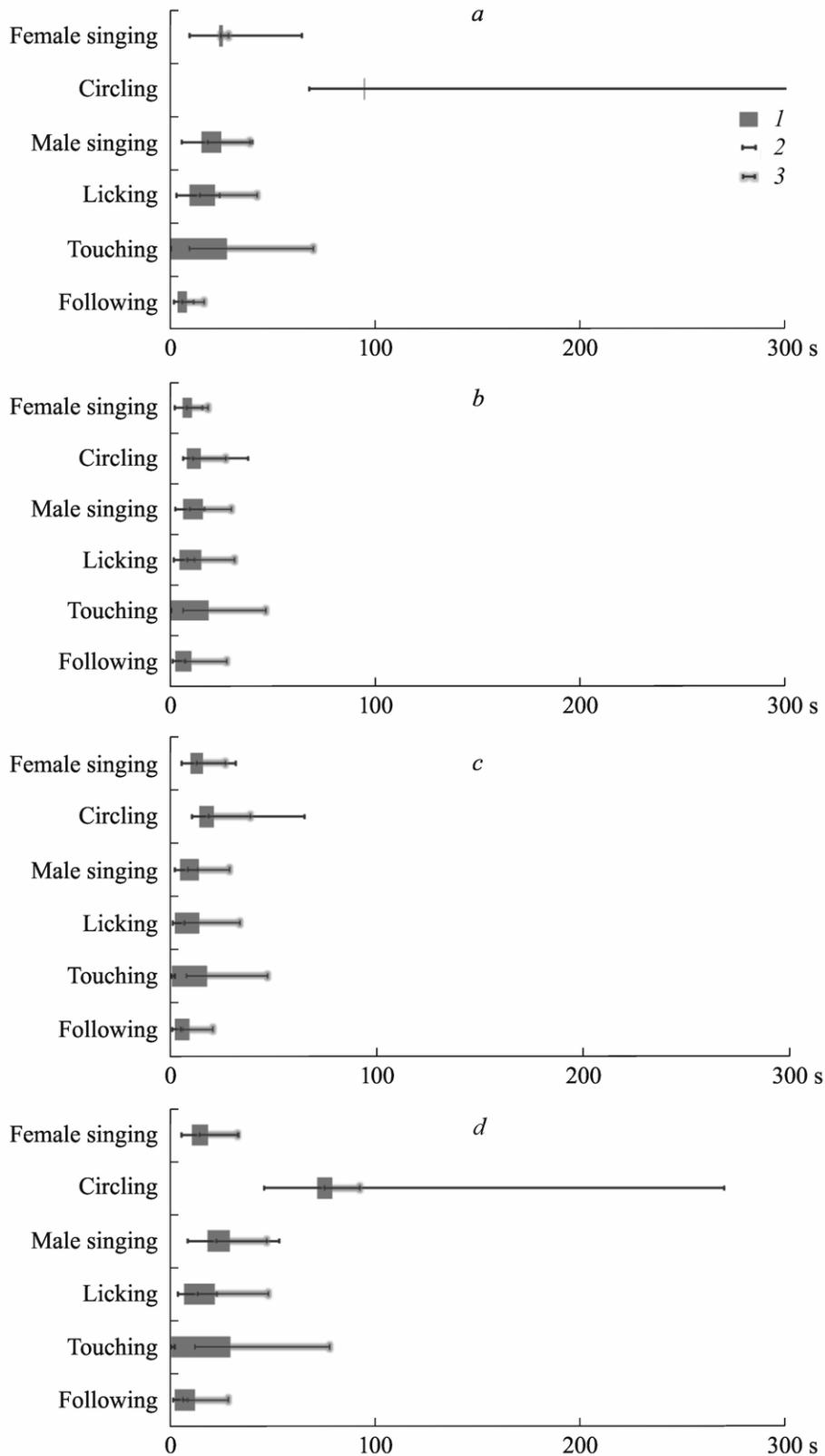


Fig. 2. Latency periods and duration of courtship elements in *D. littoralis* (northern and southern races): (a) ♀ *D. littoralis* (NR) + ♂ *D. littoralis* (NR); (b) ♀ *D. littoralis* (SR) + ♂ *D. littoralis* (SR); (c) ♀ *D. littoralis* (NR) + ♂ *D. littoralis* (SR); (d) ♀ *D. littoralis* (SR) + ♂ *D. littoralis* (NR): 1 (boxes), the duration of the given element (the mean timing of its beginning and end); 2 (bolder lines), 95% confidence interval for the mean latency; 3 (highlighted thin lines), 95% confidence interval for the mean duration.

Table 2. Duration of courtship elements (in seconds; mean \pm standard error) in three sibling species of the *Drosophila virilis* group

Combination	Following	Touching	Licking	Male singing	Circling	Copulation attempts	Copulation	Female singing
♀ + ♂ <i>virilis</i>	9.59 \pm 0.11	82.6 \pm 0.11	66.04 \pm 0.11	24.60 \pm 0.08	12.35 \pm 0.13	6.66 \pm 0.17	151.77 \pm 0.02	26.52 \pm 0.08
♀ + ♂ <i>lummei</i>	7.26 \pm 0.07	108.2 \pm 0.10	94.95 \pm 0.10	35.62 \pm 0.09	9.92 \pm 0.10	4.75 \pm 0.09	178.29 \pm 0.01	15.85 \pm 0.12
♀ + ♂ <i>littoralis</i> (NR)	4.01 \pm 0.08	24.14 \pm 0.11	28.74 \pm 0.17	18.74 \pm 0.13	11.54 \pm 0.20	3.00 \pm 0.30	406.54 \pm 0.02	16.74 \pm 0.14
♀ + ♂ <i>littoralis</i> (SR)	7.88 \pm 0.15	18.59 \pm 0.08	10.59 \pm 0.08	9.50 \pm 0.08	6.94 \pm 0.10	4.14 \pm 0.27	286.42 \pm 0.03	4.66 \pm 0.09
♀ <i>virilis</i> + ♂ <i>lummei</i>	1.71 \pm 0.13	8.66 \pm 0.11	12.82 \pm 0.18	11.69 \pm 0.24	7.01 \pm 0.26	6.61 \pm 0.14	182.74 \pm 0.03	8.46 \pm 0.17
♀ <i>lummei</i> + ♂ <i>virilis</i>	1.00	8.43 \pm 0.09	14.83 \pm 0.19	9.91 \pm 0.18	—	1.83 \pm 0.15	65.00	6.48 \pm 0.14
♀ <i>littoralis</i> (NR) + ♂ <i>littoralis</i> (SR)	7.06 \pm 0.09	17.22 \pm 0.11	11.96 \pm 0.10	9.04 \pm 0.10	7.05 \pm 0.16	2.00	286.43 \pm 0.02	5.81 \pm 0.12
♀ <i>littoralis</i> (SR) + ♂ <i>littoralis</i> (NR)	9.95 \pm 0.09	28.96 \pm 0.11	14.97 \pm 0.11	11.03 \pm 0.09	7.36 \pm 0.11	2.45 \pm 0.14	311.17 \pm 0.02	7.97 \pm 0.11
♀ <i>lummei</i> + ♂ <i>littoralis</i> (NR)	7.71 \pm 0.07	63.58 \pm 0.12	45.61 \pm 0.12	35.68 \pm 0.11	10.58 \pm 0.12	3.53 \pm 0.10	—	14.69 \pm 0.12
♀ <i>littoralis</i> (NR) + ♂ <i>lummei</i>	—	2.19 \pm 0.04	9.00	3.00	—	—	204.00	1.00
♀ <i>lummei</i> + ♂ <i>littoralis</i> (SR)	5.04 \pm 0.06	29.87 \pm 0.12	30.88 \pm 0.14	21.07 \pm 0.12	14.71 \pm 0.11	2.64 \pm 0.13	—	5.34 \pm 0.10
♀ <i>littoralis</i> (SR) + ♂ <i>lummei</i>	1.00	3.05 \pm 0.07	8.79 \pm 0.11	2.46 \pm 0.23	—	—	—	7.00
♀ <i>virilis</i> + ♂ <i>littoralis</i> (NR)	9.76 \pm 0.11	55.50 \pm 0.09	28.54 \pm 0.11	19.98 \pm 0.08	8.71 \pm 0.11	4.27 \pm 0.13	214.94 \pm 0.03	18.10 \pm 0.10
♀ <i>littoralis</i> (NR) + ♂ <i>virilis</i>	1.83 \pm 0.15	4.30 \pm 0.07	7.12 \pm 0.43	2.17 \pm 0.20	—	—	—	7.38 \pm 0.26
♀ <i>virilis</i> + ♂ <i>littoralis</i> (SR)	6.79 \pm 0.11	69.36 \pm 0.10	51.29 \pm 0.10	33.96 \pm 0.08	18.14 \pm 0.09	5.61 \pm 0.11	343.00	20.71 \pm 0.11
♀ <i>littoralis</i> (SR) + ♂ <i>virilis</i>	2.99 \pm 0.07	10.78 \pm 0.09	8.60 \pm 0.25	7.91 \pm 0.21	1.00	—	—	8.16 \pm 0.17

Dash indicates the absence of the given courtship element; NR, northern race of *D. littoralis*; SR, southern race of *D. littoralis*.

Table 3. Latency of courtship elements (in seconds; mean \pm standard error) in three sibling species of the *Drosophila virilis* group

Combination	Following	Touching	Licking	Male singing	Circling	Copulation attempts	Copulation	Female singing
♀ + ♂ <i>virilis</i>	6.8 \pm 0.22	0.61 \pm 0.09	2.24 \pm 0.12	5.78 \pm 0.12	72.6 \pm 0.29	142.30 \pm 0.17	150.31 \pm 0.13	5.63 \pm 0.12
♀ + ♂ <i>lummei</i>	32.55 \pm 0.19	0.04 \pm 0.02	1.14 \pm 0.07	5.04 \pm 0.08	88.96 \pm 0.22	78.38 \pm 0.19	140.89 \pm 0.19	14.52 \pm 0.15
♀ + ♂ <i>littoralis</i> (NR)	2.59 \pm 0.11	1.76 \pm 0.13	15.61 \pm 0.15	17.03 \pm 0.15	98.99 \pm 0.32	117.32 \pm 0.68	57.25 \pm 0.10	13.93 \pm 0.15
♀ + ♂ <i>littoralis</i> (SR)	2.62 \pm 0.11	0.24 \pm 0.04	4.68 \pm 0.08	6.5 \pm 0.09	8.13 \pm 0.24	15.73 \pm 0.27	24.26 \pm 0.12	6.10 \pm 0.09
♀ <i>virilis</i> + ♂ <i>lummei</i>	3.16 \pm 0.19	0.00	1.71 \pm 0.17	10.09 \pm 0.30	22.12 \pm 0.16	105.03 \pm 0.18	33.38 \pm 0.14	3.80 \pm 0.17
♀ <i>lummei</i> + ♂ <i>virilis</i>	5.19 \pm 0.32	0.00	4.59 \pm 0.21	5.18 \pm 0.23	–	43.90 \pm 0.33	7.00	7.61 \pm 0.20
♀ <i>littoralis</i> (NR) + ♂ <i>littoralis</i> (SR)	2.20 \pm 0.09	0.69 \pm 0.09	2.19 \pm 0.12	4.75 \pm 0.11	14.14 \pm 0.23	25.00	18.04 \pm 0.12	9.96 \pm 0.15
♀ <i>littoralis</i> (SR) + ♂ <i>littoralis</i> (NR)	2.71 \pm 0.14	0.79 \pm 0.11	7.16 \pm 0.15	18.62 \pm 0.13	72.10 \pm 0.20	50.16 \pm 0.25	50.68 \pm 0.14	11.00 \pm 0.15
♀ <i>lummei</i> + ♂ <i>littoralis</i> (NR)	11.96 \pm 0.16	0.08 \pm 0.02	13.32 \pm 0.16	20.36 \pm 0.15	132.84 \pm 0.22	216.68 \pm 0.17	–	25.36 \pm 0.17
♀ <i>littoralis</i> (NR) + ♂ <i>lummei</i>	–	0.00	2.00	3.00	–	11.00	9.00	9.00
♀ <i>lummei</i> + ♂ <i>littoralis</i> (SR)	9.04 \pm 0.15	0.16 \pm 0.06	7.41 \pm 0.15	15.54 \pm 0.19	83.04 \pm 0.24	126.45 \pm 0.23	–	30.61 \pm 0.16
♀ <i>littoralis</i> (SR) + ♂ <i>lummei</i>	1.00	0.00	7.80 \pm 0.59	3.47 \pm 0.35	–	–	–	25.00
♀ <i>virilis</i> + ♂ <i>littoralis</i> (NR)	14.12 \pm 0.21	0.21 \pm 0.06	18.15 \pm 0.16	23.53 \pm 0.15	57.52 \pm 0.20	96.78 \pm 0.21	72.84 \pm 0.20	12.32 \pm 0.13
♀ <i>littoralis</i> (NR) + ♂ <i>virilis</i>	1.45 \pm 0.09	0.00	58.00	18.62 \pm 0.50	–	–	–	11.71 \pm 0.36
♀ <i>virilis</i> + ♂ <i>littoralis</i> (SR)	14.12 \pm 0.21	0.21 \pm 0.06	18.15 \pm 0.16	23.53 \pm 0.15	57.52 \pm 0.20	67.32 \pm 0.16	17.00	12.32 \pm 0.13
♀ <i>littoralis</i> (SR) + ♂ <i>virilis</i>	5.12 \pm 0.17	0.15 \pm 0.04	5.84 \pm 0.22	30.58 \pm 0.28	1.00	–	–	15.34 \pm 0.19

Dash indicates the absence of the given courtship element; NR, northern race of *D. littoralis*; SR, southern race of *D. littoralis*.

males. The reciprocal variant ♀ *D. lummei* + ♂ *D. virilis* showed significantly shorter durations of all the courtship elements ($p < 0.0326$) except copulation attempts, as compared with the conspecific tests of *D. virilis* and *D. lummei* (Table 2; Fig. 1d). Besides, females of *D. lummei* paired with males of *D. virilis* had a significantly shorter duration of singing than females of *D. virilis* in the conspecific test ($t = -3.70$, d.f. = 39, $p = 0.0007$). The number of copulations was significantly smaller in heterospecific than in conspecific tests (Table 1). No differences in element latency were revealed between the con- and heterospecific tests (Table 3), and no significant differences were observed between the reciprocal tests either.

Analysis of correlations between female singing and male courtship elements showed significant and high correlations between the duration of female singing and that of male licking and singing (0.78–0.97). As concerns element latencies, such a correlation was observed only for females of *D. lummei* paired with males of *D. virilis* (0.93–0.95).

Heterospecific Tests with *D. lummei* and *D. littoralis*

Unlike the above heterospecific tests, considerable differences were observed between the reciprocal combinations of *D. lummei* and *D. littoralis*. As a rule, when *D. lummei* males were paired with *D. littoralis* females, courtship stopped at the touching stage and the other courtship elements were recorded only in single trials (Table 1). By contrast, *D. littoralis* males usually demonstrated a complete courtship ritual toward *D. lummei* females. This was observed in both races of *D. littoralis*. The difference between *D. lummei* and *D. littoralis* males in the duration of touching was found to be highly significant ($p < 0.001$) (Table 2; Fig. 3). At the same time, the number of copulations was very small in all the four heterospecific tests with *D. lummei* and *D. littoralis* (Table 1).

Comparison of heterospecific and conspecific tests of *D. lummei* and *D. littoralis* showed that *D. littoralis* males courted *D. lummei* females longer than conspecific females (Table 2). This trend was more pronounced in males of the northern race of *D. littoralis*, which showed longer durations of touching, licking, and singing when paired with *D. lummei* females than with conspecific females of the same race ($p < 0.018$). Females of *D. lummei* also demonstrated a greater duration of singing ($p = 0.000001$) in heterospecific tests than in conspecific ones. Males of the southern

race licked *D. lummei* females significantly longer than conspecific females ($p = 0.007$). By contrast, males of *D. lummei* courting females of *D. littoralis*, especially those of the southern race, showed much lower activity than in conspecific tests with *D. lummei*. All the courtship elements demonstrated by *D. lummei* males in heterospecific tests were considerably shorter than in conspecific tests ($p < 0.009$). The singing duration in females of *D. lummei* and both races of *D. littoralis* was the same in conspecific tests, whereas *D. lummei* females sang significantly less actively when paired with males of the southern race of *D. littoralis* ($p = 0.009$).

Heterospecific Tests with *D. virilis* and *D. littoralis*

The structure of heterospecific courtship of *D. virilis* and *D. littoralis* was largely the same as in the preceding pair of species. Particularly notable was the sharp distinction between the results of reciprocal tests. Similar to *D. lummei*, males of *D. virilis* stopped courting females of both races of *D. littoralis* immediately after the first touching acts. Males of *D. virilis* were also less active toward females of the northern than of the southern race of *D. littoralis*. By contrast, males of both races of *D. littoralis* remained active and demonstrated a complete courtship ritual when paired with *D. virilis* females. Males of *D. virilis* showed a significantly shorter duration of nearly all the courtship elements with females of *D. littoralis* ($p < 0.017$), as compared with males of *D. littoralis* courting females of *D. virilis* ($p < 0.029$; see Table 2, Fig. 4). Males of the southern race of *D. littoralis* were more active than those of the northern race.

It is interesting that the duration of singing in *D. virilis* females courted by *D. littoralis* males was almost the same as in the conspecific combination, whereas females of the northern race of *D. littoralis* sang even more actively when paired with *D. virilis* males than with conspecific males. The number of copulations was very small in all the four heterospecific tests with *D. virilis* and *D. littoralis* (Table 1).

Comparison of heterospecific tests of *D. virilis* and *D. littoralis* with the corresponding conspecific tests demonstrated considerable differences both in duration and in latency. In particular, nearly all the courtship elements were longer in *D. littoralis* males paired with *D. virilis* females as compared with conspecific females (Table 2).

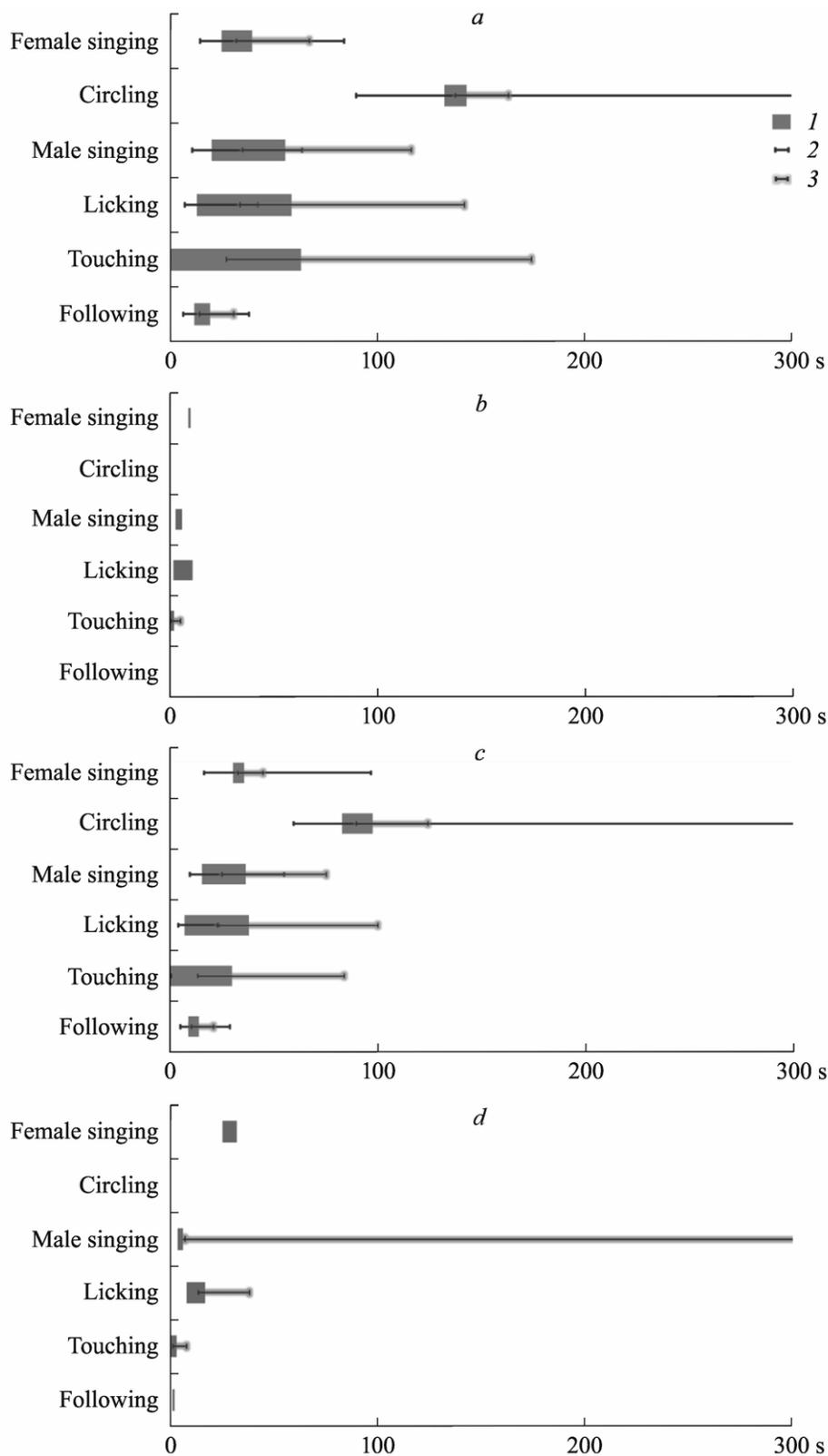


Fig. 3. Latency periods and duration of courtship elements in *D. lummei* and *D. littoralis*: (a) ♀ *D. lummei* + ♂ *D. littoralis* (NR); (b) ♀ *D. littoralis* (NR) + ♂ *D. lummei*; (c) ♀ *D. lummei* + ♂ *D. littoralis* (SR); (d) ♀ *D. littoralis* (SR) + ♂ *D. lummei*: 1 (boxes), the duration of the given element (the mean timing of its beginning and end); 2 (bolder lines), 95% confidence interval for the mean latency; 3 (highlighted thin lines), 95% confidence interval for the mean duration.

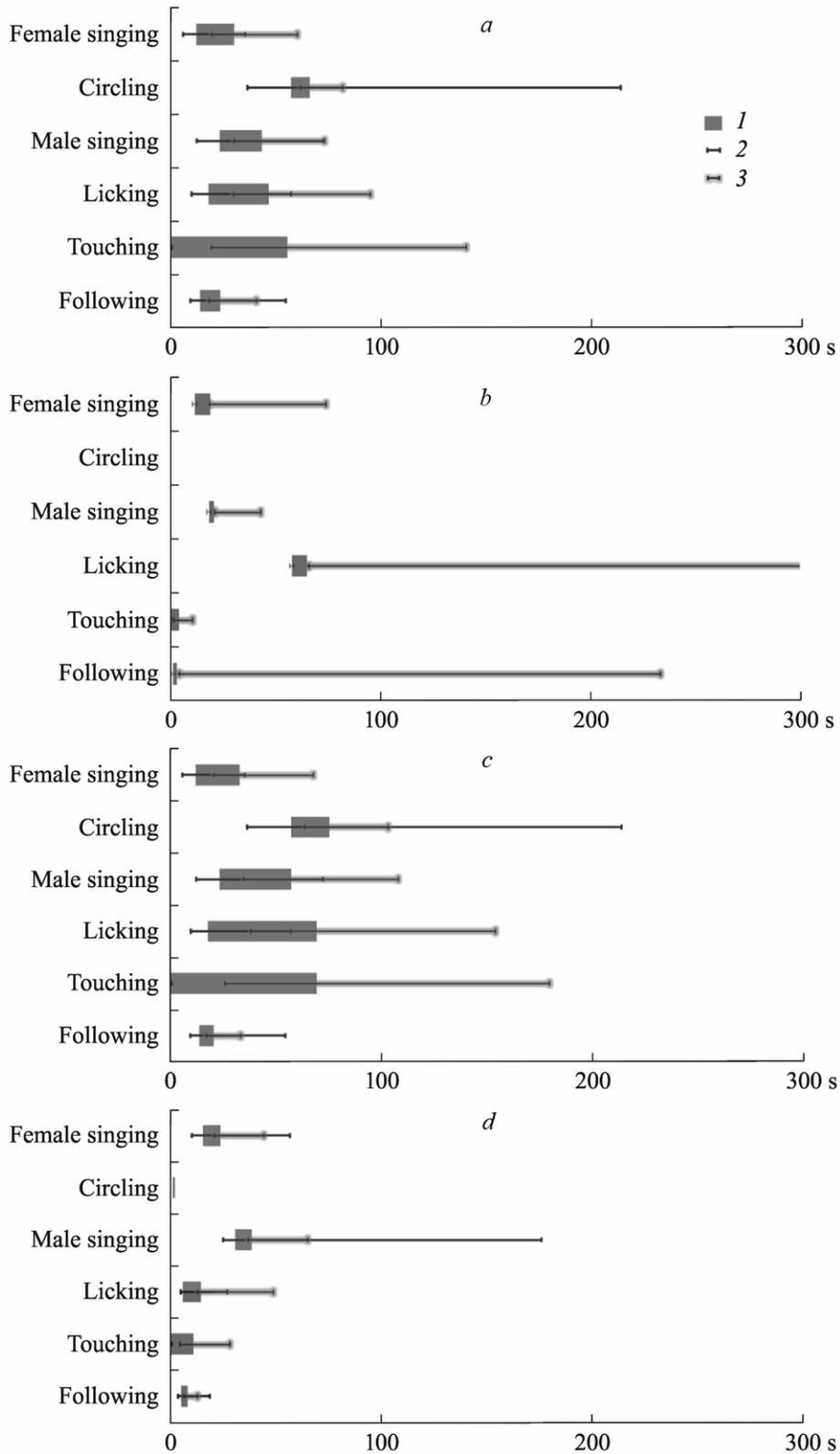


Fig. 4. Latency periods and duration of courtship elements in *D. virilis* and *D. littoralis*: (a) ♀ *D. virilis* + ♂ *D. littoralis* (NR); (b) ♀ *D. littoralis* (NR) + ♂ *D. virilis*; (c) ♀ *D. virilis* + ♂ *D. littoralis* (SR); (d) ♀ *D. littoralis* (SR) + ♂ *D. virilis*: 1 (boxes), the duration of the given element (the mean timing of its beginning and end); 2 (bolder lines), 95% confidence interval for the mean latency; 3 (highlighted thin lines), 95% confidence interval for the mean duration.

Analysis of correlations between female singing and male courtship elements showed significant and relatively high correlations between female singing and male licking and singing, both in latency (0.50–0.83) and in duration (0.65–0.84), but only in the combinations ♂ *D. littoralis* + ♀ *D. virilis* or *D. lummei*. In reciprocal tests licking and male singing rarely occurred, and the corresponding data were not sufficient for analysis.

DISCUSSION

Comparative analysis of the courtship structure in three closely related species *D. virilis*, *D. lummei*, and *D. littoralis* showed that touching and licking were the longest elements of the courtship ritual in all the conspecific combinations. These results agree with the data obtained earlier for other species and strains (Spieth, 1951; Vedenina et al., 2013; Belkina et al., 2016). The two dominant elements were usually demonstrated simultaneously and were accompanied by the courtship songs of both sexes, which formed the second longest pair of courtship elements. The same was observed earlier for other strains of *D. virilis* (Saarikettu et al., 2005). Male and female singing started considerably later than touching and licking in all the studied species, and male singing usually did not interrupt touching and licking. The shortest courtship elements in the three studied species were following and circling. The above trends were common to all the studied species and races. At the same time, *D. virilis* and *D. lummei*, belonging to the same phylad, significantly differed from both races of *D. littoralis* (a species from a different phylad) in practically all the courtship elements.

The Role of Courtship Signals in Reproductive Isolation of D. virilis and D. lummei

Analysis of the results of conspecific tests of *D. virilis* and *D. lummei* revealed no significant differences in the duration or latencies of individual courtship elements. Therefore, the two species have a similar structure of the courtship ritual. At the same time, analysis of heterospecific courtships in the combinations ♀ *D. virilis* + ♂ *D. lummei* and ♀ *D. lummei* + ♂ *D. virilis* showed, first, that courtship stopped at the touching stage in more than half the trials and, second, that courtship elements at the touching and licking stage were much shorter. In our earlier experiments (Vedenina et al., 2013), males of these two species were more active in heterospecific tests; only in the

combination ♀ *D. virilis* + ♂ *D. lummei* the duration of touching and licking was significantly shorter while the frequency of licking was considerably lower than in conspecific tests. However, the earlier experiments were carried out with different fly strains, which may explain such discrepancy in the results.

According to the previous data (Spieth, 1951; Vedenina et al., 2013), the male touching the female's abdomen performs back-and-forth movements with its forelegs. The abdominal segments of *Drosophila* flies bear numerous mechanosensory setae (Fabre et al., 2008) which may activate during male touching. The function of prolonged licking is less clear. Since copulation is never attempted without previous licking, we may suppose that during this courtship element the male receives some important signal from the female. It is known for *D. melanogaster* that in case of successful courtship the female releases a droplet of secretion at the tip of its ovipositor, and it is from this secretion that the male receives the signal required for attempting copulation (Lasbleiz et al., 2006).

Spieth (1951) and Vedenina with co-authors (2013) showed that males of sibling species of the *D. virilis* group often intensively licked not only the female's genitalia but also its abdominal sternites; this was confirmed in the present study. Such active mechanical stimulation may trigger the release of pheromones from some still unknown gland positioned close to the ovipositor, in the same way as in *D. melanogaster*. The absence of an acceptance signal from a heterospecific female may have caused considerable reduction of the licking stage and subsequent courtship elements in the heterospecific tests with *D. virilis* and *D. lummei*. The noticeable shortening of the touching stage in heterospecific tests indicates that the signal is received by the male soon after the beginning of this stage.

It may be concluded that chemical signals play the key role in reproductive isolation between *D. virilis* and *D. lummei*. The most likely signal molecules are cuticular hydrocarbon compounds since their profiles are species- and sex-specific in *D. virilis* and *D. lummei* (Bartelt et al., 1986; Oguma et al., 1992; Liimatainen and Jallon, 2007). These low-volatile hydrocarbons are detected by contact chemoreceptors located on the foreleg tarsi, palps, and proboscis of flies. Besides, they are known to be present on the front edge of the wings and near the ovipositor (Stocker, 1994). It is quite possible that during prolonged touch-

ing and licking the female receives strong tactile stimulation and possibly also chemical one (at least that of the chemoreceptors surrounding its ovipositor), which may increase its receptivity.

*Is Divergence Taking Place in *D. littoralis*?*

Comparison of conspecific tests with the northern and southern races of *D. littoralis* showed differences in the length of copulation and the latencies of some courtship elements: these elements started earlier in flies of the southern race. The observed differences in the courtship structure partly agree with the results of studying other characters in the two races of *D. littoralis*. For example, variation in some quantitative characters, such as the shape of the wing and the male phallus, indicates intraspecific divergence between the northern and southern races of *D. littoralis* (Mitrofanov and Poluektova, 1982; Goncharenko et al., 1984, 2004; Kulikov et al., 2004; Temkina, 2005; Andrianov et al., 2008). The cited authors suggested that the differences could be determined both by adaptive trends and by genetic isolation, since the populations are separated by the Caucasus Mountains (Andrianov et al., 2008).

However, our analysis of reciprocal heterospecific tests with the northern and southern races of *D. littoralis* showed the absence of obstacles to successful copulation. Thus, despite differentiation in the courtship structure, no precopulatory barriers were found between the geographic races of *D. littoralis*. Future studies of the postcopulatory barriers will probably shed light on the situation. In particular, the fertilization success rate may depend on the morphological differences in the male phalli.

*The Role of Courtship Signals in Reproductive Isolation between Members of Different Phylads of the *D. virilis* Group*

All the eight heterospecific combinations of *D. lummei* + *D. littoralis* and *D. virilis* + *D. littoralis* were characterized by very low incidence of copulation. At the same time, males behaved in essentially different ways in the reciprocal trials. Males of the *D. virilis* phylad lost interest in *D. littoralis* females immediately after the beginning of touching. By contrast, males of two races of *D. littoralis* performed the complete courtship ritual when paired with females of both *D. lummei* and *D. virilis*. This result demonstrates low abilities of *D. littoralis* males to recognize females from a different phylad. In the study of Liimatainen

and Hoikkala (1998), males of *D. littoralis* also actively courted females of *D. lummei* but showed much higher selectivity with respect to females of *D. montana* belonging to the same phylad. It was earlier determined that the hydrocarbon profiles of *D. littoralis* and *D. lummei* were similar while the profile of *D. montana* was different from either of them (Bartelt et al., 1986). However, in the case of *D. littoralis* and *D. lummei* one would expect low selectivity in males of both species, rather than asymmetrical courtship that was observed in our reciprocal tests. No sex dimorphism in hydrocarbon composition was revealed for these species (Bartelt et al., 1986). At the same time, asymmetrical courtship was also observed in these species by Liimatainen and Hoikkala (1998). It should be also noted that *D. littoralis* and *D. lummei* co-occur in the same natural biotopes; therefore we had every reason to expect equally high selectivity of the two species in reciprocal tests. The current results will probably be interpreted only after identification of the key hydrocarbons involved in conspecific recognition in these species. In our opinion, asymmetry in the reciprocal tests of *D. virilis* + *D. littoralis* can be more easily explained. The former species is characterized by sex dimorphism in the cuticular hydrocarbon profiles (Bartelt et al., 1986); moreover, the difference between the profiles of *D. virilis* males and *D. littoralis* females is greater than the difference in the reciprocal combination. It is therefore quite natural that in our tests, *D. virilis* males lost interest in *D. littoralis* females immediately after the start of touching.

In any event, we may conclude that divergence in signals of different modalities proceeds in different ways even within one group of *Drosophila* flies. Besides, the similar results of heterospecific tests of *D. virilis* + *D. littoralis* (not contacting in the nature) and *D. lummei* + *D. littoralis* (contacting in the nature) indicate that in this particular case sympatry does not influence the evolution of courtship signals.

*The Role of Female Acoustic Signals in the Courtship Ritual in the *D. virilis* Group*

There are conflicting opinions concerning the function of female acoustic signals in species of the *D. virilis* group. Some authors believe that females emit acoustic signals when refusing the courting male while others, on the contrary, consider the female song to be a signal stimulating further courtship (Donegan and Ewing, 1980; Liimatainen et al., 1998). The estimated occurrence of female songs in species of *D. vi-*

rilis group also varies. According to some data, females of the species studied herein have low acoustic activity, the total duration of singing comprising only 2 to 7% of the whole courtship ritual (Satokangas et al., 1994). By contrast, according to the relatively recent study of acoustic duetting in *D. virilis* (LaRue et al., 2015), females sing very actively and respond to the male songs. A study of the courtship behavior in *D. montana*, *D. lummei*, and *D. littoralis* showed that the female started singing only in response to licking when courted by a conspecific male, and already during touching when courted by a heterospecific male (Liimatainen and Hoikkala, 1998).

These discrepancies may be related to the fact that acoustic signals in species of the *D. virilis* group are emitted differently by the two sexes. Males move one wing sideways almost at 90° to the body axis and vibrate it producing a relatively clear sound pattern. Females slightly open both wings and produce a more variable pattern of lower intensity (Satokangas et al., 1994; LaRue et al., 2015); still, the moments of female singing were always easily detectable in our video records. In our experiments, females sang very actively in all the conspecific tests and also in those heterospecific tests in which males performed prolonged licking and singing. Our analysis showed that female songs were well correlated with male licking and singing in practically all the trials where these elements occurred with sufficient durations. Thus, our results do not exactly agree with the data of Liimatainen and Hoikkala (1998) but correspond to the results of LaRue and co-authors (2015). During licking the female most likely received some nonspecific signal from the male, although we do not know which receptors on the female abdominal sternites were stimulated in the process: only mechanoreceptors or also chemoreceptors. However, since the male acoustic signal is certainly species-specific, it is strange that the female responded to the con- and heterospecific male with the same level of activity. In any case, our results and the literature data (LaRue et al., 2015) indicate that the female song most likely stimulates further courtship by the male. Nevertheless, our heterospecific tests in which males demonstrated prolonged licking and singing while females also sang actively were characterized by very low frequency of copulations. Since males did attempt copulation after long courtship, the low rate of mating success was determined by the female's choice at the very last stage of courtship. The mechanisms of this choice remain to be studied.

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