J. Appl. Biosci., 50(2): 179-188, December, 2024
doi: 10.61081/joab/50v2i115

ISSN (Print) 0975-685X
ISSN (Online) 0975-864X

ORIGINAL ARTICLE

Female Perception of Mating Status of Male is Important for Paternity Success of a Male: A Study in a Ladybird Beetle, *Cheilomenes sexmaculata* (Fabricius)

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ABSTRACT

Multiple matings enable females to store sperm from multiple mates and select good-quality sperms for paternity. Hence, the quality of males and the pattern of sperm precedence shape mate choice and modulate female investment in mate assessment. Male ejaculate supply varies with age, mating status, and severity of sperm competition affecting the perception of females for good quality mates. In insects, cuticular hydrocarbons play an important role in identifying and assessing mates. Ladybirds exhibit last male sperm precedence but will the last male be able to win the competition if it is mated? Also, can females distinguish males when the males' elytra are washed or perfumed with CHCs from males of different mating statuses? This was testedin the ladybird beetle, *Cheilomenes sexmaculata*by subjecting females to double matings where the matingstatus of the last male was varied from unmated to once mated to twice mated. The experiment was performed at three levels (i) untreated, (ii) elytra of last males wiped using n-hexane, and (iii) males were perfumed with the CHCs of males of different mating status. Mated males were not able to outcompete the sperms of unmated males. Also, the effect of CHCs was evident in courtship behaviour and paternity share. Females showed preference for unmated males over mated males and this was prevalent in washed treatment also. Perfuming of males resulted in the exchange of CHCs, and the response of females to such "perfumed" males demonstrated that females' perception of male status is CHC-driven.

KEY WORDS: Cuticular hydrocarbons, sexual selection, mate choice, sperm precedence, surface chemicals.

INTRODUCTION

Sexual selection would likely favour the development of features to discriminate between sexes based on their reproductive state to enhance reproductive success (Ridley, 1983). Identifying the status of mates before investing in the utilization of gametes is crucial as neither sperm are free nor ova are inexpensive to fertilize. Ejaculates are not only costly to produce (Dewsbury, 1982; Svärd & Wiklund, 1986) but are limited (Svärd & Wiklund, 1986; Simmons & Beveridge, 2011). Therefore, differential reproductive fitness of the two sexes gives rise to promiscuous males and choosy females (Trivers, 1972; Andersson, 1994). Over the years, it is clear that the expenditure on matings or gametes is strategically adjusted (Arnqvist & Nilsson 2000; Moore, 2014). Thus, mating experience may impact aspects of mate quality as a mate. From this perspective, a female should discriminate among males and choose the male which renders maximum fitness benefits to her and her offspring. For instance, in cockroach, *Nauphoeta cinerea* (Olivier), females were found to discriminate against multiply mated males as subsequent matings may lead to low fertility caused by sperm exhaustion (Harris & Moore, 2005; Moore 2014).

The mating history of an organism is regarded as the key component in determining the expenditure of resources and allocation and utilisation of sperm (Lupold *et al.*, 2011). Mating with previously mated males comes at a high cost to females (Dewsbury, 1982; Thornhill & Alcock, 1983; Wedell, 1993a, 1997; Vahed, 1998, 2006; Kokko *et al.* 2003; Wedell *et al.*,2002; Wedell & Ritchie, 2004) in terms of

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*Corresponding author email: omkar.lkouniv@gmail.com ejaculate received (Kaitala & Wiklund, 1995; Wedell *et al.*, 2002; Torres-Vila & Jennions, 2005; Lauwers & Van Dyck, 2006; McNamara*et al.,* 2007; Dowling & Simmons, 2012) and decreased paternity share (Simmons, 2001a). Studies have found that females who mate with sexually experienced males have lower lifetime fecundity than females mating with unmated males (Perez & Aluja, 2004; McCartney & Heller, 2008). Females of the fruit fly, *Drosophila melanogaster* (Markow *et al.,* 1978), the leaf roller *Cnephasia jactatana* Walker (Jimenez-Perez & Wang, 2004), the tobacco budworm *Heliothis virescens* (Fabricius) (Klepetka & Gould, 1996), and the parasitoid wasp, *Spalangia endius* Walker (King & Fischer, 2010) mate with unmated males to gain increased spermatophores to enhance the reproductive output (Markow *et al.,*1978; Klepetka & Gould, 1996; Jimenez-Perez & Wang, 2004; King & Fischer, 2010; Jiao *et al.,* 2011). The amount of sperm transferred to multiply mated females is very essential in determining the paternity share (Simmons & Fitzpatrick, 2012). Furthermore, males are not always ready to supply high ejaculate which varies with age, size, mating status and severity of sperm competition risk (Li *et al.*, 2023). Females are affected by this variation in terms of low reproductive output and their fitness is impacted (Dubey *et al.,* 2018). Therefore, it is important for females to recognize the quality of the males she is mating with, for which insects are likely to utilize surface chemicals or cuticular hydrocarbons (CHCs) (Ingleby *et al.,* 2014; Fasotte*et al.,* 2016; Lane *et al.,* 2016; Isa *et al.,* 2019; Legrand *et al.,* 2019a; Verheggen *et al.,* 2020).

Insects use CHCs for assessing the sexual status of males, species identification, and gender identification (Howard & Blomquist, 2005; Johansson & Jones, 2007; Pattanayak *et al.,* 2014; Sharma *et al.,* 2015; Ginzel & Blomquist, 2016). Studies have reported the use of hydrocarbons in signalling age (Wakonigg *et al.,* 2000; Cuvillier-Hot *et al.,* 2001; Pattanayak *et al.,* 2014; Xue *et al.,* 2016), ovarian activity (Cuvillier-Hot *et al.,* 2001), nutritional condition (Liang & Silverman, 2000), protection against infection (Pattanayak *et al.,* 2015), and mating status (Jones & Elgar 2004; Dubey *et al.,* 2018) of mates. Hence, CHCs play an essential role in chemical communication and mate selection in insects (Ingleby *et al.,* 2014; Lane *et al.,* 2016; Isa *et al.,* 2019; Legrand *et al.,* 2019a; Verheggen *et al.,* 2020).

Thomas (2011) has reported a link between hydrocarbons and mating behaviour, that impacts mating success and sperm precedence. These hydrocarbons are predominantly experienced through touch stimuli (Thornhill & Alcock, 1983; Howard & Blomquistq, 2005). Many invertebrate and vertebrate species use surface chemical cues to communicate during courting (Fasotte *et al.,* 2016). Various insect orders, such as Hemiptera (Guarina *et al.,* 2008), Blattodea (Eliyahu *et al.,* 2008), and Coleoptera (Francke & Dettner, 2005), have been shown to use surface chemicals for successful matings. CHCs can transmit fertility and sex cues, allowing for close-range mate detection (Ginzel & Blomquist, 2016; Xue *et al.,* 2016; Chen *et al.,* 2020). As a tactic for sexual competitiveness, hydrocarbons are frequently exchanged between mating mates (Thomas, 2011; Ingleby, 2015) and have been shown to elicit behavioural responses to sperm competition risk (Everaerts *et al.,* 2010; Lane *et al.*, 2015; Snellings *et al.*, 2018). For instance, in *D. melanogaster*, experimental exchange of CHCs between unmated and mated females, induced longer mating duration by males (Scott 1986; Friberg, 2006). Furthermore, males can recognize the risk and intensity of sperm competition from the profile of rival males left behind on the cuticle of female as evident in Australian field crickets (*Teleogryllus oceanicus*) (Thomas & Simmons, 2009). Another study in a broad-horned flour beetle, *Gnatocerus cornutus,* showed that males exhibited increased courtship efforts and transferred more sperm to females perfumed with 1-3 rival males (Lane *et al.,* 2015). Likewise, CHCs can aid in the female perception of mate as well and act as cues of male mating status.

Adults of both sexes rely on CHC-based informative signals to assess the reproductive value of mates and then make the decision to choose mates (Smith & Harper, 2003; Lane *et al.,* 2015; Verheggen *et al.,* 2020). The importance of CHCs in reproduction and gender recognition has also been elucidated in ladybird beetles (Singer, 1998; Ingleby, 2015; Fasotte *et al.,* 2016; Legrand *et al.*, 2019a). Ladybirds from the order Coleoptera are potent biocontrol agents that are known to exhibit mate

Table 1: Results of two-way ANOVA showing the effect of mating status and treatment given to males on reproductive parameters and paternity share (P2 value) of *Cheilomenes sexmaculata*

Dependent Factors	Independent Factors		
	Mating status	Treatment	Interaction
Oviposition	F=32.25; P<0.0001; df= 2,89	F=49.52; P<0.0001;df = 2,89	F= 4.17; P<0.05;df = 4,89
Percent egg viability	F= 11.22; P<0.05; df= 2,89	F=58.68; P<0.0001;df = 2,89	F= 7.54; P<0.05;df = 4,89
P2 value	F=45.27; P<0.0001; df= 2,89	F=77.45; P<0.0001;df = 2,89	F= 16.06; P<0.05;df = 4,89

choice and multiple matings (Omkar & Mishra, 2005; Haddrill *et al.,* 2008; Omkar *et al.,* 2010; Mishra & Omkar, 2014; Dubey *et al.,* 2016a, b; Dubey *et al.,* 2018; Saxena *et al.,* 2021), and mate guarding (Chaudhary *et al.,* 2016). As it happens in many animal species, mating in coccinellids is also a result of interaction between complex multicomponent signalling (Bro-Jørgensen, 2010). A study in *Adalia bipunctata* showed how CHCs play an important role in mate recognition (Hemptinne *et al.,* 1998). Another study showed that the female sex pheromone attracts males in coccinellids via CHCs (Fassotte *et al.,* 2014).

Organisms have evolved mechanisms to avoid mating with "low quality" males that have a history of multiple matings (Harris & Moore, 2005; Moore, 2014), as they may have depleted sperms. As aforementioned, females tend to prefer unmated males over mated males and CHCs may have a role to play in this. Although, there have been many studies focusing male perception of female status (Frigberg, 2006; Steiner *et al.,* 2006; Ruther, 2013; Wurf *et al.,* 2020), not many studies focus on female response to the perception of males through CHCs. Therefore, this study was conducted to determine female responsiveness to contact-derived CHCs in the ladybird beetle, *Cheilomenes sexmaculata* (Fabricius) and the impact of male mating status on the sperm precedence based on how the female will respond to changes in the CHCs of males with different mating statuses.

The ladybirds are known to exhibit the last male sperm precedence (Chaudhary *et al.*, 2016), *i.e.* the last male to mate has the maximum paternity share. Considering the females' preference for unmated males, will the last male be able to win the competition if it is already mated? To test this, it was hypothesised that when females mate with mated and unmated males, the unmated male is likely to win the sperm competition race despite its sequence of mating. Further, to investigate whether females will be able to distinguish the males' mating status driven by CHCs, we manipulated the chemical profile of unmated males by (i) washing the elytra of males to restrict any interaction between the chemical cues of the male with the female, and, (ii) perfuming males with the CHCs of different mating status males, thereby changing their chemical profiles.For this, we hypothesised that washing-off the CHCs and perfuming of males may affect the precopulatory response of females eventually affecting sperm precedence. This study deciphers the effect of increasing the number of matings of the last male on the sperm precedence and also the role CHCs could play for females in identifying the mated and unmated males.

METHODS AND MATERIALS

Experimental Organism

Cheilomenes sexmaculata is a generalist predator (Agarwala & Yasuda, 2000) that exhibits polymorphism (Dubey *et al.*, 2016a; Dubey *et al.*, 2018) and is a potent biocontrol agent. It is widely distributed in Asia (Omkar & Bind, 1993) and has a wide aphid prey range (Agarwala & Yasuda, 2000; Omkar & Pervez, 2004). The abundantly available beetle has three main morphs: typical with zig-zag lines on orangish-red elytra, melanic with entirely black elytra and pronotum, and intermediates with an extensive fusion of zig-zag lines due to some to no melanisation of pronotum. True melanic morphs are not found in the field but reared in laboratories after mating the intermediate morphs for several generations (Dubey *et al.*, 2018). These beetles exhibit mate choice (Dubey *et al.*, 2016 a,b; Saxena *et al.*, 2021) at the precopulatory level and show last male sperm precedence (Chaudhary *et al.*, 2016) at the postcopulatory level that is independent of the morph of the last male (Dubey *et al.*, 2018).

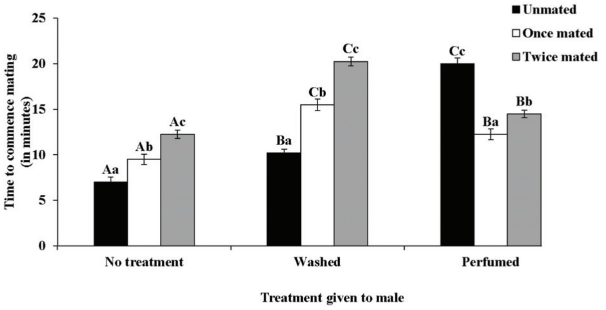

Fig. 1. The effect of mating status of last male and treatments, *i.e.* no treatment, washing and perfuming, on time to commence mating in *C. sexmaculata*. Values are Mean ± SE. Small and large letters denote the comparison of means within and across the treatments. Similar letters indicate lack of significant difference at P>0.05.

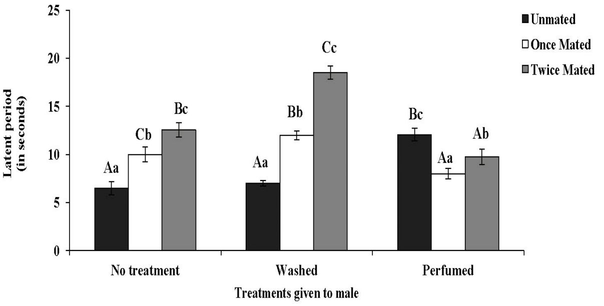

Fig. 2. The effect of mating status of last male and treatments, *i.e.* no treatment, washing and perfuming, on latent period in *C. sexmaculata*. Values are Mean ± SE. Small and large letters denote the comparison of means within and across the treatments. Similar letters indicate lack of significant difference at P>0.05.

Stock Maintenance

The adults of both typical and intermediate morphs of *C. sexmaculata* were collected from the agricultural fields of Lucknow, India (26°50'N, 80°54'E) and homomorphic pairs were formed in plastic Petri dishes (9.0 × 2.0 cm). The adults were daily fed with the *ad libitum* supply of cowpea aphid, *Aphis craccivora* (Koch) obtained from infestations on cowpea *Vigna unguiculata* L., reared in a polyhouse (at 25±2°C, 65±5% R.H.). The Petri dishes were daily hecked for oviposition and eggs laid were separated into different Petri dishes. The hatched larvae were fed with *ad libitum* supply of aphids till adult emergence. This was repeated for both morphs for three generations to create laboratory lines of pure typical and intermediate forms.

Morph as Phenotypic Markers

The morphs of the offspring were used as the phenotypic marker for the determination of paternity in the assessment of the last male sperm precedence. A previous study has confirmed using morphs as markers in this ladybird species (Chaudhary *et al.,* 2016). The mating of homomorphs always gives offspring of the same morph but mating between heteromorphs results in intermediate offspring. This helped us in assigning the father of the offspring in double mating treatments.

For all the experimental treatments, the 10-day-old typical female was allowed to first mate with10-days-old typical male and post-natural disengagement female was subjected to second mating with 10 to 15-day-old intermediate males.

Experimental Design

The experiment involved analysing the effect of two factors, mating status of males and their CHCs on the last male sperm precedence. For this, the experiment was performed at three levels.

(i) No treatment : The effect of mating status of males on last male sperm precedence was observed by subjecting the typical (T) female to double mating with males of typical and intermediate (I_3) morphs in the following set of combinations: (1) Unmated T♀ × Unmated T × Unmated I_3♂ (2) Unmated T♀ × Unmated T × Once mated I_3♂ (3) Unmated T♀ × Unmated T × Twice mated I_3♂. The males of once and twice mated mating status were prepared by subjecting them to 1 (when 9-day-old) and 2 matings (when 8 and 9 days old), respectively. At this level, the elytra were not subjected to any treatment and only the effect of mating status of last males was observed on mating parameters and sperm precedence. This was called the untreated set (No treatment).

(ii) Washing of elytra (Washed treatment) : To observe if the washing of elytra of males belonging to different mating status results in no response from female, the elytra of last male was washed with n-hexane to wipeout CHCs present and then observed their impact on sperm precedence. The females were subjected to a similar set of mating combinations, as mentioned above, but the elytra of the intermediate males which were to mate as second males in the double mating experiment was wipedout with cotton dipped in 95% n-hexane before placing them in the experimental setup. As these hydrocarbons act as the semiochemicals used as cues in sex and species-specific recognition, the washing of elytra may clear all such signals influencing the response of females at pre- and post-copulatory levels. This was called the washed treatment.

(iii) Perfuming of elytra (Perfumed treatment) : To determine the potential of females in detecting cues about the mating status of male, the chemical profile of the mated and unmated males was interchanged. The CHCs were interchanged by vortexing the males with different mating status and then the impact at pre-copulatory level and on sperm precedence was recorded. The perfuming was done following the protocol of Lane *et al.* (2015). The mating status of males was manipulated by vortexing these males with the males of the same age but opposite mating status. Vortexing helped in the exchange of CHCs and was referred to as perfuming.The 10-day-old intermediate unmated males were taken in an Eppendorf tube (1.5 mL) and vortexed with same age mated males for 30 seconds at a low setting in a minispin centrifuge allowing sufficient time to stay in contact with other males so that CHCs can be transferred from the elytra of one male to another (Lane *et al.,* 2015). Similarly, the last unmated males were vortexed with multiply mated males to see the effect of perfuming. The experimental intermediate males were allowed to relax for 30 minutes before introducing them into the experimental arena. The other male used in perfuming was discarded.

The mating pairs were allowed to mate till they disengaged naturally. The pairs were observed for mating parameters, such as time to commence mating(time duration between the introduction in the arena and first mounting of male over female), latent period (time duration between mounting and intromission of aedeagus), and mating period (time duration from intromission to natural disengagement).

Paternity Share Evaluation

Post double mating the females were isolated and placed in Petri dishes with *ad libitum* food. They were checked daily for oviposition and eggs laid were isolated and observed for hatching. The larvae on hatching were counted and reared individually in a separate Petri dish

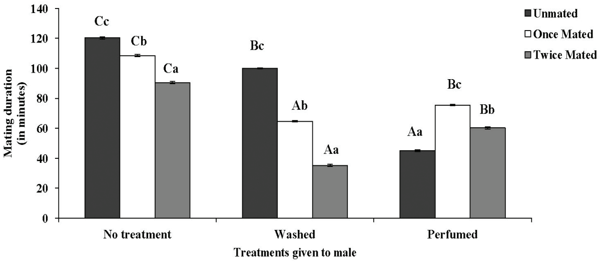

Fig. 3. The effect of mating status of last male and treatments, *i.e.* no treatment, washing and perfuming, on mating duration in *C. sexmaculata*. Values are Mean ± SE. Small and large letters denote the comparison of means within and across the treatments. Similar letters indicate lack of significant difference at P>0.05.

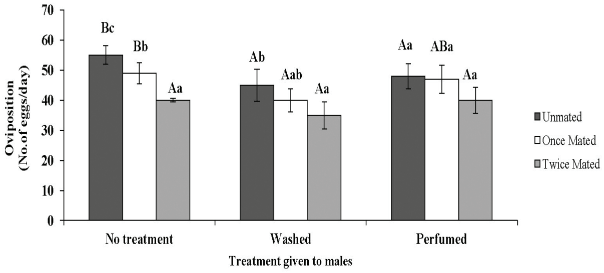

Fig. 4. The effect of mating status of last male and treatments, *i.e.* no treatment, washing and perfuming, on oviposition in *C. sexmaculata*. Values are Mean ± SE. Small and large letters denote the comparison of means within and across the treatments. Similar letters indicate lack of significant difference at P>0.05.

(one larva per Petri dish to avoid any chances of cannibalism) with *ad libitum* supply of food till pupation. The morph of the adults emerging from pupa was recorded and P2 value was calculated, which is the proportion of offspring sired by the last male. The last male sperm precedence was represented by P2>0.5, while P2<0.5 represented first male sperm precedence. However, P2=0.5 showed that the paternity was shared equally by both males and indicated random mixing as the paternity share did not incline towards either side.

Statistical Analysis

Normality and homogeneity of variance of data for time to commence mating, latent period, mating duration, oviposition, percent egg viability and paternity share (P2 values) were checked using Kolmogorov–Smirnov's test and Bartlett's test, respectively. The data were found to be normally distributed and homogenous in variation, and were subjected to two-way ANOVA where the treatments given and the mating status of males were the independent factors. All the analyses were followed by comparing the means using post hoc Tukey's honest significance test at 5%. All statistical analyses were conducted using MINITAB-16 statistical software (Minitab Inc., State College, Pennsylvania, USA).

RESULTS

Time to commence mating was found to be significantly affected by the mating status of the last male (F= 5.69; P< 0.0001; df= 2, 89) and by the treatment these males were subjected to (F= 20.58; P< 0.0001; df=2, 89). The interaction of the two factors, *i.e.* mating status and treatment, was also significant (F= 2.54; P< 0.05; df= 4, 89). The time to commence mating has increased with the increasing mating status in all the groups except in the perfumed treatment. The longest duration to commence mating was recorded for twice-mated males with washed elytra and for unmated males whose elytra had been perfumed with CHCs from a multiple-mated male. The shortest duration to commence mating was observed in an unmated male with no treatment (Fig. 1).

The latent period was also found to be significantly affected by the mating status (F= 11.25; P<0.05; df=2, 89) of males, the treatment given (F= 58.68; P<0.0001; df=2, 89) to them and by the interaction of the two factors (F= 7.54; P<0.05; df= 4, 89). The duration increased with increasing mating status of the male in all treatments except the perfumed treatment. The longest latent period was observed in twice-mated males whose elytra were wiped with hexane extract. In contrast, the shortest latent period was recorded in unmated males whoreceived no treatment (Fig. 2).

Two-way ANOVA revealed that mating duration was significantly affected by the mating status of the males (F = 54.50; P < 0.0001; df = 2, 89), the treatment (F = 77.50; P <0.0001; df = 2, 89), and their interaction, which was also found to be significant (F = 6.41; P < 0.05; df = 4, 89). Mating duration decreased with changing mating status; the highest duration was observed in pairs where the last male was unmated, followed by once-mated and twice-mated males across all treatments except the perfumed treatment. In the perfumed treatment, the highest mating duration was recorded for once-mated males, followed by twice-mated and unmated males (Fig. 3).

Two-way ANOVA shows that the oviposition, percent egg viability and last male sperm precedence are significantly affected by the mating status of the last male and by the treatments given to males (Table 1). The highest average number of eggs was laid by females that mated with unmated males receiving no treatment in their second mating. In contrast, the lowest number of eggs was laid by females that mated with twice-mated males whose elytra had been washed (Fig. 4). Similarly, the highest percentage of egg viability was observed in females that mated with untreated and unmated second males, while the lowest egg viability was found in females that mated with twice-mated males subjected to the perfumed treatment (Fig. 5).

The untreated treatment showed clear last male sperm precedence when the last male to mate was unmated (P2= 0.85 ± 0.02) or once mated (P2= 0.70 ± 0.04), however when the last male was twice mated (P2=0.50 ± 0.04) there was no sperm precedence but sperm mixing was visible(Figure 6).This is indicative of female showing a preference for unmated males at post copulatory level and changing the sperm precedence accordingly. For the washed treatment where CHCs of the last male were wiped off using n-hexane, there was sperm mixing for unmated (P2= 0.55 ± 0.01) and once mated male (P2= 0.50 ± 0.03) and first male sperm precedence for twice mated males (P2= 0.40 ± 0.02). The mating combinations of the perfumed set showed that there was sperm mixing when the last male was unmated but perfumed with the mated male (P2= 0.50 ± 0.03); while there was a case of first male sperm precedence when the last male was once (P2= 0.48 ± 0.04) and twice mated and perfumed with unmated (P2= 0.40 ± 0.03). These results suggest the dynamic nature of the last male sperm precedence and paternity being affected by the mating status of the male as perceived by the female irrespective of its sequence of mating.

DISCUSSION

This study demonstrates the role of CHCs in female perception of mates and assessing the mating status of males. It was found that females showed preference for unmated males over mated males and modulated the last male sperm precedence in favour of unmated males. Thus, the last male sperm precedence was found to be a condition dependent phenomenon subjected to change depending upon the quality of the last male. The washing of elytra would have resulted in no mating but it was found that the male elytral washing only delayed the onset of mating and reduced the mating duration.Also, perfuming males with the CHCs of the males of different mating status successfully tricked the females into perceiving mated males as unmated and vice versa, but could not change the pattern of sperm precedence.

Our results convincingly showed that females avoided mating with mated males owing to their lower reproductive fitness (Price & Hansen, 1998; Jones *et al.,* 2000; Bonduriansky & Brassil, 2002; Jones & Elgar, 2004; Beck & Promislow, 2007; Omkar *et al.,*2010). Even when the male had attained access to females, their resistance and avoidance behaviour possibly prevented males from inserting their aedeagus, which was responsible for the prolonged latent period. A previous study by Dubey *et al.* (2018) on *M. sexmaculatus* also reported that females showed a preference for unmated males, and earlier commencement of mating was observed with unmated males irrespective of the mating status of females. Due to the loss of ejaculate with each mating and exhaustion from previous matings, mating with an already-mated male can be unfavourable for females. For instance, subsequent matings may cause transfer of lesser number of sperm, smaller nutrients from nuptial gifts, increased risk of unsuccessful fertilization, or shorter subsequent lifespan (Kaitala & Wiklund, 1995; Watanabe *et al.,* 1998; Hughes *et al.,* 2000; Wedell & Ritchie, 2004; Jones *et al.,* 2006; Lauwers & Van Dyck, 2006). In addition to having less reproductive value, mated males may release less CHCs than unmated males. For instance, in *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae), multiply mated males produced less of the pheromone reducing their

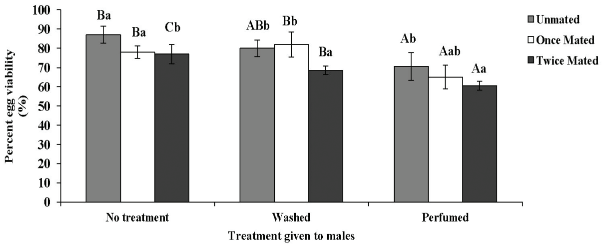

Fig. 5. The effect of mating status of last male and treatments, *i.e.* no treatment, washing and perfuming, on percent egg viability in *C. sexmaculata*. Values are Mean ± SE. Small and large letters denote the comparison of means within and across the treatments. Similar letters indicate lack of significant difference at P>0.05.

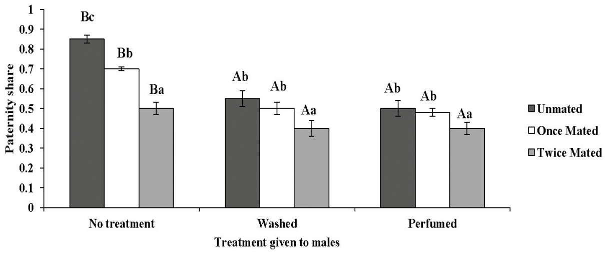

Fig. 6. The effect of mating status of last male and treatments, *i.e.* no treatment, washing and perfuming, on Paternity share in *C. sexmaculata*. Values are Mean ± SE. Small and large letters denote the comparison of means within and across the treatments. Similar letters indicate lack of significant difference at P>0.05.

attractiveness to females (Ruther *et al.,* 2009). Therefore, mating possibly results in physiological and behavioural modifications in CHCs that perhaps render them being less attractive to mate (Snellings *et al.,* 2018).

The involvement of CHCs in the identification and assessment of mate quality (Singer, 1998; Howard & Blomquist, 2005) and its crucial role in sexual communication (Bontonou & Thomas, 2014) was evident from the washing and perfuming of elytra. The delayed time to commence mating in washed treatment corresponds to weaker communication in comparison to the one with no treatment. The longer time to commence mating for males when their elytra were washed with n-hexane depicts the difficulty faced by females to interact and identify the mating status of males (Durieux *et al.,* 2010; Sloggett *et al.,* 2011). For instance, washed female elytra of dummy models of *A. bipunctata* (L.) did not elicit any mating response by males (Hemptinne *et al.,* 1998; Hemptinne & Dixon, 2000).

The manipulation in the CHCs was found to alter the female perception of the mating status of the males.The delayed time to commence mating of perfumed unmated males depicts that the females perceived them as males with mating history, resulting in resistance to mate. This demonstrates that CHCs involvement in the females' perception of males mating status. Empirical evidences suggest that the organisms can evaluate the mating status of the mate and depending upon this, the decision to mate is considered (Thomas, 2011; Marie-Orleach *et al.,* 2013; Dubey *et al.*, 2018; Singh *et al.,* 2020). In ladybirds and other insects, the interaction of pheromones/semio-chemicals partake in the mating process; however, most studies are focused towards the male perception of a female (Fasotte *et al.,* 2016; Legrand *et al.,* 2019a,b). This study was focussed on the CHCs of males and their perception by females in evaluating the status of males was found to play a role in it.

The mating duration was decreased with increasing number of matings of males. The possible reasons for the reduced duration of mating may be attributed to (i) male exhaustion from subsequent matings (Omkar & Srivastava, 2002a; Omkar & James, 2005), and (ii) exhaustion of ejaculate supply with subsequent matings and thus males did not have enough resources to transfer to females (Jimenez-Perez & Wang, 2004; Torres-Vila & Jennions, 2005; Lauwers & Van Dyck, 2006; Simmons, 2014; Iwata *et al.,* 2021; Cordero-Rivera, 2022). In ladybird beetles, it has been already reported that mating duration tends to decrease with subsequent matings (Bind, 2007; Dubey *et al.,* 2018; Shandilya *et al.,* 2021). Various other studies have also suggested that males invest heavily in first mating and investment keeps decreasing with subsequent matings (Oliver & Cordero, 2009; Wedell *et al.,* 2010; Dowling & Simmons, 2012; Alavi *et al.,* 2016). However, in washed and perfumed treatments, a lower duration of copulation for unmated males in comparison to no treatment group can possibly be attributed to the response of females towards the surface body secretions or CHCs. For instance, in the Indian sweet potato weevil, *Euscepes postfasciatus* (Fairmaire), adults responded to the surface hydrocarbons and changed their behaviour as per the change in the quality of these surface chemicals (Isa *et al.,* 2018).

The reproductive output in promiscuous females is closely related to the supply of spermatophores. Smaller spermatophores are believed to supply a lower amount of substances capable of stimulating oviposition (Morrow & Gage, 2000). Studies in insects, birds, mammals, and humans have shown that the seminal fluid proteins (SFPs) also have a significant influence on the reproductive physiology and behaviour of females and subsequent matings lead to a reduction in SFPs and ejaculate supply (Avila *et al.,* 2011; Hopkins *et al.,* 2017; McGraw *et al.,* 2015; Ratto *et al.,* 2012; Bath *et al.,* 2021). Therefore, one could say that the subsequent mated males may lack sufficient resources to enhance reproductive output in comparison to the unmated males. Our results of reduced reproductive output by mated males also stand in accordance with this. In addition, a study on lepidopterans also elucidated reduced reproductive output based on mating history of males (Torres-Vila & Jennions, 2005). Another possible reason for reduced reproductive output in females can be the supply of apyrene sperm by males who have mated recently (Cook & Wedell, 1996). This explains the necessity of females to identify the mating status of the males at the pre-copulatory level to avoid fitness costs.

The reasons attributed to the reduced reproductive output of mated males can also stand in line with the reasons for altering the last male sperm precedence pattern. This may result in females being biased towards the sperm of fitter males as perceived by females. Also, the increasing mating status resulted in a decreased amount of sperm and SFPs; lesser sperms were unable to outcompete the sperm of the first unmated male in the reproductive tract of females. Mating status of the mates thus played a decisive role in mate choice and eventually determined the reproductive success (Lane *et al.,* 2015; Verheggen *et al.,* 2020).

The probable reason for the shifting of paternity share from the last male sperm precedence to sperm mixing in the unmated last males of washed and perfumed treatment could be the females' perception of CHCs. The interaction of females with the CHCs of mated males probably resulted in a lower duration of copulation, which may be responsible for a reduced supply of sperm. Even though in the perfumed treatment females showed a preference for mated males (perfumed with the CHCs of unmated males), a lesser number of sperms and depleted nutrient content from subsequent matings could not outcompete the sperms of the first male. However, mixed sperm precedence in unmated washed and unmated perfumed treatments indicates that the quantity of sperm transferred is only one factor of reproductive success. Other factors like the female perception of male mating status via CHCs act as additional cues, resulting in equal sperm precedence of first and second males. To sum up, deviation in the paternity share can be attributed to the combined effects of (i) shorter mating duration, (ii) the sperm transferred being inferior in quality and quantity, and (iii) perception of mates at pre-copulatory level inducing cryptic female choice at post-copulatory level.

Our hypotheses were thus accepted that sperm precedence is not determined by the sequence in which male mates (*i.e.,* the last male winning the reproduction race) but is governed by several factors including the mating status of the mate, therefore last male sperm precedence appears to be a plastic phenomenon.

CONCLUSION

The competency to assess mates forms a key aspect of social interaction and helps in increasing the reproductive success of individuals. This study demonstrates that the number of matings of the last male reduced the mating performance and reproductive output. In double mating treatments, the mating status of the last male altered the paternity share, which was found to be biased towards the last unmated male, *i.e.* P2>0.5. Secondly, the interaction of male and female CHCs was perhaps responsible for eliciting the response of a female towards a male and regulating pre- and post copulatory behaviours depending upon the mating status of males. The perception of male mating status by the female and cryptic female choice is responsible for eliciting are sponse different from normal mating. The perfuming experiments altered the surface chemicals and changed the females'perception accordingly, modulating reproductive parameters, mating parameters and sperm precedence. Further studies regarding GC-MS analysis of CHCs, sperm count and paternity analysis using molecular markers will be needed to verify and confirm the results of this study in detail.

ACKNOWLEDGEMENT

AS acknowledges BSR Fellowship by the University Grant Commission, New Delhi, India. SR acknowledges the UGC Fellowship by the University Grants Commission, New Delhi, India (F.No.16-9 (June 2019)/ 2019 (NET/CSIR)) dated August 9, 2019.

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