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Multiple post-mating barriers to hybridization in field crickets

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Abstract

Mechanisms that prevent different species from interbreeding are fundamental to the maintenance of biodiversity. Barriers to interspecific matings, such as failure to recognize a potential mate, are often relatively easy to identify. Those occurring after mating, such as differences in the how successful sperm are in competition for fertilisations, are cryptic and have the potential to create selection on females to mate multiply as a defence against maladaptive hybridization. Cryptic advantages to conspecific sperm may be very widespread and have been identified based on the observations of higher paternity of conspecifics in several species. However, a relationship between the fate of sperm from two species within the female and paternity has never been demonstrated. We use competitive microsatellite PCR to show that in two hybridising cricket species, *Gryllus bimaculatus* and *G. campestris*, sequential cryptic reproductive barriers are present. In competition with heterospecifics, more sperm from conspecific males is stored by females. Additionally, sperm from conspecific males has a higher fertilisation probability. This reveals that conspecific sperm precedence can occur through processes fundamentally under the control of females, providing avenues for females to evolve multiple mating as a defence against hybridization, with the counterintuitive outcome that promiscuity reinforces isolation and may promote speciation.

Keywords: competitive microsatellite PCR, conspecific sperm precedence, cryptic female choice, reproductive isolation, speciation, sperm competition

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Introduction

Reproductive isolation between species has long been studied within the classic dichotomous framework of barriers occurring before insemination, such as availability or recognition of potential mates, and those occurring after zygote formation, such as viability and fertility of hybrid offspring (Dobzhansky 1937). Only relatively recently has attention been paid to the role of cryptic selection mechanisms acting between mating and the fertilisation of eggs. This category of mechanisms termed postmating–prezygotic (Howard *et al.* 2009) will reduce gene flow between distinct populations of individuals or species and thus act to maintain species boundaries if the success of conspecific matings is relatively greater than that of heterospecific matings. These barriers are now acknowledged to be important contributors to reproductive isolation, and there are a growing number of studies showing that in closely related species where females will mate to both conspecific and heterospecific males, the heterospecific males do not sire as many offspring, a phenomenon known as conspecific sperm precedence (CSP). Examples have been recorded across a broad range of taxa, with insect and marine invertebrate species most prevalent in the literature (Howard *et al.* 2009). Traits associated with postmating–prezygotic processes have been shown to

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have the potential to diverge rapidly, suggesting they could play an important role in speciation (Civetta & Singh 1995; Pitnick *et al.* 2003; Andrés *et al.* 2006). What we are missing is evidence for the mechanism by which conspecific sperm gain a greater share of fertilisations. This is particularly interesting because if it is something that females can influence, then sexual selection can increase reproductive isolation, which would tend to increase the rate of speciation.

Although widely reported, little is known of the underlying processes involved in CSP (reviewed by Howard 1999). Studies have followed the progress of ejaculates through the female tract without relating this to siring success in the same female (Price *et al.* 2001) or have relied upon counts of offspring displaying phenotypic markers without elucidating the cryptic processes determining the success of ejaculates within the same female (e.g. Fricke & Arnqvist 2004). It is also difficult to demonstrate that CSP is due to competition between gametes rather than differential fitness of hybrid embryos or offspring (but see Price 1997).

To overcome the problems usually associated with the study of CSP, we use a competitive microsatellite PCR (CM-PCR) technique (Wooninck *et al.* 2000; Bussière *et al.* 2010), which enables us to determine the relative contribution of an individual to mixed DNA samples. To date, this technique has successfully been used to investigate patterns of sperm storage in twice-mated dung flies, *Scathophaga stercoraria* (Bussière *et al.* 2010), the relationship between spermatophore attachment time and sperm storage in twice-mated crickets, *Teleogryllus commodus* (Hall *et al.* 2010), and the effect of relatedness of mating partners on sperm storage and paternity in twice-mated *Gryllus bimaculatus* (Bretman *et al.* 2009). We apply this technique to study the hybridizing field crickets *G. bimaculatus* and *G. campestris*, species in which CSP potentially acts as a reproductive barrier. We first determine the representation of sperm from competing males in the spermathecae of doubly mated females and second relate this to the success of each ejaculate in siring nymphs. The ability to directly observe sperm storage translating to siring success within the same female makes CM-PCR a powerful tool in the study of CSP, and to our knowledge, this is the first time that it has been employed in this context.

Gryllus bimaculatus and *G. campestris* live in grazed or mown grassland habitats and have overlapping ranges in southern Europe (Pardo *et al.* 1993; Gorochov & Llorente 2001). The two species will interbreed in captivity (Cousin 1933; von Hörmann-Heck 1957; Veen *et al.* 2011). Interbreeding is unidirectional, with *G. campestris* females almost never accepting *G. bimaculatus* males as mates (but see Cousin 1933). Although mate choice in *G. bimaculatus* is well studied, less is known of mate choice in the context of reproductive isolation between species, that is, the relationship between intra- and interspecific mate choice. While recent work has revealed reproductive barriers between *G. bimaculatus* and *G. campestris* in terms of mate choice before mating, as well as hybrid viability and sterility (Veen *et al.* 2011, 2013), to date, nothing is known of potential cryptic barriers in this system. This is a recurrent situation in the study of Gryllidae. Despite intensive study of reproductive isolation at several hybrid zones around the globe (reviewed in Veen *et al.* 2013), to our knowledge, CSP has only previously been examined in *Allonemobius* species, where there is strong CSP (Gregory & Howard 1994; Marshall 2004), and between *G. pennsylvanicus* and *G. firmus*, where no evidence of CSP was found (Larson *et al.* 2012).

The mating systems of *G. bimaculatus* and *G. campestris* are similar. Prior to mating, a male provisions a spermatophore with sperm. The number of sperm that a male invests into each spermatophore does not decline over at least the first five matings in *G. bimaculatus* (Simmons 1986, 1987); however, they may alter their investment depending on the perceived quality of potential mates (Hall *et al.* 2000). Both species are polyandrous with females mating with a number of males during their lifetime (Bretman & Tregenza 2005; Rodríguez-Muñoz *et al.* 2011). Mating takes a few seconds, consisting of the female mounting the male and the male externally attaching a spermatophore to her. After mating, sperm begin to transfer from the spermatophore to the female reproductive tract. This process takes around an hour and is occasionally terminated by early removal of the spermatophore (Simmons 1986), although removal is often prevented by the male through guarding behaviour (Simmons 1991). *Gryllus bimaculatus* females can also exert cryptic control, biasing the paternity of offspring through differential uptake of conspecific sperm (Bretman *et al.* 2009), potentially through muscular control (Simmons & Achmann 2000). Transferred sperm are stored in the spermatheca and once in storage are not displaced by subsequent matings; rather the spermatheca expands to store multiple ejaculates (Simmons 1986). It is spherical in form, a shape which is likely to promote mixing of ejaculates rather than stratified sperm storage (Walker 1980; Simmons 1986). The lack of stratified storage means there is no last male sperm precedence in this species (Simmons 1987; Bretman *et al.* 2009). Instead, success in siring offspring is likely determined as a raffle (Parker 1982), whereby the more sperm a male has in storage, the greater the chance his sperm will be used to fertilize eggs. Indeed, Bretman *et al.* (2009) found a direct relationship between the amount of sperm individual males had in storage and their subsequent paternity

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when *G. bimaculatus* females were mated to both a related and an unrelated male.

As precopulatory barriers to hybridization are relatively weak between *G. bimaculatus* females and *G. campestris* males (Veen *et al.* 2011), it is possible that postcopulatory barriers play a role as reproductive isolating mechanisms between the two species. Coupled with the knowledge that *G. bimaculatus* females are capable of cryptic female choice in terms of uptake and storage of sperm (Simmons & Achmann 2000; Bretman *et al.* 2009), we predict that CSP will be present in this system and so expect to find a greater representation of conspecific sperm in the spermathecae of multiply mated females.

While an increasing number of studies have considered cryptic barriers in terms of the overall sperm competition success of males of one species vs. another, little attention has been paid to the repeatability of success of individual males. This is an important issue because such repeatability would indicate that success in these contexts is at least partly a male trait. (Tregenza *et al.* 2009). Additionally, if the same traits are associated with success whether sperm competition is intra- or interspecific, this would indicate that mechanisms of sperm competition are conserved across species. In our experimental design, we mate each of the males twice, allowing within-individual success to be compared when competing intra- and interspecifically.

Finally, based upon the assumption that sperm mixing occurs within the spermatheca (Walker 1980; Simmons 1986) and Parker's 'raffle principle' of sperm competition (Parker 1982), we predict a direct relationship between representation in the spermatheca and subsequent paternity. Deviation from this predicted relationship could occur through biased success in poststorage sperm competition or ability to fertilize eggs or through differential mortality of hybrid offspring. To disentangle these potential mechanisms, we monitor egg laying and hatching success.

Methods

The crickets were sampled from allopatric locations (Gorochov & Llorente 2001). *Gryllus campestris* were collected from near Gijon, northern Spain (N43 27.193 W5 50.407), as nymphs, and the majority were reared to adulthood in the laboratory. Those that became adult before reaching the laboratory were allowed to adjust to standard laboratory conditions for at least 8 days prior to use in trials. We used wild-caught individuals because this species has an obligatory diapause, which makes them difficult to rear in larger numbers in the laboratory. *Gryllus bimaculatus* were collected from Valencia, southern Spain (N39 35.936 W0 34.087), and have subsequently been reared for 6 years in the laboratory. Crickets were provided with food and water *ad libitum* and maintained under a 16L/8D photoperiod at 28 °C. Individuals were separated into small plastic tubs prior to becoming adult to ensure virginity and were a minimum of 7 days old posteclosion before being used in mating trials. Mating trials were conducted over a period of 2 years.

Mating trials

Prior to mating trials, almost all males (75%) (see Data S1, Supporting information) were exposed to nonexperimental conspecific females to stimulate spermatophore development and courtship behaviour. These individuals were separated by wire mesh so that the female could be detected but not mated with. Males were monitored for the onset of courtship behaviour, indicating that a spermatophore has been produced and is ready to be transferred. Mating trials were carried out in 11 × 11 cm plastic containers lined with paper for traction. Only *G. bimaculatus* females were used, as they mate both intra- and interspecifically (Veen *et al.* 2011). Each pair was given 2 h to mate, if they had not done so within this time, the pair was trialled again on subsequent days (including re-exposing the male to a nonexperimental conspecific female) for a maximum of 5 days before being discarded. Mating pairs were observed following successful mating, and spermatophore attachment time was standardized to 1 h, the period of time required for almost all contents of the spermatophore to be transferred to the female (Simmons 1986). Females can bias paternity through early removal of the spermatophore (Simmons 1986), but this was prevented through male guarding behaviour (Simmons 1991). If the male's behaviour was not sufficient to prevent attempts by the female at early spermatophore removal, the female was moved into a small vial to restrict her movement.

Only *G. bimaculatus* females were used. They were mated twice, to a conspecific *G. bimaculatus* (B) and a heterospecific *G. campestris* (C) in either order (BC/CB) so that competition between males was interspecific or mated to two males of the same species (BB or CC) so that competition between males was intraspecific. We aimed to pair each male twice, each time facing a different competitive treatment (either intra- or interspecific), but in the same order as first or second male to mate on both occasions. No male was used more than once in either intra- or interspecific treatments (Table 1). In all, 70 triads of individuals were mated.

After mating, males were preserved in 100% ethanol or frozen at −20 °C, until DNA extraction. After their second mating, females were allowed to lay eggs in a

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Table 1 Example triad design. Only *Gryllus bimaculatus* females were used, and each of which was mated twice to either two conspecifics, two heterospecifics or one of each. We aimed to mate each male twice so that he appeared in both interspecific and intraspecific competitive contexts. B males were conspecific to the female, whereas C males were heterospecific. Competition between BC or CB pairs of males was interspecific, and competition between BB or CC males was intraspecific

Triad	*G. bimaculatus* female	1st male to mate	2nd male to mate	Competition between males
BC	1	B.1	C.2	Interspecific
CB	2	C.1	B.2	Interspecific
BB	3	B.1	B.2	Intraspecific
CC	4	C.1	C.2	Intraspecific

small container of damp sand for 48 h before preservation in ethanol. Eggs were removed from the sand and counted. A random sample of 100 of the eggs (or fewer if the total number laid was <100) was incubated at 28 °C on damp cotton wool. Upon hatching, nymphs were counted and collected twice daily and either frozen or stored in ethanol.

Molecular analysis

DNA was extracted from adult legs and whole nymphs using a salt extraction protocol (see Bretman & Tregenza 2005 for details). Thirty nymphs (or fewer depending on hatching success) were sampled from each triad, a number chosen to maximize accurate representation of each male's siring success, without becoming an unmanageable amount of tissue to extract DNA from. Extractions carrying pigment from the cuticle were cleaned prior to PCR using a DNA clean-up kit (Genomic DNA Clean & Concentrator, Zymo Research). To estimate the amount of sperm stored from both males, the spermathecae (containing DNA from the female as well as from each male's sperm) were dissected from females and the DNA extracted using a chelex protocol (see Bretman & Tregenza 2005). DNA from adult legs was standardized to 10 ng/μL using a NanoVue (GE Healthcare).

The CM-PCR technique requires the identification of a unique microsatellite allele marker in each of the two males that comprise a mating triad, that is, one not shared by the other male or the female. To identify unique alleles, adults were genotyped at up to 10 microsatellite loci [*Gbim*04, 15 (Dawson *et al.* 2003); *Gbim*21, 29, 48, 49, 52, 57, 66 and 72 (Bretman *et al.* 2008)] (MJ Research Thermal Cycler PTC-200) on an ABI 3130XL sequencer (Applied Biosystems), and allele sizes scored using GeneMapper v3.7 (Applied Biosystems). For details of PCR conditions for these microsatellite loci, see Data S2 (Supporting Information). Unique alleles were identified for 55 of the 70 triads. Thirty-two triads (of the 55) were made up of females mated to interspecifically competing males (BC/CB), 12 to two *G. bimaculatus* males (BB) and 11 to two *G. campestris* males (CC). Of the 55 triads, 17 did not produce nymphs. A total of 76 individual males were used, with equal numbers of each species. Of these males, 36 featured in both an intraspecific and an interspecific competition triad.

A standard curve was made for each of the 55 triads (following Bretman *et al.* 2009), from which to determine a male's representation in the spermatheca and nymph samples. Each standard contained a mix of DNA from the two males in varying proportions, such that the focal male (the B male in BC/CB triads, the first male to mate in BB or CC triads) accounted for 6.25%, 12.5%, 25%, 50%, 75%, 87.5% and 93.75% of the mix. As female DNA will be present in the nymphs and could potentially contaminate the sperm samples, we made a second set of standards following Bretman *et al.* (2009), including the DNA from the female in a 1:1 ratio with the DNA mixture from the two males. The standards, as well as the spermatheca and nymph samples corresponding to each triad, were then genotyped at the relevant locus identified for that triad as possessing unique male alleles. The use of a unique standard curve for each triad, rather than for all the samples as a whole (as in Bussière *et al.* 2010; Hall *et al.* 2010), avoids potential problems such as preferential amplification of smaller alleles and so does not require any statistical adjustment for such effects.

We scored the unique alleles for each triad in GeneMapper and extracted their total peak areas. The relative peak area of the focal male was then calculated as (area of focal male/area of focal male + area of other male) and then plotted against the proportion of focal male DNA in the standard mix to create a standard curve for each triad. We repeated this process for the standard samples containing 50% female DNA. The inclusion of female DNA in the standard mixes made a marked difference to relative peak heights (in most cases changing the fit of the standard curve from linear to nonlinear), so it was these values that were used to create the standard curves. Curves were fitted as linear, logarithmic or polynomial. Best fit could not be chosen based upon ANOVA, as the linear and logarithmic models contain the same number of parameters (comparisons require models to differ in the number of parameters they contain, see Statistical analyses). Instead, best fit was selected based

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upon AIC, whereby the AIC delta scores of each model were compared and considered to be different if >2 (Burnham & Anderson 2002), with the requirement that the curve must increase and not asymptote through the range of the data. The fit of the standard curve to each of the sets of standards was high (mean $R^2 = 0.976 \pm$ SE 0.004). The relative peak area of the allele from the focal male in the spermatheca and nymph samples was calculated using the formula from the standard curve to determine that male's representation in both samples.

Repeatability of the quantification of the proportion of male DNA in spermatheca and nymph samples was assessed by randomly selecting a subset of samples (eight spermathecae, nine nymphs and seven standards), and repeating the PCR and genotyping to yield a duplicate estimate of proportion of DNA. The repeatability of the selection of samples re-amplified and genotyped was high ($R^2 = 0.982$, see Data S3, Supporting information). An outlier in the data set, a *G. bimaculatus* male featuring in only one triad, where almost all of the sperm in the spermatheca were his but where he sired none of the offspring, was excluded prior to the analysis on the grounds this male was almost certainly infertile.

Statistical analyses

All analyses were carried out using R v2.14.1 (R 2011). We used the package 'lme4'(Bates *et al.* 2011) to fit generalized linear mixed models (GLMM) to assess factors affecting success in sperm storage, the relationship between representation in the spermatheca and success in siring nymphs and individual male success across contexts. In analyses where data from a focal male from each triad were used, the focal male was taken to be the *G. bimaculatus* males in interspecific triads or chosen haphazardly to randomly include equal numbers of first- and second-position males in intraspecific triads (note the difference in choice of focal male relative to the molecular analyses). Hybrid offspring have reduced hatching success; hence, measures of siring success based on counts of nymphs need to be adjusted appropriately so that we can disentangle the success of each male due to fertilization success and due to embryonic survival. To do this, we multiplied the proportion of offspring observed from heterospecific males by a correction factor based on the mean observed hatching rate of pure and hybrid offspring from eggs laid by females that only mated to one type of male (correction factor = the ratio of the hatching success of purebred offspring (from BB triads), to the hatching success of hybrid offspring (from CC triads)). Significance of terms was assessed by likelihood ratio tests between nested models (one containing the term of interest and one with that term removed) (Crawley 2007). General linear models (GLM) using ANOVA-based model selection were used to analyse the differences in egg laying across triad types, the differences in hatching success across triad types and the relationship between the amount of *G. campestris* sperm in storage and hatching success. For detailed analytical methods and model output, see Data S4 (Supporting information).

Results

Representation of competing males in the spermatheca

The contribution of sperm from a particular male to spermathecal storage depended upon both competition type (intraspecific or interspecific) and male species, but there was no effect of whether a male was first or second to mate (lmer; competition × species interaction; $\chi^2_{1,7} = 27.85$, $P < 0.001$, mating order; $\chi^2_{1,8} = 0.34$, $P = 0.562$). When competition was interspecific, much more sperm was stored from *the G. bimaculatus* male (Fig. 1).

Individual male success across contexts

Individual male success in sperm storage across contexts (intraspecific vs. interspecific competition) was repeatable; *G. campestris* males that were more successful in having their sperm stored when competition was intraspecific were also more likely to be successful in having their sperm stored when competition was interspecific (lmer; $\chi^2_{1,6} = 3.90$, $P = 0.048$). As already shown in earlier analyses, overall *G. bimaculatus* males did much better than *G. campestris* males (lmer; $\chi^2_{1,6} = 22.63$, $P < 0.001$, Fig. 2).

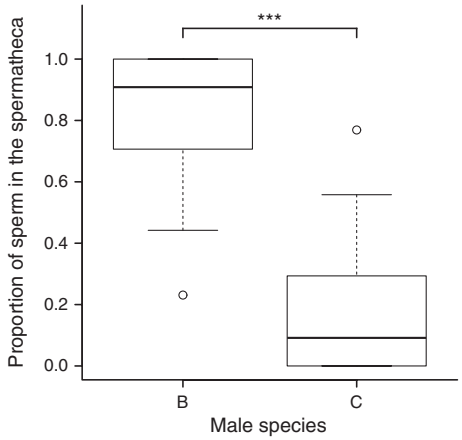

Fig. 1 Success of *Gryllus bimaculatus* and *G. campestris* males competing interspecifically, in terms of the proportion of sperm stored in the spermatheca. Boxes show the upper and lower quartiles, and central lines show medians. Statistical significance: ***$P < 0.001$.

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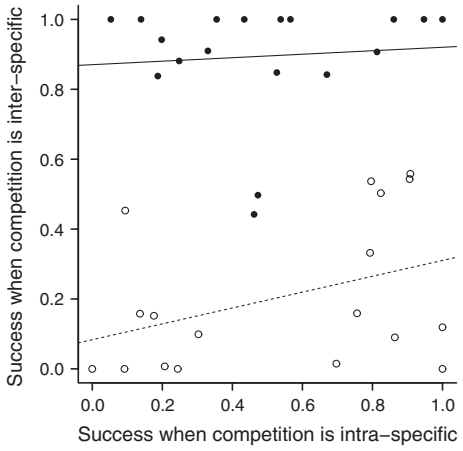

Fig. 2 Individual male success in sperm storage across different mating contexts, plotted as the proportion of the sperm stored by a female that came from a male when competition was intraspecific vs. success when competition was interspecific. Filled points and solid line show *Gryllus bimaculatus* males, and open points and dashed line show *Gryllus campestris* males.

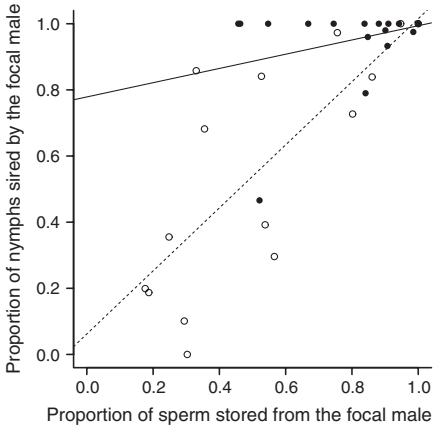

Fig. 3 The relationship between the proportion of sperm in storage and the subsequent proportion of nymphs sired by each focal male. The proportion of nymphs sired by *Gryllus campestris* males was corrected to account for the lower hatching success of hybrid offspring (see text). In interspecific pairings, the focal male was always *Gryllus bimaculatus*. Open points and dashed line show males competing intraspecifically, and filled points and solid line show males competing interspecifically.

Success of competing males in siring nymphs

When competition was intraspecific, a male's success in siring nymphs was dependent upon his representation in the spermatheca. However, when competition was interspecific, almost all nymphs were sired by the *G. bimaculatus* male, regardless of representation in the spermatheca (lmer; competitor type × sperm storage interaction $\chi^2_{1,6} = 3.96$, $P = 0.047$, Fig. 3). Neither species identity of the focal male nor mating order had an effect (lmer; species $\chi^2_{1,11} = 0.17$, $P = 0.681$, mating order; $\chi^2_{1,7} = 2.05$, $P = 0.153$).

Egg laying and hatching success

We found no evidence for differing success in the number of eggs laid among triad combinations (females mating to one male from each species or to two males of either species) (GLM; $F_{2,52} = 0.77$, $P = 0.47$, Fig. 4a). However, egg-hatching success differed greatly among

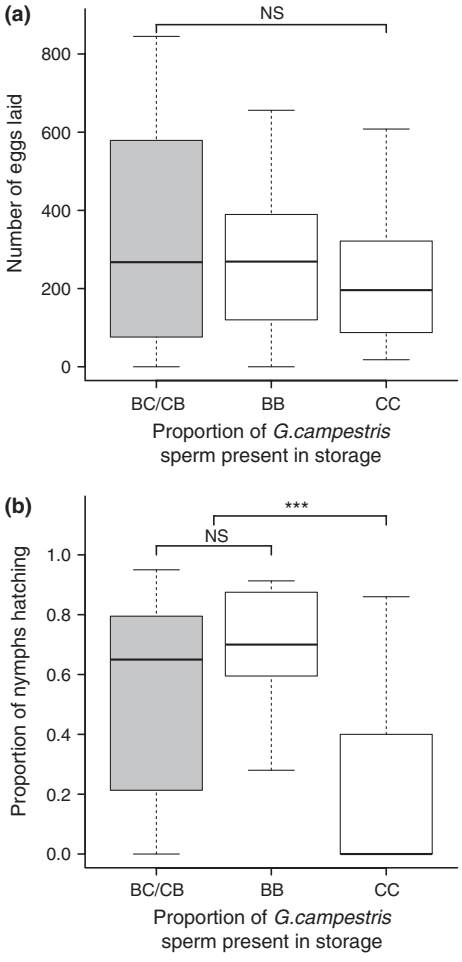

Fig. 4 The total number of eggs laid by females in the different triad types (a) and the proportion of nymphs hatching from a sample of eggs laid by females in the different triad types (b). BC/CB triads (shaded in grey) are those comprised of a *Gryllus bimaculatus* female mated to a *G. bimaculatus* male and a *Gryllus campestris* male in either order, competing interspecifically. BB triads are those comprised of competing *G. bimaculatus* males, and CC comprised of *G. campestris* males. Boxes show the upper and lower quartiles, and central lines show medians. There were no differences among groups in the total number of eggs laid (a). Fewer nymphs hatched in the CC triads than in the other triad types; between the BB and BC/CB triads, there was no difference (b). Statistical significance: NS $P > 0.05$; ***$P < 0.001$.

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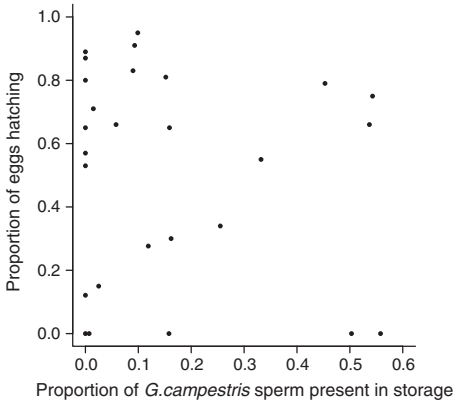

Fig. 5 The relationship between the proportion of eggs hatching and the proportion of *Gryllus campestris* sperm present in storage for intra- and interspecific triads.

triads (GLM; $F_{2,48} = 6.99$, $P = 0.002$, Fig. 4b). *Post hoc* model comparison showed that this difference was due to lower hatching success in the CC triads (GLM; $F_{1,48} = 12.87$, $P < 0.001$)—there was no difference between the BB and BC/CB triads in the proportion of nymphs (GLM; $F_{1,47} = 1.06$, $P = 0.308$). In triads where competition was interspecific, the proportion of *G. campestris* sperm present in storage did not predict hatching success ($F_{1,26} = 0.58$, $P = 0.452$, Fig. 5). Of the four females that did not lay eggs, all but one had genetic material from the male in the spermatheca.

Discussion

We demonstrate strong CSP in these closely related species, with obvious potential to create a significant post-mating–prezygotic reproductive barrier. When males of both species competed, there was a strong bias in sperm stored by *G. bimaculatus* females in favour of the *G. bimaculatus* male. Additionally we found that individual male success in getting sperm into storage was repeatable whether they were competing with a conspecific or a heterospecific. When males competed intraspecifically, representation in the spermatheca predicted success in siring offspring; however, this was not the case for males competing interspecifically. In these triads, the conspecific male sired almost all of the emerging offspring regardless of representation in the spermatheca, suggesting there are also mechanisms determining CSP poststorage.

There are a number of possible mechanisms that could create CSP. Bias in storage could occur as a result of differential investment of sperm into the spermatophore by males in response to the perceived quality of available mating partners (Gage & Barnard 1996). Although crickets may engage in this sort of manipulation (Hall *et al.* 2010), our study was designed to prevent such effects by housing males with a conspecific female during spermatophore production in all but a minority of cases (see Data S1, Supporting information). In our study, it is more likely that bias in spermathecal representation is mediated by a female response such as assistance or inhibition of ejaculate uptake through female muscular control (Simmons & Achmann 2000), acting as a form of cryptic female choice (Hall *et al.* 2010). Or that the low representation of *G. campestris* sperm in the spermatheca is due to morphological incompatibility between the spermatophore and the female reproductive tract, inhibiting sperm transfer (Dufour 1844), or differences between ejaculates in stimulating uptake by the female. *Gryllus campestris* sperm might be less able to traverse the long spermathecal duct due to poor motility in an environment which they have not evolved with (Gregory & Howard 1994), or ejaculate components may actively inhibit rival sperm (Price 1997).

While overall *G. bimaculatus* males had greater success in sperm storage than *G. campestris*, each species showed variation in success among individual males. Interestingly, we found that individual success in gaining representation in the spermatheca was moderately repeatable, even across competitive contexts. Those that were successful when competing against a male of their own species were more likely to be successful when competing interspecifically. This suggests that traits that confer a competitive advantage in sperm competition when competing intraspecifically may also increase the chances of success when competing interspecifically. Examples of repeatability in reproductive success are scant in the literature (but see Tregenza *et al.* 2009), and we encourage research to explicitly investigate this across a range of species.

We found mating order to have no effect on representation in sperm storage or second on subsequent success in siring nymphs. The first observation suggests that last male precedence, a phenomenon recorded in many other insect species (Simmons & Siva-Jothy 1998), is not found in *G. bimaculatus* in line with previous studies (Simmons 1987; Bretman *et al.* 2009). Females may be equally motivated to store sperm when virgin as when already mated, and sperm displacement by competing males does not occur (Simmons 1986). The second observation supports the idea that sperm storage is not stratified to create a 'last in, first out' system, rather sperm mixing occurs in the spermatheca (Walker 1980; Simmons 1986).

Based upon Parker's (1982) 'raffle principle', and the assumption of sperm mixing in the spermatheca, we predicted that success in siring nymphs would directly relate to the amount of sperm in storage. When a male competed against another of the same species, we found

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this prediction to hold true. However, when males of the two species competed, we found that almost all nymphs were sired by the conspecific male, regardless of their sperm representation in the spermatheca. This 'poststorage' bias against heterospecific males suggests that success in sperm competition in these crickets is not simply a 'raffle' determined by sperm number, instead CSP may act at multiple stages in this system: first at the stage of sperm uptake and storage and second after sperm have left the spermatheca.

In the cases of interspecifically competing males, deviation from our prediction that representation in the spermatheca determines siring success may be driven by a number of factors. Although heterospecific sperm are able to traverse the reproductive tract as far as the spermatheca, they may be less able to survive storage than conspecific sperm. Further work, in which spermathecal contents are stained to differentiate between live and dead sperm (Damiens *et al.* 2002), might allow us to assess the survival of heterospecific sperm in storage. However, to replicate the disadvantage that heterospecific sperm experience when competing interspecifically, conspecific ejaculate would also need to be present, perhaps through artificial introduction of seminal fluids to the spermatheca. Another potential driver of poststorage bias against heterospecific sperm might be their ability to leave storage and traverse the reproductive tract to the eggs. If they are able to reach the site of fertilization, they may be less able to attach to and penetrate the eggs (Shaw *et al.* 1994). Eggs could be stained soon after laying to assess presence or absence of sperm (Sarashina *et al.* 2005).

Alternatively, the failure to predict a male's success in siring nymphs from his representation in the spermatheca may be due to postzygotic hybrid mortality. Although not often reported, instances of hybrid embryo mortality have been found across a range of species (for example, Kinsey 1967; Elinson 1981; Álvarez & Garcia-Vazquez 2011). Arrest of embryogenesis occurs at a range of developmental stages and may be driven by genetic incompatibilities, for example differences in chromosomal rearrangements, alleles not functioning together or infection by different endosymbionts (Coyne & Orr 2004). However, if the differences in offspring sired that we observed were due to hybrid embryonic mortality, we would predict that females storing more *G. campestris* sperm should have lower egg-hatching success. We found no such relationship within the interspecific triads suggesting that CSP is determined earlier than embryonic development.

In *Drosophila* species, egg laying is stimulated by seminal proteins present in the ejaculate (Gillott 2003), potentially acting as a species isolating mechanism if heterospecific ejaculate fails to stimulate laying, especially as seminal proteins evolve very rapidly (Swanson *et al.* 2001). However, this is unlikely to play a role in this system; we observed no difference in the number of eggs laid among triads of different species combinations, corroborated by Veen *et al.* (2013), who found no difference in the number of eggs laid by female *G. bimaculatus* singly mated to *G. bimaculatus* or *G. campestris* males.

Our *G. bimaculatus* crickets were from a laboratory stock, reared over many generations, and it is likely that this population had lower genetic variability than the wild population. Our difficulty in identifying allelic mis-matches among *G. bimaculatus* individuals is consistent with this suggestion. Despite the costs to offspring fitness usually associated with inbreeding (Charlesworth & Charlesworth 1987; Tregenza & Wedell 2002), we found a strong bias in sperm storage and paternity in favour of the *G. bimaculatus* males.

Conspecific sperm precedence acts as a strong but not complete barrier to hybridization in this system and is likely to be complemented by other barriers. Prior to mating, females can choose mates based upon cues such as calling song, courtship song or pheromones (Tregenza & Wedell 1997; Veen *et al.* 2011, 2013). *Gryllus campestris* females strongly discriminate against *G. bimaculatus* males, almost never interbreeding (Cousin 1933; von Hörmann-Heck 1957; Veen *et al.* 2011). *Gryllus bimaculatus* females, however, are less choosy and are known to interbreed in captivity, although less readily so than to males of their own species (Veen *et al.* 2011). This difference between the species in female response to heterospecific mating partners may be indicative of differential costs of interbreeding, and it is possible that the relative strength of barriers to interbreeding differ also. It is possible that CSP acts to strengthen the relatively weak precopulatory barriers observed in *G. bimaculatus*. Traits associated with postmating–prezygotic sexual selection can evolve relatively quickly (Civetta & Singh 1995; Pitnick *et al.* 2003; Andrés *et al.* 2006). These traits may diverge in allopatry and subsequently act to isolate species upon secondary contact. Alternatively these traits may have diverged following isolation owing to other barriers—the current strength of isolating mechanisms does little to inform us of their historical significance in speciation (Schluter 2001; Coyne & Orr 2004). The mechanisms involved in CSP can only act as barriers if a female mates with a conspecific as well as a heterospecific male. Females may have evolved multiple mating to prevent interbreeding, and so promiscuity might, counter intuitively, reinforce isolation and promote speciation. Both *G. bimaculatus* and *G. campestris* are highly polyandrous in the wild. Bretman & Tregenza (2005) found that the mean number of males repre-

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sented in the spermatheca of each female in a Spanish population of *G. bimaculatus* was 4.5, and video observation of a natural population of *G. campestris* (Rodríguez-Muñoz *et al.* 2011) revealed frequent polyandry in that species as well. Therefore, it is likely that in natural populations, a heterospecific ejaculate might compete with multiple conspecific ejaculates, leading to an even stronger precedence than reported here.

Since the introduction of concepts such as sperm competition (Parker 1970) and cryptic female choice (Thornhill 1983; Eberhard 1996), there has been a growing interest in cryptic processes, and the development of molecular techniques has allowed these processes to be more rigorously investigated. Through the use of such techniques, we come closer to understanding which of the many processes involved in insemination, sperm movement and fertilisation govern CSP in *Gryllus*. We suggest that CSP acts at multiple cryptic stages, potentially acting as a strong but not complete barrier to hybridization in this system, with potential to have been involved in the process of speciation.

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All authors contributed to the design of the study. F.T., X.H. and T.V. performed the research, with technical advice from AB. Field work was carried out by F.T., R.R.M., T.V. and T.T. F.T. analysed the data and wrote the paper, with contributions from all other authors.

Data accessibility

The GeneMapper output for the standards, spermathecal and nymph samples, detail of the standard curves and information relating to each triad can be found in Dryad (doi:10.5061/dryad.rd304).

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 Exposure to non-experimental females prior to mating trials.

Data S2 PCR conditions for microsatellite loci.

Data S3 Repeatability of CM-PCR.

Data S4 Detail of statistical analyses and model output.

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