

# Molecular markers reveal reproductive strategies of non-pollinating fig wasps

JAMES M. COOK,<sup>1,2</sup> CAROLINE REUTER,<sup>1,3</sup> JAMIE C. MOORE<sup>4</sup>

and STUART A. WEST<sup>5</sup> <sup>1</sup>School of Biological Sciences, University of Reading, Reading, U.K., <sup>2</sup>Hawkesbury Institute for the Environment, Western Sydney University, Penrith, Australia, <sup>3</sup>Wolfson Institute of Preventive Medicine, Queen Mary, University of London, London, U.K., <sup>4</sup>Department of Social Statistics and Demography, University of Southampton, Southampton, U.K. and <sup>5</sup>Department of Zoology, University of Oxford, Oxford, U.K.

**Abstract.** 1. Fig wasps have proved extremely useful study organisms for testing how reproductive decisions evolve in response to population structure. In particular, they provide textbook examples of how natural selection can favour female-biased offspring sex ratios, lethal combat for mates and dimorphic mating strategies.

2. However, previous work has been challenged, because supposedly single species have been discovered to be a number of cryptic species. Consequently, new studies are required to determine population structure and reproductive decisions of individuals unambiguously assigned to species.

3. Microsatellites were used to determine species identity and reproductive patterns in three non-pollinating *Sycoscapter* species associated with the same fig species. Foundress number was typically one to five and most figs contained more than one *Sycoscapter* species. Foundresses produced very small clutches of about one to four offspring, but one foundress may lay eggs in several figs.

4. Overall, the data were a poor match to theoretical predictions of solitary male clutches and gregarious clutches with  $n - 1$  females. However, sex ratios were male-biased in solitary clutches and female-biased in gregarious ones.

5. At the brood level (all wasps in a fig), a decrease in sex ratio with increasing brood size was only significant in one species, and sex ratio was unrelated to foundress number. In addition, figs with more foundresses contain more wasp offspring.

6. Finally, 10–22% of females developed in patches without males. As males are wingless, these females disperse unmated and are constrained to produce only sons from unfertilised eggs.

**Key words.** Behavioural ecology, clutch size, hymenoptera, local mate competition, sex ratio.

## Introduction

Insects display a great diversity of mating systems, providing many opportunities to test evolutionary theory (Choe & Crespi, 1997; Shuker & Simmons, 2014). However, the study of insect mating systems can be challenging. First, insects are typically small and highly mobile, so they are difficult to study in the field under natural selective regimes. Second, recent studies have found cryptic species – morphologically indistinguishable but genetically distinct – in many insect taxa (Hebert *et al.*,

2004; Bickford *et al.*, 2007; Pfenninger & Schwenk, 2007). This suggests that many previous studies of mating systems may have inadvertently pooled data from multiple species (Molbo *et al.*, 2003). Fortunately, molecular techniques now provide tools to deal with the second problem (Molbo *et al.*, 2003; Cook *et al.*, 2015), while careful selection of study species can address the first.

Fig wasps are tiny insects whose offspring develop, and in most species also mate, inside the figs (inflorescences) of *Ficus* trees (Cook & West, 2005). For this reason, despite their small size (typically <5 mm long), we can collect accurate data on the size and composition of fig wasp mating groups under natural field conditions (Hamilton, 1979; Herre *et al.*, 1997). Moreover,

Correspondence: James M. Cook, School of Biological Sciences, University of Reading, Whiteknights Reading, RG6 6AS, U.K. E-mail: james.cook@reading.ac.uk

in most *Ficus* species, several wasp species develop and mate alongside each other in a fig, creating excellent opportunities for comparative studies (Hamilton, 1979; Greeff & Ferguson, 1999; Cook, 2005). Each of the >750 *Ficus* species is pollinated by one to five species (Darwell *et al.*, 2014) of fig-pollinating wasps (family Agaonidae), most of which are host-specific to a single *Ficus* species (Weiblen, 2002; Cook & Rasplus, 2003). In addition, a given *Ficus* species may host up to about 30 non-pollinating species from diverse lineages in several other wasp families (Cook & Rasplus, 2003). Most non-pollinators are also associated with just one *Ficus* species (Cook & Segar, 2010), but some lay eggs in a few closely related *Ficus* species (Marussich & Machado, 2007; McLeish *et al.*, 2010).

Female pollinator wasps (foundresses) enter receptive figs through a narrow tunnel, the ostiole, at the apex of the fig. Inside, a foundress lays many eggs (often >100, depending on species) before dying inside the fig. She thus lays 'all her eggs in one basket' (Herre *et al.*, 1997). Foundress number is typically only one to five (Herre *et al.*, 1997), so figs contain many pollinator offspring from a few mothers. Some weeks later the offspring mature and mate inside the fig. This causes local mate competition (LMC; Hamilton, 1967), leading to selection for female-biased offspring sex ratios that increase (more males) with foundress number per fig. These predictions have been tested in several species by counting live offspring and dead foundresses in the same figs and assuming that foundresses produce equal numbers of offspring (Hamilton, 1967; Herre, 1985, 1987; Herre *et al.*, 1997). More recent studies have gone further and used molecular markers to assign offspring to sibships and establish the clutch sizes and sex ratios of individual foundresses (Molbo *et al.*, 2003; Moore *et al.*, 2005). This allows tests of more nuanced LMC models that incorporate clutch size variation, and of predictions for individual foundresses, as well as the emergent patterns at the fig level (Molbo *et al.*, 2003). Importantly, molecular markers have also revealed cryptic genetic species within what was thought in previous work to be a single pollinator species (Molbo *et al.*, 2003; Sutton *et al.*, 2017). Together, these improvements in data quality have increased the fit between theory and data considerably.

In contrast, we have limited understanding of individual reproductive strategies in non-pollinating fig wasps, which vary greatly in their sex ratios (West & Herre, 1998; Fellowes *et al.*, 1999). In addition, they may have winged, wingless or dimorphic males (Hamilton, 1979; Cook *et al.*, 1997) and, in some species, lethal male combat (Hamilton, 1979; Murray, 1990). Importantly, most non-pollinators lay eggs through the fig wall, without entering the fig, providing the opportunity to lay 'a few eggs in many baskets' (Herre *et al.*, 1997). However, this also removes the possibility for researchers to count foundress bodies inside figs and test for relationships between foundress number, sex ratio and clutch size. Models of optimal strategies for non-pollinator foundresses (Greeff, 1997) allow for very small clutches, which can severely limit offspring sex ratio options (Green *et al.*, 1982; Morgan & Cook, 1994). They predict that, when a foundress lays one egg in a fig (solitary clutch) it should be a male and that larger clutches (gregarious) should generally contain one male and  $n - 1$  females. Broadly

similar predictions (more males in smaller clutches) also arise from standard LMC models where clutch size is not limiting (Werren, 1980; Stubblefield & Seger, 1990).

Adaptive allocation of offspring to clutches can also scale up to predictable patterns at the brood level (all conspecifics in a fig). Greeff (1997) developed models for foundresses that either do (informed) or do not (uninformed) use information on other foundresses laying eggs in the same fig. The models predict that brood sex ratio should be correlated positively with brood size for informed females, but negatively for uninformed females. Further, these fig level patterns are important for the evolution of constrained sex allocation (Godfray, 1990). Many fig wasp species have wingless, non-dispersing males, so females developing in a fig without males remain unmated (West *et al.*, 1997), but can still potentially produce male (haploid) offspring from unfertilised eggs. Comparative data support the correlated evolution of large brood size and male winglessness across species (Cook *et al.*, 1997), but unrecognised cryptic species could lead to underestimates of constrained sex allocation and potentially confound this pattern.

In this study, we used microsatellites on three *Sycoscapter* species that coexist and mate in the same figs and provide the first genetic estimates of reproductive patterns for any non-pollinating fig wasp species. We had two broad aims. First, we estimated the aspects of population structure that are expected to influence the evolution of reproduction behaviour. Specifically, we measured foundress numbers, clutch sizes and offspring sex ratios, as well as the proportion of females that will disperse unmated. Second, we used our data to establish the reproductive decisions of individual females, to test whether females adjust their offspring sex ratio in response to their own clutch sizes and to the number of females laying eggs in a fruit. These analyses would have been impossible without molecular markers, as we cannot place individuals in sibships, or even identify species based on morphology alone.

## Materials and methods

### *Study species and field sampling*

We studied three *Sycoscapter* species that develop only in the syconia of *Ficus rubiginosa* and are endemic to eastern Australia. These species have not been described formally and, following Moore *et al.* (2008) we refer to them as *Sycoscapter* species A, B and C. Females of species A are distinguished by their longer ovipositors, but we cannot distinguish between B and C based on morphology. The males are wingless and very difficult to identify, because there is great intraspecific variation in morphology (Moore *et al.*, 2008). However, all wasps are identified consistently into the three species using either mitochondrial DNA sequences (Moore *et al.*, 2008) or multilocus microsatellite genotypes (Bouteiller Reuter *et al.*, 2009; Cook *et al.*, 2015).

We collected 55 figs from four *F. rubiginosa* trees in Brisbane, Australia, in 2004–2005. Female wasps were allowed to emerge from figs and then preserved in 80% ethanol. Each fig was then placed in 80% ethanol and dissected under a light microscope to count the males and any remaining female wasps. All wasps

were subsequently genotyped using the nine microsatellite markers described in Bouteiller Reuter *et al.* (2009). In this paper, we use the term ‘clutch’ to refer to the offspring of one female in one fig and ‘brood’ to refer to all conspecific wasp offspring developing in one fig (i.e. mating patch).

### Molecular methods

DNA was extracted from the abdomen of each insect in a 100 µl volume of extraction solution (5% Chelex, 0.01% proteinase K) on 96-well plates. We incubated the plates at 56 °C for 35 min then at 96 °C for 15 min and centrifuged for 5 min at 3500 g. We genotyped the 387 *Sycoscapter* wasps at nine microsatellite markers (locus 5, 48, 68, 71, 82, 85, 89, 95, 96) developed by Bioprofiles (Newcastle, UK) and described in Bouteiller Reuter *et al.* (2009). Polymerase chain reaction (PCR) was carried out on 96-well microplates in 15 µl volumes containing 5 µl of DNA extract, 0.4 U of Taq polymerase (Qiagen, Hilden, Germany), 0.4 µM of each primer, 0.2 mM of dNTPs (Qiagen), 1× Buffer (Qiagen) and 2.5 mM of MgCl<sub>2</sub>. Two negative controls containing the PCR mix without DNA were set on each plate. PCR cycling conditions were 12 min of initial denaturation at 95 °C followed by 10 cycles of 15 s at 94 °C, 15 s at the specific annealing temperature [50 °C for all loci except locus 68 (48 °C), loci 48, 82 and 96 (52 °C) and locus 5 (54 °C)], 15 s at 72 °C and 30 cycles of 15 s at 89 °C, 15 s at the specific annealing temperature (as earlier), 15 s at 72 °C and a final extension at 72 °C for 10 min in Techgene (Techne, Stone, U.K.) and Eppendorf (Stevenage, U.K.) thermal cyclers. Samples were analysed on an ABI 3700 machine and fragment sizes scored using GENESCAN 3.5 and GENOTYPER 2.5 software (ABI, Warrington, U.K.).

### Basic population genetics

Basic characteristics of these loci for our three study species have already been described in (Bouteiller Reuter *et al.*, 2009). However, we report here the number of alleles per locus ( $N_a$ ) and their mean polymorphic informative content (PIC). Polymorphic informative content measures the information content of the loci for parentage analysis and is related to their expected heterozygosity (Marshall *et al.*, 1998). We also calculated  $F_{IS}$  values for each species and  $F_{ST}$  between the different pairs of species using the FSTAT software (Goudet, 1995).  $F_{IS}$  is the inbreeding coefficient and we compared this between the three *Sycoscapter* species.  $F_{ST}$  was used to measure genetic differentiation between the three cohabiting *Sycoscapter* species. Polymorphic informative content and  $F$  statistics were calculated based on  $n = 140, 47$  and  $54$  females for species A, B and C, respectively.

### Matriline reconstruction

We first used STRUCTURE to assign each individual *Sycoscapter* wasp to species, as in a study of male fighting behaviour in these species (Cook *et al.*, 2015). We then

estimated the number of matriline (sibships; i.e. offspring of one foundress) in the population (for each species) using a maximum likelihood method in COLONY (Wang, 2004). This software uses a group likelihood approach to reconstruct matriline allowing for typing errors in marker data. Genotyping errors can cause severe biases in sibship inference if they are ignored (Wang, 2004) and two classes of errors are taken into account. Class I errors are allelic dropouts, i.e. when PCR fails to amplify one of an individual’s two alleles at a locus. Class II errors include a variety of possible stochastic typing errors. These can come from various sources, including mutations, false alleles, miscalling, contaminant DNA and data entry (Wang, 2004). We reconstructed the matriline for each species separately with an allelic dropout rate for the nine loci varying between 0.001 and 0.2 and a stochastic error rate between 0 and 0.1.

### Foundress numbers, clutch size and clutch sex ratios

After assigning individuals to matriline, we then determined offspring production patterns for individual foundresses. This allowed us to calculate foundress number distributions for each species and also how many offspring (clutch size), and what sex ratio, each foundress produced in a given fig.

### Statistical analyses of sex ratios and offspring numbers

We explored reproductive patterns at different biological levels – species, clutch (offspring of one female in a fig) and brood (all conspecific wasps in a fig). For each species, we determined the foundress number distribution and sex ratio at the population level and tested for sex ratio bias. We also calculated the proportion of females developing in patches without conspecific males.

At the clutch level, we tested whether individual females adjust their sex ratios to their own clutch sizes to produce solitary male clutches and gregarious clutches with  $n - 1$  females, as predicted by models (Greeff, 1997). Due to poor support for these predictions, we further tested a simpler model to determine whether mean clutch sex ratio (proportion males) decreased with the transition from solitary to gregarious clutches.

At the brood (fig) level, we tested whether sex ratio increased or decreased with brood size – alternative predictions of models that assume females are either informed (increase) or uninformed (decrease) about other foundresses laying eggs in a fig (Greeff, 1997). In addition, we tested whether the overall brood size increased with the number of foundresses per fig.

All statistical analyses were performed in R (<http://www.Rproject.org>). We used general linear models (GLMs), with binomial errors for sex ratio (proportion) data and Poisson errors for low offspring number counts. We checked for overdispersion by comparing the residual deviance with residual degrees of freedom. If necessary, we then applied quasi-binomial or quasi-Poisson error structure and tested significance using  $F$ -tests or  $t$ -tests (Crawley, 2005). Finally, we used exact binomial tests to determine if predicted clutch compositions for solitary (prediction = male) and gregarious clutches (prediction = one male plus  $n - 1$  females) were more or less likely than

**Table 1.** Population- and fig-level data for the three wasp species.

Species	Occupied figs	Total wasps	Mean wasps per fig	Total foundresses	Mean foundresses per fig	Population sex ratio	Constrained females (%)
A	39/55	202	5.18	104	2.7	0.31	10
B	29/55	82	2.83	54	1.9	0.43	21
C	39/55	82	2.10	68	1.7	0.48	22

the alternatives, and a two-sample test for equality of proportions (the `prop.test` function in R) to test whether sex ratios differed between solitary and gregarious clutches.

## Results

### Basic population genetics

All nine loci were amplified and used successfully in all three species and the number of alleles per locus varied from three to 21 (Supporting Information, Tables S1 and S2). The mean PIC of loci was high for *Sycoscapter* species B (0.60) and C (0.68) and medium for species A (0.42). Genetic differentiation ( $F_{ST}$ ) was significant between all species pairs (randomisation tests with Bonferroni correction, all  $P < 0.001$ ). It was similar and very high between species A and either B (0.44) or C (0.41), and reduced but still high (0.23) between the two short ovipositor species, B and C. All three species also showed significant inbreeding (randomisation tests, all  $P < 0.001$ ), with  $F_{IS}$  values of 0.31 for A, 0.24 for B and 0.30 for C.

### Species-level results

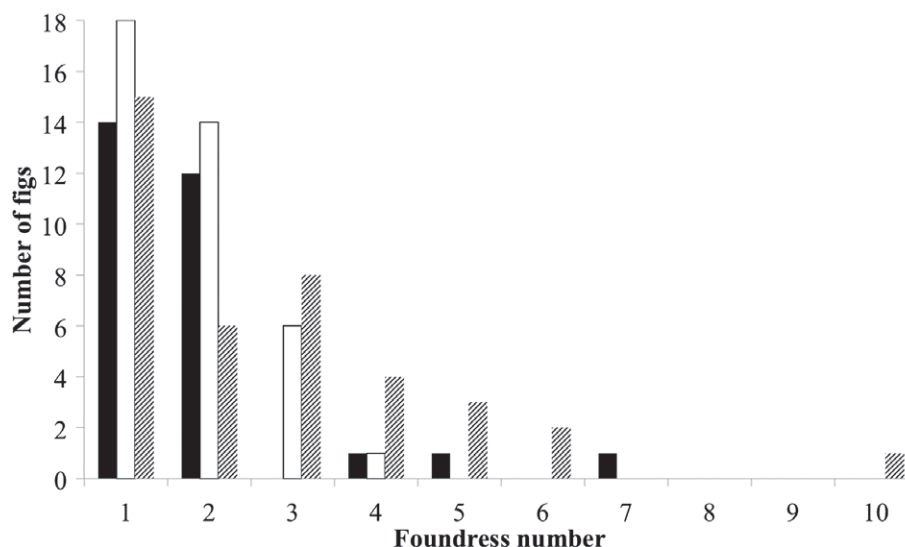
Our sample of 55 figs contained 202 *Sycoscapter* A wasps (one to 25 per occupied fig) in 39 syconia, 82 *Sycoscapter* B wasps (one to 10 per fig) in 29 figs, and 103 *Sycoscapter*

C wasps (one to seven per fig) in 39 figs (Table 1). Roughly equal numbers of figs contained wasps from one ( $n = 19$ ), two ( $n = 20$ ) or all three ( $n = 16$ ) *Sycoscapter* species (Supporting Information, Table S3). Consequently, most wasps occurred in figs that also contained close congeners, making molecular species delimitation an essential first step.

The sibship reconstruction revealed 104 (A), 54 (B) and 68 (C) matriline, and we estimated mean foundress numbers (SE) to be A = 2.7 (2.0), B = 1.9 (1.4) and C = 1.7 (0.8). Typically, there were two to three foundresses per fig (Fig. 1), but with up to 10 for A, seven for B and four for C. All three species have female-biased population sex ratios (Table 2), but the bias is only significant in *Sycoscapter* A (sex ratio = 0.31,  $P < 0.001$ , binomial test; *Sycoscapter* B,  $P = 0.43$ , NS; *Sycoscapter* C,  $P = 0.48$ , NS). The distribution of males and females across figs results in 10–22% of females developing in patches without mates, leading to constrained sex allocation (Tables 1 and S2).

### Clutch sizes and sex ratios

In all three species females lay very small clutches, typically only one to three eggs per fig (Table 2), and solitary clutches are more common than gregarious ones. Clutches very rarely contained more than one male (Table 2 and Supporting Information, Table S3). The mean number of females per clutch was also very low (Table 2 and Supporting Information), but reached



**Fig. 1.** The distribution of foundress number per fig (species A, hatched; species B, white; species C, black).

**Table 2.** Clutch sizes and sex ratios in the three wasp species.

Species	Mean clutch size	Total clutches	Solitary male clutches	Gregarious clutches with $n-1$ females
A	1.9	99	34/64	9/33#
B	1.5	59	22/38	7/21
C	1.5	69	33/47**	10/22

Solitary clutches containing males and gregarious clutches with  $n-1$  females are predictions from theoretical models. There is a significant excess (\*\* $P < 0.01$ ) or deficit (# $P < 0.05$ ) of the predicted clutch sex ratio compared with alternatives in some cases.

maxima of 11, eight and five for species A, B and C (Supporting Information, Table S2).

Observed clutch sex ratios differ considerably from the basic predictions of solitary male clutches and gregarious clutches with  $n-1$  females (Tables 2 and S2). In fact, solitary clutches are only more likely to contain a male (33/47 cases) than a female in species C (Table 2). In gregarious clutches, cases with  $n-1$  females are actually significantly less common than alternative compositions in species A (Table 2). Deviation from the  $n-1$  prediction can be due to either no males or multiple males (Supporting Information).

Mean sex ratio does, however, differ between solitary and gregarious clutches (Table 3), and this leads to a decrease in sex ratio with clutch size when tested with logistic regressions: species A ( $t = -2.75$ ;  $P = 0.007$ ; 7% deviance explained;  $n = 103$  clutches); species B ( $t = -2.36$ ;  $P = 0.02$ ; 11% deviance explained;  $n = 53$  clutches); species C ( $t = -3.66$ ;  $P = 0.0005$ ; 19% deviance explained;  $n = 67$  clutches). In all cases, there was slight overdispersion so we used quasi-binomial errors and  $t$ -tests for significance.

In addition, females may lay eggs in more than one fig. Females of species A, B and C were found to have laid eggs in up to three, two and four figs on the same tree (Supporting Information). These are minimum estimates, as the number of figs sampled is far lower than the number of figs on a tree.

#### Brood sizes and sex ratios

At the brood (fig) level, there is considerable sex ratio variation, but no correlation with foundress number for any of the species: species A ( $z = -0.32$ ;  $P = 0.75$ ; d.f. = 1, 37); species B ( $z = -0.07$ ;  $P = 0.95$ ; d.f. = 1, 27); species C ( $z = 0.06$ ;  $P = 0.95$ ; d.f. = 1, 37). Brood sex ratio decreases with brood

size (all conspecifics in a fig) in all three species (Fig. 2), but the relationship is only significant in species C: species A ( $t = -1.41$ ;  $P = 0.17$ ; d.f. = 1, 37); species B ( $t = -1.53$ ;  $P = 0.13$ ; d.f. = 1, 27); species C ( $t = -2.582$ ;  $P = 0.014$ ; d.f. = 1, 37). In addition, there is a highly significant positive correlation between brood size and foundress number (Fig. 3) in all three species: species A ( $z = 5.83$ ;  $P < 0.0001$ ; 58% deviance explained); species B ( $z = 3.67$ ;  $P < 0.001$ ; 49% deviance explained); species C ( $z = 2.78$ ;  $P < 0.006$ ; 51% deviance explained).

## Discussion

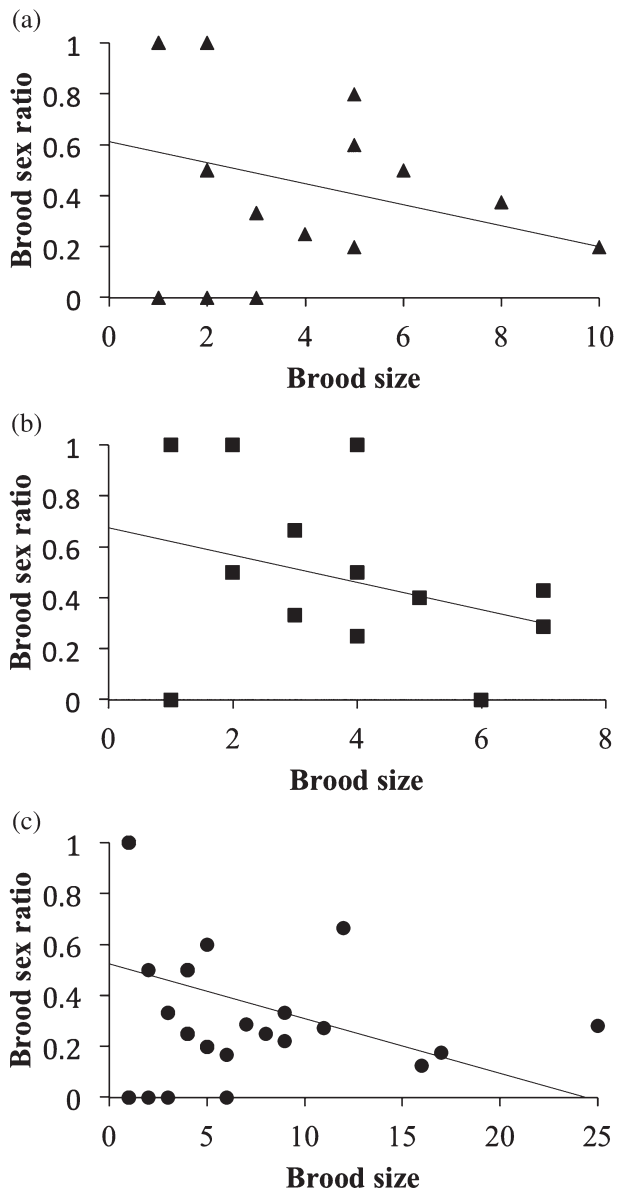
We applied microsatellite markers to three species of *Sycoscapter* wasps that coexist in *F. rubiginosa* figs to establish their offspring allocation patterns. Foundresses of all three species produced very small clutches, typically laying only two to three eggs per fig (Tables 1 and 2). Theoretical models by Greeff (1997) predicted that solitary clutches would be male and gregarious clutches would contain  $n-1$  females. These predictions were not met by species A and B, although solitary clutches were more likely to be male in species C (Table 3). Brood level patterns result from combined offspring allocation by multiple foundresses. The emergent patterns are a strong increase in brood size with foundress number in all species (Fig. 3), and a weaker decrease in sex ratio with brood size, significant only in species C (Fig. 2). Brood sex ratio is not correlated with foundress number. At the population level, all three species have female-biased population sex ratios (Table 1), but the bias (sex ratio = 0.31) is only significant in species A. Meanwhile, 10–22% of females develop in patches without mates (Table 1), leading to a corresponding level of constrained sex allocation by females dispersing unmated.

The very small clutches produced by *Sycoscapter* wasps severely constrain their sex allocation options relative to fig-pollinating wasps, which typically have large clutch sizes of tens of eggs, or even >100 eggs. Greeff's (1997) offspring allocation models for non-pollinating fig wasps assume that they lay a few eggs in each of several figs. Our data provide supporting evidence for both very small clutches and several cases where a given female laid clutches in two to four different figs (Supporting Information). Although parameter values such as fecundity and foundress distributions across patches influence the details, Greeff's models essentially predict two types of clutches: (i) solitary males, and (ii) larger clutches with one male and  $n-1$  females. Overall, our data do not support prediction (i) as singletons were equally likely to be male or

**Table 3.** Comparison of sex ratios (SR) of solitary and gregarious clutches.

Species	Solitary clutches			Gregarious clutches			$\chi^2$
	Males	Females	SR	Males	Females	SR	
A	34	30	0.53	19	91	0.17	22.89***
B	22	16	0.58	13	30	0.30	5.21*
C	33	14	0.67	16	38	0.31	12.09***

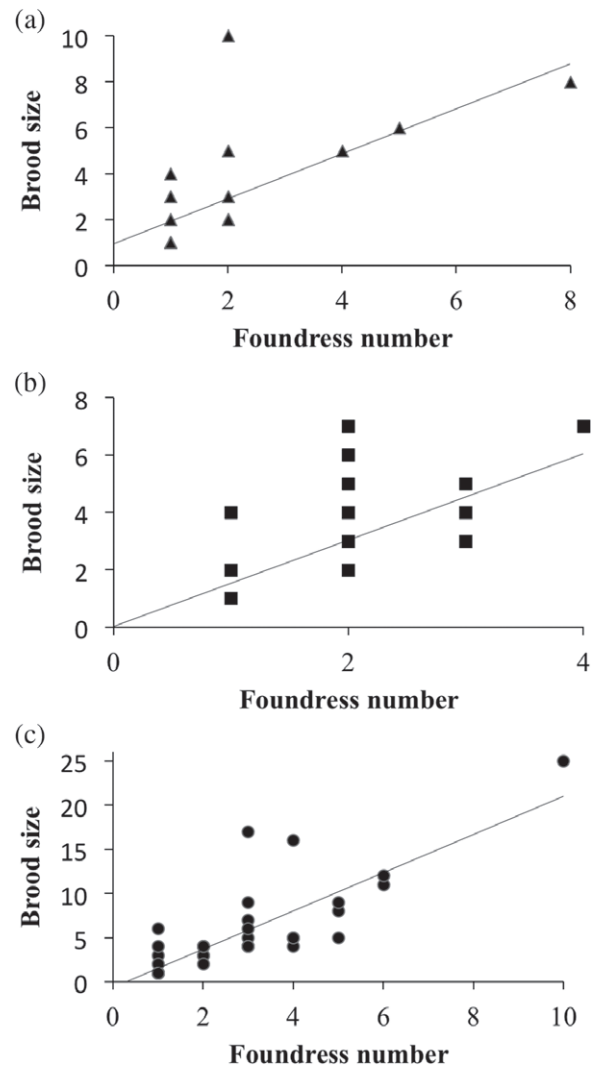
\* $P < 0.05$ ; \*\*\* $P < 0.001$ .



**Fig. 2.** Brood sex ratio (proportion of males) decreases with brood size number in *Sycosapter* sp. (a) Species A; (b) species B; (c) species C. The relationship is only significant in species C – see text for details.

female in species A and B, and there were still many singleton females in species C (Table 3). The fit to prediction (ii) is worse, with a significant excess of clutches that do not have  $n-1$  females in species A (Table 3).

These patterns may reflect a genuine lack of fit to model predictions, e.g. because wasps adopt strategies that differ in detail from the alternatives considered in the game theoretic simulations. Another possibility is that developmental mortality may lead to a difference between primary and secondary sex ratios, e.g. singleton females may have originally had a brother in the same fig, but he died. Such patterns have been detected and explored considerably in distantly related bethylid wasps (Hardy & Cook, 1995; Hardy *et al.*, 1998) that also have small



**Fig. 3.** Brood size increases significantly with foundress number in *Sycosapter* sp. (a) Species A; (b) species B; (c) species C – see text for details.

clutches and local mating. A further possibility is sex allocation 'mistakes' by ovipositing females, and the three explanations considered in this paragraph are not mutually exclusive.

At the brood level, sex ratio decreased with brood size in all three species (Fig. 2), but the decrease was only significant for species C. This decrease is predicted by Greeff's (1997) model for uninformed foundresses, whereas his model for informed foundresses predicts an increase. The limited correlation with brood level model predictions is unsurprising given that these are driven by underlying clutch allocation patterns that are also weak or absent in our data. Overall, species C shows some fit to predictions of the model for uninformed females, while species A and B do not.

Small brood sizes resulted in 10–22% of female wasps (Table 2) developing in patches without mates. These unmated females are subject to constrained sex allocation but can still potentially produce male offspring from unfertilised eggs

(Godfray, 1990). However, Bean and Cook (2001) estimated that 17% of males exit from their natal fig in *Sycoscapter australis*. A few might successfully enter other figs to search for mates, but the likelihood of dispersing and obtaining mates successfully appears very low. The high incidence of single sex patches should favour winged males (Hamilton, 1979) and this is supported by a comparative study across fig wasp species (Cook *et al.*, 1997). However, winged males are unknown in the genus *Sycoscapter* and these wasps may be subject to phylogenetic inertia and unable to respond to this selection pressure (Cook *et al.*, 1997; West *et al.*, 1997).

Our data revealed very few clutches of more than four eggs (Supporting Information, Table S2). *Sycoscapter* wasps are thought to be synovigenic, continually maturing eggs, so immediate fecundity might limit their clutch sizes. However, dissections revealed that females typically have 10–25 eggs available (J.C. Moore, unpublished) and other factors could also favour small clutches, e.g. bet-hedging on offspring survival by spreading risk of offspring mortality across patches (Rubenstein, 1982; Greeff, 1997). Additionally, adult females may be selected to make only short visits to each fig to reduce their own risk of predation by spiders, and especially ants, a pervasive threat to ovipositing fig wasps (Schatz *et al.*, 2006; Wang *et al.*, 2014).

In conclusion, we believe this to be the first study to use molecular markers to determine sex ratio and clutch size patterns in non-pollinating fig wasps and to test directly predictions from theory. Molecular markers were crucial to assign individual wasps both to cryptic species and to sibships within these. We show that clutches are very small in all three species. Species A and B show little fit to predictions of sex ratio models, although gregarious broods are more female-biased than solitary ones. Species C fits some predictions of a model of sex allocation by uninformed foundresses. There are several taxonomically disparate non-pollinating fig wasp lineages (Cook & Rasplus, 2003) and similar molecular ecology studies of other species could test how clutch size varies across taxa, and if species less constrained by small clutch sizes display different sex allocation patterns.

## Acknowledgements

We are grateful to Paul Cunningham, Mike Furlong and Myron Zalucki in Australia and Sue Rumsey in the UK for assistance with field and laboratory logistics, respectively. We thank Jaco Greeff, Sally Power and Tim Sutton for comments on the manuscript. This research was supported by the BBSRC, Swiss National Funds and the Royal Society. JMC, CR, JCM and SAW conceived the study. JCM conducted the field sampling and insect sorting. CR conducted the microsatellite studies. CR and JMC analysed the data. JMC wrote the paper with input from CR, JCM and SAW. The authors have no conflicts of interest to declare.

## Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12433

**Table S1.** Contents of each occupied fig for each of the three *Sycoscapter* species. Mating is constrained (constraint = 1) when females are in a fig with no conspecific males.

**Table S2.** Microsatellite genotypes for all wasps. Note that males have only one allele per locus as they are haploid.

## References

- Bean, D. & Cook, J.M. (2001) Male mating tactics and lethal combat in the nonpollinating fig wasp *Sycoscapter australis*. *Animal Behaviour*, **62**, 535–542.
- Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K., Meier, R., Winker, K. *et al.* (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, **22**, 148–155.
- Bouteiller Reuter, C., Hale, M.L. & Cook, J.M. (2009) Characterization of microsatellite markers for *Sycoscapter* nonpollinating fig wasps. *Molecular Ecology Resources*, **9**, 832–835.
- Choe, J.C. & Crespi, B.J. (1997) *The Evolution of Social Behaviour in Insects and Arachnids*. Cambridge University Press, Cambridge, U.K.
- Cook, J.M. (2005) Alternative male mating tactics in fig wasps. *Insect Evolutionary Ecology* (ed. by M. D. E. Fellowes, G. J. Holloway and J. Rolf), pp. 83–109. CABI Publishing, Wallingford, U.K.
- Cook, J.M. & Rasplus, J.Y. (2003) Mutualists with attitude: coevolving fig wasps and figs. *Trends in Ecology & Evolution*, **18**, 241–248.
- Cook, J.M. & Segar, S.T. (2010) Speciation in fig wasps. *Ecological Entomology*, **35**, 54–66.
- Cook, J.M. & West, S.A. (2005) Figs and fig wasps. *Current Biology*, **15**, R978–R980.
- Cook, J.M., Compton, S.G., Herre, E.A. & West, S.A. (1997) Alternative mating tactics and extreme male dimorphism in fig wasps. *Proceedings of the Royal Society of London Series B: Biological Sciences*, **264**, 747–754.
- Cook, J.M., Reuter, C., Moore, J.C. & West, S.A. (2015) Fighting in fig wasps: do males avoid killing brothers or do they never meet them? *Ecological Entomology*, **40**, 741–747.
- Crawley, M.J. (2005) *Statistics: An Introduction using R*. John Wiley & Sons, Chichester, U.K.
- Darwell, C.T., al-Beidh, S. & Cook, J.M. (2014) Molecular species delimitation of a symbiotic fig-pollinating wasp species complex reveals extreme deviation from reciprocal partner specificity. *BMC Evolutionary Biology*, **14**, 189.
- Fellowes, M.D.E., Compton, S.G. & Cook, J.M. (1999) Sex allocation and local mate competition in Old World non-pollinating fig wasps. *Behavioral Ecology and Sociobiology*, **46**, 95–102.
- Godfray, H.C.J. (1990) The causes and consequences of constrained sex allocation in haplodiploid animals. *Journal of Evolutionary Biology*, **3**, 3–17.
- Goudet, J. (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity*, **86**, 485–486.
- Greeff, J.M. (1997) Offspring allocation in externally ovipositing fig wasps with varying clutch size and sex ratio. *Behavioral Ecology*, **8**, 500–505.
- Greeff, J.M. & Ferguson, J.W.H. (1999) Mating ecology of the nonpollinating fig wasps of *Ficus ingens*. *Animal Behaviour*, **57**, 215–222.
- Green, R.E., Gordh, G. & Hawkins, B. (1982) Precise sex ratios in highly inbred parasitic wasps. *American Naturalist*, **120**, 653–665.
- Hamilton, W.D. (1967) Extraordinary sex ratios. *Science*, **156**, 477–488.
- Hamilton, W.D. (1979) Wingless and fighting males in fig wasps and other insects. *Reproduction, Competition and Selection of Insects* (ed. by M. S. Blum), pp. 168–220. Academic Press, New York, New York.
- Hardy, I. & Cook, J.M. (1995) Brood sex-ratio variance, developmental mortality and virginity in a gregarious parasitoid wasp. *Oecologia*, **103**, 162–169.

- Hardy, I.C., Dijkstra, L.J., Gillis, J.E. & Luft, P.A. (1998) Patterns of sex ratio, virginity and developmental mortality in gregarious parasitoids. *Biological Journal of the Linnean Society*, **64**, 239–270.
- Hebert, P.D., Penton, E.H., Burns, J.M., Janzen, D.H. & Hallwachs, W. (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 14812–14817.
- Herre, E.A. (1985) Sex-ratio adjustment in fig wasps. *Science*, **228**, 896–898.
- Herre, E.A. (1987) Optimality, plasticity and selective regime in fig wasp sex-ratios. *Nature*, **329**, 627–629.
- Herre, E.A., West, S.A., Cook, J.M., Compton, S.G. & Kjellberg, F. (1997) Fig-associated wasps: pollinators and parasites, sex-ratio adjustment and male polymorphism, population structure and its consequences. *The Evolution of Mating Systems in Insects and Arthropods* (ed. by J. C. Choe and B. J. Crespi), pp. 226–239. Cambridge University Press, Cambridge, U.K.
- Marshall, T.C., Slate, J.B.K.E., Kruuk, L.E.B. & Pemberton, J.M. (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.
- Marussich, W.A. & Machado, C.A. (2007) Host-specificity and coevolution among pollinating and nonpollinating new world fig wasps. *Molecular Ecology*, **16**, 1925–1946.
- McLeish, M.J., van Noort, S. & Tolley, K.A. (2010) African parasitoid fig wasp diversification is a function of *Ficus* species ranges. *Molecular Phylogenetics and Evolution*, **57**, 122–134.
- Molbo, D., Machado, C.A., Sevenster, J.G., Keller, L. & Herre, E.A. (2003) Cryptic species of fig-pollinating wasps: implications for the evolution of the fig-wasp mutualism, sex allocation, and precision of adaptation. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 5867–5872.
- Moore, J.C., Zavodna, M., Compton, S.G. & Gilmartin, P.M. (2005) Sex ratio strategies and the evolution of cue use. *Proceedings of the Royal Society of London B: Biological Sciences*, **272**, 1287–1294.
- Moore, J.C., Obbard, D.J., Reuter, C., West, S.A. & Cook, J.M. (2008) Fighting strategies in two species of fig wasp. *Animal Behaviour*, **76**, 315–322.
- Morgan, D. & Cook, J.M. (1994) Extremely precise sex-ratios in small clutches of a bethylid wasp. *Oikos*, **71**, 423–430.
- Murray, M.G. (1990) Comparative morphology and mate competition of flightless male fig wasps. *Animal Behaviour*, **39**, 434–443.
- Pfenninger, M. & Schwenk, K. (2007) Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. *BMC Evolutionary Biology*, **7**, 121.
- Rubenstein, D.I. (1982) Risk, uncertainty and evolutionary strategies. *Current Problems in Sociobiology* (ed. by D.I. Rubenstein), pp. 91–111. Cambridge University Press, Cambridge, U.K.
- Schatz, B., Proffitt, M., Rakhi, B.V., Borges, R.M. & Hossaert-McKey, M. (2006) Complex interactions on fig trees: ants capturing parasitic wasps as possible indirect mutualists of the fig-fig wasp interaction. *Oikos*, **113**, 344–352.
- Shuker, D.M. & Simmons, L.W. (2014) *The Evolution of Insect Mating Systems*. Oxford University Press, Oxford, U.K.
- Stubblefield, J.W. & Seger, J. (1990) Local mate competition with variable fecundity: dependence of offspring sex ratios on information utilization and mode of male production. *Behavioural Ecology*, **1**, 68–80.
- Sutton, T.L., DeGabriel, J.L., Riegler, M. & Cook, J.M. (2017) Local coexistence and genetic isolation of three pollinator species on the same fig tree species. *Heredity*, **118**, 486–490.
- Wang, J.L. (2004) Sibship reconstruction from genetic data with typing errors. *Genetics*, **166**, 1963–1979.
- Wang, B., Geng, X.Z., Ma, L.B., Cook, J.M. & Wang, R.W. (2014) A trophic cascade induced by predatory ants in a fig-fig wasp mutualism. *Journal of Animal Ecology*, **83**, 1149–1157.
- Weiblen, G.D. (2002) How to be a fig wasp. *Annual Review of Entomology*, **47**, 299–330.
- Werren, J.H. (1980) Sex ratio adaptations to local mate competition in a parasitic wasp. *Science*, **208**, 1157–1159.
- West, S.A. & Herre, E.A. (1998) Partial local mate competition and the sex ratio: a study on non-pollinating fig wasps. *Journal of Evolutionary Biology*, **11**, 531–548.
- West, S.A., Herre, E.A., Compton, S.G., Godfray, H.C.J. & Cook, J.M. (1997) A comparative study of virginity in fig wasps. *Animal Behaviour*, **54**, 437–450.

Accepted 2 May 2017

Associate Editor: Dirk Mikolajewski