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Molecular markers reveal reproductive strategies of non-pollinating fig wasps

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Abstract

- 25 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 supposed single species have been
30 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. We used microsatellites to determine species identity and reproductive patterns in three non-pollinating *Sycoscapter* species associated with the same fig species. Foundress number
35 was typically 1-5 and most figs contained more than one *Sycoscapter* species. Foundresses produced very small clutches of about 1-4 offspring, but one foundress may lay eggs in several figs.
4. Overall, the data show poor match to theoretical predictions of solitary male clutches and gregarious clutches with $(n-1)$ females. However, sex ratios are male-biased in solitary
40 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 is only significant in one species, and sex ratio is unrelated to foundress number. In addition, figs with more foundresses contain more wasp offspring.
6. Finally, 10-22% of females develop in patches without males. As males are wingless, these
45 females disperse unmated and are constrained to produce only sons from unfertilized eggs.

Introduction

Insects display a great diversity of mating systems, providing many opportunities to test
50 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 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
55 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.

60 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, in most *Ficus* species, several wasp species develop and mate alongside each other
65 in a fig, creating excellent opportunities for comparative studies (Hamilton, 1979; Greeff &
Ferguson, 1990; Cook, 2005). Each of the >750 *Ficus* species is pollinated by 1-5 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
70 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
75 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 1-5 (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
80 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 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
85 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
90 (Molbo et al., 2003; Sutton et al. 2017). Together, these improvements in data quality have
increased 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
95 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 unrecognized cryptic species could lead to underestimates of constrained sex allocation and potentially confound this pattern.

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In this study, we use microsatellites on three *Sycoscapter* species that co-exist and mate in the same figs and provide the first genetic estimates of reproductive patterns for any non-

pollinating 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 foundress numbers, clutch sizes, and offspring sex ratios, as well as the proportion of females that will disperse unmated. Second, we use 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 be impossible without molecular markers, since 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 *Sycosapter* 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 *Sycosapter* 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

150 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

155 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

160 (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

165 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 software (ABI).

Basic population genetics

170 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 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
175 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 co-habiting *Sycoscapter* species. PIC and F statistics were calculated based on $n = 140, 47$ and 54 females for species A, B, C respectively.

180 *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
185 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
190 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.

195 *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.

200

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
205 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
210 predicted by models (Greeff, 1997). Due to poor support for these predictions, we further tested a simpler model of 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 –
215 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 if 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
220 linear models (GLMs), with binomial errors for sex ratio (proportion) data and Poisson errors for low offspring number counts. 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 (a) exact binomial tests to test if predicted clutch compositions for solitary
225 (prediction = male) and gregarious clutches (prediction = one male plus (n-1) females) were more or less likely than alternatives, and (b) a 2-sample test for equality of proportions (the the prop.test function in R) to test if sex ratios differed between solitary and gregarious clutches.

230 **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 (Supporting Information). The mean polymorphic informative content (PIC) of loci was high for *Sycoscapter* species B (0.60) and C (0.68) and
235 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
240 0.30 for C.

Species 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
245 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).

Consequently, most wasps occurred in figs that also contained close congeners, making molecular species delimitation an essential first step.

250 The sibship reconstruction revealed 104 (A), 54 (B), and 68 (C) matrilineal lines 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 2), but the bias is only significant in *Sycoscapter* A (sex ratio = 0.31, $p < 0.001$, binomial test; 255 *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 & S2).

Clutch sizes and sex ratios

260 In all three species females lay very small clutches, typically only 1 to 3 eggs per fig (Table 2), and solitary clutches are more common than gregarious ones. Clutches very rarely contained more than one male (Tables 2 & Supporting Information). The mean number of females per clutch was also very low (Table 2 & Supporting Information), but reached maxima of 11/8/5 for species A/B/C (Supporting Information).

265 Observed clutch sex ratios differ considerably from the basic predictions of solitary male clutches and gregarious clutches with $n-1$ females (Table 2 & Supporting Information). 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 270 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: 275 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 over-dispersion so we used quasi-binomial errors and t-tests for significance.

280 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 (Supporting Information). These are minimum estimates, as the number of figs sampled is far lower than the number of figs on a tree.

285 *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: 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 290 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 (Figure 3) 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).

295

Discussion

We applied microsatellite markers to three species of *Sycoscapter* wasps that co-exist in *Ficus rubiginosa* figs to establish their offspring allocation patterns. Foundresses of all three species produced very small clutches, typically laying only 2-3 eggs per fig (Tables 1 & 2).
300
Theoretical models by Greeff (1997) predict that solitary clutches should be male and gregarious clutches 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
305 patterns are a strong increase in brood size with foundress number in all species (Figure 3), and a weaker decrease in sex ratio with brood size, significant only in species C (Figure 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
310 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
315 10s 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 2-4 different figs (Supporting Information). Although parameter values such as fecundity and foundress distributions across patches influence the details, Greeff's models essentially
320 predict two types of clutches: (a) solitary males and (b) larger clutches with one male and (n-1) females. Overall, our data do not support prediction (a) since singletons were equally likely

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

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The above 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 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.

335

At the brood level, sex ratio decreased with brood size in all three species (Figure 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.

340

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 &

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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
350 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).

355 Our data revealed very few clutches of more than four eggs (Tables S2, Supporting Information). *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 (JMM, unpubl. data) and other factors could also favour small clutches; e.g. bet-hedging on offspring survival by spreading risk of offspring mortality
360 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).

365 In conclusion, we believe this to be the first study to use molecular markers to determine sex ratio and clutch sizes patterns in non-pollinating fig wasps and 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
370 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.

375

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380

Contribution of authors

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.

385

Supporting Information Short Legends

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.

390 Table S2. Microsatellite genotypes for all wasps. NB males have only one allele per locus as they are haploid.

Conflict of Interest

The authors have no conflicts of interest to declare.

395

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.
- 400 Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K., Meier, R., Winker, K., Ingram, K.K. & Das, I. (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**, 832-405 835.
- Choe, J.C. & Crespi, B.J. (1997) The evolution of social behaviour in insects and arachnids Cambridge University Press.
- Cook, J.M. (2005). Alternative male mating tactics in fig wasps. In *Insect Evolutionary Ecology* (ed. by M.D.E. Fellowes, G.J. Holloway & J. Rollf), pp. 83-109. CABI 410 Publishing, Wallingford, UK., Wallingford, UK.
- 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. & Rasplus, J.Y. (2003) Mutualists with attitude: coevolving fig wasps and figs. 415 *Trends in Ecology & Evolution*, **18**, 241-248.
- 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.
- 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.
- 420 Crawley, M.J. (2005) *Statistics: An Introduction using R* John Wiley & Sons, Chichester, UK.

- 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.
- 425 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. *J. Heredity*, **86**, 485-486.
- 430 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. and Ferguson, J.W.H. (1999) Mating ecology of the nonpollinating fig wasps of *Ficus ingens*. *Animal Behaviour*, **57**, 215-222.
- 435 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. In *Reproduction, Competition and Selection of Insects* (ed. by B. M.S.), pp. 168-220.
- 440 Academic Press, 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*
- 445 *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.
- 450 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,
- 455 population structure and its consequences. In *The Evolution of Mating Systems in Insects and Arthropods* (ed. by J.C. Choe & B.J. Crespi), pp. 226-239. Cambridge University Press, Cambridge.
- 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.
- 460 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.
- 465 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.

- 470 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.
- 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.
- 475 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
480 among taxa and biogeographical regions. *BMC Evolutionary Biology*, **7**, 121.
- Rubenstein, D.I. (1982) Risk, uncertainty and evolutionary strategies. *Current Problems in
Sociobiology*, 91-111.
- Schatz, B., Proffit, 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
485 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.
- Stubblefield, J.W. & Seger, J. (1990) Local mate competition with variable fecundity:
dependence of offspring sex ratios on information utilization and mode of male
490 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, B., Geng, X.Z., Ma, L.B., Cook, J.M. & Wang, R.W. (2014) A trophic cascade
495 induced by predatory ants in a fig–fig wasp mutualism. *Journal of Animal Ecology*, **83**,
1149-1157.
- Wang, J.L. (2004) Sibship reconstruction from genetic data with typing errors. *Genetics*, **166**,
1963-1979.
- Weiblen, G.D. (2002) How to be a fig wasp. *Annual Review of Entomology*, **47**, 299-330.
- 500 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
505 comparative study of virginity in fig wasps. *Animal Behaviour*, **54**, 437-450.

510 **Figure and Table Legends**

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

515

Figure 2. Brood sex ratio (proportion males) decreases with brood size number in *Sycoscapter* sp. A (top, triangles), B (middle, squares) and C (bottom, circles). The relationship is only significant in species C – see text for details.

520 Figure 3. Brood size increases significantly with foundress number in *Sycoscapter* sp. A (top, triangles), B (middle, squares) and C (bottom, circles) – see text for details.

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

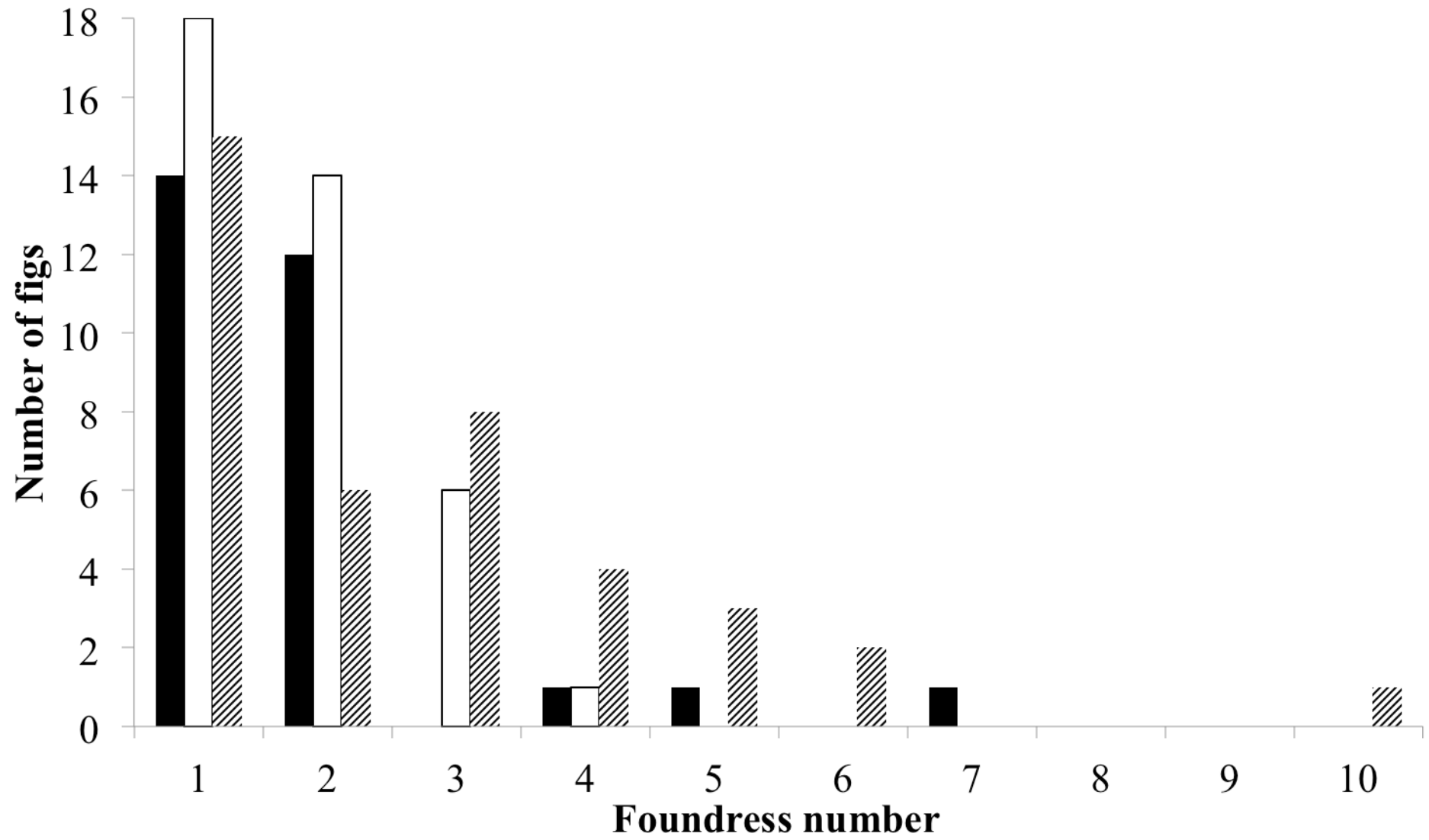
525 Table 2. Clutch sizes and sex ratios in the three wasp species.

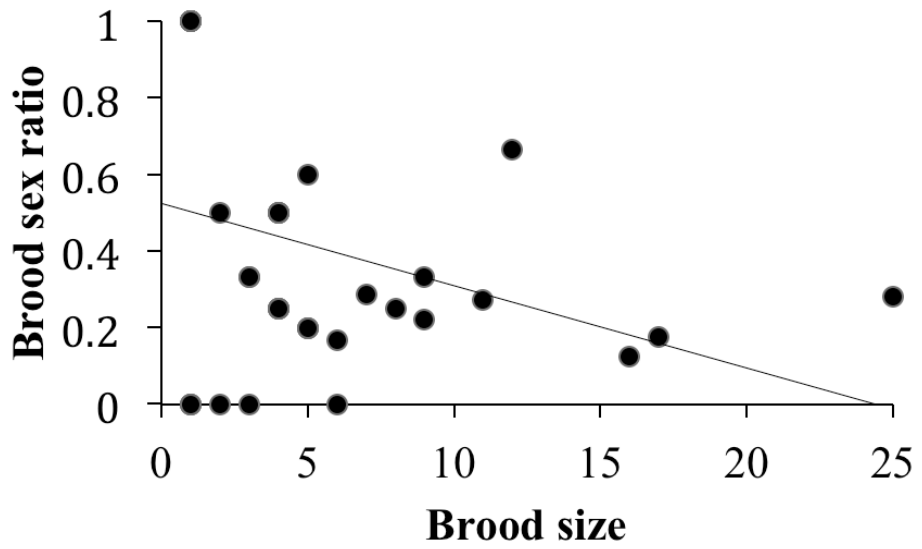
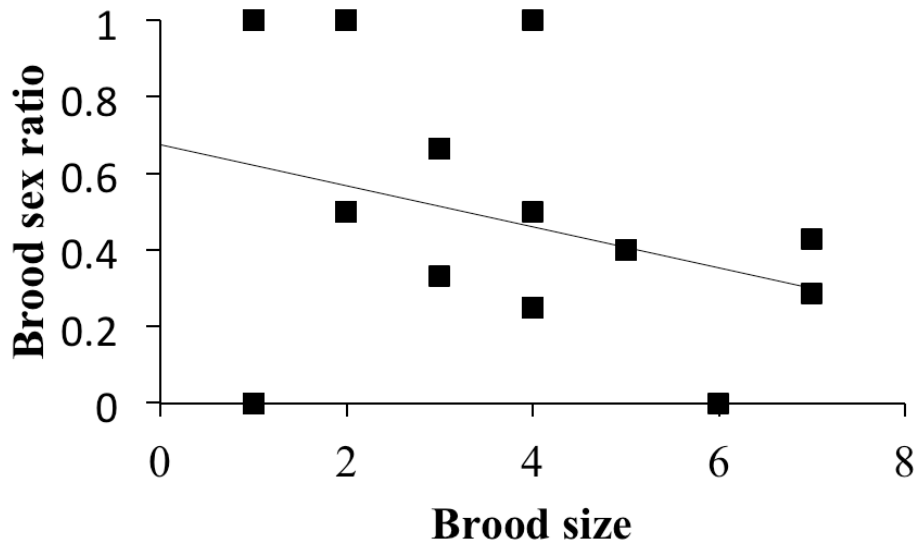
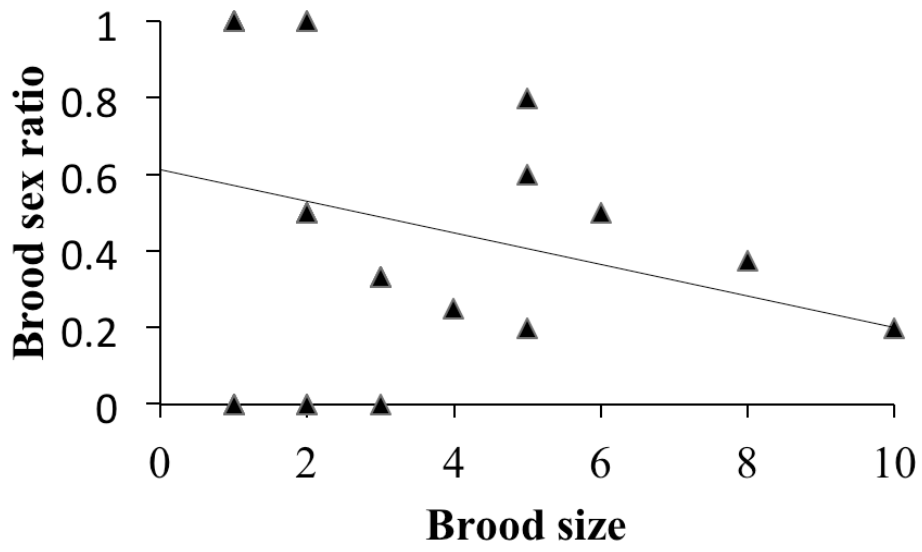
Solitary clutches containing males and gregarious clutches with n-1 females are predictions from theoretical models. A significant ($p < 0.05^*$, 0.01^{**} , 0.001^{***}) excess (*) or deficit (#) of the predicted clutch sex ratio compared to alternatives occurs in some cases.

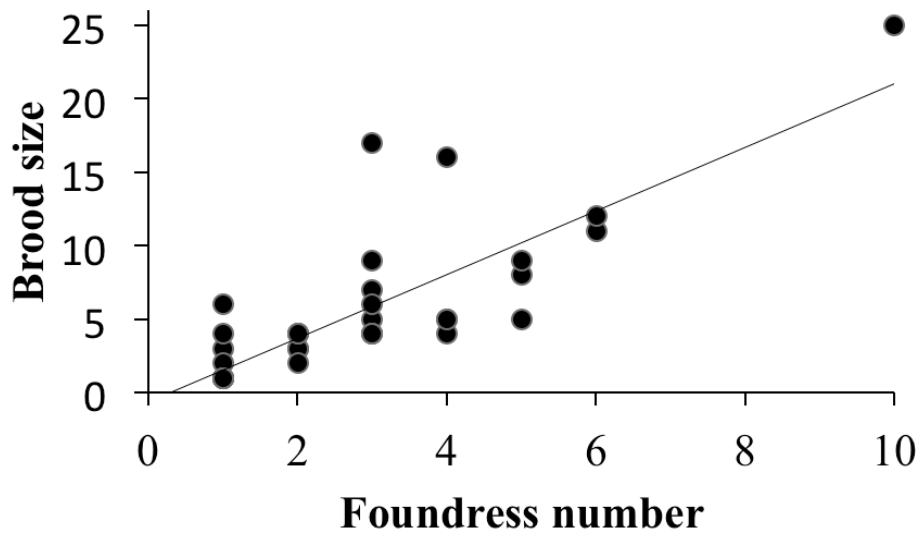
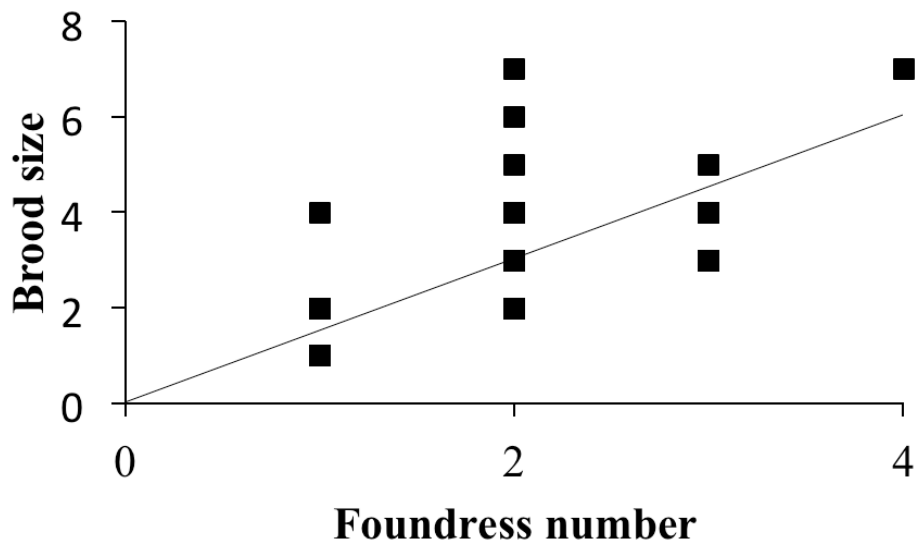
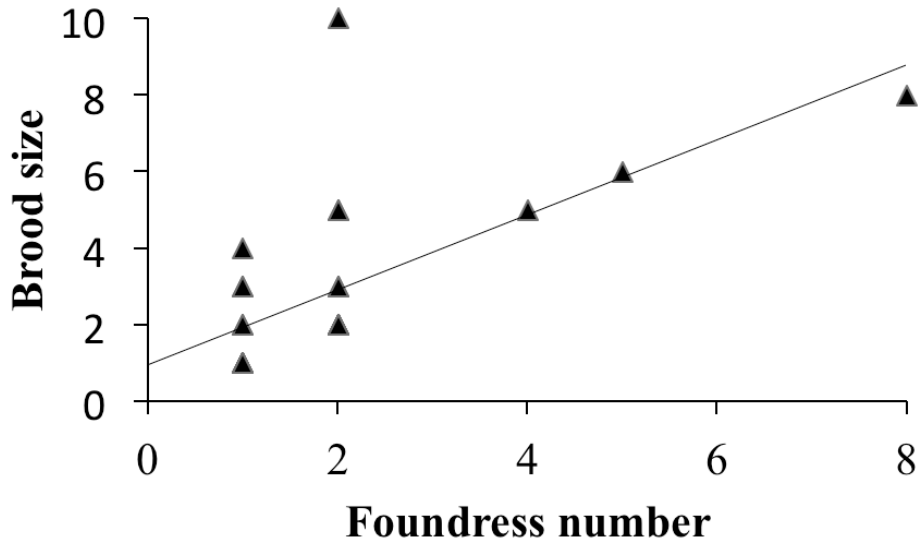
530 Table 3. Comparison of sex ratios (SR) of solitary and gregarious clutches.

$P < 0.05$ (*), 0.001 (***)).

535







Supporting information

This document provides detailed supporting information for the main document.

First, we provide a summary of allelic diversity at the microsatellite loci in Table S1. The individual genotypes for all individuals at all loci are then provided in Table S2. Finally, Table S3 provides the raw data on brood composition (all conspecific wasps in a fig) for all three wasp species and all figs.

Table S1. Allele numbers per locus in the three wasp species

Locus	Sp. B	Sp. C	Sp. A
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

Table S1b then lists all genotypes for all individual wasps on the following 7 pages.

1128	5369	f	218	218	148	148	218	218	199	199	218	225	166	166	153	161	95	95	142	142	1128	1111	C
1128	5370	f	209	217	140	142	218	218	179	179	209	209	168	170	137	139	97	97	128	132	1128	2122	A
1129	5371	m	210	0	152	0	212	0	197	0	222	0	170	0	155	0	93	0	128	0	1129	1107	C
1129	5372	m	206	0	142	0	218	0	181	0	210	0	168	0	139	0	97	0	132	0	1129	2105	A
1129	5373	m	223	0	150	0	220	0	199	0	222	0	168	0	157	0	93	0	146	0	1129	1109	C
1129	5374	f	0	0	142	142	210	218	179	181	0	0	170	178	139	139	97	97	130	138	1129	2130	A
1129	5375	f	214	214	142	142	218	218	181	181	0	0	168	176	139	139	97	97	132	132	1129	2105	A
1129	5376	f	210	218	150	152	212	212	199	203	220	222	170	172	153	155	93	93	128	128	1129	1107	C
1129	5377	f	207	210	142	142	218	218	179	179	209	209	170	170	137	139	97	97	132	136	1129	2121	A
1130	5378	m	208	0	142	0	218	0	181	0	209	0	170	0	137	0	97	0	132	0	1130	2121	A

fig	species	male	female	total	constraint	unmated
1001	A	3	2	5	0	0
1004	A	1	0	1	0	0
1015	A	0	1	1	1	1
1021	A	0	1	1	1	1
1022	A	1	0	1	0	0
1023	A	1	0	1	0	0
1024	A	0	3	3	1	3
1031	A	0	2	2	1	2
1032	A	2	2	4	0	0
1037	A	2	5	7	0	0
1038	A	2	2	4	0	0
1040	A	0	1	1	1	1
1043	A	1	2	3	0	0
1101	A	1	0	1	0	0
1102	A	1	4	5	0	0
1105	A	3	6	9	0	0
1106	A	1	0	1	0	0
1108	A	3	14	17	0	0
1109	A	2	14	16	0	0
1110	A	1	5	6	0	0
1111	A	1	0	1	0	0
1113	A	1	3	4	0	0
1114	A	1	2	3	0	0
1115	A	1	0	1	0	0
1116	A	1	4	5	0	0
1117	A	2	2	4	0	0
1118	A	7	18	25	0	0
1119	A	3	8	11	0	0
1120	A	2	6	8	0	0
1121	A	8	4	12	0	0
1122	A	1	3	4	0	0
1123	A	2	2	4	0	0
1124	A	2	7	9	0	0
1126	A	1	1	2	0	0
1127	A	1	4	5	0	0
1128	A	1	3	4	0	0
1129	A	1	3	4	0	0
1130	A	1	0	1	0	0
1222	A	0	6	6	1	6
Totals		62	140	202	6	14

fig	species	male	female	total	constraint	unmated
1001	B	3	3	6	0	0
1003	B	3	2	5	0	0
1004	B	0	3	3	1	3
1005	B	2	8	10	0	0
1011	B	2	0	2	0	0
1016	B	1	1	2	0	0
1018	B	3	5	8	0	0
1019	B	1	2	3	0	0

1022	B	2	0	2	0	0
1031	B	0	1	1	1	1
1032	B	0	2	2	1	2
1033	B	1	2	3	0	0
1034	B	1	3	4	0	0
1037	B	4	1	5	0	0
1038	B	1	0	1	0	0
1048	B	1	4	5	0	0
1105	B	1	0	1	0	0
1107	B	1	0	1	0	0
1109	B	0	2	2	1	2
1110	B	1	2	3	0	0
1111	B	1	1	2	0	0
1113	B	1	0	1	0	0
1114	B	0	1	1	1	1
1116	B	1	1	2	0	0
1118	B	0	1	1	1	1
1120	B	1	1	2	0	0
1121	B	1	0	1	0	0
1127	B	1	0	1	0	0
1128	B	1	1	2	0	0
Totals		35	47	82	6	10

fig	species	male	female	total	constraint	unmated
1001	C	1	3	4	0	0
1003	C	1	2	3	0	0
1004	C	2	2	4	0	0
1005	C	2	5	7	0	0
1007	C	0	1	1	1	1
1010	C	1	1	2	0	0
1011	C	1	1	2	0	0
1015	C	1	0	1	0	0
1018	C	0	1	1	1	1
1019	C	2	1	3	0	0
1022	C	0	6	6	1	6
1030	C	2	0	2	0	0
1032	C	2	3	5	0	0
1033	C	2	1	3	0	0
1035	C	1	0	1	0	0
1037	C	1	3	4	0	0
1038	C	1	0	1	0	0
1046	C	1	0	1	0	0
1101	C	1	0	1	0	0
1105	C	0	1	1	1	1
1109	C	1	3	4	0	0
1110	C	2	0	2	0	0
1114	C	1	1	2	0	0
1115	C	0	1	1	1	1
1116	C	1	1	2	0	0
1117	C	2	1	3	0	0
1118	C	1	0	1	0	0
1119	C	2	1	3	0	0

1120	C	2	1	3	0	0
1121	C	1	3	4	0	0
1122	C	4	0	4	0	0
1123	C	0	1	1	1	1
1124	C	0	1	1	1	1
1125	C	1	0	1	0	0
1126	C	1	0	1	0	0
1127	C	3	4	7	0	0
1128	C	1	1	2	0	0
1129	C	2	1	3	0	0
1222	C	2	3	5	0	0
Totals		49	54	103	7	12