Effects of male age, body size and mating status on female mate preference and offspring sex ratio in *Pachycrepoideus vindemmiae* (Hymenoptera: Pteromalidae)

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Abstract. Mate choice is one of the main components of sexual selection, with females usually considered to be the selective sex. Male status is an important factor that affects female choice and to a large extent female mating preference. *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae) is an important solitary ectoparasitic idiobiont parasitoid that attacks several fly species and thus can be used as a biological control agent. We explored the influence of male status on mate selection, fecundity and offspring sex ratio. The results show that (1) *P. vindemmiae* females prefer to mate with young and large males and tended to choose males with only one mating experience; (2) the age, body size and mating status of males did not significantly affect female fecundity; and (3) the proportion of male offspring was larger when females mated with older, small and more often mated males. These results show that *P. vindemmiae* females prefer to mate with young and large males that had mated few times, and that mating with these males results in a higher proportion of female offspring. In addition, this study also indicates the way of rearing *P. vindemmiae* that produce more female offspring for use as biological control agents.

INTRODUCTION

Sexual selection arises from variation in competition for mates or competition for fertilisation more generally (Shuker, 2010) and has been a major field of study since the 1970s (Andersson & Iwasa, 1996). Following Darwin (1871), sexual selection has traditionally been divided into intra-sexual contest competition (male-male or female-female competition) and inter-sexual mate choice. Mate choice is an important component of sexual section and has been widely studied in many animals (Setchell & Huchard, 2010; Willis et al., 2011; Mingju et al., 2021). Typically, mate choice can involve female or male choice among mating partners or it can be the outcome of male competition (Joyce et al., 2009). In most animals, the sex that invests the most in reproduction, generally the female, is the sex that expresses mate choice (Prado & Haddad, 2005; Creighton et al., 2009; Harris & Uller, 2009). Females, which usually need to mate only once to fertilize their eggs, are typically considered to be the selective sex in mate choice (O'Neill, 2000).

In many species of insects, male status, such as age, body size and mating history, are important factors that affect

female choice and, to a large extent, affect female mating preference (He & Wang, 2006; King & Fischer, 2010; Liu et al., 2011; Sawadogo et al., 2013). However, female mate preference may be the result of active choice, passive choice, or both (Blanckenhorn et al., 2000). Male competition is also a key component of sexual selection. Usually, large or old males tend to win against small or young males because large males possess greater resource-holding potentials than small males (Arnott & Elwood, 2009; Hardy et al., 2013; Tsai et al., 2014) and old males have less time left to mate or obtain resources and may be more willing to act aggressively. When competing males have different genetic or developmental origins (Lacoume et al., 2006), it can be difficult to separate male competitive ability from female choice. Haplodiploid parasitoid species are important and unique insects that parasitize other organisms (Bianchi et al., 2006; Heraty, 2009), in which males develop from unfertilized (haploid) eggs and females from fertilized (diploid) eggs. Mated female parasitoids can store sperm in their spermathecae to control progeny sex at oviposition (Abe, 2019). Typically, females manipulate the sex of their progeny according to environmental factors (Liu et al.,

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2017; Cochard et al., 2019). Parasitoids have been extensively used to reduce the population sizes of pest species and employed as important study systems in behavioural ecology, molecular biology and evolutionary biology (Ode & Heimpel, 2016; Weber et al., 2021). To date, many studies have examined female mate choice in many parasitoids (Gu & Dorn, 2003; Martel et al., 2008) and found it to be influenced by male age (Cheng et al., 2003; He & Wang, 2008) and body size (Joyce et al., 2009). Consequently, maternal fitness may also be affected by the result of female mate choice (Jones et al., 1998; Ruther et al., 2009; Liu et al., 2011). Many studies reveal that male status has no influence on mated females' fecundity but alters their offspring sex ratio (Steiner et al., 2008; King & Fischer, 2010; Pérez-Lachaud, 2010). However, in *Diaeretiella rapae* (M'Intosh) (Hymenoptera: Braconidae), female fecundity is reduced by mating with males that have mated many times (Kant et al., 2012).

Pachycrepoideus vindemmiae (Rondani) (Hymenoptera: Pteromalidae) is an ectoparasitic idiobiont and solitary parasitoid that parasitizes various flies, including flies in the genera *Drosophila*, *Musca*, *Anastrepha* and *Calliphora* (Marchiori & Borges, 2017; Bezerra et al., 2019; Gowton et al., 2020). *P. vindemmiae* is widely distributed around the world, occurring in the United States, Italy and Brazil. It was introduced to Hawaii and several South American countries for the control of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) and *Anastrepha* spp. (Diptera: Tephritidae) (Purcell, 1998; Ovruski et al., 2000) and mass released for the control of *C. capitata* in Costa Rica (Ovruski et al., 2000). In recent years, *P. vindemmiae* has been identified as an important natural enemy of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), which is a significant pest of soft- and thin-skinned fruit in North America, Europe and South America (Walsh et al., 2011; Burrack et al., 2012; Cini et al., 2012, 2014). In addition, the biological characteristics of *P. vindemmiae*, such as its fecundity, development time and mating behaviour, have been extensively studied (Stacconi et al., 2013; Gabarra et al., 2015). However, there are few studies on mate selection in this species. Thus, this study examined female mate choice in *P. vindemmiae*, with males differing in status (age, body size and number of times mated). This study provides a means of further advancing the knowledge of sexual selection in parasitoid wasps.

MATERIALS AND METHODS

Insects

In May 2015, pupae of *Musca domestica* (Linnaeus) (Diptera: Muscidae) were placed in the wild at Anhui Normal University, Wuhu City, Anhui Province, China (31.34°N, 118.38°E), for approximately 3 days and then brought back indoors and reared at a temperature of $25 \pm 1°C$. After approximately 16 days, adults of *P. vindemmiae* emerged from the pupae and were maintained on *M. domestica* pupae. In addition, adults of *M. domestica* were collected at Anhui Normal University and reared under laboratory conditions to acquire pupae.

To acquire wasps for this study, thirty *M. domestica* pupae were placed with one mated female wasp in a plastic container (25 mm in diameter, 50 mm in height). After 24 h, the hosts and wasp were isolated. Then, each *M. domestica* pupa was placed in a 2-ml plastic tube with 150-µm pore size nylon mesh seal around the lid to prevent emerging parasitoids encountering and mating with other individuals. All wasps were reared at a temperature and relative humidity of $25 \pm 1°C$ and $60 \pm 5\%$, respectively, under a 14L : 10D photoperiod. After eclosion, each adult wasp was supplied with cotton wool soaked with 30% honey for 24 h to ensure that reached sexual maturity.

Choice experiments were carried out at a temperature of $25 \pm 1°C$ and relative humidity of $60 \pm 5\%$.

Male age

To investigate the effect of male age on female choice, one female (2 days old) was introduced into a Petri dish (5.5 cm in diameter, 1.3 cm in height) including a 1-day-old male and a 3-day-old male, a 1-day-old male and a 5-day-old male, or a 1-day-old male and a 7-day-old male. For 5 min, the results of mating, e.g., whether mating behaviour occurred and the outcome of mate choice, were recorded. Mating in *P. vindemmiae* usually includes courtship, pre-copulation, copulation and post-copulation. Therefore, we recorded only these events. There were 20 replicates for each treatment.

To determine whether male age affected female fecundity and offspring sex ratio, one 2-day-old female was provided with a 1-day-old, 3-day-old, 5-day-old, or 7-day-old male. After successful mating, each female was separately placed plastic vials. Thirty fresh *M. domestica* pupae and honey-soaked cotton wool were provided and replaced daily until the female died. The pupae were then placed into a Petri dish. After eclosion, the number and sex of progeny produced by females were recorded. There were 5 replicates for each male age treatment.

Male size

To investigate the effect of male size on female choice, one female (2 days old) was introduced into a Petri dish including two males (2 days old) that differed in body size. After 5 min, the results of mating behaviour were recorded as described above. Posterior tibial length is commonly used as a proxy for body size in parasitoid wasps (Godfray, 1994). Thus, after the experiment ended, the posterior tibial length of parasitoids in each Petri dish were measured (Table S1) using a stereomicroscope (Leica M205A, Wetzlar, Germany) with a Leica DFC295 camera. Measurements were taken from photographs using Leica Application Suite v.3.7 software. Posterior tibial length was measured from the junction of the femur with the tibia to the junction of the tibia with the tarsus. The parasitoids were all unmated, and females had not laid eggs before being used in the experiments.

To determine whether male size affected female fecundity and offspring sex ratio, one 2-day-old female was provided with either a large male or small male. After successful mating, the posterior tibial length was measured and each female was separately placed in plastic vials containing 30 fresh *M. domestica* pupae and honey-soaked cotton wool, which were replaced daily until the female died. The number and sex of progeny produced by females were recorded. In addition, all male sizes were determined as described above and recorded.

Male mating status

To acquire males that had mated different numbers of times (i.e., once, fifteen and thirty times), sufficient unmated females (2 days old) were provided seriatim to a male, and the number of times it mated recorded. Then, one female (unmated, 2 days old) was introduced into a Petri dish including either an unmated male and a once-mated male, an unmated male and a fifteen-times-mated male, or an unmated male and a thirty-times-mated male

(all males were 2 days old). After 5 min, the results of mating behaviour were recorded as described above. There were 40, 40 and 12 replicates for unmated males with mated once males, unmated males with fifteen times mated males and unmated males with thirty times mated males, respectively, and all females had not laid eggs before the experiment.

To determine whether male mating status affected female offspring number (i.e., fecundity) and offspring sex ratio, one female (unmated, 2 days old) was mated with either a male mated once, fifteen times or thirty times. Then, thirty fresh *M. domestica* pupae and honey-soaked cotton wool were provided to unmated females (as a control treatment) or females that had mated with males that had previously mated different numbers of times and replaced daily until the females died. The number and sex of progeny produced by females were recorded. In total, there were 7, 6, 5 and 5 replicates for females mated with unmated males, males that had mated once, fifteen and thirty times, respectively. The females had not laid eggs before being used in this experiment.

Statistical analysis

All of the analyses were conducted in R 2.13.0 (R Core Team, 2018). In the choice experiments, the preferences for males of different ages, body sizes and mating status were analysed using sign tests. Generalized linear model (GLM) and analysis of deviance were used to analyse the effects of male age, size and mating status on offspring number. Proportional data have typically nonnormally distributed error variances, so GLM analysis of this data was used, assuming Poisson errors and a log link function for count data and binomial errors and a logit link function for proportional data. We replaced Poisson or binomial error distributions with quasi-Poisson or quasi-binomial error distributions in the analyses when there was a relatively large residual deviance after fitting the explanatory variables. When more than one explanatory variable was considered, a full model including explanatory variables and their interactions was initially fitted to the data. Terms were then removed from the full models by stepwise deletion. For evaluating the trend in the difference among the treatments, we calculated pairwise contrasts using Tukey's method using the emmeans package. The final models were tested using an F-test (Crawley, 2007). In addition, the male body size data were analysed using an independent-sample t-test.

RESULTS

Male age

In the choice experiment, when a female was simultaneously provided with one 1-day-old and one 3-day-old male, there was no significant mating preference based on male age (Table 1). Similar results were recorded in the treatment in which one 1-day-old male and one 5-day-old male were simultaneously provided to one female (Table 1). However, when one 1-day-old male and one 7-day-old male were simultaneously provided, females prefer to choose the younger male (Table 1).

The number of offspring produced by females was not affected by male age ($F_{3, 16} = 0.489$, $P = 0.695$, Fig. 1A). However, the offspring sex ratio, i.e., male %, increased significantly with male age ($F_{3, 15} = 2.718$, $P < 0.05$, Fig. 1B).

Male size

In the choice experiment, there was a significant tendency for females to mate with large males. Large males copu-

Table 1. Female preference for males of different ages, sizes and mating experience.

Factors	Male condition	Number of males mated [1]	N	Z-value	P-value
Age	1-day-old	9	20	–0.447	0.655
	3-day-old	11			
	1-day-old	10	20	0.00	1.000
	5-day-old	10			
	1-day-old	16	20	–2.683	0.007
	7-day-old	4			
Size	larger	12	15	–2.324	0.020
	smaller	3			
Mating status	unmated	11	40	–2.846	0.004
	1 time	29			
	unmated	24	40	–1.265	0.206
	15 times	16			
	unmated	9	12	–1.732	0.083
	30 times	3			

[1] A female was caged with two males of different condition (age, size or mating status).

lated with females in 12 replicates, whereas small males copulated with females in only 3 replicates (Table 1).

The number of offspring was not significantly associated with male size ($F_{1, 22} = 1.756$, $P = 0.199$, Fig. 1C). However, the offspring sex ratio (male %) decreased significantly with increase in male size ($F_{1, 22} = 5.236$, $P = 0.032$, Fig. 1D).

Male mating status

In the choice experiment, when one unmated male and one once-mated male were simultaneously provided to a female, the female was more likely to choose the once-mated male (Table 1). For unmated males and males that had mated fifteen times or thirty times (Table 1), females had no significant preference.

The number of times that males had previously mated had no significant effect on the number of progeny ($F_{3, 19} = 0.806$, $P = 0.506$, Fig. 1E). However, when females mated with males that had mated thirty times, the offspring sex ratio was significantly higher than that for females mated with unmated or once-mated males (unmated and thirty-times mated: $F_{1, 9} = 10.73$, $P = 0.005$; once mated and thirty-times mated: $F_{1, 8} = 9.048$, $P = 0.006$; Fig. 1F).

DISCUSSION

Consistent with the findings of many other studies (Beck & Promislow, 2007; Rezaei et al., 2015), the results presented show that females prefer to mate with young and large males. In addition, in *P. vindemmiae*, compared with males that had previously mated fifteen, thirty or zero times, those with only one previous mating experience were preferred by females. Age, body size and mating status did not significantly affect female fecundity; however, the proportion of male offspring significantly increased when females mated with older, smaller or more often mated males.

Mate choice is a critical component of sexual selection and females are usually predicted to be the selective sex (O'Neill, 2000). Female mate decisions can be influenced by male status, which has been widely studied in many
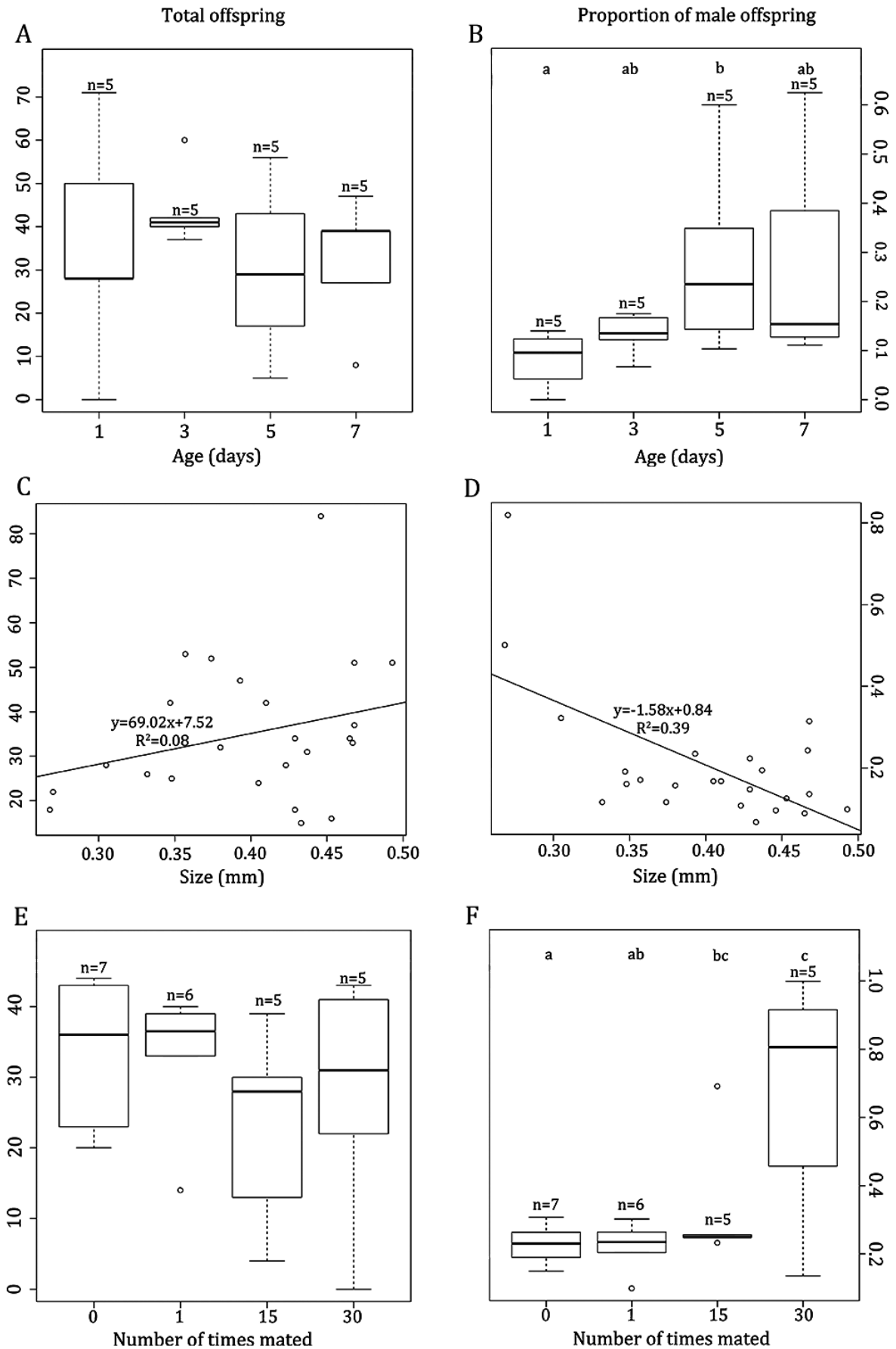
Fig. 1. The influence of male age, size and number of times mated on the number and sex ratio of offspring. Box – 25th and 75th percentiles; heavy line – median; whiskers – 1.5 times the interquartile range of the data; dots – outliers. Different letters above columns in a given plot indicate significant differences between treatments (Tukey's test, P < 0.05).

species (Byers et al., 2010; Liu et al., 2011; Giunti, 2018; Aich et al., 2020), including parasitoid wasps (Cheng et al., 2004; Joyce et al., 2009; King & Fischer, 2010). In *P. vindemmiae*, consistent with the findings for many other species, e.g., *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) (Joyce et al., 2009) and *Aphidius ervi* (Haliday) (Hymenoptera: Aphidiidae) (He & Wang, 2008), the results presented show that females prefer to mate with young and large males. Female preference for large or young male mates may be an active or passive choice, when a female mates with the winner of male-male competition. In this study, small males of *P. vindemmiae* may have been the offspring resulting from superparasitism (even though sufficient hosts were provided). Therefore, the differences in male offspring resulting from superparasitism should be studied in the future. In addition, in many species, compared with mated males (i.e., those with mating experience), unmated males are more likely to be chosen by females (He & Wang, 2008; King & Fischer, 2010; Kant et al., 2012; Kant, 2013). Mated males may have less pheromone left to release than unmated males. In *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae), males that had mated seven or more times produced less of the pheromone that attracts females (Ruther et al., 2009). However, in *P. vindemmiae*, in the comparison of unmated males and males that had mated many times, males with only one mating experience tended to be chosen by females. This result is consistent with the results of Wittman & King (2019) for the parasitoid wasp *Urolepis rufipes* (Ashmead) (Hymenoptera: Pteromalidae). A possible explanation is that males that had mated only once were more attractive, which is associated with pheromone production (Ruther et al., 2009; Steiner & Ruther, 2009; Blaul & Ruther, 2012). Usually, males are sperm-limited when mated many times (Abe, 2019) and sexually immature and sperm-depleted males produce less pheromone (Ruther et al., 2009). Furthermore, male vigour is an indicator of male attractiveness. In *Trichogramma evanescens* (Westwood) (Hymenoptera: Trichogrammatidae), there is a significant increase in the duration of copulation with increase in the number of times a male has previously mated, which may be due to loss of male vigour (Damiens & Boivin, 2005).

Similar to many other studies (He & Wang, 2006; Heinze et al., 2018), this study shows that the age, size and mating status of males have no significant effect on female fecundity. In parasitoids, female fecundity is typically determined by female body size (He & Wang, 2006; Heinze et al., 2018), which is mainly related to host size in solitary parasitoids (Mackauer & Chau, 2001). As *P. vindemmiae* females were reared on similar sized hosts in this study, body size upon emergence did not differ significantly. In addition, in many insects, females usually use the nutrients provided by males during copulation for producing eggs and increasing their fecundity (Huignard, 1983; Simmons & Gwynne, 1993; Jiménez-Pérez & Wang, 2004). However, other results indicate that female parasitoids do not obtain any nutrients from males during copulation (Fau-

vergue et al., 1998; Godfray, 1994), which might explain the lack of an effect of male status on female fecundity in this study.

Hymenopteran parasitoids are unique in terms of their haplodiploid sex-determination system, meaning that males develop from unfertilized (haploid) eggs and females from fertilized (diploid) eggs (Heimpel & De Boer, 2008). After mating, female parasitoids can store sperm in their spermathecae and adjust the sex of eggs by retaining or releasing sperm (Charnov, 1982; Ode & Hardy, 2008; Mateo Leach et al., 2009). Thus, the quantity and quality of sperm stored by females are important factors influencing the offspring sex ratio in these wasps (Damiens et al., 2002) and these features of sperm are significantly affected by male status (e.g., age and mating experience) (Damiens et al., 2003; Bressac et al., 2009). Similar to the patterns recorded in many other species (He & Wang, 2006; Kant, 2013), females that mated with large, young or unmated males produced significantly more female progeny. Large and young males may have a greater insemination capacity (Boulton et al., 2015) and males that have previously mated several times may be sperm depleted (Damiens & Boivin, 2005; Bressac et al., 2009; Abe, 2019), which might result in them ejaculating less sperm and production by females of more male offspring.

Sex ratio distortion can be a major problem in mass-rearing programmes of parasitoid wasps, as male-biased sex ratios in offspring are detrimental for mass-rearing (Heimpel & Lundgren, 2000; Santolamazza-Carbone et al., 2007). Therefore, it is necessary to study sex allocation in parasitoids and measures should be taken to avoid male-biased offspring in mass-rearing procedures. *P. vindemmiae,* an important pupal ectoparasitic wasp, is widely used for biological control. To acquire more female offspring in mass rearing, our results indicate that young, large and less frequently mated males should be offered to females as mates.

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Supplementary Table S1 follows on next page.

Table S1. Posterior tibial length (mm).

Large male	Small male	Female
0.417	0.300	0.434
0.449	0.392	0.429
0.460	0.352	0.427
0.417	0.312	0.457
0.470	0.336	0.454
0.391	0.287	0.445
0.405	0.370	0.442
0.361	0.278	0.448
0.436	0.336	0.434
0.437	0.364	0.466
0.536	0.369	0.464
0.345	0.312	0.461
0.442	0.303	0.458
0.514	0.378	0.455
0.437	0.293	0.455