



Last male wins the egg fertilization fight: A case study in ladybird, *Menochilus sexmaculatus*



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ABSTRACT

Sexual selection and the mechanisms involved in sperm competition have not been greatly explored in ladybird beetles. The present study was conducted to investigate the processes of sperm competition and the role of mate guarding behaviour in its regulation in ladybird beetles. We investigated these questions in polyandrous females of the ladybird, *Menochilus sexmaculatus* (Coleoptera: Coccinellidae) using a phenotypic marker (typical and intermediate morph) to assess paternity of offspring; to determine sperm competition. We conducted two double mating experiments: (i) complete first and second matings, and (ii) disrupted first and complete second matings each using homomorphic and heteromorphic pairing in alternation. Males which mated last were found to sire up to 72% of the offspring produced, indicating last male sperm precedence. Morph itself, independent of mating order, did not have a significant effect on proportion of offspring sired. Paternity share of the last male was negatively associated with mating duration of the first male; mating duration of the first male being indicative of mate guarding. This therefore indicates that prolonged matings by first males are essentially examples of post-copulatory mate guarding to prevent last male sperm precedence.

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1. Introduction

In organisms that undertake polyandrous matings alongside precopulatory sexual selection, two forms of postcopulatory sexual selection also occur i.e. sperm utilization and sperm competition (Parker, 1970; Ridley, 1989). Sperm utilization is a female driven process in which sperm from multiple males are differentially selected by the female to fertilize her eggs (Parker, 1970). On the other hand, sperm competition is a process in which sperm from one or multiple males compete to fertilize the given set of ova from a single female. Sperm competition is known to be influenced and modified by a large number of factors and processes including: (i) sperm precedence – non-random utilization of sperm from a particular male when the sperm of multiple males compete within a female (Watson, 1991; Birkhead and Møller, 1998; Kehl et al., 2013); (ii) sperm mixing – mixing of sperm from multiple males (Simmons and Parker, 1992); (iii) sperm flushing – indirect removal of the sperm of previously mated males from the female's sperm storage organ(s) by the incoming ejaculate of a copulating male (Birkhead and Møller, 1998; Gwynne, 2012; Yan et al., 2013); (iv) the number

of mating males and the mating interval (Drnevich, 2003); (v) ejaculate manipulation or number of sperms transferred (Sirota et al., 2011; Baer, 2014), and (vi) sperm quality (Boschetto et al., 2011).

A number of studies have observed 'last male sperm precedence' in polyandrous insects, in which the last male that mates sires most of the offspring (Bissoondath and Wiklund, 1996; De Jong et al., 1998; Simmons, 2001; Friedlander et al., 2005; Solensky and Oberhauser, 2009). With extensive support for last male sperm precedence in insects, the emphasis of studies has now shifted to the investigation of various tactics (mentioned below) evolved by competing males to lower the risk of it occurring. Some males of organisms that display last male sperm precedence attempt to reduce the probability of female remating, and subsequent sperm competition, by placing a genital plug. Genital plugs are formed by coagulation of ejaculate in the female reproductive tract to reduce access for other males (Alcock, 1994; Wigby and Chapman, 2004; Seidemann, 2015). In other cases, sperm competition can also be reduced by post-copulatory mate guarding where genital contact between male and female is maintained after sperm transfer (Simmons, 2014; Chaudhary et al., 2015). Additionally, some other factors, like the age of both partners (Mack et al., 2003; Kehl et al., 2013) and successive matings with two different males (Singh et al., 2011) are known to regulate the level of sperm precedence.

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Post-copulatory mate guarding in insects has been regarded as a major means for securing paternity (Sherman, 1989; Sakaluk, 1991; Komdeur et al., 1999). Mate-guarding *per se* is regarded as a sperm protection mechanism (Gwynne, 1997; Wedell, 1998) in which a male, by remaining in copula (with aedeagus inserted) post sperm transfer, reduces last male sperm precedence and increases its paternity (Parker, 1979; Telford and Dangerfield, 1990; Chaudhary et al., 2015).

Until recently, attempts to document patterns of sperm precedence have been constrained by methods available for discriminating between sires. Though in the past few years various measures have been adopted for assigning paternity such as irradiated male technique in *Cadra cautella* (McNamara et al., 2009), molecular markers in *Pectinophora gossypiella* (Higginson et al., 2005), chemosterilization in *Ephestia kuehniella* (Xu and Wnag, 2010a,b), and phenotypic markers in some hymenoptera and lepidoptera (Brakefield et al., 2001; Kehl et al., 2013). In the ladybird beetle, *Adalia bipunctata*, phenotypic markers (De Jong et al., 1998), allozymes (Ransford, 1997) and microsatellites (Haddrill et al., 2008) have been used to assign paternity following multiple matings.

In ladybird beetles, previous studies have established that they are polyandrous, exhibit multiple matings (Haddrill et al., 2008), and display mate choice (Mishra and Omkar, 2014). In case of multiple matings, ladybirds are known to store sperm in the spermatheca where sperm survives for extended periods (Hodek, 1973), thus increasing chances for sperm competition. Previous studies in *A. bipunctata* have revealed the occurrence of last male sperm precedence (De Jong et al., 1993; Ransford, 1997; Haddrill et al., 2008). A recent study in another ladybird, *Menochilus sexmaculatus* (Fabricius) has revealed the occurrence of mate guarding behaviour (Chaudhary et al., 2015). However, it is not known if mate guarding is a means of reducing last male sperm precedence in ladybirds, as has been reported in many other organisms (Allen et al., 1994; Sutter and Lindholm, 2016).

In view of the scarcity of information on these processes in ladybirds, we decided to take our previous study of mate guarding in *M. sexmaculatus* (Chaudhary et al., 2015) forward and investigate if observed mate guarding is a means of manipulating sperm precedence. We hypothesize that (i) differential sperm utilization in the form of sperm precedence will be observed, and (ii) the first male will increase his fertilization success by reducing sperm precedence through post copulatory mate guarding.

2. Materials and methods

2.1. Study species

Menochilus sexmaculatus commonly known as the zigzag ladybird is a locally abundant, generalist (Agarwala and Yasuda, 2000), polymorphic (Kawakami et al., 2015; Dubey et al., 2016), fast developing ladybird with high reproductive output (Omkar et al., 2005). Typical, intermediate and melanic morphs have been observed, though the latter is not found in nature and was reared in the laboratory through selection studies (Dubey et al., 2016). Typical morphs have black zigzag lines on yellowish-red elytra, while the pure melanic morph have entirely black elytra and a highly melanized pronotum with very pale visible streaks (Plate 1). Intermediate morphs of varying degrees (I_1 , I_2 and I_3 ; Dubey et al., 2016), which have extensive fusions of the zig-zag lines, and none to some melanization on the pronotum, are found in nature. I_3 morphs are very abundant, second only to typical morphs (personal observation). For the current study, field collected typical and intermediate I_3 morphs were used (Plate 1) and for sake of convenience, the latter will be referred to as intermediates (I) throughout the manuscript.

2.2. Stock maintenance

Adults of typical (T) and Intermediate (I) morphs of *M. sexmaculatus* were collected from the agricultural and horticultural fields surrounding Lucknow, India ($26^{\circ}50'N$, $80^{\circ}54'E$) and brought to the laboratory for rearing. Homomorphic pairs were formed from the field collected adults and placed in transparent plastic Petri dishes ($14.5 \times 1.5 \text{ cm}^2$; one pair per dish) and provided with pea aphids, *Acyrtosiphon pisum* (Harris) reared on host plant *Vicia faba* L. in greenhouse (maintained at $22 \pm 3^{\circ}C$; $65 \pm 5\%$ R.H. and 14L: 10D photoperiod), which were replenished daily. Petri dishes were then placed at $27 \pm 1^{\circ}C$; $65 \pm 5\%$ R.H.; 14L: 10D in BOD incubators (Yorco Super Deluxe, YSI-440, New Delhi, India). The pairs were checked daily for oviposition. The eggs laid were collected every 24 h and incubated under above abiotic conditions until hatching. The larvae were reared individually until adult emergence in plastic Petri dishes (size as above). 10-day-old adults of both morphs (second generation) were taken from the stock culture for experiments.

2.3. Experimental design

2.3.1. Sperm precedence

This experiment was designed to investigate the occurrence of sperm precedence through double mating experiments using two different male morphs so that paternity could be assigned. A previous study conducted in our laboratory reveals that homomorphic pairs produce progeny of the same morph at $25 \pm 2^{\circ}C$, while heteromorphic pairs of typical and melanic adults result in an intermediate morphs that are lighter than the melanic parent and darker than the typical parent (Dubey et al., 2016). Thus, in this study two different morphs of the ladybird are used to ascertain paternity of the offspring.

Well fed, 10-day-old, unmated adults were paired for a single mating and allowed to mate until they disengaged naturally. The mating duration was recorded manually. Mated females were each paired with a second unmated male of different morph immediately after her first mating. The pair were placed in new Petri dishes and again allowed to mate until they disengaged naturally. Immediate pairing with the second male was required as an interval between first and second mating may allow the female to eject the previous spermatophore, thus potentially removing any effect of last male sperm precedence. The occurrence of mating was ascertained by observing genital contact under a stereoscopic binocular microscope (Magnus, at 16 X magnification). All matings were conducted at $27 \pm 2^{\circ}C$ under 14L:10D conditions. Time to commence second mating and mating duration was recorded. Second mating occurred in all pairs and started on average within 4.00 ± 0.35 min of pairing. Following double mating setups were designed on the basis of morph of ladybird beetle (Table 1).

Each treatment had 10 replicates. Post mating, the females were isolated and kept on *A. pisum*, replenished daily and oviposition and egg viability recorded every 24 h for the next five days. After hatching, all neonates were transferred and reared individually in Petri dishes (to avoid cannibalism) on an ad libitum supply of *A. pisum* until pupation and after adult emergence (abiotic conditions similar to stock culture). On emergence, the morph of the offspring for each parental pair was recorded. Offspring of each parental pair were considered as a single replicate.

2.3.2. Effect of mate guarding by first male on sperm precedence

In control treatments, well fed, 10-day-old, unmated, typical morph adults were paired for a single mating in separate plastic Petri dishes (size as above) and allowed to mate until they disengaged naturally. Immediately, the mated female was paired with a second unmated intermediate male in a new Petri dish and again allowed to mate until they naturally disengaged. In experimen-

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