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ZOOLOGY

The Role of Acoustic Signals in Courtship Behavior of *Drosophila virilis*

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Abstract—The role of the male acoustic signal in courtship behavior in *Drosophila virilis* was studied by a video-typing method. Three series of the experiments were performed: tests with intact flies, with wingless males and intact females, and with intact males and females with the aristas removed. It was demonstrated that touching and licking were the most prolonged elements of the male courtship. It was noted that removal of the wings in males or aristas in females resulted in an increase in the duration of almost all elements of the courtship behavior (following, touching, licking, singing, and circling) and in a significant decrease in the portion of successful copulations. It was demonstrated that the courtship structure in the experiments with females without aristas changed to a greater extent than in tests with wingless males.

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INTRODUCTION

Courtship behavior is one of the most important factors of reproductive isolation to prevent hybridization and gene exchange between closely related species. The courtship behavior in *Drosophila* is an exchange of signals of different modality (chemical, acoustic, visual, and tactile). Two types of signals, including acoustic signals 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 chemical signals received by the male during female touching and licking (Bartelt et al., 1986; Oguma et al., 1992; Liimatainen and Jallon, 2007), were mainly studied during investigation of the *Drosophila virilis* group species. In particular, it was demonstrated that the male "songs" generated as a result of wing vibrations differ in closely related species by both temporal parameters (the duration of pulses and intervals between pulses) and the carrier frequency. The important role of various parameters of the male song in the female choice was detected in experimental studies of behavior (Hoikkala and Aspi, 1993; Aspi and Hoikkala, 1995; Hoikkala et al., 1998; Ritchie et al., 1998; Päällysaho et al., 2003; Saarikettu et al., 2005b; Klappert et al., 2007).

The mating ritual in the *D. virilis* group significantly differs from that in *D. melanogaster*. In the latter species, there is a relatively strict sequence of courtship elements and the elements themselves are rather short. In the *D. virilis* group, several elements are as a rule performed simultaneously, and the courtship itself is longer and less stereotyped (Spieth, 1951; Vedenina et al., 2013). In addition, the sequences of the main courtship acts also differ in these *Drosophila* groups: for example, the *D. mela-nogaster* males first touching the female abdomen by the front pair of legs, then produce an acoustic signal, after which they lick the female genitals (Sawamura and Tomaru, 2002). The *D. virilis* males touch and lick the female abdomen almost simultaneously and only then produce the acoustic signal (Spieth, 1951; Vedenina et al., 2013). The male song is the longest element of courtship behavior in *D. melanogaster* (Lasbleiz et al., 2006), while licking is in *D. virilis* (Saarikettu et al., 2005a; Vedenina et al., 2013).

In spite of the fact that acoustic signals in the *D. virilis* group have been studied thoroughly, little attention has been paid to the way how eliminating of the acoustic communication channel influenced the female choice, courtship behavior in general, and certain elements of it. For example, only the effect of surgical organ removal on the result of female insemination was studied during the selective blocking of the receptor of the same modality, but no video-typing of the courtship behavior was conducted (Hoikkala, 1988). The effect of elimination of the acoustic channel on the duet organization in *D. virilis* was studied recently (LaRue et al., 2015). It is necessary to note that acoustic signals are generated not only by males, but also by females in the *D. virilis* group (as opposed

to the *D. melanogaster* group). However, it was demonstrated that female singing is not necessary for copulation, but favors it (Satokangas et al., 1994). At the same time, the researchers estimated the percentage of copulating pairs after the removal of wings in males or aristas (the hearing organ) in females, while the effect of these surgical operations on certain courtship elements was not studied (LaRue et al., 2015).

Most of researchers use so-called kinetographs (that illustrate the transitions between different elements of the male courtship and appropriate female behavior) for description of the *Drosophila* courtship behavior (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 description of the courtship allows us to represent the relative frequency of each element and the transition between them. However, such a method is based on the assumption of a successive change of elements. Meanwhile, it was demonstrated that some elements are simultaneously performed even in *D. melanogaster* (a species known for its rather stereotypical courtship behavior) (Lasbleiz et al., 2006). In the *D. virilis* group, at least three different elements can be performed simultaneously. Therefore, in our work the drawing method of the courtship diagram (Vedenina et al., 2013) differs from those used by other authors (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).

The aim of this study was clarification of the role of the male acoustic signal in the courtship behavior and recognition of the *D. virilis* sexual partner by a videotyping method, as well as study of the effect of the removal of the male wings and female aristas on the courtship behavior and courtship efficiency in general.

MATERIALS AND METHODS

The *D. virilis* line no. 102 was used from the collection of the Institute of Developmental Biology (Russian Academy of Sciences). This line originates from adult individuals caught in Berlin in 1967 (Germany). The flies were kept on semolina–yeast medium in tubes 100 mm in height and 25 mm in diameter (5–10 mL feed) in a temperature-controlled room at a daily cycle 12 h light/12 h dark. Imagoes at the age of 1–2 days after emerging were immobilized by means of cold and sexed. The virgin females and males were kept separately in tubes of the indicated size with standard medium. Tests were conducted in the same tubes. Each individual was involved in only one test. The male wings or female aristas were removed by microsurgical scissors. Both procedures are minor, and the imagoes of both sexes remain active after them. The wing and arista removal were performed 2–3 days before the behavioral tests. The tests themselves were conducted when the flies reaching two weeks of age.

The courtship behavior was registered by means of a videotaping method; all interactions between the female and male were filmed on a Sony HDR-SR 12E video camera (Japan) and then analyzed by means of the Virtual Dub 1.10.3 software. The flies were separated in the case when the male demonstrated no interest in the female for 30 min after the beginning of the experiment. In the case of courtship, behavior was recorded till copulation or for 30 min after the beginning of courtship. The total duration of each behavioral act and the total duration of the courtship from the beginning of the first act to the beginning of copulation (excluding long pauses) were measured for each pair. We distinguished eight courtship elements: following of the female by the male, touching of the female abdomen by the male, licking of the female genitals by the male, the male singing and the female singing, the male circling around the female, attempts a copulation, and copulation (Table 1). We referred those active male operations that did not end with copulation to attempts a copulation.

The latencies (the time from the beginning of courtship to the beginning of each element) and the courtship elements themselves were calculated by means of the Microsoft Excel program. Data analysis was conducted by two researchers. The comparison of the results that they obtained gave no significant differences. Data obtained were statistically treated by means of the Microsoft Excel and Statistica programs.

Three series of the experiments were conducted: tests with intact flies (control), with wingless males and intact females, with intact males and females without aristas. More than 30 pairs were tested in each series of experiments (Table 1).

RESULTS

Three courtship elements (touching, licking, and male singing) were registered in each tested pair in the control (intact flies) and in the series with females without aristas (Table 1). Following was registered in approximately half of tests with intact flies and in the higher number of tests with wingless males and with females without aristas. However, these differences were insignificant. Males courting females without aristas significantly more often demonstrated circling as compared with wingless and control males (Fisher's exact test: $p = 0.0017$, taking into account the correction for the false discovery rate $q = 0.033$ (corrected probability value)). The number of copulations was significantly higher in the control than in tests with wingless males and females without aristas ($p = 0.036$ and 0.02, respectively; taking into account the correction for the false discovery rate $q = 0.05$ (corrected probability value for multiple comparisons)).

Table 1. Frequency of courtship elements in *Drosophila virilis* in three series of tests

Test variant	Sample size, number of tests	Number (percentage, %) of tests in which courtship elements are performed							
		following	touching	licking	male song	circling	copulation attempts	copulation	female song
♀ + ♂ intact	30	17 (53.1)	30 (100)	30 (100)	30 (100)	12 (40)	9 (30)	26 (86.7)	29 (96.7)
♀ intact + ♂ wingless	30	18 (60)	30 (100)	30 (100)	–	15 (50)	11 (36.7)	19 (63.3)	30 (100)
♀ without aristas + ♂ intact	30	23 (76.6)	30 (100)	30 (100)	30 (100)	24 (80)	16 (53.3)	18 (60)	30 (100)

Table 2. Comparison of duration of courtship behavior elements in *Drosophila virilis* in different testing variants

Comparison test variants	Parameter	Following	Touching	Licking	Male song	Circling	Copulation attempts	Copulation	Female song
Control, wingless males	*t*	−1.4	−2.13	−2.57	–	−0.8	−0.89	−1.03	−0.83
	df	58	58	58	28	58	58	58	58
	p	0.17	0.04*	0.01*	–	0.43	0.38	0.31	0.41
Control, females without aristas	*t*	−3.24	−1.95	−2.19	−3.63	−3.02	−1.61	−0.79	−2.35
	df	58	58	58	58	58	58	58	58
	p	0.002*	0.06	0.03*	0.0006*	0.004*	0.11	0.43	0.02*
Wingless males, females without aristas	*t*	−1.69	0.45	0.53	–	−2.06	−0.66	0.23	−1.38
	df	58	58	58	28	58	58	58	58
	p	0.1	0.65	0.6	–	0.04*	0.51	0.82	0.17

t, Student's criterion; df, degrees of freedom; *p,* probability; "–", absence of this element of courtship behavior due to the absence of wings: for Tables 2 and 3.

* $p < 0.05$; for Tables 2 and 3.

Touching and licking were the most prolonged courtship elements in all tests (Fig. 1). Touching and licking were performed almost simultaneously, and the male and female produced acoustic signals against the background of these acts. However, the duration of singing was significantly lower than the duration of touching and licking (Table 2). The duration of touching and licking was significantly greater in tests with wingless males and females without aristas than in the control. Moreover, males sang longer, followed females longer, and circled them longer in tests with females without aristas than males in the control; females without aristas also sang longer than females in the control.

The durations of the latencies did not differ in different testing variants (Table 3). Intact males in pairs with females without aristas on average started singing faster than males in the control, while wingless males started circling later as compared with intact males. The wingless males also started licking and circling later than intact males in tests with females without aristas.

DISCUSSION

The partial blocking of the acoustic channel by the removal of wings in males and aristas in females of *D. virilis* we conducted in this study resulted in a decrease in the courtship efficiency. To be specific, the number of copulation attempts increased and the number of successful copulations significantly decreased. These results correspond to data obtained recently on

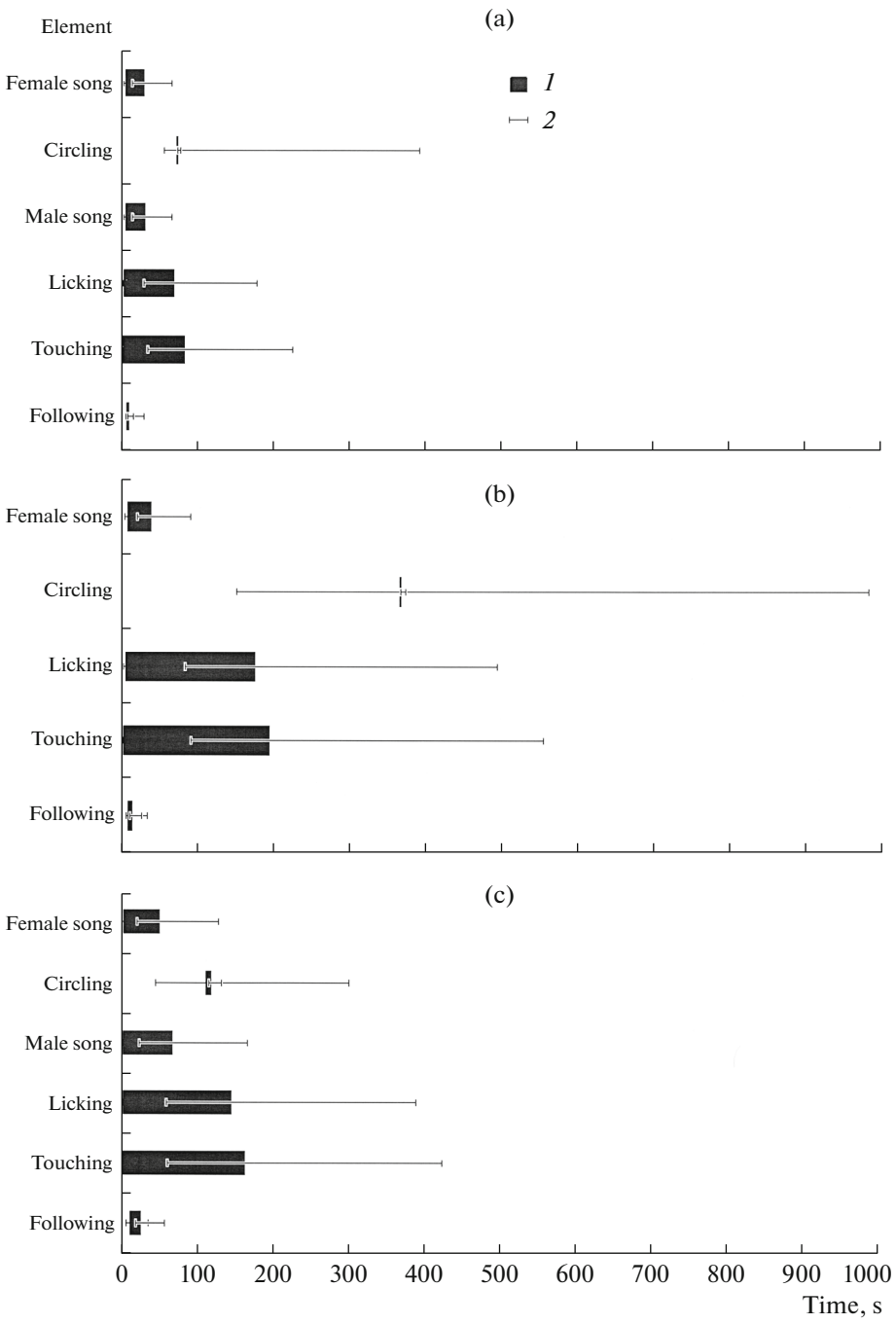

Fig. 1. Latency and duration of courtship behavior elements of studied *Drosophila virilis* twin species in different testing variants. (a) intact flies; (b) tests with wingless males and intact females; (c) tests with intact males and females without aristas. (*1*) Average duration of the element; (*2*) 95% confidence interval.

D. virilis by other authors (LaRue et al., 2015). Thus, the male song is required for successful courtship. At the same time, estimation of the number of inseminated females in the experiments in which wingless males were with intact females or intact males were with females without aristas for 24 h demonstrated that arista removal in females had more critical consequences than wing removal in males (Hoikkala, 1988). This fact apparently reflects the importance of sound contact for the female and deficiency of other modality signals produced by the male. The courtship structure in our experiments with females without aristas also changed more than in tests with wingless males: males followed females and circled around females more often and longer, while females sang more. At the same time, the question why the removal of aristas

Table 3. Comparison of latencies of courtship behavior elements in *Drosophila virilis* in different testing variants

Comparison test variants	Parameter	Following	Touching	Licking	Male song	Circling	Female song
Control, wingless males	*t*	−0.13	−0.74	−1.29	–	−2.44	−0.42
	df	33	58	58	28	25	57
	p	0.9	0.46	0.2	–	0.02*	0.68
Control, females without aristas	*t*	−0.67	1.31	1.34	2.78	−0.73	1.55
	df	38	58	58	58	33	57
	p	0.51	0.2	0.18	0.007*	0.47	0.13
Wingless males, females without aristas	*t*	−0.49	1.89	2.82	–	3.17	1.92
	df	39	58	58	28	36	58
	p	0.62	0.06	0.007*	–	0.003*	0.06

in females has a greater effect on the courtship than wing removal remains open. It is possible that the female no longer perceives not only the male acoustic signal (as a result of the removal of aristas), but also other low-frequency environmental oscillations (for example, caused by the male circling around the female). In addition, the removal of aristas may be a more traumatic procedure for Drosophila than wing removal (which affects female behavior in general).

The increased acoustic female activity in the absence of the male song, which was shown in our study and by other authors (LaRue et al., 2015) is explained by the significance of the acoustic duet for courtship success. The latter authors also suggested the importance of female singing: the absence of song in the experiments with wingless females reduced the percentage of successful courtships. At the same time, the function of the female acoustic signal remains not very clear and requires further study.

The main result of our study is that the partial blocking of the acoustic communication channel results in an increase in the duration of almost all courtship elements. This is consistent with data obtained by Hoikkala and Aspi (1993) on three other species of this group (*D. littoralis*, *D. montana*, and *D. ezoana*) during the measurement of total courtship duration, which was increased in the wingless males as compared with the control.

The roles of acoustic signals in courtship behavior are not the same in different species from the *D. virilis* group (Hoikkala, 1988; Hoikkala and Aspi, 1993). For example, the percentage of copulations decreases only insignificantly in the absence of the male acoustic signal in *D. littoralis*, while elimination of the acoustic signal decreases this portion to zero in *D. montana* (Hoikkala and Aspi, 1993). Subsequently, we are planning to study the effect of the removal of male wings and female aristas on courtship behavior in other sibling species from this *Drosophila* group.

REFERENCES

Aspi, J. and Hoikkala, A., Male mating success and survival in the field with respect to size and courtship song characters in *Drosophila littoralis* and *D. montana* (Diptera: Drosophilidae), *J. Insect Behav.*, 1995, vol. 8, pp. 67–87.

Bartelt, R.J., Arnold, M.T., Schaner, A., and Jackson, L.L., Comparative analysis of cuticular hydrocarbons in the *Drosophila virilis* species group, *Comp. Biochem. Physiol.*, 1986, vol. 83, pp. 731–742.

Brown, R.G.B., Courtship behavior in the *Drosophila obscura* group. Pt II: comparative studies, *Behavior*, 1965, vol. 25, pp. 281–323.

Cobb, M., Burnet, B., and Connolly, K., The structure of courtship in the *Drosophila melanogaster* species sub-group, *Behavior*, 1985, vol. 97, pp. 182–212.

Cobb, M., Burnet, B., Blizard, R., and Jallon, J.-M., Courtship in *Drosophila sechellia*: its structure, functional aspects, and relationship to those of other members of the *Drosophila melanogaster* species subgroup, *J. Insect Behav.*, 1989, vol. 2, pp. 63–89.

Dankert, H., Wang, L., Hoopfer, E.D., Anderson, D.J., and Perona, P., Automated monitoring and analysis of social behavior in *Drosophila, Nat. Meth.*, 2009, vol. 6, pp. 297–303.

Hoikkala, A., The importance of different courtship stimuli in the mating behavior of european species of the *Drosophila virilis* group, *Ann. Zool. Fennici*, 1988, vol. 25, pp. 257–263.

Hoikkala, A. and Aspi, J., Criteria of female mate choice in *Drosophila littoralis, D. montana* and *D. ezoana, Evolution*, 1993, vol. 47, pp. 768–777.

Hoikkala, A., Aspi, J., and Suvanto, L., Male courtship song frequency as an indicator of male genetic quality in an insect species, *Drosophila montana, Proc. Roy. Soc. L. B*, 1998, vol. 265, pp. 503–508.

Hoikkala, A. and Crossley, S.A., Copulatory courtship in *Drosophila*: behavior and songs of *D. birchii* and *D. serrata*, *J. Insect Behav.*, 2000, vol. 13, pp. 71–86.

Hoikkala, A. and Lumme, J., The genetic basis of evolution of the male courtship sounds in the *Drosophila virilis* group, *Evolution*, 1987, vol. 41, pp. 827–845.

Klappert, K., Mazzi, D., Hoikkala, A., and Ritchie, M.G., Male courtship song and female preference variation

between phylogeographically distinct populations of *Drosophila montana, Evolution*, 2007, vol. 61, pp. 1481–1488.

LaRue, K.M., Clemens, J., Berman, G.J., and Murthy, M., Acoustic duetting in *Drosophila virilis* relies on the integration of auditory and tactile signals, *eLife*, 2015, vol. 4, p. e07277.

Lasbleiz, C., Ferveur, J.-F., and Everaerts, C., Courtship behaviour of *Drosophila melanogaster* revisited, *Anim. Behav.*, 2006, vol. 72, pp. 1001–1012.

Liimatainen, J.O. and Hoikkala, A., Interactions of the males and females of three sympatric *Drosophila virilis*-group species, *D. montana, D. littoralis*, and *D. lummei*, (Diptera Drosophilidae) in intra- and interspecific courtships in the wild and in the laboratory, *J. Insect Behav.*, 1998, vol. 11, pp. 399–417.

Liimatainen, J.O. and Jallon, J.M., Genetic analysis of cuticular hydrocarbons and their effect on courtship in *Drosophila virilis* and *D. lummei, Behav. Genet.*, 2007, vol. 37, pp. 713–725.

Manning, A., The sexual behavior of two sibling *Drosophila* species, *Behavior*, 1959, vol. 15, pp. 123–145.

Oguma, Y., Nemoto, T., and Kuwahara, Y., (*z*)-11-Pentacosene is the major sex pheromone component in *Drosophila virilis* (Diptera), *Chemoecology*, 1992, vol. 3, pp. 60–64.

Päällysaho, S., Aspi, J., Liimatainen, J.O., and Hoikkala, A., Role of X chromosomal song genes in the evolution of species-specific courtship songs in *Drosophila virilis* group species, *Behav. Genet.*, 2003, vol. 33, pp. 25–32.

Ritchie, M.G., Townhill, R.M., and Hoikkala, A., Female preference for fly song: playbacks confirm correlational evidence of the targets of sexual selection, *Anim. Behav.*, 1998, vol. 56, pp. 713–717.

Saarikettu, M., Liimatainen, J.O., and Hoikkala, A., Intraspecific variation in mating behavior does not cause sexual isolation between *Drosophila virilis* strains, *Anim. Behav.*, 2005a, vol. 70, pp. 417–426.

Saarikettu, M., Liimatainen, J.O., and Hoikkala, A., The role of male courtship song in species recognition in *Drosophila montana, Behav. Genet.*, 2005b, vol. 35, pp. 257–263.

Satokangas, P., Liimatainen, J.O., and Hoikkala, A., Songs produced by the females of the *Drosophila virilis* group of species, *Behav. Genet.*, 1994, vol. 24, pp. 263–272.

Sawamura, K. and Tomaru, M., Biology of reproductive isolation in *Drosophila*: toward a better understanding of speciation, *Popul. Ecol.*, 2002, vol. 44, pp. 209–219.

Spieth, H.T., Mating behavior and sexual isolation in the *Drosophila virilis* species group, *Behavior*, 1951, vol. 3, pp. 105–145.

Suvanto, L., Hoikkala, A., and Liimatainen, J.O., Secondary courtship songs and inhibitory songs of *Drosophila virilis* group males, *Behav. Genet.*, 1994, vol. 24, pp. 85–94.

Vedenina, V.Y., Ivanova, T.I., and Lazebny, O.E., Analysis of courtship behaviour in closely related species of *Drosophila virilis* group: a new approach arises new questions, *J. Insect Behav.*, 2013, vol. 26, pp. 402–415.

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