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Sperm competition and melanic polymorphism in the 2-spot ladybird, *Adalia bipunctata* (Coleoptera, Coccinellidae)

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Sperm precedence was investigated in the 2-spot ladybird, *Adalia bipunctata* by utilizing a di-allelic colour marker gene. Non-melanic (homozygous recessive) virgin females were mated once with a non-melanic male and after subsequent laying of fertile eggs they were mated with a melanic male of known genotype. Frequencies of colour morphs in the offspring provided evidence for almost complete second male sperm precedence, although the data from certain matings do not completely exclude the possibility of first male sperm precedence. The results are discussed in the light of the hypothesis of thermal melanism.

Keywords: *Adalia bipunctata*, female rejection, sexual selection, sperm competition, thermal melanism.

Introduction

Sperm competition has been defined as the competition within a single female between sperm from two or more males for the fertilization of the ova (Parker, 1970). It is, therefore, of particular significance in species in which females mate more than once. Many different patterns of sperm use in the successful fertilization of eggs from different male mating partners have been described in insects (Walker, 1980; Thornhill & Alcock, 1983). There are examples of sperm competition resulting in (almost complete) first male or in last male sperm precedence, as well as in some degree of sperm mixing (Ridley, 1989).

Female ladybird beetles (Coccinellidae) are highly polygamous and it is known that they can store sperm in a spermatheca (Hodek, 1973; de Gunst, 1978; P. W. de Jong, personal observation). Those of the 2-spot ladybird, *Adalia bipunctata* (L.), may mate many times during the peak spring period of mating which lasts several weeks; an average of 23.5% of the adult population on *Rosa* shrubs were *in copula* on any one sampling occasion in a field study in The Netherlands (Brakefield, 1984a). *A. bipunctata* exhibits genetic polymorphism for melanism: non-melanics are red with two black spots while melanics, which are determined by a dominant allele (Lus, 1928, 1932; M. E. N. Majerus & P. M. Brakefield, unpublished data), are black with red spots. Field data have provided evidence for different forms of sexual selection associated with the different colour morphs: (i) a female preference for melanic males has been described in some British populations (Majerus *et al.*, 1982; O'Donald & Majerus, 1988; but see Kearns *et al.*, 1990, 1992); (ii) a frequency-dependent mating advantage for melanics has been recorded (Muggleton, 1979; Kearns *et al.*, 1990); (iii) a general mating advantage to melanics over non-melanics was found in The Netherlands (Brakefield, 1984c). An understanding of the extent of sperm competition in this species is necessary to interpret the consequences of any deviation from random mating. If for example, melanic beetles tend to mate more frequently but earlier than non-melanics, as in the Dutch study, then the genetic consequences will depend on the pattern of sperm precedence and the timing of egg laying. The mating advantage of melanics will only increase their fitness relative to non-melanics when eggs are laid at the beginning of the season or when sperm transferred in early matings can fertilize eggs laid after later matings.

This paper describes laboratory experiments to investigate sperm competition after double matings. Paternity was determined by examining the ratio of the melanic morphs in the offspring of the non-melanic females (the homozygous recessive) which had been

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mated initially to a non-melanic male and subsequently, to a melanic.

Materials and methods

Adult beetles were collected in October 1990 on plane trees (*Platanus* x *hybrida* Brot.) and lime trees (*Tilia* spp.) in Breda (Netherlands). These were bred for one generation to obtain virgins and to increase the proportion of homozygous melanics. The 180 wild caught melanic ladybirds were divided into groups of about 15 individuals and kept in 9 cm diameter plastic petri dishes containing a 7 cm diameter filter paper. Eighty *typica* insects were similarly divided up. The whole experiment was carried out under constant conditions (20°C and a L:D ratio of 18:6). Clean petri dishes were provided daily to prevent disease transmission, to promote egg-laying and to minimize egg cannibalism by the adults. Petri dishes containing eggs were kept separate until hatching. Ladybirds and larvae were fed daily with an ample supply of pea aphids (*Acyrthosiphon pisum* Harris) from a laboratory stock. After the second moult, larvae were separated into groups of 5 per petri dish, to reduce cannibalism. After the third moult, larvae were kept individually in 5.5 cm petri dishes with 4.5 cm diameter filter papers, until pupation. Emerging adults were kept separately and fed for 3 days before being held at 9°C.

The proportion of melanic ladybirds in the original Breda sample was 48% (326 *typica*, 300 melanic). Assuming Hardy–Weinberg equilibrium, the frequency of homozygous melanics in the F_1 generation of melanics (i.e. those used in this experiment) was 41%, more than double that in the field (16.7% of all field melanics are expected to be melanic homozygotes). The use of homozygous insects was preferred since the results following sperm competition are much easier to interpret than if a heterozygous parent is involved in a cross.

The F_1 ladybirds were used for the actual experiment. The ladybirds were sexed using characters described in Majerus & Kearns (1989) and de Jong *et al.* (1991). Seven days before the start of the experiment all ladybirds were put at 20°C and their petri dishes labelled individually. They were fed daily with pea aphids. *Typica* males and females were randomly selected and placed in pairs in clean petri dishes at time 0. They were observed continuously and the time elapsed until the start and end of mating was scored (minutes) for each pair. Immediately after mating the male was taken out of the petri dish; they were thus allowed to mate only once (mating A). The experimental females were then fed daily and egg batches were collected to determine whether they had been fertilized. After 4 or 5 days these females were remated with a melanic male (mating B) and, again, they were allowed to mate only once. No distinction was made between melanic males of the forms *quadrimaculata* and *sexpustulata* with four and six red spots, respectively. The time elapsed until the start and end of mating was scored. Eggs were collected and emerging larvae were raised through to adult. The procedure to reduce cannibalism described above was followed. The morph of each resulting adult F_2 offspring was scored.

To discover whether the melanic F_1 males used were homozygous or heterozygous, they were remated with a virgin *typica* female. They were left together and further remating was possible. Egg batches were collected, and larvae raised as above. It was necessary to rear at least seven offspring for each test-male to determine its genotype with 99% certainty.

Control experiments were carried out to see whether sperm-exhaustion could play a role over the period of 4–5 days between matings A and B. Virgin *typica* females were mated once with either *typica* or melanic males. Conditions were as described above. Numbers and the fertility of daily egg batches were scored over the female life span.

Results

The experiment was initiated with 51 *typ* × *typ* pairs (mating A). Of these, 22 females produced fertilized eggs within 5 days and these females were used for the remating with melanic males (mating B). Of these second matings 12 resulted in fertilized eggs, but one produced only a single offspring so was excluded from the analysis. Mating failures were either due to successful copulation but failure of fertilization of the eggs, or to no copulation at all. The latter was caused by rejection of the male by the female involving running away, raising the abdomen, kicking the male with the hind legs, rolling over to one side (Majerus & Kearns, 1989) or retracting the abdomen as far as possible under the elytra (M. D. Verhoog, personal observation). Failure of copulation occurred in eight of 51 mating A pairs and in four of 22 pairs of mating B (Fisher exact test, $P = 1.00$, two tailed, ns).

The females for which both copulations were successful could be divided into two categories: those in which mating B was with a homozygous melanic male, and those where it was with a heterozygous melanic male. Details of these pairings are given in Table 1.

A successful copulation with a homozygous melanic male resulted in exclusively melanic offspring in four crosses (Table 1: females 4, 5, 6, 7). These crosses yielded a total of 104 melanics. A further cross resulted in five melanic and one *typica* offspring (female 8). The

crosses involving females 1–3 yielded only *typica* offspring following mating B. With a heterozygous melanic male in mating B, female 9 produced only *typica* offspring, and females 10 and 11 produced a ratio of melanic: *typica* which was not significantly different from 1:1 ($\chi^2 = 0.061$, ns, and binomial test: $P = 0.227$, respectively).

Table 1 Frequencies of *typica* (typ) and melanic (mel) offspring produced following mating B with a melanic male (homozygote: hom; heterozygote: het), the duration of copulations and the occurrence of rejection behaviour.

Male genotype	Female	Offspring Mel	Typ	Duration mating (min) A	B	Rejection behaviour in mating A	B
hom	1	–	10	126	181	–	+
hom	2	–	66	80	118	–	+
hom	3	–	17	121	107	–	+
hom	4	9	–	161	163	–	–
hom	5	51	–	174	144	+	–
hom	6	35	–	129	161	–	–
hom	7	9	–	100	150	–	–
hom	8	5	1	111	109	–	+
het	9	–	69	77	136	–	+
het	10	34	32	116	298	–	–
het	11	2	5	115	147	–	–

Figure 1 shows that those females producing melanic offspring, except female 7, did so immediately following mating B and continued doing so until they ceased producing fertile eggs.

The proportion of failed inseminations after successful copulation did not differ significantly between mating A and B (A = 22 successful inseminations, 21 unsuccessful; B = 8:10; ,$\chi^2 = 0.039$, ns). The unsuccessful inseminations after mating B included four females in Table 1 that continued to produce only *typica* offspring and six cases in which only unfertilized eggs were laid after mating B.

Using only successful first inseminations with successful rematings in the comparison, copulation times (see Table 1) were not significantly different between matings A and B (mating A: median = 122.5 min, range = 100–174 min; mating B: median = 148.5 min, range = 88–298 min; Wilcoxon matched-pairs signed-ranks test, $T = 11.5$; $n = 8$, ns). Also, no significant difference was found in copulation times between successful and unsuccessful inseminations (Mann–Whitney U test, mating A: successful: median = 119.5 min, range = 77–174 min; unsuccessful: median = 123

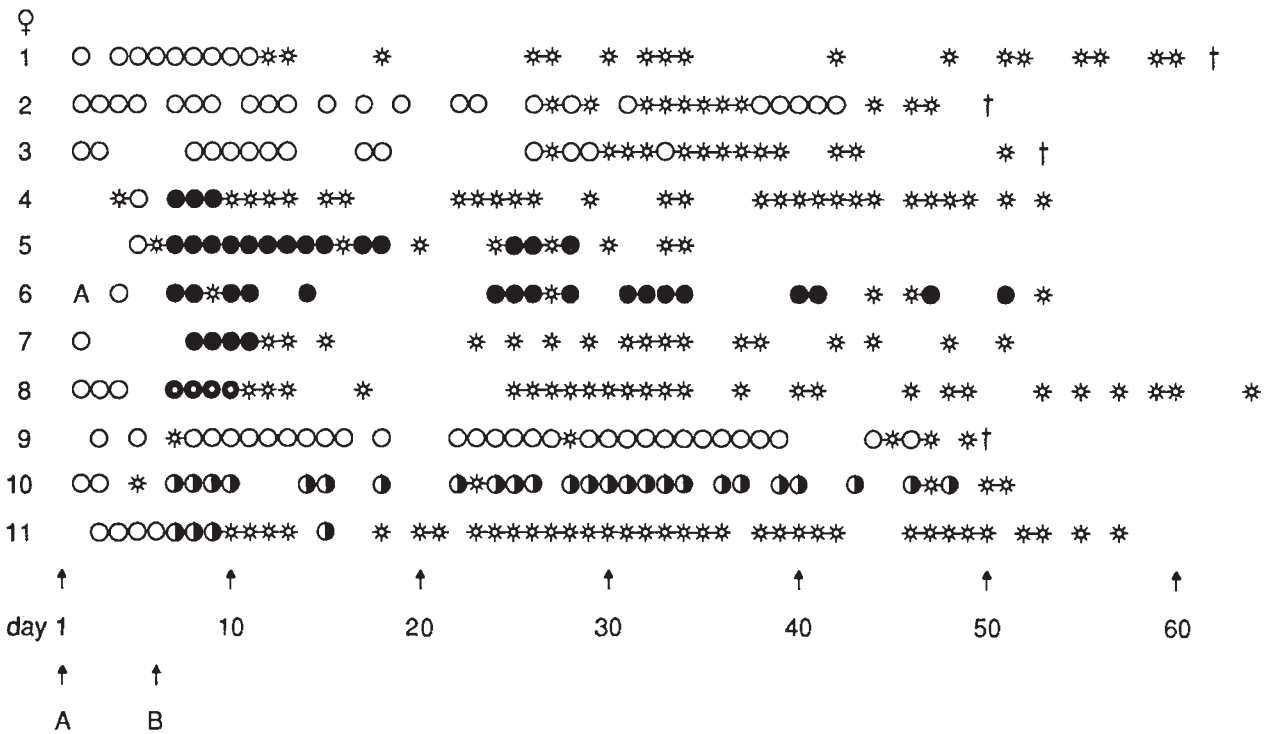

Fig. 1 Egg laying history of females 1–11 through time (days). The two successive matings A and B are indicated, as well as death of female (†); if not indicated, date of death unknown. For female 6, mating A took place on day 2. ✲: unhatched egg batches, ○: *typica* offspring, ●: melanic offspring, ⊙: melanic offspring and one *typica*, ◑: offspring with a ratio of *typica*: melanic ≈ 1:1.

min, range = 0.3–172 min; $z = 0.23$; $n_1 = 22$, $n_2 = 21$, ns; mating B, preceded by successful mating A: successful: median = 148.5 min, range = 88–298 min; unsuccessful: median = 132.5 min, range = 42–215 min; $U = 27.5$; $n_1 = 8$, $n_2 = 10$, ns; preceded by unsuccessful mating A: successful: median = 151.5 min, range = 121–170 min; unsuccessful: median = 128 min, range = 28–174 min; $U = 26.5$; $n_1 = 8$, $n_2 = 13$, ns).

Time until copulation was not significantly different between successful matings A and B (mating A: median = 444 s, range = 37–863 s; mating B: median = 140 s, range = 54–416 s; Wilcoxon matched-pairs signed-ranks test, $T = 8$; $n = 8$, ns), or between successful and unsuccessful inseminations (Mann–Whitney U test, mating A: successful: median = 319.5 s, range = 37–1008 s; unsuccessful: median = 165 s, range = 40–1973 s; $z = 1.13$; $n_1 = 22$, $n_2 = 21$, ns; mating B, preceded by successful mating A: successful: median = 140 s, range = 54–416 s; unsuccessful: median = 221 s, range = 48–2146 s; $U = 28$; $n_1 = 8$, $n_2 = 10$; preceded by unsuccessful mating A: successful: median = 120.5 s, range = 51–379 s; unsuccessful: median = 92 s, range = 59–5228 s; $U = 53$; $n_1 = 8$, $n_2 = 13$, ns).

To control for sperm exhaustion, females were mated once to either melanic or *typica* males, and the period of time during which fertilized egg batches were laid was recorded. For this purpose, the results from Table 1 were also used. The average period of time over which fertilized eggs were laid was 23.5 ± 12.8 (S.D.) days ($n = 6$, range = 5–39 days).

Discussion

With the exception of one female, the results of this study fall into two categories: after having mated initially with a *typica* male and then a melanic, either (i) the *typica* female continues laying eggs fertilized by only the first (*typica*) male, or (ii) after the second mating she immediately starts laying eggs fertilized exclusively by the second (melanic) male. The latter is substantiated for four of the homozygous melanic males (100% melanic offspring) and is consistent with the two heterozygous males which produced a 1:1 ratio of melanics: *typica*. We found only one exception, where in a family of only six offspring after the second mating with a homozygous melanic male, one *typica* offspring was found.

The most likely explanation of the results where mating B resulted in 100% *typica* offspring is that, perhaps due to rejection by the female, the inseminations failed. Inspection of Table 1 shows that there is a significantly higher tendency for rejection behaviour by the female to occur immediately prior to mating when there is no evidence from progeny of successful B mating (Fisher exact test, $P < 0{\cdot}05$; note all pairs were definitely *in copula*). Of course, the occurrence of first male sperm precedence in these matings cannot be ruled out; this might occur, for example, through the deployment of mating plugs, although no direct evidence for such plugs exists in *Adalia*.

Since the interval between mating A and B was much shorter than the average period of time in which the control females laid fertile eggs (5 days versus, on average, 23.5 days), the chance that complete sperm exhaustion or sperm depletion played any role in the chance of sperm transfer is extremely low. However, examination of Fig. 1 indicates that the four B matings (females 4–7) with homozygous melanics which produced subsequently only melanic offspring were preceded by only single fertile egg batches from the first mating A (the presence of gaps in the laying of fertile egg batches in most of the female histories suggests that the short gaps for females 4–7 prior to the second mating are not indicative of complete sperm exhaustion). This might indicate that last male sperm precedence and successful insemination is more likely to occur when the first mating involves transfer of a small amount of sperm. The rejection behaviour by females observed prior to unsuccessful second male copulations might also be indicative of a high remaining sperm load and, therefore, a low 'motivation' to remate. This might in turn increase the probability that a second male cannot successfully transfer sperm. However, the proportion of successful copulations which did not result in fertilization of eggs was not significantly different between mating A (when no females contained sperm) and B (when all females contained sperm).

Our results provide evidence that the 2-spot ladybird shows (almost) complete last male sperm precedence. However, different patterns of sperm precedence may apply under other experimental conditions, for example when three or more matings occur or when the time interval between matings is (much) shorter (N. Canham & M. E. N. Majerus, personal communication). The mechanism of last male sperm precedence in the 2-spot ladybird is unknown. Several mechanisms have been described for insects (Thornhill & Alcock, 1983; Birkhead & Hunter, 1990). Probably the most prominent among these is sperm displacement. Sperm from previous matings can be repositioned or removed by a male, but the female can also influence sperm precedence. Evidence has been found in some species that a female can, possibly stimulated by a copulating male, remove sperm from her storage organs (Birkhead & Hunter, 1990; Villavaso, 1975). In another ladybird species, *Harmonia axyridis* Pallas, sperm is transferred

from male to female by a spermatophore, and after migration of the sperm to the spermatheca, the remains of the spermatophore are ejected by the female, and eaten (Obata & Hidaka, 1987). If transfer of sperm through a spermatophore also occurs in *A. bipunctata*, a mating male may be able to stimulate a female to eject a spermatophore containing sperm from a previous mating. If the time interval between the two matings is short, this could result in sperm displacement. In any case, a 'last in, first out' mechanism, in which the sperm of the first male fertilizes the later eggs, does not seem to operate in the 2-spot ladybird, since once switched from *typica* to melanic offspring, females continue to produce melanic offspring until they cease producing fertile eggs.

Interestingly, in six out of 18 successful B copulations, the female laid only infertile eggs after mating B, whereas in two of these six copulations she laid fertilized eggs until the day before mating B. This is further evidence of the influence of a copulation on previously stored sperm. These data suggest a two-phase copulation, in which previously stored sperm is manipulated first, and insemination with new sperm follows. If this is true, in the six pairings mentioned above the first phase could have been successful, but the insemination failed. The copulation times in these six pairings were not significantly different from those successful B matings which followed successful A matings (Mann–Whitney U test: $U = 15.5$, $n_1 = 6$, $n_2 = 8$, ns). This suggests that the insemination phase is very short compared with the time needed for manipulation of the sperm from previous males.

No significant differences were found in time until copulation or in length of copulation between successful first matings and successful rematings. Furthermore, no significant differences were found for these parameters between successful and unsuccessful matings within each class of matings (A or B). No evidence has, therefore, been found for an influence of experience, female preference, external disturbances or colour morph *per se* on the results. This apparent independence of mating time and colour morph suggests that the evidence for non-random mating in the field is not an artefact of different mating times (Brakefield, 1984c). However, our data set for rematings is small and all pairings involving melanic males were B matings. Therefore, an additional experiment involving a comparison of first matings of females by *typica* and melanic males remains necessary.

When sperm precedence occurs in the 2-spot ladybird, adaptations are expected to prevent subsequent males from successfully inseminating the female. Mating plugs have already been mentioned as a possible mechanism. During the experiments, while continuously observing a number of mating pairs of ladybirds, it was noted that usually, when a pair broke up, the male immediately mounted the female again. Although this was not investigated in any detail, this observation might indicate mate guarding or remating as a mechanism to prevent or reduce the effect of subsequent matings with competing males.

Knowing that sperm precedence is operating in the 2-spot ladybird, we may ask the question why do females mate more than once? Females should gain by doing so, or there would be a selection pressure against multiple matings. In numerous insects a female receives enough sperm from one insemination to potentially fertilize all of her eggs, and given that she can store this sperm, why would she waste time and energy to mate again? Several advantages have been suggested (Thornhill & Alcock, 1983; Halliday & Arnold, 1986). They have been divided into four classes by Thornhill & Alcock (1983): sperm replenishment, material benefit, genetic benefit and convenience. Although in most ladybird species one copulation is generally reported to be sufficient for the female to fertilize all the eggs she lays during her life (Hodek, 1973), evidence has been found in *A. bipunctata* that remating increases the rate of egg-laying or viability of eggs (Ellingsen, 1969; Sem'yanov, 1970). In the present study, however, ladybirds were able to lay fertilized eggs over a relatively long period of time. A second selection pressure favouring remating by females might be that the male provides the female with nutritional benefits while mating. It has already been mentioned that in at least one ladybird species sperm is transferred through a spermatophore, which is subsequently eaten by the female (Obata & Hidaka, 1987). A similar use of nutrients is also known from other insects, for example butterflies (Boggs & Watt, 1981) and grasshoppers (Butlin *et al.*, 1987). Spermatophores, or quantities of other non-genetic material transmitted from the male to the female via the penis may equal as much as 40% of male body weight (Butlin *et al.*, 1987). However, these quantities apparently do not need to be particularly large to influence female fecundity. This might outweigh disadvantages of remating in terms of loss of time and energy in relation to feeding activity or the risk of predation.

Sperm precedence potentially has important consequences for the population biology of the 2-spot ladybird. An hypothesis about the way in which selection influences the colour polymorphism involves thermal melanism, in which the melanic morph has an advantage relative to the *typica* morph under circumstances of limited sunshine levels (Lusis, 1961; Muggleton *et al.*, 1975; Brakefield, 1984b; Brakefield & Willmer, 1985; Brakefield & Lees, 1987). An essential feature

of this hypothesis is the presence of food (aphids) for the ladybirds. Earlier activity (including mating) of the melanic morph under these conditions increases reproductive success only if food is available at the same time to produce viable eggs, or if last male sperm precedence does not occur. The synchronization of food availability and activity of ladybirds becomes even more important if complete sperm precedence is operating. A potential advantage for melanic ladybirds due to earlier mating would be reduced or could even disappear if eggs could only be laid by the time *typica* males have also become active. The *typica* males would then have an increasing chance of inseminating females and thereby displacing melanics' sperm.

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