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Why do male *Callosobruchus maculatus* beetles inseminate so many sperm?

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Abstract Male *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) inseminate more sperm than females can effectively store in their spermathecae. This study examines the adaptive significance of "excess" sperm transfer by measuring components of male and female reproductive success in response to manipulating the number of sperm inseminated. The number of sperm transferred during copulation was reduced from 56,000 ±4,462 to 8,700±1,194 by sequentially mating males to virgin females. Reducing the number of sperm inseminated by the first male to mate had no effect on the extent of sperm precedence, but reducing the number of sperm inseminated by the second male resulted in a significant reduction in the extent of sperm precedence. When large numbers of sperm are inseminated the remating refractory period of females is increased. These results indicate that males transferring large numbers of sperm during copulation have a two-fold advantage at fertilization; they are more effective at preempting previously stored sperm and they are likely to father more offspring by delaying the time of female remating. The transfer of "excess" sperm does not appear to serve as nonpromiscuous male mating effort; the number of eggs laid, their fertility and the subsequent survival of zygotes were unaffected by manipulating the number of sperm inseminated. The underlying mechanisms of sperm precedence were also examined. Simple models of sperm displacement failed to accurately predict the patterns of sperm precedence observed in this species. However, the results do not provide conclusive evidence against the models but rather serve to highlight our limited understanding of the movement of sperm within the female's reproductive tract.

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Key words Bruchidae · Sperm competition · Sperm number manipulation

Introduction

Parker et al. (1972) and Parker (1984) argued that anisogamy evolved in response to gamete competition and later sperm competition. Using the game theoretical approach of Maynard-Smith (1974), Parker demonstrated that sperm competition may have acted to maintain small sperm size in order to maximise sperm productivity and consequently fertilization success. This scenario is easy to envisage in an ancestral sessile animal with external fertilization; males that produce most sperm are likely to fertilize most ova. A similar situation is envisaged with internal fertilizers that lack specialized sperm storage organs; maximization of sperm production maximizes male fertilization success (Gomendio and Roldan 1993). However, the situation for internal fertilizers that possess female sperm storage organs (e.g. insects) is less clear. It appears wasteful for a male insect to inseminate large numbers of sperm if only a small proportion of these are actually stored. Excess sperm transfer appears to be a common feature of males' reproductive strategies (Bedford 1970; Brillard and Bakst 1991). Whether the production of excess sperm is functional or a non-adaptive consequence of sperm production remains a contentious issue (Cohen 1973; Baker and Bellis 1988, 1989; Harcourt 1989, 1991). The insemination of excess sperm may function to preempt previously stored sperm (Parker 1984) or reduce the likelihood of one's own sperm being preempted, or it could be an adaptation to ensure that enough sperm reach the site of fertilization despite the prevailing hostile conditions within the female reproductive tract. Large inseminations might also function as a form of parental

investment, increasing either female and/or zygote fitness (Gwynne 1984a; but see Wickler 1985).

It has been shown that male *Callosobruchus maculatus* inseminate on average 7 times more sperm than females can effectively store in their spermathecae (Eady 1994b). This paper investigates the function of these "excess" sperm by examining the effect of manipulating the number of sperm inseminated on sperm precedence (sperm preemption), female receptivity (anti-sperm preemption), female fitness (fecundity and fertility) and zygote survival in *C. maculatus*. In addition this paper examines the underlying mechanisms of sperm precedence in *C. maculatus* by comparing the predictions of mechanistic models of sperm competition (Lessells and Birkhead 1990, Parker et al. 1990; Parker and Simmons 1991) with observed patterns of sperm precedence. A close fit between predicted and observed results would indicate that the mechanism being modelled was operative. Maximising the number of observed and predicted results increases the statistical rigour of such a comparison. The number of observed and predicted P_2 values (the proportion of offspring sired by the second male to mate) was maximised for this purpose by combining the results of the present study with those of Eady (1991, 1994b).

Methods

General methods

The maintenance of stock cultures of *C. maculatus* and a description of the black and tan colour morphs used to assign paternity in multiple mating experiments are described in Eady (1991). All means are given ±1SE.

Manipulation of sperm transfer

During copulation sperm are transferred to the female via a spermatophore, deposited in the female's bursa copulatrix. Five minutes after copulation sperm begin to migrate from the bursa copulatrix into the spermatheca (Eady 1994b). By counting the number of sperm in the bursa copulatrix immediately following copulation the number of sperm inseminated can be determined.

Initially virgin males were sequentially mated to virgin females in 25-ml clear perspex cells under constant environmental conditions at 30°C. Immediately following a copulation the mated female was replaced by another virgin female. This process was continued for 1 h. All sequential matings were performed within 1 h of the first mating. Immediately following copulation inseminated females were immersed in alcohol and the number of sperm in their bursa copulatrix (i.e. the number of sperm inseminated) determined using the methods described in Eady (1994b). Sperm counts were performed upto 12 h after the original copulation. Preserving female beetles in alcohol immediately after copulation prevented sperm migration into the spermatheca and the degradation of sperm in the bursa copulatrix (P.E. Eady, unpublished work). The number of sperm transferred during the first, third and fourth copulations in sucession were determined for 10, 11 and 8 males respectively. No males completed five copulations within 1 h. The number of sperm inseminated declined with the number of copulations (see Results). Therefore the sequential mating of males to females provides a method for manipulating the number of sperm transferred. Elytral length (an estimate of male size, Wilson and Hill 1989) of copulating males was measured to the nearest 0.01 mm using a microscope linked to an image analyser, to determine the effect of size on the number of sperm transferred.

The effect of reduced sperm transfer on sperm precedence

Paternity was determined using the genetic marker technique with tan and black morph beetles the genetic markers (Eady 1991). Two experiments were performed in which P_2 was measured in response to manipulation of the number of sperm transferred by (1) the first male to mate and (2) the second male to mate.

1. Manipulating first male sperm transfer. Initially virgin tan males were sequentially mated to virgin tan females using the same experimental protocol as above. Following insemination females were isolated and permitted 48 h of oviposition on 50 cowpeas (*Vigna unguiculata*) before being remated to virgin black males. Following the second mating the females were isolated and allowed to oviposit on fresh cowpeas. Thus P_1 (the proportion of offspring fertilized by the first male to mate) could be determined for virgin, once, twice, and thrice previously mated males (n=26, 27, 17 and 9 respectively).
2. Manipulating second male sperm transfer. Virgin black males were mated to virgin tan females. These females were isolated and permitted 48 h of oviposition on 50 cowpeas before being remated to tan males that were either virgin, twice or three times previously mated (n=28, 31 and 13 respectively). Paternity was determined as above.

The effect of reduced sperm transfer on female receptivity

Virgin females were individually mated to either virgin, once mated or twice mated males as described above. These females were then split into two groups; in one group females were allowed 16 h of oviposition on 20 cowpeas each and in the other group females were allowed 24 h of oviposition on 20 cowpeas each. Following oviposition, females were placed in separate 25-ml clear perspex cells containing a single virgin male. As a measure of female receptivity the proportion of females remating within 30 min was recorded.

The effect of reduced sperm transfer on fecundity, fertility and zygote survival

Females were allowed one copulation with either virgin, once, twice or thrice previously mated males (n=25, 20, 20 and 7 respectively). Immediately following insemination females were placed in 25-ml gauze-covered pots containing 20 cowpeas as oviposition sites. Cowpeas were changed daily over the following 4 days (15, 10, 7 and 5 cowpeas provided each day respectively). Female fecundity, fertility and zygote survival were determined daily using the methods described in Eady (1991).

Results

Manipulation of sperm transfer

The sequential mating of males with virgin females over a short period of time resulted in males inseminating significantly fewer sperm than if virgin [Fig. 1; number of sperm inseminated by virgin males 56,199± 4,462

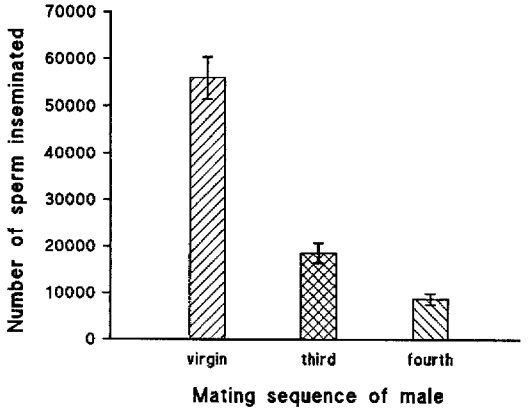

Fig. 1 The mean number of sperm inseminated by virgin, previously twice mated (3rd copulation) and previously three times mated (4th copulation) males. *Error bars*±1SE

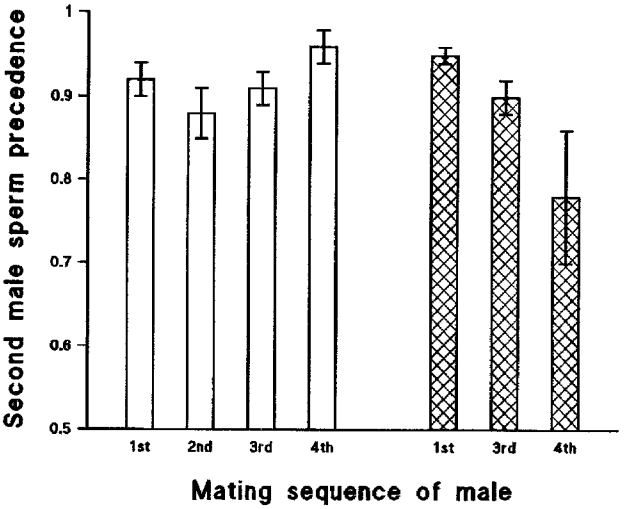

Fig. 2 The effect of male mating sequence on sperm precedence. *Open bars* represent the mating sequence of the first male to mate, *hatched bars* represent the mating sequence of the second male to mate. *Error bars*±1SE

(n=10), previously twice mated males (copulating for the third time) 18,563±2,166 (n=11) and previously three times mated males (copulating for the fourth time) 8,700±1,194 (n=8); one-way ANOVA: $F_{(2,\ 26)}$=64.7, $P < 0.0001$].

Males that mated three or more times were not significantly larger than males that mated only once (mated once male size=1.83±0.02 mm, n=10, mated three times=1.85±0.01 mm, n=11 and mated four times =1.81±0.03 mm, n=8; one-way ANOVA: $F_{(2,\ 26)}$=1.4, $P > 0.05$). The number of sperm inseminated was independent of male size (first copulation r=0.14, n=10, $P > 0.05$; third copulation r=0.5, n=11, $P > 0.05$; fourth copulation r=0.36, n=8, $P > 0.05$).

The effect of reduced sperm transfer on sperm precedence

Reducing the number of sperm inseminated by the first male to mate from a mean of 56,000 to an average of 8,700 (see above) had no effect on P_2 (Fig. 2; Spearman rank correlation coefficient: $r_s = -0.03$, n=79, $P > 0.05$). However, reducing the number of sperm inseminated by the second male caused a significant decrease in P_2 (Fig. 2; Spearman rank correlation coefficient: $r_s = -0.37$, n=72, $P < 0.001$). Therefore, sperm precedence is in part determined by the number of sperm inseminated by the second male, but is largely unaffected by the number of sperm inseminated by the first male to mate.

The effect of reduced sperm transfer on female receptivity

Females mated to virgin males were less likely to remate after either 16 h or 24 h than females mated to non-virgin males (Table 1). Therefore, inseminating large numbers of sperm reduces the likelihood that females will remate within a given period of time. Males mating for the third time in succession, with separate females, fill the spermatheca to capacity (Eady 1992). Therefore, it is likely that female receptivity is determined by the volume of seminal fluid in the bursa copulatrix rather than the number of sperm present in the spermatheca.

The effect of reduced sperm transfer on fecundity, fertility and zygote survival

The number of sperm inseminated had no effect on lifetime female fecundity, fertility or zygote survival (Fig. 3; Table 2). Therefore, ejaculates containing large numbers of sperm do not appear to function as male parental investment or nonpromiscuous mating effort (Gwynne 1984a).

Mechanisms of sperm precedence

The relationship between P_2 and the number of sperm inseminated can be used to test whether patterns of sperm precedence, predicted by mathematical models, correspond to observed patterns of sperm precedence. A close fit between observed and predicted results would indicate that the mathematical model generating the predictions describes the underlying mechanism of sperm precedence. In order to increase the statistical rigour of such a test it is necessary to maximise the number of comparisons between observed and predicted results for each model. This was achieved by combining the results of the present study with those of Eady (1991) in which P_2=0.83 when 24 h separated single inseminations and Eady (1994b) in which the number of sperm inseminated was measured at 46,000 ±3,169, and the number of sperm in the spermatheca

Table 1 The proportion of females remating after 16 and 24 h when originally mated to virgin, once mated or twice mated males

Initial male mating status	Proportion of females remating	
	16 h later	24 h later
Virgin	0.10 (4/40)	0.37 (7/19)
Mated once	0.15 (6/40)	0.58 (11/19)
Mated twice	0.43 (16/37)	0.84 (16/19)
χ^2 (df=2)	14***	8.9*

* $P < 0.05$, *** $P < 0.001$

Table 2 The effect of recent male mating history (whether mated once, twice, three or four times) on fecundity, fertility and zygote survival

	Spearman rank correlation coeff.		
	n	r_s	P
Fecundity	357	−0.03	ns
Fertility	331	−0.07	ns
Zygote survival	331	−0.04	ns

ns $P > 0.05$

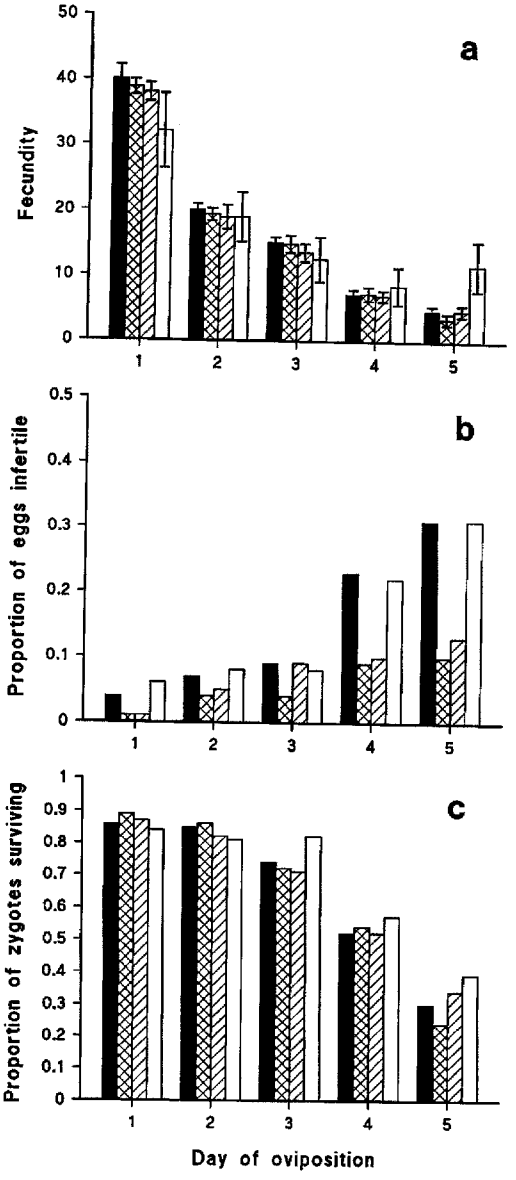

Fig. 3 The effect of manipulated sperm transfer (through the sequential mating of males) on female reproductive success: **a** daily fecundity, **b** daily fertility and **c** daily zygote survival. *Solid, cross hatched, hatched* and *open bars* represent males copulating for the first, second, third and fourth times respectively

estimated at 3,600 (58% of capacity) at the time of the second insemination, 24 h after the first copulation (i.e. immediately before the second insemination).

Parker et al. (1990) developed prospective models for analysing sperm competition data in order to predict the underlying mechanisms of sperm precedence.

Model 1: the fair raffle

Each sperm from each male has an equal chance of entering the fertilization set (spermatheca) (Parker et al. 1990). P_2 simply depends on the relative proportion of male 1 to male 2 sperm in the fertilization set. Thus:

$$P_2=S_2/(S_1+ S_2) \quad (1)$$

where S_1=the number of sperm inseminated by male one and S_2=the number of sperm inseminated by male two. A simple linear version of the raffle is given by rearranging the equation into the following form:

$$1/P_2=(S_1/S_2) + 1 \quad (2)$$

If the fair raffle was operative, a plot of $y=1/P_2$ against $x=S_1/S_2$ would yield a regression with a slope of +1 and an intercept of +1. P_2 values from the present study and those of Eady (1991) and Eady (1994b) were fitted to the linear version of the fair raffle model. The intercept of the regression was significantly different from 1 (t=6.8, df=2, $P < 0.05$) and the slope significantly different from +1 (t=66.0, df=2, $P < 0.001$). Therefore, the fair raffle does not explain the patterns of sperm precedence in *C. maculatus*.

Model 2: sperm displacement

Parker et al. (1990) and Parker and Simmons (1991) have described three mechanisms by which sperm are displaced from the female's sperm stores. No mixing until after displacement is complete (NMAD) describes a mechanism in which every male 2 sperm that enters the fertilization set (the spermatheca in the case of *C. maculatus*) displaces a male 1 sperm. Instantaneous mixing during displacement (IMDD) means that every male 2 sperm that enters the fertilization set displaces

a previously stored sperm, which could be either a male 1 or male 2 sperm. Constant random sperm displacement (CRSD) describes the volumetric displacement of seminal fluid and sperm contained within from the fertilization set. The NMAD model was not tested in the present study as it was considered too unrealistic. The IMDD model was modified to account for an incompletely full spermatheca at the time of the second insemination (Eady 1994b). In their original format, linear versions of each model were derived in order to compare predicted P_2 values against observed P_2 values using standard regression techniques. However, no simple linear versions of the "modified" IMDD (Eady 1994b) or the CRSD models used in this analysis could be derived, therefore an alternative method of testing the predictions of models was applied. If a model accurately describes the displacement of sperm then the regression of observed P_2 against predicted P_2 should have an intercept of 0 and a slope of +1. Observed and predicted P_2 values are from the present study and those of Eady (1991, 1994b). Assuming all inseminated sperm enter the spermatheca (i.e. p=1) the intercept of observed versus predicted P_2 values from the CRSD model was not significantly different from zero (t=0.7, df=2, $P > 0.05$) and the slope not significantly different from +1 (t=1, df=2, $P > 0.05$). However, the CRSD model did not explain a significant amount of the variation in P_2 as the regression of observed versus predicted was non significant ($F_{(1, 260)}$=0.7, $P > 0.05$). Regression analysis was also applied to the observed versus predicted P_2 values obtained from the "modified" IMDD model of sperm displacement. Again the intercept was not significantly different from zero (t=2.25, df=2, $P > 0.05$) and the slope not significantly different from +1 (t=2.8, df=2, $P > 0.05$), but the modified IMDD model did not explain a significant amount of the variation in P_2 ($F_{(1, 260)}$=2.9, $P > 0.05$). Therefore, assuming p=1, both the CRSD and IMDD models of sperm displacement fail to accurately predict the observed patterns of sperm precedence.

However, it appears that not all inseminated sperm enter the spermatheca, therefore $p < 1$ (Eady 1994b). If we assume that either the CRSD or the IMDD model do describe the process of sperm displacement and ultimately sperm precedence in *C. maculatus*, then it is possible to solve for p in each model. Solving for p in both the CRSD and IMDD models suggests that if the models are correct p must change in accordance with the number of sperm inseminated (Fig. 4). Whether p actually changes when different numbers of sperm are inseminated is not known. Therefore, based on our current understanding of the dynamics of sperm movement between the bursa copulatrix and the spermatheca it is not possible to evaluate whether the CRSD or IMDD models of sperm displacement are operative.

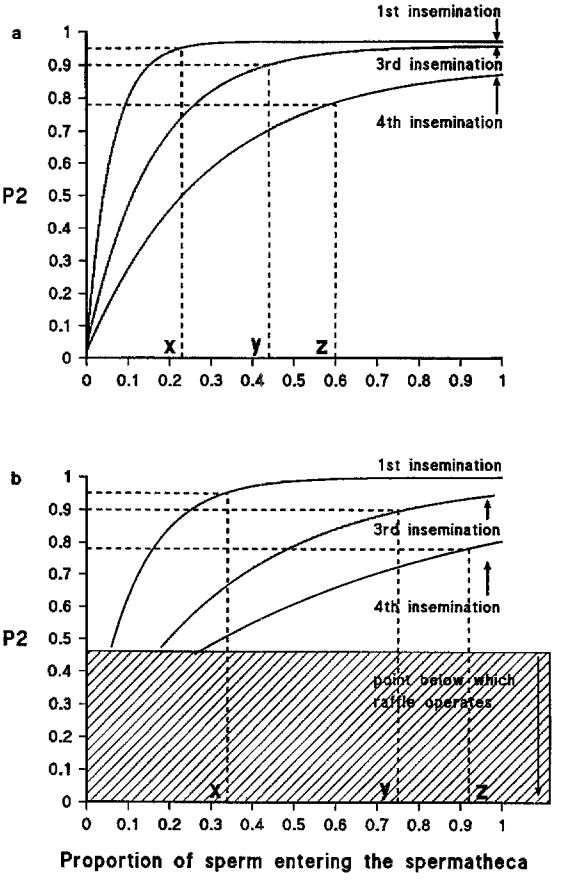

Fig. 4 Estimates of p (the proportion of inseminated sperm entering the spermatheca) from **a** the CRSD model of sperm displacement and **b** the "modified" IMDD model of sperm displacement. The curves depict changes in P_2 with p when different numbers of sperm are inseminated (i.e. during the first, third or fourth copulations). Points x, y and z correspond to the values of p necessary to account for the observed P_2 values. The *hatched area* of **b** is the point in which the "modified" IMDD model no longer operates because the spermatheca is incompletely full and so no displacement occurs

Discussion

This paper investigated the effect of manipulating the number of sperm inseminated on sperm precedence, female mating refractory period and female reproductive success. In addition it examined the effectiveness of sperm competition models in predicting the extent of sperm precedence.

Sperm competition

An 84% reduction in the number of sperm inseminated by the first male (56,000 to 8,700) had no effect on P_2. However, an 84% reduction in the number of sperm inseminated by the second male to mate resulted in a significant reduction in P_2. This suggests that the insemination of large numbers of sperm is, in part, an

adaptation to preempt previously stored sperm. Because P_2 was affected by the manipulation of the second males' ejaculate and not the first males' and the fact that last-male sperm precedence is not the result of direct sperm removal, sperm stratification or the passive loss of sperm between matings (Eady 1994a, 1994b), indicates that last-male sperm precedence in *C. maculatus* is the result of indirect sperm displacement. However, the sperm precedence results of the present study could be an artefact of the experimental design. Because the probability of a female remating was affected by male mating status it is possible that females receiving fewer sperm (or females that utilized sperm at a greater rate or that had smaller sperm reserves) were more likely to remate. This would bias the sperm precedence results by elevating the measured P_2 of females initially mated to virgin males. This could obscure a correlation between male mating status and P_2 when the number of sperm transferred by the first male to mate was manipulated. However, if operative, it would also tend to obscure the relationship between second male mating status and P_2. The fact that there was a negative relationship between second male mating status and P_2 indicates that the extent of sperm precedence is influenced by the number of sperm transferred by the second male. Inseminating large numbers of sperm also had the effect of delaying the time of female remating. This is likely to be a male adaptation to prevent or reduce the likelihood that their sperm will be preempted. This study therefore provides evidence that males inseminating large numbers of sperm have a twofold advantage at fertilization; they fare better at preempting previously stored sperm and they reduce the likelihood that their sperm will be pre-empted. The two opposing selection forces related to paternity (preemption and anti-preemption mechanisms) identified by Parker (1970) appear to be resolved in one adaptation; the production of excess sperm. Whether this one adaptation is in fact many (i.e., the production of different sperm morphs to serve different functions) remains an untested possibility.

Do large inseminations serve as nonpromiscuous mating effort?

Female reproductive success was not affected by the number of sperm inseminated. Therefore, there is no evidence to suggest that large ejaculates function as parental investment. This appears to contradict Brauer (1944), Ouedraogo (1978), Fox (1993) and T. Tufton and P.E. Eady (unpublished work) in which the reproductive success of female *C. maculatus* beetles has been demonstrated to increase with multiple inseminations. However, the discrepancies between the methods (multiple inseminations compared to the manipulation of single inseminations) suggests that some of the mechanisms by which female reproductive success is elevated are more likely than others. For example, the actual process of repeated copulation could increase female reproductive success (Eberhard 1985, 1990; Huck et al. 1985). This hypothesis is compatible with both the present study and those listed above. Another possibility is that the quality, quantity or importance of limiting resources may not be reflected in ejaculate size (Marshall and McNeil 1989), or that female nutritional state might determine the relative importance of male investment (Turner and Anderson 1983; Gwynne 1984b; Svard and Wiklund 1991). T. Tufton and P.E. Eady (unpublished work) demonstrated that the reproductive success of female *C. maculatus* was elevated when females were inseminated multiply, irrespective of female nutritional state. A characteristic feature of Tufton and Eady's study was an increase in fecundity soon after additional copulations, suggesting copulation and/or sperm transfer stimulates egg production (see also Huignard 1974; Huignard et al. 1977; Ouedraogo 1978). The exact mechanism responsible for this increased fecundity in *C. maculatus* is unknown, although in *Drosophila funebris* egg production is stimulated by "active factors" (e.g. paragonial substance PS-2, a compound contained within the seminal fluid that stimulates oogenesis, Baumann 1974). Some active factors are known to have relatively short half-lives (e.g. PS-2, Baumann 1974), therefore, a female strategy of multiple mating could be driven by a dependence on "oviposition stimulants" such as PS-2. Under such circumstances it would be beneficial for a female to remate when the circulating level of active factor fell below that necessary for optimal oviposition. What the optimal rate of egg production is will depend in part on the environment. For example, in *C. maculatus* the availability of oviposition sites affects the rate of egg maturation (Wilson and Hill 1989). If optimal egg production rate is environmentally determined and copulation affects egg production, one could envisage female mating strategy to be in part environmentally determined. A preliminary investigation supports this hypothesis; female *C. maculatus* are less likely to remate if they have few oviposition sites (P.E. Eady unpublished work).

The effects of ejaculate size on male and female reproductive success in *C. maculatus* are similar to those recorded for the European swallowtail butterfly *Papilio machaon* by Svard and Wiklund (1991). Virgin males of *P. machaon* transfer ejaculates twice as large as any subsequent ejaculate they might produce. Svard and Wiklund (1991) demonstrated that ejaculate size had little effect on female reproductive output and they concluded that large ejaculate production was maintained primarily by selection on males to induce long inter-mating refractory periods in females. A proximate explanation for this link between inter-mating refractory periods and spermatophore size was demonstrated by Sugawara (1979), in the cabbage white butterfly

Pieris rapae crucivora. In this species stretch receptors in the bursa copulatrix were more active (in terms of afferent nerve impulses) when the bursa contained a large ejaculate compared with a small ejaculate. This neuronal complex probably defines the information responsible for the observed behavioural change from acceptance to refusal of mating (see also Baumann 1974; Gwynne 1986). The bursal "valves" of *C. maculatus* (Mukerji and Bhuya 1973; Eady 1994a) could function in a similar manner to the stretch receptors of *P. rapae crucivora*. Such a mechanism provides a theoretical, proximate explanation for the increase in female refractory period with increased ejaculate size.

Mechanisms of sperm precedence

The results of the present study and those of Eady (1994a, 1994b) suggest that last-male sperm precedence in *C. maculatus* is the result of indirect sperm displacement. Only displacement can explain the fact that reducing the size of the first male's ejaculate had no effect on P_2, while reducing the size of the second male's ejaculate, by the same amount, resulted in a significant reduction in P_2. The fair raffle model also predicted a decline in P_2 when the number of sperm inseminated by the second male was reduced. However, the model also predicted a decrease in P_2 when the number of sperm inseminated by the first male was increased; no such decline in P_2 was observed. Therefore the mechanism of sperm precedence in *C. maculatus* is unlikely to be a simple lottery based on the number of sperm transferred. However, sperm precedence is likely to be determined by a lottery based on the number of sperm in the spermatheca, although what determines which sperm enter the lottery (spermatheca) remains unclear. The number of sperm inseminated and the number stored in the spermatheca are probably linked; increasing the number of sperm inseminated is likely to result in increased numbers of sperm in the spermatheca, up until the maximal spermathecal capacity is reached. How sperm enter a full spermatheca and what determines the rate at which they enter requires further investigation (Eady 1994b).

Exactly how sperm are displaced remains elusive. The CRSD and IMDD models of sperm displacement failed to accurately predict patterns of sperm precedence in *C. maculatus*. However, rejection of these mechanistic models would be premature for three reasons. Firstly, it is difficult to perform sufficient sperm transfer manipulations to test the models of sperm displacement using regression analysis. This is especially true when the interruption of copulation, as a means of manipulating sperm transfer, is not a possibility. The results of studies that use interrupted copulation as a means of manipulating sperm transfer should be interpreted with care because 'copulatory courtship' (Eberhard 1985) may influence the fate of inseminated sperm. Secondly, in testing the displacement models P_2 was predicted from the average number of sperm transferred. Some of the unexplained variation in the models may be explained by variation in the number of sperm delivered by individual males, although the variation within the treatments was considerably less than the variation between the treatments. Finally, accurate measurement of the proportion of inseminated sperm entering the spermatheca (p) was not possible. This value is essential if we are to understand the mechanisms and extent of sperm displacement. In this respect the models of sperm competition analysed in this paper have served a crucial role in highlighting our current lack of understanding of the dynamics of sperm movement within the reproductive tracts of females.

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