

Dominance effects strengthen premating hybridization barriers between sympatric species of grasshoppers (Acrididae, Orthoptera)

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Abstract

Sexual selection can lead to the rapid evolution of premating hybridization barriers and allows accelerated diversification and speciation within an evolutionary lineage. Especially during early stages of divergence, hybridization may impede further divergence, which strongly depends on the reproductive success of hybrids. Behavioural sterility of hybrids can limit or even prevent homogenizing gene flow. In this study, we investigated the attractiveness of male courtship songs for females of the grasshopper species *Chorthippus biguttulus* and *C. brunneus* and their interspecific F1 and F2 hybrids. Song preferences of females of both species are highly species specific and differ in three parameters: shape of the preference function, preference for syllable pattern and phrase duration. F1 hybrid females of both reciprocal crosses as well as F2 hybrid females resembled closely pure *C. biguttulus* females in respect of shape of the preference function and preference for syllable pattern, while preference for phrase duration showed an intermediate expression. This resulted in song preferences of hybrid females that closely resembled those of one parental species, that is *C. biguttulus* females. Such strong dominance effects were rarely reported so far. They represent an effective barrier limiting gene flow between the two species, since hybrid females will backcross to only one parental species and discriminate against hybrid males, which are behaviourally sterile. Such taxon-specific modes of inheritance may have facilitated the rapid divergence of acoustically communicating grasshoppers of the species group of *Chorthippus biguttulus*. Our findings have novel implications on the expression of neuronal filters and the evolution of complex courtship signals.

KEYWORDS

acoustic communication, behavioural sterility, *Chorthippus*, female preference, inheritance, speciation

1 | INTRODUCTION

Speciation is an intensely studied topic in evolutionary biology addressing one of the most complex processes in the evolution of biodiversity. In several taxa, premating behaviour is involved early in speciation (Hollocher, Ting, Pollack, & Wu, 1997; McMillan, Jiggins, & Mallet, 1997; Richman & Price, 1992). Populations can diverge rapidly in sexually selected traits of males and in female preferences thereof leading to premating hybridization barriers (Coyne & Orr, 2004; Janicke, Ritchie, Morrow, & Marie-Orleach, 2018; Panhuis, Butlin, Zuk, & Tregenz, 2001). However, especially during early stages of sexual isolation, hybrid mating can occur if populations meet. Due to the lack of usually more slowly evolving post-zygotic hybridization barriers, hybrids are likely to be viable and able to reproduce. Accordingly, hybrids act as a link between the parental lineages, mediating gene flow among them and thus preventing further divergence. Hence, the reproductive success of hybrids is essential for the future evolutionary development, that is whether populations admix or will diverge further.

Sexual selection itself can cause behavioural sterility, and thus, post-mating mechanisms are not necessarily required for hybrid sterility. Hybrid males are behaviourally sterile if females of both parental lineages as well as female hybrids discriminate against them. Despite its important role in rapid speciation by sexual selection, discrimination of females against hybrid males and thus behavioural sterility of hybrid males was studied only in a small number of species. Two generally distinct types of behavioural sterility are observed. First, intrinsic behavioural hybrid sterility occurs, when hybrids have behavioural anomalies like deficiencies that prohibit courting or mating (Coyne, 1989; Wu & Hollocher, 1998; : Coyne & Orr, 2004). The second is extrinsic behavioural hybrid sterility, which is mainly caused by hybrids exhibiting intermediate behaviour, which is not attractive to the choosing sex (Servedio, 2009; Servedio & Noor, 2003). For example, Stratton and Uetz (1986) showed that both sexes of F1 hybrids between two wolf spider species were completely sterile through their intermediate behaviour. A study of *Heliconius* butterflies revealed that hybrids mate readily with each other, but not with parentals (Naisbit, Jiggins, & Mallet, 2001). Most studies showing behavioural sterility of hybrids also show that ecological factors like environmental cues or predation contribute to the extent how hybrids suffer from disadvantages compared to parental species (Naisbit et al., 2001; Nosil, Crespi, Gries, & Gries, 2007; Vamosi & Schluter, 1999).

Gomphocerinae grasshoppers comprise several rapidly diverging species groups that evolved effective premating hybridization barriers in the absence of strong post-zygotic barriers (Mayer, Berger, Gottsberger, & Schulze, 2010). Closely related species are frequently morphologically cryptic but males exhibit species-specific songs that match female preferences. Males produce calling songs, and receptive conspecific females answer by producing response songs. This allows the male to localize the female and approach her. The probability of a female replying to a sound stimulus (e.g. a male song) with a response song is a good predictor of the female's preference

for a male song and also for its willingness to mate with this male (Klappert & Reinhold, 2003; Perdeck, 1958; von Helversen & von Helversen, 1994). In this study, we investigated the preference function (i.e. pattern of female response across variation in male sexual signals (Reichert & Ronacher, 2014)) of parental and hybrid females of *Chorthippus biguttulus* and *C. brunneus*. Both species occur sympatrically over a wide range of Europe and frequently also syntopically in the same habitats (Ragge & Reynolds, 1998; Ragge, Reynolds, & Willemse, 1990). In the field, hybrids between the two species occur very rarely (Perdeck, 1958; Faber, 1957; Ragge, 1976; Ingrisch, 1995; H. Kriegbaum and O. v. Helversen, unpublished data), showing that the premating hybridization barrier is not complete. As hybrids are viable and fertile (Perdeck, 1958), no obvious intrinsic post-zygotic incompatibilities are known.

We addressed three specific questions by behavioural playback experiments: (a) Do females of the two parental species as well as of the F1 and F2 hybrids discriminate against male hybrid songs? (b) What parameters of male songs are crucial for the females' response behaviour? (c) Do the response songs generated by females differ between the parental species and their hybrids? In addition, comparing the preferences of the parental species with those of the hybrids may provide also first insights into the inheritance of female song preferences and female response songs. Finally, we discuss our results in the context of genetic mechanisms that allow rapid evolution of sexual isolation and thus may lead to high speciation rates driven by behaviour.

2 | MATERIAL AND METHODS

2.1 | Study species

We collected third- and fourth-instar larvae of *C. biguttulus* and *C. brunneus* in southern Germany (Erlangen and Seewiesen, Bavaria) and Austria (Kühtai, Tyrol). At all three locations, both species occur syntopically. Animals were kept in plastic breeding cages (44 × 44 × 44 cm) and were fed with orchard grass (*Dactylis glomerata*) and annual bluegrass (*Poa annua*) ad libitum. Light and heat were provided for 12 hr each day with a 40 W bulb inside the cage. Cages were monitored daily. Directly after imaginal moult, both sexes were separated and housed in different cages. Animals were marked individually on their pronotum and/or wings with paint markers (Edding 780).

2.2 | Interspecific crossing experiments

Crossing experiments were done in small gauze cages (8 × 7 × 6 cm) at temperatures between 35 and 40°C. We only used singing males and virgin females, which were responding to conspecific male songs. As with intact animals barriers were so strong, that no interspecific copulation was achieved thereupon males were muted by cutting off the fore- and back wings. Males were still able to move their legs normally but no sound was emitted. The abscission of the wings did not influence the courtship behaviour of males. Before and during

crossing experiments, females were stimulated by conspecific male songs from a recorder or from real males, which were placed near the female's cage. Six copulations were obtained in a total of 186 experiments (i.e. 3%) with a *C. biguttulus* female and a mute *C. brunneus* male. Three reciprocal crosses occurred in a total of 143 experiments, representing only 2%. After copulation females were kept isolated, and egg pods were collected. Egg pods were embedded in moist sand in Petri dishes for about 2 months at room temperature and thereafter at 6°C for at least 6 months for diapause. Hatching was initiated by incubating the egg pods at room temperature.

Throughout the text, the first mentioned species in hybrids is always the mother, and the second mentioned one is the father species. F2 hybrids were only obtained from the crosses of *C. brunneus* × *C. biguttulus* F1 animals. Other F2 mating combinations were not obtained, due to the small number of available F1 animals and the reluctance of old females to mate after playback experiments.

2.3 | Female preference tests

All female preference tests were performed using virgin females at an age of at least 6 days after imaginal moult as from then on females start to react to the species-specific male songs (Kriegbaum & von Helversen, 1992). The behavioural tests were performed in a sound-attenuated thermostatic chamber with a constant temperature of $30 \pm 1^\circ\text{C}$. The chamber was equipped with a loudspeaker for acoustic playback experiments and a condenser microphone (Type MCE-101; 50–12,000 Hz) for recording the female's response song (von Helversen, 1979; von Helversen & Helversen, 1983). During experiments, a female was kept 15 cm from the loudspeaker in a small gauze cage and was provided with some blades of grass and moist sand as egg-laying substrate. A computer-controlled set-up played back synthetic sound stimuli and registered female reply songs automatically. Thus, there was no disturbance and no observer bias during tests. Song stimuli were synthesized by generating an amplitude envelope for a song pattern (see below) that was filled with white noise. The resulting amplitude-modulated broadband song stimuli were played back with custom-built power amplifiers and emitted by tweeter loudspeaker with a flat response from 2 to 40 kHz (Dynaudio D21/2, Skanderborg Denmark). Signal amplitude was calibrated to a constant intensity level of 70 ± 2 dB SPL (peak) with a Brüel & Kjær sound level meter (Type 2231 and a Brüel & Kjær $\frac{1}{2}$ inch condenser microphone 4133) at the position of the animal (von Helversen, 1979; von Helversen & Helversen, 1983).

2.4 | Song patterns

The amplitude modulation of the sound was altered in two main parameters creating five different song patterns (Figure 1 for schemes of song patterns 1–4). By means of these song patterns, we simulated a transition from *C. biguttulus* until *C. brunneus* male calling songs with intermediate stages. The first varied parameter was the syllable and pause duration, which was given in five different combinations: a typical *C. biguttulus* song pattern with 80 ms long syllables

separated by 12 ms pauses (pattern 1), two intermediate syllable/pause durations of 40/8 ms (pattern 2) and 20/6 ms (pattern 3), a *C. brunneus* song pattern that consisted of 10 ms syllables with 4 ms pauses (pattern 4) and one pattern without any pauses (pattern 5), which served as a negative control (not shown).

The second altered parameter was phrase duration. The five song patterns were played back to females in phrases of 11 different durations: 90, 130, 180, 250, 350, 500, 700, 1,000, 1,400, 2,000 and 2,800 ms, resulting in a total of 55 different sound stimuli per test session. As songs in *C. biguttulus* and *C. brunneus* consist of repeated phrases, we played back each sound stimulus three times. Pauses between these phrases were set to 6 s, when females did not reply and 2 s when females answered with a response song. The order of stimuli was randomized. After playbacks of all 55 stimuli (one session), playbacks were paused for 1 min. If no response song of a female was registered within one complete session, presentation was stopped for 30 min. When females responded, the programme immediately continued with a next session. Each female was tested on average with 21 sessions (total females $N = 78$; $SD = 6.2$) meaning that each female was on average tested 1155 times, resulting in a very strong database.

2.5 | Data analysis

The response probability was calculated by dividing the response rate by 100. Accordingly, its values ranged from zero to one. In total, we analysed 27 *C. biguttulus*, 13 *C. brunneus*, 17 F1 hybrids of *C. biguttulus* × *C. brunneus*, 12 F1 hybrids of *C. brunneus* × *C. biguttulus* females and 9 F2 females. When females are not in a receptive state, they do not answer at all. Females can also be overmotivated and begin to sing themselves even without any kind of stimulus. Therefore, we excluded unmotivated or overmotivated and hence unselective females from further analysis, if the maximum response probability to song stimuli over 500 ms duration was below 15% or when they responded to a control song stimulus (lacking pauses) longer than 500 ms (song pattern 5 tests, data not shown) in more than 15% of all sessions. The 500 ms threshold was set as limit as *C. brunneus* females often responded to short song stimuli of the control pattern (lacking pauses within phrases). To control for differences in motivation levels among days, females and species, we normalized each response profile by setting the maximum number of responses of a female to a stimulus to 100%.

The female response data consist of a series of repeated tests of the same female, and the response probabilities were binomially distributed. To account for this data structure, we used generalized estimation equations (GEE) to test whether different song parameters affect female response probabilities and thus whether preference function curves differ among the five groups of animals (Reichert, 2015; Reichert & Ronacher, 2014). The test model consisted of the terms group (of females, i.e. *C. biguttulus*, *C. brunneus*, both reciprocal F1 hybrids, and F2 hybrids), phrase, and an interaction-term group*phrase as well a term for the individual female as a random factor to account for repeated measurements of the same female.

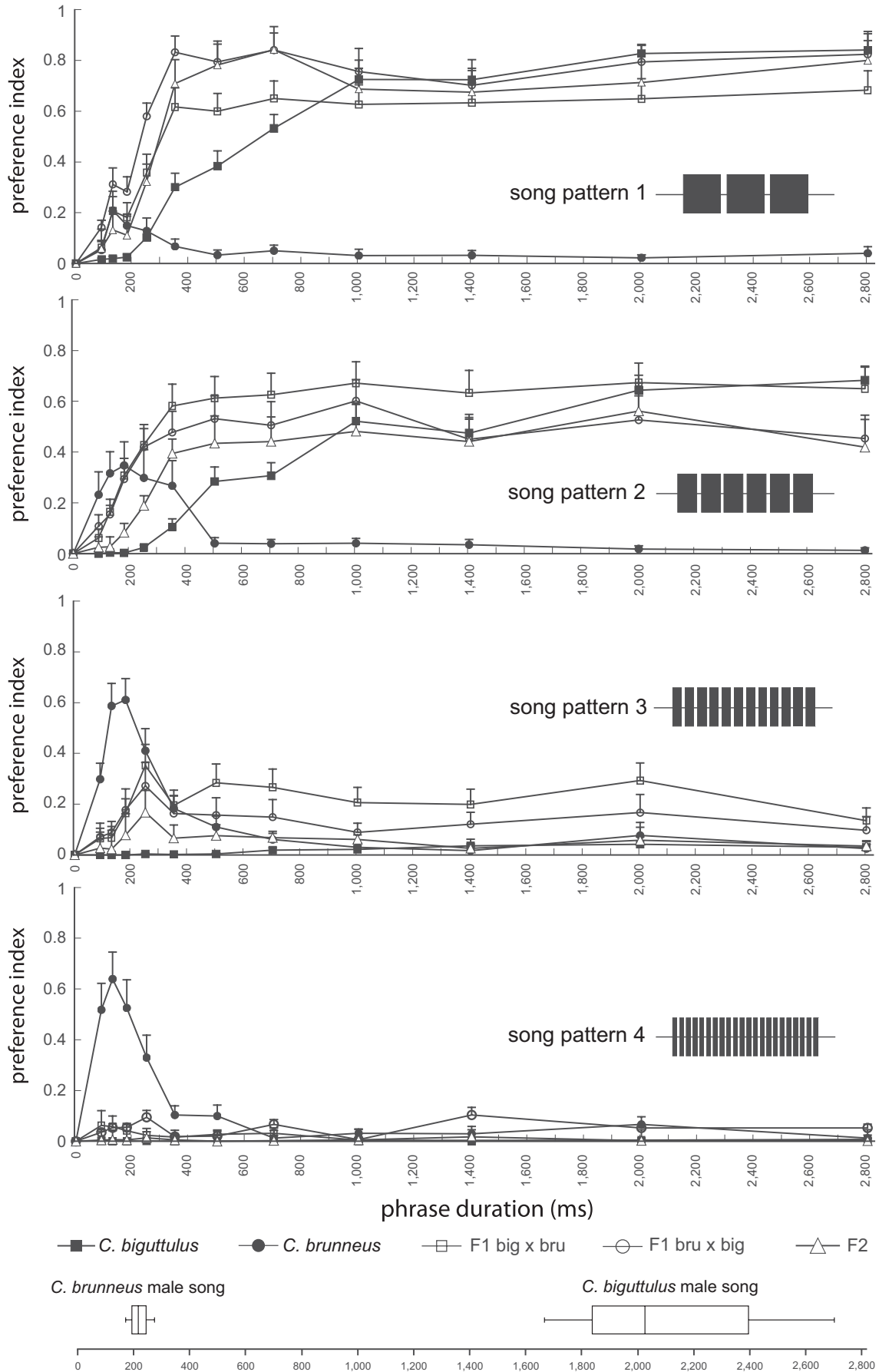


FIGURE 1 Response probabilities of female grasshoppers tested with four different song patterns and varying the phrase durations: *Chorthippus biguttulus* (filled square), *C. brunneus* (filled circle), bigXbru F1 hybrid (open squares), bruXbig F1 hybrids (open circles) and F2 hybrids (open triangles). Mean preference + standard error bars are shown. Song pattern 1 consists of syllable to pause duration of 80 to 12 ms (characteristic for *C. biguttulus*), song pattern 2 of 40 to 8 ms, song pattern 3 of 20 to 6 ms and song pattern 4 of 10 to 4 ms (characteristic for *C. brunneus*). The boxplots in the bottom represent phrase duration of calling songs of *C. biguttulus* males (mean = 2.17 s; SD = 0.435) and *C. brunneus* males (mean = 0.22 s; SD = 0.05)

The four song patterns were always tested separately. First, all five groups of females were tested (Table 1) followed by pairwise comparisons of groups of females (Table S1). To evaluate the statistical significance of model terms, we report the Wald chi-square tests. For analysing the models, we used the `geeglm` function in the `geepack` package (Højsgaard, Halekoh, & Yan, 2006) for the R 3.2.3 software.

2.6 | Female songs

The duration of the female's response songs was registered automatically in the sound-attenuated thermostatic chamber (von Helversen, 1979). The number of recorded response songs differed among females and ranged from 60 to 5,000 per female; therefore, we calculated a weighted mean for each female. Phrase durations among the five groups of females (Figure 2; two parental species, two reciprocal F1 hybrids and F2 hybrids) were analysed using ANOVA and compared with Tukey HSD post hoc test for unequal sample size (Table 2).

3 | RESULTS

The female song preference curves were significantly different among the five groups of females for all four song patterns tested (Figure 1, Table 1). For patterns 1 and 2, all three terms "group," "phrase" and the interaction-term "group*phrase" were significantly different. For pattern 3, only the term group and for pattern 4 the terms "phrase" and "group*phrase" showed significant differences. Post hoc pairwise comparisons of female preference curves were used to infer differences among the five groups of females for each of the four song patterns (Table S1).

3.1 | Song preferences of parental species

Females of the two parental species *Chorthippus biguttulus* and *C. brunneus* showed highest response probabilities to sound stimuli

resembling conspecific male songs and no or very low response to those of heterospecific songs (Figure 1). Accordingly, female preference curves of both species differed significantly for all four song patterns (Table S1). Mean preferences of *C. biguttulus* females to song pattern 1 (*biguttulus*-type) were highest (above 70%) if phrases had durations of at least 1,000 ms. It remained high until the longest phrase duration tested covering the range of natural male calling songs. Shortening syllable duration towards syllable durations of *C. brunneus* reduced response probabilities of *C. biguttulus* females drastically and were close to zero for song patterns 3 and 4 (*brunneus*-type) irrespective of phrase duration (Figure 1, Figures S1–S4). In contrast, response rates of *C. brunneus* females continuously increased from song patterns 1–4 and showed highest response rates for song patterns 3 and 4 (Figure 1, Figures S1–S4). The increase by *C. brunneus* females was limited to short phrases (≤ 250 ms), to which females of *C. biguttulus* never showed high response rates. For phrase durations of more than 500 ms, the mean response rates were close to zero irrespective of the song pattern.

3.2 | Song preferences of hybrids

Female preference curves of the three groups of hybrids (reciprocal F1 and F2) were in general similar among each other for all four song patterns tested (Figure 1). The twelve pairwise comparisons of preference curves among hybrids were not significant taking multiple testing into account (Bonferroni correction for 40 pairwise comparisons).

Although the mean preference curves of all three groups of hybrid females were significantly different from those of the two parental species, they always showed a curvature rather similar to those of *C. biguttulus* females among all four song patterns tested (Figure 1). Variation within each group of hybrids was larger than within each of the two parental species (Figures S1–S4). For song pattern 1, mean response probabilities of hybrid females generally stayed high ($>60\%$) towards longer phrase durations as in females of *C. biguttulus*. Such high response probabilities were already attained

TABLE 1 Statistical tests of effects of phrase duration and five groups of animals (*C. biguttulus*, *C. brunneus*, both reciprocal F1 hybrids, and F2 hybrids) on female response probabilities using generalized estimation equations (GEE) for each of the four song patterns tested

Term	Df	Song pattern 1		Song pattern 2		Song pattern 3		Song pattern 4	
		χ^2	P(> Chi)	χ^2	P(> Chi)	χ^2	P(> Chi)	χ^2	P(> Chi)
Group	1	43.3	$4.7 \times 10^{-11}^{***}$	6.2	0.01301*	4.86	0.028*	0.95	0.3296 ns
Phrase	1	136.6	$<2 \times 10^{-16}^{***}$	50.8	$1 \times 10^{-12}^{***}$	3.82	0.051 ns	8.01	0.0046**
Group*phrase	1	14.1	0.00018***	11.5	0.00068***	0.86	0.353 ns	5.67	0.0172*

Note: Significance levels are visualized as ns (not significant), * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$ or *** $p < 0.001$.

at lower phrase durations by hybrid females than by females of *C. biguttulus* (≥ 350 ms compared to $\geq 1,000$ ms in *C. biguttulus* females). Interestingly, some hybrid individuals deviated from this general pattern and either resembled females of *C. brunneus* or very rarely showed a bimodal preference curve with high response rates for short and long phrase durations but not for intermediate phrase durations. For song pattern 1, *C. brunneus*-like preference curves were found in all three groups of hybrids (1 out of 17 $F1_{big \times bru}$, 3 out of 12 $F1_{bru \times big}$ and 1 of 9 F2 hybrids) and only two out of 12 $F1_{bru \times big}$ females had a bimodal preference and responded to short as well long phrase durations (Figure S1). The proportion of hybrid females expressing a preference curve similar to *C. brunneus* was highest for song pattern 3 but limited to the F1 hybrid females. Six out of 17 $F1_{big \times bru}$ (35%) and 3 out of 12 $F1_{bru \times big}$ females (25%) showed high response rates (>0.5) to short phrase durations that usually (not always) decreased towards longer phrase durations (Figure S3). For the *C. brunneus*-like pattern 4, the response probability decreased to almost zero in all hybrid females with the exception of one $F1_{bru \times big}$ hybrid female. This female

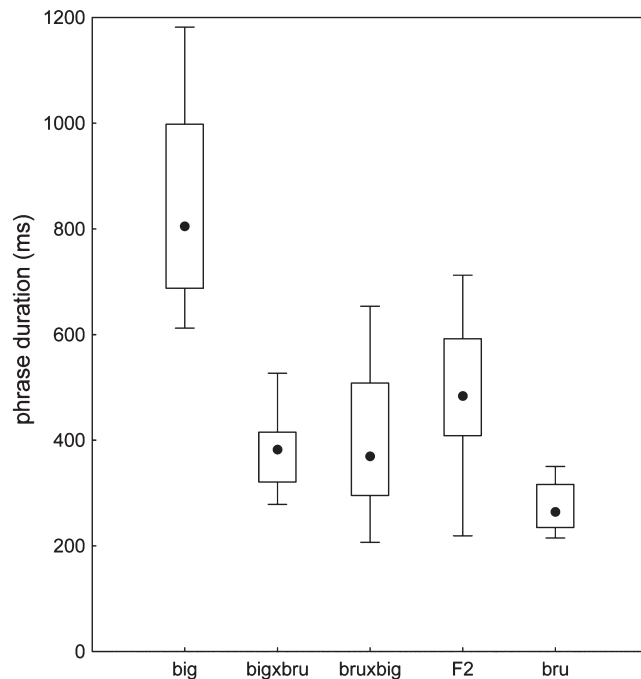


FIGURE 2 Phrase duration of female response songs of *Chorthippus biguttulus* (big), F1 hybrids (bigXbru and bruXbig), F2 hybrids (F2) and *C. brunneus* (bru). The mean (dot), standard error (box) and standard deviation (bar) is shown

TABLE 2 Unequal N HSD post hoc test of phrase duration of female songs of *C. biguttulus* (big), *C. brunneus* (bru), both reciprocal F1 hybrids (bigXbru, bruXbig), and F2 hybrids (F2)

Group	big	bigXbru	bruXbig	F2
bigXbru	473 ms, $p_{adj} < 1 \times 10^{-6}$			
bruXbig	441 ms, $p_{adj} < 1 \times 10^{-6}$	32 ms, $p_{adj} = 0.97$		
F2	363 ms, $p_{adj} = 2.6 \times 10^{-8}$	110 ms, $p_{adj} = 0.30$	78 ms, $p_{adj} = 0.69$	
bru	569 ms, $p_{adj} < 1 \times 10^{-6}$	97 ms, $p_{adj} = 0.37$	129 ms, $p_{adj} = 0.14$	207 ms, $p_{adj} = 0.01$

Note: Values refer to the differences between the mean phrase duration and the p value (p_{adj}) for each pairwise comparison.

was the only hybrid female that showed a preference curve typical for *C. brunneus* for all for song patterns tested.

3.3 | Response songs of females

Songs of *C. biguttulus* females responding to a song stimulus were characterized by long phrases, whereas they were very short in females of *C. brunneus* (Figure 2). In all three groups of hybrid females (two reciprocal F1 and F2 hybrids), phrase duration of response songs was intermediate between those of the two parental species (Figure 2), suggesting additive effects of multiple loci. Phrase duration of female response songs differed highly significant among the five groups of females (ANOVA $F_{4,71} = 52.7$, $p = 2 \times 10^{-16}$). Unequal Tukey HSD post hoc tests showed that *C. biguttulus* always differed highly significant from the other four groups of females ($p < 0.001$; Table 2). Phrase duration of *C. brunneus* partly overlapped with those of hybrid females. Only F2 hybrid females differed significantly from *C. brunneus* females ($p = 0.02$).

4 | DISCUSSION

Song preferences of F1 and F2 hybrid females of both reciprocal crosses between the grasshopper species *Chorthippus biguttulus* and *C. brunneus* were not intermediate between the two parental species. Instead, they largely resembled female preferences of only one parental species, that is *C. biguttulus*. Accordingly, *Chorthippus biguttulus* as well as F1 and F2 hybrid females showed highest response rates for the *biguttulus*-like syllable pattern and phrase durations. Response rates of hybrids decreased substantially towards songs with short phrases, which are characteristic for songs of *C. brunneus* males. The major difference between hybrid females and females of *C. biguttulus* is a slight shift of high response rates towards shorter phrase durations in hybrid females.

Our results show that a haploid set of the *C. biguttulus* genome is sufficient to express a functional neuronal filter for song recognition in a F1 hybrid female that closely resembles those of *C. biguttulus* females in respect to both, the preference of syllable structure (song pattern) and an open-ended preference for phrase duration. Surprisingly, this was even observed in F2 hybrids, in which homozygous loci for *C. brunneus* are expected due to recombination. However, we cannot exclude the possibility of biased F2 hybrid mortality, which could cause the lack of specific genotypes among F2

hybrids and thus the lack of specific (e.g. *C. brunneus*-like) song preferences in F2 hybrid females.

The response behaviour of females can be dissected into three preference traits for songs in which the two parental species differ. First, the shape of the preference function is open-ended in *C. biguttulus* but unimodal in *C. brunneus*. Most, but not all F1 and F2 hybrid females expressed an open-ended preference function (Figures S1–S4) and thus resembled *C. biguttulus*. Second, preference for syllables within phrases is characteristic for *C. biguttulus*. F1 and F2 hybrid females also preferred songs resembling those of *C. biguttulus* over those of *C. brunneus*. In contrast, for the third song trait, that is phrase duration, hybrid females expressed preferences for intermediate values and thus did not resemble one parental species. The increase in response rates occurred in F1 and F2 hybrid females at phrase durations, which are in-between very short phrases of *C. brunneus* and long phrases of *C. biguttulus*. This intermediate preference for phrase duration argues for additive genetic effects involving multiple genes with minor effects.

A study similar to ours involved also *C. biguttulus*, which was hybridized to *C. mollis*. Both, songs as well as female preferences of hybrids, were investigated (Finck & Ronacher, 2017; von Helversen & von Helversen, 1975a,1975b). All three species, *C. mollis*, *C. biguttulus* and *C. brunneus*, are characterized by species-specific songs that differ in several song parameters and song preferences of females (e.g. von Helversen & von Helversen, 1994). Surprisingly, hybrids of both interspecific crosses differ substantially in their modes of inheritance. In contrast to the dominance effects in our study, von Helversen and von Helversen (1975a) found strong maternal effects in song preferences in F1 females of crosses between *C. biguttulus* and *C. mollis*. Hybrid females of both reciprocal crosses on average strongly preferred songs of their mother species. This response behaviour was rather stable throughout a female's life, and only a minority of individuals responded equally to the songs of both parental species. Since the F1 hybrids did not show an intermediate phenotype and females are homogametic, the authors hypothesized that neuronal filters of both parental species are expressed in hybrid females and their activation is individual-specific and is driven by maternal effects.

4.1 | Parallel expression of neuronal filters

Despite the striking differences between the two hybrid studies (strong dominance effects in hybrids of *C. biguttulus* and *C. brunneus* and strong maternal effects in hybrids of *C. biguttulus* and *C. mollis*), the mechanism of parallel expression of both parental neuronal filters in hybrids proposed by von Helversen and von Helversen (1975b) can also explain the results of our study. The dominant expression *C. biguttulus*-like preference functions in our *C. biguttulus/C. brunneus*-hybrid females could result from the different role of calling and courtship behaviour of both species. Courtship song is well developed in *C. biguttulus* and receptive females readily respond to calling males. In contrast, calling in *C. brunneus* results frequently in

male–male communication (rivalry songs) whereas courtship song is less developed and females are less likely to respond acoustically (Jacobs, 1953; Perdeck, 1958; Ragge & Reynolds, 1998; Weih, 1951). Accordingly, in our study the proportion of responding females was higher among females of *C. biguttulus* than in *C. brunneus* (86% and 38%, respectively). Superposition of such song-dependent activation of parallel expressed neuronal filters would result in a more likely acoustic response of a hybrid female to a *C. biguttulus*-like song than to a song typical for *C. brunneus*. This expectation matches nicely with our observations.

In line with this scenario of parallel expression of both parental neuronal filters for song preferences is the result that few hybrid females showed preference curves that rather resembled those of *C. brunneus* (i.e. high response rates for a small range of short phrase durations) than those of *C. biguttulus* (Figure S1). This was found for only few individuals for tests with *C. biguttulus*-like syllable patterns (1 and 2). The proportion of such *C. brunneus*-like behaving individuals increased to 35% for tests with syllable pattern 3 but surprisingly not for those with *C. brunneus*-like song pattern 4.

Despite the fact that few hybrid females showed unimodal preference functions resembling more *C. brunneus*, they never showed high response rates to very short phrase durations that are characteristic for *C. brunneus*. Highest response rates of females with a unimodal preference curve were observed for phrase durations that were intermediate between *C. biguttulus* and *C. brunneus*, which provides additional support for intermediate inheritance of preference for phrase duration.

4.2 | Nonhomology of neuronal filters

Nonintermediate inheritance of female preferences in hybrids but instead a rather pure expression of both parental neuronal filters for song recognition argues for separate, nonhomolog genetic networks for the species-specific behavioural traits of the preference function and the preference for syllable structure (see also von Helversen & von Helversen, 1975a,1975b). These species-specific neuronal filters for the general shape of the preference curve for phrase duration (open-ended in *C. biguttulus* and unimodal in *C. brunneus*), for syllable structure within phrases (characteristic for *C. biguttulus*) and for different phrase durations (both species) must have evolved within a rather short evolutionary time since the divergence of the closely related species that lack obvious post-zygotic barriers. Nothing is known about possible underlying genetic mechanisms, but enormous genome sizes may have played a role. Acridid grasshoppers including the genus *Chorthippus* are known to have very large genome sizes of more than 10 GB and thus several times bigger than the human genome (<http://www.genomesize.com>, Bensasson, Petrov, Zhang, Hartl, & Hewitt, 2001). Duplications of genomes, large regions of the genome or genes may have allowed neo-functionalization of genes involved in acoustic communication and may have led to the evolution of additional neuronal filters. Such a scenario of gene duplication and modification could possibly also explain the evolution of complex, that is multi-element songs in

acidid grasshoppers, although this interpretation is speculative. In many grasshopper species, courtship songs are composed of different song elements that must be presented by a male in a rather stereotyped and ordered manner in order to release a positive response of the female. Accordingly, in the female, neuronal filters for the different song elements must be expressed simultaneously for the recognition of conspecific songs.

4.3 | Modes of inheritance

Mate choice of hybrid females was investigated in several taxonomic groups including several acoustically communicating orthopterans of grasshoppers, crickets and bushcrickets. In almost all of these studies, intermediate phenotypes or strong maternal or sex-chromosomal effects were observed (reviewed by Bakker & Pomiankowski, 1995). Within-Orthoptera female preferences for intermediate phrase durations respectively for numbers of syllables were found among hybrid females of crosses between the grasshopper species *Chorthippus brunneus* and *C. jacobsi* (Bridle, Saldamando, Koning, & Butlin, 2006) or two races of the bushcricket *Ephippiger ephippiger* (Ritchie, 1992). Other studies, including again some on Orthoptera, suggest that X chromosomal genes can have a significant effect on sexually selected traits (Reinhold, 1998; Ritchie & Phillips, 1998). In the species group of *Chorthippus parallelus*, the inheritance patterns of female preferences seem also to indicate sex-linked or maternal effects (Butlin & Hewitt, 1988). Similar results were also found in F1 hybrid females from crosses between the crickets species *Teleogryllus oceanicus* and *T. commodus* (Bentley & Hoy, 1972) and in hybrid females among races within the bush cricket genus *Poecilimon* (Reinhold, 1994).

A strong dominant inheritance of female preferences in hybrids as reported here was rarely found in other taxa. Extensive crossing experiments between *Drosophila ananassae* and *D. pallidosa* showed that F1 hybrid females showed the same mating pattern as *D. ananassae* females and that possible loci controlling females' discrimination behaviour are located in a single region on the second chromosome (Doi, Matsuda, Tomaru, Matsubayashi, & Oguma, 2001). Mate preference studies in *Heliconius* butterflies revealed a dominant allele for female preference of a white band in the forewing (Kronforst et al., 2006). Finally, coloration-based mate choice in Lake Malawi cichlid fishes showed preference of F1 hybrid females for one parental species, although the strength of preference differs between both reciprocal crosses and diminished in the F2 generation (Ding et al., 2014). Interestingly, all three studies with obvious dominance effects have in common that one major or very few genetic factors control mate choice. Such a simple genetic control cannot be excluded but seems unlikely for the *Chorthippus* grasshoppers we investigated, since a large variation would be expected in the recombining F2 generation. Instead, preferences of F2 hybrid females were rather homogeneous and closely resembled those of both reciprocal F1 hybrids. However, sample size of F2 females was small (nine individuals).

5 | PREMATING REPRODUCTIVE ISOLATION: ARE MALE HYBRID SONGS DISCRIMINATED AGAINST BY PARENTAL AND HYBRID FEMALES BETWEEN BOTH SPECIES?

The dominance effects in terms of high similarity between the preferences of *C. biguttulus* females and both reciprocal F1 hybrid females suggest that hybrid females will readily and most exclusively backcross to males of *C. biguttulus*. Both reciprocal F1, F2 and backcross hybrid males are unattractive to F1 hybrid females and to females of *C. biguttulus* because their songs contain, if at all, only occasionally a syllable structure and syllables are never regular and rather long compared to the syllables of *C. biguttulus* (Gottsberger & Mayer, 2007). In addition, intermediate song durations of hybrid males (Gottsberger & Mayer, 2007) are unattractive for females of *C. brunneus*. This results in a restriction of F1 hybrid reproduction to one sex (females) that backcrosses to one parental species (*C. biguttulus*) limits unidirectional gene flow from *C. brunneus* to *C. biguttulus*. Rare interspecific hybridization in nature (Perdeck, 1958; Ragge, 1976; O. von Helversen, personal communication) in combination with dominant expression of song preferences in F1 hybrid females likely represents an effective evolutionary mechanism that allows occasional hybridization without merging of the two species, although both species largely overlap in their distribution range and occur syntopically at many locations (Ragge & Reynolds, 1998; Ragge et al., 1990).

6 | CONCLUSIONS

Two hybridization studies involving two species pairs of *Chorthippus* grasshoppers have revealed strikingly different modes of general inheritance of female preferences, dominant inheritance in *C. biguttulus/brunneus*-hybrid females (this study) and strong maternal effects in *C. biguttulus/mollis*-hybrid females (von Helversen & von Helversen, 1975a,b). Nevertheless, the underlying mechanisms may be quite similar. All three species have evolved neuronal filters for the detection of the different species-specific song parameters. Both studies indicate that hybrid females express preferences rather similar to those of the parental species instead of intermediate preferences. Nonintermediate inheritance suggests that neuronal filters are not homologous and are expressed simultaneously in hybrid females. These neuronal filters activate via command neurons the motor pattern generator in the thoracic ganglion that releases the response song of the female (Hedwig, 1994). Although maternal effects influence the activation of command neurons in *C. biguttulus/mollis*-hybrid females, the dominant expression of preferences for *C. biguttulus*-like songs in *C. biguttulus/brunneus*-hybrid females can be explained by species-specific differences in the role of acoustic response behaviour in *C. biguttulus* (pronounced) and *C. brunneus* (less pronounced), that is command neurons releasing a

female's acoustic response are more likely activated by *C. biguttulus*-like songs than by *C. brunneus*-like songs.

Thus, the rapid evolution of independent neuronal filters is likely a key innovation of acoustically communicating grasshoppers that allows on the one hand the evolution of complex multi-element songs and at the same time establishes hybridization barriers since hybrid females will backcross to the parental species and discriminate against hybrid males. Both may have contributed to the high species richness especially among acoustically communicating grasshoppers with complex songs (Mayer et al., 2010; Vedenina & Mugue, 2011).

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