

Post-copulatory male mate-guarding in a solitary parasitoid wasp impedes re-mating in the female

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ABSTRACT

Background: If the sperm from two or more males fertilize the ova of a single female, sperm competition results. Among mostly solitary parasitoid wasps, females often lose receptivity after an initial mating.

Organism: *Pachycrepoideus vindemmiae* (Pteromalidae), a solitary parasitoid wasp. After copulation, *P. vindemmiae* males remain mounted on females for some time.

Questions: Does the post-copulatory behaviour of mate-guarding impede re-mating in females? If re-mating does occur, does it result in more female offspring or a higher proportion of females?

Methods: We removed males of copulating pairs at various times after copulation. We provided sufficient hosts to females who mated twice. We measured the number of offspring and the sex ratio during the period in which a female oviposited. We then analysed the effects of re-mating.

Results and conclusions: The post-copulatory mate-guarding ritual of *P. vindemmiae* males reduced female receptivity. No females were receptive after copulation when we allowed males to perform the entire ritual. The shorter the ritual allowed, the higher the frequency of females that permitted re-mating. Compared with once-mated females, twice-mated females had similar numbers of male and female offspring, as well as a similar proportion of males throughout female oviposition.

Keywords: mate-guarding, mate system, sex ratio, sperm competition.

INTRODUCTION

Polyandry occurs when a female mates with two or more males, while monandry occurs when a female only mates with a single male, especially in true monandrous females, who become completely unreceptive after initial copulation or remain receptive to only

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one individual (Ringo, 1996; Boulton and Shuker, 2013). Females in almost all animal groups are polyandrous, copulating with multiple males (Pizzari and Wedell, 2013). DNA fingerprinting has shown polyandry to be extremely widespread among animals, which reveals that the patterns of mating that biologists have observed directly do not match the true patterns of paternity among offspring, and females across a very broad range of species have been found to be mating with multiple males (Boulton and Shuker, 2013). Females of the majority of insects also mate with multiple partners (Arnqvist and Nilsson, 2000; Shuker and Simmons, 2014).

In some taxa, such as parasitoid wasps, studies have shown that monandry is common in solitary species where only one offspring can emerge from a single host, and most females of solitary parasitoids can mate only once with a single male, storing sperm from copulation throughout their reproductive lives (King, 1987; Ridley, 1993; Godfray, 1994). For example, females of *Spalangia endius* (Pteromalidae) usually mate only once, even if they lack sperm in a later bout during oviposition (King *et al.*, 2005; King and Bressac, 2010; King and Fischer, 2010). Monandry seems to be a successful strategy for males where sperm competition can be avoided throughout female oviposition (Simmons, 2001).

However, some female parasitoid wasps commonly believed to be monandrous can engage in multiple matings. A solitary egg parasitoid, *Ooencyrtus kuvanae* (Encyrtidae), had previously been considered monandrous because females mate only once and then become unattractive to males (Ablard *et al.*, 2012). But females of *O. kuvanae* can sometimes be multiple-mated while still receptive shortly after initial copulation, and the presence of more males enhances the mating frequency (Ablard *et al.*, 2014). Females of *Anisopteromalus calandrae* (Pteromalidae) can accept another mating in the simultaneous presence of two males, with both males mounting the female together and copulating with the female one after the other (Khanh *et al.*, 2005). Parasitoid wasps usually have a female-biased sex ratio predicted by the local mate competition (LMC) theory, but the sex ratio would be less female-biased when more females oviposit together (Hamilton, 1967; Werren, 1987). A higher proportion of males would result in a higher frequency of multiple matings (Boulton and Shuker, 2016).

The benefits of polyandry to females have been discussed extensively. One common benefit is the acquisition of sufficient sperm to fertilize all the ova during the reproductive period (Stockley, 1997; Boulton and Shuker, 2013). Sperm number limitation might occur when males partition ejaculates between sequential copulations, and females' sperm stores are only partially filled after a single copulation, as described for some *Drosophila* (Pitnick and Markow, 1994). A negative effect of re-mating on female longevity in species without nuptial feeding has been found, but the positive effects (e.g. increased egg production and fertility) more than outweigh this negative effect at moderate mating frequencies (Arnqvist and Nilsson, 2000). Hymenoptera, as parasitoid wasps, have a haplodiploid genetic system, in which males are usually haploid, developing from unfertilized eggs, whereas females are diploid and develop from fertilized eggs. Research on parasitoid wasps has shown that more male offspring are laid at the end of an oviposition bout, such as in *Uscana semifumipennis* (Trichogrammatidae) (Henter, 2004), *Anaphes nitens* (Mymaridae) (Santolamazza-Carbone *et al.*, 2007), and *Pachycrepoideus vindemmiae* (Pteromalidae) (Hu *et al.*, 2012). The higher male-biased offspring sex ratio shows that sperm in the spermatheca of mated female parasitoids should be limited (King and Bressac, 2010). The haplodiploid genetic system would favour multiple mating when females are under strong selection to produce an optimal sex ratio.

Pachycrepoideus vindemmiae is a solitary idiobiont ectoparasitoid wasp, whose hosts include the pupal stage of a range of cyclorrhaphous dipteran species (Morris and Fellowes, 2002). The wasp attacks host pupae and lays eggs on the surface of the pupal body within the

puparium. Newly hatched larvae pupate within the host before emerging from the host's shell after eclosion (Tormos *et al.*, 2009). Often, one adult offspring emerges per host (Nadel and Luck, 1985). The longevity of adult females can reach 11 days, and a single female can produce up to 62 offspring, 21% of which are male (Sun *et al.*, 2013). Males can mate with dozens of females in rapid succession, but females usually mate only once. Females are unable to mate again just after mating or after having laid eggs for 8 days. Thus, the parasitoid is often monandrous, regardless of whether it lacks sperm (Sun *et al.*, 2013). A female-biased sex ratio was recorded for *P. vindemmiae*, in line with the theoretical prediction of the LMC model, and a less female-biased sex ratio was obtained when more females oviposited in the same patch (Li *et al.*, 2014).

To understand mating behaviour when multiple matings occur, we used *P. vindemmiae* as our model species. The post-copulation ritual seems to incur a cost for males, considering the ability of males to find additional receptive females. However, a fitness advantage would exist if the behaviour could prevent the female from mating a second time. We addressed two questions. First, is post-copulatory behaviour a mate-guarding behaviour meant to impede re-mating of the female? Second, if it does occur, does re-mating result in more female offspring or a higher proportion of females?

MATERIALS AND METHODS

Origin and rearing conditions of the insects

Pachycrepoideus vindemmiae was obtained on the campus of Anhui Normal University, Wuhu, Anhui province, China and was maintained on pupae of the housefly, *Musca domestica*, for approximately one year. Previous results have shown that the parasitoid can use frozen housefly pupae as hosts (Rueda and Axtell, 1987; He *et al.*, 2013). With frozen pupae as hosts, no houseflies would emerge, and the hosts could be preserved for longer (He *et al.*, 2013). Housefly pupae purchased from Zhonghe Biotech Co. were used in mass culture of the parasitoid. The housefly pupae were frozen at -20°C for approximately 2 weeks in a refrigerated cabinet. Before use in the culture, pupae were thawed out for approximately one hour. Parasitoid wasps were reared in glass vials (25 mm in diameter, 50 mm high) with housefly pupae and honey-soaked cotton wool as food in an incubator at a temperature and relative humidity of $25 \pm 1^{\circ}\text{C}$ and $60 \pm 5\%$, respectively; the photoperiod was 14 hours light/10 hours dark. Before the experiments, housefly pupae were measured with a ruler, and individuals of a similar length (about 5.5–6.0 mm) were selected to ensure that parasitoids were similar in body size. Parasitized housefly pupae were selected and laid individually in a plastic tube with a 150 μm pore size nylon mesh sealed around the lid to ensure that emerging parasitoids would not meet or mate. Emerging parasitoids were fed with a little honey on the nylon mesh. Parasitoids emerging in less than 12 hours were used in subsequent experiments.

Durations of different mating stages

Mating of *P. vindemmiae* includes the stages of courtship, pre-copulation, copulation, and post-copulation (Sun *et al.*, 2013). Mating was conducted between one female and one male in a 9 cm (diameter) Petri dish. We observed the mating behaviour continuously and recorded the duration of the above four mating phases with an electronic stopwatch (from the

moment when the aedeagus of the male was pulled out until the male dismounted from the female's back). Twenty pairs of male and female wasps were observed.

Receptivity of females after copulation

We placed a virgin female in a 9 cm (diameter) Petri dish and provided one virgin male as M1. We observed the mating behaviour continuously and removed M1 after copulation during post-copulatory mate-guarding. Three treatments were considered: removing M1 at approximately 5, 15, and 25 seconds after copulation, respectively. In each treatment, 30 mated females were obtained and randomly divided into three repeat groups: 10 mated females were tested in each group. Just after M1 had been removed, the receptivity of mated females to re-mating was tested. A second male (M2) was immediately provided. Receptivity of a mated female was identified as complete copulating behaviour with three other males. If one of the males finished copulation, the female was taken as a twice-mated female.

In the 10 repeat control groups, the post-copulation ritual ceased without artificial control. Immediately after M1 dismounted from the female's back, the receptivity of the female was also tested.

Offspring of twice-mated females throughout oviposition

Females in the above experiment were collected and placed individually into a 30 mL transparent bottle, with a 150 μm pore size nylon mesh sealed around the lid and honey-soaked cotton wool inside as food. Thirty frozen-preserved housefly pupae were laid in the bottle as hosts and changed daily until the female died. The exposed hosts were placed individually and inspected daily until parasitoid offspring emerged, and all of the hosts were dissected under a microscope to collect the parasitoid offspring that failed to emerge within approximately one week after offspring emergence. All specimens were examined under a stereomicroscope for sex determination.

Statistical analyses

We used a generalized linear model (GLM) analysis of deviance for the data, assuming a Poisson distribution of errors, a log link function for count data, assuming a binomial distribution of errors, and a logit link function for proportional data. We assessed the appropriateness of the assumption of Poisson or binomial errors by comparing the residual deviance with the residual degrees of freedom after fitting the explanatory variables. Large relative values of residual deviance indicate over-dispersion, which may result in an over-estimation of significance levels, and we replaced Poisson or binomial with quasi-Poisson or quasi-binomial distributions in the analyses. When more than one explanatory variable was considered, a full model was initially fitted to the data, including explanatory variables and their interactions. Terms were then removed from the full models by stepwise deletion. Whether the removal of a term caused a significant increase in deviance was assessed with a χ^2 test. The final models were tested using an *F*-test (Crawley, 2007). All of the analyses were conducted in R v.2.13.0 (R Development Core Team, 2011).

RESULTS

Duration of mating

After two parasitoids met, total mating behaviour of *P. vindemmiae* lasted 74.68 ± 29.94 seconds, including courtship, pre-copulation, copulation, and post-copulation. Once introduced into the Petri dish with a virgin female, the male began to chase the female with his wings occasionally vibrating (Fig. 1a). Courtship lasted 15.25 ± 19.26 seconds, until the male mounted her dorsally (Fig. 1b). Copulation always occurred after a pre-copulatory interaction between them, with their antennae touching. The duration of pre-copulation was 26.93 ± 19.15 seconds. When the female was receptive, her body became still, and then she opened her genital orifice. The male backed up, and they copulated, which lasted only 2.58 ± 0.60 seconds (Fig. 1c). After copulation, the post-copulatory attendance began, during which the female kept still and the male remained mounted but not in copula, the precopulatory-like interactions with antennae and mouth resumed, and her genital orifice began to close (Fig. 1d). Post-copulatory attendance lasted 29.93 ± 12.53 seconds. The female woke up and began to move, and the male eventually dismounted and left. The four stages differed significantly in duration ($F_{3,76} = 13.88$, $P < 0.01$), and the longest stage was post-copulation (Fig. 2).

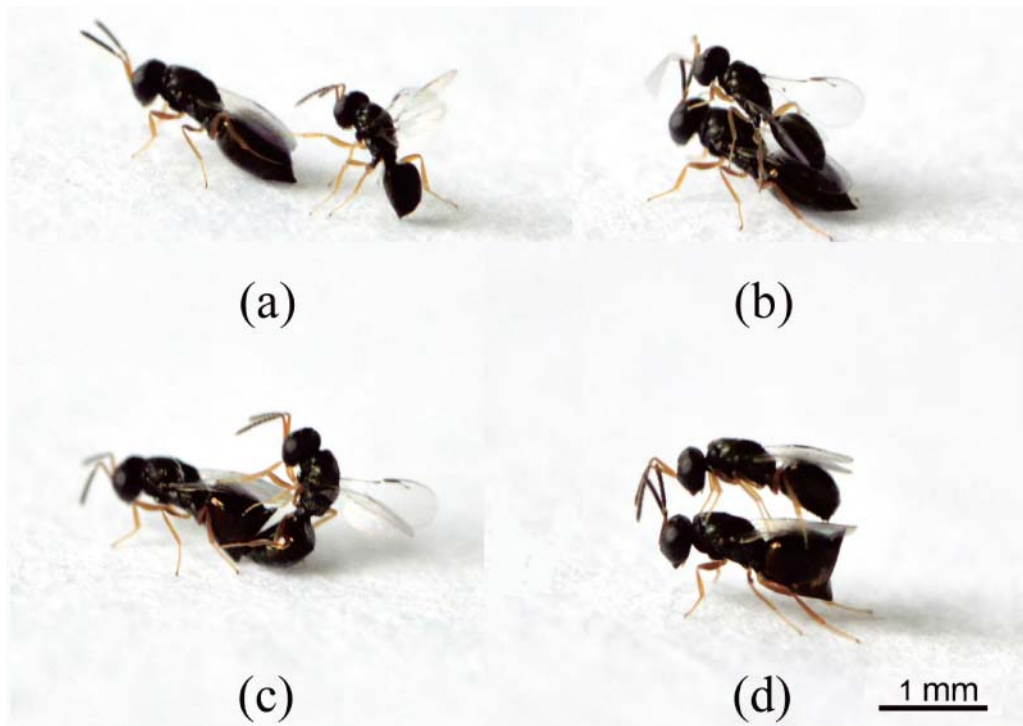


Fig. 1. Main stages of mating behaviour in *P. vindemmiae*: (a) courtship, (b) pre-copulation, (c) copulation, and (d) post-copulation. The wasp behind (in a) and the upper wasps (in b–d) are the males.

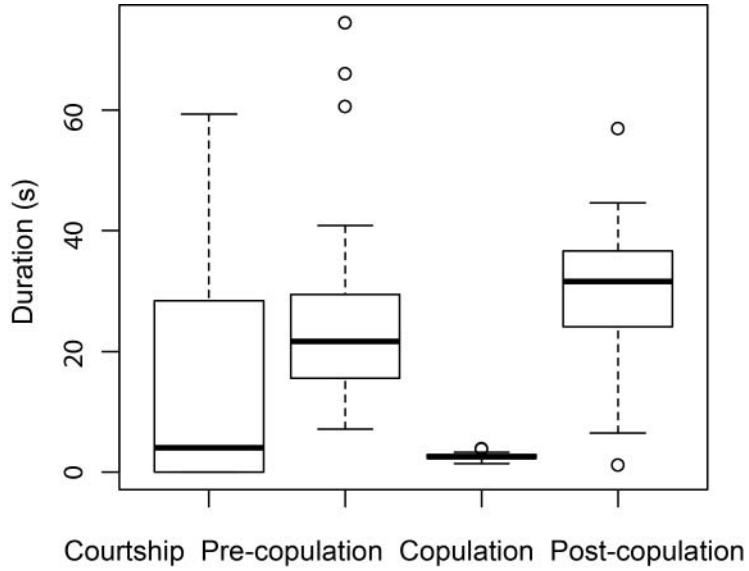


Fig. 2. Box plots showing the durations of mating stages in *P. vindemmiae*.

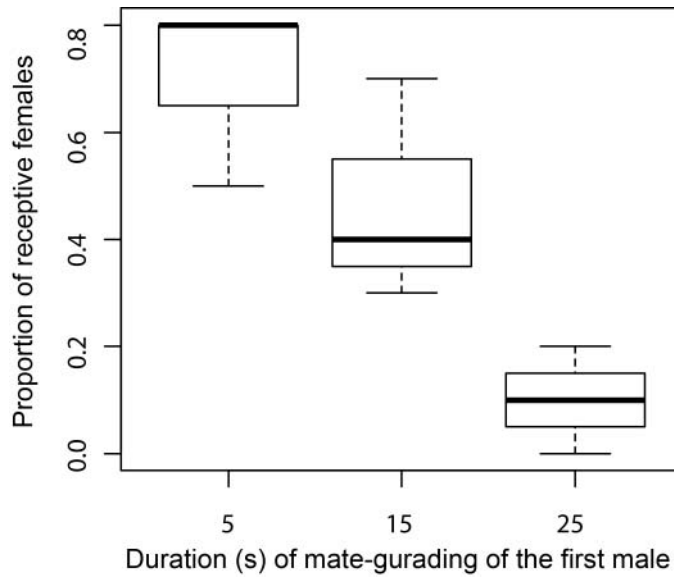


Fig. 3. Box plots showing the reduced proportion of receptive females of *P. vindemmiae* with the duration of mate-guarding.

Receptivity of females after copulation

When the post-copulation ritual by M1 ceased at 5, 15, and 25 seconds, M2 began to pursue the mated females. The proportion of receptive females was reduced significantly with increasing ritual duration ($F_{1,7} = 17.51$, $P = 0.004$) (Fig. 3). In treatments with only a 5 second ritual, most (70%) females were receptive. But when M1 moved after 25 seconds, only 10% of mated females remained receptive. In the control groups, after the post-copulation ritual ceased, none of the females re-mated. Although all of the M2 males could pursue the mated females, the genital pores of the females did not open again, and no further copulations were observed.

Comparison of broods between females mated once and mated twice

Compared with once-mated females, twice-mated females had similar numbers of total offspring ($F_{1,88} = 2.44$, $P = 0.88$), female offspring ($F_{1,20} = 0.19$, $P = 0.67$), and male offspring ($F_{1,20} = 0.14$, $P = 0.71$) (Fig. 4). The proportion of males was 0.20 ± 0.14 for offspring of twice-mated females, with paternity from both males, which was similar to that of offspring from females in the control group (0.23 ± 0.14) ($F_{1,20} = 0.51$, $P = 0.48$).

The daily proportion of male offspring differed during oviposition in broods from once-mated (Fig. 5a) and twice-mated (Fig. 5b) females. The proportion of male offspring increased significantly in broods of once-mated females ($F_{1,446} = 4.05$, $P = 0.04$) and in broods of twice-mated females ($F_{1,285} = 2.67$, $P = 0.10$). Twice-mated females did not produce a higher number of female offspring than once-mated females throughout the female oviposition period.

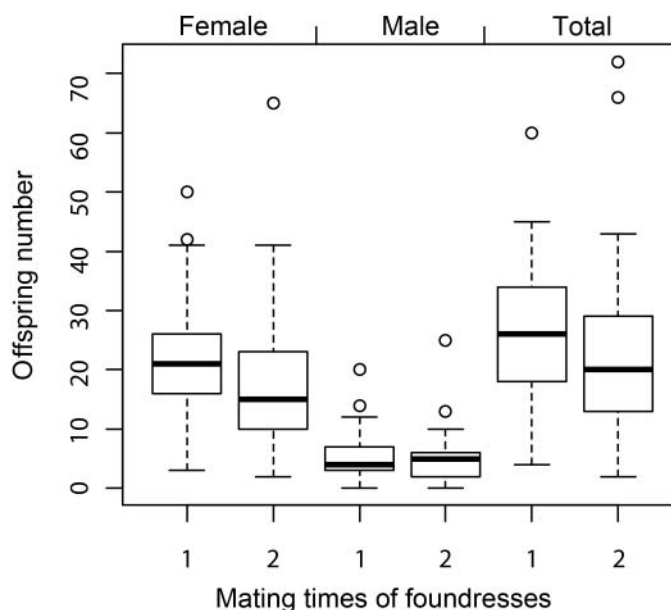


Fig. 4. Box plots showing similar numbers of male, female, and total offspring for *P. vindemmiae* females mated once and females mated twice.

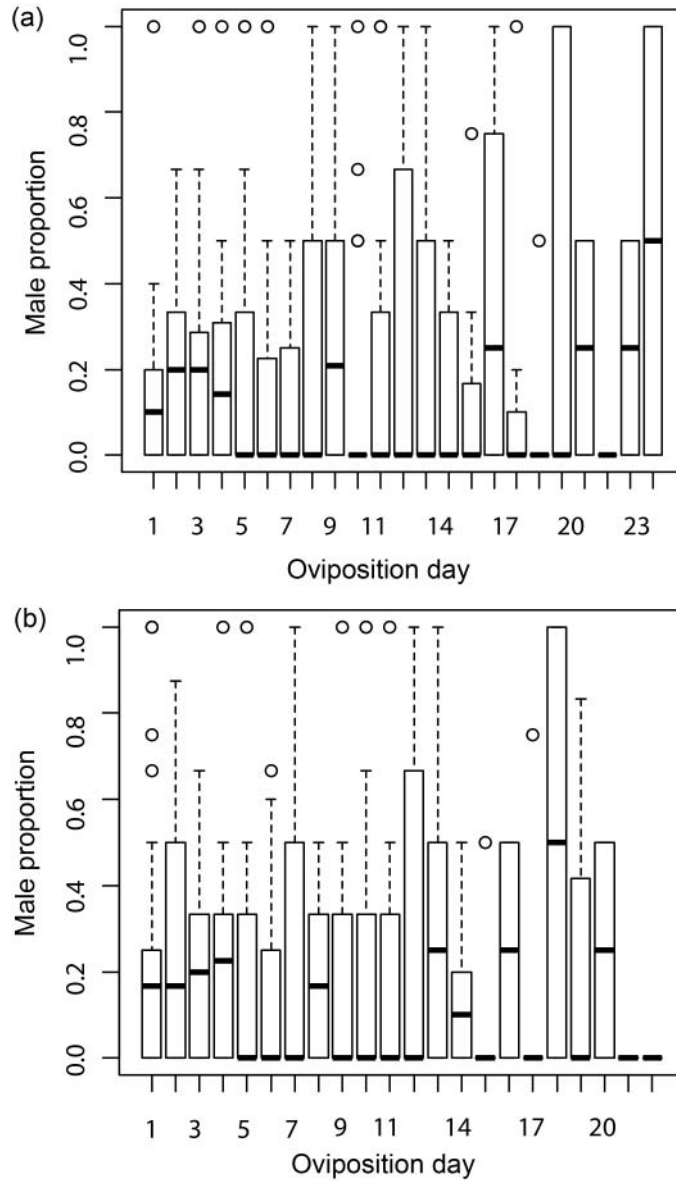


Fig. 5. Box plots showing the daily proportion of males for *P. vindemmiae* foundresses mated once (a) and twice (b).

DISCUSSION

Our results show that a post-copulation ritual existed when males finished copulating with females. When the ritual was prevented, females would remain receptive. If the ritual had finished, none of the females could re-mate, and all females were monandrous. Thus, the post-copulation ritual could be interpreted as mate-guarding behaviour on the part of

P. vindemmiae males, which could dramatically reduce the receptivity of females. The earlier the interruption of post-copulatory mate-guarding, the higher the frequency of receptive females.

Some authors have pointed out that the post-copulatory behaviour described for parasitoid wasps is not yet fully understood (Ruther *et al.*, 2000); however, mate-guarding is a common explanation. The post-copulation ritual has been shown to serve a mate-guarding function to prevent the receptivity of mated females of a number of parasitoid wasps, including *Aphytis melinus* (Aphelinidae) (Allen *et al.*, 1994), *Lariophagus distinguendus* (Pteromalidae) (Ruther *et al.*, 2000), *Aphidius ervi* (Braconidae) (McClure *et al.*, 2007), *S. endius* (King *et al.*, 2005; King and Kuban, 2012; King, 2018), and *O. kuvanae* (Ablard *et al.*, 2014). But it also seemed ineffective for some species; for example, *Urolepis rufipes* (Pteromalidae) females soon lost receptivity even when copulation and post-copulation were prevented, and female receptivity was not significantly affected by the absence of post-copulation (King and Kuban, 2012). Further research is needed to determine the prevalence of post-copulatory mate-guarding among parasitoid wasp species.

Mate-guarding has been interpreted widely as an effective method of reducing female receptivity. The mate-guarding hypothesis predicts that a male remains with his mate to increase the number of fertilized eggs because, in this way, he prevents his partner from re-mating (Alcock, 1994). Shuker and Simmons (2014) highlighted two types of mate-guarding: biochemical and behavioural. Biochemical mate-guarding occurs with the production of accessory gland secretions such as sex peptide in ejaculate (Shuker and Simmons, 2014), anti-aphrodisiac pheromone (Brent *et al.*, 2017), and counter-perfume (Malouines, 2016). Behavioural mate-guarding occurs before or after mating. As mentioned above, the post-copulation ritual could be interpreted as mate-guarding. Behavioural mate-guarding prior to mating is often associated with monandry; for example, the males of *Heliconius* butterflies guard pupal females prior to copulation (Shuker and Simmons, 2014). When solitary parasitoid wasps emerge from clumped hosts, *P. vindemmiae* could be described as quasi-gregarious, and male offspring emerge earlier than females (Nadel and Luck, 1985). In such circumstances, mating often occurs in the native patch, males may also guard the pupal females, and behavioural mate-guarding prior to mating would be expected.

Re-mating sometimes leads to greater offspring production by females, even in species that do not carry out nuptial feeding (Arnqvist and Nilsson, 2000). However, it has been shown that other females, such as those of *N. vitripennis*, that mate twice do not produce more daughters (Beukeboom, 1994); they may even produce fewer daughters (Assem and Bruijn, 1977). Multiple mating has no significant effects on the offspring sex ratio in, for example, *D. fuscipennis* and *Macrocentrus ancylivorus* (Braconidae) (King, 1987). Similar results have been found in *A. calandrae*, which also gains no advantage in either sperm stock or offspring quantity by mating twice (Khanh *et al.*, 2005). Our results showed that twice-mated females have similar numbers of female and total offspring to those that mate only once. If monandrous females cannot gain obvious benefits from polyandry, there will be few forces motivating the parasitoid wasp to break its mate-guarding habit and exchange the monandrous mating system for a polyandrous one. This would help to explain the maintenance of monandry in mostly solitary parasitoid wasps.

In Hymenoptera, both fertilized and unfertilized ova can develop offspring; therefore, re-mating would mainly affect the offspring sex ratio. Due to the haplodiploid genetic system, additional sperm from a secondary partner would provide the female with more female offspring. Ejaculates from both males would be used during oviposition, as by the

females of *Diachasmimorpha longicaudata* (Braconidae) (Martinez-Martinez *et al.*, 1993). Based on the present results, it is clear that the prospective benefit of sufficient sperm from an additional copulation might be non-existent. However, the males used here were all virgins that would have transferred sufficient sperm to their partners. Because of the female-biased sex ratio, males often mate with several females. If the female has primarily mated with males that have mated with several females in rapid succession, she would probably receive less sperm, and a secondary mate would provide her with the necessary sperm to fertilize her ova. Remated *S. endius* females have been shown to obtain such benefits as a higher proportion of females among their offspring if their first mate had previously mated multiple times (King, 2018). The benefits of polyandry in *P. vindemmiæ* females when mated with sperm-insufficient males would be interesting and would help to further understand the forces promoting the evolution of monandrous and polyandrous mating systems.

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