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SPECIAL ISSUE: FEMALE MATING FAILURES

Why do so many flour beetle copulations fail?

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Abstract

Copulations that fail to result in fertilisations are common across a broad range of species, and remain enigmatic given the inherent costs that are being paid by both partners. The determinants of failures are varied and can take effect before, during, or after copulating. In the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), population estimates of copulations failing to result in fertilisations as high as 55% have been reported, suggesting that the causes of these failures play an important role in the mating system, or that the costs experienced by those determining failure, in this system likely the females, is low. Here, we show that failure is determined peri-copulation; successful spermatophore transfer (indicated by change in mass of mating individuals) typically results in offspring production, suggesting that mechanisms of post-copulatory female choice do not cause outright failure. The proportion of copulations that failed to result in fertilisations was apparently not influenced by the relatedness of mating pairs, the age, mating status, or mass of the female, or the interval she experienced between matings. This suggests that alternative adaptive explanations are responsible for the prevalence of copulations that fail to result in fertilisations in this species, or that the costs involved are sufficiently small, so that selection against such failures is weak.

Introduction

Copulations failing to result in fertilisations are a curious phenomenon observed across a range of taxa, including insects. The proportion of matings that do not succeed can vary dramatically; a review of 30 insect species showed a mean failure rate of 22%, ranging from 0 to 63% (García-González, 2004). Upon initial consideration, the prevalence of copulations failing to result in fertilisations is surprising as individuals who mate unsuccessfully bear costs; resources such as time and energy are wasted (Dewsbury, 1982; Forsyth et al., 2005), and exposure to predators, pathogens, and parasites is increased (Magnhagen, 1991; Sheldon, 1993). If the assumption were made that failure serves no purpose, its prevalence should be reduced through selection; however, despite the apparent costs borne by individuals, considerable levels persist in many species.

Many documented cases of inability to reproduce are due to lack of opportunities to mate (Rhainds, 2010), or due to infertility or sterility (García-González, 2004). Here, we examine failures to produce offspring from behaviourally normal copulations between fertile individuals. In these cases, there are two principle behavioural strategies that might influence the likelihood of copulations failing to result in fertilisation: decisions by the male to transfer few or no sperm during copulation, and decisions by the female not to accept sperm during copulation, or not to use sperm post-copulation. Alternatively, copulation failures may not be the result of strategic decisions at all, but may be non-adaptive failures arising from physical or chemical incompatibilities, or out of compromises between failure rate and other aspects of mating, such as speed and energetic costs.

Males of a number of species are able to manipulate the size of their investment through strategic sperm allocation (Wedell et al., 2002). If the level of allocation is particularly low, sperm numbers might be too few for successful fertilisation to occur, potentially explaining a number of cases of failures. The likelihood of insemination can also be influenced through manipulation of copula duration. Many species have been shown to have a minimum time requirement to allow the

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successful transfer of sperm (Lorch et al., 1993; Engqvist & Sauer, 2003), and if copula duration is too short then insemination may not be possible. Donors and recipients can therefore manipulate the chance of successful insemination by halting copulation.

Often, a male's decision to allocate sperm is imposed upon a female through coercion or forced copulations (Thornhill & Sauer, 1991; Smuts & Smuts, 1993; McKinney & Evarts, 1998). In situations such as these, a female might have some bearing on the outcome of the copulation after insemination has taken place. There are now a number of recorded examples of species in which female adaptations have developed increasing post-copulatory control over their offspring's paternity, a phenomenon known as cryptic female choice (Thornhill, 1983; Eberhard, 1996). In these cases, a female is able to favour an individual on the basis of either phenotypic traits, or traits of his sperm. Variations of sperm choice have been recorded in a range of species, e.g., fruit flies, *Drosophila melanogaster* (Meigen), weevils, *Gonipterus scutellatus* (Gyllenhal), and decorated field crickets, *Gryllodes supplicans* (Walker) (Santolamazza Carbone & Cordero River, 1998; Clark et al., 1999; Stockley, 1999).

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), is highly polygamous, engaging in copulations every few minutes, and has a reproductive lifespan of many months (Sokoloff, 1974; Lewis, 2004). Each individual therefore has many opportunities to mate during their lifetime, and consequently reduced pressure for each mating event to be successful. This species exhibits significant levels of failure – estimates of copulations not leading to the production of offspring range from 15 to 55% (Bloch Qazi et al., 1996; Lewis & Iannini, 1995). Many of the failures can be accounted for by unsuccessful intromission. However, it is not known, which male traits affect the ability to accomplish this, or for what reasons a female might refuse a male (Pai & Yan, 2003). Sperm depletion is unlikely to be a cause of failure; although declining stores reduce the number of eggs a male can fertilise, matings involving sperm-depleted males are no more likely to completely fail to produce offspring than those involving non-depleted males (Bloch Qazi et al., 1996). Instead, copulation failure might be a behavioural strategy to compensate for the apparent absence of pre-copulatory mate choice in this species. *Tribolium* lack visual and acoustic signals, and signals of male quality such as competition or courtship of females are not used (Wojcik, 1969; Sokoloff, 1974). Assessment of mates might be carried out through pheromone and other chemical cues (Levinson & Levinson, 1995; Howard & Blomquist, 2005); however, this might rely upon close proximity or physical contact. In the absence of pre-copulatory mate choice, their range of behaviours during and after a mating could provide them with the opportunity to influence its outcome, through deliberate prevention of insemination or fertilisation.

Matings take place on the surface of flour. The male mounts the female and rubs the sides of her body with his legs, then transfers a spermatophore into the female's bursa copulatrix. Once the male has mounted, the female usually adopts a distinct posture during a period of quiescence, and there is evidence that this female cooperation is required for successful sperm transfer (Bloch Qazi, 2003). After copulation, females play an active role in moving sperm from the site of insemination into storage (Bloch Qazi et al., 1998), and can expel the spermatophore, which often still contains sperm (Fedina, 2007). Both of these represent possible mechanisms for cryptic female choice.

The first aim of this research was to identify whether failure is initiated during or after the mating. To achieve this, individuals were weighed before and after copulation to assess whether or not spermatophore transfer had taken place, anticipating a decrease in male mass and an increase in female mass with successful insemination, as demonstrated in previous studies (Savalli & Fox, 1999; Edvardsson & Tregenza, 2005). If instigated during copulation, failure should be associated with lack of spermatophore transfer. However, if failures are a result of post-copulatory mechanisms, such as cryptic female choice, then offspring would not be expected to be produced despite successful spermatophore transfer. In pinpointing the stage of peri- or post-copulation in which failure occurs, we might be brought closer to identifying specific behaviours or mechanisms involved.

The next aim of this research was to investigate potential determinants of copulations that fail to result in fertilisation: genetic incompatibility has been shown to affect the behaviour and mating success of a broad range of species. *Tribolium castaneum*, a species known to exhibit inbreeding depression (Gaur & Rao, 1997), might prevent insemination or fertilisation to avoid investing in sub-optimal, inbred offspring, and so we predict that failure to produce offspring will occur more frequently when mating pairs are close kin. Another potential determinant of copulations failing to result in fertilisation is the mating status of the individuals involved. We predict that those subjected to a longer interval between matings will be more motivated to acquire sperm, and so failures will be less likely to occur. We also consider the extent of female cooperation (quiescence), copula durations, the mass of both partners, and their ages.

Materials and methods

General protocol

Three strains of *T. castaneum* were used in the trials, GA-1, Rd (bearing antler-like antennae, a dominant phenotypic marker visible to the naked eye; both supplied by Richard Beeman, Center for Grain & Animal Health Research, Manhattan, KS, USA), and Oz, a wild-type strain, collected from a flour store in Canberra, Australia. All individuals were sexed as pupae, and isolated to ensure virginity upon eclosion. Pupae were kept separately in 2-cm^2 cells containing 1 g of flour mix (90% flour, 10% yeast), and incubated at 30 °C. Upon reaching sexual maturity, adults were used in three mating trials, described below.

For ease of identification, males were marked with a small dot of blue paint prior to each trial. All trials were carried out in 2-cm^2 grid cells, which were lined with paper to provide traction, at 20–23 °C under standard laboratory electrical lighting. A male was given a maximum of 30 min to mate once with the female, but they were allowed multiple mounting attempts during this time. Only after remaining mounted for 30 s was this counted as a copulation (copulations less than 30 s long are unlikely to result in spermatophore transfer under any circumstances). Time to initiation of copulation and copula duration were recorded. If the female spent more than 20 s motionless, her behaviour was recorded as quiescent. Males were removed as soon as the pair separated, or if they did not copulate within 30 min. Each female was then kept in a nursery pot containing 30 g of flour mix, and incubated at 30 °C.

Spermatophore transfer

Virgin individuals were shaken across the surface of a fine mesh to remove as much flour dust as possible, and then weighed prior to and after mating using a Mettler Toledo XS3DU micro-balance. Each weighing was repeated 3× and a mean mass was calculated. Spermatophore transfer was considered to have taken place if the female gained more than 0.01 mg between the pre- and post-copulatory weighings. This threshold value was used so that weighing errors, or transfer of grains of flour onto the females, were not mistaken for spermatophore transfer. Nineteen pairs of Oz strain individuals were used, aged 9–19 days post-eclosion. Six pairs of Rd strain were used, aged 8–10 days post-eclosion. Success or failure of the copulation was assessed through presence or absence of larvae in the nursery pots after 15 days.

Relatedness

Fertilised GA-1 females from general stock were isolated in pots and allowed to lay eggs. Pupae from each family pot were sexed and isolated (as described above). Paternity of the offspring was unknown, but the majority from each family will have been full-siblings, because in this species, the percentage of offspring sired by the last male to mate is ca. 90 (Arnaud et al., 2001). Whether full-sibs or half-sibs, all were considered ‘related’. Males from other families were used as ‘unrelated’ males. Each female was mated once; half of the sample by a related male and half by an unrelated male. Fifty replicates of each treatment were carried out, using combinations of individuals from 38 families, aged 17–32 days post-eclosion. Success or failure of the copulations was assessed through presence or absence of larvae in the nursery pots after 15 days.

Inter-copulation interval

GA-1 females were each mated twice; firstly to a wild-type GA-1 male and secondly to an Rd male. The interval between the two matings was either 2 days (‘short’) or 14 days (‘long’). Nineteen replicates of the short duration treatment were carried out, and 18 of the long treatment. Success or failure of the copulations was assessed through presence or absence of offspring in the nursery pots bearing wild-type or Rd-antennae 35–40 days after the second mating.

Statistical analysis

The relationship between change in male and female mass due to spermatophore transfer was analysed using linear regression. Logistic regression was used to assess copulation success according to change in female mass during copulation. The effects of spermatophore transfer on frequencies of successes and failures to produce offspring were analysed using a χ^2 test, likewise the effects of relatedness of mating partner and inter-copulation interval. The effects of female quiescent behaviour, copula duration, latency to initiation of copulation, and mass and age of individuals were analysed in a generalised linear mixed model with binomial errors, including ‘strain’ as a random effect. Model selection was based on ANOVAs using the χ^2 distribution. All analyses were conducted using R 2.12.2 software (R Development Core Team, 2011).

Results

Spermatophore transfer

Each individual’s mass was determined from three repeated measures (r = 0.99 ± 0.0001). The increase in a female’s mass between weighings is dependent on the decrease in male mass (linear regression: $R^2 = 0.312$, $F_{1,29} = 14.58$, $P<0.001$; Figure 1), indicating that mass is transferring from male to female through insemination. An increase in female mass can therefore be used as a proxy for successful spermatophore transfer. The few cases of increase in a male's mass with decrease of a female's are likely due to transfer of particles of flour from the female to the male. There is a marginally non-significant relationship between copula duration and amount of sperm transferred (Pearson correlation: $t = -1.53$, d.f. = 29, $P = 0.068$).

The majority of copulations failing to result in fertilisation occur when there is a lack of spermatophore transfer. When spermatophores are successfully transferred, offspring production is unlikely to fail ($\chi^2 = 17.0$, d.f. = 1, $P<0.001$; Figure 2). This suggests that failures are not brought about through post-copulatory behaviours or mechanisms, but during copulation.

Likelihood of success changes significantly with increased change in female mass (Logistic regression: $z = 2.71$, $P<0.05$; Figure 3). There appears to be a threshold change in mass at which copulations become successful; the mean change in female mass at which copulations fail is −0.003 mg, whereas the mean change at which they succeed in offspring production is 0.019 mg.

Relatedness

The outcome of copulations is not affected by the relatedness of mating pairs ($\chi^2 = 0.011$, d.f. = 1, $P = 0.92$; Figure 4), suggesting that the effects of inbreeding are not costly in this species, or that kin recognition does not occur.

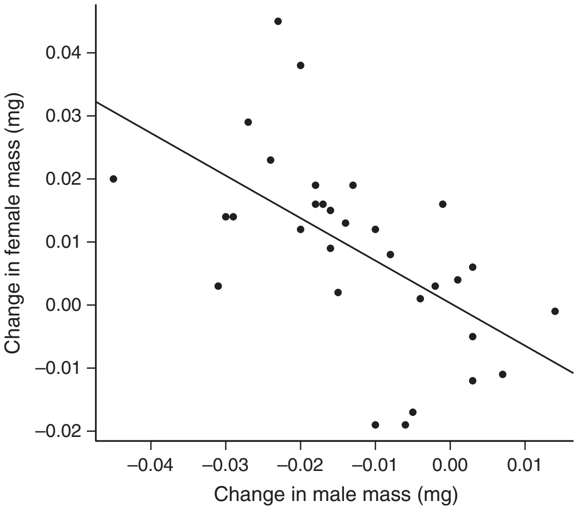

Figure 1 Relationship between the change in mass of male and female *Tribolium castaneum* resulting from copulation. Decrease in male mass is associated with increase in female mass, which can be attributed to the transfer of a spermatophore from the male to the female.

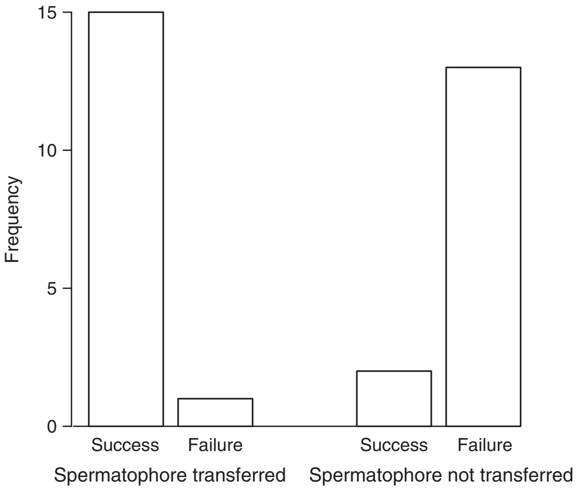

Figure 2 Success and failure of offspring production according to spermatophore transfer inferred from mass change in female *Tribolium castaneum*.

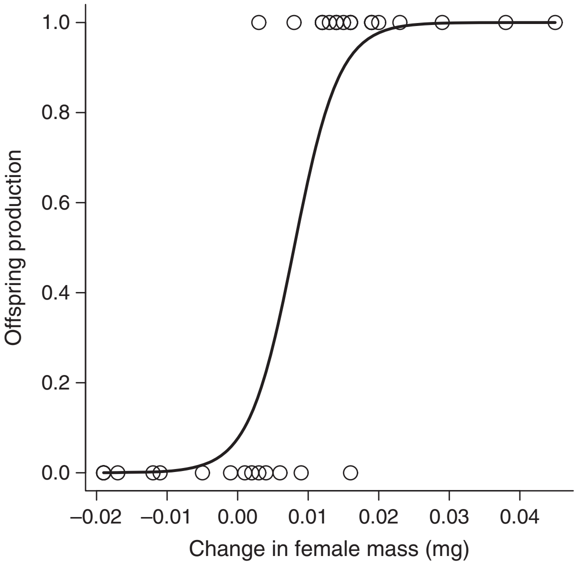

Figure 3 Likelihood of production of offspring depending on female *Tribolium castaneum* mass change during copulation. Mass change is a good predictor of production of offspring, indicating that mass change is associated with sperm transfer.

Inter-copulation interval

The number of failures to produce offspring from post-interval matings was extremely high, regardless of treatment ($\chi^2 = 0.073$, d.f. = 1, $P = 0.79$; Figure 5). The high failure rate might be attributed to the Rd strain of males used, which were comparatively smaller and less vigorous

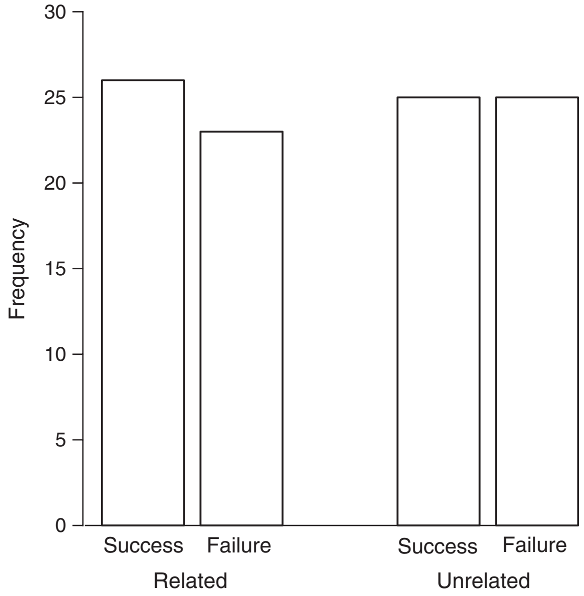

Figure 4 Frequency of copulations succeeding and failing to produce offspring depending on relatedness of mating pairs in *Tribolium castaneum*.

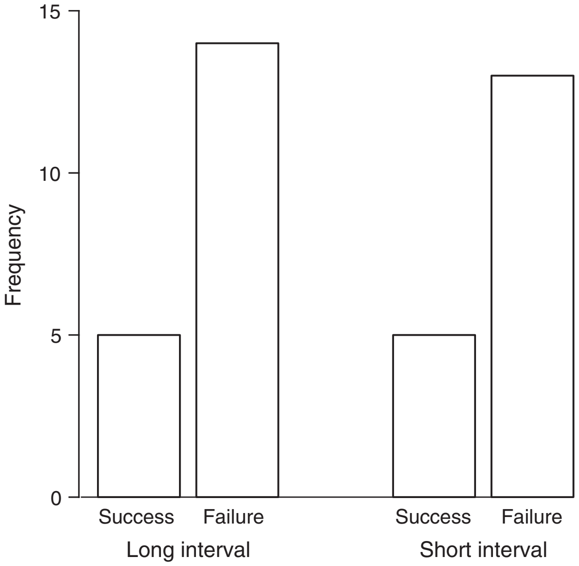

Figure 5 Frequency of copulations succeeding and failing to produce offspring depending on inter-copulation interval in *Tribolium castaneum*. The high number of failures may be due to the genetic health of the stock, but this rate does not differ with the interval.

than wild-type beetles. The first male to mate sired significantly fewer offspring when the second male successfully fertilised the female (Wilcoxon rank sum test: W = 235, P<0.001). This effect was greater in the long interval treatment group (W = 25, P = 0.012).

Analyses of the effects of female quiescence, copula duration, latency to initiation of copulation, age, and mass were carried out using the data combined from the spermatophore transfer and relatedness experiments. Of these factors, male mass, female quiescence, and copula duration had effects on success rates (GLMM with binomial errors; female quiescence: $\chi^2 = 4.3$, d.f. = 1, P = 0.038; copula duration: $\chi^2 = 5.21$, d.f. = 1, P = 0.023; male mass: $\chi^2 = 10.96$, d.f. = 1, P<0.001).

Discussion

A large percentage of *T. castaneum* copulations is unsuccessful. Anywhere between 15 and 55% of mating attempts do not lead to the production of offspring (Lewis & Iannini, 1995; Bloch Qazi et al., 1996). This high failure rate is combined with a relatively long adult lifespan (Sokoloff, 1974) and a highly polygamous mating system: both sexes will mate many times in quick succession (Lewis, 2004). Each individual therefore has the opportunity to engage in many copulations. Once females have been inseminated, they are able to store and use sperm to fertilise eggs for up to 3 months (Attia, 2004), and so females that have already mated experience a rapid decline in the costs of mating failure. Males, on the other hand, experience the same loss of reproductive opportunity with any mating that fails to result in offspring production. This suggests that either males are interfering with the copulation success of the future mates of females they mate with, or more likely that females are directly responsible for the high failure rate in this species. However, little is known of the proximate mechanisms driving failure, or the factors involved in determining the outcome of a mating.

Spermatophore transfer

The 'spermatophore transfer' experiment was designed to establish whether failures arise peri- or post-copulation. The results from these trials provide no evidence for failures occurring after spermatophore transfer has occurred. If mechanisms of post-copulatory cryptic female choice were involved, then a significant proportion of copulations would fail to result in fertilisations despite successful spermatophore transfer. Mechanisms of cryptic female choice have been shown to be involved in determining the number of offspring that result from each insemination (Fedina, 2007). However, in agreement with the present research, in no cases is there mention of outright failure. Instead, the results demonstrate that failures occur peri-copulation, and are due to the female not receiving or uptaking ejaculate.

The technique of weighing individuals to determine whether females are inseminated does not appear to have been used before in published studies of *Tribolium*, although it has been used successfully with another beetle species, *Callosobruchus maculatus* (Fabricius) (Savalli & Fox, 1999; Edvardsson & Tregenza, 2005). Unlike the traditional methods of dissecting females after copulation, this technique leaves them unharmed and so post-copulatory behaviours that might affect offspring production can also be considered. It has been noted that until recently, sexual selection in *Tribolium* has been studied in isolation from other sources of selection (Fedina & Lewis, 2008). This technique might be useful in allowing peri- and post-copulatory behaviours to be considered more comprehensively.

Relatedness

In *Tribolium,* the number of offspring produced from inbred matings is significantly lower than from outbred matings, and they are known to exhibit inbreeding depression (Pray & Goodnight, 1995; Gaur & Rao, 1997). Theory predicts that species that experience costs of inbreeding should avoid mating with relatives, so as not to invest in the production of sub-optimal offspring (Thornhill, 1993). Contrary to predictions, we find no relationship between relatedness of mating partners and copulations failing to result in the production of offspring. This suggests that the costs associated with inbreeding depression are not sufficiently great for individuals to wholly avoid producing offspring with kin, or that *Tribolium* are unable to recognise kin. There is evidence that males are capable of kin recognition, whereas females are not (Attia, 2004). The lack of effect of relatedness on copulations failing to result in fertilisation found in this study may be due to the females' inability to discern kin, indicating that instances of failure are determined by females.

The lack of discrimination may have been exaggerated by the mating status of the individuals. Had the females been previously mated, they would already have enough sperm stored to fertilise large numbers of eggs (females can continue to lay fertilised eggs for up to 3 months after insemination; Attia, 2004), and thus have been more choosy regarding their subsequent mating partners. Alternatively, the virgins used in these tests may have been more motivated to mate, and so less discriminatory (Gabor & Halliday, 1997; Jennions & Petrie, 2000).

Inter-copulation interval

The 'inter-copulation interval' experiment was designed to investigate differences in success of copulations depending on the time since the female last mated. Females are able to use sperm to fertilise eggs for up to 3 months after insemination (Attia, 2004). However, the inevitable decline of these stores suggests that those females that have not mated for some time may be less choosy regarding subsequent mating partners, and be more motivated to mate (Gabor & Halliday, 1997; Jennions & Petrie, 2000). Theory therefore predicts that females subjected to a longer inter-copulation interval would be less likely to initiate copulation failure. It is difficult to draw clear conclusions from our data; regardless of treatment, the rate of failure for post-interval matings was unusually high; only 31% led to the production of offspring. This may be explained by the strain of male beetles used in post-interval matings, but strain of mating partner has previously been shown to have no effect on success of sperm transfer (Attia, 2004). The explanation might therefore lie in the genetic health of the particular stock of Rd used, which may have reduced the sperm transfer capacity of males.

Of the other factors examined that might influence the outcome of a mating, the extent of female cooperation, copula duration, and male mass all had a bearing on the likelihood of success. Female cooperation (quiescence) is important in sperm transfer in *Tribolium*, and consistent with Bloch Qazi (2003), the assessment of quiescence made during these trials was a good predictor of insemination success. Despite this, there are several cases of offspring being produced from copulations during which no quiescence was recorded. This suggests that although important in sperm transfer, quiescence is not essential.

The length of copulation had a significant effect on the likelihood of its success, with failures more likely to occur with shorter durations, although duration has previously been shown to have no effect on the number of sperm transferred (Bloch Qazi et al., 1996). Combined these two pieces of information suggest that a longer copulation increases the time that a female has to become quiescent and for the male to achieve intromission; the transfer of the sperm itself is unaffected, indicating that this is a relatively fast process. Likewise, male mass, found to have a significant effect on the likelihood of successful production of offspring in this study, has previously been shown to have no effect on the number of sperm transferred (Bloch Qazi et al., 1996). This might suggest that smaller size decreases a male's ability to achieve intromission, or that females are less likely to accept spermatophores from smaller males; although when a spermatophore is transferred, the absolute number of sperm transmitted is unaffected.

Conclusions

Not all failures are associated with short durations or lack of female cooperation, suggesting that there may be a further mechanism through which failure is brought about.

Additional research is required to quantitatively describe male and female behaviours during copulation, and to relate these to the likelihood of spermatophore transfer. It also remains unclear what ultimate factors are involved in determining the outcome of a mating. Future research might include a more detailed assessment of the effects of male phenotypic traits, and other potential factors such as differences in the conditions that individuals experience, for example, rearing density or resource availability. Studies specifically examining copulations failing to result in fertilisations in a range of different species should be encouraged, so that broader explanations for the prevalence of failure might be identified.

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