ISSN 0962-1083

VOLUME 17
NUMBER 13
JULY
2008

# MOLECULAR ECOLOGY

Published by
Wiley-Blackwell

Molecular Ecology (2008) **17**, 3189–3197 doi: 10.1111/j.1365-294X.2008.03812.x

# Female multiple mating in wild and laboratory populations of the two-spot ladybird, *Adalia bipunctata*

PENELOPE R. HADDRILL,*‡ DAVID M. SHUKER,‡ WILLIAM AMOS,† MICHAEL E. N. MAJERUS* and SEAN MAYES*§

*Department of Genetics, †Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EH–3EJ, UK, ‡Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Ashworth Laboratories, King's Buildings, Edinburgh EH9 3JT, UK, §Division of Agricultural and Environmental Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, LE12 5RD, UK

**Abstract**

**Female mating rate is an important variable for understanding the role of females in the evolution of mating systems. Polyandry influences patterns of sexual selection and has implications for sexual conflict over mating, as well as for wider issues such as patterns of gene flow and levels of genetic diversity. Despite this, remarkably few studies of insects have provided detailed estimates of polyandry in the wild. Here we combine behavioural and molecular genetic data to assess female mating frequency in wild populations of the two-spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae). We also explore patterns of sperm use in a controlled laboratory environment to examine how sperm from multiple males is used over time by females, to link mating with fertilization. We confirm that females are highly polyandrous in the wild, both in terms of population mating rates (~20% of the population found *in copula* at any given time) and the number of males siring offspring in a single clutch (three to four males, on average). These patterns are consistent across two study populations. Patterns of sperm use in the laboratory show that the number of mates does not exceed the number of fathers, suggesting that females have little post-copulatory influence on paternity. Instead, longer copulations result in higher paternity for males, probably due to the transfer of larger numbers of sperm in multiple spermatophores. Our results emphasize the importance of combining field and laboratory data to explore mating rates in the wild.**

*Keywords*: ladybird, microsatellites, paternity analysis, polyandry, sexual conflict, sperm competition.

*Received 24 January 2008; revision received 29 March 2008; accepted 18 April 2008*

## Introduction

Insects are some of the best-studied organisms in terms of their mating systems and patterns of mating behaviour, including their behaviour in the wild (Thornhill & Alcock 1983; Eberhard 1996; Choe & Crespi 1997; Simmons 2001). As such, insects have played an important role in developing and testing ideas about sexual selection and sexual conflict (Arnqvist & Rowe 2005). One aspect of reproduction that has received a lot of attention in both insects and other taxa has been the number of times females mate, and whether those matings are with the same or different males (i.e. multiple mating and true polyandry). The level of polyandry is an important variable for understanding the role of females in mating competition, including postmating, prefertilization competition (Eberhard 1996), and in part defines the possible sexual conflicts of interest with males over mating (Arnqvist & Rowe 2005; Chapman 2006). It will also influence, for example, patterns of gene flow (Chesser & Baker 1996), levels of genetic diversity (Zeh *et al*. 1997), and has important implications for the effective population size ($N_e$) of the X chromosome vs. the autosomes, the ratio of which will depart from expectations if there is greater variance in reproductive success in males than in females (Charlesworth 2001).

Although females of many species are known to mate multiple times in the laboratory environment, quantitative

Correspondence: Penelope R. Haddrill, Fax: (+44) 131 650 6564; E-mail: p.haddrill@ed.ac.uk

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

estimates of polyandry are still relatively uncommon from natural populations. Without the benefits of long-term data sets from marked individuals that are increasingly available to vertebrate ecologists, studying insect mating behaviour in the wild may be logistically challenging (e.g. following specific individuals), or have to rely on indirect methods (as in, for example, seaweed flies; Blyth & Gilburn 2007). As such, field observations may give unreliable estimates of the number of females mating multiply, as has been seen in studies of wild bird populations (reviewed in Griffith *et al*. 2002). However, quantitative estimates of the level of polyandry are needed if we are to draw more concrete conclusions from patterns of mating in the laboratory, and also design more realistic and relevant experiments. This is particularly important because experimental design can influence the pattern of costs and benefits of female multiple mating in the laboratory (Arnqvist & Nilsson 2000).

With the increasing availability of molecular markers have come a number of studies looking for multiple paternity in the offspring of wild-caught females. The majority of these studies have focused on social insects, perhaps unsurprisingly given the importance of female multiple mating for patterns of intracolony relatedness and the resulting impact on social evolution (e.g. Evans 1993; Estoup *et al*. 1995; Goodnight *et al*. 1996; Boomsma & Van der Have 1998; Chapuisat 1998; Sumner *et al*. 2004). Female multiple mating in the wild has also been investigated in some economically and medically important species (Bonizzoni *et al*. 2002; Tripet *et al*. 2003; Song *et al*. 2007), since female multiple mating is likely to compromise efforts to control pest species through the sterile insect technique (Kraaijeveld & Chapman 2004). A number of studies have also examined levels of multiple paternity in wild-caught *Drosophila*, and all found that females produced offspring sired by more than one male (Harshman & Clark 1998; Imhof *et al*. 1998; Bundgaard *et al*. 2004; Schlötterer *et al*. 2005; Good *et al*. 2006).

Recently, in a study of wild-caught *Gryllus bimaculatus* crickets, Bretman & Tregenza (2005) expanded on the above studies in two ways. As well as estimating the number of males to sire the offspring of seven wild-caught females using microsatellite analysis, they also genotyped sperm stored in the spermathecae of 24 additional wild-caught females to estimate the number of males a female had mated with (assuming sperm from all mates is stored). This allowed them to draw some conclusions about whether females are exercising any postcopulatory/prefertilization choice over which males sire their offspring. As well as using a straightforward allele-counting method to get a minimum estimate of the number of males siring offspring (the number of male alleles divided by 2 gives a minimum number of male contributions), they also used methods that took into account allele frequencies in the population from which the females were collected, to give a more reliable estimate (Emery *et al*. 2001; Jones 2001; Bretman & Tregenza 2005). Similarly, Song *et al*. (2007) used population allele frequencies to assess the reliability of their estimates of the number of fathers siring the offspring of 22 females in a natural population of the wild tobacco fly, *Bactrocera cacuminata*.

Here we examine patterns of female mating in the wild in the two-spot ladybird, *Adalia bipunctata*. In the laboratory, female two-spots readily re-mate, both with the same male and with different males, throughout their lives (Majerus 1994a; Ransford 1997; Haddrill *et al*. 2007). Recent work in the laboratory has also suggested that there may be an optimum number of matings for females in terms of the hatchability of their eggs (mating with around five to six different males; Haddrill *et al*. 2007). Much less is known about mating in the wild, however. Using observations of mating individuals in the field, Brakefield (1984) estimated that, on average, 23.5% of all adults were mating at any one time, with a maximum of around 44%. Again using behavioural observations from a wild Polish population, Webberley *et al*. (2006) estimated mating rates of between 0.075 and 0.75 matings per individual per day across a period of 10 weeks. These estimates indicate a high frequency of polyandry, but they do not consider how many of these matings result in fertilized eggs, or whether postcopulatory selection reduces the number of males per female that contribute genes to the next generation.

To address this, we combine three approaches to consider the extent and consequences of female multiple mating in *A. bipunctata*. First, we estimate female mating frequency in the wild by behavioural observations. This provides a link between previous estimates of mating frequency and our next two approaches. Second, we estimate the number of males that successfully father a female's offspring in the wild by using microsatellite markers to genotype offspring. By sampling two clutches, we are also able to examine whether there are any changes in patterns of paternity over time. Third, in the laboratory, we explore patterns of sperm use in multiply-mated females to examine how sperm is used over time, in order to link mating with fertilization.

## Materials and methods

### *Direct observation of mating rate in the wild*

Observations were carried out along a 750-m fixed transect around Fen Causeway in Cambridge, UK. The site was predominantly grassland, with hedge parsley (*Torilis arvensis*) and stinging nettles (*Urtica dioica*) being the other main vegetation present. The transect was walked by P.R.H. a total of 21 times over a period of 4 weeks and the number of single and mating two-spot ladybirds observed was recorded, and their elytral spot/colour-pattern phenotype noted, along with the time of day. Transects

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

were carried out in the morning (0830–1230 h) or in the afternoon (1230–1630 h). Two additional transects were also carried out; one very early in the morning (0525–0705 h) and one late in the evening (1930–2100 h).

### *Patterns of paternity in the wild*

A large sample of wild two-spot ladybirds was collected from Coe Fen in Cambridge, UK and from the banks of the River Seine in central Paris, France in July 2000. Individuals were brought into the laboratory, sexed and separated into single sex groups. Ten females were randomly selected from each of the samples and were housed individually in 9-cm Petri dishes and fed excess pea aphids (*Acyrthosiphon pisum*). If the last male to mate with a female was available (if the pair were collected *in copula*), he was also housed individually. Eggs were collected daily and larvae from the first and fourth clutches laid by a female were raised to at least the fourth instar stage and were placed individually in 1.5 mL Eppendorf tubes, killed by submergence in liquid nitrogen and stored at –70 °C for molecular analysis. No larvae died before reaching this stage. Genomic DNA was extracted from 512 offspring, 20 experimental females and seven males using methods modified from Sambrook *et al*. (1989) and Vogler & Desalle (1993). All individuals were genotyped for three microsatellites (Ab11, Ab32 and Ab35) using methods described previously (Haddrill *et al*. 2002). These microsatellite loci are highly variable; within the sample of wild parents and offspring genotyped, we observed 20, 10 and 14 alleles in Cambridge and 20, 9 and 16 alleles in Paris for locus Ab11, Ab32 and Ab35, respectively.

For each offspring at each locus, the maternal allele was identified and excluded. The number of paternal alleles present in each clutch was then determined for each locus separately and the minimum number of male contributions was calculated, based on all individuals being heterozygous. The highest value across the three loci was taken as the number of males siring offspring in each clutch, although it was sometimes possible to increase the minimum number of males by combining data across two or more loci, or if the last male to mate with the female was known to be homozygous.

This method is likely to be an underestimate of the number of males contributing to each clutch, since it is based on all individuals being heterozygous and does not take into account the frequencies of different alleles in the populations. We therefore also used the program DADSHARE (http://www.zoo.cam.ac.uk/zoostaff/amos/#Computer%-20Programs) to calculate a more accurate estimate by utilizing data on allele frequencies at the three microsatellite loci in a larger sample of two-spot ladybirds from each of the two populations (Haddrill *et al*. 2002). DADSHARE provides a method for assessing the number of paternal contributions to a group of offspring with known mothers. The program begins by removing maternal alleles and inferring the paternal alleles for all offspring genotypes, and then uses these to construct a similarity matrix, and thence a tree based on paternal similarity. In general, the smaller the number of fathers involved, the higher will be the average relatedness between individuals on adjacent terminal branches. To assess the meaning of the observed value for average relatedness, the program conducts extensive Monte Carlo simulations, based on a range of conditions from one male fathering all offspring through to each offspring having a different father. The observed value is then interpreted in terms of the effective number of fathers, i.e. the number of equally successful males who would generate a similar level of sibling relatedness, by reference to the simulated data. Although DADSHARE takes into account how likely it is for an individual male to be homozygous/heterozygous at any individual locus, based on allele frequencies in the population, this method may still be an underestimate, since it is not possible to distinguish between two fathers with identical genotypes. However, in the large sample ($n$ = 76) screened in Haddrill *et al*. (2002), only two individuals were genotypically indistinguishable using these three microsatellite loci.

### *Patterns of sperm usage in the laboratory*

A sample of 10 virgin males and 10 virgin females was taken from a laboratory stock (Cambridge origin) of two-spot ladybirds, housed individually in 9 cm Petri dishes and fed excess pea aphids for seven days to ensure reproductive maturity. All males were mated once to nonexperimental females from the same laboratory stock, to control for any difference in sperm allocation between a male's first and subsequent matings (Ransford 1997). The following day, four females and six males were randomly selected for mating in experimental crosses. Each female was mated daily to one of the males in a randomised block design, so that each female mated with each male, in a different order. Male two-spot ladybirds produce spermatophores, with one spermatophore being passed to the female per 'mating cycle' (terminology as in Ransford 1997). A single copulation may comprise up to three cycles of mating, i.e. up to three spermatophores may be passed to the female per copulation. Male genitalia are not disengaged between cycles, and the male and female remain *in copula* throughout. A single mating cycle can be identified by distinctive male behaviours during the cycle, and a single copulation is defined as beginning when the male genitalia are engaged, and ending when they are disengaged (Ransford 1997). The female therefore seems to have little control over the number of cycles of mating the male carries out, although rejection-like behaviour before mating begins suggests females may have some control over whether mating

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

**Table 1** Relative representation of elytral colour-pattern phenotypes in the Cambridge population

| Phenotype | Population sample* | Mating pairs† |
|---|---|---|
| *typica* | 3751 (92.5) | 801 (92.5) |
| Weak *annulata* | 235 (5.8) | 48 (5.5) |
| Intermediate *annulata* | 31 (0.8) | 4 (0.5) |
| Melanic‡ | 28 (0.7) | 9 (1.0) |
| Others§ | 12 (0.3) | 4 (0.5) |
| Total | 4057 | 866 |

*Population sample, number observed in the total population (percentage). †Mating pairs, number observed in mating pairs (percentage). ‡Melanic includes *sexpustulatus* and *quadrimaculata* forms. §Others include bar *annulata*, zigzag spotty, *duodecempustulata* and some recombinant *annulata* forms.

occurs in the first place (Majerus 1994b). The number of cycles of mating and the time spent *in copula* were recorded for every mating. One male died before mating with one of the females, so was replaced by another male.

After each mating, males and females were separated into individual clean 9 cm Petri dishes and fed excess pea aphids. All of the eggs laid between each mating were collected for each female, as well as one further clutch, 48 h after the final mating. Larvae were all reared to at least the fourth instar stage and were prepared for molecular analysis as above. Once all matings were completed, males and females were weighed and their pronotum widths measured before being prepared for molecular analysis. The 11 parents and 312 offspring were genotyped for three microsatellites as described above. Using these three loci, we were able to assign paternity to each offspring with 100% certainty. For each clutch, we calculated the proportion of offspring that were sired by the male who mated prior to the production of that clutch. The first mating and clutch were excluded from subsequent analyses due to the absence of sperm competition when only one ejaculate is present, leaving a total of 20 clutches. However, all clutches laid before the second mating were confirmed to have been sired by the first male to mate. Data were analysed using parametric statistics when the assumptions of the tests were met. Proportion data were arcsine square root transformed prior to analysis.

## Results

### *Direct observation of mating rate in the wild*

More than 4000 sightings of two-spot ladybirds were observed during the 21 transect walks, and the percentage of individuals observed mating was calculated for each. The samples were divided into morning (0830–1230 h) and afternoon (1230–1630 h), and a mean percentage of individuals mating calculated for both time periods. The percentage (number) of individuals mating in the morning ranged from 12.80% (16) to 25.91% (66), with a mean of 18.56% (38.2). The afternoon range was between 17.32% (20) and 30.57% (92), with a mean of 23.59% (42.3). The additional early (0525–0705 h) and late (1930–2100 h) walks produced values of 7.48% (8) and 7.79% (6) mating, respectively. Overall, the percentage of individuals mating between the hours of 0830 h and 1630 h was 21.34%. However, the Cambridge population is known to be infected with a male-killing *Rickettsia* bacteria at low prevalence (approximately 7.3% of females infected; Hurst *et al*. 1993), so the sex ratio in the population will be female biased. Our estimate of the proportion of individuals mating in the population is therefore likely to be a slight overestimate for females. We can correct for this, using an estimate of the sex ratio bias for this population (proportion of males = 0.465, Hurst *et al*. 1993), and this results in a value of 19.92% of females mating.

In terms of elytral phenotypes, the *typica* phenotype is at a very high frequency in the Cambridge population, and this is reflected in its representation in mating pairs (Table 1). All phenotypes appear at the same frequencies in mating pairs as in the population as a whole (*G*-test of heterogeneity: $G = 3.12$, $P = 0.54$), indicating that there is no excess of any phenotype in mating individuals.

### *Patterns of paternity in the wild*

Table 2 shows the number of paternal contributions in each clutch, for each female from the two populations, based on the two methods for estimating the number of fathers. Using the minimum number of male contributions, multiple paternity is detected in all but three of the 40 clutches, with between one and four males siring each clutch [mean number of paternal contributions (with standard errors): Cambridge first clutches = 2.50 (0.27), fourth clutches = 2.40 (0.31); Paris first clutches = 2.50 (0.17), fourth clutches = 2.60 (0.16); Fig. 1]. By taking into account the fact that it is unlikely that all males are heterozygous at each microsatellite locus, the DADSDHARE program consistently estimates a greater number of paternal contributions (Wilcoxon signed rank test: $Z = -6.29$, $P < 10^{-4}$), detecting multiple paternity in all but two of the 40 clutches, with between one and six males siring each clutch [mean number of paternal contributions (with standard errors): Cambridge first clutches = 3.70 (0.42), fourth clutches = 3.50 (0.50); Paris first clutches = 3.80 (0.20), fourth clutches = 3.80 (0.25), Fig. 1].

Using the DADSHARE estimates of the number of paternal contributions, there was no difference in the number of male contributions to the two clutches (paired *t*-test: Cambridge $t_9 = 0.48$, $P = 0.64$; Paris $t_9 = 0.00$, $P > 0.99$), or in the variation in the number of male contributions to each

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

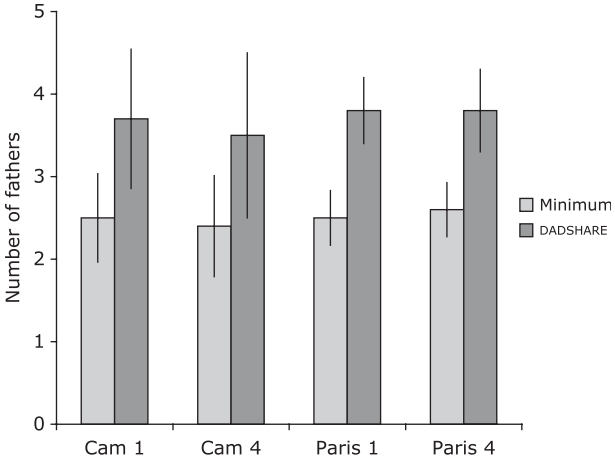


**Fig. 1** Mean number of fathers estimated for the first (1) and fourth (4) clutches laid by wild-caught females from two populations [Cambridge (Cam) and Paris] using two methods: the minimum number of paternal contributions and the DADSHARE method (see text for more details). Error bars indicate two standard errors.

**Table 2** Number of paternal contributions to two egg clutches laid by females from Cambridge (C) and Paris (P), using the minimum number of paternal contributions$^{M}$ and DADSHARE$^{D}$ methods

| Female | Clutch 1$^{M}$ | Clutch 4$^{M}$ | Clutch 1$^{D}$ | Clutch 4$^{D}$ |
|---|---|---|---|---|
| C5 | 1 | 1 | 1 | 1 |
| C6 | 3 | 3 | 4 | 4 |
| C7 | 3 | 2 | 3 | 3 |
| C10 | 3 | 3 | 4 | 5 |
| C12 | 4 | 2 | 6 | 4 |
| C13 | 2 | 2 | 4 | 2 |
| C15 | 2 | 3 | 4 | 3 |
| C16 | 2 | 4 | 3 | 5 |
| C17 | 2 | 1 | 3 | 2 |
| C18 | 3 | 3 | 5 | 6 |
| P2 | 3 | 2 | 3 | 3 |
| P3 | 2 | 3 | 3 | 5 |
| P5 | 2 | 2 | 5 | 5 |
| P7 | 2 | 3 | 4 | 4 |
| P8 | 3 | 2 | 4 | 3 |
| P10 | 2 | 2 | 4 | 4 |
| P11 | 2 | 3 | 3 | 4 |
| P12 | 3 | 3 | 4 | 4 |
| P13 | 3 | 3 | 4 | 3 |
| P14 | 3 | 3 | 4 | 3 |

clutch (*F*-test of variance ratio: Cambridge $F_{9,9} = 0.72$, $P = 0.63$; Paris $F_{9,9} = 0.64$, $P = 0.52$), in either population. There was no difference between the two populations in the number of males contributing to each clutch (unpaired *t*-test: first clutches, $t_{18} = -0.21$, $P = 0.83$; fourth clutches, $t_{18} = -0.54$, $P = 0.59$), although there was some evidence for greater variation in the number of male contributions in the Cambridge population compared to the Paris population (first clutches, $F_{9,9} = 4.47$, $P = 0.036$; fourth clutches, $F_{9,9} = 4.02$, $P = 0.050$).

### *Patterns of sperm use in the laboratory*

Figure 2 shows the proportion of offspring sired by each male in each clutch produced by two of the females, and indicates the order in which the males mated (similar patterns are seen for all females). Most clutches (82.61%) were sired by multiple males and there is evidence of sperm from males mating early in the series remaining in the spermatheca and being used throughout the experiment. The proportion of offspring sired by the male mating prior to the production of each clutch ranged from 0 to 1, with a mean (standard error) of 0.67 (0.07). We examined whether mating order affected the proportion of offspring sired by each male. Since each female mated six times, males that mated earlier in the series might be expected to sire a higher proportion of offspring, since their sperm would be in less competitive conditions and there is evidence of sperm being stored across the entire experimental period. For example, the second male to mate initially competes with only one other, whereas the final male competes with the remains of five other ejaculates. There was an effect of mating order (the number of the copulation for the female) on the proportion of offspring sired (mixed model ANOVA with male and female identity fitted as random effects: $F_{4,15} = 3.38$, $P = 0.037$), although post hoc tests (Fisher's PLSD) revealed that this was only an effect of the mean value for the third mating being lower than the second ($P = 0.007$) and fourth ($P = 0.014$).

There was no difference in the number of cycles of mating carried out by each male ($F_{6,12} = 1.79$, $P = 0.18$) or received by each female ($F_{3,16} = 0.75$, $P = 0.54$). Across all mating interactions in the experiment, there was a positive correlation between the length of mating (time spent *in copula*) and the proportion of offspring sired (Pearson's correlation: $r_{20} = 0.62$, $P = 0.003$), with the proportion of offspring sired increasing with longer matings (Fig. 3a). This was confirmed by analysis of the proportion of offspring sired after matings involving different numbers of cycles of mating ($F_{1,18} = 5.03$, $P = 0.04$, Fig. 3b).

## Discussion

This study provides the first direct evidence that female two-spot ladybirds are highly polyandrous in the wild and that they store sperm from multiple males to fertilize their eggs. The results of controlled paternity studies in the laboratory indicate that the number of mates is not greater than the number of fathers, suggesting that our estimate of the number of males that share paternity of a female's eggs

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

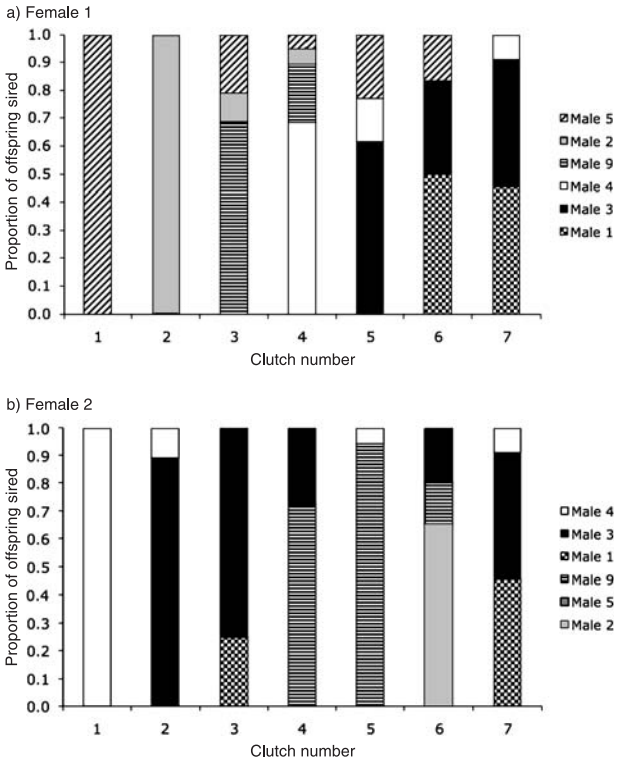


**Fig. 2** Patterns of paternity, in terms of the proportion of offspring sired by different males, across seven clutches for two females mated to six males in a controlled laboratory mating experiment. The legend indicates the order in which the males mated (with the first male at the top), the numbers are identification labels for individual males.

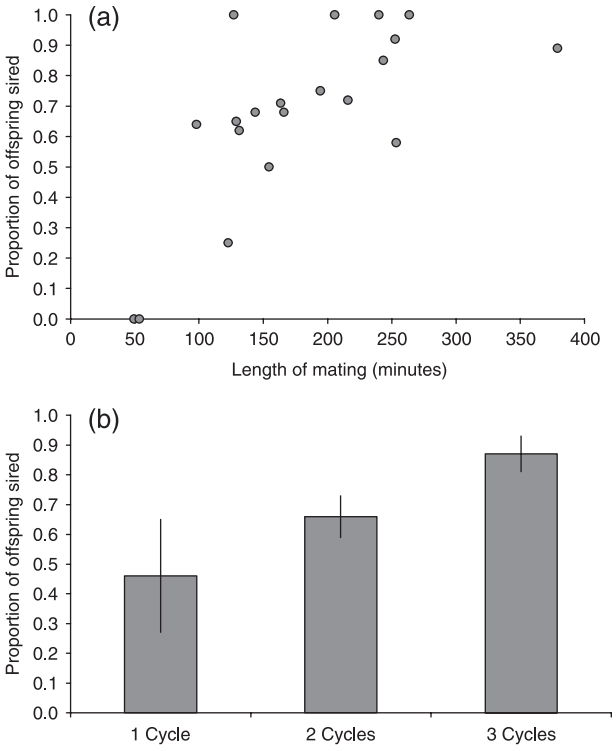


**Fig. 3** Male paternity was positively related to mating duration in terms of both (a) the time spent *in copula* and (b) the number of cycles of mating.

represents the number of males that the female has mated with. This is the first study to our knowledge to bring together these different techniques to quantify and interpret patterns of polyandry in the wild.

Female two-spot ladybirds are known to mate at a high rate in the laboratory, but this has not been directly confirmed in the wild (Majerus 1994a; Ransford 1997; Haddrill *et al*. 2007). Brakefield (1984) and Webberley *et al*. (2006) both used the proportion of individuals observed mating in natural populations (Dutch and Polish, respectively) as an estimate of the mating rate in the wild. We therefore used the same approach in the Cambridge population, to assess whether mating rates were comparable between this and other populations and to provide greater context for our molecular analysis. Our results show that approximately 20% of individuals were observed copulating on any given day, which is comparable to previous observations (Brakefield 1984; Webberley *et al*. 2006). We find no over-representation of any elytral phenotype in mating pairs, suggesting that there is no mating advantage to particular male elytral phenotypes, as has been reported previously for this population (Muggleton 1979; O'Donald & Muggleton 1979; O'Donald *et al*. 1984; but see Kearns *et al*. 1990).

We also examined levels of multiple mating and patterns of paternity in natural populations by genotyping the offspring of wild-caught females from the Cambridge population and from a Parisian population, allowing us to compare patterns of paternity between populations. By genotyping the offspring of the first and fourth clutches laid by females, we were also able to examine patterns of paternity over time (Simmons 2001). For example, if the amount of sperm mixing increases over time, then the first clutch may be sired by fewer males than the fourth clutch. We used two methods for estimating the number of paternal contributions to a clutch, including one incorporating information about population allele frequencies (Bretman & Tregenza 2005; Song *et al*. 2007). There was a very high level of multiple paternity in the offspring of wild-caught females. The percentage of females mating with more than one male was 95%, and multiple paternity within clutches was identified in 92.5% and 95% of the 40 clutches examined, using the allele counting and DADSHARE methods, respectively. We found no evidence for differences in patterns of paternity between populations, or between the first and fourth clutches, with the mean number of paternal contributions being estimated as approximately 2.5 and 3.7 per clutch, using the allele-counting and DADSHARE methods, respectively (see Fig. 1). We can link these data back to our behavioural observations by estimating how much time

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

females should be spending in the mating pool. In order to mate between three and four times, females would have to spend around two-and-a-half weeks in the mating pool (with a daily probability of mating $P = 0.2$, binomial mean number of matings for 14 days in the mating pool is 2.8, and for 21 days is 4.2 matings). This kind of approach suggests that molecular estimates of polyandry may also be used to generate hypotheses about a number of aspects of natural mating systems (above and beyond female mating frequency) that could be tested with further field observations and experiments.

The levels of polyandry we observe are very similar to those estimated by Bretman & Tregenza (2005) in a sample of wild-caught female crickets, using the same types of methods. However, they note that although estimates of the number of paternal contributions using a method incorporating population allele frequencies are likely to be much more accurate than those based on allele counting, they may still be underestimates. First, inbreeding will cause the number of fathers to be underestimated, since male genotypes will be more similar than expected (Bretman & Tregenza 2005). However, as noted previously, the three microsatellite loci used were variable enough that in a sample of 76 individuals from the two populations studied here, only two individuals were genotypically indistinguishable (Haddrill *et al*. 2002); thus, this is unlikely to have a major effect on the estimated number of paternal contributions. Second, the fact that we only examine a small fraction of the lifetime reproductive output of a female may cause us to underestimate the number of fathers. However, when the number of offspring sampled is considerably larger than the number of fathers, this will be a minor problem (Bretman & Tregenza 2005). The sizes of the clutches we examined (mean 12.8 offspring per clutch) suggest that this should not affect our results to any great degree. Finally, substantial levels of sperm clumping may lead to underestimates of the number of fathers, if sperm stored in the spermatheca becomes less stratified and more mixed over time (Harvey & Parker 2000). However, since we estimated the number of fathers in both the first and fourth clutches produced by each female and found no change over time, we can be confident that our results are not biased in this way. As such, this result is consistent with Ransford (1997) who suggested that the model of sperm competition most likely to apply to the two-spot ladybird was one which predicted a fairly high rate of mixing soon after sperm transfer.

In the final part of our study, we examined patterns of paternity over time in a controlled laboratory mating experiment, in order to link the number of fathers with the number of mates. Genotyping the offspring of wild-caught females gives an estimate of the number of fathers siring each clutch, but does not directly estimate the number of males a female has mated with, since she may not store sperm from all matings, and may not use all sperm that is stored to fertilize her eggs. Comparing the number of mates with the number of fathers also provides some insight into postmating/prefertilization processes occurring in the female reproductive tract (Eberhard 1996; Simmons 2001).

Although the sample size for this part of our study is necessarily constrained by the number of offspring that need to be genotyped (over 300), and thus is small in terms of the number of females tested, the data clearly do not support the hypothesis that the number of mates greatly differs from the number of fathers. Our results show that sperm from all mates is stored, and that all males get at least some share of the paternity, even after several other males have mated. This has a number of implications. First, it suggests that our estimates of the level of polyandry in the wild might actually be reasonably good. Second, these results also suggest that female influence on patterns of paternity after insemination may be limited, and there does not appear to be any evidence of females mating with males and then discarding their sperm, as would be predicted by some models of the evolution of polyandry by cryptic female choice (Eberhard 1996). In fact, our data suggest that, although the most recent male to mate gains a transient fertilization advantage (see Fig. 2), copulation duration (in absolute terms and in terms of the number of spermatophores formed inside the female) is the only factor that is positively associated with male paternity success (see Fig. 3). It is therefore likely that males that are more successful at fertilizing eggs pass more sperm to females. Given the size of our experiment, we are wary of making strong claims about patterns and mechanisms of sperm precedence (notoriously difficult to do in any case: Simmons 2001). However, Ransford (1997) found a similar relationship between mating duration and paternity in the Cambridge population, and suggested that male two-spot ladybirds control the number of sperm they deliver to females by altering the number of spermatophores, not the number of sperm transferred per spermatophore (see also de Jong *et al*. 1993, 1998). More generally, these results also emphasize the need to consider sperm competition among multiple males (rather than just two competing males), and over longer time periods than just one clutch of offspring (Eady *et al*. 2000; Simmons 2001).

Finally, our work does leave open the question of how mating duration is determined in *Adalia bipunctata*; males may be able to increase their paternity share by forcing longer copulations, or females may be able to bias sperm use towards particular males by allowing longer copulations (Eberhard 1996). Copulation duration is therefore one potential source of sexual conflict between males and females (Arnqvist & Rowe 2005). Another possible conflict is over mating rate. While males are likely to maximize their reproductive success by mating as many times as possible, previous work has suggested that egg hatch rates

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

are highest after females have mated with approximately five to six different males, meaning that optimal mating rates are likely to be lower for females than for males (Haddrill *et al*. 2007). The data presented here suggest that females have mated with three to four males. To what extent this represents male or female control will require further studies determining which sex has the greatest influence over female mating rate.

## Acknowledgements

We thank Dominique Bertrand, Martha Chyb, Roger Day, Denis Farrington and Ian Wright for technical assistance with ladybird work, and Jeremie Fant for technical assistance with molecular work. We thank Dan Halligan for useful comments on the manuscript. This work was supported by a BBSRC studentship to P.R.H. and a NERC Advanced Research Fellowship to D.M.S.

## References

Arnqvist G, Nilsson T (2000) The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*, **60**, 145–164.

Arnqvist G, Rowe L (2005) *Sexual Conflict*. Princeton University Press, Princeton, New Jersey.

Blyth JE, Gilburn AS (2007) Extreme promiscuity in a mating system dominated by sexual conflict. *Journal of Insect Behavior*, **19**, 447–455.

Bonizzoni M, Katsoyannos BI, Marguerie R *et al*. (2002) Microsatellite analysis reveals remating by wild Mediterranean fruit fly females, *Ceratitis capitata*. *Molecular Ecology*, **11**, 1915–1921.

Boomsma JJ, Van der Have TM (1998) Queen mating and paternity variation in the ant *Lasius niger*. *Molecular Ecology*, **7**, 1709–1718.

Brakefield PM (1984) Ecological studies on the polymorphic ladybird *Adalia bipunctata* in the Netherlands. I. Population biology and geographical variation of melanism. *Journal of Animal Ecology*, **53**, 761–774.

Bretman A, Tregenza T (2005) Measuring polyandry in wild populations: a case study using promiscuous crickets. *Molecular Ecology*, **14**, 2169–2179.

Bundgaard J, Barker JSF, Frydenberg J, Clark AG (2004) Remating and sperm displacement in a natural population of *Drosophila buzzatii* inferred from mother-offspring analysis of microsatellite loci. *Journal of Evolutionary Biology*, **17**, 376–381.

Chapman T (2006) Evolutionary conflicts of interest between males and females. *Current Biology*, **16**, 744–754.

Chapuisat M (1998) Mating frequency of ant queens with alternative dispersal strategies, as revealed by microsatellite analysis of sperm. *Molecular Ecology*, **7**, 1097–1105.

Charlesworth B (2001) The effect of life-history and mode of inheritance on neutral genetic variability. *Genetical Research*, **77**, 153–166.

Chesser RK, Baker RJ (1996) Effective sizes and dynamics of uniparentally and diparentally inherited genes. *Genetics*, **144**, 1225–1235.

Choe JC, Crespi BJ (1997) *The Evolution of Mating Systems in Insects and Arachnids*. Cambridge University Press, Cambridge, UK.

Eady PE, Wilson N, Jackson M (2000) Copulating with multiple mates enhances female fecundity but not egg-to-adult survival in the bruchid beetle *Callosobruchus maculatus*. *Evolution*, **54**, 2161–2165.

Eberhard WG (1996) *Female Control*: *Sexual Selection by Cryptic Female Choice*. Princeton University Press, Princeton, New Jersey.

Emery AM, Wilson IJ, Craig S, Boyle PR, Noble LR (2001) Assignment of paternity groups without access to parental genotypes: multiple mating and developmental plasticity in squid. *Molecular Ecology*, **10**, 1265–1278.

Estoup A, Scholl A, Pouvreau A, Solignac M (1995) Monoandry and polyandry in bumble bees (Hymenoptera: Bombinae) as evidenced by highly variable microsatellites. *Molecular Ecology*, **4**, 89–93.

Evans JD (1993) Parentage analyses in ant colonies using simple sequence repeat loci. *Molecular Ecology*, **2**, 393–397.

Good JM, Ross CL, Markow TA (2006) Multiple paternity in wild-caught *Drosophila mojavensis*. *Molecular Ecology*, **15**, 2253–2260.

Goodnight KF, Strassmann JE, Klingler CJ, Queller DC (1996) Single mating and its implications for kinship structure in a multiple-queen wasp, *Parachartergus colobopterus*. *Ethology Ecology and Evolution*, **8**, 191–198.

Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology*, **11**, 2195–2212.

Haddrill PR, Majerus MEN, Mayes S (2002) Isolation and characterization of highly polymorphic microsatellite loci in the two-spot ladybird, *Adalia bipunctata*. *Molecular Ecology Notes*, **2**, 316–319.

Haddrill PR, Shuker DM, Mayes S, Majerus MEN (2007) Temporal effects of multiple mating on components of fitness in the two-spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae). *European Journal of Entomology*, **104**, 393–398.

Harshman LG, Clark AG (1998) Inference of sperm competition from broods of field-caught *Drosophila*. *Evolution*, **52**, 1334–1341.

Harvey IF, Parker GA (2000) 'Sloppy' sperm mixing and intraspecific variation in sperm precedence (P-2) patterns. *Proceedings of the Royal Society of London Series B: Biological Sciences*, **267**, 2537–2542.

Hurst GDD, Majerus MEN, Walker LE (1993) The importance of cytoplasmic male killing elements in natural populations of the two spot ladybird, *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae). *Biological Journal of the Linnean Society*, **49**, 195–202.

Imhof M, Harr B, Brem G, Schlötterer C (1998) Multiple mating in wild *Drosophila melanogaster* revisited by microsatellite analysis. *Molecular Ecology*, **7**, 915–917.

Jones AG (2001) GERUD 1.0: a computer program for the reconstruction of parental genotypes from progeny arrays using multilocus DNA data. *Molecular Ecology Notes*, **1**, 215–218.

de Jong PW, Brakefield PM, Geerinck BP (1998) The effect of female mating history on sperm precedence in the two-spot ladybird, *Adalia bipunctata* (Coleoptera, Coccinellidae). *Behavioral Ecology*, **9**, 559–565.

de Jong PW, Verhoog MD, Brakefield PM (1993) Sperm competition and melanic polymorphism in the two-spot ladybird, *Adalia bipunctata* (Coleoptera, Coccinellidae). *Heredity*, **70**, 172–178.

Kearns PWE, Tomlinson IPM, O'Donald P, Veltman CJ (1990) Non-random mating in the two-spot ladybird (*Adalia bipunctata*): I. A reassessment of the evidence. *Heredity*, **65**, 229–240.

Kraaijeveld K, Chapman T (2004) Effects of male sterility on female remating in the Mediterranean fruitfly, *Ceratitis capitata*. *Proceedings of the Royal Society of London Series B: Biological Sciences*, **271**, S209–S211.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Majerus MEN (1994a) Female promiscuity maintains high fertility in ladybirds (Col., Coccinellidae). *Entomologist's Monthly Magazine*, **130**, 205–209.

Majerus MEN (1994b) *Ladybirds*. Harper Collins, London.

Muggleton J (1979) Non-random mating in wild populations of polymorphic *Adalia bipunctata*. *Heredity*, **42**, 57–65.

O'Donald P, Derrick M, Majerus MEN, Wier J (1984) Population genetic theory of the assortative mating, sexual selection and natural selection of the two-spot ladybird, *Adalia bipunctata*. *Heredity*, **52**, 43–61.

O'Donald P, Muggleton J (1979) Melanic polymorphism in ladybirds maintained by sexual selection. *Heredity*, **43**, 143–148.

Ransford MO (1997) *Sperm competition in the two-spot ladybird, Adalia bipunctata*. PhD Thesis, University of Cambridge, Cambridge, UK.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schlötterer C, Reiss M, Schneider A, Imhof M (2005) Similar mating and sperm displacement patterns in two highly divergent *D. simulans* populations from Africa and Europe. *Molecular Ecology*, **14**, 1511–1515.

Simmons LW (2001) *Sperm Competition and its Evolutionary Consequences in the Insects*. Princeton University Press, Princeton, New Jersey.

Song SD, Drew RAI, Hughes JM (2007) Multiple paternity in a natural population of a wild tobacco fly, *Bactrocera cacuminata* (Diptera: Tephritidae), assessed by microsatellite DNA markers. *Molecular Ecology*, **16**, 2353–2361.

Sumner S, Hughes WOH, Pedersen JS, Boomsma JJ (2004) Ant parasite queens revert to mating singly. *Nature*, **428**, 35–36.

Thornhill R, Alcock J (1983) *The Evolution of Insect Mating Systems*. Harvard University Press, Cambridge, Massachusetts.

Tripet F, Touré YT, Dolo G, Lanzaro GC (2003) Frequency of multiple inseminations in field-collected *Anopheles gambiae* females revealed by DNA analysis of transferred sperm. *American Journal of Tropical Medicine and Hygiene*, **68**, 1–5.

Vogler AP, DeSalle R (1993) Phylogeographic patterns in coastal North American tiger beetles (*Cicindela dorsalis* Say) inferred from mitochondrial DNA sequences. *Evolution*, **47**, 1192–1202.

Webberley KM, Buszko J, Isham V, Hurst GDD (2006) Sexually transmitted disease epidemics in a natural insect population. *Journal of Animal Ecology*, **75**, 33–43.

Zeh DW, Zeh JA, Bermingham E (1997) Polyandrous, sperm-storing females: carriers of male genotypes through episodes of adverse selection. *Proceedings of the Royal Society of London Series B: Biological Sciences*, **264**, 119–125.

---

This work forms part of Penelope Haddrill's PhD thesis, examining the mating system of two-spot ladybirds, with particular emphasis on female multiple mating. David Shuker is a NERC Advanced Research Fellow at the University of Edinburgh studying the evolutionary ecology of insect reproductive behaviour. Bill Amos has a long standing interest in the realisation of mating behaviour, but usually in larger species such as marine mammals. Mike Majerus is an evolutionary geneticist, focusing mainly on sexual strategies, sex ratio biases, melanism and invasive species in the Lepidoptera and ladybirds. Sean Mayes is a molecular geneticist and has been interested in population structure and genome evolution for a number of years, although more usually in crop plants than in insects.

---

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd