Measuring polyandry in wild populations: a case study using promiscuous crickets

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Abstract

Mating rate has important implications for patterns of sexual selection and sexual conflict and hence for issues such as speciation and the maintenance of genetic diversity. Knowledge of natural mating rates can provide insights into the factors driving female mating behaviour. We investigated the level of polyandry in a Spanish population of the field cricket *Gryllus bimaculatus* using microsatellite markers. Two approaches were employed: (i) genotyping the offspring of wild-caught gravid females to determine the number of males siring the brood and (ii) genotyping sperm stored in the spermathecae of females mated in the wild to estimate the number of mating partners. We compared existing methods for inferring the minimum and probable number of fathers and described a novel probabilistic technique estimating the number of mates by genotyping stored sperm. Using the most conservative allele-counting method, 71% of females produced offspring sired by at least two males (a minimum mean of 2.4 fathers per clutch), and all females had mated to at least two males with minimum mean estimates of 2.7–5.1 mates per female. Our study reveals high levels of polyandry in the wild and suggests that females mate with more males than sire their offspring.

Keywords: cryptic female choice, genotyping sperm, *Gryllus bimaculatus*, mating rate, microsatellites, sperm competition

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Introduction

To understand the evolutionary importance of polyandry (females mating with more than one male) it is necessary not only to establish whether a species is polyandrous or not, but also measure the level of polyandry. The number of males a female typically mates with has implications for gene flow (e.g. Chesser & Baker 1996), speciation (e.g. Parker & Partridge 1998; Gavrilets 2000) and maintenance of genetic variation (e.g. Zeh *et al*. 1997). Varying levels of polyandry can lead to the evolution of complex male behaviours including differential sperm allocation when faced with variable risk and intensity of sperm competition (Parker 1998; Pizzari *et al*. 2003) and alternative mating strategies (Arak 1984). Establishing the proportion of females mating to multiple males within a species can reveal whether there is a mixed mating strategy with some monandrous and some polyandrous females. Determining the degree of polyandry may also shed light on the reasons for observed female mating patterns, because different explanations predict different levels of polyandry. For instance, if polyandry occurs because of the risk of only mating with an infertile male, females are expected to mate more than once, but not to mate many times. Similarly if females only remate if they encounter a male superior to their previous mate (Halliday 1983), a low level of polyandry is likely. Alternatively, if polyandry is driven by direct benefits of matings, such as the acquisition of nuptial gifts, female fitness may increase with each mating so a high degree of polyandry is expected. If females mate repeatedly because of sperm depletion we predict a large number of mates over the female's reproductive lifespan but few sires in any one brood. Finally, the possibility that females exercise mate choice after copulation (Eberhard 1996) could lead to females storing sperm from many mates concurrently but only using a subset as sires for their offspring.

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A further reason to estimate mating rates in the wild is to assess whether experimental designs reflect what happens in nature. Laboratory experiments impose different conditions on the species of interest, but to be relevant they must to some extent represent the study organism's natural behaviour. For example, in the laboratory female *Gryllus bimaculatus* (De Geer) crickets will readily mate with almost any male they encounter. However, in the laboratory the female does not have to spend time looking for mates, is perhaps not given the opportunity to choose and, depending on the experimental mating arena, cannot move away from the male. This does not imply that females do not or cannot refuse to mate in the laboratory, but refusal is rare. In their study of inbreeding depression in *G. bimaculatus*, Tregenza & Wedell (2002) found only 5 out of 114 females that refused to mate. With the cost of mate searching altered and the removal of choice, females may mate more frequently in the laboratory than they would in the wild. When performing experiments, do we use realistic parameter ranges? For example, in Tregenza & Wedell's (1998) study of the benefits of polyandry, one group of females were mated with four males; is this fewer or more males than females would normally mate with in the wild?

Assessing degree of polyandry by directly observing mating in the field is often challenging, particularly for species such as *G. bimaculatus* that are small, nocturnal, and mate in burrows. Even behavioural studies of mating in more easily observed species have been found to be unreliable. Until recently over 90% of bird species were thought to be monogamous (Birkhead 1987), yet the use of genetic techniques to assign paternity has revealed that nearly 60% are polyandrous (Petrie *et al*. 1998). There are now numerous studies of multiple paternity in birds (150 studies are reviewed by Griffith *et al*. 2002), usually from populations where most breeders are sampled and aiming to assign paternity to particular individuals. Studies of invertebrates present different challenges and opportunities. Larger brood sizes increase the upper limit for multiple paternity. Also, species that store sperm (most insects) can be used to compare estimates of number of mates with number of sires. For some invertebrate species female mating rate can be assessed directly, for example in some Lepidoptera remnants of each spermatophore remain within the female reproductive tract throughout her lifetime (Drummond 1984). This method has been used to show variation in degree of polyandry within species (Wedell *et al*. 2002), between populations (Wiklund & Forsberg 1991) and between species (Gage 1994). However, whilst this is a direct measure of number of copulations, each spermatophore does not necessarily equate to a different mate.

A number of molecular methods have been used to assess multiple paternity in the wild: allozymes (Allen *et al*. 1994; Gregory & Howard 1996), karyotyping (Lopez-Leon *et al*. 1995), and more recently microsatellites. The former methods are less informative because of the low power with which they can identify different genotypes. Imhof *et al*. (1998) report that their analysis of multiple paternity in *Drosophila melanogaster* using microsatellites suggests a higher level of multiple paternity than earlier studies in the same species. The level of polyandry in their study was 100% (although the sample size was only 4) compared with 50% in the allozyme study (Ochando *et al*. 1996), though this study had a much larger sample size (92–419, depending on the locus).

Three separate issues are involved when measuring degree of polyandry: what proportion of females mate multiply, how many fathers typically contribute to a brood of offspring and how many males do females mate with. Previous studies of polyandry in insects tend to address one or the other of these issues. The approach typically used is that of genotyping offspring (Allen *et al*. 1994; Lopez-Leon *et al*. 1995; Gregory & Howard 1996; Zeh *et al*. 1997; Boomsma & Van der Have 1998; Harshman & Clark 1998; Imhof *et al*. 1998; Boomsma *et al*. 1999; Bonizzoni *et al*. 2002; Sumner *et al*. 2004), which can reveal the number of males siring a brood but not necessarily the number of males a female mated with. A growing number of studies have genotyped stored sperm to assess the number of mates (Peters *et al*. 1995; Goodnight *et al*. 1996; Gertsch & Fjerdingstad 1997; Chapuisat 1998; Haberl & Tautz 1998; Krieger & Keller 2000; Hammond *et al*. 2001; Fernandez-Escudero *et al*. 2002; Tripet *et al*. 2003), but comparsion of this estimate with the number of sires has only been made in species in which males are haploid and hence show essentially no genetic variation within an ejaculate (Chapuisat 1998; Haberl & Tautz 1998; Hammond *et al*. 2001; Fernandez-Escudero *et al*. 2002). Previous studies have estimated a minimum number of fathers (Zeh *et al*. 1997; Boomsma & Van der Have 1998; Chapuisat 1998; Imhof *et al*. 1998; Boomsma *et al*. 1999; Bonizzoni *et al*. 2002), but only in haplo-diploid insects have population allele frequencies or offspring relatedness been used to infer the probable number of fathers (Boomsma & Van der Have 1998; Chapuisat 1998; Boomsma *et al*. 1999; Fernandez-Escudero *et al*. 2002).

In this study, we use two strategies to measure degree of polyandry in field crickets, and propose a methodology that may be employed in other animals. We genotype offspring of wild-caught gravid females to estimate how many males typically sire the offspring and additionally genotype stored sperm from different but simultaneously captured females to estimate the number of males with whom they have mated. Using both methods goes some way to distinguishing between number of fathers and number of mates, though is limited because sperm from each male may not ultimately be stored by the female. We also compare different methods for inferring number of fathers and mates, both minimum numbers by counting

Locus	T_a (°C)	MgCl$_2$ (mM)	BSA	DMSO	Dilution	Dilution (sperm)
Gbim04	65	1.5	+	+	1:15	–
Gbim06	57	1.0	+	–	1:10	–
Gbim09	62	1.5	+	–	1:8	1:4
Gbim11	65	1.5	+	–	1:6	–
Gbim14	57	1.5	+	–	1:3	1:2
Gbim15	65	1.0	–	+	1:20	1:10

Table 1 PCR conditions and product dilutions

T_a, annealing temperature; BSA, addition of 1 µL 0.5% bovine serum albumin (BSA: Sigma); DMSO, addition of 1 µL 1% dimethyl sulphoxide (DMSO: Sigma); dilution, dilution factor of PCR product.

nonmaternal alleles and methods using population allele frequencies to determine the probable number of fathers and mates. We describe a straightforward new approach to assessing the probable number of males contributing to an allele array in stored sperm.

Materials and methods

Field collection

Crickets were collected in October 2002 from an area 1 km west of Colinas, near Seville, southern Spain. In total 31 males and 35 females were caught and genotyped at six loci, in order to calculate population allele frequencies. Seven females were kept alive and allowed to lay eggs in the laboratory. DNA was extracted from 16 nymphs from each female. A further 24 females were preserved in alcohol so that sperm from their spermathecae could be extracted later. In the laboratory, crickets were kept at 29 °C and given free access to food and water. Moist cotton wool was provided as egg laying substrate. Nymphs were collected 24–48 h after hatching and immediately frozen at −20 °C until DNA extraction.

Microsatellite genotyping

Microsatellite analysis was carried out using loci described by Dawson *et al.* (2003). DNA was extracted from adult hind leg muscles and whole nymphs using a salting-out method (Strassmann *et al.* 1999). Pellets were eluted in 30 µL ddH$_2$O at 4 °C, then run on agarose gels (2 µL DNA in 5 µL 1% orange G, all loaded). Amount of DNA was estimated and dilutions made to approximately 10 ng/µL. Each 10-µL polymerase chain reaction (PCR) contained 0.1–10 ng of genomic DNA, 1 µM of each primer, 0.2 mM of each dNTP and 0.25 unit *Taq* DNA polymerase (Thermo-prime^{Plus}, ABGene) in the manufacturer's buffer (final concentrations 20 mM (NH$_4$)$_2$SO$_4$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween), including 0.6–1.5 mM MgCl$_2$ (see Table 1). PCR amplification was improved by the addition of 0.5% bovine serum albumin, fraction V (BSA: Sigma)

and 1% dimethyl sulphoxide (DMSO: Sigma) (see Table 1), and by reducing the amount of template DNA to 1.0 ng or 0.1 ng. PCR amplification was performed in a Hybaid Touchdown thermal cycler (Thermo Hybaid). The reaction profile was 94 °C for 2 min, then 94 °C for 1 min, T_a °C for 30 s, 72 °C for 30 s for 5 cycles, then 94 °C for 30 s, T_a °C for 30 s, 72 °C for 30 s for 30 cycles, then 72 °C for 3 min, where T_a is the annealing temperature and can be found in Table 1.

PCR products were diluted and multiplexed into two sets: set A *Gbim04*, *Gbim06*, *Gbim11* and *Gbim14*, set B *Gbim09* and *Gbim15*. The size ranges of the loci did not allow all loci to be multiplexed together. Dilutions are shown in Table 1. Gels were run on an ABI Prism 377 Sequencer and analysed using GENESCAN 3.1.2 and GENO-TYPER (Applera PerkinElmer). Failures were re-run with increased MgCl$_2$ and a 2 °C lower annealing temperature. If the reaction still failed, more dilute (1 ng/µL) and neat DNA extractions were run.

Sperm were extracted from the spermathecae using the method described by Tripet *et al.* (2003). DNA was extracted using a modified version of the Chelex extraction of DNA from sperm (Walsh *et al.* 1991). The whole female was rehydrated in ddH$_2$O overnight (approximately 16 h). This made finding and removing the spermatheca easier, and allowed the sperm to be removed from the spermatheca. The sperm coagulates in ethanol and, when rehydrated remains as a ball which can be removed from the spermatheca. However, if rehydrated for too long the sperm will not remain in a ball (Tripet *et al.* 2003). Sperm was digested in 200 µL 5% Chelex with 4 µL proteinase K and 7 µL 1 M dithiolreitol (DTT) at 56 °C for 1–2 h or overnight at 37 °C. Samples were then vortexed for 30 s, centrifuged for 20 s and boiled for 8 min. They were centrifuged for 5 min and the supernatant transferred to a new tube. DNA was precipitated in 500 µL 100% EtOH overnight at −20 °C, then centrifuged for 5 min, the ethanol removed and washed in 70% EtOH. Pellets were dried and eluted in 10 µL ddH$_2$O. As the amount of DNA extracted was inevitably small, extractions were not run on agarose gels to estimate the amount present.

Sperm extractions were run eight times each with three loci (*Gbim09*, *Gbim14*, *Gbim15*), four times under ordinary conditions with a 1:5 dilution of the extraction, and four times with neat DNA under less stringent conditions (increased MgCl$_2$ and a 2 °C lower annealing temperature). Products were not multiplexed, to allow easier scoring, and the products were not diluted as much as for DNA samples from individual nymphs or adult muscle tissue (see Table 1).

Results

Number of fathers

In the genotypes of the offspring (16 offspring per female, seven females), maternal alleles were identified and disregarded, and the remaining alleles counted. Homozygotes were counted as having a father with the same allele as the mother. The number of alleles, from the locus with the most nonmaternal alleles, was divided by two (as each father could potentially donate two different alleles) to give an estimate of the number of fathers present. The number of fathers ranged between 1 and 4 with a mean of 2.8 ± 0.34 (all means are given with their standard error).

Allele counting gives a simplistic and conservative estimate of the number of fathers. To calculate a more realistic estimate using the frequency of alleles in the population, two methods were applied. First, for each female, the mean relatedness of offspring was calculated using MER2 (Wang 2002). This program has low sensitivity to sampling error in estimation of allele frequencies compared with other estimators such as that of Queller & Goodnight (1989) (Blouin 2003). The mean offspring relatedness was then applied to the formula of Ross (1993)

$$r_s = (r_{fs}) \cdot (1/M_e) + (r_{hs}) \cdot [(M_e - 1)/M_e] \qquad \text{(eqn 1)}$$

where r_s is the mean relatedness of offspring, r_{fs} is the mean relatedness of full siblings, r_{hs} is the mean relatedness of half siblings and M_e is the effective number of mates. For a diploid system, $r_{fs} = 0.5$ and $r_{hs} = 0.25$. Hence the effective number of mates can be found from

$$M_e = (0.5 - 0.25)/(r_s - 0.25) \qquad \text{(eqn 2)}$$

By this method, mean effective number of mates across all seven broods is 2.03 fathers. After the removal of potentially spurious alleles (discussed later) this reduces to 1.79 fathers. This method assumes zero inbreeding, equal male mating rates and that all males contribute equally to paternity within a brood

The program PARENTAGE (Emery *et al.* 2001) was used to infer the probable number of fathers using population allele frequencies (available from www.maths.abdn.ac.uk/

~ijw). This program simulates the likely number of fathers for a given set of offspring using initial probabilities derived from what is already known about the parentage of the offspring and the population from which they are drawn. Each known mother's genotype is specified (though the program can deal with a range of possible mothers or unknown maternity) and the maternal alleles identified in the offspring. Initial probabilities are set for the number of possible fathers represented in the brood (in this case 1–16), the share of paternity between fathers and the population allele frequencies (in this study from the genotypes of 66 adults). The probability that two offspring share a father is then determined. This is applied across all offspring in the sample, such that further offspring either share the same father as previous offspring or add a new father, until the number of males contributing to the clutch is determined. This is repeated for a specified number of iterations (a sufficiently large number so that further iterations do not change the outcome) and the most likely number of fathers (the most commonly returned number of fathers) and the confidence in this estimate are determined.

Although in this instance it was impossible that the offspring had actually come from a female other than that assigned as the mother, the statistical properties of the model are improved if an initial probability of maternity share (α) is set. Consequently we set the maternity share such that there was a very low probability that there was more than one mother (a gamma distribution with shape parameter 1 and mean 0.25). As 16 offspring were genotyped, potentially each could have a different father and hence the range of number of fathers was set as 1–16. A further parameter allows the share of paternity between males to be estimated (the paternity share β). This enables an allowance to be made for either strong first or last male sperm precedence, or for the possibility that the number of offspring sampled is not sufficiently larger than the number of potential fathers so be confident that all actual sires will be represented in the sample of offspring genotyped. There is a limit to the number of males a female can mate with, but the number of offspring sampled is low compared to the total number of offspring so that if indeed there were 16 sires of the brood, a sample of 16 offspring would make it unlikely that all fathers are sampled. Therefore, we set the paternity share using a probability distribution that meant that fewer fathers were more likely (a gamma distribution with shape parameter 1 and mean 0.005). Nevertheless, because we did not know how many fathers were likely to be found, the effect of changing the paternity share was tested. Runs where a higher number of fathers (a gamma distribution mean of 0.02) and a lower number of fathers (a gamma distribution mean of 0.002) were more likely were tried but did not change the outcome. As the results were robust to changes in paternity share, the initial distribution was used for all seven clutches.

Female	Mean	SE	Minimum	Maximum	Mode	*P* (mode)†	Range‡
1	4.01	0.001	3	5	4	0.989	4
2	8.29	0.012	6	11	8	0.434	7–10
3	1	0.000	1	1	1	1.0	1
4	3.04	0.003	3	5	3	0.963	3
5	1	0.00	1	1	1	1.0	1
6	3.83	0.006	2	5	4	0.808	3–4
7	3.99	0.002	3	5	4	0.98	4

Table 2 Summary of results for number of fathers inferred by PARENTAGE, after the removal of potentially spurious genotypes (details in text)

SE, standard error of the mean. †Observed proportion of samples with the modal number of fathers from 5000 simulations. ‡Range of number of fathers that includes at least 95% of simulations.

A mutation rate (μ) was also set (a gamma distribution with a shape parameter of 2 and a mean 0.001), so that 95% of the mutation rates lie between 0.00014 and 0.0028 mutations per generation, which is in agreement with observed mutation rates for microsatellite markers (Weber & Wong 1993). The output highlights genotypes at which it is more likely that a mutation occurred in the individual offspring rather than adding a further father. In practice, a mutation probably indicates a miss scored band and it would be preferable to re-amplify the individual for that locus. Instead, where more than 50% of simulations resulted in a mutation, the genotype was removed and coded as missing data. This affected about 10% of the all the alleles scored (mean number of alleles per female across six loci = 21.14 ± 3.43, mean number removed per female across six loci = 2.39 ± 0.52). The allele-counting method was also repeated with the spurious bands removed, the mean number of fathers becoming 2.4 (± 0.43).

Runs for all seven clutches contained 5000 iterations (with a burn-in of 5000 iterations and a thinning interval of 400). Table 2 summarizes the results of PARENTAGE analysis for each clutch. The range of modal values is 1–8 fathers per clutch (mean 3.8 ± 0.90 fathers per cutch). For six of the clutches, 95% of simulations result in one or two estimates of number of fathers. However, for the clutch with the greatest number of fathers (female 2, mode 8), the outcome is less certain and 7–10 fathers must be included to achieve 95% of simulations.

A further method for inferring the number of fathers was employed using the program GERUD (Jones 2001; executables for GERUD and GERUDSIM can be downloaded from www.biology.gatech.edu/professors/labsites/joneslab/parentage.html). This is a more sophisticated approach than allele counting, as it uses multiple loci simultaneously, but does not use population allele frequencies or relatedness of offspring and so gives a minimum number of fathers rather than a probable number. The program removes maternal alleles from the offspring genotypes then simulates all possible paternal genotypes. It then calculates combinations of these genotypes that require the fewest individual males
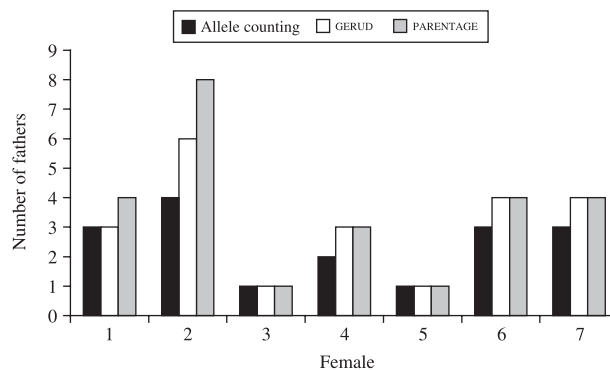

Fig. 1 Estimated number of fathers from the allele counting, GERUD and PARENTAGE methods. For the allele count, the estimate is derived from the locus with the greatest number of nonmaternal alleles. For the GERUD estimate only data from loci *Gbim06*, *Gbim14* and *Gbim15* have been used. The genotypes identified as potentially spurious by PARENTAGE have been removed from all estimates. The PARENTAGE estimate is the modal number of fathers from 5000 simulations (details in Table 2).

resulting in the observed offspring genotypes. As the problem of possible misscored alleles had been highlighted by PARENTAGE, these alleles were removed. However, because GERUD has no facility for dealing with missing data, this required the removal of individual offspring entirely (mean number of offspring per clutch = 13.86 ± 0.67). As computing time required increases exponentially with number of loci, only data from the three most polymorphic loci were used. GERUD gives a range of 1–6 fathers per clutch (mean = 3.14 ± 0.67, Fig. 1). However, the estimate of 6 fathers for the clutch of female 2 must be treated with caution because this is the maximum number of fathers that GERUD allows. The results of the three methods of allele counting, PARENTAGE and GERUD are presented in Fig. 1.

GERUD's sister program, GERUDSIM (Jones 2001) allows the estimation of the power of a set of loci to detect multiple paternity for a given sample of offspring genotyped. We

used this program to assess whether a female whose offspring actually had two fathers could have been mistakenly assigned only one father. Using the population allele frequencies GERUDSIM simulates sets of offspring genotypes, draws a sample of offspring and then estimates the number of sires contributing to the sample. The number of offspring in the subsample must be specified, as must the actual number of fathers as GERUD would. Only the three most polymorphic loci were used (as for GERUD above). Parameters were set as 16 offspring sampled (as in our methodology) and two sires equally contributing to the offspring as would be assumed if there is sperm mixing as suggested for *Gryllus bimaculatus* (see Simmons 1987a; Morrow & Gage 2001). In three independent runs of 5000 simulations two sires were detected in 100% of simulations. Hence, it is unlikely that multiple paternity was mistaken as single paternity.

Number of mates

Sperm samples were far more difficult to score than individual genotypes because of the number of alleles present. All bands with a microsatellite profile were scored. From this, three band scores were taken: (i) all alleles scored in at least one of the eight replicate reactions that we ran of the sperm sample from each female, (ii) the number of alleles that appeared in at least 50% of reactions, and (iii) of those that appeared in at least 50% of reactions, the number that appeared at least once without another band one repeat unit (2 bp) either side of them. Hence, there were three independent estimates of number of mates, from the three loci, and for each locus there were three band scores that had a low, moderate and high level of stringency. Again, the number of alleles was converted into number of mates by dividing by two. The mean number of mates at each locus for all three band scores are shown in Fig. 2. The minimum estimate from the high stringency band score comes from locus 2 with a mean of 1.9 ($\pm$ 0.18) mates, and the highest with the low stringency band score is locus 3 with a mean of 4.5 ($\pm$ 0.22) mates. Under the most conservative estimate all females have sperm from more than one male in their spermathecae, therefore 100% of females had mated with multiple males. Contamination by maternal DNA is unlikely because no array contained the female's alleles at all three loci.

As with the number of fathers, counting the number of alleles present gives a simplistic estimate of number of mates. Another estimate was made using the allele frequencies in the population to determine the probability of observing an array of alleles if a different number of males contributed to the sample. The probability of not observing an allele is

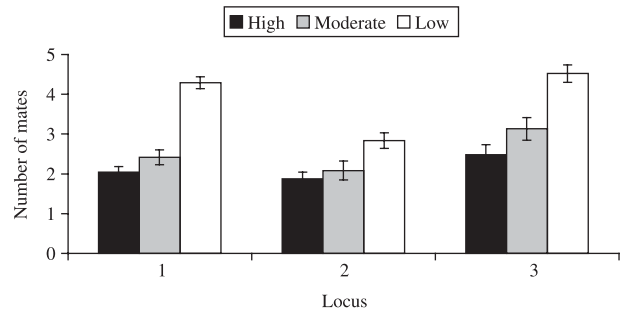$$P_{(not)} = [1 - f(a)]^t \qquad \text{(eqn 3)}$$

Fig. 2 Mean ($\pm$ 1 SE) number of mates as inferred from genotyping of sperm from the spermatheca, by dividing the number of alleles by two. There are three independent estimates from three loci (1) *Gbim09* (2) *Gbim14* and (3) *Gbim15*. As genotyping samples with many alleles is problematic, three estimates are made with low (all probable alleles), moderate (alleles that appeared in at least 50% of reactions, that is 4 out of 8 reactions), and high stringency [of those that appeared in 50% of reactions, the number that appeared at least once without a band one repeat unit (2 bp) either side].

where $f(a)$ is the allele frequency and t is the number of trials, or attempts at observing the allele, which is twice the number of males sampled. Hence the probability of observing an allele is

$$P_{(obs)} = 1 - P_{(not)} \qquad \text{(eqn 4)}$$

The probability of obtaining the observed array of alleles is the product of the $P_{(obs)}$ for alleles in the array and $P_{(not)}$ for those alleles in the population that are not observed in the array. This figure was calculated for $t = 2$ to 70, representing 1 to 35 males (a number thought to be sufficiently high to exceed the maximum number of males a female could have mated with or could be detected). The number of trials with the highest probability indicates the most likely number of males represented in the array. This process was repeated for all three band-scoring methods at all three loci for all females. The mean number of mates for the most conservative band score is 3.0 ($\pm$ 0.18), for the moderately conservative band score is 4.2 ($\pm$ 0.2), and when including all probable bands is 10.0 ($\pm$ 0.83) (Fig. 3). This is derived from the most polymorphic locus estimate for each individual, which is not necessarily the same locus for all individuals.

Comparison of number of fathers and number of mates

The estimates of number of mates and number of fathers were compared, using allele counting from the locus indicating the greatest number of fathers or mates, which is not necessarily the same locus for each individual. As Fig. 4 illustrates, there is evidence that females mate with more males than father their offspring. There is no significant
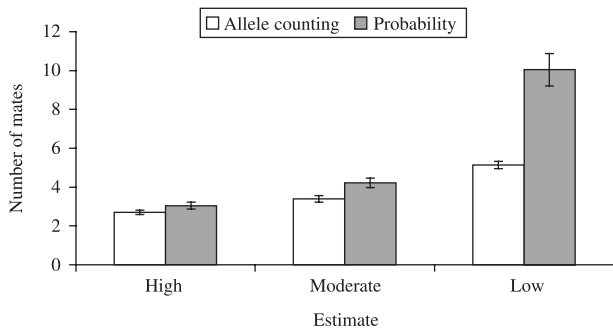
Fig. 3 Mean (± 1 SE) number of mates from the allele-counting and probability methods, for the three band scores with high, moderate and low levels of stringency. This was calculated from the locus with the greatest number of alleles, and so the locus may vary between females and between estimates.
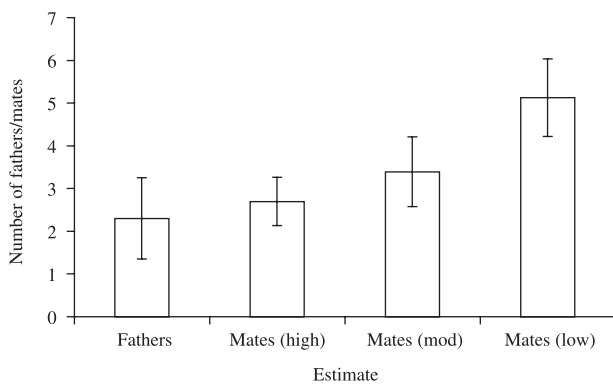

Fig. 4 Number of mates inferred by the two methods, genotyping offspring (fathers) and genotyping sperm (mates with high, moderate and low levels of stringency) (mean ± 1 SE). Both estimates are from the locus giving the highest number of mates, which is not necessarily the same locus for each female. There is no significant difference between the number of fathers and the most conservative estimate of number of mates. However, there is a significant difference between number of fathers and number of mates using the moderately conservative band scores and all probable bands.

difference between the number of fathers and the most conservative estimate of number of mates (Mann–Whitney $U = 57.5$, $N = 31$, $P = 0.165$). However, there is a significant difference between number of fathers and number of mates using the moderately conservative band scores (Mann–Whitney $U = 34.5$, $N = 31$, $P = 0.014$) and all probable bands (Mann–Whitney $U = 4.0$, $N = 31$, $P < 0.001$). After Bonferroni corrections, comparisons with both band scores remain significant.

Discussion

This study provides the first evidence that *Gryllus bimaculatus* are highly polyandrous in the wild. Females

store sperm from different males and have offspring sired by different males. The estimates of the frequency of polyandry, or the proportion of females mating with at least two males, were 71% from the number of fathers method and 100% from the number of mates method. Other studies of insect species using microsatellites have found 100% of females mating to more than one male in leaf-cutting ants *Acromyrmex echinatior* (Sumner *et al.* 2004), army ants *Anomma* sp. (Kronauer *et al.* 2004) and *Eciton burchellii* (Denny *et al.* 2004), harvester ants *Pogonomyrmex badius* (Rheindt *et al.* 2004) and Himalayan giant honeybees *Apis laboriosa* (Paar *et al.* 2004); approximately 60–80% polyandrous females in *Drosophila melanogaster* (Harshman & Clark 1998), *D. buzzatii* (Bundgaard *et al.* 2004), and the harlequin beetle riding pseudoscorpion *Cordylochernes scorpioides* (Zeh *et al.* 1997), and 20–30% for the wood ant *Formica paralugubris* (Chapuisat 1998), *Bombus* sp. bumble bees (Payne *et al.* 2003) and the leaf-cutting ant *Acromyrmex insinuator* (Sumner *et al.* 2004).

Genotyping offspring

Using data from genotyping offspring, we have employed four methods to infer the number of fathers contributing to these broods. The most simplistic method is allele counting, which is conservative both because a homozygote father will only donate one unique allele to the offspring, and because different males may carry the same allele, particularly if they are related. Data from several loci at once can be combined as implemented in the program GERUD. This approach is more powerful than counting alleles, but at present is limited by the computational demands of deriving estimates from large numbers of loci and by GERUD being limited to a maximum of six fathers and not allowing missing data.

Population allele frequencies can be incorporated into our estimates by using the mean relatedness of half-sibs (Ross 1993), and by using the program PARENTAGE (Emery *et al.* 2001). The former method gives the mean effective number of fathers per female across the population, and has been used in a number of studies (Boomsma & Van der Have 1998; Chapuisat 1998; Boomsma *et al.* 1999; Zane *et al.* 1999; Fernandez-Escudero *et al.* 2002). However, it is not suited to comparing differences between females within a population, and assumes zero inbreeding, equal male mating rates and that all males contribute equally to paternity within a brood. It seems likely that all of these assumptions may be violated, given that in our study, this method returns a lower average than the minimum mean number of fathers derived from allele counting. The mean number of fathers per clutch derived from PARENTAGE provides the highest estimate of number of fathers. This is the most sophisticated approach, incorporating population allele frequencies and providing confidence limits. It assumes

that all males in the population have an equal probability of fathering a given offspring, and that there is no inbreeding. If inbreeding occurs, the degree of polyandry will be underestimated, as individual genotypes will be more similar than would be expected if investigated by pedigree and hence many males could be providing similar genotypes to the offspring.

Our estimate of the power of the three most polymorphic loci to detect multiple mating calculated using the program GERUDSIM (Jones 2001), indicates that it is highly unlikely that a multiply mated female would have been misclassified as singly mated and errors of nondetection of fathers will be very rare. Nevertheless, it is likely that the percentage of females found to be polyandrous (71%) is an underestimate of wild populations. We only genotyped 16 offspring, a small fraction of the potential fecundity of a female *G. bimaculatus* which can be as high as 4000 eggs (R. Rodríguez-Muñoz, unpublished). Nonsampling error is therefore possible and may cause the number of fathers to be underestimated, although this problem will be slight when the number of offspring sampled is substantially larger than the number of mates. A second issue that could lead to an underestimate of the degree of polyandry is the potential for sperm precedence or sperm clumping (Harvey & Parker 2000). Imhof *et al.* (1998) genotyped the offspring of four female *D. melanogaster*, all of which had offspring sired by 4 to 6 males. In two females, all paternal genotypes were detected within the first 24 h of laying, in the other two, new genotypes were detected over the subsequent oviposition period. We only genotyped nymphs from the first few days of oviposition, and hence if sperm tended to be stratified in the spermatheca or displaced by each subsequent mating, the first eggs to be laid might tend to be fertilized by sperm from the first or last male to mate, reducing the overall estimate for number of sires. However, two independent studies have found approximately equal paternity of first and second males in *G. bimaculatus*, and no evidence for clumping (such as a bimodal P2 distribution) suggesting that sperm mix in the spermatheca (Simmons 1987b; Morrow & Gage 2001).

Genotyping mixed samples of sperm

We obtained a more accurate measure of how many males females mate with by genotyping sperm stored in the spermathecae of females than by genotyping offspring. This method assumes that females store sperm from all the males with which they have mated, which may not be the case and will therefore be a conservative estimate of actual number of copulations. Nevertheless, estimating number of mates by genotyping sperm from the spermatheca is an improvement on the method of inferring number of mates from the number of fathers of a brood of offspring. This method will suffer in the same way as the genotyping of

offspring if the population is inbred. Less related males will contribute similar sperm with similar genotypes, and hence the number of mates could be underestimated.

Genotyping mixed samples of sperm is undeniably challenging. There is a small amount of DNA to work with, limiting scope for re-runs and increasing the chance of allelic dropout caused by alleles being present in low copy numbers (Walsh *et al.* 1992). An attempt was made to overcome this problem by amplifying eight subsamples of each DNA extraction to limit PCR sampling artefacts. Nevertheless, the possibility remains that alleles with high copy number in the PCR could have swamped alleles represented by a few copies. The stochasticity of PCR is illustrated by the difference between the estimates scoring all probable alleles and only those alleles that appeared in 50% of reactions. From the most polymorphic locus a mean of 5.13 (± 0.19) males is given by counting all bands, but this is reduced to 3.39 (± 0.17) males from bands appearing in 50% of reactions.

Another potential source of error comes from stutter bands one or more repeat units shorter (or more rarely longer) than the real band caused by DNA slippage during PCR amplification (Goldstein & Schlotterer 1999). When running DNA from one individual this is not usually a problem, as the stutter bands are fainter than the real band. However, when samples from different individuals are run together, stutter from one band can disguise another or combine to appear to be a real band. We reduced errors of this type by only scoring bands that had already been scored in the 66 adults run singly and by making a more conservative estimate by only counting bands that appeared at least once without a band 2 bp either side. Using microsatellite loci of tri- or tetranucleotide repeats would cut down this error, as longer repeats are less prone to stutter. Alternatively, information of which of our estimates is the most accurate could be provided by genotyping sperm from females mated to known numbers of males, and manipulating sperm contributions through varying spermatophore attachment time.

Previous studies that have genotyped sperm from spermathecae either simply state that alleles from at least two males are observed (Tripet *et al.* 2003), or are often from species in which females typically mate with either one or two males (Peters *et al.* 1995; Chapuisat 1998; Krieger & Keller 2000; Hammond *et al.* 2001; Tay & Crozier 2001; Fernandez-Escudero *et al.* 2002) Indeed, in the opinion of Chapuisat (1998), this method is only useful for distinguishing between singly and doubly mated females. We have shown that although this method is not without its difficulties, it can provide an estimate of number of mates, even when there are a large number of alleles in the array. Also, rather than simply counting the number of alleles present, we suggest a technique for inferring the probable number of mates from population allele frequencies. Haberl

& Tautz (1998) found evidence for seven mates in the honeybee *Apis mellifera*, although they only use this data to ask whether additional alleles are present in the sperm that are not present in the offspring. It is more straightforward to genotype the sperm of haplo-diploid species such as honey bees as each male typically only contributes one allele to the array. Using this method for diploid species is more complicated but can be informative.

Comparison of number of fathers and number of mates

The distinction between number of mates and number of fathers is sometimes not made in the literature (e.g. Allen *et al.* 1994; Lopez-Leon *et al.* 1995; Gregory & Howard 1996; Harshman & Clark 1998; Bonizzoni *et al.* 2002), yet is an important issue. If females can bias paternity between males from whom they have already received sperm, or if sperm competition results in a similar bias, we expect the number of mates to exceed the number of fathers.

We found that all females had sperm from more than one male, but only 71% of females had more than one father siring their offspring. When comparing the number of fathers and number of mates statistically, there is some evidence that females may mate with more males than are used to father their offspring, which is necessary if there is to be postcopulatory female choice. However, there is also the possibility that offspring of genetically less compatible or lower quality males do not hatch and so were not genotyped. Genotyping eggs rather than hatchlings may distinguish between these hypotheses. As the females that were allowed to lay eggs were different to those used to genotype sperm, our evidence that females mate with more males than are represented in their offspring is indirect, though it is difficult to envisage how the observed pattern could otherwise be achieved.

Polyandry creates the opportunity for differences in male fertilization success to occur postmating (Parker 1970; Eberhard 1996). Females may choose to preferentially store or dump sperm from particular males, or alternatively they could store sperm from all their mates but preferentially use sperm from particular males to fertilize their eggs. There may also be sperm–egg interactions such that certain sperm phenotypes are more likely to fertilize eggs. Our findings suggest that sperm is stored from more males than sire a female's offspring, a necessary condition for females to exercise choice amongst stored sperm, and for paternity biases due to sperm–egg interactions. Our method of assessing the number of alleles present in the sperm in the spermatheca does not allow us to estimate copy number, and hence how much of each males' sperm is stored. If females can vary the amount of sperm stored, then sperm from undesirable males may be present in the spermatheca but in low numbers, making them unlikely to be used in fertilization. It may be that by imposing criteria

on the scoring of bands from the sperm, those present in low numbers are removed from the analysis, making the estimate of number of mates much more similar to the number of fathers. Additionally, males may differentially allocate sperm to different females (Parker 1998), and so be represented by fewer sperm in the spermatheca and be less likely to sire offspring. Quantitative PCR could be employed to assess the relative amounts of sperm of different genotypes present. Evidence that females may exert some postcopulatory choice was found in a previous study (Bretman *et al.* 2004), where previously mated females appeared to discriminate against sperm from their brothers. This study is consistent with the hypothesis that females can employ postcopulatory choice to use sperm from particular males, but further work is needed to test this rigorously.

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Amanda Bretman has recently completed her PhD in sexual selection, focusing on the evolution of polyandry. Tom Tregenza is a Royal Society Fellow studying sexual selection and conflicts of interest between the sexes using insects as model systems. This study is part of an ongoing project into sexual selection and the benefits of polyandry in the cricket *G. bimaculatus*.