Sperm-limited males continue to mate, but females cannot detect the male state in a parasitoid wasp

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Abstract

Female mating frequency and male ejaculate allocation are likely to interact. Females may adjust their propensity for remating based on the amount of provided sperm to ensure a sufficient sperm supply, and males may determine sperm allocation based on female availability and female mating frequency. In this study, I investigated male and female mating behaviors in the parasitoid wasp *Anisopteromalus calandrae*. The wasp exhibits the haplo-diploid sex determination, in which sperm-depleted females are constrained to produce only sons by laying unfertilized eggs. The first experiment showed that a rapid succession of male mating decreased the production of daughters (fertilized eggs) by the inseminated females, suggesting that sperm-limited males provided an insufficient amount of sperm to the females. Although the males appeared to replenish their sperm store after 6 h, they mated upon encountering females despite their sperm shortage. The second experiment showed that copulation reduced the subsequent mating receptivity of the inseminated females irrespective of whether the females received a sufficient amount of sperm. Moreover, although approximately 26% of females accepted a second mating and recovered a certain degree of daughter production, remating was independent of the mating status of their first mating partner or the social environment. These results suggest that sperm-limited males may benefit from continuing to mate because their copulation prevents competing males from reproducing with their mates. Females incur a cost from not remating depending on the amount of sperm provided, which may result from weak environmental selection pressure or manipulation by the initial mate.

Significance statement

Male and female mating strategies are likely to evolve interdependently. Specifically, in species that produce limited numbers of sperm, the mating behavior of males and females is likely to be influenced by sperm transfer. The results of the present study suggest that males of the parasitoid wasp *Anisopteromalus calandrae* can replenish their sperm stores over time. However, sperm-limited males that encounter a potential mating partner mate rather than wait for sperm recovery. Females do not discriminate the male state before copulation, and those that mate with sperm-limited males suffer from sperm shortage. Although females can augment their sperm supply by remating, only a portion of females do so, regardless of sperm supply. The lack of a female facultative response suggests weak environmental selection on the species or the existence of sexual conflict over sperm transfer.

Keywords *Anisopteromalus calandrae* · Mating strategies · Parasitoid wasp · Polyandry · Reproductive behaviors · Sexual conflict

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Introduction

The ubiquity of polyandry in nature implies that multiple mating yields benefits that can offset the costs imposed on females (Arnqvist and Nilsson 2000; Pizzari and Wedell 2013; Parker and Birkhead 2013). In turn, female multiple mating causes postcopulatory male competition over fertilization (sperm competition; Parker 1998). Although the ultimate cost of reproduction is smaller for males than for females, male

resources for reproduction are far from limitless and are expected to be optimally allocated among successive matings (Wedell et al. 2002; Parker and Pizzari 2010). Males may strategically vary the amount of their ejaculate based on the risk and intensity of sperm competition, which are dictated by the degree of polyandry (Parker 1990; Parker et al. 1996). Therefore, female mating frequency and male sperm allocation are likely to be interdependent and to establish coevolutionary feedbacks (Abe and Kamimura 2015; Bocedi and Reid 2016).

To examine the interaction between male and female reproductive strategies, Abe and Kamimura (2015) proposed a novel framework called the “sperm economy” theory and developed a model that investigated the coevolutionary dynamics between male ejaculate allocation and female remating frequency (see also Bocedi and Reid 2016). The interaction between these strategies is likely to be influenced by factors that determine the level of sperm limitation in the population, such as operational sex ratios. Consequently, the model predicted that under an even sex ratio, females mate only once, as in traditional models. However, under female-biased conditions, males are predicted to favor decreasing the amount of ejaculate in each mating to partition their reproductive budget, such as sperm, among many females, which could lead to sperm limitation for their mates. In contrast, females are predicted to favor multiple matings to acquire sufficient male reproductive budgets to prevent reproductive constraint (Abe and Kamimura 2015). While this and other theoretical works have incorporated coevolutionary feedbacks, the sperm economy theory emphasizes the importance of simultaneously examining both male ejaculate allocation and female mating frequency to reveal the balance of sperm supply in empirical investigations.

Parasitoid wasps are a taxonomic group in which the level of sperm limitation may influence male and female reproductive strategies (Godfray 1994; Abe and Kamimura 2015). These wasps show a variety of sex ratios, including various female-biased sex ratios (Godfray 1994), so the females can suffer from sperm depletion and be constrained in their reproduction (Hardy and Godfray 1990). Hymenopteran insects have haplo-diploid sex determination, in which fertilized and unfertilized eggs develop into female and male individuals, respectively. Therefore, although sperm-depleted females can still reproduce, they can be constrained to produce non-optimal sex ratios. Most species of parasitoid wasps are characterized by monandry (Ridley 1993), but there is interspecific variation in female mating frequencies across species (Hardy 1994). Ridley (1993) explored the literature on female mating frequency among parasitoid species and found a tendency of polyandry to appear more frequently in gregarious species than in solitary species. One explanation could be sperm shortage due to female-biased sex ratios (Godfray 1994). In contrast to solitary species, whose individuals

disperse to mate globally, the mating of gregarious species typically occurs among individuals who emerged from the same hosts, and these species show female-biased sex ratios, as predicted by local mate competition (LMC) theory (Hamilton 1967). Under such a female-biased condition, females are expected to mate multiple times to obtain sufficient sperm to be able to adaptively allocate sex, because the amount of ejaculate by a single male may be insufficient due to sperm depletion or ejaculate partitioning (Godfray 1994; Abe and Kamimura 2015). Although these predictions were originally made to explain the interspecific variation in female mating frequency resulting from an evolutionarily adaptive response, a similar prediction is likely to be made for facultative response by individual females (Charlat et al. 2007; Abe and Kamimura 2015). Females are likely to remate, when they recognize that the ejaculate provided by the first mating is insufficient or when they are in a situation in which males are expected to provide a small amount of sperm.

Anisopteromalus calandrae (Hymenoptera: Pteromalidae) is an ectoparasitoid wasp that attacks the larvae and pupae of a wide range of coleopteran pest insects that infest several grains (van den Assem et al. 1984). Although it is a solitary parasitoid, the wasp parasitizes spatially clumped hosts, so its population structure is characterized by a clumped distribution (it is categorized as a quasi-gregarious parasitoid). Its sex ratios change largely in accordance with the predictions both of Hamilton's (1967) LMC model (Lebreton et al. 2010) and Charnov et al. (1981) host quality model (van den Assem et al. 1984; Ji et al. 2004) and vary between 0% female and nearly 90% female (Nishimura and Jahn 1996). Considering the variable sex ratios and the intermittent emergence timing of males, the frequency of encounters with the opposite sex is likely to fluctuate greatly, and mating opportunities may be biased in favor of specific males (Martel and Boivin 2007). Moreover, although male *A. calandrae* are synspermatogenic (i.e., they continue producing sperm after emergence), they exhibit reduced amounts of sperm in seminal vesicles over consecutive matings (Bressac et al. 2009). If the reduction of sperm influences the reproductive performance of females, then females may make decisions regarding mating acceptance or adjust their remating tendency depending on the mating status of the males (Boivin 2013; Boulton et al. 2015).

In this study, I examine both male sperm allocation among successive matings and female remating behavior to reveal the transfer of sperm from males to females in *A. calandrae*. In the experiments below, the amount of sperm transferred to the females was estimated based on the proportion of daughters (fertilized eggs) produced by the females. The rationale for this approach is that studies of *A. calandrae* that have directly counted the number of sperm in female spermathecae have revealed that females efficiently use the stored sperm when they fertilize eggs in oviposition (Khanh et al. 2005; Bressac et al. 2009; Kasamatsu and Abe 2015). In our previous study,

we confirmed that after laying clutches with mixed sexes (demonstrating that they have been inseminated), females produce only sons (unfertilized eggs), suggesting that they have run out of sperm. This finding was confirmed by dissecting such females, which had no or very few sperm in their spermathecae (Kasamatsu and Abe 2015; unpublished data).

In the first experiment, I assess how males allocate their ejaculate among successive matings and how this allocation impacts the fecundity and offspring sex ratio of inseminated females. In the second experiment, I investigate whether females can distinguish sperm-limited males before or after copulation. If females recognize the status of males upon encounter and have an absolute preference for the male state, they might accept copulation at a higher rate after courtship by virgin males than after courtship by sperm-limited males. If females recognize the amount of ejaculate after copulation and can replenish their sperm supply through additional matings, they might adjust their tendency to remate depending on the status of their first partner. I consider the possibility that females not only directly recognize the amount of ejaculate provided but also indirectly estimate it based on the social environment in terms of the operational sex ratios. Finally, based on the results of the two experiments, I discuss the evolutionary consequences for sperm transfer between males and females, specifically in relation to the selection pressure experienced in the environment and the context of sexual selection.

Materials and methods

Parasitoid wasps

The strain of *A. calandrayae* used in the experiments was created from wild-caught wasps collected from Kyoto, Japan, in 1938 and maintained in laboratories. The wasps for the experiments and in the stock cultures were reared on one of their host species, *Callosobruchus chinensis* (Coleoptera: Bruchidae), which infests the cowpea *Vigna unguiculata* (Fabales: Fabaceae). Stock cultures were initiated every 2 weeks, and each culture was founded by introducing approximately 20 females and some males from the previous culture into a new petri dish, in which beans were provided containing 14-day-old hosts. Under this condition, roughly 70% of individuals develop into females (M. Shimada and Y. Nagase, personal communication). To obtain the individuals used in the following experiments, subcultures were created, in which five males and five females were moved from the stock culture into a new petri dish with 120 beans, with each bean containing a few hosts. To standardize the size of the experimental individuals, hosts that had developed for more than 14 days were used (Kasamatsu and Abe 2015). Prior to the emergence of the offspring generation, the 120 beans in each subculture were divided into units of three or four beans each that were

kept in plastic cases ($40 \times 30 \times 20 \text{ mm}^3$). The cases were checked on a daily basis, and newly emerged virgin individuals who did not coexist with the opposite sex after emergence were collected and kept individually in 0.2-ml plastic tubes or with groups of the same sex in new plastic cases ($40 \times 30 \times 20 \text{ mm}^3$) until they were used in the mating treatments described below. Therefore, individuals were ready for the experiments within 24 h after emergence. Adults are already sexually mature on the day of emergence: females are ready to accept copulation, and males possess a sufficient amount of sperm to inseminate females (Bressac et al. 2009; Kasamatsu and Abe 2015). All the experiments were conducted under a constant temperature of 28 °C and a photoperiod of L16:D8 h. To minimize observer bias, blind methods were used when recording all behavioral data in the following experiments.

Experiment 1: male mating potential and the impact on female reproduction

In the first experiment, I investigated male mating behavior across successive matings, the males' recovery capacity from sperm shortage, and the impact on female reproduction. Twenty males were sequentially subjected to eight mating trials and an additional two subsequent mating trials conducted 6 h and 24 h later. In each mating trial, a male was placed with a virgin female in a mating chamber (8 mm diameter, 20 mm width) composed of a transparent plastic cylinder with silicon caps on both ends. The courtship behavior and copulation success were directly observed for 10 min or until copulation occurred, whichever came first. Even if a courtship did not result in a successful copulation, the pair was allowed to have other mating opportunities within 10 min. The trial time of 10 min was considered sufficient because, among copulating pairs, most copulations occurred within 10 min in continuous observations of more than 30 min (H. Ayabe, personal communication). Regardless of whether mating occurred in the first mating trial, the male was immediately transferred to a clean mating chamber after the end of copulation for the mated males or after the end of the trial time for the refused males. In the new mating chamber, the male was provided another virgin female, and his mating behavior was observed again; this procedure was repeated a total of eight times. After the eighth mating trial, the males were moved and individually kept in the 0.2-ml tube. The males were provided with two more subsequent mating opportunities 6 h and 24 h later in the same manner described above. The last two mating trials were conducted to examine whether and when their sperm stocks are replenished and how the males behave.

To evaluate male insemination capacity and its influence on female reproduction, a subset of inseminated females was allowed to produce offspring. Offspring production was examined for the first, third, fifth, and seventh females among those that mated successfully in the first eight successive

mating and for females that mated 6 h and 24 h later. The detailed procedures for measuring offspring production are described elsewhere (Kasamatsu and Abe 2015). Briefly, a lone inseminated female was allowed to produce a clutch just after the 24 h later mating trial by laying eggs on a batch of cowpea seeds containing at least 100 bean beetle hosts for 4 days. To allow the female to produce subsequent clutches, the cowpea seeds with the hosts were renewed every 4 days until the death of the female. The females were checked for mortality every 4 days, and the number of clutches produced by a female reflected her longevity. After the offspring were allowed to develop into adults, the number of male and female offspring was counted. Lifetime fecundity was measured as the total number of offspring that emerged from all the clutches produced by a single female, and the sex ratio was represented as the proportion of female offspring. Under this condition, a female is predicted to produce a clutch with approximately 60% females if she mates with a virgin male (Kasamatsu and Abe 2015). One female out of those that were mated with the males a third time produced only one offspring and was excluded from analysis (the final number of replicates used to analyze offspring production is presented in Fig. 2).

Experiment 2: influence of male state and social environment on female mating propensity

In the second experiment, I investigated whether females are able to recognize the male state either before or after copulation and adjust their mating behavior accordingly. Females might perceive the mated status of males during courtship or copulation or directly recognize the amount of ejaculate provided after copulation. Alternatively, they might use cues from the social environment to determine the mated status of encountered males; in an environment with abundant females, males are likely to become sperm limited after repeated copulations. We may expect that females recognize that they might be supplied a smaller amount of ejaculate when they frequently encounter other females or must wait a long time for a mating opportunity compared to easily finding a potential mating partner. Therefore, this experiment consisted of two treatments regulating the state of male mates and females' experience before mating. First, to vary the amount of ejaculate transferred to females in their first mating, a virgin female was allowed to mate with either (i) a virgin male or (ii) a sperm-limited male (male state treatment). Based on the results of the first experiment, males that mated four or more times (up to seven times) in rapid succession were used as sperm-limited males. Second, to vary the social environment that females experienced prior to mating, a virgin female was allowed to mate (i) on the day of emergence, (ii) after being kept alone for 2 days, or (iii) after being maintained with a group of 10 females for 2 days (social environment treatment). Females were placed in a 0.2-ml plastic tube or in a plastic case ($40 \times 30 \times 20 \text{ mm}^3$) for 2 days when

kept alone (category ii) or in a group (category iii), respectively. In categories (i) and (ii), females were used that did not coexist with any other individuals after emergence. For category (iii), the females in the groups were randomly collected from a single subculture. After females experienced one of the three environmental contexts, they were exposed to a virgin or sperm-limited male in the mating chamber, and their courtship and copulation success were recorded for 10 min, as described in experiment 1.

After performing each treatment above, females that mated in the first mating attempt were then randomly assigned to one of two groups. One set of mated females was used to investigate offspring production by once-mated females. After the first mating, the females that mated with virgin or mated males were allowed to produce offspring on the host batches, which were successively provided following the same procedure as in experiment 1, and their fecundity and offspring sex ratios were examined. The other set of mated females was used to investigate the female propensity to remate. Once a pair finished their copulation in the first mating, the mated female was moved to a new mating chamber, and isolated from males for 60 s. Then, a new virgin male was introduced into the mating chamber, and the mating behavior of the pair was observed for 10 min to determine copulation success. The interval length of 60 s was selected to contrast the dual mating reported in the same species by Khanh et al. (2005) and Bressac et al. (2009), which occurs within 10 s after the end of the first copulation without providing additional courtship. If the females accepted the second mating attempt, they were also used to examine offspring production. Offspring production was examined for once-mated and twice-mated females in the present experiment. Note that once-mated females are not those that rejected a second mating attempt but those that were offered only one mating attempt. Two once-mated and one twice-mated females produced no offspring, and one once-mated female had only five offspring. Therefore, these females were excluded from the analysis (the final number of replicates used to analyze offspring production is presented in Fig. 4).

Statistics

All data analyses were performed by generalized linear mixed models (GLMMs) using R 3.3.1 software (R core team 2016). Binary data such as mating success and proportional data such as sex ratios were fitted with a binomial error structure and a logit link function, and count data such as clutch size and the number of clutches produced were fitted with a Poisson error structure and a log link function. For analysis of mating acceptance data, a male was provided with mating attempts with different females in experiment 1, a female was provided with mating attempts with up to two males in experiment 2, and a female was allowed to sequentially produce multiple clutches

in experiments 1 and 2. In these cases, the terms male individual, female individual, and female individual, respectively, were added to the models as random effects to avoid pseudo-replication by using the same individuals. In addition, for proportional and count data, additional terms that represent the block of data were also added as random factors to resolve the problem of overdispersion. In each analysis, a full model was conducted, including all fixed effects of interest, the second-order interaction terms, and the random effects described above. The statistical significance of each fixed main effect and interaction was evaluated by the likelihood ratio test. Non-significant terms ($\alpha > 0.05$) were removed to acquire minimal adequate models following the backward stepwise deletion procedure (Crawley 2007).

Data availability

All data generated and analyzed during this study are included in the supplementary information file.

Results

Experiment 1: male mating potential and the impact on female reproduction

Male mating success was independent of the order of the male mating trials (Fig. 1) when the mating order was analyzed as a continuous variable within the first eight successive mating trials ($\chi^2 = 0.014$, $df = 1$, $P = 0.91$) or when it was treated as a categorical variable in the overall mating trials, including the

Fig. 1 Mating success rate is independent of the order of male mating trials. The mating success rate is shown as the percentage of successful mating out of the mating trials of an examined 20 males. Each male was provided with eight virgin females in succession and subsequently two additional virgin females 6 h and 24 h later. Error bars represent 95% binomial confidence intervals

two additional trials after the intervals ($\chi^2 = 3.84$, $df = 9$, $P = 0.92$). This tendency persisted when the same data set was analyzed with the explanatory variable being the male prior mating frequency instead of the mating trial order (Fig. S1).

All females laid smaller, more male biased clutches after ovipositing on multiple host batches, suggesting that they ran out of sperm. This apparent sperm depletion started earlier when females had mated with males that had mated multiple times already (see Fig. S2 for the analyses and results). The lifetime fecundity of the females over all their clutches was not influenced by male mating order within the first successive mating ($\chi^2 = 0.43$, $df = 1$, $P = 0.51$) or in the overall mating ($\chi^2 = 4.6$, $df = 5$, $P = 0.47$; Fig. 2). However, the lifetime sex ratios decreased significantly within the first successive mating ($\chi^2 = 52.8$, $df = 1$, $P < 0.001$) and were significantly different in the overall mating ($\chi^2 = 87.7$, $df = 5$, $P < 0.001$), suggesting that although the amount of sperm provided by males decreased over the first successive mating, it had recovered in the males that mated with females after 6 or 24 h (Fig. 2; Fig. S2).

Experiment 2: influence of male state and social environment on female mating propensity

Female receptivity significantly decreased between the first and second mating attempts ($\chi^2 = 60.5$, $df = 1$, $P < 0.001$; Fig. 3). However, female receptivity to mating was not significantly influenced by the male state in the first mating ($\chi^2 = 0.10$, $df = 1$, $P = 0.75$), the social environment ($\chi^2 = 1.46$, $df = 2$, $P = 0.48$), or their interactions (all $p > 0.16$; Fig. 3).

Fig. 2 Lifetime fecundity and offspring sex ratio (proportion of females) of mated females depending on male mating order. Bars indicate the mean lifetime fecundity ($\pm$ SE) on the left axis, and a thick line indicates the mean sex ratio ($\pm$ SE) on the right axis. The first, third, fifth, and seventh mated females among the successfully mated females in the eight successive mating trials and mated females in the additional mating trials after 6 h and 24 h were allowed to produce offspring

Fig. 3 Mating success rate of virgin females (a) and once-mated females (b). All the females examined were provided with a first mating opportunity either with virgin or sperm-limited males and either within 24 h after emergence (day 0; white bars), after being kept alone for 2 days

(alone; light gray bars), or after being kept in a group of 10 females (group; dark gray bars). Part of the females that accepted the first mating was offered a new virgin male to provide a second mating attempt. Error bars represent 95% binomial confidence intervals

Lifetime fecundity was not different depending on female mating frequency ($\chi^2 = 1.20$, $df = 1$, $P = 0.27$), male state in first mating ($\chi^2 = 0.23$, $df = 1$, $P = 0.63$), social environment ($\chi^2 = 1.55$, $df = 2$, $P = 0.46$), or their interactions (all $p > 0.10$; Fig. 4). However, lifetime sex ratios had a significant interaction term between female mating frequency and male state in first mating ($\chi^2 = 9.42$, $df = 1$, $P < 0.01$), although neither the main effect of social environment nor other interaction terms were significant (social environment $\chi^2 = 0.46$, $df = 2$, $P = 0.80$; interactions all $P > 0.22$; Fig. 4). The significant interaction suggests that females that mated once with sperm-limited

males produced clutches with a smaller proportion of females than females that mated once with virgin males or females that subsequently remated with virgin males (Fig. 4). When the once-mated and twice-mated females were analyzed separately, the sex ratios of both types of females were not significantly affected by social environment (once-mated $\chi^2 = 0.54$, $df = 2$, $P = 0.76$; twice-mated $\chi^2 = 0.30$, $df = 2$, $P = 0.86$) or by the interaction between social environment and male state in the first mating (once-mated $\chi^2 = 1.75$, $df = 2$, $P = 0.42$; twice-mated $\chi^2 = 2.72$, $df = 2$, $P = 0.26$); however, there was a significant effect of male state in the first mating for both types of

Fig. 4 Lifetime fecundity and offspring sex ratio (proportion of females) of once-mated females (a) and twice-mated females (b) in varying social environments prior to mating attempts and the state of male mates. Bars indicate the mean lifetime fecundity ($\pm$ SE) on the left axis, and a thick line indicates the mean sex ratio ($\pm$ SE) on the right axis. A female was provided with a first mating opportunity with either a virgin or a sperm-limited male either within 24 h after emergence (day 0; white bars), after

being kept alone for 2 days (alone; light gray bars), or after being kept in group of 10 females (group; dark gray bars). Once-mated females were those that accepted the first mating attempt and were allowed to produce offspring. The other set of once-mated females was offered a new virgin male to provide a second mating attempt, and females that accepted the second mating were examined for offspring production as twice-mated females

females (once-mated $\chi^2 = 21.8$, $df = 1$, $P < 0.001$; twice-mated $\chi^2 = 5.72$, $df = 1$, $P < 0.05$). These results indicate that although females that initially mated with sperm-limited males could recover their sex ratios by remating with virgin males, their sex ratios were less female biased than those of twice-mated females that initially mated with virgin males (Fig. 4). When only the data set of females that had mated once with a virgin male was analyzed, the sex ratios were not significantly influenced by the social environment experienced by the females ($\chi^2 = 3.04$, $df = 2$, $P = 0.22$; Fig. 4a). This suggests that although the social environment could be a cue used by the females to strategically alter their offspring sex ratios (Hamilton 1967; Charnov et al. 1981), this was not the case in the present experiment.

Discussion

The present study revealed that sperm limitation could be a restricting factor that regulates the reproduction of male and female *A. calandreae*. The results of the first experiment showed that over successive male matings, the inseminated females produced a lower proportion of female offspring (fertilized eggs); the production of daughters was progressively reduced and only male offspring were laid in the late reproductive stage. This suggests that male sperm stores were reduced over successive matings, and females that mated later in the succession were constrained in daughter production due to haplo-diploid sex determination. Although the male sperm store appeared to be replenished after a period, males continued to mate upon encountering females, even in later succession despite the sperm shortage. The results of the second experiment showed that females accept copulation without distinguishing the male mating status. When females first mated with a sperm-limited male, they could recover some degree of daughter production by remating with a virgin male, suggesting that their sperm supply was replenished. However, female remating was not influenced by the mated status of the first male or social environmental conditions.

Why was conditionally dependent remating not observed in the females of *A. calandreae*? One of possible explanations could be the weakness of environmental selection pressure. If individuals in nature do not experience the female-biased sex ratio experienced by the wasps in the present experiments, then male and female mating strategies to counteract sperm shortage may not evolve. In a less female-biased condition, the abundance of males in the environment reduces the competition between females over mating opportunities to acquire sperm, and the females do not require a large number of sperm to produce female-biased clutches. The present study suggested that male mating in quick succession with more than four females leads to sperm limitation in the inseminated females. Considering the variable sex ratios in the species (0–

90% females; van den Assem et al. 1984; Nishimura and Jahn 1996; Ji et al. 2004) and spatially and temporally aggregated mating opportunities (Martel and Boivin 2007), such sperm-limiting situations may occur in natural populations of *A. calandreae*, as documented in other parasitoid species in the field (Ode et al. 1997; Henter 2004). However, the longevity of females may be shorter under natural conditions compared to in the laboratory, or the availability of hosts may be restricted in the wild. In such cases, females may not be constrained by sperm shortage in nature as much as indicated in the present study. Moreover, the male and female reproductive behaviors observed in the present experiments may have evolved under laboratory conditions (Burton-Chellew et al. 2007; Boulton and Shuker 2015). The laboratory cultures were founded by multiple females over multiple generations, which reduce the LMC and leads to a less female-biased condition in the stocks. Under such a circumstance, selection pressure on sperm shortage may have been mitigated (Boulton and Shuker 2015; Boulton et al. 2017).

Alternatively, the results of the present experiments could be explained as a consequence of sexual conflict (Chapman et al. 2003; Arnqvist and Rowe 2005). Under a female-biased condition, sperm shortage is likely to cause sexual conflict over male and female mating strategies. If single ejaculates from sperm-limited males are not sufficient to produce optimal sex ratios, then female counterstrategies are expected to evolve so that females can avoid being constrained. First, females may make a decision to accept copulation based on the male sperm store state. The only evidence of female choosiness for virgin males in parasitoids was documented in *N. vitripennis*, in which males release lower amounts of a sex pheromone as their sperm stores decrease and females are more attracted to males that release higher amounts of the pheromone (Ruther et al. 2009; Blaul and Ruther 2011). However, similar to other parasitoid species (Jacob and Boivin 2004; Steiner et al. 2008; King and Fischer 2010), the present research on *A. calandreae* suggested that females do not discriminate between virgin and sperm-limited males. Second, if females cannot distinguish sperm-limited males before copulation, they are expected to remate to compensate for the shortage in sperm supply (Godfray 1994; Abe and Kamimura 2015). The present study showed that approximately 26% of females accepted a second mating and recovered their sperm supply to some extent. However, the decision to remate was not associated with the state of the male from the first mating. Studies of other parasitoid wasps have reported that females under sperm limitation indiscriminately remate and recover the number of stored sperm (Chevrier and Bressac 2002; Damiens and Boivin 2006; Steiner et al. 2008; Pérez-Lachaud 2010). Even if females do not facultatively remate depending on the conditions, indiscriminate multiple mating by females could be advantageous by ensuring against the risk of sperm depletion (Boivin 2013).

Males, however, will be selected to inhibit females from remating to prevent sperm competition. In insects, males can physiologically manipulate the inseminated female to reduce subsequent mating receptivity by transferring certain proteins in seminal fluids together with sperm (Chapman 2001; Gillott 2003; Avila and Sirot 2011). The present study showed that in *A. calandreae*, previously mated females are less receptive to matings than virgin females. Moreover, even if they accepted a second mating, they could not produce daughters in the same amount as females that mated with a virgin male in the first mating, suggesting that these females were affected by a lower level of sperm supply. Similarly, mated females of *Trichogramma evanescens* accept subsequent mating, but a first mating with a sperm-depleted male inhibits the females from fully replenishing their sperm supply through subsequent matings, which is likely because the seminal fluids from a previous copulation physically block sperm from subsequent matings from entering the spermatheca of the female (Damiens and Boivin 2006).

Similar to *A. calandreae* males, males of many parasitoids are reported to transfer only small amounts or no sperm to females after a succession of matings and to continue to engage in matings, resulting in sperm shortages in females (Nadel and Luck 1985; King 2000; Jacob and Boivin 2004; Damiens and Boivin 2005, 2006; Bressac et al. 2008; Ruther et al. 2009; Steiner et al. 2008; Pérez-Lachaud 2010; Kant et al. 2012; Chirault et al. 2016). For sperm-limited males, continuing to mate could be adaptive because it increases their relative fitness by inhibiting females from remating and decreasing the paternity of competing males (Damiens and Boivin 2006; Boivin 2013). This may be particularly applicable to pro spermatogenic species, where males emerge with full sperm stores but do not produce any more sperm; such males have no opportunities to directly increase their fitness by producing offspring after sperm depletion. However, the present study showed that sperm-limited males continue to mate in a syn spermatogenic species despite the fact that they can recover their sperm store after a period.

Finally, the present study provided results that contradict the reports of previous studies for the same parasitoid species. First, in *A. calandreae*, females have previously only been shown to mate once except for dual mating, which occurs only within a short time interval (< 10 s) when a second male copulate immediately after the end of the first copulation without providing additional courtship (Khanh et al. 2005; Bressac et al. 2009). However, the present study showed that several females accepted a second mating after being provided with another courtship with an interval of at least 1 min after the previous copulation, which is consistent with the report in Baker et al. (1998). Second, in previous work, paternity after dual mating was considered proportional to the amount of sperm ejaculated by each male, suggesting that there was no sperm precedence (Khanh et al. 2005; Bressac et al. 2009). In

contrast, the present study showed that a second mating incompletely recovered the sperm supply from the first copulation, suggesting first male precedence as demonstrated in a variety of other parasitoid species (Boulton et al. 2015). This discrepancy might be due to differences in the sperm-processing mechanism. In the dual mating investigated in previous studies, the ejaculates from two males were thought to be mixed in the uterine cavity of the females before their migration to the spermatheca (Bressac et al. 2009). However, in the present study, before the ejaculate from the second male was passed to the female, the ejaculate from the first male likely started to migrate to the spermatheca and blocked additional sperm from entering (the details of sperm processing are discussed for *N. vitripennis*, which belongs to the same family as *A. calandreae*, in Boulton et al. 2017).

In conclusion, the present study of *A. calandreae* showed that although potentially sperm-depleted males appeared to be able to replenish their sperm stock by resting, they continue to mate, which constrains females from producing an optimal sex ratio throughout their reproduction. However, the females do not respond facultatively by avoiding mating with sperm-limited males or remating after such mating. These results may arise from sexual conflict over sperm transfer between males and females, which are likely dictated by the selection pressure that the species experiences in nature or in the laboratory environment. Future studies are suggested to produce different selection lines with varying sex ratios (Macke et al. 2011) and to examine the evolutionary consequences of the reproductive behavior of males and females to identify the relative importance of sexual conflict. More generally, the present results emphasize the usefulness of simultaneously examining male and female mating strategies to reveal the economics of sperm transfer between the sexes. A better understanding in this area could be obtained by considering the interplay between theoretical and empirical works. Theoretical works have recently been launched to reveal the coevolutionary feedback between male and female reproductive behaviors (Abe and Kamimura 2015; Bocedi and Reid 2016). In the future, existing models should be extended to incorporate the empirical findings, such as mating by sperm-depleted males and antagonistic coevolution between the sexes, which were newly suggested in the present study.

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Compliance with ethical standards

Conflict of interest The author declares that there is no conflict of interest.

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