



Multiple mating does not benefit females of a polyandrous pollinating fig wasp

Jaco M. Greeff^{*}, Duncan V.K. Newman

Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, 0002, South Africa

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ABSTRACT

In some species, females vary in the numbers of times they mate. While polyandry will always be beneficial to a male that mate with a previously mated female, the effect on female fitness is unclear. From females' perspective, variation in matedness can reflect adaptive differences in females' requirements for mating or non-adaptive chance factors. Pollinating fig wasps have been considered to be mostly monandrous although polyandry has been confirmed in a number of species. Here we first show that the pollinating fig wasp, *Platyscapa awekei*, is polyandrous. Second, we show that intraspecific variation in female matedness may be explained best by chance encounters between males and receptive females. The mean number of offspring does not increase with polyandry. Nor is there evidence of sperm limitation. These observations rule out direct benefits to females. Despite evidence for multiply-mated females having mated with less compatible males, multiple mating is not combined with selective preference for more compatible males' sperm, ruling out indirect benefits. Therefore variation in female matedness seems to have no fitness benefit to females and from the females' perspective may be best explained by chance variation in encounter rates between males and receptive females.

1. Introduction

Bateman (1948) suggested that males benefit from mating with multiple females whereas females do not benefit from polyandry. However, female insects that mated twice have on average 28% more offspring than if they have been mated once only (Arnqvist and Nilsson, 2000). But this is not the case for non-eusocial female hymenopterans where the benefit of polyandry is unclear (Arnqvist and Nilsson, 2000; Boulton et al., 2018; Simmons and Siva-Jothy, 1998). Matedness vary in some pollinating fig wasps giving us the opportunity to test potential explanations for why females mate multiple times.

First, intraspecific variation in matedness may not affect female fitness and can simply be a consequence of variability in chance mate encounters (Bleu et al., 2012; Jacob and Boivin, 2005; Kokko and Mappes, 2013; Taylor et al., 2014; but see Gowaty, 2013). It is thus important to compare matedness to appropriate null models (Kokko and Mappes, 2013). Second, polyandry may be advantageous to females. Polyandry can improve genotypic compatibility of a partner (Jennions and Petrie, 2000) and can avoid sperm limitation (Simmons, 2005). For genotypic compatibility to be the explanation, sperm from more compatible males should have a higher fertilization success (Tregenza

and Wedell, 2000). Variation in matedness of non-eusocial wasps suggests another benefit and does not clarify the situation: monogamy predominates in solitary species while gregarious species are more likely to be polyandrous (Ridley, 1993). Ridley (1993) suggested that polyandry may reduce competition between gregarious sibs (genetic variability) while Godfray (1994) suggested that gregarious species may require more sperm. Females from gregarious species are likely to have more encounters with males in their short receptive windows resulting in multiple mating.

In many insects, the second male to mate with a female fertilizes the majority of the eggs (Simmons and Siva-Jothy, 1998). This may be the result of sperm displacement and/or it can be that females trade up in second matings and mate with better/more compatible males (Jennions and Petrie, 2000). Chalcid wasps have an unusual sperm precedence pattern compared to other Insecta. Namely, second males typically fertilize fewer eggs, not more (Simmons and Siva-Jothy, 1998; Allen et al., 1994: *Aphytus melinus* 14.2%; Holmes, 1974: *Nasonia vitripennis* 1.8%; Wilkes, 1966: *Dahlbombinus fuscipennis* 32.5%; but *Anisopteromalus calandreae* use sperm randomly (Do Thi Khanh et al., 2005; Bressac et al., 2009)). Trading up may thus not be possible for chalcid wasps. In line with this idea, females' reproductive organs are very

^{*} Corresponding author.

E-mail address: jaco.greeff@up.ac.za (J.M. Greeff).

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simple (King, 1962; Wilkes, 1965) possibly precluding cryptic female choice (Boulton et al., 2018) and females tend to use received sperm so efficiently (Chevrier and Bressac, 2002; *Dinarmus basalis*, Pteromalidae) that selection (=waste) of sperm seems unlikely.

Hymenoptera are haplodiploid and sperm are only used to produce daughters whereas unfertilized eggs develop into sons. Therefore, sperm limitation should not result in smaller clutches, but in more sons (=constrained sex allocation; Godfray, 1994). Hymenopteran fathers will benefit from manipulating their mates to have more daughters (Hawkes, 1992). We can thus expect male ejaculates to contain substances to make their mates have more daughters (Henter, 2004; Shuker et al., 2006). On the other hand, sperm blocking can result in multiply-mated females having male biased ratios (Boulton et al., 2018; van den Assem and Feuth-De Bruijn, 1977).

Pollinating fig wasps are similar to gregarious parasitoids in that many wasps become sexually mature simultaneously in each fig. However, each wasp matures in its own gall inside the fig. Since one or a few mothers lay eggs in a fig, sibs frequently mate with each other (Hamilton, 1979; Herre et al., 1997; Kjellberg et al., 2005b; Murray, 1990). We summarise Kjellberg et al.'s (2005b) three broad pollinating fig wasp mating patterns. Pattern 1. Males eclose from their galls, they search for galls containing conspecific females, gnaw a small mating hole through the gall wall, insert their telescopic aedeagus and mate with the female inside. They continue mating until most females are mated and then switch to chew emergence holes into galls (Zammit and Schwarz, 2000) and an exit tunnel through the fig wall to release the females from the fig. Pattern 2. In figs inhabited by wasps from the genus *Ceratosolen*, males mate as in group 1, but females enlarge the mating holes by themselves and terminate mating when they emerge *en mass* into the lumen (the cavity in the centre of the fig). Males are not able to mate with enclosed females and then switch from mating to chewing the exit tunnel. Pattern 3. In figs with large lumens, males sometimes pull females out of their galls upon mating thereby preventing polyandry (Greeff et al., 2003).

While females of the third mating syndrome cannot be mated multiple times, females of the first two life history patterns can potentially be polyandrous. Two species that fall in group 1 mate only once, *Kradibia tentacularis* (Zavodna et al., 2005b) and *Pleistodontes imperialis* (Zammit and Schwarz, 2000). On the other hand, the females of two *Ceratosolen* species frequently mate multiple times (Murray, 1990; Peng et al., 2014).

The males of the pollinating fig wasp, *Platyscapha awekei*, disperse between figs and do not pull females from their galls (Greeff et al., 2003) so multiple mating is possible in this species. However, they inhabit small figs (Nelson and Greeff, 2009) that may tend to host monogamous wasps (Zavodna et al., 2005b). *Platyscapha awekei* has outbreeding and possibly inbreeding depression (Greeff et al., 2009) suggesting a very simple form of genotypic incompatibility that may favour multiple mating. Molecular tools developed for this species can be used to quantify female matedness and sperm use (Jansen Van Vuuren et al., 2006). Given *P. awekei*'s rate of sibmating, band sharing reflects genetic similarity (Greeff et al., 2009). Here, we report on 2698 genotyped progeny representing the lifetime reproductive output of 61 *P. awekei* mothers ovipositing in pairs in 30 figs (one fig contained 3 mothers). We determined if and how frequently multiple mating occurred. We compared data of matedness, sperm usage, mate compatibility and fitness to various models using an information-theoretic approach (Anderson, 2008) to understand why *P. awekei* females are polyandrous.

2. Methods

2.1. Study population and approach

We reanalysed data used to study facultative sex ratio adjustment in *P. awekei* (Greeff and Newman, 2011). In short, we introduced unrelated *P. awekei* females in pairs to oviposit in thirty figs from three trees (10

figs on each tree). The females came from figs that we harvested the previous evening and that we left intact so that matings were not interrupted. We checked the ripening figs regularly and collected all the wasp offspring when they emerged from their figs. We genotyped the offspring at 6 microsatellite loci to assign each offspring to one of the two mothers. These 6 microsatellites are highly informative and was developed for the species ($H_e = 0.895$, mean number of alleles per locus = 18 (Jansen van Vuuren et al., 2006)); this study found substantially more variation, see below). Since males get DNA from their mothers only, their genotypes can be used to reconstruct the mothers' genotypes. The haploid fathers, each of whom produce genetically identical sperm, can then be worked out by looking at the daughters. We confirmed these family identification results with COLONY (Wang, 2004).

Here we analyse these data in terms of matedness and its consequences using an information-theoretic approach to measure support for alternative hypotheses (Burnham et al., 2011) in the R environment (R Core Team, 2021). AICc is the corrected Akaike Information Criterion calculated from the log-likelihood (L), the number of parameters estimated in models (=K) and sample size (n) as $AICc = AIC + 2K(K+1)/(n-K-1)$, with $AIC = -2L + 2K$ being the Akaike Information Criterion (Bolker, 2008), or from the AIC given in generalized linear models (Burnham et al., 2011). In these cases, if data were overdispersed, we calculated the comparable QAICc as $(AIC - 2K_1)/c + 2K_2 + 2K_2(K_2+1)/(n-K_2-1)$ (Anderson, 2008), where AIC was taken from the standard model, c was the dispersion parameter (estimated in the overdispersed most detailed model), K_1 was the number of parameters estimated in the model, $K_2 = K_1 + 1$ (because c was estimated) and n was the sample size. We calculated AIC differences (Δ_i) relative to the smallest AICc (or QAICc) for each model *i* by subtracting the smallest AICc (or QAICc) from all values and used these to calculate the Akaike weights ($w_i = \frac{e^{-0.5\Delta_i}}{\sum_j e^{-0.5\Delta_j}}$) given the set of models considered (Burnham et al., 2011). Lower AICc (and QAICc) values indicate better fits to data.

2.2. Can matedness be inferred from offspring genotypes?

The number of genetically identifiable fathers of a clutch can underestimate a female's matedness because she may have mated more than once with one male, mated with males with identical genotypes, or she may not have used the sperm of all the males she mated with. The chance that a male's sperm is not detected declines rapidly as the number of offspring increases and increases as the paternity skew increases. For two males the binomial distribution gives the confidence that both fathers sired offspring given a certain number of daughters (*d*) and a certain paternity skew (s ; $= \binom{d}{0}(s^d) + \binom{d}{d}(1-s)^d = s^d +$

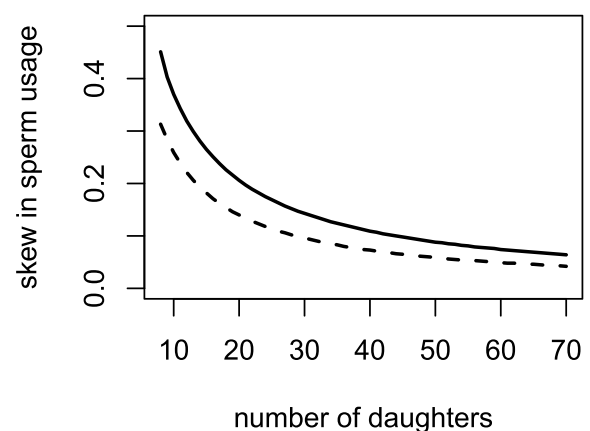


Fig. 1. Sensitivity of molecular detection of multiple fathers. The skew in sperm usage that can be detected with 99% (solid) or 95% (dashed) certainty given a certain number of daughters (in haplodiploids sons do not have fathers).

$(1 - s)^d$; Fig. 1). Fig. 1 shows that with a fertilization skew of 1:4 one can be almost 99% confident that both fathers sired offspring when the clutch is about 20 offspring (daughters in haplodiploids). Similarly, considering 20 offspring (daughters in haplodiploids), one can be 95% confident that both fathers sired offspring if the skew is as much as $1:5^{2/3}$. When larger clutches are considered, the certainty and/or the skew that can be detected are higher. Where appropriate, we will use only clutches having at least 20 daughters.

If enough offspring were produced and genotyped for us to have a reasonable chance of detecting two fathers, we may not detect these fathers if they are genetically identical. Genetic uniqueness depends on the genetic markers and the organism's breeding system. In fig wasps that are haplodiploid and frequently inbred the genetic system needs to be taken into account. A male's sperm are all identical to each other and to himself. Thus, if two males differ at one locus their offspring can always be told apart. *Platyscapa awekei* inbreeds and have an $F = 0.4$ (Erasmus, 2006; Jansen Van Vuuren et al., 2006), which implies a sib-mating rate of almost 80% (Greeff et al., 2009). Note however that only after about six generations of uninterrupted inbreeding do we start to see an appreciable number of mothers that are homozygous at all 6 loci (Greeff et al., 2009; in the current study the average mother was heterozygous at 4.1 loci). If the two males that mated with a female are brothers, as is often the expectation with fig wasps, it will be difficult to tell them apart. To determine how frequently brothers can be told apart given *P. awekei*'s markers and mating system we went through all clutches we identified and calculated how frequently brothers differ from one another.

2.3. How many times do females mate?

We estimated the number of males a female wasp mated as the number of genetically identifiable males that sired the female's daughters. We may thus overlook some matings and we do not know the mating sequence of males. Two scenarios can result in one or a few daughters differing at one locus from all their other sisters. These novel alleles may be mutations, which are expected in a study of this

magnitude, or may be a different, but related father, with one or a few daughters only. Data were analysed considering both possibilities.

2.4. Is the number of matings females have the result of a random discovery process?

We consider two models: in model 1 the number of matings is explained entirely as a random process that has a Poisson distribution with parameter λ_1 , but because no females were unmated we will use a zero-truncated Poisson distribution (all models considered in this study are summarised in Table 1). Model 2 assumes that a fraction of mothers, p , will mate once only and the remaining $(1 - p)$ mothers will mate again, with additional matings following a zero-truncated Poisson distribution with parameter λ_2 (Table 1). We first calculated the parameters (model 1: λ_1 ; model 2: λ_2 and p) that maximises the probability of the observed data. We did so considering novel alleles as mutations (models 1.1 and 2.1) and as extra matings (1.2 and 2.2; Table 1).

2.5. Are poorly matched females more likely to re-mate?

If a female re-mates to improve the genetic match with her mate we can expect that the probability of re-mating should increase as the compatibility between a female and her mate decreases. Greeff et al. (2009) showed that if a female is homozygous at η loci, then her fitness, $v(\eta)$, will be equal to a constant $+ 9.71\eta - 1.31\eta^2$. Hence, the expected fitnesses of daughters (=females) can be calculated as an expectation of their homozygosity. In turn, the expected homozygosity of a daughter can be calculated from the genetic similarity between the mother and father. Specifically, if the number of loci at which a male is identical to both his female partners' alleles is z , and to one of her alleles is y , then the female's daughters will have z loci that must be homozygous and a probability of $\binom{y}{i} (1/2)^i$ that an additional i loci will be homozygous. If we multiply the probabilities of having $z + i$ loci homozygous with the expected fitness ($v(z + i)$) and sum over all possible i , we can calculate the daughter's expected fitness (v) if that male's sperm was used as:

Table 1
Summary of models. See Materials and methods for details of variables.

Model	Submodel ^a	Equation ^b	Method ^c	n	Distribution	Biological meaning
1	1	# matings $\sim \lambda_1$	mll	56	zero-truncated Poisson	mating is a random Poisson process
	2					
2	1	# matings $\sim p + \lambda_2$	mll	56	zero-truncated Poisson	fraction, p , of females are monandrous
	2					
3		Pr(re-mating) $\sim \min(\Delta E(v))$	glm	56	quasi-binomial	probability of re-mating depends on male's compatibility
4		Pr(re-mating) ~ 1	glm	56	quasi-binomial	probability of re-mating is independent on compatibility
5		Pr(re-mating) $\sim \text{mean}(\Delta E(v))$	glm	56	quasi-binomial	probability of re-mating depends on compatibility
6		Pr(re-mating) ~ 1	glm	56	quasi-binomial	probability of re-mating does not depend on compatibility
7	3	Pr(fertilized) $\sim q_1$	mll	9	truncated & folded binomial	binomially distributed fraction, q , of eggs fertilized by 1 male
	4			13		
8	3	Pr(fertilized) $\sim q_2 + \theta$	mll	9	truncated & folded beta-binomial	beta-binomially distributed fraction, q , of eggs fertilized by 1 male
	4			13		
9	3	Pr(fertilized) $\sim \Delta E(v)$	glm	9	quasi-binomial	fraction of eggs fertilized depends on male's compatibility
	4			13		
10	3	Pr(fertilized) ~ -1	glm	9	quasi-binomial	fraction of eggs fertilized independent on male's compatibility
	4			13		
11	1	clutch size $\sim \text{tree}$	glm	54	quasi-Poisson	fitness affected by tree ID
	2					
12	1	clutch size $\sim \text{tree} + \text{matedness}$	glm	54	quasi-Poisson	fitness affected by tree ID and matedness
	2					
13	1	# daughters $\sim \text{tree}$	glm	54	quasi-Poisson	# of daughters affected by tree ID
	2					
14	1	# daughters $\sim \text{tree} + \text{matedness}$	glm	54	quasi-Poisson	# of daughters affected by tree ID and matedness
	2					

^a Decimal indicated in models: 1 = novel alleles considered as mutations; 2 = novel alleles considered as extra matings; 3 = excludes 4 clutches with one unambiguous daughter each; 4 = includes 4 clutches with one unambiguous daughter each.

^b Model equations with dependent variable on the left of the \sim and the independent variables on the right; # = number of; Pr(x) = probability of x; min indicates the minimum value of a set; mean indicates the mean of a set; 1 on the ~ 1 = only an intercept was estimated; ~ -1 = no intercept was estimated, zero-intercept assumed.

^c Two methods were used to estimate parameters: mll = maximum log-likelihood and glm = generalized linear models.

$$E(v) = \sum_{i=0}^y \binom{y}{i} \left(\frac{1}{2}\right)^y v(z+i).$$

Note that the constant in v is irrelevant and set to 0. The expectation is for the fitness of a daughter using the sperm of a specific male and we can link each $E(v)$ to a specific father, given a mother. Fig. 2 gives a worked example. This expectation varies between 0 and 17.88. We calculated females' daughters' expected fitnesses for each identified male the mother had offspring with. We fitted two kinds of models to these data. In models 3 & 4 we assumed that the lowest $E(v)$ value of multiply-mated females is that of the first male, and in models 5 & 6 we assumed that the mean of $E(v)$ is a better estimate of the first male's fit (Table 1). We tested if either of these measures of $E(v)$ had an effect on the likelihood of mating by fitting generalized linear models explaining the probability of multiple mating as a function of $E(v)$ (models 3 & 5) and just an intercept (models 4 & 6; Table 1).

2.6. Is one male's sperm used more?

We cannot determine which one of the two males in the 14 doubly mated females mated first. It is thus impossible to calculate the fraction of eggs fertilized by the second male (P_2). However, we fitted two models to fertilization fractions of doubly mated females to see if one male's sperm is favoured over the other. We modelled the fraction of eggs fertilized by each of the two males as a binomial (model 7) and a beta-binomial distribution (model 8; Table 1). Because it was impossible to detect clutches where all or no eggs were fertilized by one of two males, we truncated the distributions at both ends. Further, because there is no way to assign matings by either male in one of two classes (failure and success) as these distributions require, we folded the distribution so that the probability of x fertilizations out of a total of y daughters and $y-x$ fertilizations out of y daughters were added together. We estimated the maximum likelihood parameters of the models, the respective probabilities in the two distributions, q_1 and q_2 , and θ , the overdispersion parameter for the beta-binomial, by fitting to these data. We consider nine doubly mated females where all daughters could be assigned to fathers (7.1 and 8.1; Table 1) as well as the assigned daughters of a further 4 clutches where only one daughter per clutch could not be assigned to either of the males (these 4 daughters accounted for less than 3% of the four clutches' daughters and never swung the fraction fertilized over 0.5; models 7.2 and 8.2; Table 1). One doubly mated female with 33 out of 36 daughters that could not be assigned was left out of the analysis.

2.7. Are more compatible sperm used more?

If more compatible males fertilize more eggs, then the fraction of eggs fertilized by him should increase as the difference in $E(v)$ between him and the other male increases. Like in 2.6, we considered the nine doubly mated mothers first and then added the additional 4 clutches with one unassigned daughter per clutch. We fitted a generalized linear model with binomial errors to the numbers of fertilizations by the two males, with a zero y -intercept and $\Delta E(v) = E(v \text{ of male 1}) - E(v \text{ of male 2})$, as the predictor (model 9) and with no independent variable (model 10; Table 1). $E(v \text{ of male } i)$ refers to the expected fitness of a daughter if male i was the father.

2.8. Are multiply-mated mothers fitter?

We showed that the fruits of one of the three trees were large enough for females to lay all their eggs while the other two trees did not always have sufficient space for both females to lay all their eggs (Greeff and Newman, 2011). To incorporate tree differences, we added tree as an independent variable. We fitted generalized linear models with Poisson errors; if these models were overdispersed, we specified quasi-Poisson errors. We considered the 54 clutches where more than 20 daughters

could be genotyped and where two mothers were identified in a fig (i.e. excluding one fig with three mothers). In model 11 "tree" was the independent categorical predictor and in model 12, "tree" and "matedness" (singly or multiply) were the two categorical predictors (Table 1). We fitted models for when novel mutations were counted as mutations (11.1 and 12.1) or extra matings (11.2 and 12.2; Table 1).

2.9. Do multiply-mated mothers have more or less female-biased sex ratios?

To test if second males affect the sex ratio, similar to section 2.8, we fitted two models to evaluate if the number of daughters changed with an increase in number of matings (models 13 and 14; Table 1). Again, we fitted models for when novel mutations were counted as mutations (13.1 and 14.1) or extra matings (13.2 and 14.2; Table 1).

To determine if sex ratios are affected by matedness we fitted a generalized linear model with binomial errors to explain offspring sex ratio as a function of matedness (singly or multiply). Since data were overdispersed, we specified quasi-binomial errors, but the errors were not normally distributed, even after leaving out outliers. As a result we compared the sex ratios of singly and multiply mated mothers with a Wilcoxon rank sum test.

3. Results

We worked on genotypes of 2203 female and 495 male wasp offspring at 6 loci each, giving a total of 29,406 scored gene copies with a mean of 26.8 alleles per locus. Between 565 and 532 gene copies did not amplify, that is less than 2%. Of the 92 fathers identified from mothers with more than 20 daughters, six males had one null allele and one had two null alleles. These were easily identified from inheritance patterns that would otherwise be extremely improbable, and were inherited simply and did not lead to any confusion. Genotypes of one of the thirty figs suggested that one fig had offspring of three mothers and it was discarded from fitness, but not matedness calculations. Apart from this genotype-based conclusion that three mothers laid eggs in this fig, its higher sex ratio, 0.36 as compared to 0.18, also suggested more than two mothers. These data on 61 mothers and that on an additional 30 mothers (Greeff and Newman, 2011) suggest that 0% (95% CI: 0–4%) of mothers were not mated. One mother with a total of 16 offspring of which 11 were male may have had too few sperm. Ignoring one mother that only had 5 offspring, the sex ratios of the other 60 mothers were: 9 between (0 and 0.1], 32 between (0.1 and 0.2], 11 between (0.2 and 0.3], 4 between (0.3 and 0.4] 3 between (0.4 and 0.5] and the one over 0.5.

3.1. Can matedness be inferred from offspring genotypes?

For the 61 mothers, the mean clutch size was 44.88 (sd = 15.18) and consisted of a mean of 36.11 (sd = 14.98) daughters and 8.11 (sd = 4.09) sons. While the majority of mothers had more than 20 daughters, 5 of the 61 identified mothers had fewer than 20 daughters and we excluded these from matedness estimates as additional males could have been overlooked (leaving 56 mothers).

Of the 2263 brother comparisons, 1900 (84 %) of brother pairs could be told apart. Considering 60 families with more than one son, the brothers from seven families were all unique from each other and the median fraction of males that could be distinguished was 91 %. However, in four families, brothers were indistinguishable. Therefore, while some multiply-mated mothers may have been missed, genotypes should reveal the majority of multiply-mated mothers.

3.2. How many times do females mate?

Twenty-three of the 56 clutches that had more than 20 daughters, had multiple fathers (41%). If the novel genotypes in daughters are

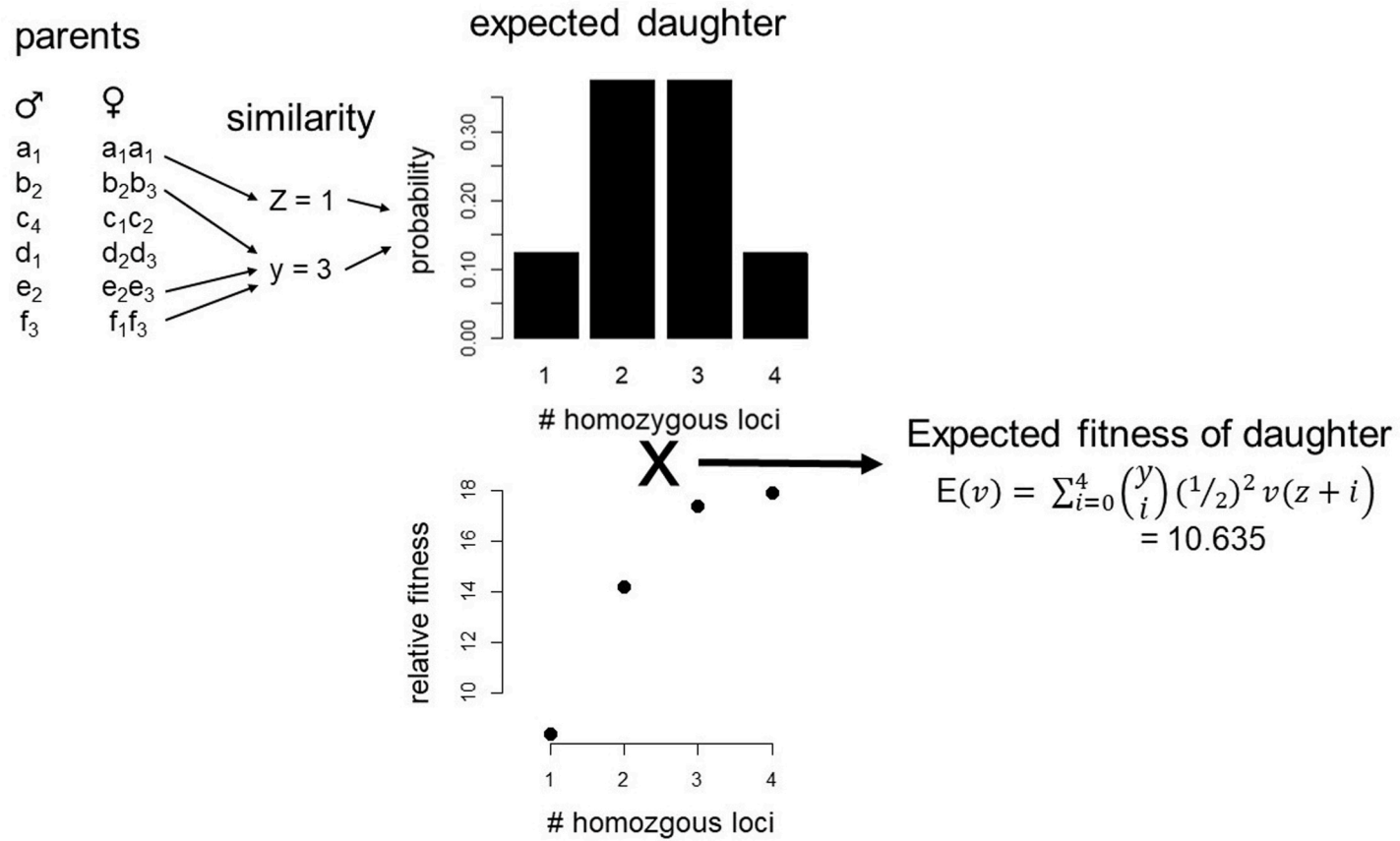


Fig. 2. Starting top left, the genotypes of the parents are compared to obtain z and y . These can be used to calculate the probability of daughters having a specific number of homozygous loci. Each of these probabilities are multiplied with the relative fitness and summed to obtain the expected fitness of a daughter sired by the specific father.

counted as extra fathers rather than mutations, 36 (64%) of the mothers mated multiple times. *Platyscapa awekei* should thus be considered a polyandrous species (Table 2; Torres-Vila et al., 2004).

3.3. Is the number of matings females have the result of a random discovery process?

The support for model 1 was higher than model 2, irrespective of whether we consider novel alleles as mutations or matings (Table 2). If novel alleles were mutations, which seems more likely, the evidence ratio is 1.8 times stronger for model 1 than model 2, but since Δ is smaller than 2 the models are virtually indistinguishable. If we counted novel alleles as extra matings the evidence ratio is 3.2 times stronger for model 1 than model 2 and here Δ is larger than 2. Although these difference are small, the close fit with observed data (Fig. 3) suggests that the number of matings females has is the result of chance discovery by males.

3.4. Are poorly matched females more likely to re-mate?

The $E(v)$ of singly-mated mothers' daughters clearly overlapped with that of the lowest $E(v)$'s of multiply-mated mothers' daughters' (Table 3). Unsurprisingly, the mean and median of the worst of groups were slightly below single samples. Both sets of $E(v)$ were left-skewed (Table 3) suggesting that most matings were with males with high compatibility.

Model 3 explained these data twice as well as model 4, while model 6 explained these data just over twice as well as model 5 (Table 4). Neither of the Δ 's are larger than 2 suggesting that there is not convincing evidence for incompatible mothers to mate more. Given the large overlap in compatibility between singly- and multiply-mated mothers, the high compatibility with mates, the *a priori* expectation that groups' minima should be less than individual values if sampled from the same distribution, the small gain in explained information (contrast 3 to 4) or reduction in information explained (contrast 5 and 6) it seems that a mating with a male with low compatibility is unlikely to be a trigger for additional matings.

3.5. Is one male's sperm used more?

In the nine clutches where all daughters could be assigned to either of two fathers, the binomial distribution was only slightly better than the beta-binomial and both suggested similar fertilization ratios with one male fertilizing almost twice as many eggs as the other (Table 5). The overdispersion factor of the beta-binomial (θ) was large, suggesting limited clumping of data. When we considered 13 clutches, very similar estimates were the most likely but the beta-binomial explained these data almost four times better than the binomial. Both sets of models suggest that one father's sperm fertilizes roughly twice as many eggs as the other father's.

Table 2

Matedness and randomness of the number of matings. Observed and predicted matedness of 56 females when novel alleles are counted as mutations (1.1 and 2.1) or as a result of additional matings (1.2 and 2.2), and fits to zero-truncated Poisson distribution without (1.1 and 1.2) and with a fraction (p) of females that mate once only (2.1 and 2.2). Maximum likelihood estimates for the models, AICc, Δ_i and Akaike weights are given.

Model	Novel alleles considered as	observed or predicted	Number of matings					Maximum likelihood estimates		AICc	Δ_i	w_i
			one	two	three	four	five	λ_1 or λ_2	p			
1.1	mutations	observed	33	14	5	4	0					
	mutations	predicted	30.7	16.9	6.2	1.7	0.4	1.1008		16.78	0	0.64
2.1	mutations	predicted	32.9	13.4	6.7	2.3	0.6	1.0072	0.588	17.91	1.12	0.36
	matings	observed	20	17	9	7	3					
1.2	matings	predicted	17.9	17.6	11.6	5.7	2.2	1.9693		18.10	0	0.76
2.2	matings	predicted	19.6	15.0	11.8	6.2	2.4	1.5737	0.3506	20.38	2.28	0.24

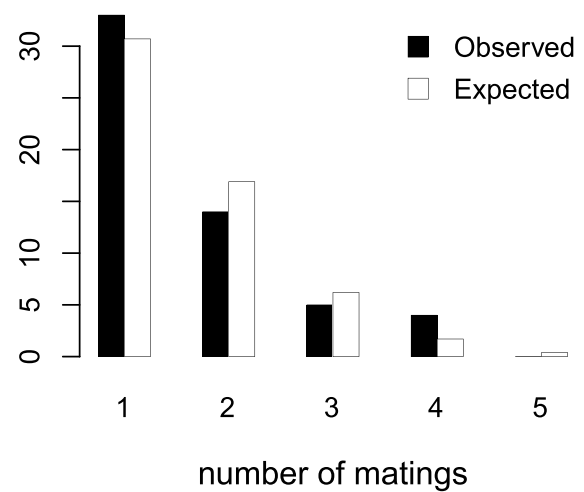


Fig. 3. Observed and expected number of matings of females according to model 1.1.

Table 3

Compatibility and matedness. Summary statistics of the $E(v)$ of singly and the lowest of $E(v)$'s of multiply mated females.

matedness	range	mean	median	standard deviation
singly	0-17.61	14.13	15.38	3.53
multiply	0-16.96	11.76	13.53	5.51

Table 4

Comparison of models explaining the probability that a female will mate again. In models 3 & 4 the independent variable is the minimum $E(v)$ (or compatibility) of all the males that mated with a female, in models 5 & 6 the independent variable is the mean compatibility ($E(v)$) of all the males that mated with a female. Note that model coefficients are for a linear model of the logits and needs to be back-transformed. Overdispersion was 1.036 for model 3 and 1.037 for model 5. QAICc, Δ_i and Akaike weights are given.

glm model coefficients					
model	intercept	$E(v)$	QAICc	Δ_i	w_i
3	1.233	-0.121	76.007	0	0.67
4	-0.361		77.401	1.394	0.33
5	0.351	-0.052	79.099	1.741	0.30
6	-0.361		77.357	0	0.70

3.6. Are more compatible sperm used more?

While the sperm of more compatible males was used more ($\Delta E(v)$ coefficient positive in model 9), there is little support for this view in these data (Table 6; Fig. 4). The pattern seems mostly the result of the outlier, left bottom (Fig. 4). Model 10 where there is no effect of genetic compatibility, had slightly more support than model 9 for nine clutches.

Table 5

Comparison of models explaining the skew in sperm usage as a folded binomial (model 7) and a folded beta-binomial distribution (model 8). The decimals in the models indicate the exclusion (7.3 and 8.3) or inclusion (7.4 and 8.4) of four clutches where one daughter could not be assigned to one of the two fathers. The AICc, Δ_i and Akaike weights are given.

Model	q	θ	AICc	Δ_i	w_i
7.3	0.355	–	50.606	0	0.51
8.3	0.378	17.088	50.667	0.061	0.49
7.4	0.354	–	74.297	2.708	0.21
8.4	0.379	18.800	71.589	0	0.79

Table 6

Comparison of models explaining the probability that a male's sperm are used given his compatibility. Note that model coefficients are for a linear model of the logits and needs to be back-transformed. The decimals in models indicate the exclusion (9.3 and 10.3) or inclusion (9.4 and 10.4) of four clutches where one daughter could not be assigned to one of the two fathers. The overdispersion was 3.734 for 0.3 models and 5.164 for 0.4 models. QAICc, Δ_i and Akaike weights are given.

Model	glm coefficient $\Delta E(v)$	QAICc	Δ_i	w_i
9.3	0.274	23.495	0.54	0.43
10.3	–	22.954	0	0.57
9.4	0.102	27.575	2.40	0.23
10.4	–	25.173	0	0.77

Table 7

Comparison of models explaining the number of offspring females had, given that they were mated once or multiple times. The decimals in models indicate counting novel alleles as mutations (1) or extra matings (2). Note that model coefficients are for a linear model of the logs and needs to be back-transformed. The overdispersion for .1 model is 1.583 and for 0.2 models is 1.574. QAICc, Δ_i and Akaike weights are given.

Model	glm coefficients for trees			glm coefficient	QAICc	Δ_i	w_i
	tree 1	tree 2	tree3	singly mated			
11.1 ^a	3.636	+0.099	+0.457	–	252.78	0	0.76
12.1	3.630	+0.094	+0.455	+0.010	255.13	2.36	0.24
11.2 ^a	3.636	+0.099	+0.457	–	254.39	0	0.74
12.2	3.627	+0.091	+0.454	+0.031	256.45	2.06	0.26

^a These models are the same.

3.8. Do multiply-mated mothers have more or less female-biased sex ratios?

Multiply mated mothers had slightly fewer daughters but model 13, where mating status played no roll, had three times as much support as model 14 (Table 8). Multiply-mated mothers did not produce a significantly different sex ratio than mothers that were mated once only (Wilcoxon rank sum test: $W = 310, p = 0.47$; Fig. 6). This suggests that males are not able to cause their mates to lay more female biased sex ratios nor does sperm blocking occur.

4. Discussion

We found that *P. awekei* females are polyandrous with at least 41% of females mating with more than one male. While polyandry must increase the number of offspring accrued by males, the same was not true for females. From the females' perspective, this seems to be purely a matter of chance with no fitness benefits to the female. We argue for this because 1) variation in mating numbers closely fit a pattern of random discovery of females by searching males, 2) sperm of more compatible males did not fertilize a greater fraction of eggs, 3) multiply-mated mothers did not produce more offspring and 4) there is no indication that mothers receive too little sperm. While one male fertilized twice as many eggs as the other it is impossible to say if it relates to mating order. Males did not influence the females' clutch composition to favour themselves.

While these data seem to make a compelling case that females do not benefit from multiple mating, we hasten to add some caveats. First, this was not a controlled experiment. Females that mated different numbers of times were not a random selection and it may be that multiply-mated mothers may have had reduced clutch sizes or have been sperm-limited if they mated once only. The required experimental manipulations is impossible in fig wasps. Second, we state again that genotyping may have missed some multiply-mated females. It is also possible that failure to amplify, may sometimes have resulted in daughters being allocated to the wrong father. However, it is unlikely that it would result in the appearance of support of a benefit to multiple mating. Third, it is possible that mothers avoid using some males' sperm so effectively that they are never used and thus never genotyped. Given that there is a skew in sperm usage, but that it is not linked to male compatibility we find this unlikely. Fourth, our estimate of compatibility that relies on six loci may be inaccurate, even though it is supported by Greeff et al. (2009).

These data failed to show an increase in the life-time reproductive success of females that mated multiple times. This suggests that multiple mating only benefits the second male to mate with a female in *P. awekei*. Such a lack of a fitness effect seems to be true for parasitoids in general (Allen et al., 1994; Bressac et al., 2009; Cheng et al., 2004; Chevrier and Bressac, 2002; Chirault et al., 2019; Do Thi Khanh et al., 2005; Jacob and Boivin, 2005; King and Bressac, 2010; Shuker et al., 2006) but can differ (Hegazi et al., 2020) and vary (Boulton and Shuker, 2015).

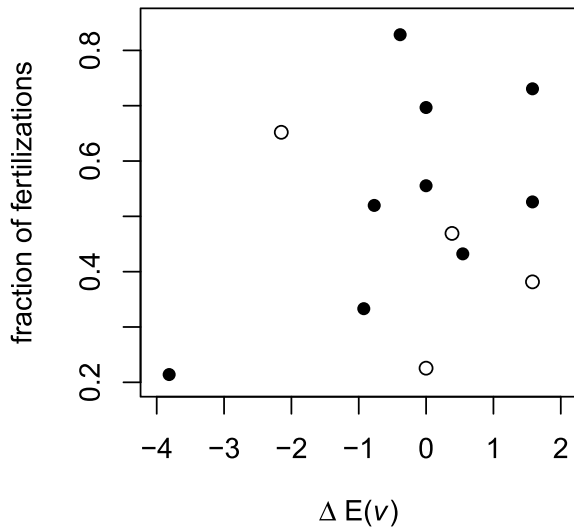


Fig. 4. Expected fitness and sperm use. The fraction of daughters fathered by dad 1 when the difference in $E(v_{dad1}) - E(v_{dad2})$ is given by $\Delta E(v)$. Filled points are nine clutches with all daughters assigned and open points are four clutches where one daughter could not be assigned.

With 13 clutches model 10 was three times more likely than model 9. Therefore these data suggest that sperm usage is independent of a male's compatibility.

3.7. Are multiply-mated mothers fitter?

The clutch sizes, which is the life-time reproductive success of these females, were slightly higher for singly mated females, not multiply mated females (Table 7). Irrespective of if unique alleles were counted as mutations or additional fathers, model 11 had three times as much support as model 12 (Table 7), meaning that whether a female was mated once or more than once did not affect their clutch size. Curiously, the variance in offspring number seems to decrease as the number of matings increased (Fig. 5).

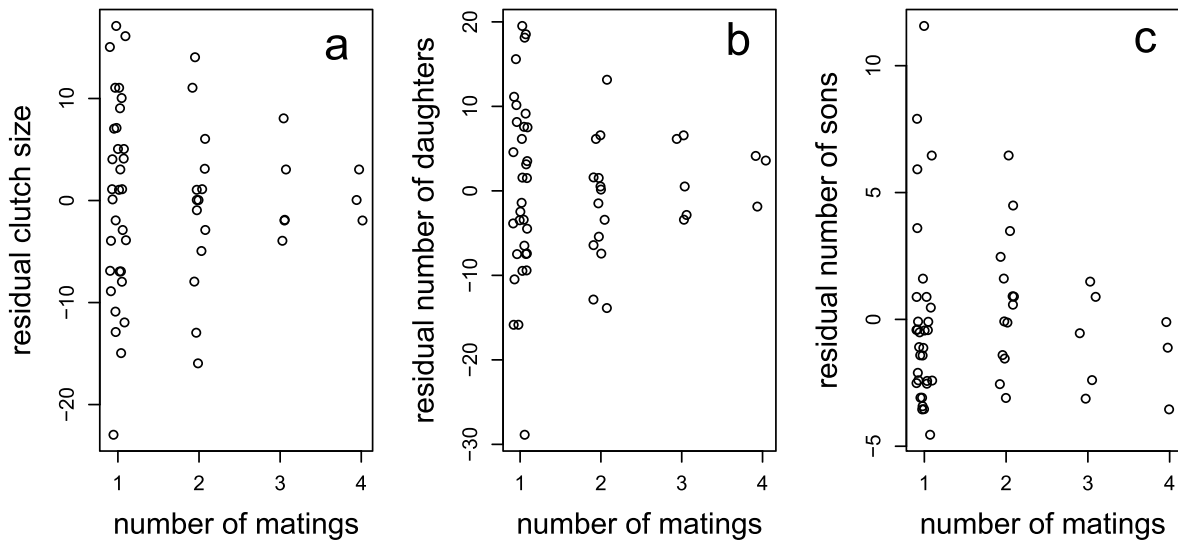


Fig. 5. Fitness and matedness. The residuals from linear models of offspring produced with tree as the only dependent variable, against the mothers' number of matings. The a) total number of offspring or clutch size, b) the number of daughters and c) the number of sons.

Table 8

Comparison of models explaining the number of daughters mothers had given that they were mated once or multiple times. The decimals in models indicate counting novel alleles as mutations (1) or extra matings (2). Note that model coefficients are for a linear model of the logs and needs to be back-transformed. The overdispersion was 2.239 for 0.1 models and 2.230 for 0.2 models. QAICc, Δ_i and Akaike weights are given.

Model	glm coefficients for trees			glm coefficient			
	tree 1	tree 2	tree3	singly mated	QAICc	Δ_i	w_i
13.1 ^a	3.447	+0.093	+0.462	-	192.09	0	0.76
14.1	3.437	+0.084	+0.458	+0.024	194.40	2.31	0.24
13.2 ^a	3.447	+0.093	+0.462	-	192.81	0	0.75
14.2	3.436	+0.084	+0.458	+0.036	194.96	2.15	0.25

^a These models are the same.

We suggest that females are initially receptive to matings, once they are mated their receptive window starts to shut down, should another male try to mate before the closing down is complete, the female will be multiply mated, if not, she will be singly mated. The coexistence of monandrous and polyandrous females in *P. awekei* may thus be the chance outcome of a very simple mating dynamic rather than females actively trying to mate multiple times (compare to [Kokko and Mappes, 2013](#)). It would be in line with solitary wasps in general where females are normally considered monandrous ([Godfray, 1994](#)), but will mate multiple times if presented with males in quick succession ([Allen et al., 1994](#); [Bressac et al., 2009](#); [Cheng et al., 2004](#); [Chevrier and Bressac, 2002](#); [Do Thi Khanh et al., 2005](#); [King and Bressac, 2010](#)). In general, a pattern of polyandry under conditions of increased male density suggests that the encounter rate may play an important and possibly a stochastic role in determining levels of polyandry in disparate taxa ([Burton-Chellew et al., 2007](#); [Ishibashi and Saitoh, 2008](#); [Janicke et al., 2013](#); [Martin et al., 2014](#); [Mayer and Pasinelli, 2013](#); [Plough et al., 2014](#); [Sandrin et al., 2015](#); [Thonhauser et al., 2014](#)).

In the case of fig wasps, females remain in their galls and are mated there by males passing through. Males are not able to mate with females who have eclosed from their galls ([Kjellberg et al., 2005b](#)), so females cannot actively pursue males, however, females can affect their mating rate by remaining attractive for shorter or longer periods. We can expect that factors such as gall location ([Peng et al., 2014](#)) and sex ratio ([Hamilton, 1979](#)) will influence matedness and it will vary across and within species ([Greeff and Kjellberg, 2022](#); [Herre et al., 1997](#)). In *Ficus hispida*, galls abutting the lumen contain more multiply-mated *C. solmsi marchali* females ([Peng et al., 2014](#)). This is presumably because males patrol here more frequently and are more likely to discover mated females here that are still receptive ([Peng et al., 2014](#); [Yu and Compton, 2012](#); [Zavodna et al., 2005a](#)).

Even though [West et al. \(1997\)](#) suggested that 4.3% of *P. awekei* mothers are not mated we did not observe any unmated mothers. One mother may have received limited sperm, although this is debateable due to her small clutch size. Apart from this female, no other females had the tell-tale signs of high sex ratios indicating no, or limited sperm, or low quality sperm ([Henter, 2004](#)). However, sperm limitation is more complex and should not be seen as a threshold trait ([Chirault et al., 2015, 2019](#); [Henter, 2004](#); [Ruther et al., 2009](#)). It is possible that multiply-mated mothers would have had too few sperm if they had not mated again. This seems unlikely as parasitoid females seem to be

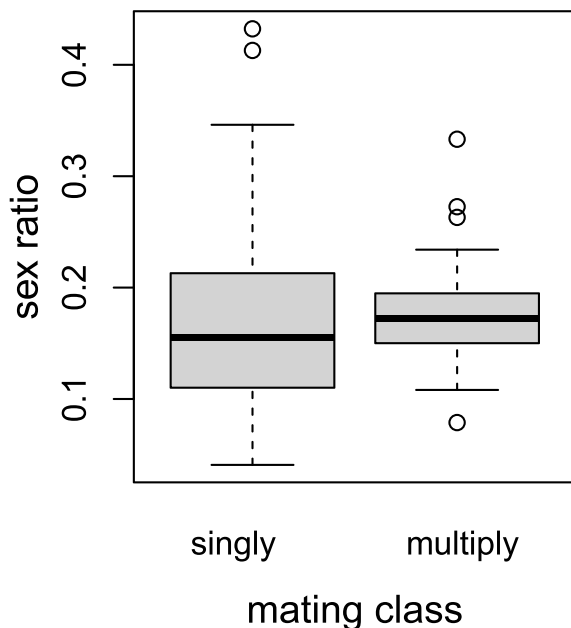


Fig. 6. Sex ratio and matedness. The sex ratio (fraction of sons) of singly- and multiply-mated mothers.

unable to detect if they are sperm limited (Bressac et al., 2008; Chirault et al., 2015, 2019; King, 2000; Steiner et al., 2008). We should also keep in mind that *P. awekei* males can disperse and mate with females from different figs (Greeff et al., 2003; Moore et al., 2006) and therefore the fitness cost of being unmated is smaller in this species.

Unlike eusocial hymenopterans that have very accurate control over the usage of sperm from distinct sources (Jennions and Petrie, 2000), non-eusocial species seems not to. It seems that when matings are in quick succession sperm mixes in the uterus before reaching the spermatheca and is used as in a fair raffle (Bressac et al., 2009; Do Thi Khanh et al., 2005). But if the first ejaculate reaches the spermatheca before the second, the first male seems to be favoured (Allen et al., 1994; Bressac et al., 2009; Holmes, 1974; Wilkes, 1966), and this is presumably a result of the spermatheca and spermathecal duct's design and operation (Boulton et al., 2018; Holmes, 1974). Another potential effect of the female reproductive tract's design, sperm blocking due to multiple mating, did not occur in *P. awekei*, suggesting an alternative setup to *N. vitripennis* (Boulton et al., 2018). Like most insects we see a fertilization bias in this pollinating species with one male, presumably the first, dominating the fertilization by a ratio of almost 2 to 1. We should thus have identified all fathers in figs with more than 20 daughters with a high confidence. This observation suggests that these females are normally re-mated after the first ejaculate already reached the spermatheca. Note however that we do not know which male mated first and we cannot be certain if this wasp has sperm precedence.

While multiply-mated females seem to have mated with a less compatible male than singly-mated females and one male's sperm is used more, sperm usage seemed independent of genetic compatibility. In other words, sometimes more compatible sperm are used more often and other times, less compatible sperm are used more frequently. This makes it very unlikely that indirect genetic benefits is an explanation for multiple mating. The great overlap in compatibility measures of single fathers and the worse fathers of groups of fathers also suggests that indirect genetic benefits are unlikely. Given frequent sib-mating in parasitoids, genetic incompatibility may generally not be important for this group (Boulton and Shuker, 2015). However, it may be that our measure of genetic compatibility is insufficient.

Even though males are only related to daughters and would benefit from biasing the sex ratio towards daughters, *P. awekei* males and hymenopteran males in general seem to have no or very little control over the clutch's sex ratio (Bressac et al., 2009; Henter, 2004; Shuker et al., 2006). In some species multiply-mated females lay more female-biased sex ratios, but this seems to be caused by singly-mated females being sperm limited (i.e. reproductive incompetence rather than male manipulation; Chevri er and Bressac, 2002; Chirault et al., 2019; Hegazi et al., 2020). While multiply mated females of the fig wasp *Ceratosolen solmsi marchali*, lay more female biased sex ratios, this is most probably because multiply mated females are larger (Greeff and Kjellberg, 2022; Peng et al., 2014); larger females have larger clutches, and larger clutches are more female biased (Kjellberg et al., 2005a). Since *P. awekei* does not harbour *Wolbachia* (Ahmed et al., 2013), there is no chance that this bacteria affected the outcome.

The third mating syndrome (Kjellberg et al., 2005a) where males remove females from their galls, presumably to avoid multiple mating (Greeff et al., 2003), must have evolved in the context of frequent multiple mating. While multiple mating is now impossible in these species, it must have been commonplace in them before.

Even though pollinating fig wasp sex ratios are very female biased they vary greatly between figs (Greeff and Kjellberg, 2022). In figs with highly female-biased sex ratios, males will benefit from focusing on discovering and mating with virgin females rather than enter into weighted fertilization sperm competitions. In such figs, males sharing a fig may frequently be related (Greeff et al., 2003; Hamilton, 1979; Herre et al., 1997) and displacing related sperm is less beneficial than displacing unrelated sperm. In addition, the chances that a subsequent mating may be more compatible is small to the female.

In conclusion, this study found no evidence that polyandry is an active strategy pursued by females to increase fitness. In fact, these data suggest that from a female's perspective, polyandry is the result of chance discovery of receptive females. Hence, at least from the females' perspective, polyandry should not be seen as functional or adaptive in the sense of Williams (1966).

5. Author contributions

JMG: Conceptualization, Formal analysis, Resources, Data curation; Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding Acquisition; DVKN: Conceptualization, Validation, Investigation, Data curation, Writing - review & editing, Project administration.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All genotypes and the summary of each fig's contents are available on figshare pending an eight month embargo.

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