

Molecular markers reveal reproductive strategies of non-pollinating fig wasps

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ABSTRACT

Molecular markers can reveal offspring allocation patterns under field conditions in insects that lay eggs in discrete patches. Fig-pollinating wasps provide a famous example, with females (foundresses) entering figs to lay eggs and their offspring later mating inside the same fig. However, diverse non-pollinating fig wasps have external oviposition strategies (EOS), laying eggs into figs without entering them. This opens up a wide range of potential reproductive strategies with different combinations of clutch size, sex ratio, and number of patches (figs) used. Developing appropriate theory is challenging unless some parameter values can be assumed or constrained, while empirical study is hindered by the coexistence of cryptic species.

I can see that you are trying to avoid the problem from referees last time, but the above felt a bit too vague to me. What is the problem we solve? Why care? How about something more like:

Fig wasps have proved extremely useful study organisms for how reproductive decisions evolve in response to population structure. In particular, they have provided text book examples of how natural selection can favour female biased offspring sex ratios, lethal combat for mates, and dimorphic mating strategies. However, much of this previous work has been challenged by the discovery that, in many cases, what were previously thought to be single species, have been discovered to be a number of cryptic species. Consequently, work is required to determine the population structure and reproductive decisions of individuals that can be unambiguously assigned to species. We exploited nine microsatellite loci to uncover reproductive patterns in three *Sycoscapter* species, that co-exist within one fig species. We show that foundress number is typically 1-5, similar to many fig-pollinating wasps, and that most figs contain wasps from more than one *Sycoscapter* species. In contrast to fig-pollinating wasps, *Sycoscapter* foundresses produce tiny clutches of typically 1-3 offspring per fig, but one foundress may lay eggs in

several figs. Single egg clutches are usually male, while multiple egg clutches are female-biased, typically with one male plus a few female offspring. However, all female clutches also exist. At the fig level, brood size (all wasps in a fig) shows a strong positive correlation with foundress number and a weaker negative correlation with brood sex ratio. However, none of these variables are indices of the relatedness of males in the patch, because foundresses rarely lay more than one son. Finally, about 20% of females develop in patches without males. As males are wingless and non-dispersing, these females disperse unmated and are constrained to produce only sons from unfertilised eggs.

INTRODUCTION

Insects are the most diverse class of the most diverse phylum of animals. There are thought to be about five million species (Stork et al., 2015), but the true figure may be much higher depending on the prevalence of morphologically cryptic species. The species diversity of insects is mirrored by their diverse mating systems (Choe & Crespi, 1997; Shuker & Simmons, 2014) and insects have both inspired theory in this area (e.g. (Hamilton, 1967)) and been used to test it (Herre, 1985). However, study of insect mating systems can be challenging for two key reasons. First, many insects are small and highly mobile, so difficult to study in the field. Second, recent molecular studies have found cryptic species in many insect taxa (Bickford et al., 2007; Hebert et al., 2004; Pfenninger & Schwenk, 2007), suggesting that many previous studies of mating systems may have inadvertently pooled data from multiple species (Molbo et al., 2003). Fortunately, molecular techniques, such as barcoding and microsatellites, provide opportunities to deal with the second problem (Cook et al., 2015; Molbo et al., 2003), while careful selection of study species can address the first.

Fig wasps (Hymenoptera) are tiny insects whose offspring develop only in the inflorescences (figs) of *Ficus* trees (Cook & West, 2005). In many species males are wingless and mate with winged females inside figs before the females disperse to lay eggs in other figs (Cook, 2005; Hamilton, 1979; Herre et al., 1997). For this reason, despite the fact that most fig wasps are <5 mm long, we can collect accurate data on the size and composition of their mating aggregations under natural field conditions (Herre et al., 1997). Moreover, in the figs of most *Ficus* species, several wasp species develop and mate alongside each other, creating excellent opportunities for comparative studies (Cook, 2005). One large radiation, the fig-pollinating wasps, makes up the family Agaonidae. Each of the >750 *Ficus* species is pollinated by 1-5 agaonid species (Darwell et al., 2014), most of which are host-specific to a single *Ficus* species (Cook & Rasplus, 2003; Weiblen, 2002). In addition, a given *Ficus* species may host up to 30 non-pollinating fig wasp (NPFW) species from diverse lineages in other wasp families (Cook & Rasplus, 2003). These are also typically host-specific to just one *Ficus* species (Cook & Segar, 2010), but again with several exceptions (e.g. (Marussich & Machado, 2007; McLeish et al., 2010)).

Agaonid wasps have an “internal oviposition strategy” (IOS) in which females typically lay “all their eggs in one basket” (Herre et al., 1997). Females (foundresses) enter receptive figs through a narrow tunnel, the ostiole, at the apex of the fig. Inside, a foundress then lays many eggs (often

100 or more, depending on species) within a few hours before dying inside the fig. Foundress number is typically only 1-5 (Herre et al., 1997), so figs contain many offspring from a few mothers. Some weeks later the offspring mature and mate inside the fig. There is local mate competition (LMC - (Hamilton, 1967)) and sib-mating is prevalent, leading to selection for female-biased sex ratios adjusted to foundress number (Herre, 1985, 1987; Herre et al., 1997).

However, NPFWs outnumber pollinators by about 5-10 species to one (Cook & West, 2005) and have more diverse mating systems. NPFW species vary considerably in their sex ratios (Fellowes et al., 1999; West & Herre, 1998) and a given species may have winged, wingless or dimorphic males (Cook et al., 1997; Hamilton, 1979). Further, in species with wingless males, some have regular lethal fighting for access to mates (Hamilton, 1979; Murray, 1990). A key difference between agaonids and most NPFWs is that NPFW females lay eggs through the fig wall, without entering the fruit. This “external oviposition strategy” (EOS) provides the opportunity to lay “a few eggs in many baskets” (Herre et al., 1997). Foundress number is not constrained by the need to enter figs, but has not been quantified in any NPFW species.

Theory for IOS wasps is both well-developed and extensively tested. IOS means that foundress behaviour should be optimised for laying all eggs in a single fig, so the main theoretical issue is to deduce optimal sex ratios (Hamilton, 1967). The predicted relationship between foundress number and offspring sex ratio has often been tested by counting live offspring and dead foundresses in the same figs and assuming foundresses produce equal numbers of offspring (Hamilton, 1967; Herre, 1985, 1987; Herre et al., 1997). More recent studies have 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 models that incorporate clutch size variation, and of predictions for individual foundresses, as well as the emergent patterns at fig level. Moreover, molecular markers have revealed cryptic species within what was thought to be a single wasp species (Molbo et al., 2003). Together, these improvements in data quality have increased fit between theory and data considerably (Molbo et al., 2003).

The challenges are greater for EOS wasps as it is hard to develop models to simultaneously optimize clutch size and sex ratio without imposing assumptions (Greeff, 1997). However, we lack empirical studies to estimate the key variables – e.g. clutch size, sex ratio, foundress

number - in any NPFW species. In addition, the “number of baskets” (figs) into which a given foundress lays eggs is also unknown. These variables all interact to determine optimal reproductive strategies (Greeff, 1997); e.g. small clutch sizes can severely limit offspring sex ratio options (Green et al., 1982; Morgan & Cook, 1994). Consequently, direct genetic estimation of key variables is essential for progress in understanding EOS reproductive strategies.

In this study, we use microsatellites on three *Sycoscapter* species in *Ficus rubiginosa* figs and provide the first genetic estimates of reproductive patterns for any EOS fig wasp species. We have two broad aims. First, we estimate the aspects of population structure that are expected to influence the evolution of reproduction behaviour. Specifically, we measure XXXX. Since male are wingless and non-dispersing, we are also able to use our population structure data to determine the proportion of females that will be unable to obtain mates, and hence be constrained to produce only (haploid) sons from unfertilised eggs (Godfray, 1990). Second, we use our data to establish the reproductive decisions of individual females, to test whether female adjust their offspring sex ratio in response to the number of females laying eggs in a fruit, and their clutch size.. This would be impossible without molecular markers, since we cannot identify individuals to species level based on morphology alone (see methods for details).

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 mtDNA sequences (Moore et al., 2008) or multilocus microsatellite genotypes (Bouteiller Reuter et al., 2009; Cook et al., 2015).

We collected 55 figs from four *Ficus rubiginosa* trees in Brisbane, Queensland, 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 ul 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 15min and centrifuged for 5 min at 3500 rpm. We genotyped the 387 *Sycoscapter* wasps at nine microsatellite markers (locus 5, 48, 68, 71, 82, 85, 89, 95, 96) developed by Bioprofiles (Newcastle) and described in (Bouteiller Reuter et al., 2009). PCR was carried out on 96-well microplates in 15 ul volumes containing 5 ul of DNA extract, 0.4 U of Taq polymerase (Qiagen), 0.4uM of each primer, 0.2 mM of dNTPs (Qiagen), 1x Buffer (Qiagen) and 2.5 mM of MgCl₂. 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 sec at 94°C, 15 sec at specific annealing T°C (50C for all loci except locus 68 (48°C), loci 48, 82 and 96 (52°) and locus 5 (54°C)), 15 sec at 72°C and 30 cycles of 15 sec at 89°C, 15 sec at specific annealing T°C (same as above), 15 sec at 72°C and a final extension at 72°C for 10 min in Techgene and Eppendorf thermal cyclers. Samples were analysed on an ABI3700 machine and fragment sizes scored using Genescan3.5 and Genotyper 2.5 softwares (ABI).

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). PIC measures the informativeness 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 and also with IOS fig-pollinating wasps. F_{ST} was used to measure genetic differentiation between the three co-habiting *Sycoscapter* species. PIC and F statistics were calculated based on $n = 140, 47$ and 54 females (i.e. the haploid males were not used) for species A, B, 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 individual foundress produced in a given fig.

Statistical analyses of sex ratios and offspring numbers

We explored reproductive patterns at different biological levels – species, brood (all wasps in a fig) and clutch (offspring of one female in a fig). For each species, we first 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; i.e. those subject to constrained offspring sex allocation due to lack of matings.

We then tested theoretical predictions for lower spatial levels. At the fig level, for each species, we tested whether brood size and brood sex ratio increased with foundress number, as predicted by simple LMC models (Hamilton, 1967). We also tested whether brood sex ratio increased or decreased with brood size, as predicted by alternative sex allocation models for EOS fig wasps with restricted clutch sizes (Greeff, 1997).

At the clutch level, we tested whether individual females adjust their sex ratios to their own clutch sizes by comparing sex ratios of solitary (single offspring) and gregarious (multiple offspring) clutches. The small clutch size LMC models of (Greeff, 1997) predict that solitary clutches should always contain males, while gregarious clutches should be female-biased, generally with only one male. Similar prediction come from standard LMC models where clutch size is variable (Stubblefield & Seger, 1990; Werren, 1980).

All statistical analyses were performed in R (<http://www.Rproject.org>). We used general linear models (GLMs), with binomial errors for sex ratio data, since these involve proportions, and Poisson errors for the low offspring counts for clutch/brood size. We checked for over-dispersion 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 the `prop.test` function in R for a 2-sample test for equality of proportions to compare the sex ratios of two size classes of clutches produced by foundresses.

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 3 to 21 (Tables 4; S1). The mean polymorphic informative content (PIC) of loci was high for *Sycoscapter* species B (0.60) and C (0.68) and medium (0.42) for species A. 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/Population level results

Our sample of 55 figs contained 202 *Sycoscapter* A wasps (1-25 per occupied fig) in 39 syconia, 82 *Sycoscapter* B wasps (1-10 per fig) in 29 figs, and 103 *Sycoscapter* C wasps (1-7 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 (Table S1). Consequently, most wasps occurred in figs that also contained close congeners, making our molecular species delimitation an essential first step.

The sibship reconstruction revealed 104 (A), 54 (B), and 68 (C) matrilineages respectively and we estimated mean foundress numbers (SE) to be: A=2.7 (2.0), B=1.9 (1.4), C=1.7 (0.8). Typically, there were 2-3 foundresses per fig (Figure 1), but with up to ten for A, seven for B and four for C. All three species have female-biased population sex ratios (Table 1), 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 some females developing in patches without mates. We estimated the proportions of unmated females to be 0.1 (14/140) in species A, 0.21 (10/47) in B, and 0.22 (12/54) in C.

Brood level patterns of reproduction

At the brood (fig) level, there is considerable sex ratio variation, but no correlation with foundress number for any of the species: A ($z=-0.32$; $p=0.75$; $df=1,37$); B ($z=-0.07$; $p=0.95$; $df=1,27$); C ($z=0.06$; $p=0.95$; $df=1,37$). Brood sex ratio decreases with brood size (all conspecifics in a fig) in all three species (Figure 2), but the relationship is only significant in species C: A ($t=-1.41$; $p=0.17$; $df=1,37$); B ($t=-1.53$; $p=0.13$; $df=1,27$); C ($t=-2.582$; $p=0.014$; $df=1,37$). In addition, there is a highly significant positive correlation between brood size and foundress number in all three species: A ($z=5.83$; $p<0.0001$; 58% deviance explained); B ($z=3.67$; $p<0.001$; 49% deviance explained); C ($z=2.78$; $p<0.006$; 51% deviance explained). In summary, the emergent patterns at the brood (fig) level are a strong increase in brood size with foundress number, and a weaker decrease in sex ratio with brood size.

Clutch level patterns of reproduction

Females of all three species lay very small clutches, typically only 1 to 3 eggs per fig (Table 1), and very rarely lay more than one son per clutch (Figure 3; Table 2). The number of daughters is more variable with maxima of 11/8/5 for species A/B/C but average numbers are much lower (Table 2). In all three species, solitary clutches are usually male, despite the female-biased

population sex ratio (Table 1), while gregarious clutches are female-biased (Tables 2, 3). This results in significant decreases in sex ratio as clutch size increases 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/C were found to have laid eggs in up to 3/2/4 figs on the same tree. These are minimum estimates of figs used, because the number of figs sampled is far lower than the total number of figs on a tree.

DISCUSSION

We applied microsatellite markers to three species of EOS *Sycoscapter* wasps that develop in the figs of *Ficus rubiginosa* to establish the reproductive strategies of individual foundresses and how these scale up to patterns at fig and population level. We found that >95% of figs receive <6 foundresses of a given species (Figure 1), which means that foundress number distributions are actually very similar to those observed in many IOS fig-pollinating wasps (Herre et al., 1997). While researchers can sometimes estimate foundress number in IOS wasps by counting dead females inside figs (Herre, 1985, 1987), this option is unavailable for EOS wasps and most other insects laying eggs in patches e.g. (Atkinson et al., 2003).

Our application of molecular markers reveals foundress numbers for the first time in any EOS fig wasps. It also allows estimation of inbreeding coefficients (F_{IS}) directly from genetic data and we found that all three *Sycoscapter* species had significant and similar inbreeding levels ($F_{IS} = 0.24-0.31$). These are much lower than global values reported for several IOS fig-pollinating wasps (e.g. *Pegoscapus hoffmeyer* = 0.87; Molbo et al., 2003; *Platyscapa awekei* = 0.423; (Van Vuuren et al., 2006). However, they are actually higher than values reported for some other pollinator species (e.g. *Pegoscapus gemellus* sp. B < 0.2; Molbo et al. 2004; *Ceratosolen fusciceps* = 0.1758; (Kobmoo et al., 2009)). An interesting comparison is with *Pleistodontes imperialis* sp. 1, the pollinator of the same *F. rubiginosa* figs used by our three *Sycoscapter* study species. Across five populations, *Pleistodontes* sp. 1 had F_{IS} values ranging

from 0.27 to 0.53 (Sutton et al., 2015), so the lower end of its range of inbreeding values is similar to those recorded here for *Sycoscapter* species. Overall, this supports the idea that sib-mating is common in these *Sycoscapter* wasps and it is presumably the only option in single foundress figs, or where unrelated males have been killed (Cook et al., 2015)

We found that *Sycoscapter* wasps produce very small clutches (Table 1) with females typically laying only 2-3 eggs per fig. This severely constrains their sex allocation options, as in some parasitoid wasps associated with other types of plant and insect hosts (Hardy & Cook, 1995; Kapranas et al., 2008; Morgan & Cook, 1994). This is in marked contrast to the co-habiting fig-pollinating wasps, which produce large clutches (often >100 eggs – e.g. (Molbo et al., 2004; Molbo et al., 2003; Raja et al., 2008) and conform well to the predictions of classical LMC models (Herre et al., 1997). The parasitoid wasp, *Nasonia vitripennis*, which attacks fly larvae in birds' nests, represents an intermediate case. Its foundress number distribution is again similar, ranging from 1-7 per patch, but mean clutch size is about 10, giving greater flexibility to adjust sex ratio adaptively (Grillenberger et al., 2008). Interestingly, the *Nasonia* case also showed that some fine adjustments of sex ratios observed in controlled laboratory experiments were not detectable in the field data (Burton-Chellew et al., 2008).

Sycoscapter wasps cannot achieve the classic LMC sex allocation patterns shown by fig-pollinating wasps because of their small clutch sizes. While there are numerous cases of adaptive sex ratio adjustment in parasitoid wasps in general (West, 2009), a lack of sex ratio adjustment has also been observed in the large clutches of *Mellitobia* parasitoids of fly larvae, although this may be due to competitive asymmetries between early and late emerging males (Abe et al., 2003a; Abe et al., 2003b; Abe et al., 2007; Innocent et al., 2007).

Greeff (Greeff, 1997) constructed offspring allocation simulation models for EOS fig wasps, assuming that they visit multiple figs and lay few eggs per fig. Our data reveal small clutches and use of multiple figs in *Sycoscapter* wasps. Although our study has limited power to detect females laying eggs in multiple figs, we found several cases where a given female laid clutches in 2-4 different figs (Table S2). (Greeff, 1997) developed two models, for females with and without information about other wasp offspring in a fig. Although parameter values such as fecundity and foundress distributions across patches influence the details, the models all generally predict two types of clutch: (1) single males and (2) larger clutches with one male and

(n-1) females. We found that many clutches fitted this dichotomy (Table 3), but there were also many single female clutches (Table 2). In addition, there are several gregarious clutches that contain only females (Table 2). Overall, about half of all clutches surveyed do not fit the expected simple dichotomy. This could show a genuine lack of fit to model predictions, perhaps because the wasp adopt strategies that differ in detail from the alternative strategies considered in the game theoretic simulations.

Another interesting possibility is that offspring mortality explains at least some of the discrepancy between models and data. As in most such studies, we actually assessed offspring numbers and sex ratios after larval mortality, and these values might differ significantly from the primary (conception) clutch sizes and sex ratios. In particular, all female clutches may have included a male at conception, but the male died. For example, the many singleton females may have originally had brothers laid in the same fig. Such patterns have been detected and explored considerably in some distantly related bethylid parasitoids ((Hardy & Cook, 1995; Hardy et al., 1998) and, if mortality is high and single foundress situations common, can actually select for laying “insurance” males (Heimpel, 1994).

Our data reveal very few clutches of more than four eggs (Table 2). The female *Sycoscapter* wasps are thought to be synovigenic, continually maturing eggs rather than having a full complement of them at adult emergence. It is therefore possible that fecundity limits clutch size to very low values. However, we consider this unlikely, since dissections revealed that *Sycoscapter* females typically have 10-25 eggs available (JMM, unpubl. data) and other studies of a range of synovigenic fig wasps also report egg numbers well above four (REF). Other factors could also select for small clutch sizes. It could be a form of bet-hedging on offspring survival by spreading eggs (and risk of egg mortality) across many patches (Greeff, 1997; Rubenstein, 1982). It could also result from selection on adult females to reduce their own predation risk, because ovipositing *Sycoscapter* females are often attacked by spiders and especially ants (JMC, pers. obs.). Indeed, there is increasing evidence that ant predation is a pervasive threat to ovipositing NPFWs (Schatz et al., 2006; Wang et al., 2014) and it may select for short visits to each fig and result in smaller clutches.

The reproductive strategies of individual females revealed by our molecular markers, and discussed above, scale up to generate patterns at the fig level. At this level, we here refer to all

conspecific wasps developing in one fig as a brood. Molecular markers remain crucial at this level as they are currently our only reliable way of discriminating between males of all three *Sycoscapter* species and between females of species B and C. We found that brood sex ratio decreased with brood size in all three species (Figure 2), but the relationship was only significant for species C. A decrease in brood sex ratio with brood size is predicted by Greeff's (1997) model for uninformed foundresses. In contrast, Greeff's model for informed females (and classical LMC models), predicts the opposite – an increase in brood sex ratio with foundress number. Our results support the idea that females are uninformed, at least for *Sycoscapter* sp. C. In addition, the relatively weak correlations revealed are unsurprising when you consider that brood level patterns scale up from clutch level patterns and our clutch level patterns show only partial match to Greeff's model predictions.

Our molecular revelation of the correlations between foundress number, brood size and sex ratio at the fig level are also important for the related topic of male reproductive strategies, and specifically the study of lethal fighting in male fig wasps (including *Sycoscapter* species – (Bean & Cook, 2001; Cook et al., 2015; Moore et al., 2008)). Some previous studies (e.g. (Bean & Cook, 2001; Murray, 1987; West et al., 2001) have used brood sex ratio as a surrogate measure for the relatedness of males competing within a fig. This has been based on the assumptions that brood sex ratio increases, and average male relatedness decreases, as foundress number increases. While this may be valid for IOS fig-pollinating wasps (Herre et al., 1997), and for comparisons across species (West et al., 2001), it is clearly invalid for comparisons within the three species studied here, because sex ratio is unrelated to foundress number. This was predicted by (Greeff, 1997) on theoretical grounds and, clearly, direct genetic estimates of male relatedness are a far superior empirical approach (Cook et al., 2015).

Interestingly, however, we found a strong positive relationship between foundress number and brood size (Fig 3). This could be taken to suggest brood size as a better surrogate index of the relatedness of competing males. However, this would also be a mistake. Brood size is a good index of foundress number, but foundresses generally only lay one son per fig so foundress number has little bearing on male relatedness! Again, this validates a theoretical prediction made by (Greeff, 1997) and emphasises that genetic analysis is required to study how relatedness influences mate competition in field situations.

Scaling up from clutch and brood level patterns, we can also examine mating system issues at the population level and compare species. We found that species C had a significantly female-biased population sex ratio (proportion males = 0.31) while the other two species had slightly, but not significantly, female-biased sex ratios (Table 1). Previous comparative studies across fig wasp species have found that the sex ratios of species with wingless males, which should experience higher levels of LMC due to lack of male dispersal, have greater female bias than species with either wing-dimorphic or fully winged males (Fellowes et al., 1999; West & Herre, 1998).

Small clutch sizes plus low foundress numbers result in many single sex broods (Table 2). Since *Sycoscapter* wasps have wingless males, this suggests that 10-22% of female wasps cannot mate in their natal patch unless either males or females disperse to other patches to look for mates. It has been assumed that mating is basically restricted to the natal patch for both sexes, because *Sycoscapter* males are wingless and mating outside of figs has not been observed. However, (Bean & Cook, 2001) estimated that 17% of males exit from their natal fig in *Sycoscapter australis* and some might enter other figs to search for mates. However, we have never seen males entering figs and the chance of dispersing and obtaining mates successfully would appear very low. We thus expect that males developing in single sex patches have zero fitness, although unmated females can still potentially produce male offspring from unfertilised eggs (Godfray, 1990). The high incidence of single sex patches should select for wings in males, and there is a strong correlation between the chance of mating in the natal patch and winged males 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).

To our knowledge, ours is the first study to determine detailed field oviposition patterns in EOS fig wasps, but there are several taxonomically disparate EOS fig wasp lineages (Cook & Rasplus, 2003). Studies of other species could test whether the important constraint of small clutch size is common, and if species with larger clutch sizes are subject to other constraints, such as availability of information cues ((Shuker & West, 2004)).

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

(*total sample = 55 figs, but each species is present in only a subset of these)

Species	Figs*	Wasps	Foundresses	Found. / fig	Sex ratio	Clutch size
<i>Sycoscapter A</i>	39	202	104	2.7 (2.0)	0.31	1.9 (1.7)
<i>Sycoscapter B</i>	29	82	54	1.9 (1.4)	0.43	1.5 (1.3)
<i>Sycoscapter C</i>	39	82	68	1.7 (0.8)	0.48	1.5 (1.0)

Table 2. Composition of all individual clutches (offspring of a given foundress).

Clutch size	Wasps	Sex ratio	A	B	C
1	F	0	30	16	14
	M	1	34	22	33
2	2F	0	10	4	4
	MF	0.5	2	3	8
	2M	1	2	3	3
3	3F	0	3	0	4
	M 2F	0.33	4	2	0
	2M F	0.66	0	0	0
	3M	1	0	0	0
4	4F	0	1	1	1
	M 3F	0.25	2	1	1
	2M 2F	0.5	0	0	0
	3M F	0.75	0	0	0
	4M	1	0	0	0
5	5F	0	1	0	0
	M 4F	0.2	1	0	0
	2M 3F	0.4	1	0	0
	3M 2F	0.6	0	0	0
	4M F	0.8	0	0	0
	5M	1	0	0	0
>=6	6F	0	2	0	0
	M 5F	0.17	0	0	1
	M 8F	0.11	0	1	0
	2M 11F	0.18	1	0	0
	2M 5F	0.29	1	0	0
	2M 4F	0.33	1	0	0

Table 3. Comparison of sex ratios (SR) of clutches with a single (solitary) or multiple offspring (gregarious). $P < 0.05$ (*), 0.001 (***).

Sp.	Solitary clutches			Gregarious clutches			X^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***

Table 4. Allele number per locus.

Locus	Sp. B	Sp. C	Sp. A
5			
48	6	10	4
68	6	9	3
71	9	15	3
82	9	16	5
85	6	12	9
89	14	8	5
95	8	8	4
96	4	21	16
Average	8.2	14.6	8.8

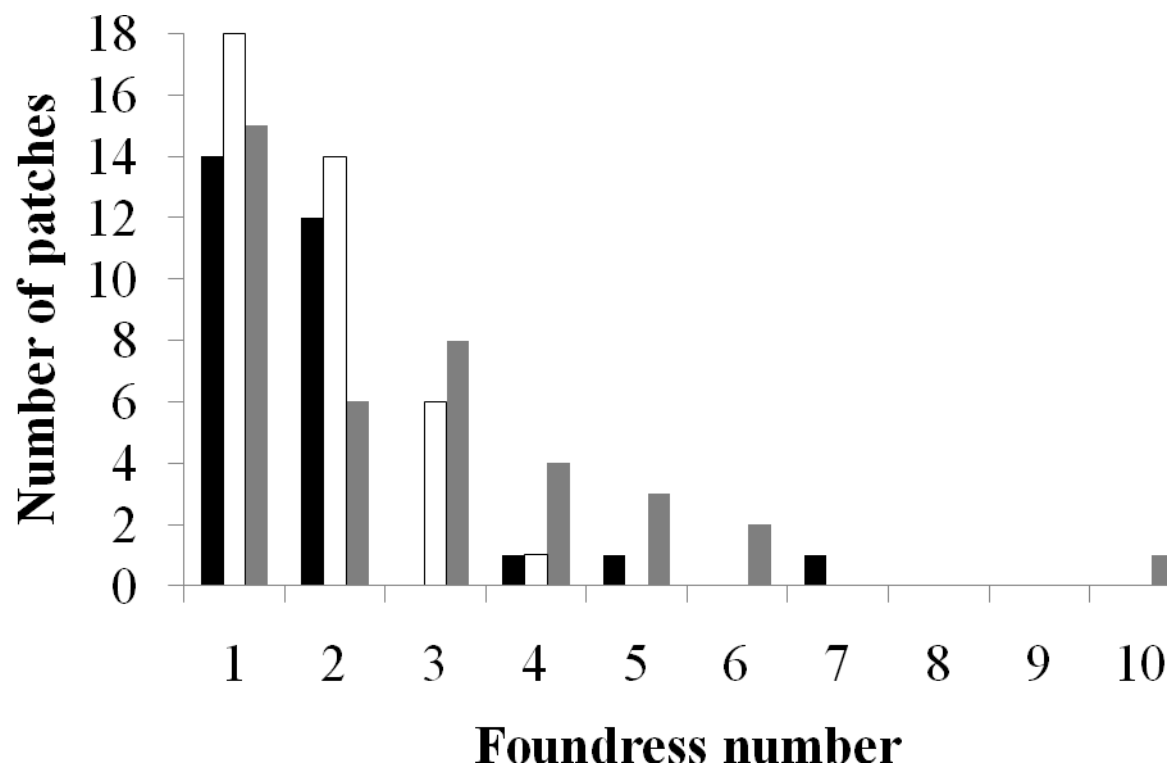


Figure 1. The distribution of foundress number per fig (Species A grey, B white and C black).

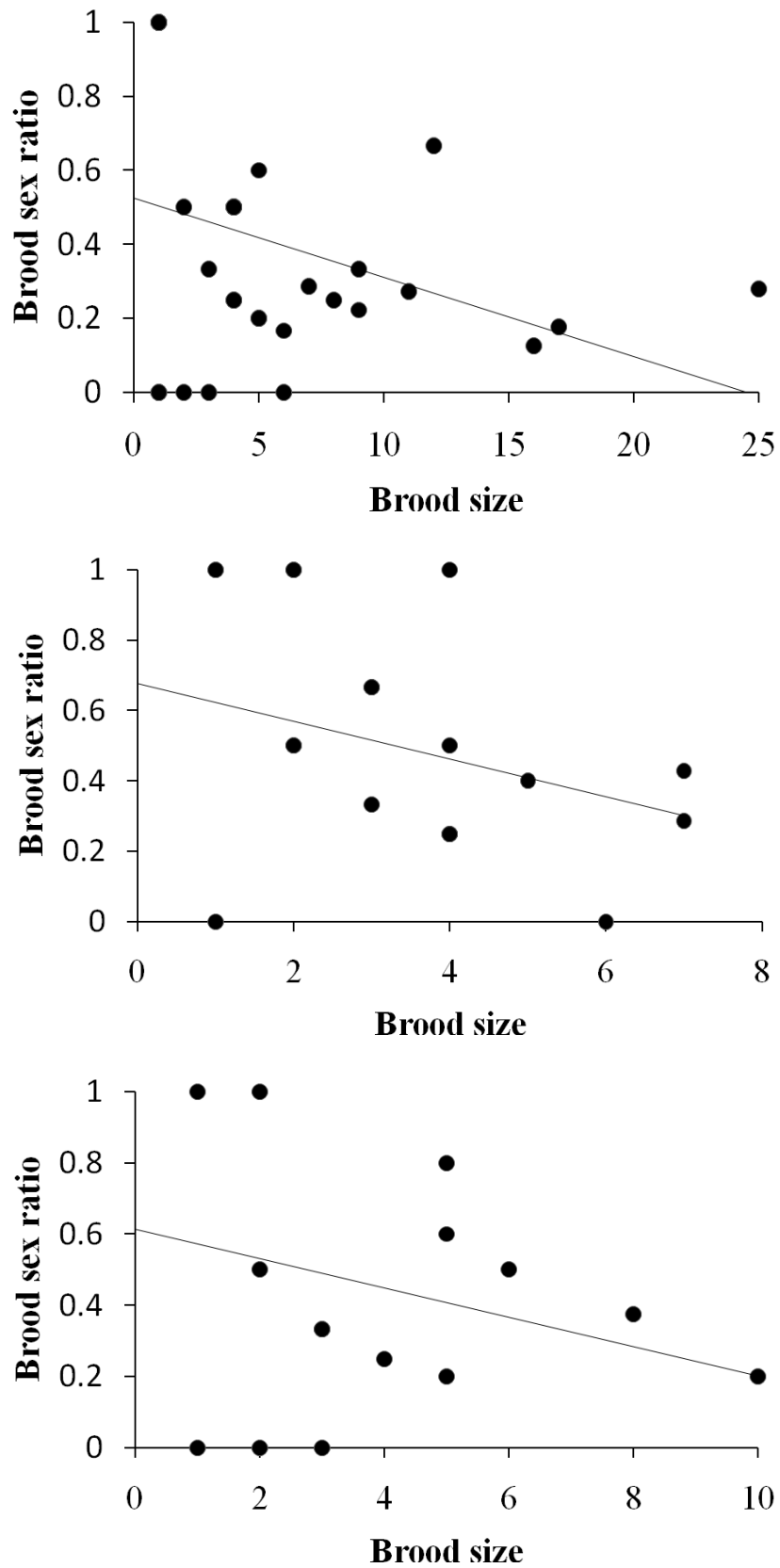


Figure 2. The decrease in brood sex ratio with brood size for *Sycoscapter* sp. A, B, C.

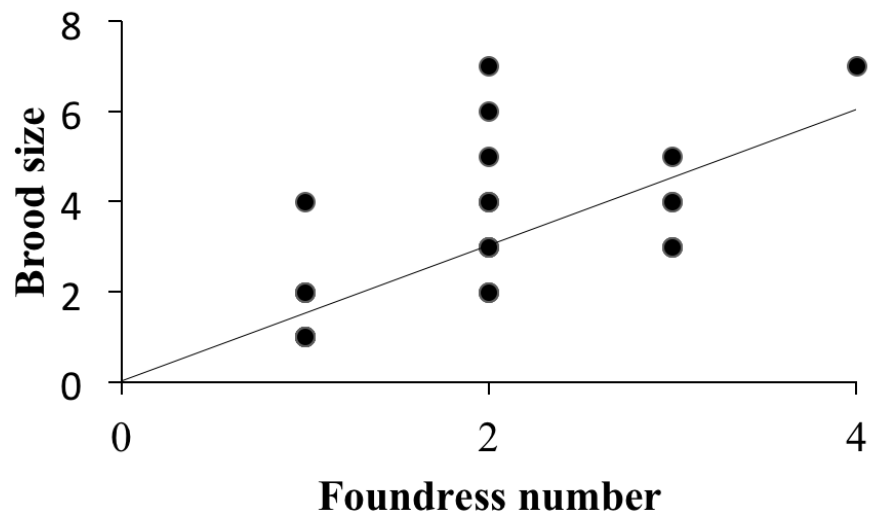
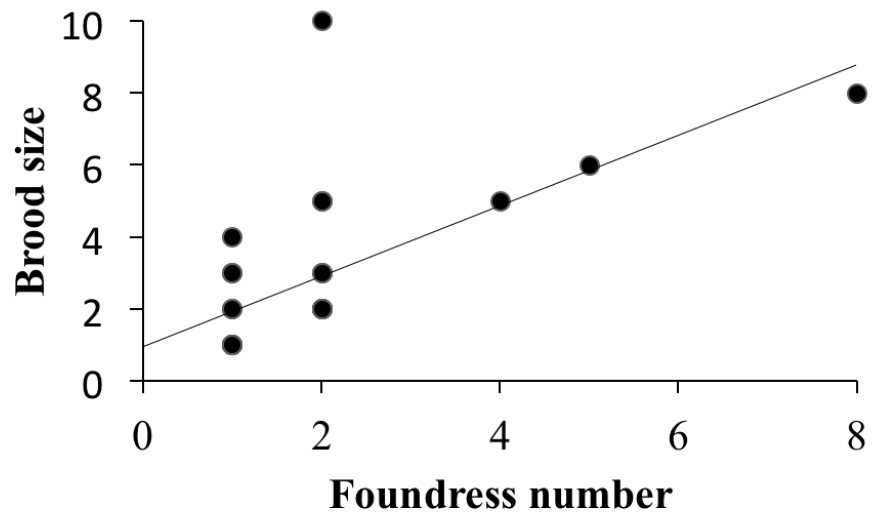
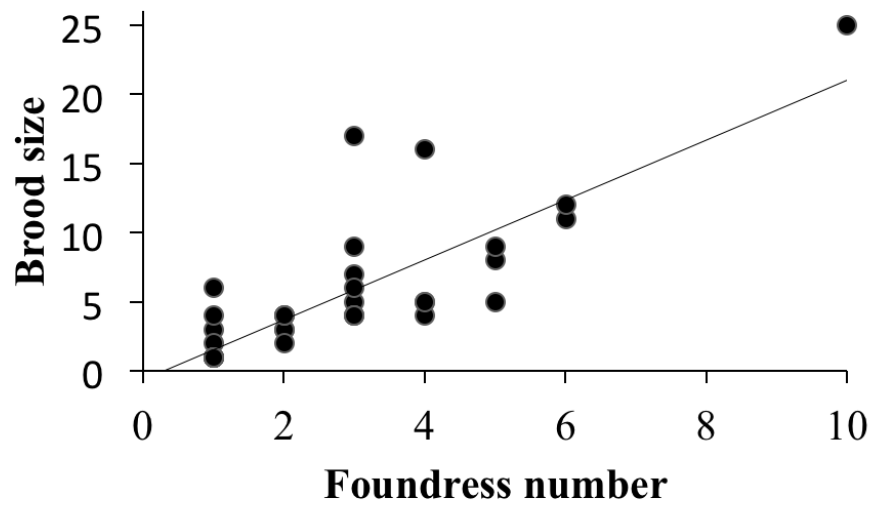


Figure 3. Brood size decreases as foundress number increases in *Sycoscapter* sp. A, B, C.

List of supplementary online material:

Table S1. Contents of each occupied fig for each of the three *Sycoscapter* species.

Mating is constrained (Constraint=1) when females are present in a fig that has no conspecific (wingless) males.

Table S2. Microsatellite genotypes for all wasps.

NB males have only one allele per locus as they are haploid.

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