Fig wasps from the centre of figs have more chances to mate, more offspring and more female-biased offspring sex ratios

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Pollinator fig wasps serve as useful empirical models for studies of sex ratio evolution because females adjust their offspring sex ratios in relation to the number of foundresses that share a fig. Placement of pollinator offspring within figs is not random and more centrally located flowers are more likely to support pollinator development. We compared components of fitness of female fig wasps developing in central and peripheral flowers and whether this influenced the sex ratios of their offspring. We used *Ceratosolen solmsi marchali*, a pollinator of the Asian dioecious fig tree, *Ficus hispida*. Mating frequency was determined from the number of mating holes in females' flowers. Most females mated once, but some had the opportunity to mate up to four times and multiple mating opportunities were more frequent among centrally located galls. Body size was not linked to flower location, although the females that mated most were significantly larger than others, and came from significantly larger galls. Females that had multiple mating opportunities produced more daughters but similar numbers of sons, resulting in more female-biased broods. These females are likely to have produced more offspring because of their larger size but may have also benefited from mating with more males. The results demonstrate that differences in the natal locations of foundresses can modify offspring sex ratios and obscure sex ratio adjustment in response to the number of foundresses sharing a fig.
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Sex allocation strategies are a productive field of study in evolutionary biology because they allow empirical, quantitative tests of theory based on optimality predictions (Flanagan, West, & Godfray, 1998; Moore, Zavodna, Compton, & Gilmartin, 2005; Orzack, 1990). Since Hamilton (1967), the proportion of male offspring in spatially structured mating populations has received particular attention. Under these local mate competition (LMC) conditions foundress females are predicted to produce a female-biased offspring sex ratio because it reduces competition between sibling males for mates, but as foundress numbers increase a less female-biased offspring sex ratio becomes optimal. Numerous empirical studies are in broad agreement with this expectation (Hardy, 2002; West, Reece, & Sheldon, 2002).

The unusual biology of the fig wasps (Hymenoptera, Agaonidae) that pollinate fig trees (*Ficus* spp., Moraceae) has led to their widespread use in studies of sex ratio selection (Hamilton, 1967; Herre, 1985, 1987; Kathuria, Greeff, Compton, & Ganeshaiah,

1999; Moore, Compton, Hatcher, & Dunn, 2002). In haplodiploid organisms such as fig wasps and most other hymenopterans, fertilization of the eggs results in the production of diploid females, whereas unfertilized eggs result in haploid males (King, 1987; Werren, 1987). Mated female fig wasps produce highly female-biased broods but, in line with theory, the extent of this bias is often found to vary between figs according to the numbers of foundress females that entered to lay their eggs. Foundress numbers determine the extent of LMC within individual figs and also, more generally, determine average levels of inbreeding for each species. Qualitative agreement has regularly been achieved between empirical data and model predictions that incorporate these factors, although some of the biological assumptions of simpler models have been questioned and the quantitative fit is often poor (Greeff, 2002; Herre, 1985, 1987; Kathuria et al., 1999; Molbo, Machado, Herre, & Keller, 2004; Moore et al., 2002; Nelson & Greeff, 2009; West, Murray, Machado, Griffin, & Herre, 2001).

Sex ratio adjustment in insects is often achieved by a 'male eggs first' strategy, whereby male eggs are laid at the start of an oviposition sequence, after which mostly female eggs are laid (Hokyo,

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Shiga, & Nakasuji, 1966; Strand, 1988; Waage, 1982; Waage & Lane, 1984). This produces sufficient males for all the females to be inseminated and results in progressively more female-biased sex ratios as the clutch size increases. An increasing number of fig wasps are now known to lay mostly male eggs first, including species of *Kradibia* (=*Liporrhopalum*) (Raja, Suleman, Compton, & Moore, 2008), *Ceratosolen* (Sun, Wang, & Hu, 2009) and *Pegoscapus* (Ramírez, Monge-Nájera, & Chavarría, 2009). This produces changes in offspring sex ratio in response to an increase in foundress number because the average number of offspring produced per foundress declines, and most of these later offspring would have been females (Moore et al., 2005; Raja et al., 2008; Yu & Compton, 2012). In species in which male eggs are laid mainly at the start of an oviposition sequence, any factors that influence clutch size, in addition to the number of foundresses sharing a patch, will also modify offspring sex ratios, and additional more direct responses to the presence of other females have been detected (Greeff & Newman, 2011). The number of eggs a fig wasp carries when emerging from a fig correlates with her body size (Ghara & Borges, 2010; Moore & Greeff, 2003) suggesting that in the absence of oviposition site limitation, larger females should produce more female-biased sex ratios. Body size can be influenced by the location of the wasp's (galled) natal flower, with smaller wasps produced from more peripheral ovules (Dunn, Yu, Ridley, & Cook, 2008).

The combination of preferential oviposition into shorter-styled flowers and the laying of mostly male eggs first leads to the relatively small numbers of male offspring tending to be concentrated towards the centre of a fig, with female offspring distributed in progressively longer-styled flowers as foundress number increases (Compton, Rasplus, & Ware, 1994). In dioecious figs, style length variation is not pronounced, but a concentration of male offspring towards the centre of the figs is achieved through the elongation of gall pedicels containing male offspring, probably because these were the first flowers to be galled (Yu & Compton, 2012) The major natural enemies of pollinator fig wasps are nonpollinating fig wasps, which either destroy pollinator larvae or compete with them for oviposition sites (Al-Beidh, Dunn, Power, & Cook, 2012; Dunn, Segar, et al., 2008; Yu & Compton, 2012). Nonpollinating fig wasps mainly oviposit from the outside of the figs, making more peripheral flowers more vulnerable to attack. As a consequence of the different distributions of pollinator offspring this can result in differential mortalities and changes in their realized sex ratio, because female offspring are more likely to be killed (Pereira & Prado, 2005).

Male fig wasps emerge from their galls before the females and mate with them while the females are still in their natal galls. Matings are often between siblings because few foundresses contribute offspring to each fig. Female fig wasps have less scope for precopulatory mate choice because they are still in the galls. They have generally been assumed to mate only once (Frank, 1985; Hamilton, 1967, 1979; Herre, 1985, 1987; West & Herre, 1998), an assumption supported by some paternity analyses (Zavodna, Compton, Raja, Gilmartin, & Van Damme, 2005). Although multiple mating has been reported in pollinating fig wasps, its influence on offspring sex ratios has not been considered (Kinoshita, Kasuya, & Yahara, 2002; Murray, 1990). Not all pollinator females succeed in mating with even one male (West, Herre, Compton, Godfray, & Cook, 1997), and those from inner galls can be more likely to mate (Dunn, Segar, et al., 2008; Dunn, Yu, et al., 2008). Body size can also be correlated with gall position and thereby fig wasp fecundity (Anstett, 2001). In this study, we examined the relationship between the location of natal flowers within figs and the fig wasps that emerged from them, and asked the following. (1) How many *Ceratosolen solmsi marchali* females only have an opportunity to mate with one male? (2) Do female fig wasps that develop in more central locations benefit in terms of having a higher frequency of mating opportunities and a larger body size? (3) Do such benefits translate into greater fecundity and changes in their offspring sex ratios? And (4) does multiple mating modify sex ratio changes in response to foundress numbers inside shared figs?

METHODS

Study System

Ceratosolen solmsi marchali Mayr is the pollinator of *Ficus hispida* L. in the Xishuangbanna area of southwestern China. *Ficus hispida* is a small to medium-sized free-standing dioecious fig tree that produces figs on leafless branchlets hanging down from the trunk and major branches. Figs are present on different trees all year round, but there are seasonal peaks in production (Patel & McKey, 1998). Mature male figs have a diameter of about 28 mm and contain 1774.33 ± 48.79 (mean $\pm$ SE, $N = 92$) female flowers.

Foundress females actively pollinate the fig flowers and are unable to leave a fig once they have entered it. The ovipositor of *C. solmsi marchali* is long enough to reach almost all the ovules in male figs of *F. hispida*. Locally, mean foundress number per male fig is 2.08 ± 0.12 (SE, $N = 182$, range 1–9), with about 53% of the figs pollinated by a lone foundress. Three species of nonpollinating fig wasps, *Philotrypesis pilosa* Mayr, *Philotrypesis* sp. and *Apocrypta bakeri* Joseph are also present. They oviposit from the outside of the figs into ovaries containing pollinator eggs or larvae. Reflecting the locations from which the fig wasps oviposit, pollinator offspring are concentrated in more central locations (mean pedicel length of occupied flowers = 0.76 ± 0.02 mm, $N = 154$), and the three species of nonpollinating fig wasps are located more towards the periphery of the figs (pedicel lengths of occupied flowers = 0.16 ± 0.01 mm, based on measurements from 134 flowers). All three nonpollinating fig wasps are parasitoids/inquilines, so pollinator larvae developing towards the periphery are more liable to be attacked.

Observations of Mating Behaviour

The behaviour of male pollinators was observed under a dissecting microscope during the period from when they first emerged from their galls until the first females emerged. The figs were broken into pieces to allow observation. Along with general observations, focal galls were marked and followed for several hours to record the numbers of visits by males, or individual males were similarly followed to record sequences of behaviour. The observation was performed during 4–5 h periods each day and continued for 1 week.

Mating Opportunities for Females

Mating frequency estimates were generated by counting the mating holes present in galls that contained female pollinators (Murray, 1990). Observations confirmed that the chewing of mating holes was almost always followed by the male inserting its abdomen into the female's gall, but direct confirmation of mating and insemination was not possible. The mating holes therefore provide a measure of the number of times that females had an opportunity to mate, rather than their number of matings per se. Figs were opened at the stage when the first females were starting to emerge from their galls and the male fig wasps were starting to produce a communal exit hole through the fig walls. Two male trees were used as sources of male-phase figs (the wasps emerged from the figs), with 15 figs collected from each. The figs were split into quarters through the ostiole and the numbers of mating holes

in all the female galls were recorded. Overall, 21–47 galls per fig and 490 and 565 galls in total were scored from the two trees.

Mating Frequency and Female Body Size

Twenty haphazardly chosen figs from one male tree where female pollinators were starting to emerge from their galls were opened and the galls were sorted according to the number of mating holes present. The pedicel length of each gall, its diameter and the head width of the female wasp that subsequently emerged were measured. Head width is a good indicator of female body size for *C. solmsi marchali* (Liu, Yang, & Peng, 2011). Subsamples of 10 galls with a single mating hole per fig were chosen at random for measurement, as this category was very abundant. All the female pollinators from galls with two, three and four mating holes were measured.

Fecundity and Progeny Sex Ratios

From February to April 2007, figs in which female fig wasps were starting to emerge from their galls were opened and occupied galls with one, two or three mating holes were separated into nylon bags. Once the females had emerged from these galls they were introduced rapidly into female-phase (receptive) figs on another tree. These figs had previously been encased in fine-mesh nylon bags to prevent oviposition. One, three or five foundresses that had mated one, two or three times were introduced into each fig. The bags were replaced after the fig wasps had been introduced into the figs. When mature, the figs were harvested and the numbers of male and female progeny were recorded. The foundresses in this experiment were not given the opportunity to collect pollen and this may have reduced their reproductive success (Jandér & Herre, 2010).

Analyses

All statistical analyses were performed in R 2.9.2 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Generalized linear models were used to analyse the effects of foundress numbers and mating opportunities on total numbers of wasps, females, males and sex ratios, with quasi-Poisson distributions for total wasps, females and males and binomial distributions for sex ratios. Generalized linear models with Poisson distributions of the error terms analysed relationships between pedicel length and frequency of mating. The effect of frequency of mating opportunities on wasp size, and the relationships between gall size and pedicel length, and between wasp size and pedicel length were analysed using linear models.

RESULTS

Mating Behaviour of Pollinator Fig Wasps

The wingless males of *C. solmsi marchali* emerged first and then crawled sluggishly around the interior of the fig on the surface of the galls, with their abdomen curved forwards under the thorax. When a male found a gall that contained a female, it chewed a hole through the gall wall to gain entry to the female. While the hole was being produced, the flexible telescopic abdomen was gradually extended forwards under the thorax and head and into contact with the gall surface. Once the hole was sufficiently large to allow the abdomen through, the male stopped chewing and copulation commenced. When completed, the male moved away and searched for other galls containing females. Each male only produced a single hole and previous mating holes in the same gall were not used by subsequent males. A new hole was produced even on the rare occasions when a male returned to a gall containing a female with which it had previously attempted mating. The behaviour of males that were producing a second mating hole appeared identical to that of males producing the first and the number of mating holes in a gall was therefore a good indication of the number of times that females had an opportunity to mate (Fig. 1). Most pollinator females only had the opportunity to mate once, but up to four mating attempts were recorded. The females emerged from their natal galls after enlarging the mating holes. In contrast to the females, individual males were observed to make mating holes in the galls of up to 12 females.

Mating Opportunities for Females

Mating frequencies were consistent across 30 figs from the two male trees, with most females mating with a single male (Fig. 2). The percentage of females mating with one male varied in different figs from 68.8 to 94.1 and averaged 81.0. The remaining 19% of the females were either unmated or had the opportunity to mate with more than one male.

Mating Frequency and Female Body Size

The number of mating holes in the galls of female wasps was strongly linked to their location within the figs. Females that had failed to mate were concentrated towards the periphery of the figs, in galls that were sessile or had very short pedicels. Conversely, females in galls with more than one mating hole were concentrated in the longer pedicel classes, towards the centre of the figs (Fig. 3a). A general linear model confirmed that there was a significant relationship between pedicel length and frequency of mating ($\beta \pm$ SE $= 0.315 \pm 0.016$, $z = 19.17$, $P < 0.001$). There was also a significant positive relationship between pedicel length and gall size (linear model: $\beta \pm$ SE $= 0.028 \pm 0.011$, $t = 2.514$, $P < 0.05$). The body size of females was not correlated with the pedicel lengths of the galls in which they developed (linear model: $\beta \pm$ SE $= 0.0008 \pm 0.002$, $t = 0.456$, $P = 0.65$), but females from galls with more mating holes, which were concentrated towards the centre of the figs, were significantly larger than females from galls with fewer matings (Fig. 3b; linear model: $\beta \pm$ SE $= 0.005 \pm 0.002$, $t = 2.195$, $P < 0.05$).

Figure 1. *Ficus hispida* fig showing the mating holes made by male *Ceratosolen solmsi marchali* in galled ovules that contain conspecific females.
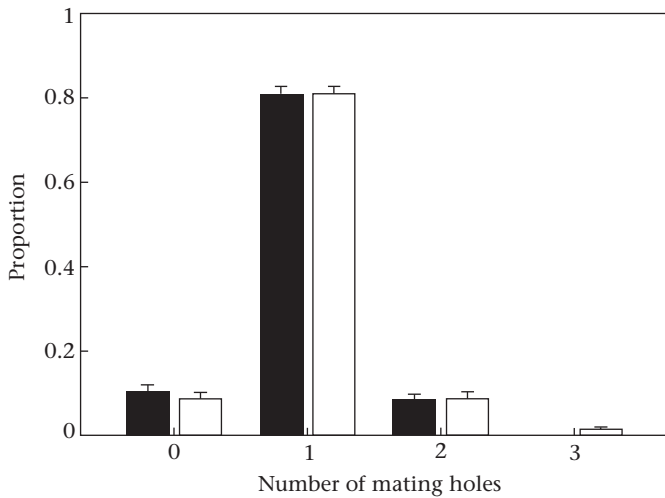
Figure 2. Frequencies of mating holes in flowers occupied by females (mean + SE). Matings only take place when females are within the flowers, so females in galled ovules with no holes remain unmated. Black and white bars represent different trees.
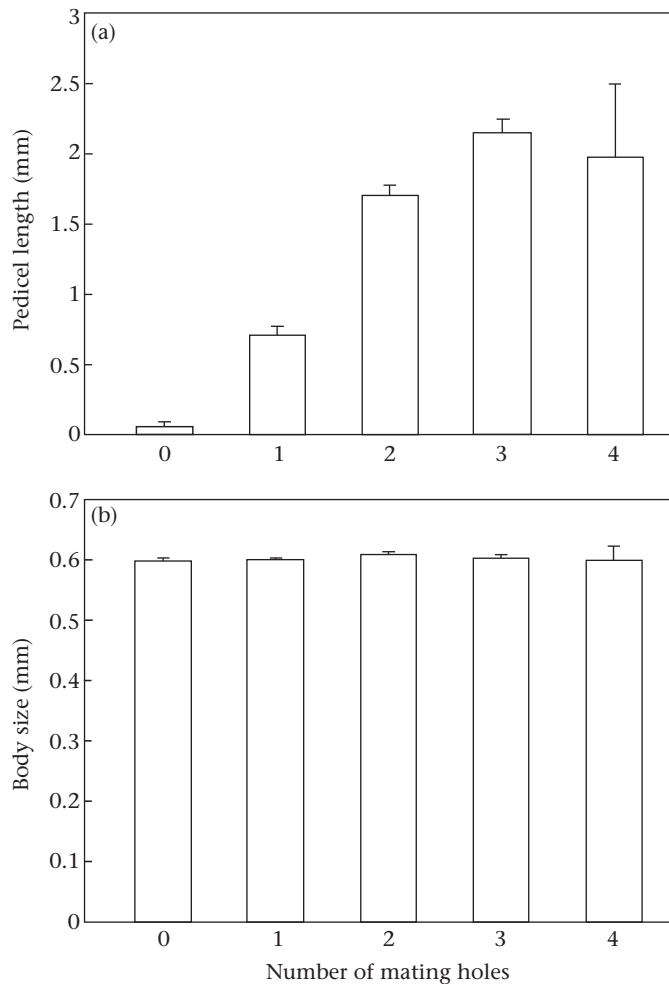
Fecundity and Progeny Sex Ratios

The relationship between mating holes, sex ratio and female offspring production was particularly clear in single-foundress figs, in which females with the opportunity to mate more than once produced more daughters but similar numbers of sons, resulting in more female-biased broods (Fig. 4, Table 1). Figs entered by three or five foundresses contained more offspring in total, but fewer offspring per female than figs entered by a single foundress, reflecting increased competition for oviposition sites. For any given foundress density, females from galls with more mating holes produced more offspring, with numbers of female and male offspring significantly increasing with foundress numbers. In contrast, number of mating holes significantly influenced numbers of female offspring but not male offspring (Table 2). This resulted in significant variation in offspring sex ratios between treatments linked to mating frequency, rather than foundress number. Although interpretation is made more difficult by a significant interaction between mating hole numbers and foundress number (Table 2), the results show that mating opportunities among foundresses modified offspring sex ratios and influenced apparent responses to foundress number.
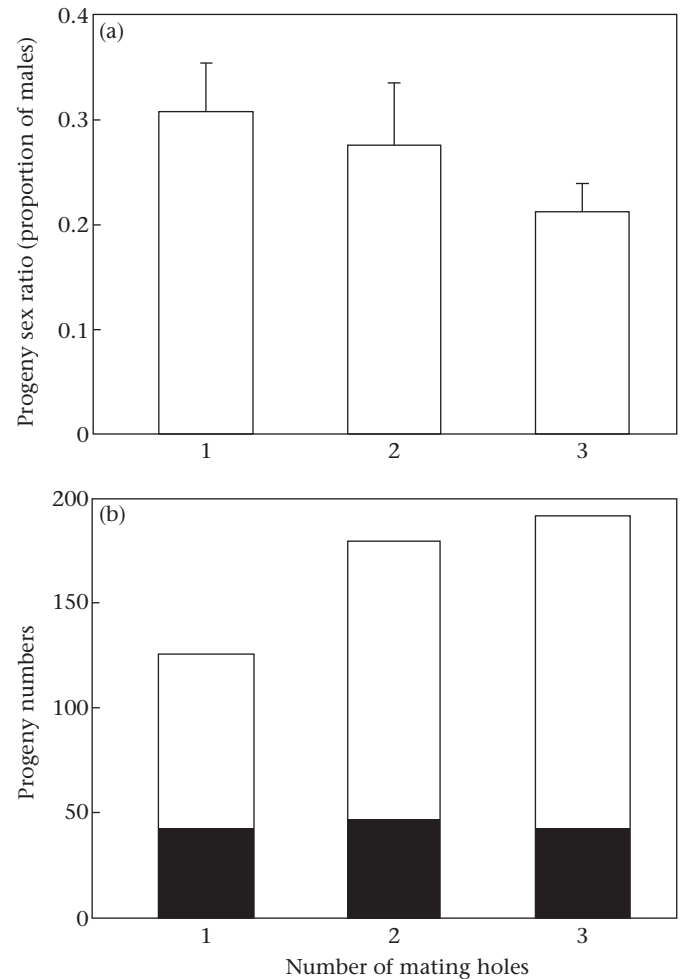

Figure 3. The relationship between the number of mating holes in flowers occupied by females and (a) the pedicel length of the flowers and (b) the body size of the females (mean + SE). Sample sizes for zero-, one-, two-, three- and four-hole groups = 107, 200, 199, 101, 5.

Figure 4. The relationship between the number of mating holes and (a) the sex ratios of offspring (mean + SE) and (b) the number of offspring (black bars: males; white bars: females) in single-foundress broods.

Table 1
Characteristics of pollinator broods in experiments in which the numbers of foundresses and how many times they had opportunities to mate were controlled independently

Foundress treatments		Sample size	Total number of offspring (mean±SE)	Offspring per foundress (mean±SE)	Male offspring per foundress (mean±SE)	Female offspring per foundress (mean±SE)	Sex ratio (mean±SE)
No. of foundresses	No. of mating holes						
1	1	21	125.3±20.4	—	41.9±11.7	83.4±14.1	0.31±0.05
	2	15	179.3±35.1	—	46.9±12.6	132.5±27.4	0.28±0.06
	3	11	191.6±43.0	—	42.1±13.1	149.5±30.7	0.21±0.03
3	1	13	271.0±40.5	90.3±13.5	23.7±3.9	66.6±10.3	0.24±0.03
	2	17	325.8±38.2	108.6±12.7	34.1±5.8	74.5±11.1	0.32±0.04
	3	15	342.9±49.0	114.3±16.3	31.6±5.3	82.7±12.0	0.28±0.02
5	1	21	336.2±33.7	67.2±6.7	22.6±3.0	44.6±5.8	0.36±0.04
	2	19	343.9±41.2	68.8±8.2	18.5±2.6	50.3±6.4	0.28±0.02
	3	14	382.2±57.2	76.4±11.4	22.9±3.8	53.6±7.9	0.29±0.01

DISCUSSION

Because of the female-biased offspring sex ratios of pollinator fig wasps, males have the opportunity to routinely mate with several females. The frequency of multiple mating among females is more variable, and is apparently absent in some species (Frank, 1985; Molbo et al., 2004; Murray, 1990; Zavodna, Compton, Biere, Gilmartin, & Van Damme, 2005). In *F. hispida*, most females of the pollinator *C. solmsi marchali* mated at most only once, as indicated by the presence of a single mating hole in their galls. Some galls, none the less, had as many as four mating holes, created by different males, providing females with the opportunity to mate up to four times, The frequency of multiple mating opportunities for individual females was lower than that recorded for the closely related *C. solmsi solmsi* in Malaysia, for which females were reported to mate up to 10 times (Murray, 1990). It was not possible to observe mating directly, because the females were still hidden in their galls, but the males spent similar lengths of time with their abdomen inserted into galls that had or had not contained previous mating holes, suggesting that mating was taking place as normal. The females have fewer opportunities to avoid mating than in other insect species, because they are tightly confined in their galls, the cavities of which are more or less the same size as the females.

The female fig wasps that developed in more central locations had a higher frequency of mating opportunities and a larger body size. These females produced more offspring and more female-biased sex ratios. There was a significant interaction between mating opportunities and foundress number inside shared figs that weakened the effect of foundress numbers on combined-brood offspring sex ratios. In contrast to some other fig tree species (Jandér & Herre, 2010; Tarachai, Compton, & Trisonthi, 2008), male figs of *F. hispida* that were entered by foundress fig wasps that lacked pollen were rarely aborted and the fig wasp offspring developed normally. The lack of pollen in the experimental figs may, none the less, have influenced the fig wasps' offspring sex ratios.

Several different advantages accrue to fig wasps that develop in more central galls within figs, including larger body size, relatively lower rates of attack by parasitoids and a higher likelihood of mating and subsequently emerging from figs (Anstett, 2001; Dunn, Segar, et al., 2008; Dunn, Yu, et al., 2008; Yu & Compton, 2012). For the pollinator of *F. hispida*, increased opportunities for multiple mating can be added to this list. In this species we failed to detect a significant relationship between gall location and female wasp body size, although the females that achieved the most matings were significantly larger than females with fewer matings. Our measure of female size (head width) may have failed to detect small but significant differences in body size that translate into greater fecundity, but there was a weak but significant relationship

between *C. solmsi marchali* gall size and location, and any nutritional benefits gained from developing in larger galls may not necessarily be reflected only by size.

Multiple mating increases the chances of mating with a high-quality male that will contribute more to female fitness (Lorch & Chao, 2003), and may promote postcopulatory mechanisms that reduce genetic incompatibility, increase offspring viability and improve performance under natural selection (Jennion & Petrie, 2000; Newcomers, Zeh, & Zeh, 1999; Pizzari, Cornwallis, Levlie, Jakobsson, & Birkhead, 2003). The high frequency of putative multiple matings recorded for individual females of *C. solmsi solmsi* and to a lesser extent *C. solmsi marchali* may result in intense sperm competition (Murray, 1990), but also provide more sperm for females to utilize. In haplodiploid organisms such as fig wasps, this will allow females to produce more daughters, and male insects can also manipulate offspring sex ratios via their ejaculates to produce more daughters (Civetta & Clark, 2000), which is advantageous to the males because they are unrelated to haploid sons (Trivers & Hare, 1976).

Greeff and Newman (2011) failed to detect an effect of number of matings on offspring sex ratios in an African fig wasp, whereas we found that females that had multiple mating opportunities produced more female-biased sex ratios than single-mated individuals. This was a consequence of the former generating more offspring in total, most or all of which were female. Single-mated females were smaller than females that may have mated several times, and smaller broods are inevitably less female-biased in species that lay mostly male eggs at the start of oviposition sequences. Male fig wasps may also contribute nutritionally to females during mating, and thereby contribute to increased clutch sizes. For example, stoichiometric studies have shown that transfer of phosphorus occurs during mating in *Drosophila*, despite females already having much higher levels of this element (Markow, Coppola, & Watts, 2001). Differences in gross chemical composition are also present between fig wasp sexes, with males much richer in nitrogen and lower in phosphorus than females (Zhang & Han, 2010), suggesting there is scope for similar transfer (Kay et al., 2005). The limited postmating life span of the females (2 days at most) and their pro-ovigenic (all eggs are mature upon adult emergence) reproduction will limit the scope of any male provision, but the absence of feeding by adult females may make any such contributions more significant than would be the case if subsequent feeding took place.

Early models of sex ratio adjustment in fig wasps assumed that single matings were ubiquitous, but if the pollinators of *F. hispida* are not atypical then some promiscuous females are likely to be found among the pollinators of other fig tree species. Multiple matings increase the number of female genotypes represented among the offspring that develop within each fig and decrease the

Table 2
Results of generalized linear models examining how offspring numbers responded to the numbers of foundresses in a fig and how often they had the opportunity to mate

Dependent variable	Factors	Slope	t	P
Total wasps	No. of mating holes	0.127±0.059	2.170	<0.05
	No. of foundresses	0.176±0.030	5.917	<0.001
Female offspring	No. of mating holes	0.155±0.064	2.437	<0.05
	No. of foundresses	0.167±0.032	5.178	<0.001
Male offspring	No. of mating holes	0.059±0.077	0.768	0.444
	No. of foundresses	0.198±0.040	5.020	<0.001
Sex ratio	No. of mating holes	−0.197±0.036	−5.514	<0.001
	No. of foundresses	−0.029±0.019	−1.214	0.225
	No. of mating holes∗No. of foundresses	0.029±0.009	3.133	<0.005

Quasi-Poisson (total wasps, females and males) and binomial (sex ratio) distributions were applied.

frequency of sib matings. In figs entered by a single foundress they result in two or more genotypes among the diploid female progeny, but the haploid males will still be full sibs. Consequently, comparisons of inbreeding levels between species that are based on mean numbers of foundresses entering figs may need to be modified if frequencies of multiple mating prove to be variable.

Foundress fig wasps typically modify their progeny sex ratios in response to the numbers of other foundresses sharing a fig (Herre, 1985; Moore et al., 2002). This results mainly from increased competition for oviposition sites when the number of females sharing the fig increases (Greeff & Newman, 2011; Ramírez et al., 2009). We failed to detect sex ratio adjustment in response to foundress number when we varied both the numbers of foundresses entering a fig and the number of times they had the opportunity to mate. This was because clutch sizes were more strongly influenced by the size of the foundresses (which covaried with the number of mating opportunities achieved by the females) than by the extent of competition between the females for oviposition sites. This effect may be inevitable among fig wasps that lay mostly male eggs at the start of an oviposition sequence, because any variable that modifies their fecundity also modifies offspring sex ratios. Our results extend the understanding of sex ratio adjustment in fig wasps, and imply that realized sex ratios among fig wasps may rarely, if ever, be truly optimal.

Acknowledgments

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