Temporal effects of multiple mating on components of fitness in the two-spot ladybird, Adalia bipunctata (Coleoptera: Coccinellidae)

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Abstract. Insects have provided much of the best evidence to date concerning possible costs and benefits of multiple mating, and here we investigate the benefits of polyandry in the two-spot ladybird, Adalia bipunctata, by attempting to replicate the highly promiscuous mating system in this species. We compared the temporal pattern of reproductive success of females mated multiple times to one male with that of females mated an equal number of times to multiple males, and found transient differences in offspring production and hatch rate over time. Our data suggest that polyandrous females benefit from multiple mating in some circumstances, but the patterns are complex. Following how both the costs and benefits to mating accrue over time will be necessary if we are to fully understand why polyandry evolves.

INTRODUCTION

Understanding the benefits that females obtain from mating with certain males is crucial to our understanding of reproductive ecology and sexual selection (Andersson, 1994). This is particularly true when females mate frequently, especially when one or a few matings are sufficient for full fertility (Arnaqvist & Nilsson, 2000). If females receive direct, material benefits from mating multiply, for instance through nuptial gifts or access to resources in the environment (Vahed, 1998; Wedell & Karlsson, 2003), then the evolution of female multiple mating (polyandry) is uncontroversial (Andersson, 1994). However, when females receive nothing but sperm from males, the only benefits that are available are indirect genetic benefits. The nature and importance of these genetic benefits have long concerned evolutionary biologists (e.g. Bateson, 1983; Kirkpatrick & Ryan, 1991; Maynard Smith, 1991; Andersson, 1994; Kirkpatrick & Barton, 1997; Jennions & Petrie, 2000; Tregenza & Wedell, 2000; Kokko et al., 2002; Zeh & Zeh, 2003).

Genetic benefits have typically been separated into “good genes” and “sexy sons” benefits, wherein the benefits are realised as genetically fit offspring, or sexually attractive male offspring, respectively (Andersson, 1994). This division need not be absolute however (Kokko et al., 2002). More recently, a third kind of genetic benefit has been considered, namely producing genetically fit offspring via gamete compatibility (Zeh & Zeh, 1996, 1997, 2003; Tregenza & Wedell, 2000). In this case, females are expected to choose to mate with males whose gametes are the most compatible with their own, thus producing offspring without negative within- or among-locus interactions between maternally and paternally inherited alleles. Females are expected to differ in terms of their preferred males in the population, since different female genotypes will vary in their compatibility with any given male. The resulting sexual selection on males will therefore not be directional, as in traditional “good genes” or “sexy sons” sexual selection. Although in some cases females may be able to use phenotypic information to estimate their likely genetic compatibility with a given male (for instance in the cases of mate choice with respect to MHC genotype: Penn & Potts, 1999; Tregenza & Wedell, 2000; Penn, 2002; Zelano & Edwards, 2002), it may not always be easy for females to score such a trait prior to copulation. Females may therefore only be able to determine compatibility through processes that occur after insemination (e.g. sperm-egg interactions).

There has recently been renewed effort to measure the direct and indirect costs and benefits of polyandry. A productive approach pioneered by Tregenza & Wedell (1998, 2002) has been to mate females multiple times with different males (true polyandry), or multiple times with the same male. In their work with the cricket Gryllus bimaculatus, Tregenza and Wedell showed that females increase offspring production via polyandry by increasing the likelihood of mating with a male with a more compatible genome, and females appear to be able to bias sperm use towards sperm from compatible males (Tregenza & Wedell, 2002; Bretman et al., 2004). Similar benefits have been shown in a number of species (e.g. Newcomer et al., 1999; Fedorka & Mousseau, 2002; Evans & Mar-
shall, 2005) but appear to be lacking in others (e.g. Eady et al., 2000; Simmons, 2001; Brown et al., 2004; Jennions et al., 2004; Maklakov & Lubin, 2004). Whilst this approach is a powerful experimental tool, especially when combined with a singly-mated female control (Ivy & Sakaluk, 2005), experiments have not always replicated the often highly promiscuous mating system of the species being studied. One problem that arises is that the balance of costs and benefits of mating may be poorly estimated. Mating costs are likely to be an important component of the costs of reproduction (Bell & Koufopanou, 1986; Roff, 1992; Stearns, 1992) and should therefore also be considered.

Here we consider the benefits of polyandry in the two-spot ladybird, *Adalia bipunctata* (Linnaeus). The two-spot ladybird is highly promiscuous, with males and females mating with many different partners throughout their lives both in the laboratory and in the wild (Majerus, 1994; Ransford, 1997; Haddrill, 2001). For example, in a Dutch population, Brakefield (1984) found that, on average, 23.5% of all adults were mating at any time, with this number rising to 44% at peak mating periods. In addition to this, Webberley et al. (2006) estimated mating rates in a Polish population as being between 0.705 and 0.75 times per individual per day across the first ten weeks of a single mating season. Understanding the evolutionary ecology of multiple mating in this species is important not only in terms of sexual selection, but also in terms of understanding the dynamics of two sets of inter-specific interactions: (1) between the ladybird and the male-killing endosymbiotic bacteria they carry (Hurst et al., 1992; Majerus et al., 2000); (2) between the ladybird and various sexually transmitted diseases (Webberley et al., 2004, 2006). Thus far, no evidence for direct nutritional benefits from multiple mating has been found in the two-spot ladybird (Ransford, 1997), although females who mate only once may eventually suffer from depleted sperm supplies (Majerus, 1994). Pre-copulatory female mate choice associated with colour pattern has been observed in some populations (Majerus et al., 1982, 1986; but see Kearns et al., 1990, 1992) but so far there is no evidence for post-copulatory female choice, with sperm competition patterns suggesting random mixing of ejaculates within a female (Ransford, 1997).

We focus on the possible benefits that accrue to females from mating ten times either with the same male or with ten different males. If mating several times increases female offspring production, for instance through males providing nutrients in the ejaculate, singly mated females should produce fewer offspring compared to females mated multiply to the same or different males. However, if benefits only accrue as a result of mating with a number of different males, as predicted by indirect genetic benefits models, then repeatedly mating with the same male should not lead to increased offspring production compared with singly mated females. In this experiment, by mating females with ten males, we also introduce potentially substantial costs of reproduction, including the costs of mating and of increased offspring production (Bell & Koufopanou, 1986; Roff, 1992; Stearns, 1992). We may therefore expect offspring production to change non-linearly over time for the multiply mated treatment, increasing and then decreasing as the costs of reproduction increase.

**MATERIAL AND METHODS**

Ladybirds were drawn from laboratory stocks of *A. bipunctata* derived from individuals collected in Moscow by Professor I. Zakharov and reared in mass culture under standard conditions (Majerus et al., 2000). To minimise genetic variation amongst the treatments, three virgin sisters, matched for size, were selected from ten different families (i.e. n = 30), and housed individually in 9cm clear Petri dishes and fed excess pea aphids (*Acyrthosiphon pisum*), for a minimum of 14 days before the experiment began. Each sister was randomly assigned to one of three treatments; singly mated females (SM, one mating with one male), repeatedly mated females (RM, ten matings with one male) and multiply mated females (MM, one mating with ten different males). All SM and RM females were randomly assigned one male each, while all MM females were each assigned ten males from ten different families. Males (all of which were non-virgins) were drawn from multiple different stock families, including the ten families from which the females were drawn, but were never paired with females from their own family. They were also matched for size where possible, housed individually and fed excess *A. pisum*. On the first day of the experiment, all females mated with their assigned male. RM and MM then mated every other day, a total of ten times; RM females with the same male each time, MM females with a different male each time. The number of cycles of mating and the time spent in copula were recorded for every mating. “Cycles of mating” refers to the fact that in the two-spot ladybird, a single mating event can consist of up to three separate inseminations, or cycles, with each cycle involving the production of a new spermatophore. Although the male does not disengage his genitalia between cycles, the transition from one cycle to the next can be identified by specific male and female behaviour and by the changing position of the male on the female (Ransford, 1997).

Each day throughout the experiment, and for five days after the final matings, eggs were collected and counted (25 days in total). Not all females survived to the end of the experimental period, and therefore some RM and MM females did not complete the planned number of matings (see Table 1). All males completed their matings as planned, assuming that the appropriate female survived. The number of eggs laid per day was used as a measure of fecundity. As the eggs hatched (three to five days after oviposition), larvae were removed to prevent consumption of un-hatched eggs. Approximately one week after oviposition, eggs were viewed under a dissecting microscope and the number of eggs that had hatched to produce offspring was recorded. One set of three sisters all failed to produce any eggs, and thus were excluded from the analyses (i.e. n = 9 for each treatment).

We compared the effect of mating treatment on female reproductive output summed across the experiment, and also over time using a general linear modelling (GLM) approach. For the first analysis (data summed across the experiment), we added number of days in the experiment as a factor, to take into account the females that did not complete the experiment. For the GLM models, we used mixed models with female identity included as a random factor to take into account the repeated measures on individual females. We fitted day as a quadratic term to test for non-linear relationships of the response variables.
with experimental day. Statistics were carried out in S-Plus 6.2 (Insightful Corporation Inc.) and Statview 5.0.1 (SAS Institute Inc.). Normality of data was tested using Kolmogorov-Smirnov tests in Statview. We arcsine-square root transformed proportion data prior to analysis.

RESULTS

The number of matings a female had did not influence reproductive output, summed across the experiment. There was no significant effect of mating treatment on lifetime egg production (F2,23 = 0.21, P = 0.81), number of hatched eggs (F2,23 = 0.61, P = 0.55), or the proportion of eggs that hatched (F2,23 = 2.30, P = 0.12). There was a significant positive effect of how many days of the experiment a female survived on egg production (b = 29.81 ± 5.00; F1,23 = 33.27, P < 0.0001) and number of offspring (b = 18.05 ± 4.32; F1,23 = 16.88, P = 0.0004) but not on proportion of eggs that hatched (F1,23 = 0.001, P = 0.98).

The patterns of female reproductive output over time were not straightforward with respect to treatment. For egg production, the mixed model confirmed no treatment effect (F2,24 = 0.24, P = 0.71), but showed linear and quadratic terms for experimental day (F1,504 = 25.36, P < 0.0001 and F2,504 = 6.24, P = 0.01; Fig. 1a and b), with daily egg production peaking after around 11 days. For offspring production (egg hatch), again there were significant linear and quadratic effects of day (F1,504 = 40.03, P < 0.0001 and F2,504 = 4.46, P = 0.04), with offspring production increasing and then decreasing over time (Fig. 1c and d). Whilst there was no treatment main effect (F2,24 = 0.74, P = 0.49), treatment interacted with the linear effect of day (F2,504 = 8.21, P = 0.003), and with the linear and quadratic effects of day (F2,504 = 3.37, P = 0.04), so that the relationship between offspring production and day varied between treatments (Fig. 1c and d). For most of the experiment, MM females produced the most offspring, while offspring production for RM females was fairly constant over time.

For the proportion of eggs that hatched, the mixed model confirmed the lack of the treatment effect (F2,24 = 2.86, P = 0.08). The linear and quadratic terms of day were again highly significant (F1,504 = 11.51, P = 0.0007 and F2,504 = 7.74, P = 0.003) with proportion of eggs hatching increasing and then decreasing across the experiment (Fig. 1e and f). Treatment again interacted with the linear and quadratic effects of day, both separately and in combination (these interactions F1,504 = 4.59–14.81, P = 0.01 or less), so that the relationships between day and proportion of offspring that hatched were complicated (Fig. 1e and f). For most of the experiment, MM females produced eggs most likely to hatch, whilst hatch rate increased over the course of the experiment for RM females.

Although the males used in this experiment were of roughly equal condition (controlled environment, matched for size and fed ad libitum), variation in their mating history due to treatment may have affected the results, for instance by influencing the number of sperm inseminated. This is because males in the MM treatment mated only once during the experimental period, whilst RM males mated ten times, although all males had mated a number of times prior to the experimental period. Ransford (1997) showed that male A. bipunctata control the number of sperm they inseminate by altering the number of cycles of mating, not by altering the number of sperm transferred in one spermatophore. Analysis of the total number of cycles of mating received by females showed that there was no difference between the RM and MM female treatments (unpaired t-test: t0 = 0.81, P = 0.43; Table 1) and no difference in the number of cycles of mating per mating received by any of the three treatments (F2,24 = 2.5, P = 0.10; Table 1). There was also no difference in the total time spent in copula by RM and MM females (unpaired t-test: t0 = 0.48, P = 0.64; Table 1) or in the time spent in copula per mating between all three treatments (F2,24 = 0.25, P = 0.78; Table 1). In addition, across all females mean time spent in copula was not correlated with egg production (r2 = 0.21, P = 0.29), number of eggs hatching (r2 = 0.23, P = 0.26) or proportion of eggs hatching (r2 = 0.36, P = 0.07). Likewise, the mean number of cycles a female experienced was not correlated with egg production (r2 = 0.18, P = 0.36), number of eggs hatching (r2 = 0.11, P = 0.59) or proportion of eggs hatching (r2 = 0.18, P = 0.36). Taken together, male mating frequency is therefore unlikely to have affected the results.

DISCUSSION

We failed to show that female two-spot ladybirds benefit consistently from polyandry. Egg production and the number of eggs hatching from broods laid across the experiment did not vary with respect to treatment, although in the latter case mating treatment did influence the effect of experimental day, indicating transient benefits for multiply mated females as expected under genetic benefits models, although these did not last for the entire experiment. Although the number of eggs hatching provides one measure of offspring production, clearly we did not measure offspring survival to reproduction, which means that any fitness differences between treatments that accrue between hatching and reproduction have not been

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean egg production</th>
<th>Mean no. of hatched eggs</th>
<th>Mean proportion of eggs hatched</th>
<th>Mean days in the experiment</th>
<th>No. of females surviving</th>
<th>Mean copulation duration</th>
<th>Mean no. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM (n = 9)</td>
<td>410.67 ± 107.12</td>
<td>245.22 ± 75.46</td>
<td>0.60 ± 0.01</td>
<td>20.00 ± 2.96</td>
<td>6</td>
<td>184.11 ± 23.10</td>
<td>1.78 ± 0.28</td>
</tr>
<tr>
<td>RM (n = 9)</td>
<td>441.78 ± 85.28</td>
<td>317.22 ± 64.87</td>
<td>0.52 ± 0.01</td>
<td>20.11 ± 2.75</td>
<td>7</td>
<td>222.41 ± 9.57</td>
<td>2.20 ± 0.09</td>
</tr>
<tr>
<td>MM (n = 9)</td>
<td>554.56 ± 95.14</td>
<td>290.67 ± 71.24</td>
<td>0.72 ± 0.01</td>
<td>22.89 ± 1.51</td>
<td>6</td>
<td>223.86 ± 9.70</td>
<td>2.31 ± 0.09</td>
</tr>
</tbody>
</table>
Fig. 1. Influence of mating treatment on female reproductive output over time. Regression lines are presented separately for clarity: a – egg production over time with respect to treatment (MM females: filled circles; RM females: open circles; SM females: grey squares); b – regression line for the three treatments pooled, eggs = 14.42871 + 1.9617*day – 0.08544*day²; c – number of eggs hatching from broods laid across the experiment with respect to treatment (symbols as before); d – regression lines for each treatment, MM offspring = 11.25890 + 1.66777*day – 0.08201*day², RM offspring = 7.803818 + 0.847492*day – 0.030512*day², SM offspring = 10.48696 + 0.95296*day – 0.05697*day²; e – arcsine-square root transformed proportion of eggs hatching over time with respect to treatment (symbols as before); f – regression lines for each treatment, MM hatching = 0.3916345 + 0.1001735 *day – 0.0040335*day², RM hatching = 0.343279 + 0.0523387*day – 0.0007125*day², SM hatching = 0.4097624 + 0.0556736*day – 0.0028518*day².
measured (e.g. Ivy & Sakaluk 2005). The proportion of eggs hatching also varied across time in a complicated way, again indicating that MM females gain a transient advantage. There was no evidence that males provide direct benefits such as nutrients to females via the ejaculate, although a clear reduction in hatch rate over time was apparent in the singly mated females. This is in agreement with Majerus (1994), suggesting that sperm were becoming limited in these females, thereby providing some possible benefit for mating more than once. This was confirmed by the increase in hatch rate over time for females mating repeatedly with the same male, although hatch rates for females mating to different males did tail off at the end of the experiment. Overall, our data suggest that female mating treatment did influence patterns of reproductive output, but not in a straightforward way.

Our data also suggest that possible reproductive costs associated with mating did not differ between treatments, as egg production did not vary between them, consistently showing a curvilinear response with time. These data are interesting given the conflicting evidence about costs of mating and the degree of promiscuity. For example, in promiscuous stalk-eyed flies, mating repeatedly does not appear to be very costly (Reguera et al., 2004). Indeed, Reguera et al. (2004) suggested that promiscuous species should perhaps be expected to have limited costs associated with mating, as is the case here. However, in the equally promiscuous seed bug *Lygaeus equestris*, frequent mating can dramatically reduce female lifetime reproductive success (Shuker et al., 2006).

Genetic benefits to polyandrous females have been shown to be lacking in various other species, including seed beetles (Eady et al., 2000), spiders (Maklakov & Lubin, 2004), and the fruit fly *Drosophila melanogaster* (Brown et al., 2004). However, our results, albeit of transient effects of mating treatment, also show some similarities to studies in which females do gain genetic benefits from mating with multiple males. For example, increased fertilisation/hatching success has been shown to benefit multiply mated females (compared to repeatedly mated females) in field crickets (Tregenza & Wedell, 1998), pseudoscorpions (Newcomer et al., 1999), ground crickets (Fedorka & Mousseau, 2002) and sea urchins (Evans & Marshall, 2005). However, unlike these studies, we found that the magnitude of the benefit to polyandrous females changed over the course of the experiment, and that female reproductive success is perhaps a trade-off between the benefits and costs of multiple mating (although see Arnqvist & Nilsson, 2000; Maklakov & Lubin, 2004; Arnqvist et al., 2005).

In this study we have attempted, albeit imperfectly, to replicate the highly polyandrous mating system of the two-spot ladybird in the wild (Brakefield, 1984; Majerus, 1994; Haddrill, 2001; Webberley et al., 2006). We are therefore trying to get a realistic picture of the complex interactions between the costs and benefits of multiple mating which other studies may miss by only providing females with two or a few matings. The transient benefit to multiply mated females may also be more relevant in the wild if levels of predation and food availability mean that early reproductive success is more important than that later in life. More work on the costs of reproduction and their mechanistic basis is therefore needed in *A. bipunctata* to determine how results found under laboratory conditions transfer to wild populations. For example, recent work has examined in detail a specific cost of mating, namely the sexually-transmitted mite *Coccipolipus hippocamptae* (Hurst et al., 1995; Webberley et al., 2004, 2006). It will also be important to establish more accurate measures of the rate of female multiple mating in the wild, to determine: (1) whether females mate substantially more than the apparent optimum, and (2) whether female mating rate is female-controlled or a result of sexual conflict with males (Haddrill, 2001; Chapman et al., 2003).

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