The Importance of Acoustic Signals in Multimodal Courtship Behavior in *Drosophila virilis, D. lummei* and *D. littoralis*

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Abstract The courtship rituals of *Drosophila* include an exchange of several signals with different modalities, chemical, visual, acoustic and tactile stimuli, between sexes. Using a video recording method, we studied the role of acoustic communication in courtship behavior in three species of the *Drosophila virilis* group, *D. virilis, D. lummei* and two populations of *D. littoralis*. Five series of experiments were performed: tests with intact flies (control), tests with mute flies (wingless males or females), and tests with deaf flies (aristaless males or females). We distinguished the two situations: either a female did not hear a male or vice versa, males did not hear females. When females did not hear males, the reduction in the copulation number was found in *D. virilis* and both populations of *D. littoralis*, but not in *D. lummei*. When males did not hear females, the reduction in the copulation number was only found in *D. littoralis*. The ablation of the male aristae in *D. virilis* and *D. lummei* even increased the mating success as compared to the control, which may be explained by the 'sensory overload' hypothesis. The changes in courtship temporal structure usually included the delayed onset of the main courtship elements (tapping, licking, and singing), and the variation in their duration and the total time of courtship. These effects were, however, more substantial in *D. virilis* and both populations of *D. littoralis* than in *D. lummei*. Thus, the effect of blocking the acoustic channel was different in the three species regardless of their phylogenetic relationship, and the role of acoustic communication in courtship behavior seemed to increase in the order *D. lummei – D. virilis – D. littoralis*.

Keywords Courtship behavior · Acoustic signals · Drosophila · Videorecording method

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Introduction

Courtship behavior is often multimodal, implying the use of multiple sensory channels to send and receive signals. In *Drosophila,* courtship communication occurs through chemical, acoustic, tactile, and visual stimuli. Courtship behavior of *Drosophila* males was usually described as stereotypical sequential elements

such as orienting towards a female, touching her with their foreleg tarsi, wing vibration, circling the female, and licking her genitalia. When touching and licking the females, both sexes receive chemical and tactile cues from each other. In the course of wing vibration, the males produce acoustic and sometimes visual cues (see, e.g., Spieth 1951, 1974; Shorey 1962; Jallon and Hotta 1979; Ewing 1983; Markow and O'Grady 2005). Both sexes detect sounds with the largest chordotonal organ (Johnston's organ) located on the second antenna segment, the pedicel. A feather-like arista is attached to the third antenna segment, the funiculus. When acoustically stimulated, the arista and the funiculus vibrate, thereby activating the neurons of Johnston's organ (e.g., Petit 1958; Manning 1967; Burnet et al. 1971; Kavlie and Albert 2013; Albert and Göpfert 2015).

Signals of same modalities may crucially differ between closely related species of *Drosophila*, as well as courtship rituals themselves may differ substantially between the species. For example, touching usually precedes the 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, and then, touching co-occurs with licking. It is necessary to note that during touching, the male usually rubs the female abdomen with alternating back and forth movements of the forelegs (Spieth 1951; Vedenina et al. 2013); therefore, we suggest the name 'tapping' to be more appropriate when describing this courtship element. Wing vibration was usually produced later and against the background of tapping and licking (Saarikettu et al. 2005a; Vedenina et al. 2013). 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 elements were shown to be tapping and licking (Saarikettu et al. 2005a; Vedenina et al. 2013; Belkina et al. 2016). In contrast to *D. melanogaster*, the females of *D. virilis* may also vibrate their wings producing the songs (Donegan and Ewing 1980; Hoikkala 1985; Satokangas et al. 1994; LaRue et al. 2015).

The group of sibling species related to *D. virilis* is a well-known model system for studies in speciation. The group consists of 11 species occurring in different regions of the world, which are capable of hybridizing under laboratory conditions (Throckmorton 1982; Spicer 1992). Genomes of the two species of this group, *D. virilis* and *D. americana*, have been fully sequenced (http://beta.flybase.org/static/sequenced_species; http://cracs.fc.up.pt/~nf/dame/). Moreover, the divergence pattern in the species of this group was reconstructed based on the studies of polymorphism in chromosomal rearrangements, proteins, and DNA (Throckmorton 1982; Spicer and Bell 2002; Caletka and McAllister 2004; Wang et al. 2006). At the same time, the detailed studies of reproductive behavior, in particular of courtship behavior, are not numerous in the *D. virilis* group (Liimatainen and Hoikkala 1998; Vedenina et al. 2013; LaRue et al. 2015). The majority of studies conducted on the species of this group are devoted to the role of acoustic signals in species recognition and mate choice. The male songs were shown to differ between the species in both temporal (pulse length, interpulse interval, pause between pulses), and frequency parameters, which play an important role in inter- and intraspecific mate choice (Hoikkala and Lumme 1987; Hoikkala and Aspi 1993; Suvanto et al. 1994; Aspi and Hoikkala 1995; Hoikkala et al. 1998; Ritchie et al. 1998; Päällysaho et al. 2003; Saarikettu et al. 2005b; Klappert et al. 2007).

It remains poorly studied, however, how acoustic communication in *Drosophila* interacts with other sensory cues. Multimodal signals have been classified as redundant (e.g., backup signals) and non-redundant (e.g., multiple messages) signals depending on whether they send similar or different information (Møller and Pomiankowski 1993; Partan and Marler 2005; Bro-Jorgensen 2010). When combined, components of a redundant signal might elicit an equivalent response (Partan 2013). By contrast, components of a non-redundant signal when combined carry different information and generate different responses in the receiver. Multiple signals may interact to provide greater honesty in signaling or to improve the efficacy of each other (Candolin 2003; Hebets and Papaj 2005). One of the methods to study whether multimodal signals are redundant or non-redundant can be an elimination of a single sensory channel and further measurements of overall reproductive success. For example, chemosensory cues were shown to be very important for both sexes of *D. subquinaria* and *D. recens,* whereas vision was found to be necessary only for males (Giglio and Dyer 2013).

In three species of the *D. virilis* group, courtship song is less important in *D. littoralis*, more important in *D. ezoana*, and crucial in *D. montana* (Hoikkala and Aspi 1993). No single sensory modality is necessary for *D. willistoni* mating success, whereas vision is essential in males of a closely related species *D. nebulosa* (Gleason et al. 2012). In these studies, either inseminated females proportion or mating frequency in choice tests, or courtship latency and duration in no-choice tests have been measured. At the same time, the influence of the elimination of single sensory channels on other sensory modalities was poorly studied in *Drosophila*. One such rare study shows that in *D. montana*, elimination of auditory channel leads to a decrease in touching duration but leads to an increase in licking duration during courtship (Liimatainen et al. 1992). This result is unexpected, considering that tapping and licking are usually positively correlated with each other in the *D. virilis* group (Vedenina et al. 2013; LaRue et al. 2015). In the comprehensive study conducted by LaRue et al. (2015) on *D. virilis*, tactile cues delivered to the female abdomen and genitalia during courtship were shown to be important in the coordination of acoustic duetting. Moreover, tapping and licking were most predictive of the occurrence of female song, whereas the male acoustic cues played a subordinate role in the female song timing. Thus, tactile and auditory cues may be classified as non-redundant signals.

In our previous paper (Belkina et al. 2016), we studied the role of male acoustic signals in the courtship behavior of *D. virilis*. It was found that removal of the wings in males or aristae in females did not cause the elimination of mating but resulted in a significant decrease in the percentage of matings. The duration of almost all courtship elements increased after the removal of either male wings or female aristae; the durations of tapping and licking, however, increased substantially. This result corresponds to the data obtained by LaRue et al. (2015). In the present study, we compared the role of acoustic signals in the courtship behavior of *D. virilis* and *D. lummei* belonging to the phylad of *D. virilis*, with that of *D. littoralis* belonging to the phylad of *D. montana* (Spicer 1992; Spicer and Bell 2002). *Drosophila littoralis* was represented by the northern and southern populations. We recorded courtship behavior by videotaping and further analyzed latency and the total duration of each courtship element. When the acoustic channel was blocked, we distinguished between the two situations: either a female did not hear a male (wingless males or aristaless females), or vice versa, a male did not hear a female (wingless females or aristaless males). We suggested that blocking of the acoustic signals would be more crucial in the situation when females do not hear males than in the reverse situation because females are expected to be more selective than males. We also assumed that elimination of the acoustic channel in *D. lummei* and *D. littoralis* could mainly involve an increase in the duration of tapping and licking, as we earlier showed in *D. virilis* (Belkina et al. 2016). However, considering the results of Hoikkala (1988), we expected to show that the acoustic channel blocking will affect the mating success and courtship structure to a lesser degree in *D. lummei* and *D. littoralis* than in *D. virilis*. Thus, we assumed a different relationship between components of multiple signals in closely related *Drosophila* species independent of their phylogenetic relationship, which implies a rapid evolution of multimodal mating signals.

Material and Methods

All sibling species used were obtained from the collection of the Koltzov Institute of Developmental Biology: *D. virilis* (strain 102 originated from Berlin, Germany 1967), *D. lummei* (strain 1109 originated from Muonio, Finland 1972), *D. littoralis* (northern strain FP 12–01 and southern strain AB-58 originated from Moscow, Russia 2012 and Pitsunda, Abkhazia 2013, respectively).

The flies were cultured on a semolina–yeast medium in glass vials (100×25 mm) at 21–24 °C under a standard 12 h light/12 h dark cycle. The one-day-old flies were immobilized under cold anesthesia and were separated by sex. The virgin females and males were kept separately in vials and used in the experiments at the age of 14–21 days. Each fly was used in only one test. The wings and the aristae were bilaterally removed by microsurgical scissors 2–3 days before the behavioral tests. Both types of ablation were not traumatic: the flies of both sexes remained active after them, and visually did not differ from the intact flies. The behavior

of flies was recorded in the period from 9 a.m. to 1 p.m. The courtship behavior was registered using a videorecording method: all interactions between one female and one male were recorded with a Sony HDR-SR 12E video camera (Japan) and then analyzed using the Virtual Dub 1.10.3 software.

The experimental procedure started by placing one female and one male in a glass vial (100×25 mm) with 7–8 ml of the standard medium, by gentle aspiration without anesthesia. If the male did not 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. We started to record the interactions between the flies when the male showed any interest in the female; usually, the interaction started with tapping. The latencies from the start of experiment to the beginning of each courtship element, and the total duration of each courtship element (excluding the long pauses lasting more than one minute) were measured for each pair. We distinguished eight courtship elements: following (the male follows the female), tapping (the male touches the female abdomen by the forelegs), licking (the male licks the end of the female abdomen), male singing (the male takes one wing aside and vibrates by it), female singing (the female vibrates by both wings being almost folded), circling (the male circles around the female), copulation attempt (the male tries to mount the female, but the attempt lasts for less than one minute), and copulation (Table 1). We also measured the total duration of courtship (time in sec from the beginning of the courtship ritual until the copulation or the expiration of 30 min) and the duration of copulation (time in sec from the copulation onset until disengagement). Considering that simultaneous recording of the video and audio signals was technically challenging, we did not record the acoustic signals. On the video recordings, the male wing extensions and the female wing fluttering were clearly visible and detectable to be interpreted as singing.

The data analysis was conducted by the two authors independently. The comparison of the results obtained by the authors gave no significant differences. Statistical analysis was made with the MS Excel 2010 and Statistica v10.0 software. The differences in the occurrence of courtship elements between the experiments were estimated by the exact Fisher's test, the latencies and duration of courtship elements and the total duration of courtship ritual were compared by using the Mann-Whitney U test. The differences were considered statistically significant at $p < 0.05$. Benjamini and Hochberg (1995) adjustment was used for multiple comparisons with false discovery rate set to 5%.

The five series of the experiments were conducted in the current study: tests with intact flies (control), with wingless males and intact females, with intact males and females without the aristae, with males without the aristae and intact females, with intact males and wingless females. In *D. virilis*, we also conducted the 6th series of experiments to estimate an effect of complete blocking of the acoustic channel: we removed the aristae and wings in all *D. virilis* flies of both sexes (negative contol). The 30 pairs were tested in each series of experiments.

To assess the copulation rate in *D. virilis* and *D. lummei*, the two experiments on multiple mate choices were conducted in the larger vials (5×11 cm) with the standard medium. Mating trials were initiated by placing of 33 females with 34 males in *D. virilis*, and 34 females with 35 males in *D. lummei*. Observations of matings lasted for one hour for each species. During these observations, we marked a place where the two flies just had mated by a point with a marker. Taking into account that the females of these species do not mate more often than once per hour (unpublished observations), we equated the number of dots on the vial to the number of the females mated. The *D. virilis* flies were tested at the age of 14 days, and those of *D. lummei* were at the age of 21 days.

Results

How Did the Blocking of the Acoustic Channel Affect the Occurrence of Courtship Elements?

The various types of ablations led to different changes in the percentage of copulations depending on the species (Table 1). In tests where *D. virilis* males could not hear the female song (with wingless females), the number of copulations significantly decreased as compared to the tests with intact *D. virilis* (Fisher's exact test; $p = 0.00001$). A similar decrease of mating success was found in the negative control. In tests with *D. virilis* wingless

Table 1 Percentage of courtship elements in different trials in three sibling species of the *Drosophila virilis* group

Combination of tests	The total number of trials	Percentage (%) of trials with the occurrence of courtship elements							
		following	tapping	licking	male song	circling	copulation attempt	copulation	female song
virilis all intact	30	23	100	100	100	50	23	100	100
virilis wingless males	30	33	100	100	–	60	37	87	100
virilis aristaless females	30	30	100	100	100	50	43	97	100
virilis wingless females	30	20	100	100	100	53	**60**	**83**	–
virilis aristaless males	30	33	100	100	100	**23**	20	100	100
virilis negative control	30	33	100	93	–	**80**	**57**	**83**	–
lummei all intact	30	63	100	100	97	57	40	33	93
lummei wingless males	30	63	100	100	–	43	23	27	87
lummei aristaless females	30	**90**	100	100	100	57	40	20	80
lummei wingless females	30	77	100	90	87	67	50	20	–
lummei aristaless males	30	53	100	97	97	53	40	**60**	87
littoralis (SP) all intact	30	60	100	100	97	40	10	77	100
littoralis (SP) wingless males	30	57	100	97	–	63	20	**50**	93
littoralis (SP) aristaless females	30	63	100	**80**	**80**	50	**30**	**33**	**83**
littoralis (SP) wingless females	30	**27**	100	**70**	83	63	3	**13**	–
littoralis (SP) aristaless males	30	73	100	93	90	53	10	**50**	90
littoralis (NP) all intact	30	70	100	97	97	20	7	87	93
littoralis (NP) wingless males	30	77	100	97	–	**43**	7	**23**	97
littoralis (NP) aristaless females	30	73	100	97	87	40	20	**60**	90
littoralis (NP) wingless females	30	77	100	**70**	90	**70**	3	**37**	–
littoralis (NP) aristaless males	30	60	100	93	83	40	13	67	90

Bold entries represent significant differences between the given element and the control, Fisher's exact test, $p < 0.05$ level

Dash indicates the absence of the given courtship element; *NP*, northern population of *D. littoralis*; *SP*, southern population of *D. littoralis*

males and aristaless females, the number of matings only slightly and insignificantly decreased. After three types of ablations in *D. lummei* (wingless males, wingless and aristaless females), the number of matings also slightly and insignificantly decreased in comparison to control. Notably, this species was characterized by a relatively low mating success even in intact flies, and this could explain insignificant difference between the percentage of copulations between the intact and operated flies. This species was also remarkable by a high mating success with aristaless males (almost twice as high ($p < 0.05$) as in the control). In both populations

of *D. littoralis,* by contrast, almost all types of ablations resulted in significant mating decrease ($p < 0.05$).

The blocking of the acoustic channel also influenced the occurrence of other courtship elements except for tapping (Table 1). The aristaless males demonstrated circling more rarely than males in the control tests (Fisher's exact test; $p = 0.002$). At the same time, *D. virilis* males tried to copulate with wingless females more often than the intact flies ($p = 0.008$). In the negative control, the percentage of circling and copulation attempts also increased ($p < 0.02$). The *D. lummei* males followed aristaless

females more often as compared to the control ($p = 0.015$). The males of the southern population of *D. littoralis* (*D. littoralis* SP) that courted wingless females followed ($p = 0.009$) and licked them ($p = 0.001$) more rarely than in the control. The *D. littoralis* SP males also licked aristaless females and sang to them more rarely ($p < 0.05$) than those in the control. In the northern population of *D. littoralis* (*D. littoralis* NP), males licked wingless females more rarely ($p < 0.023$) than in the control as well. However, the circling frequency increased ($p < 0.05$) after the wing ablations in both sexes.

How Did the Blocking of the Acoustic Channel Affect the Latencies to Courtship Elements?

The acoustic channel blocking affected the latencies to various courtship elements to a larger extent in *D. lummei* and *D. littoralis* SP than in *D. virilis* and *D. littoralis* NP. In the majority of cases, the courtship started with tapping; thus, the values for tapping latencies were too small to show them in Figs. 1, 2, 3 and 4. In *D. virilis*, female singing started earlier in tests with aristaless males than in the control (Mann–Whitney U test; $p = 0.001$; Table 1S, Fig. 1). In the negative control, copulation started later than in the control ($p = 0.006$).

The *D. lummei* males started to follow wingless females (Mann–Whitney U test; $p = 0.005$) and aristaless females ($p = 0.0006$) earlier than in the control (Table 1S, Fig. 2). However, in tests with wingless females, males started to tap ($p = 0.022$), lick ($p = 0.00001$) and sing ($p = 0.006$) later than males in the control. The aristaless males also started to lick females later ($p = 0.02$) than the control males.
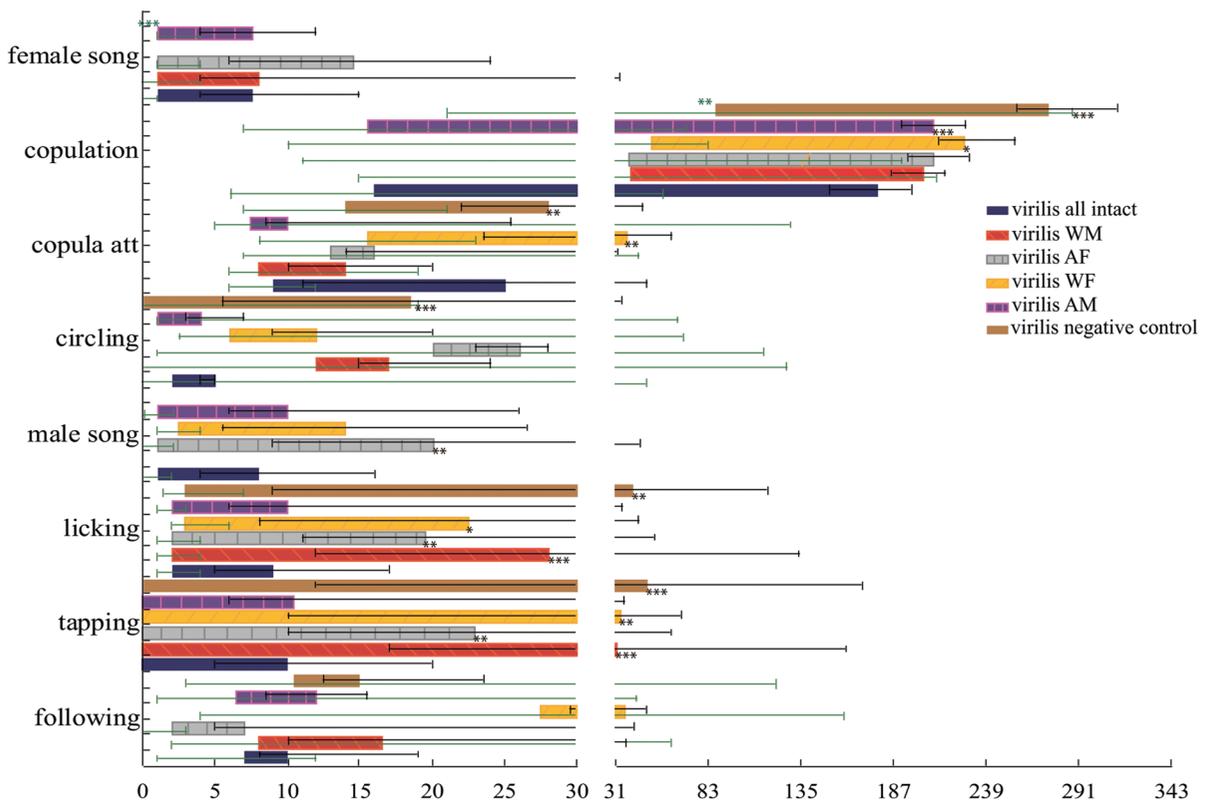
Fig. 1 The median latencies and the median durations (s) of the courtship elements in *Drosophila virilis*. WM – wingless males, AF – aristaless females, WF – wingless females, AM – aristaless males, negative control – aristaless & wingless females + aristaless & wingless males. Whiskers show lower and upper quartiles (green for latencies, black for durations). Asterisks indicate statistical significance of the differences between the given element and the control (Mann-Whitney's test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)
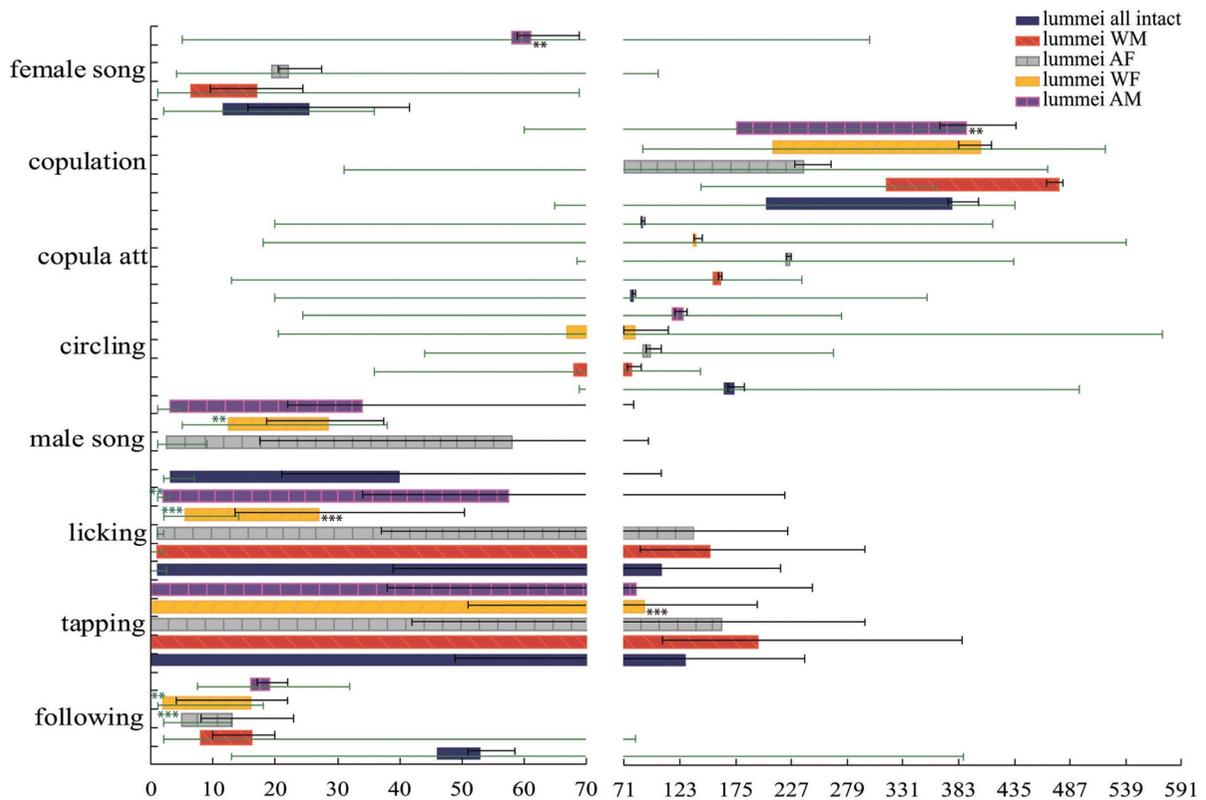
Fig. 2 The median latencies and the median durations (s) of the courtship elements in *Drosophila lummei*. WM – wingless males, AF – aristaless females, WF – wingless females, AM – aristaless males. Whiskers show lower and upper quartiles (green for latencies, black for durations). Asterisks indicate statistical significance of the differences between the given element and the control (Mann-Whitney's test; *p < 0.05; **p < 0.01; ***p < 0.001)

The latencies changed to a larger extent in the tests where *D. littoralis* SP males did not hear females than in the reversed tests; notably, the latencies in *D. littoralis* SP usually increased in comparison to the control (Table 1S, Fig. 3). In tests with *D. littoralis* SP wingless females, licking (Mann–Whitney U test; p = 0.00004), male singing (p = 0.003) and copulation (p = 0.011) started later than in the control. Aristaless males also started to follow (p = 0.009) and circle (p = 0.017) the females later than the control males. Similarly, aristaless *D. littoralis* SP females started to sing later (p = 0.003) in comparison to the control. Only tapping started earlier in both tests with *D. littoralis* SP wingless females and aristaless males (p = 0.011).

In *D. littoralis* NP, the acoustic channel blocking affected the latencies to courtship elements to a minor extent. We found the only significant

difference in licking in tests with wingless females, which started later (p = 0.00003) than in the control (Table 1S, Fig. 4).

The latencies to licking, male and female singing often correlated with each other. These correlations were similar in the control and after different ablation types, being, however, not very high (Spearman rank correlation; r = 0.4–0.7, p < 0.02).

How Did the Blocking of the Acoustic Channel Affect the Durations of Courtship Elements?

Almost all ablation types in *D. virilis* (except of ablation of the male aristae) increased the durations of tapping (Mann–Whitney U test; p < 0.004) and licking (p < 0.025) in comparison to the control (Table 2S, Fig. 1). Similarly, the increase in duration was found in tests with aristaless *D. virilis* females
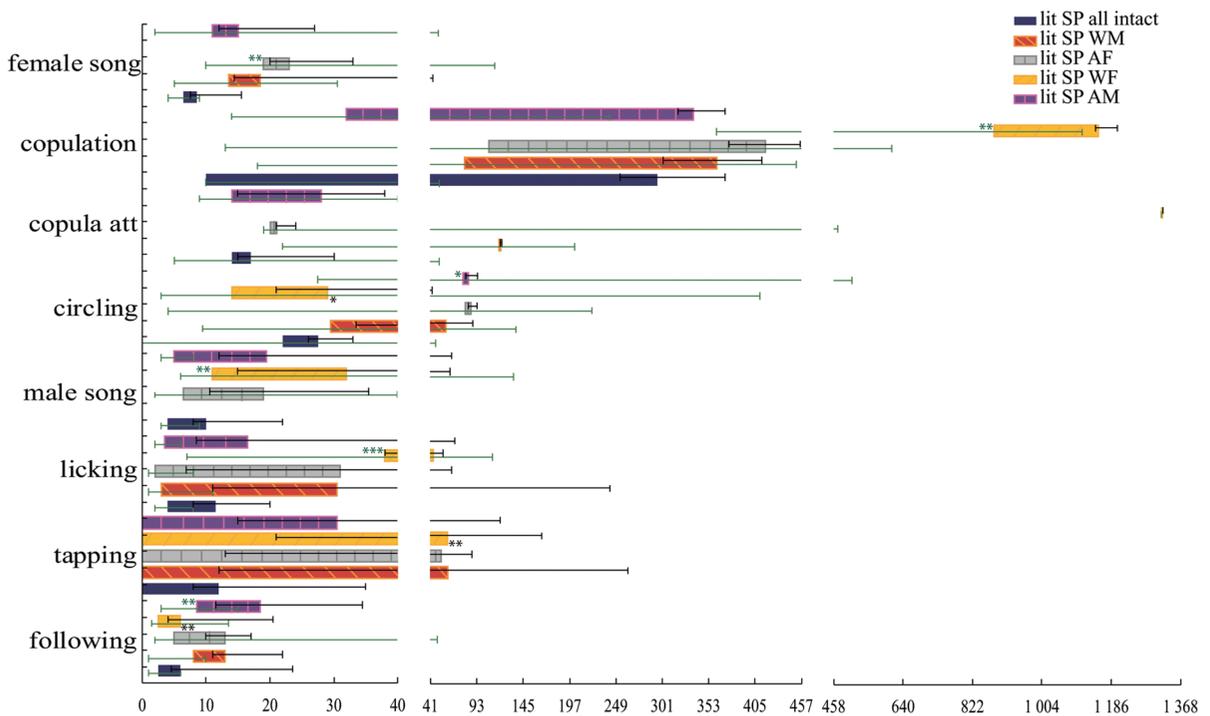
Fig. 3 The median latencies and the median durations (s) of the courtship elements in the southern population (SP) of *Drosophila littoralis.* WM – wingless males, AF – aristaless females, WF – wingless females, AM – aristaless males.

Whiskers show lower and upper quartiles (green for latencies, black for durations). Asterisks indicate statistical significance of the differences between the given element and the control (Mann-Whitney's test; *p < 0.05; **p < 0.01; ***p < 0.001)

for male singing ($p = 0.003$), in tests with wingless females for copulation attempts and copulation ($p = 0.005$ and $p = 0.02$, respectively), in tests with aristaless males for copulation ($p = 0.00007$). in the negative control for circling ($p = 0.00007$), copulation attempts ($p = 0.01$) and copulation ($p = 0.0007$). The total courtship duration also increased ($p < 0.024$) in all tests except of tests with aristaless males in comparison to the intact *D. virilis* flies (Fig. 5).

In tests with *D. lummei*, the acoustic channel blocking affected the durations of courtship elements to the minor extent (Table 2S, Fig. 2). In tests with wingless females, males licked (Mann–Whitney U test; $p = 0.00002$) and sang ($p = 0.002$) for a shorter time than in the control. When aristaless males demonstrated courtship, the duration of female song ($p = 0.008$) decreased, while the duration of copulation ($p = 0.007$) increased. In tests with wingless males, by contrast, the duration of copulation ($p = 0.004$) decreased. The total courtship duration did not change significantly (Fig. 5).

In tests with *D. littoralis* SP, the durations only changed in tests with wingless females: durations of tapping (Mann–Whitney U test; $p = 0.005$) and circling ($p = 0.017$) increased, while duration of following ($p = 0.009$) decreased as compared to the control (Table 2S, Fig. 3). At the same time, the total courtship duration increased ($p < 0.03$) in all tests except of tests with aristaless females in comparison to the control (Fig. 5).

In tests with aristaless females of *D. littoralis* NP, the duration of copulation (Mann–Whitney U test; $p = 0.003$) decreased in comparison to the control (Table 2S, Fig. 4). In tests with wingless females, males tapped ($p = 0.00001$) and circled ($p = 0.0002$) longer but licked ($p = 0.00004$)) for a shorter time than in the control. The total duration of courtship was found to increase only in tests with wingless females ($p = 0.00007$) as compared to the control (Fig. 5).

In the control tests, the duration of four main courtship elements (tapping, licking, male and
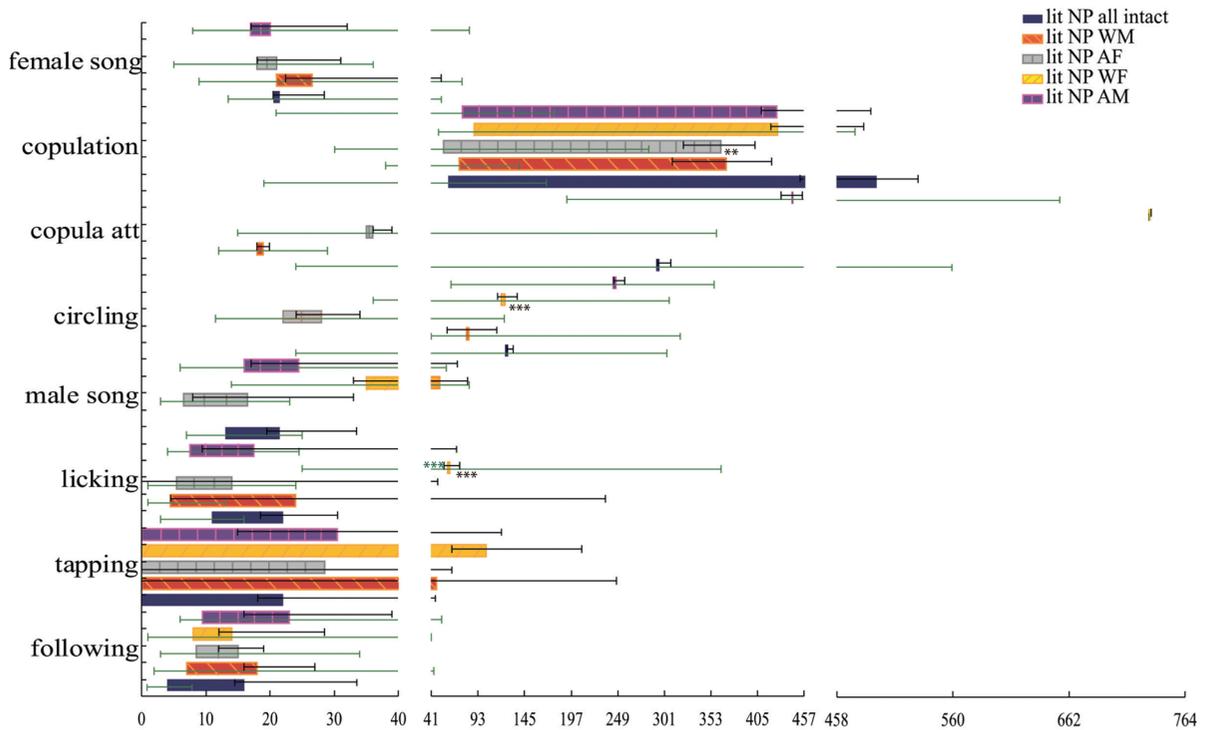
Fig. 4 The median latencies and the median duration of the courtship elements in the northern population (NP) of *Drosophila littoralis*. WM – wingless males, AF – aristaless females, WF – wingless females, AM – aristaless males. Whiskers show lower and upper quartiles (green for latencies, black for durations). Asterisks indicate statistical significance of the differences between the given element and the control (Mann-Whitney's test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

female singing), highly correlated between each other (Spearman rank correlation, $r = 0.74–0.99$, $p < 0.001$). In almost all series of experiments with ablations, the correlations between the durations of tapping, licking and male singing remained as high as in control. These three elements showed lower but still significant correlations with female singing in many tests with ablations, especially in all tests with *D. lummei* ($r = 0.58–0.99$, $p < 0.001$). In tests with aristaless *D. littoralis* NP females, the correlations between the durations of these four elements appeared to be high but non-significant.

Comparison of the Copulation Rate between *D. virilis* and *D. lummei*

To check for the low percent of copulations in intact *D. lummei* (Table 1), we performed the multiple mating choice experiments in *D. virilis* and *D. lummei*. A comparison of the copulation rate in *D. virilis* and *D. lummei* showed the dramatic difference between the two species (Fig. 6). In 30 min from the test beginning, the number of *D. virilis* copulations was more than 90%, while in *D. lummei* this number was about 50%. In one hour, the number of *D. lummei* copulations still did not reach 90%. Thus, *D. lummei* requires more time for successful courtship than *D. virilis*.

Discussion

The acoustic channel blocking had a larger effect on the duration of elements than on their latencies: 34 significant changes in the element durations versus 17 significant changes in the element latencies. Generally, the acoustic channel blocking led to increasing rather than decreasing of the courtship element durations. The tests when a male do not hear a female showed more changes as compared to the control than the reverse tests. This result
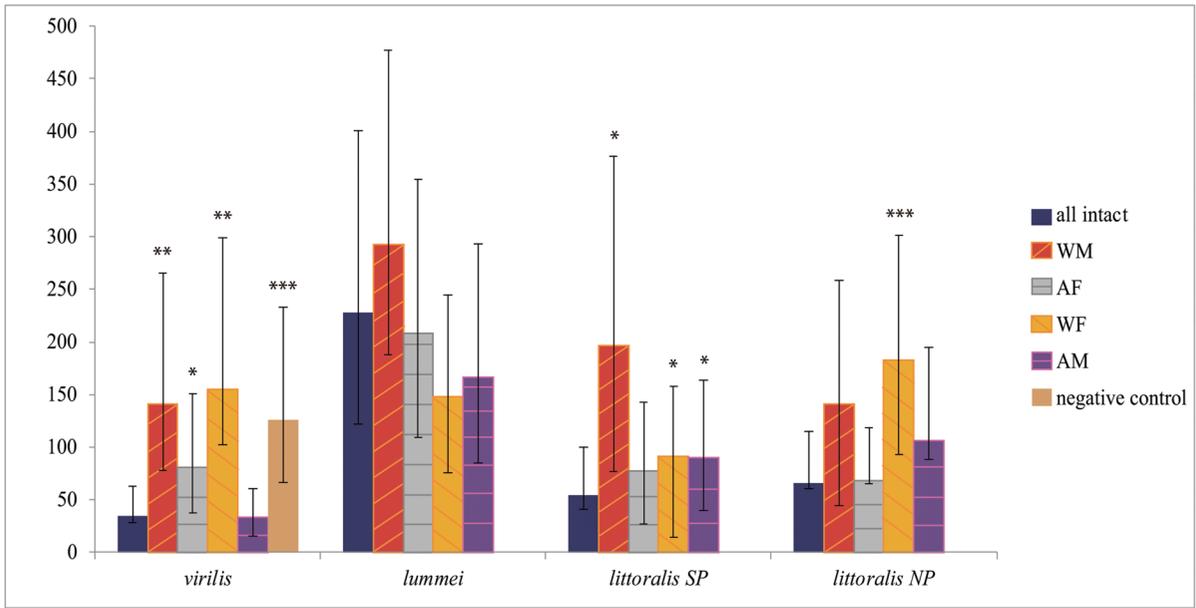
Fig. 5 The mean durations of total courtship in three sibling species of *Drosophila virilis* group. Asterisks indicate statistical significance of the differences between the given element and the control (Mann-Whitney's test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Whiskers show lower and upper quartiles

Fig. 6 The rate of copulation in *D. virilis* (33 females and 34 males) and *D. lummei* (34 females and 35 males) in two multiple mate choice experiments
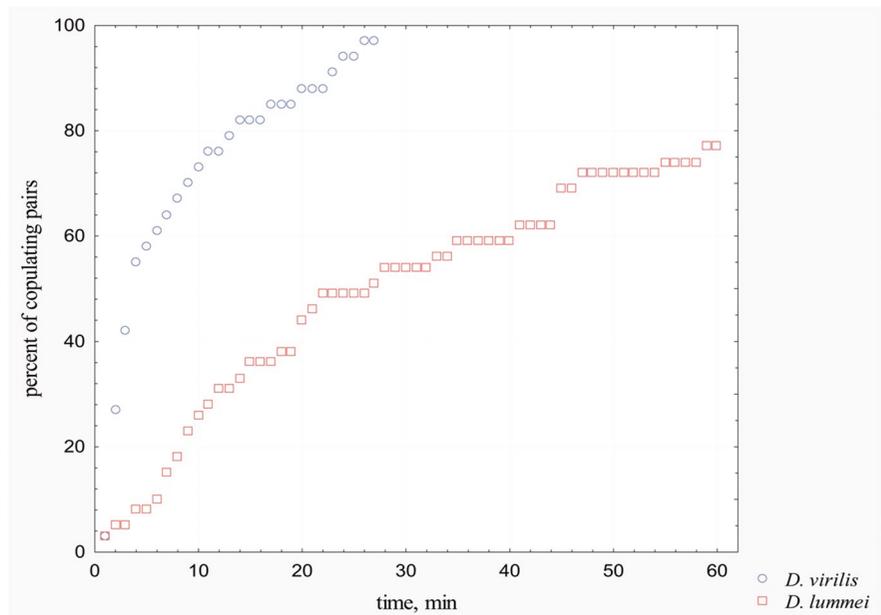
was unexpected since we anticipated more pronounced effects after blocking of the female auditory channel.

The Relationship between Acoustic Channel and Other Courtship Signals Differs in Closely Related Species

In the study of Hoikkala (1988), removal of the female aristae reduced the percentage of inseminated females in *D. virilis* but not in *D. littoralis* and *D. lummei*. We, therefore, expected that the acoustic channel blocking we made in our experiments would affect the mating success largely in *D. virilis* and to a less extent in *D. lummei* and *D. littoralis*. However, the reduction in the copulation number was found in *D. virilis* and *D. littoralis*, but not in *D. lummei*. We can explain this discordance by the differences in methods used by various authors. Hoikkala (1988) tested the flies in multiple-choice experiments for 24 h, whereas we studied each pair no longer than 30 min in no-choice tests. Besides, Hoikkala (1988) used the percentage of females inseminated as an indicator of mating success; we used the copulation number as such indicator. Our multiple choice tests showed the lower mating success in the intact *D. lummei* males compared to the intact *D. virilis* males (Fig. 6). We suggest that the *D. lummei* males spent more time for warming up and courting before mating than the *D. virilis* males did. As a result, only half of the *D. lummei* males would mate by the end of 30-min tests, whereas all *D. virilis* males would mate during this period.

We expected that elimination of acoustic channel in *D. lummei* would lead to the increase in duration of tapping and licking, as we earlier found in *D. virilis* (Belkina et al. 2016). According to the data of LaRue et al. (2015) on *D. virilis*, tactile cues received by a female during tapping and licking are important for coordination of acoustic duetting. We expected that when a female would not hear the male song, she could, nevertheless, produce the song in response to the tactile cues. We also assumed an increased duration of the total courtship, which could be explained as a compensation of the absence of the male song. Such a result was obtained in *D. saltans* by Colyott et al. (2016). The increased duration of courtship has occurred in *D. virilis* and both populations of *D. littoralis,* but not in *D. lummei*. At the same time, the latencies of licking and female singing usually correlated in all species, which suggests that tactile cues may be important in coordination of acoustic duetting in all species studied.

The results obtained in most our experiments suggest the redundancy of male or female songs with respect to other courtship signals. The acoustic signal blocking did not completely eliminate the mating success, but mating success either decreased or remained to be unchanged. The redundant multimodal signals can elicit the same response as the separate components or an enhanced response (Bro-Jorgensen 2010; Partan 2013). The redundant signals are also positively correlated with each other, which corresponds to our data. There are several evolutionary hypotheses explaining the redundancy of multiple signals. The 'backup' hypothesis maintains that multiple equivalent signals compensate for errors during information coding (Møller and Pomiankowski 1993; Tanner and Visscher 2008). According to the 'alerting signal' hypothesis, attention grabbers can improve signal detection by alerting receivers to other, more informative, signal components (Rowe 1999; Grafe and Wanger 2007). The 'receiver psychology' hypothesis suggests that redundant signal components enhance the accuracy and speed of receivers in discrimination and learning tasks (Smith and Evans 2008). To test which hypothesis explains our results, it would be necessary to conduct experiments with blocking other sensory modalities.

In tests with the aristaless males of *D. lummei*, males copulated with females significantly more often than males in the control. This could mean that the female song prevents a male from a fast copulation thus stimulating him to court longer. According to the 'sensory overload' hypothesis (Hebets and Papaj 2005), the neural suppression of incongruent multimodal stimuli might be exploited by signalers when the interest of signaler and receiver diverge. There are some examples illustrating this hypothesis (Skals et al. 2005; Thompson et al. 2008), but empirical evidence that signalers benefit from manipulating receivers by sensory overloading is unknown. In our experiments, females may be interested in males that court longer, thus testing their quality. According to the 'good genes' hypothesis of sexual selection (Zahavi 1987; Smith 1991), the females testing the quality of a male must prefer the most costly features of the signal. Production of a long courtship probably requires much energy from a singing male and thus may be used by a female as an indicator of his vigour.

The Effect of the Acoustic Channel Blocking Differs between the Sexes

We suggested that blocking of the acoustic signals would be more crucial in the situation when females

do not hear singing of males than in the reverse situation. This, however, was not shown in *D. virilis*: copulation success decreased only after ablations of the female wings. Therefore, the blocking of the female auditory channel (a mute male or a deaf female) decreased copulation success, while the blocking of the male auditory channel (a mute female or a deaf male) had the opposite effect. In *D. lummei*, no significant reduction in the copulation number was found in all tests, whereas in *D. littoralis*, all ablations led to the reduction in the copulation number. The question arises of whether the function of the female song varies between the species of the *D. virilis* group.

Wings can be used for various purposes in different sexes: to produce acoustic signals, as a visual stimulus, as a chemical stimulation (propelling air flows with pheromones with wing oscillations), or some combination of these (Spieth 1952). Although the behavior of the *Drosophila* females during courtship was first described more than 60 years ago (Spieth 1952), the question remains whether the wing fluttering emitted by females could be a signal of acceptance or refusal. Does a female respond to the courting male by the wing fluttering or the reverse, does she stimulate a male in this way (Donegan and Ewing 1980; Liimatainen and Hoikkala 1998)? LaRue et al. (2015) demonstrated that the *D. virilis* female actively emitted sound signals in response to the courtship song of a conspecific male, thus forming an acoustic duet that resulted to successful copulation. Females of *D. montana*, *D. lummei* and *D. littoralis* were shown to start singing only in response to licking by a conspecific male (Liimatainen and Hoikkala 1998). Previously, we also revealed the correlation of female singing with licking and male singing when both male and female were intact (Belkina et al. 2018). In the current study, males started licking later and for a shorter time in tests with blocking of the male auditory channel than in the control. A similar effect was found for the male singing. For female singing, however, no such pattern was found. Moreover, after the acoustic channel blocking, the correlations between the main male courtship elements and female singing became weaker. We suggest that in tests with wingless females, the males might be unsuccessful in mating because they did not get any response from the females, which, in turn, could stimulate them to lick. During singing, the females can transmit not only acoustic and visual signals, but can also spread sex

pheromones to stimulate males. How then one could explain an increase in the copulation number in tests with the aristaless males of *D. lummei*? In such tests, the males did not hear the female song, but continued to perceive visual and chemical signals emitted by movements of the female wings. We suggest that in *D. lummei*, the female song can prevent a male from a fast copulation, thus stimulating him to court longer. The data of other authors obtained on *D. saltans* (Colyott et al. 2016) support this idea. In *D. littoralis*, by contrast, the female song may have another function, e. g., signaling about an acceptance of the female to mate. It is also possible that females produce different song types (Liimatainen et al. 1992). The functions of the different song types produced by females are not clear and could vary depending on the species (Hoikkala 1985; Satokangas et al. 1994).

The spreading of the wings by the *Drosophila* females is a common signal of acceptance preceding the subsequent copulation (Spieth 1952; Hoikkala et al. 1982; Liimatainen et al. 1992; Colyott et al. 2016). We found that the males that courted wingless *D. virilis* females made the copulation attempts more often than in the control; similar result was obtained in the negative control. In tests with double ablations in both sexes, courtship behavior was also characterized by longer durations of the main elements and the significant copulation delay. Notably, about a quarter of males in the negative control systematically tapped and licked the female's head, after which they tried to copulate with the very head. We suggest that males could use the female's wings to visually locate her abdomen and coordinate an appropriate behavior to achieve copulation. At the same time, males are suggested to use some additional signals to determine the receptivity of a female, such as an extension of the female genital plates that is performed during licking (Spieth 1952; Hoikkala et al. 1982; Ewing 1983). It could explain why the males of *D. virilis* and *D. lummei* that courted wingless females had a similar mating success as in the control.

The Similar Effect of the Acoustic Channel Blocking in Two Populations of *D. littoralis*

In our previous study (Belkina et al. 2018), we found some differences in the courtship behavior between the northern and southern populations of *D. littoralis*, but in intact flies. In the current study, we, however,

found no principal differences in the impacts of the acoustic channel blocking between the two populations.

It was previously shown that *D. littoralis* from Caucasus and *D. littoralis* from Scandinavia and Central-European Russia may be considered as different subspecies according to the data on inversion polymorphism (Mitrofanov and Poluektova 1982) and protein polymorphism at 15 loci (Goncharenko et al. 1984, 2004). The estimated time of divergence calculated according to the protein polymorphism is 250–300 thousand years (Goncharenko et al. 1989). The study of the *D. littoralis* local population from the Don River (Rostov region, Russia) revealed a stable mtDNA polymorphism with a fixation of more than one haplotype (Andrianov et al. 2008).

Surprisingly, the divergence in the phallus shape was shown to be greater between the two subspecies of *D. littoralis* than between some species of the *D. virilis* group like *D. montana, D. flavomontana, D. lacicola*, and *D. borealis* (Kulikov et al. 2004). On the basis of this data, one could suggest even the status of different species for the two subspecies/populations of *D. littoralis*. These data, however, do not match our recent data on comparison between con- and heterospecific courtships of the northern and southern populations of *D. littoralis* (Belkina et al. 2018). In the latter study, the authors found only moderate differences in courtship structure between the two populations, and showed no mating reduction in heterospecific courtships as compared to conspecific ones. In the current study, we also found the minor differences in the impacts of the acoustic channel blocking between the two populations. In particular, the most crucial effect on the percentage of copulations was observed after the ablation of the female wings in the southern population of *D. littoralis*, and after the ablation of the male wings in the northern population of *D. littoralis* (Table 1). The acoustic channel blocking resulted in the more essential increase of courtship duration in the southern population of *D. littoralis* than in the northern population of *D littoralis* (Fig. 5).

The distribution areas of the two subspecies are geographically separated (Gontcharenko and Emelianov 1992), hence they can be considered as allopatric populations. The lack of overlapping areas does not lead to a rapid development of reproductive isolation due to the absence of a secondary contact zone (Jennings et al. 2014; Humphreys et al. 2016). It is well known that the most rapid changes in courtship behavior tend to occur between sympatric species but not between allopatric relatives (Coyne and Orr 1997). For example, there is no sexual isolation between the two allopatric species, *D. lini* and *D. ogumai*, whose males generate similar sine songs with a high carrier frequency (about 250 Hz) (Wen et al. 2011). Likewise, three allopatric species of the *D. eremophila* species complex, *D. eremophila*, *D. micromettleri* and *D. mettleri*, have similar courtship behavior and song parameters (Alonso-Pimentel et al. 1995). The minor differences in courtship behavior between the *D. littoralis* populations in intact flies (Belkina et al. 2018) and after the blocking of acoustic modality are in concordance with the data on other *Drosophila* species. At the same time, the significant differences in the genitalia structure between the *D. littoralis* subspecies remain a puzzle.

Changes in the Copulation Duration after the Acoustic Channel Blocking

In *D. melanogaster,* the duration of copulation is regulated by a male, while in the species of the *virilis* group, it is determined by both sexes through interactions between females and males (MacBean and Parsons 1967; LaRue et al. 2015). Thus, the copulation duration may be the subject of conflict of interest between the sexes. On the one hand, males need to transfer more sperm and accessory gland proteins (the sex peptides and ovulin) to decrease the risk of the subsequent sperm competition. On the other hand, a female attempts to dismount a male in the last stage of copulation to leave a possibility of remating with another male. Females invest more in egg production than males in sperm production because of the difference in size between these two types of gametes. Nevertheless, production of sperm is suggested to be energetically more costly (Bateman 1948).

During courtship, the male can evaluate the female quality using signals of different modalities. Our results showed that the *D. littoralis* NP males mated for a shorter time in tests with aristaless females than in the control. It is very likely that aristaless females could be identified by the males as the imperfect mates. Thus, a shorter copulation gives a male an

opportunity to economize the high cost resources like time, sperm, and accessory gland proteins. It was shown that each copulation reduces the number of future matings, which a male can potentially have (Bonduriansky 2001).

The males of many *Drosophila* species produce songs not only during courtship. According to Spieth (1952), some species are known to produce songs also in the beginning of mounting and during copulation. These data are supported by our observations: we noticed similar copulatory behavior in all species of the *virilis* group studied. Taking this into account, the decrease of the copulation duration in wingless males of *D. lummei* can be due to the absence of wings and, as a result, the inability to sing.

We also noticed that during copulation, the male was often standing between the female wings and holding his forelegs on them. The male was grabbing the female abdomen with his middle legs, putting the forelegs at the base of the female wings. Therefore, one would assume that males mated with wingless females could be in an unstable position, leading to the reduced copulation duration. On the contrary, we found the increased copulation duration of *D. virilis* males in tests with wingless females; obviously, the lack of the female wings did not interfere with mating in this species. In addition, the increased duration of copulation in aristaless males of *D. virilis* and *D. lummei* was in concordance with the high percentage of matings in these tests. Moreover, the increased copulation duration was found in the negative control. It has been shown recently that *D. melanogaster* females sing by wing vibration being in copula (Kerwin et al. 2020). We therefore assume that females can regulate copulation duration through an acoustic signal. Thus, the results support our hypothesis that the female song has different functions in the three species studied: the courtship inhibition in *D. virilis* and *D. lummei,* but the courtship stimulation in *D. littoralis.*

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