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Multiple barriers to gene exchange in a field cricket hybrid zone

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Data on patterns of variation within hybrid zones, combined with studies of life history, mate choice, and hybrid performance, allow estimates of the contribution of different pre-zygotic and post-zygotic barriers to reproductive isolation. We examine the role of behavioural barriers to gene exchange in the maintenance of a hybrid zone between North American field crickets *Gryllus firmus* and *Gryllus pennsylvanicus*. We consider these barriers in the context of previous studies that documented temporal and ecological isolation and a one-way post-mating incompatibility (i.e. *G. firmus* females do not produce offspring when they mate only with heterospecific males). Based on no-choice mating experiments in the laboratory, we demonstrate strong behavioural pre-mating barriers between the two species, but no apparent fecundity or fertility costs for *G. firmus* females when they mate with both conspecific and heterospecific males. Furthermore, we show that *G. firmus* females do not discriminate between hybrids and conspecifics, whereas *G. pennsylvanicus* females do. This observation could explain the asymmetric allele introgression observed in the hybrid zone. We also document a failure of heterospecific males to induce normal oviposition in *G. firmus* females, which may be due to rapid evolution of accessory gland proteins and may serve as an additional barrier to gene exchange. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **97**, 390–402.

ADDITIONAL KEYWORDS: failure to mate – fecundity – fertility – *Gryllus firmus* – *Gryllus pennsylvanicus* – mate choice – oviposition – reproductive isolation – time to mate.

INTRODUCTION

Hybrid zones provide valuable insights into the operation and evolution of barriers to gene exchange between closely-related species (Hewitt, 1988; Harrison, 1990). Many hybrid zones represent secondary contact between populations or species that have diverged in allopatry, and differences between hybridizing taxa will only persist if one or more barriers are strong enough to counteract the homogenizing effects of gene flow. Data on patterns of variation within hybrid zones, combined with studies of life history, mate choice, and hybrid performance in the laboratory, allow estimates of the contribution of both pre-zygotic and post-zygotic barriers.

In many taxa, multiple reproductive barriers contribute to isolation (Coyne, 1992; Schluter, 2001; Price & Bouvier, 2002; Ramsey, Bradshaw & Schemske, 2003), but the relative contribution of each barrier, as well as its importance in the speciation process, often remain unknown. Only in a few model systems (e.g. sympatric species of *Mimulus* in North America; Ramsey *et al.*, 2003) have quantitative estimates of individual 'barrier strengths' been made.

Despite the obvious importance of and emphasis on post-zygotic barriers (e.g. the extensive literature on Dobzhansky–Muller incompatibilities and Haldane's Rule; Coyne & Orr 2004), cases in which species

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differences persist only because of post-zygotic barriers are thought to be relatively rare (Kirkpatrick & Ravigne, 2002). Pre-mating barriers act early in the life cycle, and thus have the potential to reduce gene flow proportionally more than barriers that act later (Coyne & Orr, 2004). Furthermore, it has been argued that bimodal hybrid zones (i.e. those in which barriers are strong and parental types predominate) 'are invariably coupled with strong assortative mating or assortative fertilization' (Jiggins & Mallet, 2000). Thus, pre-zygotic barriers (and especially behavioural barriers) may be responsible for the deficiency of heterozygotes or 'intermediate' individuals and the strong linkage disequilibrium characteristic of bimodal hybrid zones (Harrison & Bogdanowicz, 1997; Jiggins & Mallet, 2000; Ross & Harrison, 2002; Vines *et al.*, 2003).

Within hybrid zones, behavioural pre-zygotic barriers may be a byproduct of local adaptation prior to secondary contact. Behavioural barriers may also arise or be strengthened in situ and will then depend on the cost to females of mating with heterospecific males. In the presence of post-zygotic barriers, less fit hybrids are produced, and costs of heterospecific matings can be high, potentially leading to reproductive character displacement (Dobzhansky, 1940; Howard, 1993; Coyne & Orr, 2004). However, when females are polyandrous and there is strong sperm precedence and/or gametic incompatibilities, few or no hybrid offspring may be produced, substantially reducing the cost of mating with heterospecific males. Females who mate multiply are more likely to receive sperm from at least one conspecific male and thus ensure fertilization. In these cases, the mating cost per se might be very low, especially if females gain direct benefits (e.g. access to resources; Andersson, 1994; Veen *et al.*, 2001) from mating with heterospecific males.

In the present study, we examine the role of pre-mating behavioural barriers to gene exchange in the maintenance of a bimodal mosaic hybrid zone between the North American field crickets *Gryllus firmus* and *Gryllus pennsylvanicus*. We consider these barriers in the context of previous studies that have documented asymmetrical allele introgression (Ross & Harrison, 2002; Maroja, 2008), temporal and ecological isolation, and a one-way gametic incompatibility between these two species (Harrison, 1983, 1985; Harrison & Rand, 1989; Ross & Harrison, 2002, 2006). We also estimate the potential cost for *G. firmus* females of mating with heterospecific males. The results obtained reveal strong behavioural pre-mating barriers, which provide a plausible mechanism for the observed directional introgression of *G. pennsylvanicus* alleles into *G. firmus*.

The study system

Female field crickets are polyandrous (Solymar & Cade, 1990; Bretman & Tregenza, 2005) and are able to store sperm from many mates in a single elastic spermatheca (Simmons, 1986; Bretman & Tregenza, 2005). Unlike many other insects, field cricket females appear to benefit from multiple mating through both direct (i.e. increased lifetime fecundity) and indirect (i.e. genetic) benefits (Simmons, 1988; Burpee & Sakaluk, 1993; Wagner *et al.*, 2001; Sakaluk *et al.*, 2002; Ivy & Sakaluk, 2005). In gryllid crickets, forced copulation is impossible because the female must mount the male; both sexes cooperate in the transfer of the spermatophore.

The field crickets *G. firmus* and *G. pennsylvanicus* form an extensive hybrid zone (Harrison & Arnold, 1982; Harrison & Bogdanowicz, 1997), in which multiple pre- and post-mating barriers to gene flow have been described. These include: (1) a one-way incompatibility, in which no offspring are produced from crosses of *G. firmus* females and *G. pennsylvanicus* males, but the reciprocal cross produces viable and fertile offspring (Harrison, 1983); (2) a habitat association in Connecticut, with *G. firmus* on sandy soils and *G. pennsylvanicus* on loam soils (Rand & Harrison, 1989; Ross & Harrison, 2002, 2006); and (3) temporal isolation of adults in Virginia, but not in Connecticut (Harrison, 1985).

Indirect evidence also suggests that behavioural barriers are present. In the laboratory, *G. pennsylvanicus* females housed with males of both species produce offspring sired primarily by conspecific males (Harrison & Rand, 1989). Because there is no evidence of conspecific sperm precedence (assortative fertilization) in these species (G. Hume, unpubl. data), the data suggest that there is positive assortative mating.

In the present study, we document behavioural barriers in this system, based on differences in time to mating and rejection rates of males by virign and singly-mated females. We also estimate fecundity and fertility of doubly-mated *G. firmus* females to investigate the costs (if any) to *G. firmus* females of mating with heterospecific males. Finally, we examine the fecundity of *G. firmus* females mated only to heterospecific males to determine whether these males trigger normal oviposition behaviour.

MATERIAL AND METHODS

Because it is impossible to distinguish pure species individuals in mixed/hybrid populations, we used individuals from 'pure species' allopatric populations. In August and September of 2003 and 2004, we collected late instar *G. firmus* nymphs in Guilford, CT

(41°15′; –72°42′) and *G. pennsylvanicus* nymphs in Ithaca, NY (42°24′; –76°31′). Crickets were sorted by sex and species and maintained in groups of six to eight individuals in plastic cages (30 × 16 × 9 cm), with food (Purina Cat Chow®), a water vial, and cardboard for shelter. The cages were maintained under a 12 : 12 h light/dark cycle at at 25 °C.

Mating trials with *G. firmus* females

Seven- to 8-day-old adult *G. firmus* virgin females were randomly assigned to one of six treatments (Fig. 1). We abbreviate treatments using up to three letters (e.g. FPF): The first letter indicates the female species (F = *G. firmus* or P = *G. pennsylvanicus*), the second letter indicates the first male species (i.e. F or P) and the third letter indicates the second male species (i.e. F or P). In treatment F, females ($N_F = 15$) were not given access to males. In treatments FF and FFF, females either mated once with a conspecific male ($N_{FF} = 15$ successful out of 15 trials) or twice, consecutively, with two different conspecific males ($N_{FFF} = 16$ successful out of 16 trials). In treatment FPP, females mated twice with two different heterospecific males ($N_{FPP} = 12$ successful out of 21 trials). In treatment FPF, females mated first with a heterospecific male (*G. pennsylvanicus*) and second with a conspecific male ($N_{FPF} = 15$ successful out of 17 trials). Finally, in treatment FFP ($N_{FFP} = 14$ successful out of 25 trials), each female was mated first to a conspecific male and then to a heterospecific male. All the males used in the mating trials had been adult for 7–12 days. Males were chosen at random and used only once to avoid pseudo-replication. All individuals were sized by measuring pronotal width to the nearest 0.1 mm using the same pair of vernier calipers.

To initiate mating trials, each virgin female was placed with a first male in a mating chamber, a 10-cm Petri dish lined with moist filter paper. We observed

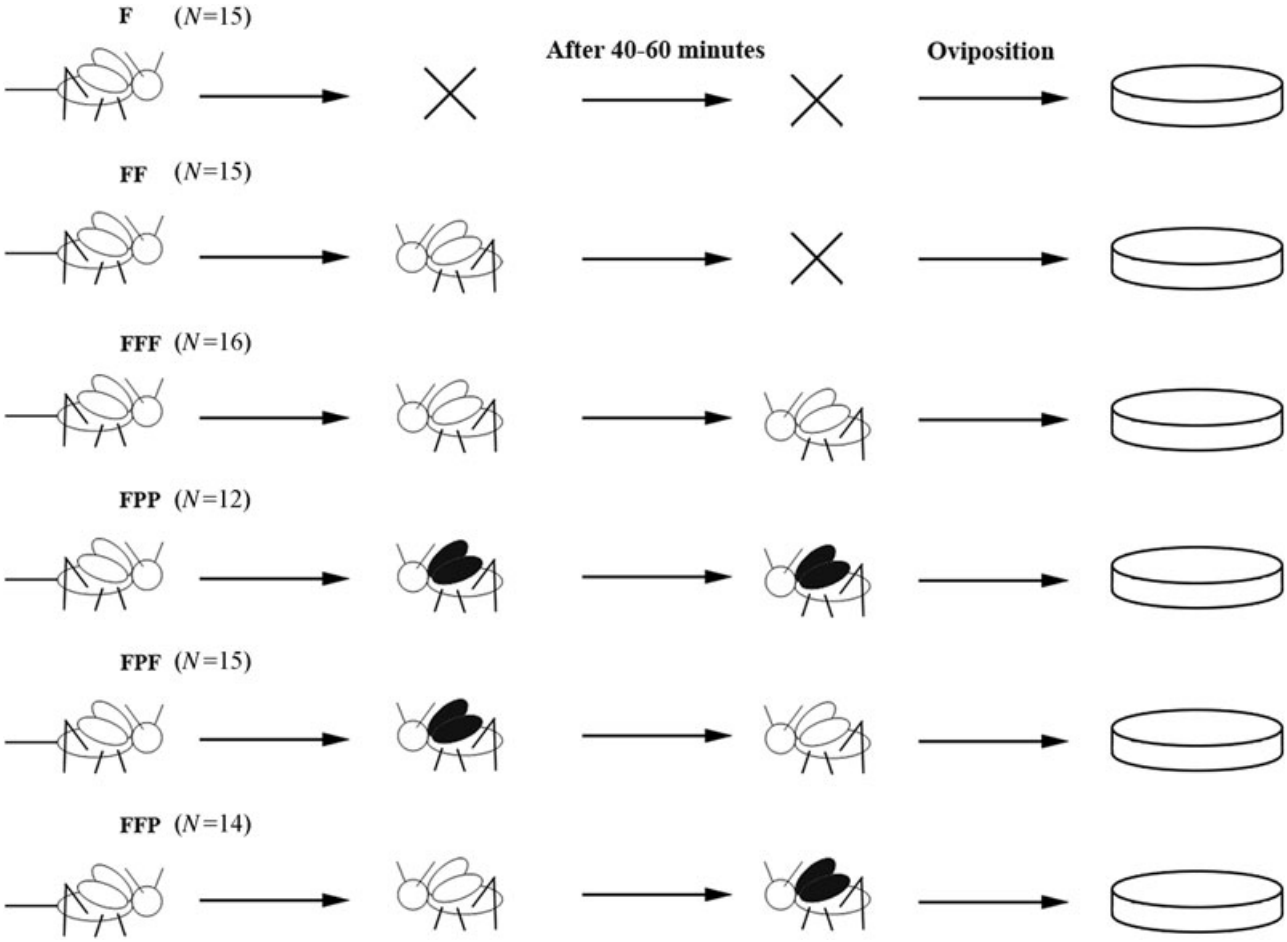

Figure 1. Experimental protocol for *Gryllus firmus* females (similar protocol for *Gryllus pennsylvanicus* females). F, *G. firmus*; P, *G. pennsylvanicus*. Crickets in the left column are females (with ovipositor). Crickets with black wings represent heterospecific males (i.e. *G. pennsylvanicus*). For further details, see text.

all mating trials and recorded the time to mate. If no spermatophore transfer occurred during the first hour, the first male was replaced by a second male of the same species for no more than 1 h. If the female remained unmated after this time, we scored the mating trial as failed and excluded both males and female from subsequent trials. Single-mated (FF) females were isolated individually in plastic oviposition chambers ($30 \times 15 \times 8$ cm), provided with food, water and a Petri dish of sterilized soil as oviposition substrate. With single-mated females from other treatments, we proceeded with the second part of the mating trials: immediately after the female detached the spermatophore from her first mating (about 40 min after mating), she was transferred to a new mating chamber and placed with a second male for 1 h. As in the first part of the mating trials, if mating did not take place during this time, females were exposed to a new second male of the same species for an additional 1 h. After mating for a second time, females were individually isolated as described above. Food and water in the individual oviposition chambers were replaced twice a week and mortality was scored every other day.

Oviposition dishes were incubated for a maximum of 40 days at 25 °C and then placed in a refrigerator at 4 °C for 102 days to ensure synchronous hatch of nymphs (Harrison, 1985). Lifetime fecundity was assessed by counting all of the eggs laid by each female. Eggs were separated from the oviposition substrate using a series of sieves and counted under a stereoscopic microscope. To estimate fertility, samples of 100 eggs were taken from each female. For those females that laid less than 100 eggs ($N_F = 13$; $N_{FF} = 1$; $N_{FFF} = 0$; $N_{FPF} = 1$; $N_{FFP} = 2$; $N_{FPP} = 6$) fertility was assessed using the entire clutch. Fertility was estimated as the proportion of eggs that successfully hatched. After removal from 4 °C, all eggs were incubated at room temperature. Hatching began 11 days after the eggs were removed from the refrigerator. Two weeks after the first nymphs hatched, the number of offspring was determined. No eggs hatched after this period.

Mating trials with *G. pennsylvanicus* females

Seven- to 8-day-old adult *G. pennsylvanicus* virgin females were randomly assigned to one of four different treatments. In treatment PPP, females were sequentially mated to two different conspecific males ($N_{PPP} = 9$ successful out of 22 trials). In treatment PFF, females mated sequentially with two different heterospecific males ($N_{PFF} = 9$ successful out of 24 trials). In treatment PFP, females mated first with a heterospecific male and subsequently with a conspecific one ($N_{PFP} = 9$ successful out of 31 trials). Finally, in the last treatment, PPF, females were first mated to a conspecific male and then to a heterospecific one ($N_{PPF} = 10$ successful out of 30 trials). The mating protocol was the same as that described above for the *G. firmus* mating trials, but both males and females were eliminated after mating (no eggs were collected).

Mating trial with hybrids

This set of mating trials included both hybrids and pure-species individuals. These experimental individuals were all lab-reared offspring of captive matings between wild-caught crickets. F_1 hybrids were produced by crossing *G. pennsylvanicus* females from Ithaca, NY, to *G. firmus* males from Guilford, CT. Pure-species individuals were offspring of within population crosses of crickets from these same localities. The mating protocol was as described above for *G. firmus* mating trials, with the exceptions that: (1) females were only given the opportunity to mate once and (2) we recorded only mating success or failure and did not measure time until mating. Using the notation above (i.e. first letter indicates the female type and second letter indicates the male type), and designating hybrids as H, the total number of experimental crosses were: $N_{FF} = 13$, $N_{FH} = 9$, $N_{PP} = 16$, $N_{PH} = 15$, $N_{HP} = 18$, $N_{HF} = 21$.

Statistical analysis

We use a Bayesian model selection approach to draw statistical inferences. This approach is grounded in likelihood theory, and has several advantages over the more traditional approach of null hypothesis testing. First, different models can be compared to one another by evaluating the relative support in the observed data for each model. Second, model averaging can be implemented to make robust estimates (Johnson & Omland, 2004).

A priori selection of candidate models

A critical first step is the selection of the appropriate set of models covering reasonable competing hypotheses. In insect species, female mating behaviour is known to be influenced by male species and size as well as female mating history (Andrés & Cordero-Rivera, 2000; Friberg, 2006). Therefore, to model female mating behaviour (i.e. 'time to mate' and 'time to remate'), initially, we considered all nested models within:

$$T_i = \alpha + \beta_1 Sp_1 + \beta_2 Sp_2 + \beta_3 Si_1 + \beta_4 Si_2 + \beta_5 (ti_1.) + \beta_6 (Sp_1 . Sp_2) + \varepsilon_1$$

where, Sp_1, Si_1 and Sp_2, Si_2 are respectively the species and size of the first and second male to mate

with the female (second male is only considered in the 'time to remate' model) and ti_1 is the time to mate with the first male (only considered in the 'time to remate' model). The most complex model considers all additive effects and the potential effect of the interaction between male species. Other interactions were not taken into account because they had no clear biological meaning.

Similarly, we modelled female fecundity and fertility (F_{ij}) as follows:

$$F_{ij} = \alpha + \beta_1 Tr_j + \beta_2 Lf_i + \beta_3 Si_1 + \beta_4 (Tr_i . Lf_i) + \beta_5 (Tr_j . Si_i) + \beta_6 (Lf_i . Si_i) + \beta_7 (Tr_j . Lf_i . Si_i) + \varepsilon_{ij}$$

where, Tr_j is female mating treatment (see above), Lf_i is female lifespan, and Si_i is female size. In this case, we considered a full model (i.e. including all possible interactions between explanatory variables). Because both virgin females (F) and females mating only with heterospecific males (FPP) have extremely low fecundity and zero fertility, we analysed these two treatment groups separately.

Model fitting

The distribution of 'time to mate' was highly skewed. Therefore, we fitted our data to generalized linear regression models (GLMs) with gamma errors and an inverse link function (Crawley, 1993). We fitted fertility and fecundity data to GLMs with negative binomial errors using a log link function. Residuals of all the performed GLMs were analysed by visual inspection and no deviations from normality were observed. No significant outliers were found using Cook's statistics values. All analyses were performed using R software (R Development Core Team, 2005).

Selection of the best set of models

We used GLMs in combination with Bayesian model averaging (BMA) to model the effect of the appropriate explanatory variables 'D' (i.e. male size, male species, female size, female lifespan) on independent variables '*I*' (i.e. female time to mate, fecundity, fertility). This combined approach allowed us not only to take into account the appropriate structure of the data, but also to incorporate uncertainty in model selection into our statistical inferences (Wintle *et al.*, 2003). BMA implementation has been shown to produce more accurate estimates than methods based on the selection of a single best model (Burnham & Anderson, 1998).

Model averaging is calculated as:

$$p(I|D) = \sum_{k=1}^{k} p(I|M_k, D)\, p(M_k, D)$$

where, $P(I|D,M_k,D)$ is the posterior prediction from model Mk, given the data, $P(M_k,|D)$ is the posterior probability of model M_k, given the data and k is the number of models considered (Hoeting *et al.*, 1999). That is, BMA provides an estimate of $P(I|D)$ as a weighted average of the posterior prediction from all models considered, where the weights are the posterior probabilities of each model (Wintle *et al.*, 2003). Integrating the posterior model probabilities for all models that include a given explanatory variable yields the conditional probability that the variable has a nonzero coefficient $p(\beta \neq 0)$.

We carried out the analyses using the 'bic.glm' function in the 'BMA' package (Raftery *et al.*, 2005) implemented in R software (R Development Core Team, 2005). This function uses the 'leaps and bounds' algorithm (Furnival & Wilson, 1974) to identify the most probable models based on the Bayesian information criterion (BIC) approximation to Bayes factors (Raftery *et al.*, 2005). The 'bic.glm' uses maximum likelihood estimation to fit individual models and weights to those models according to BIC values (Raftery, Madigan & Volinsky, 1995; Wintle *et al.*, 2003).

RESULTS

Time to mate and mating trial failures

Gryllus firmus females took significantly longer to mate with heterospecific males (42.9 ± 4.4 min) than with conspecific males (7.4 ± 1.0 min; Fig. 2). In matings involving virgin females (mating for the first time), the first male species (but not male size) made a strong contribution to time to mate prediction [$\Pr(\beta \neq 0) = 1$; Fig. 3, see also Supporting information]. In matings involving previously mated females, only the second male species made a strong contribution to time to mate prediction [$\Pr(\beta \neq 0) = 1$; Fig. 3, see also Supporting information]. Furthermore, significantly more virgin *G. firmus* females failed to mate with heterospecific males (21.1% of 38 females) than with conspecific males (0% of 41 females; Fisher's exact test, $P = 0.0018$). By contrast, laboratory-reared *G. firmus* females did not appear to differentiate between F_1 hybrid and conspecific males; they never failed to mate with either sort of male (0% of nine and 0% of 13 females respectively).

For *G. pennsylvanicus* females, time to mate with heterospecific males (43.2 ± 4.8 min) was not significantly different than time to mate with conspecific males (39.6 ± 5.7 min; Fig. 4). Furthermore, none of the variables measured made a substantial contribution to the time to mate prediction (Fig. 3); this was

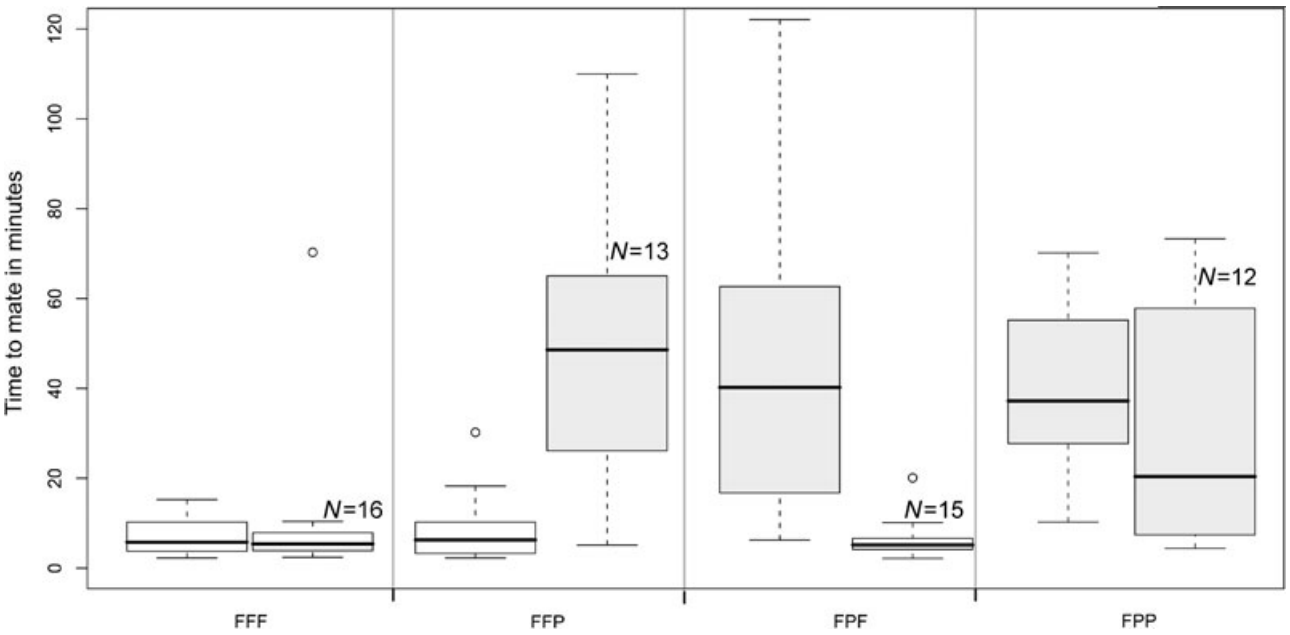

Figure 2. Box plot of *Gryllus firmus* female time to mate with first and second male for each double mating treatment (*N*, sample size). White boxes represent conspecific males. Grey boxes represent heterospecific males.

true both for virgin females and those that had previously mated. Independent of the species of the male, virgin *G. pennsylvanicus* females failed to mate (38.3% of 107 females) more often than virgin *G. firmus* females (10.4% of 77 females; Fisher's exact test, $P < 0.0001$). However, as with *G. firmus*, significantly more virgin females failed to mate heterospecific males (47.3% of 55 females) than conspecific males (28.5% of 52 females; Fisher's exact test, $P = 0.02$). Similarly, the proportion of laboratory-reared *G. pennsylvanicus* females that failed to mate with F_1 hybrid males (46.7% of 15 females) was greater than the proportion that failed to mate with conspecific males (12.5% of 16 females; Fisher's exact test, $P = 0.038$).

F_1 hybrid females more often failed to mate with *G. pennsylvanicus* males (38.9% of 18 females) than *G. firmus* males (19% of 21 females), but the difference was not significant (Fisher's exact test, $P = 0.11$).

Fertility and fecundity in *G. firmus* females

There was no difference in lifetime fecundity (Fig. 5) or fertility (Fig. 6) for *G. firmus* females that mated at least once with a conspecific male (treatments FF, FFF, FPF, and FFP). Only female lifespan made a contribution to the lifetime fecundity (i.e. total number of eggs laid) prediction [$\Pr(\beta \neq 0) = 0.55$; Fig. 7, see also Supporting information]. None of the variables measured made strong contributions to predicting fertility (i.e. number of hatchlings in a sample of 100 eggs) (Fig. 7).

The numbers of eggs deposited by *G. firmus* virgin females (F; 47.5 ± 31 eggs) and by females mated only to heterospecific males (FPP; 181.7 ± 53.7 eggs) were much lower than numbers of eggs from females mated to at least one conspecific male (FF, FFF, FPF, and FFP, 702.0 ± 61.7 eggs; Fig. 5). There were also significant differences between the two treatments (F and FPP). Both female lifespan and treatment contributed to the prediction of lifetime fecundity in females from the F and FPP treatment [$\Pr(\beta \neq 0) = 0.42$ and $\Pr(\beta \neq 0) = 0.38$, respectively; Fig. 8, see also Supporting information]. The contribution of treatment alone was not as strong as we would expect given the striking differences in fecundity (47.5 versus 181.7 eggs). It appears that the interaction of treatment and size, which also contributed to female lifetime fecundity [$(\Pr(\beta \neq 0) = 0.48$; see Supporting information], decreased the contribution of treatment alone. This is probably a consequence of small sample size because it is clear that *G. firmus* females mated to heterospecific males deposited more eggs than virgin females (Fig. 5). As expected, neither virgins nor females mated only to heterospecific males produced any offspring (Fig. 6).

DISCUSSION

The present study reports previously uncharacterized behavioural pre-mating barriers to gene exchange between *G. firmus* and *G. pennsylvanicus*. These two cricket species exhibit very little differentiation in

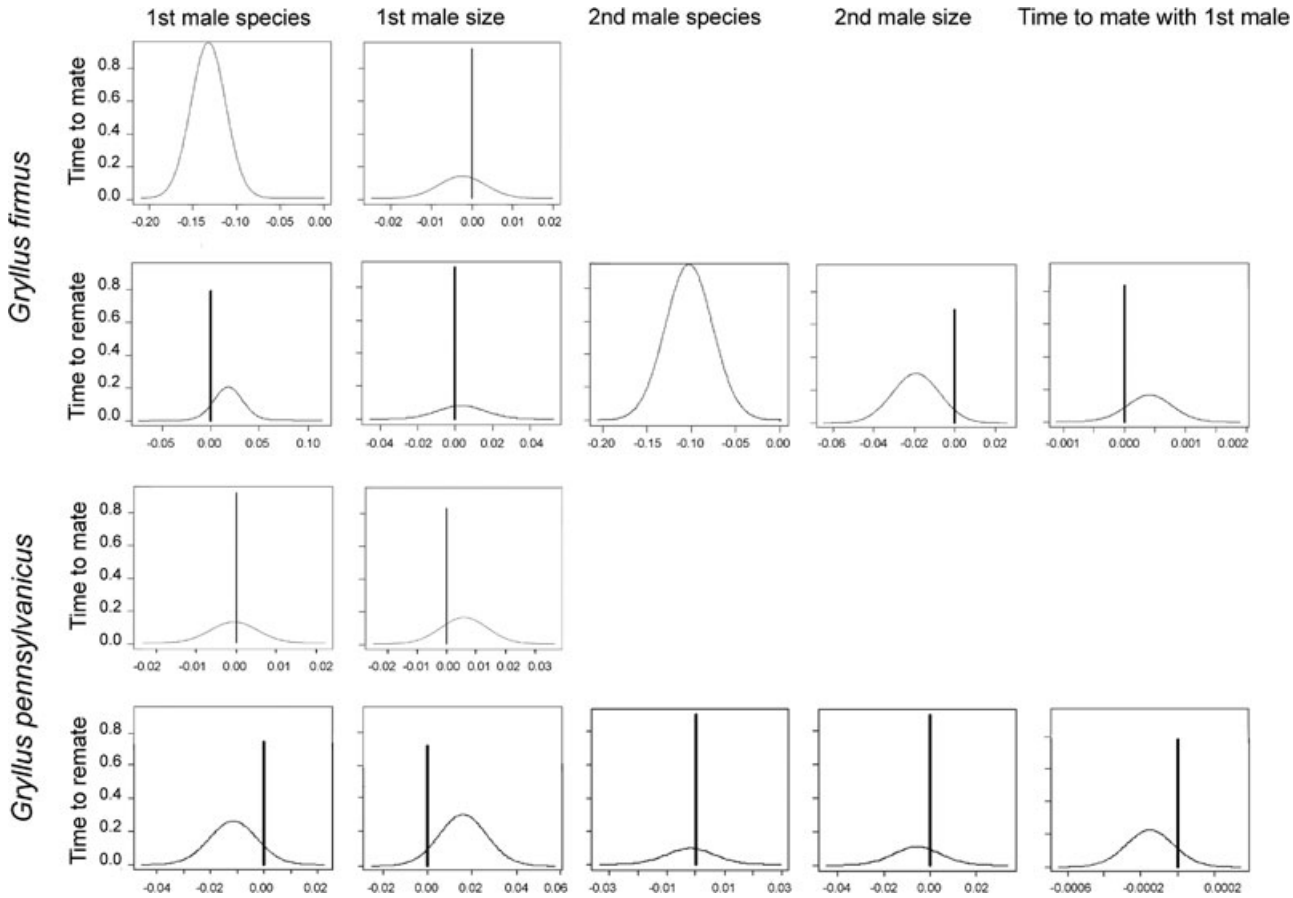

Figure 3. Posterior distribution of the main effects coefficients produced by model averaging of time to mate. The posterior probability that the coefficient is zero is represented by a solid line at zero, with height equal to the probability. The nonzero part of the distribution is scaled so that the maximum height is equal to the probability that the coefficient is nonzero. Only two variables (i.e. first male species and second male species) contributed strongly to *Gryllus firmus* time to mate and time to remate predictions.

morphology or DNA sequence and are estimated to have diverged approximately 200 000 years ago (Willett, Ford & Harrison, 1997; Broughton & Harrison, 2003; Maroja, 2008). Analyses of molecular markers [pallozymes, mitochondrial (mt)DNA sequences, nuclear restriction fragment length polymorphisms, nuclear gene intron sequences] have uncovered few diagnostic differences (Harrison & Arnold, 1982; Harrison & Bogdanowicz, 1997), and gene genealogies often reveal absence of exclusivity and haplotype sharing between the species (Willett *et al.*, 1997; Broughton & Harrison, 2003; Maroja, 2008). By contrast to the similarities in morphology and gene sequences, the ecology, behaviour, and development of the two cricket species have apparently diverged substantially. These differences, including the differences in mating behaviour reported in the present study, result in multiple barriers to gene exchange that act throughout the life history of *G. firmus* and *G. pennsylvanicus* (Table 1).

Many insect hybrid zones are reported to have multiple trait differences that restrict gene flow (Mendelson & Shaw, 2002; Ross & Harrison, 2002; Bailey, Thomas & Butlin, 2004). In the geographically extensive *G. firmus–G. pennsylvanicus* hybrid zone, some barriers operate throughout the zone (e.g. one-way incompatibility), whereas others vary geographically (e.g. temporal isolation) (Table 1). None of these barriers acting alone is complete but, together, they appear to severely restrict gene exchange; very few F_1 individuals are found in mixed populations and the hybrid zone remains clearly bimodal (Harrison, 1986; Harrison & Bogdanowicz, 1997; Ross & Harrison, 2002).

In our no-choice experiments, we used two measures of mate preference: time to mate and the proportion of trials in which spermatophore transfer failed to occur. Using the latter criterion, females of both species 'prefer' conspecific males. However, only in *G. firmus* did the male species make a strong

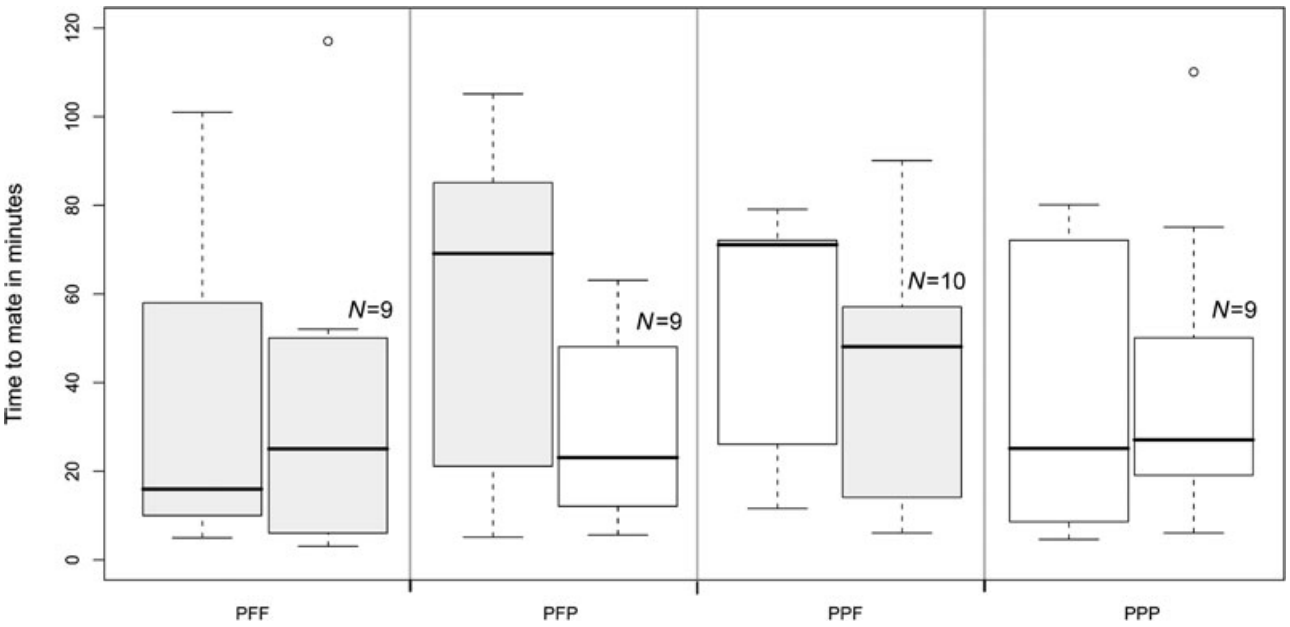

Figure 4. Box plot of *Gryllus pennsylvanicus* female time to mate with first and second male for each double mating treatment (*N*, sample size). White boxes represent conspecific males. Grey boxes represent heterospecific males.

Table 1. List of known pre- and post-mating barriers to gene exchange between *Gryllus firmus* and *Gryllus pennsylvanicus*

	Barrier	Likely mechanism	References
Pre	Ecogeographic isolation	Association with different soils	Rand & Harrison (1989); Ross & Harrison (2002, 2006)
	Temporal isolation	Differences in time of adult appearance (due to differences in development times)	Harrison (1985)
	Acoustic isolation	Differences in calling song	Alexander (1957); Doherty & Storz (1992)
	Time to mate	Differences in time to mate with conspecific and heterospecifics	Present study
Post	One-way incompatibility	Gametic incompatibility in the heterospecific cross between *G. firmus* female and *G. pensylvanicus* male	Harrison (1983)

contribution to the time to mate prediction; *G. firmus* females mated readily with conspecific males, but took far longer to mate with heterospecific males. *Gryllus pennsylvanicus* females were generally more reluctant to mate; the frequency of spermatophore transfer, but not time to mate, differed depending on the species of male with which they were paired.

In addition to demonstrating mate preference, we also documented that matings with *G. pennsylvanicus* males did not trigger normal oviposition in *G. firmus* females. Females mated only to heterospecific males deposited more eggs than virgin females but significantly fewer eggs than females mated to conspecific males. This difference could be due to rapid evolution of accessory gland proteins in *G. firmus* and/or *G. pennsylvanicus* (Andrés *et al.*, 2006) because transfer from male to female of accessory gland proteins is known to influence oviposition in other insects (Neubaum & Wolfner, 1999; Chapman *et al.*, 2003; Liu & Kubli, 2003). Failure to stimulate oviposition may therefore serve as an additional barrier to gene exchange.

The differences in mating behaviour between the two crickets can be invoked to explain asymmetrical introgression, with alleles flowing more readily from *G. pennsylvanicus* into *G. firmus*. Asymmetry in intro-

gression is predicted because: (1) *G. pennsylvanicus* females discriminate against F_1 hybrid males, whereas *G. firmus* females do not, and (2) F_1 hybrid females appear to 'prefer' *G. firmus* males over *G. pennsylvanicus* males, although this difference was not significant.

Indeed, asymmetric introgression between *G. firmus* and *G. pennsylvanicus* has been reported for mtDNA (Harrison, Rand & Wheeler, 1987; Harrison & Bogdanowicz, 1997; Willett *et al.*, 1997) and allozymes (Harrison & Arnold, 1982). More recently, the clear signature of asymmetrical gene flow has also been reported for nuclear genes. In a fine-scale study of the hybrid zone in Connecticut, Ross & Harrison (2002) observed differential introgression at nuclear loci, with alleles moving from *G. pennsylvanicus* into *G. firmus*. Similar results have recently been observed over a much broader geographic scale, with significant difference in the directional introgression rates of neutral loci (Maroja, 2008). Because F_1 hybrids are only produced by *G. pennsylvanicus* females, asymmetrical introgression of mtDNA alleles

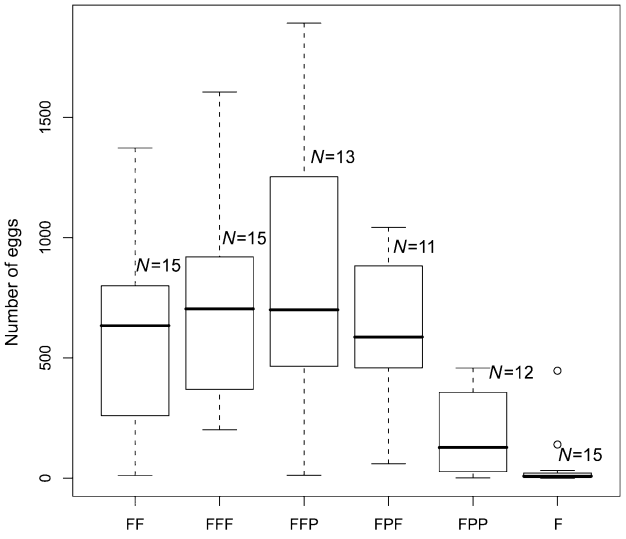

Figure 5. Box plot of *Gryllus firmus* fecundity for each treatment (*N*, sample size).

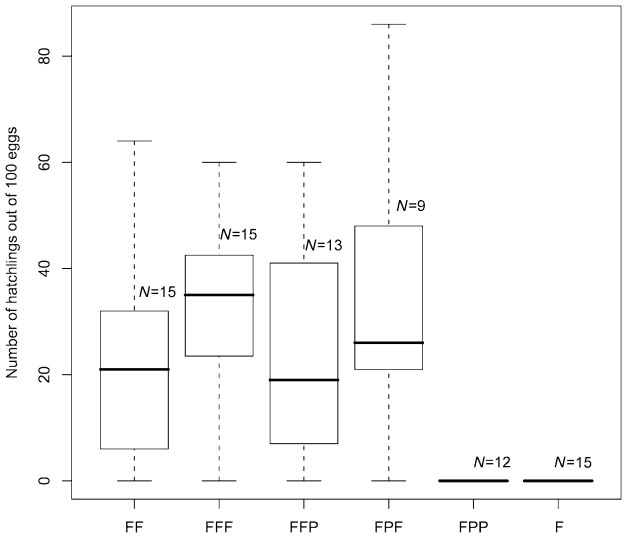

Figure 6. Box plot of *Gryllus firmus* fertility for each treatment (*N*, sample size).

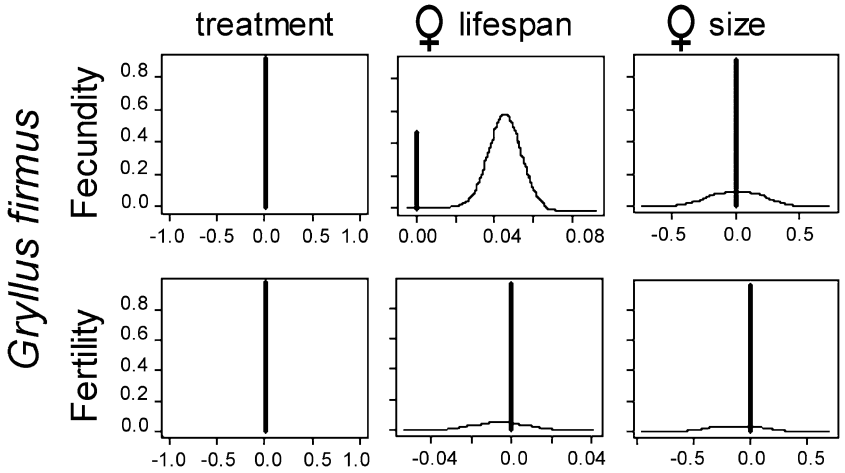

Figure 7. Posterior distribution of the main effects coefficients produced by model averaging of fecundity and fertility of *Gryllus firmus* females mated to at least one conspecific male [excluding treatments where females were not given access to males and females were mated twice with two different heterospecific males]. The posterior probability that the coefficient is zero is represented by a solid line at zero, with height equal to the probability. The nonzero part of the distribution is scaled so that the maximum height is equal to the probability that the coefficient is nonzero.

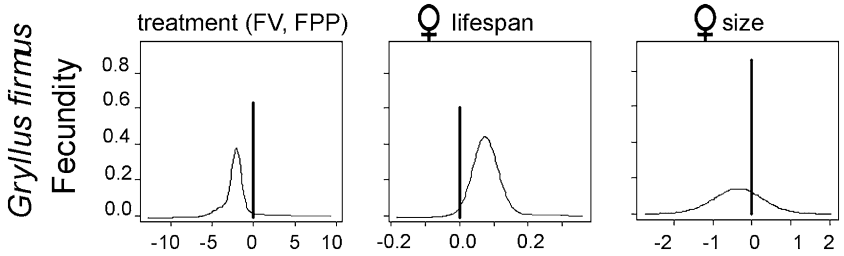

Figure 8. Posterior distribution of the main effects coefficients produced by model averaging of fecundity and fertility of virgin and *Gryllus firmus* females mated only to heterospecifics [virgin females (FV) and females mated twice with two different heterospecific males (FPP)]. The posterior probability that the coefficient is zero is represented by a solid line at zero, with height equal to the probability. The nonzero part of the distribution is scaled so that the maximum height is equal to the probability that the coefficient is nonzero.

is not surprising. However, the one-way incompatibility cannot explain the asymmetrical introgression of nuclear alleles; the behavioural barriers identified in the present study are more likely to explain this pattern.

The phenotypic differences responsible for the observed mate choice remain unclear. Acoustic signals in Orthoptera have been shown to play a role in pre-mating isolation and female choice (Wells & Henry, 1998; Mendelson & Shaw, 2002; Bridle *et al.*, 2006). In some crickets, females clearly respond preferentially to conspecific male song (Mendelson & Shaw, 2002; Holzer, Jacot & Brinkhof, 2003; Saldamando *et al.*, 2005), and it has been argued that differences in calling song could be important in the maintenance of the *G. firmus–G. pennsylvanicus* hybrid zone (Doherty & Storz, 1992). Although there are slight differences in the calling song of these species (Alexander, 1957; Harrison & Rand, 1989; Doherty & Storz, 1992), the courtship song of North American *Gryllus* species does not vary (Alexander, 1968). Because each male–female pair in our experiments was housed in a small confined space, in which females were exposed only to courtship song, the pre-mating barriers reported in the present study are not due to differences in calling song. Variation in chemical cues (e.g. cuticular hydrocarbons) has been shown to differentiate closely-related species of other insects (Hardy & Shaw, 1983; Howard & Blomquist, 2005; Nagamoto, Anonuma & Mituhiko, 2005; Mullen *et al.*, 2007) and are heritable in the cricket *Teleogryllus oceanicus* (Thomas & Simmons, 2008). Cuticular hydrocarbons may thus play an important role in mate recognition in *Gryllus*.

The laboratory, no-choice experiments reported in the present study show that females of both species prefer to mate with conspecific males, although the evidence is stronger for *G. firmus* females. However, such experiments obviously fail to mimic situations in natural populations. Given the local abundance of field crickets in hybrid zone populations (L. S. Maroja, pers. observ.), females are rarely in no-choice situations. In the presence of multiple males, female reluctance (or failure) to mate with heterospecific males should serve as a substantial barrier and make heterospecific matings rare in the wild. Indeed, the difficulty of finding F_1 individuals in the hybrid zone (Harrison & Bogdanowicz, 1997) confirms the importance of pre-mating barriers in preventing gene flow, given that there is no conspecific sperm precedence (G. Hume, unpubl. data). Although habitat isolation no doubt serves to reduce encounter rates between the two crickets, adults of both species are found together within single populations in Connecticut (Harrison, 1986; Harrison & Bogdanowicz, 1997), and behavioural barriers provide the only explanation for the persistent bimodal nature of the hybrid zone.

Individuals from hybrid zones are expected to evolve stronger assortative mating if there are costs to mating with heterospecifics (Liou & Price, 1994). Furthermore, mosaic bimodal hybrid zones may facilitate reproductive character displacement by providing an initial level of assortative mating by habitat use (Jiggins & Mallet, 2000). However, because nonrandom mating is costly (Andersson, 1994), females are expected to be choosy only if the cost of mating with the 'wrong' male is high. We did not find any costs in fecundity or fertility for *G. firmus* females mated to both conspecific and heterospecific males (Figs 1, 2). Because these females do not produce hybrid offspring, they do not suffer the associated costs and could even benefit from heterospecific matings if there are any direct benefits (e.g. nutritional) associated with multiple mating (Wagner *et al.*, 2001). However, as is evident from the strong preference for conspecific males, *G. firmus* females are choosy despite the apparent absence of fecundity or fertility costs and the possible benefits of multiple matings.

In the present study, we only measured fecundity and fertility costs; but females may experience other

costs in mating with heterospecific males. In moving toward calling males, females are subjected to predation and parasitism, and crickets are known to alter their mating behaviour in conditions of high predation (Hedrick & Dill, 1993). Furthermore, acoustically oriented parasitoids might pose risks for females remaining in close proximity to singing males (Cade, 1975; Wagner, 1996). In addition, there is a very severe cost to *G. firmus* females that mate only with heterospecific males, namely a failure to produce progeny. Although failure to mate with any conspecific males is unlikely to occur in the wild, in localities where *G. pennsylvanicus* is far more abundant, *G. firmus* females might find few or no conspecific males.

The field-collected crickets used in the present study were all pure species from allopatric populations. In spite of no direct exposure to heterospecifics, these crickets showed strong assortative mating, especially for the cross that produces no hybrid offspring (*G. firmus* female and *G. pennsylvanicus* male). Furthermore, both F_1 hybrids and parental species individuals reared in the laboratory showed assortative mating behaviour. It is possible that these behavioural barriers are a byproduct of divergence in allopatry and that the relevant trait differences were already present before secondary contact. It is also possible that secondary character displacement spread from the hybrid zone into the pure species populations adjacent to the hybrid zone. Studies of mating behaviour of crickets within and very far from the hybrid zone are needed to resolve this issue.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Model selection for *Gryllus firmus* time to mate (first male).
Table S2. Model selection for *Gryllus firmus* time to remate (second male).
Table S3. Model selection for *Gryllus pennsylvanicus* time to mate (first male).
Table S4. Model selection for *Gryllus pennsylvanicus* time to remate (second male).
Table S5. Model selection for *Gryllus firmus* fecundity of female mated to at least one conspecific (FF, FFF, FFP, FPF).
Table S6. Model selection for *Gryllus firmus* fertility of females mated to at least one conspecific (FF, FFF, FFP, FPF).
Table S7. Model selection for Fecundity of *Gryllus firmus* females from F and FPP treatments.

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