# Isolation Mechanisms in Closely Related Grasshopper Species
## *Chorthippus albomarginatus* and *Ch. oschei* (Orthoptera: Acrididae)

### V. Yu. Vedenina,[a] N. K. Kulygina,[b] and A. K. Panyutin[a]

*[a]Institute for Information Transmission Problems, RAS, Moscow, 127994 Russia*
*[b]Moscow State University, Biological Department, Moscow, 119899 Russia*

**Abstract**—Two closely related grasshopper species *Chorthippus albomarginatus* and *Ch. oschei* are known to hybridize in the narrow contact zone at the territory of Ukraine and Moldova. Different isolaton mechanisms providing reproductive isolation between the two species were studied. In choice mating experiments, females of the both species demonstrated a strong assortative mating (80–90% preference for the conspecific males). Comparison of the parental and hybrid viability revealed a reduced hatching and increased larval mortality in $F_1$ and $F_2$ hybrids. In choice mating experiments, the hybrid females mated less assortatively than the parental females. An assymmetry was found in mating preferences and in viability of hybrids. The results demonstrate the existence of pre- and postmating isolation between *Ch. albomarginatus* and *Ch. oschei*. A possible fate of the hybrid zone is discussed.

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The ways species evolve reproductive isolation and isolating mechanisms develop in the process of speciation have been the central questions in evolutionary biology. It is customary to distinguish two main forms of isolation mechanisms: premating and postmating reproductive isolation (Dobzhansky, 1937; Mayr, 1974). Premating isolation evolves as a result of assortative mating caused by various environmental (seasonal, habitat) changes (Harrison, Rand, 1989; Mac-Callum et al., 1998) or may be a consequence of sexual selection when competition for mates leads to divergence in mating signals (Andersson, 1994).

Postmating isolation is mostly provided by inviability and sterility of hybrids (Haldane, 1922; Orr, 1996; Naisbit et al., 2002). Quite recently it has been shown that premating isolating barriers can evolve more rapidly than postmating ones. Closely related species that don't crossbreed or very rarely hybridize in nature successfully hybridized under laboratory no-choice conditions and produced fertile offspring (Helversen and Helversen, 1975; Shaw, 1996; Monti et al., 1997; Mendelson and Shaw, 2002; Mendelson, 2003; Saldamando et al., 2005). However, during the last decades there appeared many data on the existence of hybrid zones between closely related species of different groups of animals. The species hybridizing in nature offer valuable experimental material for studying the ways of the establishment of reproductive isolation. There are many examples of hybridizing species demonstrating at least partial assortative mating and producing at the same time viable and fertile hybrids (Panov, 1989, 1993; Kryukov and Gureev, 1997; Jiggins et al., 1997; Rolan-Alvarez et al., 1997; Bailey et al., 2004). In some instances the hybrids are viable under laboratory conditions but lose to parental species in fitness with regard to specific ecological conditions or are less successful in attracting mates (Hatfield and Schluter, 1999; Naisbit et al., 2001). There are examples when, on the contrary, assortative mating of parental species is absent, the hybrids are viable and fertile, but the isolation mechanisms are manifested at the level of zygote formation, i.e., the egg cells are selectively fertilized by the sperm of conspecific males (Howard et al., 1998). The fate of hybrid zones may be different. Isolation mechanisms may become more pronounced due to the reinforcement of assortative mating (Saetre et al., 1997; Jiggins et al., 2001), which is, however, difficult to reveal and demonstrate convincingly. Hybrid zones may enlarge due to the increase in the hybridization intensity when introgression involves not only the contact zone but also the population beyond it. Such examples were described for birds (Panov, 1989, 1993), however, as a rule, it is hardly possible to reveal the changes not only in isolation mechanisms but also in the spatial structure of the

hybrid zone during the period under study. In such cases the hybrid zone is considered to be stable, which could be provided by the balance between gene exchange and selection against hybrids (Blinov et al., 1993; Harrison, 1983; Barton and Hewitt, 1989; Saldamando et al., 2005).

We investigated pre- and post-mating isolation mechanisms in two closely related grasshopper species *Chorthippus albomarginatus* (De Geer 1773) and *Chorthippus oschei* Helversen 1986. The ranges of the two species are rather broad: *Ch. albomarginatus* inhabits Northern and Central Europe and in the east its range stretches through Siberia to Central Yakutia and the Baikal environs. The range of *Ch. oschei* extends from the Balkans eastward to Ukraine (Bei-Bienko and Mishchenko, 1951; Helversen, 1986; Vedenina and Helversen, 2003).

*Ch. albomarginatus* and *Ch. oschei* are very similar in morphology and calling songs but are easily distinguished by their courtship songs. These species have an extremely complex and elaborate courtship behavior: the sound signal consisting of several elements with different amplitude and temporal patterns is accompanied with spectacular movements of hind legs and antennae (Helversen, 1986; Vedenina, 2005). The two species were shown to hybridize in the territory of Ukraine and Moldova within a relatively narrow contact zone about 200 km wide (Vedenina and Helversen, 2003). However, the hybrid males producing more or less intermediate courtship song comprised less than 10% of the natural population. In all localities of the hybrid zone, males producing songs similar to one or another parental type dominated. The song analysis of laboratory hybrids showed that some males obtained from backcrosses produced parental-like songs (Vedenina et al., 2007). Hence the true proportion of hybrids in the population of the hybrid zone remains unknown.

Among the processes running in the hybrid zone, of particular interest are the isolation mechanisms evolved by the hybridizing species. How can the isolation mechanisms develop in the contact zone, or does the hybrid zone remain relatively constant in space and time?

In this work we studied, first, whether there exists premating ethologic isolation between *Ch. albomarginatus* and *Ch. oschei*, i.e. if there are reliable differences in mate preferences given the choice between this or that species. Secondly, we studied the occur-

rence of postmating isolation between *Ch. albomarginatus* and *Ch. oschei*. We compared the fitness of $F_1$ and $F_2$ hybrids with that of parental species under the laboratory conditions. We also studied the ability of hybrids to compete with parental species for the mate partner given the choice between hybrid and parental species.

## MATERIALS AND METHODS

Specimens of *Ch. albomarginatus* were collected in the following localities: Ukraine, the border between Dnepropetrovsk and Donetsk provinces, 25 km W of Krasnoarmeisk, Kamenka, near Byk River, 22 July 2000; Ukraine, Poltava Prov., 24 km NE of Mirgorod, Velikie Sorochintsy, 13 July 2001; Moscow, Bittsa forest-park, 3 July 2001; Germany, 30 km N of Nurnberg, environs of Effeltrich, 25 July–25 August 2002.

Specimens of *Ch. oschei* were collected in the following localities: Ukraine, Kherson Prov., Skadovsk Distr., near Tarasovka., 18 July 2000; Ukraine, Odessa Prov., beach of Dnestrovskii liman, near Semenovka, 8 July 2001; Bulgaria, 50 km SW of Plovdiv, meadow near Lake Batak, 28 June 2002; Hungary, Bekes Prov., 7 km NW of Battonya, 16 July 2002. The localities were chosen considering that all populations in Germany and Bulgaria were allopatric while all Ukrainian sampling sites were situated at the border or inside the contact zone of the two species. From the site in Kherson Province, we recorded hybrid songs, but in behavioral experiments only the males producing the song of *Ch. oschei* were used.

To assess the fitness of the collected specimens, equal number (15–20) of males and females from the same locality were placed in cages measuring $50 \times 25 \times 30$ cm and allowed to mate for 2–4 weeks. The females were supplied with moist sand for oviposition. Egg pods were collected and placed in plastic Petri dishes containing moist sand (10–20 pods per dish). These dishes were stored for 4–6 months in a refrigerator at 4°C for diapause. In spring, egg pods were transferred from the fridge to room temperature (22°C) and after 3–4 weeks started to hatch for about 10 days. Nymphs were placed in plastic cages and fed with fresh grass, *Poa annua*, nymphs of the last instars and adults, with *Dactylus glomerata* under 12 light : 12 dark cycles. The development from hatching to adult took 4–6 weeks. Immediately after molting to adults, males and females were separated and kept in different cages for behavioral experiments.

On completion of the nymph hatching, the pods were opened to count the number of unfertilized eggs and dead embryos. The eggs were counted as unfertilized if their content was liquid and homogenous or if the eggs were solid or dry but lacking embryos. The total number of eggs in a pod was estimated by summing up hatched and dead nymphs (the latter were periodically removed from the cage). We assume that part of dead nymphs, especially those of instars I–II that were not removed in time from the cage, could be eaten and remained unaccounted for.

Two series of reciprocal crosses were conducted: *Ch. albomarginatus* (Moscow) $\times$ *Ch. oschei* (Odessa Prov.) and *Ch. albomarginatus* (Germany) $\times$ *Ch. oschei* (Bulgaria). In 2002–2003, $F_1$ and $F_2$ reciprocal hybrids were obtained from crosses between the Russian and Ukrainian populations, i.e. $F_1(a \times o)RU$, $F_1(o \times a)RU$, $F_2(a \times o)RU$, $F_2(o \times a)RU$ (female $\times$ male). In 2003–2004, reciprocal F1 and F2 hybrids were obtained from crosses between the German and Bulgarian populations as well as the backcrosses between the hybrid males and the female of pure species, i.e., $F_1(a \times o)GB$, $F_1(o \times a)GB$, $F_2(a \times o)GB$, $F_2(o \times a)GB$, and BC a $\times$ (a $\times$ o), BC o $\times$ (o $\times$ a) (female $\times$ male). $F_3$ hybrids were obtained from crosses in only one direction (F2(a $\times$ o)GB between one another), other crosses were not conducted.

To study behavioral preferences, 7–14 females of one species or of one type of crosses were placed in a cage together with an equal number of males of different species or different types of crosses. Each series of experiments was conducted for 6–8 hours each day during 10–14 days. All the specimens were marked with paint on the back of the pronotum. The presence of copulating pairs was checked at intervals of 20–30 min. In the majority of *Chorthippus* species, in particular in the *Ch. albomarginatus*-group, the copulation process lasts for more than 1 h, therefore we were able to record all successful matings. Thirteen series of behavioral experiments were conducted with the following groups: *Ch. albomarginatus* from Ukraine, Dnepropetrovsk Prov. in 2000, Poltava Prov. in 2001 and from Germany in 2002; *Ch. oschei* from Ukraine, Kherson Prov. in 2000, Odessa Prov. in 2001 and from Hungary in 2002; the $F_1$ generation of *Ch. albomarginatus* from Germany and the $F_1$ generation of *Ch. oschei* from Bulgaria in 2003; $F_1$, $F_2$ hybrids and BC a $\times$ (a $\times$ o) between German *Ch. albomarginatus* and Bulgarian *Ch. oschei* in 2003–2004. The design of the experiment was slightly different in different se-

ries. Females of parental species were allowed to choose between two types of males: the males of *Ch. albomarginatus* and *Ch. oschei* or among conspecific and hybrid males. Thus, 12 females of *Ch. albomarginatus* could choose among 6 males of *Ch. albomarginatus* and 6 males of *Ch. oschei.* The hybrid females were allowed to choose among males of three types, i.e. the males of both parental species and the hybrid males. Thus, 10 females $F_1(a \times o)$ could choose among 3 hybrid males, 3 males of *Ch. albomarginatus* and 3 males of *Ch. oschei.* The females of $F_2$ hybrids were placed together with $F_2$ hybrid males and BC hybrid males were used instead of the males of *Ch. albomarginatus* and *Ch. oschei.* The courtship songs of the BC hybrids were preliminarily recorded and analyzed. The males producing songs similar to the songs of parental species were used in the experiment.

## RESULTS

### Viability of Parental Species and Hybrids

The number of unfertilized eggs (UE) varied in parental species and in hybrids from 6 to 49% and from 18 to 67%, respectively (Table 1, figure). This parameter was rather low in $F_1$ of parental species from three populations, while in $F_1$ of *Ch. oschei* from Hungary and *Ch. albomarginatus* from Russia it achieved 42–49%. The UE also varied in hybrids obtained from crosses between the parents originating from different populations. In $F_1(o \times a)UR$ the proportion of UE was much greater (50%) than in the reciprocal hybrids (23%). In $F_2$ generation, this parameter was rather similar in reciprocal crosses and was lower (32–41%) than in $F_1(o \times a)UR$. In $F_1$ hybrids obtained from crosses between German and Bulgarian parents, the proportion of UE was very high (65–67%) regardless of the cross direction. In $F_2$ hybrids UE was also rather high while in the hybrids obtained from backcrosses UE was similar to this parameter in the parental species (18–26%).

The number of dead embryos (DE) in parental species and in hybrids varied within a similar range (figure, b). Among the parental species, the high proportion of DE was found in *Ch. oschei* from Bulgaria (10%), among the hybrids a similar DE proportion was recorded in Russian-Ukrainian $F_2$ hybrids and in most German-Bulgarian hybrids. The embryo mortality was higher when *Ch. oschei* served the maternal species, though the difference between the reciprocal hybrids was not significant. The highest embryo mortality was recorded in $F_3$ hybrids.

**Table 1.** Survival of eggs, nymphs and adults of *Chorthippus albomarginatus*, *Chorthippus oschei* and their hybrids

| Species or cross type | Total number of eggs | Number of unfertilized eggs | Number of dead embrios | Number of dead nymphs of I–II instars | Number of dead nymphs of III–VI instars | Number of individuals, reaching stage of adults | Appearance of first nymphs, day number | Appearance of first adults, day number |
|---|---|---|---|---|---|---|---|---|
| $F_1$ alb., Russia | 33 | 16 (49) | 0 | 2 (6) | 1 (3) | 14 (42) | 17 | 30 |
| $F_1$ alb., Germany | 70 | 4 (6) | 1 (1) | 19 (27) | | 46 (66) | 26 | 31 |
| $F_1$ osch., Bulgaria | 125 | 21 (17) | 12 (10) | 20 (16) | | 72 (58) | 29 | 43 |
| $F_1$ osch., Hungary | 119 | 51 (43) | 3 (3) | 33 (28) | 2 (2) | 30 (25) | no data | no data |
| $F_2$ osch., Hungary | 122 | 19 (16) | 1 (1) | 60 (49) | 12 (10) | 30 (25) | 23 | 43 |
| $F_1$ (a × o) RU | 79 | 18 (23) | 0 | 5 (6) | 5 (6) | 51 (65) | 22 | 31 |
| $F_1$ (o × a) RU | 321 | 161 (50) | 5 (2) | 27 (8) | 26 (8) | 102 (32) | 22 | 29 |
| $F_2$ (a × o) RU | 75 | 24 (32) | 6 (8) | 4 (5) | 15 (20) | 26 (35) | 30 | 27 |
| $F_2$ (o × a) RU | 239 | 99 (41) | 32 (13) | 60 (25) | 31 (13) | 17 (7) | 33 | 51 |
| $F_1$ (a × o) GB | 191 | 127 (67) | 2 (1) | 25 (13) | | 37 (19) | 28 | 31 |
| $F_1$ (o × a) GB | 297 | 193 (65) | 23 (8) | 27 (9) | | 54 (18) | 30 | 41 |
| $F_2$ (a × o) GB | 212 | 142 (67) | 12 (6) | 17 (8) | 4 (2) | 37 (17) | 20 | 33 |
| $F_2$ (o × a) GB | 420 | 215 (51) | 44 (11) | 133 (32) | 10 (2) | 18 (4) | 20 | 34 |
| BC a ×(a × o) | 80 | 21 (26) | 1 (1) | 32 (40) | 5 (6) | 21 (26) | 20 | 30 |
| BC o ×(o × a) | 148 | 27 (18) | 9 (6) | 91 (62) | 9 (6) | 12 (8) | 23 | 53 |
| $F_3$ (a × o) GB | 92 | 38 (41) | 17 (18) | 37 (40) | 0 | 0 | 25 | – |

Notes: Percentage of the total number for each species or cross type is shown in parentheses. RU, hybrids, obtained from the crosses of Russian *Ch. albomarginatus* and Ukrainian *Ch. oschei*; GB, hybrids, obtained from the crosses of German *Ch. albomarginatus* and Bulgarian *Ch. oschei*.

Nymph mortality in parental species from different populations varied from 9 to 59%, the highest proportion was found in $F_2$ of Hungarian *Ch. oschei*. The same regularity was observed in the Russian-Ukrainian hybrids: nymph mortality in $F_2$ (25–38%) was higher than in $F_1$ (12–16%). The German-Bulgarian hybrids demonstrated a somewhat different regularity: the $F_2$ hybrids originating from the maternal species *Ch. albomarginatus* showed low nymph mortality comparable with that in $F_1$ hybrids. Basically, the trend has been toward higher nymph mortality in the hybrids originating from the maternal species of *Ch. oschei*. However in *Ch. oschei* from Bulgaria, the proportion of dead nymphs was relatively low with this parameter being essentially higher for instars I–II than for instars III–IV (Table 1; figure, *c*).

The maximum number of specimens that reached the adult stage was revealed in $F_1$ of *Ch. albomarginatus* from Germany (66%) and in $F_1$ of *Ch.oschei* from Bulgaria (58%) (Table 1; figure, *d*). In their $F_1$ and $F_2$ hybrids, the share of specimens that developed to adult stage was significantly lower, 17–18% and 4–19%, respectively. Basically, the same tendency was observed as the one described above for nymph mortality: the survival was lower in hybrids originating from *Ch. oschei* as a maternal species. Fitness was higher in $F_1$ and $F_2$ hybrids originating from *Ch. albomarginatus* as a maternal species. Not a single nymph developed to adult in the hybrids $F_3$(a × o)GB.

The time for appearance of the first nymphs since the end of the "winter diapause" similarly varied in parental species and in hybrids, 17–29 and 20–33 days, respectively. In parental species, the development time from hatching of the first nymphs to adults ranged from 30 to 43 days, and the nymphs of *Ch. albomarginatus* developed faster than those of *Ch. oschei*. In hybrids, variations in the development time were wider, from 27 to 51 days. The hybrids with *Ch. oschei* as a maternal species, $F_2$(o × a)UR, $F_1$(o × a)GB and BC o × (o × a) developed as long as the $F_1$ generation of *Ch. oschei*, or considerably longer.

## Mate Preferences in Ch. albomarginatus and Ch. oschei

In six series of experiments on mate preference, the specimens from six populations were used. In each
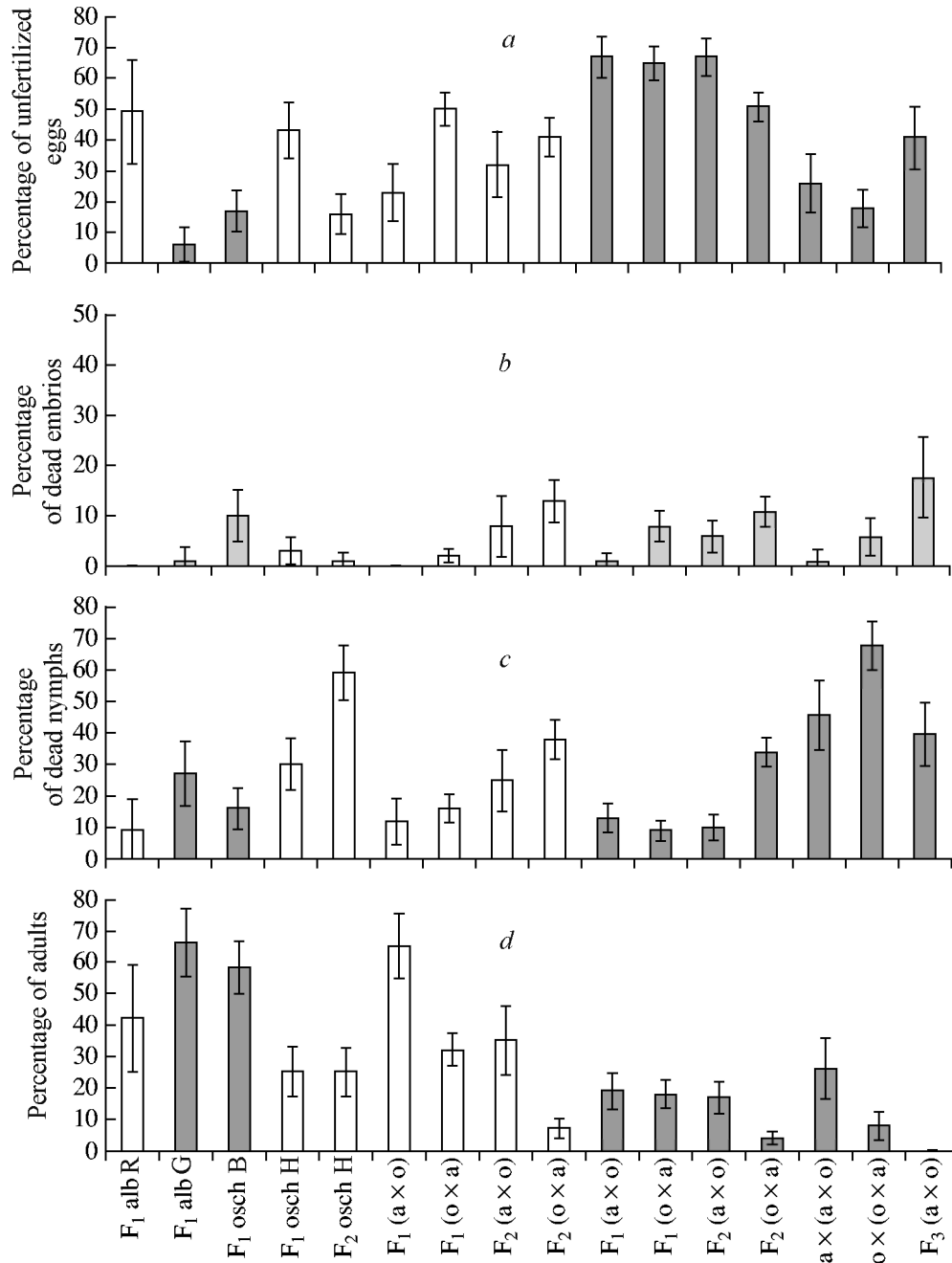
Parameters of viability of *Chorthippus albomarginatus* (alb) and *Ch. oschei* (osch) from different populations (R, Russia; G, Germany; B, Bulgaria; H, Hungary) and their $F_1$ and $F_2$ hybrids (for original data, see Table 1): (*a*) unfertilized eggs, (*b*) dead embryos, (*c*) dead nymphs, (*d*) specimens reaching the adult stage (German and Bulgarian parental species and their hybrids are marked with gray color; the 95% confidence interval is indicated on the histograms).

series the number of matings varied for different females (Table 2). Thus, of 12 *Ch albomarginatus* females from Poltava Province during a 11-day experiment, seven females mated once, four females, twice, and one female, three times. Of 12 *Ch. oschei* females from Hungary during a 13-day experiment, five females were recorded to mate once, three females, twice, two females, three times, and one female, four times.

Under the laboratory conditions of choice between equal numbers of con- and heterospecific males, females of both species mated significantly more frequently (binomial test, $p < 0.005$) with conspecific males (Table 2). In all the six series of experiments conducted on parental species, the share of heterospecific matings ranged from 10 to 20%. No regularities were found in heterospecific matings. In the cases of single matings, six females mated with hetero-

**Table 2.** Results of behavioral experiments with females of *Chorthippus albomarginatus* and *Ch. oschei* from different populations

| Females from different populations | Total number of females | Number of females mated | | | | Duration of experiment (h) | Mating frequency per hour | Total number of matings | Number of matings with | | | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 time | 2 times | 3 times | 4 times and more | | | | conspecific males | heterospecific males | hybrid males | |
| *Ch. albomarginatus,* Ukraine, Dnepropetrovsk Prov. | 13 | 8 | 5 | – | – | 103 | 0.013 | 18 | 15 | 3 | n/p | 0.001 |
| *Ch. albomarginatus,* Ukraine, Poltava Prov. | 12 | 7 | 4 | 1 | – | 90 | 0.017 | 18 | 14 | 4 | n/p | 0.004 |
| *Ch. albomarginatus,* Germany | 14 | 5 | 1 | 2 | 3 | 110 | 0.021 | 32 | 28 | 4 | n/p | 0 |
| *Ch. oschei,* Ukraine, Kherson Prov. | 11 | 4 | 3 | 3 | 1 | 107 | 0.020 | 24 | 21 | 3 | n/p | 0 |
| *Ch. oschei,* Ukraine, Odessa Prov. | 11 | 2 | 6 | 3 | – | 90 | 0.023 | 23 | 21 | 2 | n/p | 0 |
| *Ch. oschei,* Hungary | 12 | 5 | 3 | 2 | 1 | 99 | 0.018 | 21 | 19 | 2 | n/p | 0 |
| $F_1$ *Ch. albomarginatus,* Germany | 10 | 3 | 2 | 3 | 2 | 94 | 0.030 | 28 | 16 | n/p | 12 | 0.172 |
| $F_1$ *Ch. oschei,* Bulgaria | 11 | 4 | 3 | 2 | 1 | 85 | 0.028 | 26 | 25 | n/p | 1 | 0 |

Notes. *p*, probability of matings with the strange males (binomial test); n/p, type of males not participated in experiments.

**Table 3.** Results of behavioral experiments with hybrid females obtained from the crosses of German *Chorthippus albomarginatus* and Bulgarian *Ch. oschei*

| Hybrid females | Total number of females | Number of females mated | | | | Duration of experiment (h) | Mating frequency per hour | Total number of matings | Number of matings with | | | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 time | 2 times | 3 times | 4 times and more | | | | hybrid males | males of *Ch. albomarginatus* | males of *Ch. oschei* | |
| $F_1$ | 18 | 7 | 5 | 4 | 2 | 82 | 0.025 | 37 | 15 | 16 | 6 | 0.5 (hybr/alb) 0.013 (hybr/osch) |
| $F_2$ | 15 | 6 | 4 | 1 | – | 114 | 0.009 | 16 | 5 | 6 | 5 | 0.5 (hybr/alb) 0.377 (hybr/osch) |
| BC a × (a × o) | 12 | 6 | 2 | 1 | – | 69 | 0.016 | 13 | 7 | 5 | 1 | 0.194 (hybr/alb) 0.004 (hybr/osch) |

Notes: *p*, probability of matings of hybrid females with males of parental species (binomial test), male types are shown in parentheses.
In each experiment, males of three types participated: *Ch. albomarginatus* (alb), *Ch. oschei* (osch) and hybrids (hybr).

specific males. No significant differences in mate preferences between the populations of one species were found.

### *Mate Preferences in Hybrids*

The females of parental species from German and Bulgarian populations were allowed to choose be-

tween conspecific and hybrid males (Table 2). In these experiments, the number of matings for different females varied just as in the series described above, but the frequency of matings with the hybrid males differed in females of different species. The females of *Ch. albomarginatus* mated equally frequently with conspecific males and hybrid males (binomial test,

$p = 0.172$), whereas the females of *Ch. oschei* significantly more frequently mated with conspecific males ($p < 0.001$).

The hybrid females in $F_1$ generation mated almost equally frequently with the hybrid males and the males producing the courtship song of *Ch. albomarginatus*, but they mated significantly less frequently with the males producing a courtship song of *Ch. oschei* ($p = 0.013$) (Table 3). The hybrid females in $F_2$ generation showed almost equal number of matings with all types of males. The females BC a × (a × o) mated with the same frequency with the hybrid males and the males of *Ch. albomarginatus*, but significantly less frequently–with the males of *Ch. oschei*.

To compare the mating frequency of females from different populations and cross types, a conventional value of the mating frequency of one female per hour was calculated. This frequency varied in different groups, being the lowest in hybrid females of $F_2$ generation and the highest in $F_1$ generation of *Ch. albomarginatus* from Germany and *Ch. oschei* from Bulgaria (Tables 2 and 3).

## DISCUSSION

### Is there a Premating Behavioral Isolation between Ch. albomarginatus and Ch. oschei?

In choice mating experiments, females of two closely related grasshopper species *Ch. albomarginatus* and *Ch. oschei* demonstrated a rather strong assortative mating: in 80–90% of the cases they mated conspecific males preferring them to heterospecific ones. We did not find significant differences in mating preferences between allopatric and parapatric populations of the same species, which may testify to the fact that no reinforcement of reproductive isolation takes place on the border of ranges.

The duration of courtship was not precisely registered in the experiments, however, it has been observed that the males did not show preference while courting females of one or another species. Before starting courtship, the male always touched the female with antennae and started to sing with no regard of the female species. It seems likely that the females of *Ch. albomarginatus* and *Ch. oschei* do not differ in the pheromone composition although this suggestion calls for further investigation. At the moment we assume that the mate choice in these species is completely determined by the female's preference and is based on the recognition of a specific acoustic signal accompa-

nied in addition by visual signals demonstrated at a certain moment of courtship. The courtship signals of two species are well distinguished by amplitude and temporal parameters (Vedenina, Helversen, 2003).

The question arises why females of both species in 10–20% of matings choose heterospecific males. Before accepting an attempt at copulation, a female has not only to choose a male of the same species, but also to evaluate the male "quality" (Andersson, 1994). This choice may be realized owing to certain parameters of the courtship signal. In spite of significant differences in courtship songs between *Ch. albomarginatus* and *Ch. oschei* they have some common elements (Vedenina et al., 2007). It may be suggested that some elements, including the common ones, play a crucial role in conspecific recognition. Other courtship parameters more likely serve as an indicator of the male quality and are essential in stimulating and inducing the female to copulation (Vedenina, 2005).

It was reported earlier (Riede, 1983) that a long-term courtship accelerated the beginning of receptive phase of the *Gomphocerippus rufus* female. Our data on the variable number of copulations of the *Ch. albomarginatus* and *Ch. oschei* females observed over the same periods of time are in conformity with the observations of Hartmann and Loher on *G. rufus* (1996, 1999). They found that secretions from the male accessory glands received together with sperm change the sexual behavior of the female and reduce the female's receptivity. It is conceivable that the occurrence of multiple matings by females observed in our experiments was determined not only by the courtship song pattern and the courting duration but also by the amount of sperm and the secretions from accessory glands received from the first male.

The results of our experiments favor incomplete premating behavioral isolation between the closely related species *Ch. albomarginatus* and *Ch. oschei*.

### Are the Hybrids between Ch. albomarginatus and Ch. oschei Less Viable than the Parental Species?

According to the Haldane rule (Haldane, 1922), heterogametic sex in $F_1$ hybrids is often completely or partially sterile. In our experiments, it could be expected that $F_2$ generation would contain either many unfertilized eggs or a high embryonic and nymph mortality would be observed. However, a great proportion of unfertilized eggs has been already found among the eggs of $F_1$ generation of German-Bulgarian hybrids

(figure, *a*). These results could be explained, first, by incompatibility between the receptors and specific proteins on the egg surface and the sperm, which prevented fertilization. Second, the females of parental species could altogether less frequently mate with heterospecific males and hence receive insufficient amount of sperm. At the same time, we did not expect the high proportion of unfertilized eggs in $F_1$ generations of *Ch. albomarginatus* from Russia and *Ch. oschei* from Hungary. The former case could be explained by the age of females collected late in summer at the end of the reproductive period. The case observed with Hungarian *Ch. oschei* is more difficult to explain. Especially surprising was very high nymph mortality in $F_2$ of *Ch. oschei*. One cannot exclude the weakening of insects in succeeding generations in laboratory culture, which should be considered when analyzing the results of all laboratory experiments.

The Russian-Ukrainian and German-Bulgarian hybrids differed not only in the number of unfertilized eggs, but also in nymph mortality. Our data demonstrate an overall higher viability of the Russian-Ukrainian hybrids, although it varied depending on the cross direction. In both series of crosses, the hybrids originating from *Ch. oschei* as the maternal species had a higher embryonic and nymph mortality. A similar asymmetry in the viability of reciprocal hybrids is described by many authors (e.g., Harrison, 1983; Orr, 1996; Naisbit et al., 2002). Genetic incompatibility that is stronger expressed in one direction of crosses points to an incompletely established isolation barrier.

The higher viability of Russian-Ukrainian hybrids is possibly due to the fact that the parental *Ch. oschei* specimens were collected at the site adjacent to the hybrid zone. It is not inconceivable that the genome of this population contained the alleles of *Ch. albomarginatus*, which enhanced the genetic compatibility of the parental genotypes.

### Can the Hybrids Compete for Mates with the Parental Species?

Under the conditions of choice between the conspecific and hybrid males, the females of parental species demonstrated different selectivity. The females of *Ch.oschei* in 90% cases copulated with the conspecific males, whereas the *Ch. albomarginatus* females did not discriminate between conspecific and hybrid males. $F_1$ hybrid females behaved in the same way as the females of *Ch. albomarginatus*: they did not distinguish the *Ch. albomarginatus* males from the

hybrid ones but reliably less frequently copulated with the *Ch. oschei* males. This interesting result may be explained in the following way: most of the courtship song parameters of the hybrid males more closely resemble those of *Ch. albomarginatus* males than those of *Ch. oschei* males (Vedenina et al., 2007). The correlation of the above courtship signal parameters with the results of behavioral experiments suggests that these parameters are really essential for recognition.

$F_2$ hybrid females did not discriminate between all the three types of males, although on the whole they mated less frequently than the males of $F_1$ hybrids. Most likely the hybrid females can compete for the mate with the females of parental species in mixed biotopes. The offspring of hybrid females could be as numerous as that of parental species if it were not for the revealed lower fitness of hybrids. A somewhat different situation occurs with the hybrid males: they can compete with the hybrid males of *Ch. albomarginatus* but lose to the *Ch. oschei* males. A correlation should be noted between the different selectivity of *Ch. albomarginatus* and *Ch. oschei* females and the different viability of the reciprocal hybrids. The females of *Ch. oschei* were found to mate more assortatively, while the hybrid offspring obtained from them survived rather poorly. In contrast, the females of *Ch. albomarginatus* mated less assortatively (i.e. they did not distinguish between conspecific males and hybrid ones), but their hybrid offspring was more viable. This correlation may indicate that the premating isolation mechanisms are reinforced in the hybrid zone. It may be suggested that during the evolution, a stronger genetic incompatibility between the females of *Ch. oschei* and the males of *Ch. albomarginatus* resulted in the development of increased selectivity of the *Ch. oschei* females. However, as noted earlier (Vedenina et al., 2007), the high selectivity of the *Ch. oschei* females may be explained by the peculiarities of perception and inheritance of the acoustic signals in the *Ch. albomarginatus* group.

In our previous studies of the hybrid zone (Vedenina and Helversen, 2003) we found only one parental species present in all hybrid biotopes investigated, and most of hybrid signals more closely resembled the song of this very species. Therefore the hybrid biotopes were conventionally named the *albomarginatus*-like and *oschei*-like biotopes. Populations of different types were next to one another, the adjacent meadows differing in plant composition and soil moisture. We have suggested that two species have different eco-

logical preferences. The results obtained in this study allow a suggestion that selection pressure against hybrids is stronger in the *oschei*-like hybrid biotopes than in the *albomarginatus*-like biotopes, which may result in the movement of the hybrid zone border.

## ACKNOWLEDGMENTS

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