

Fighting in fig wasps: do males avoid killing brothers or do they never meet them?

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Abstract. 1. In many fig wasp species, armoured wingless males regularly engage in lethal fights for access to females inside figs, which act as discrete mating patches.

2. Kin selection generally opposes killing brothers, because their reproductive success provides indirect genetic benefits (inclusive fitness). However, siblicide may be avoided if (i) brothers do not occur in the same figs, or (ii) males avoid fighting brothers in the same fig. Alternatively, (iii) siblicide may occur because intense mate competition between brothers at the local scale overcomes kin selection effects, or (iv) males do not recognise kin.

3. A fig may also contain wasps from other closely related species and it is not known if males also fight with these individuals.

4. Nine microsatellite loci were used in the first genetic analysis of fighting in fig wasps. We assigned species and sibling identities to males and tested alternative fighting scenarios for three *Sycoscapter* wasp species in figs of *Ficus rubiginosa*.

5. Approximately 60% of figs contained males from more than one *Sycoscapter* species and approximately 80% of fights were between conspecifics, but a surprising 20% were between heterospecific males.

6. Within species, few figs contained brothers, suggesting that females typically lay one son per fig. Overall, most males do not compete with brothers and all fights observed were between unrelated males.

Key words. Competition, fighting, genetics, kin selection, microsatellites, relatedness.

Introduction

Insects, like animals more widely, display an amazing variety of mating systems (Thornhill & Alcock, 1983; Choe & Crespi, 1997; Shuker & Simmons, 2014). To identify drivers of this variation, we need to understand and measure costs and benefits to individuals and also identify constraints to adaptation. Many insects are hard to study in the wild, because of their small size and high mobility. However, fig wasps offer some excellent study opportunities (Cook, 2005) because (i) many species mate inside figs, distinct patches that permit behavioural observations and analysis of entire mating groups; and (ii) each fig species

typically hosts multiple wasp species with diverse mate competition systems (Hamilton, 1979; Cook *et al.*, 1999; Greeff & Ferguson, 1999; Cook, 2005).

Fig wasps have been used extensively to test theories on sex ratios (Herre, 1985; Fellowes *et al.*, 1999; Molbo *et al.*, 2003; Gardner & West, 2004; Greeff & Newman, 2011), alternative mating strategies (Hamilton, 1979; Cook *et al.*, 1997; Pienaar & Greeff, 2003), and male fighting for access to mates (Hamilton, 1979; Murray, 1989; Bean & Cook, 2001). Fatal fighting during mate (or another resource) competition is generally rare in animals, which often have assessment behaviours that help reduce contest escalation (Enquist & Leimar, 1990). However, aggressive fighting is the norm in many non-pollinating fig wasp (NPFW) species, where it has evolved in several different chalcid wasp lineages. Although assessment of rivals has been reported in some *Idarnes* NPFW species (Pereira & do Prado, 2005), it has not been reported in other taxa, and males of

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many species regularly injure or kill rivals for limited mating opportunities inside figs (Hamilton, 1979; Enquist & Leimar, 1990; Cook, 2005). Male fights also occur regularly in a few African fig-pollinating wasp species (Greeff *et al.*, 2003; Nelson & Greeff, 2009), where fighting is common but rarely severe or fatal.

Resource competition theory predicts that fatal fighting should evolve only when the currently contested resource is of greater value than expected future gains (Murray & Gerrard, 1985; Enquist & Leimar, 1990). Fighting males of NPFW species appear to fit this general prediction as they are wingless and most do not disperse and have no mating opportunities beyond the small number of females developing in the same fig (Hamilton, 1979; West *et al.*, 1997, 2001). Indeed, the comparative study of West *et al.* (2001) found that the number of females (potential mates) in a fig was the best predictor of fatal fighting across species with wingless males.

The situation is complicated by kin selection theory (Hamilton, 1964), which predicts that under most circumstances a male should avoid killing his brothers, because their offspring share many of his genes (Hamilton, 1979). However, the new generation of kin selection models predict that kin selection effects can be overcome by resource competition, if this occurs at a local scale so that siblings compete for the same resources (Taylor, 1992; West *et al.*, 2002). Meanwhile, a model of fighting behaviour based closely on fig wasp biology (Reinhold, 2003) predicts that, under some circumstances, males may be selected to direct aggression towards unrelated males, if they compete in patches containing a mixture of brothers and unrelated males.

To date, these theoretical models have been little tested in fighting fig wasps and, in particular, nobody has applied genetic markers to assess relatedness of competing males directly. Instead, either the sex ratio (Murray, 1987, 1989; West *et al.*, 2001) or the number of females laying eggs in a fig (Nelson & Greeff, 2009) has been used as a proxy for the relatedness of competing males. These studies have not found a correlation between relatedness and fighting, but it is unclear if this is a true pattern or not, because the proxies (especially sex ratio) may be poor indicators of underlying genetic relatedness (Nelson & Greeff, 2009). Consequently, there is an urgent need for studies that include direct assessment of relatedness using genetic markers.

An important biological reality with fig wasp systems is that a single fig often contains more than one wasp species with fighting males (Hamilton, 1979; Greeff & Ferguson, 1999; Pereira & do Prado, 2008; Moore *et al.*, 2008). In some cases, there may be fighting males from multiple closely related species in the same fig and this raises the question of whether fights also occur between heterospecific males. In particular, many figs host multiple species of *Sycoscapter* or *Philotrypesis* wasps with fighting males (Cook, 2005; Moore *et al.*, 2008). We suspect that this situation is far more common than realised, as co-occurrence of closely related congeneric fig wasps may only be recognised after genetic analysis (Molbo *et al.*, 2003; Bouteiller-Reuter *et al.*, 2009; Darwell *et al.*, 2014).

Here, we conducted the first genetic analysis of fighting behaviour in fig wasps. We used microsatellite data to determine species identity, identify brothers, and explore patterns of fighting in three closely related *Sycoscapter* species that fight and mate inside the figs of *Ficus rubiginosa* Desf. ex Vent. in Australia. Males of these species engage in fights that may lead to decapitation, limb removal or evisceration of rivals (Moore *et al.*, 2008). We first test the hypothesis that males fight only with conspecifics, despite the presence of close congeners. We then investigate three alternative hypotheses for why kin selection does not prevent fighting within species: (i) brothers do not co-occur in the same figs (Greeff, 1997); (ii) brothers compete in the same patches but only fight unrelated males (Reinhold, 2003), (iii) brothers compete and fight, implying that either (a) local competition negates kin selection (see Taylor, 1992) or (b) males do not discriminate between brothers and non-kin.

Methods

Study species

The three *Sycoscapter* species all develop and mate only in the figs of *F. rubiginosa*. None has a valid formal name and according to Moore *et al.* (2008) we refer to them as species A, B, and C. Females of species A have relatively longer ovipositors (Segar *et al.*, 2014), but we cannot distinguish morphologically between females of B and C. Male morphology often varies considerably within *Sycoscapter* species (Bean & Cook, 2001; Moore *et al.*, 2008) and we cannot reliably identify males of the three species based on morphology. However, all wasps can be readily assigned to species A, B or C using either mtDNA (Moore *et al.*, 2008) or microsatellite markers (Bouteiller-Reuter *et al.*, 2009). There has been no phylogenetic study including these three species and a range of other *Sycoscapter* species, but B and C diverge by only about 6% in cytochrome b and are probably sister species (Darwell, 2012), whereas A differs by about 12% from B or C at this locus but is still closely related to them relative to most other *Sycoscapter* species studied to date (Segar *et al.*, 2012). Most wasps in the genus *Sycoscapter* are probably parasitoids of fig-pollinating wasps. This has been demonstrated directly from the larval-feeding habit in one species (Tzeng *et al.*, 2008) and inferred from statistical patterns in some other species (Segar & Cook, 2012; Suleman *et al.*, 2013), including those studied here (Segar *et al.*, 2014).

Field sampling and fighting observations

We sampled 58 figs, just before the wasps emerged, from trees in Brisbane, Australia between January 2004 and March 2006. Figs were sampled haphazardly, and in succession, as it was only possible to observe the behaviour in one fig at a time. Each fig was carefully sliced open and male behaviour was observed under a dissecting microscope at 10–20× magnification. In all three species, males often fight until serious injury or death (typically through decapitation or evisceration) occurs (Moore

et al., 2008). On the first occasion that we observed a fight leading to visible injury, we captured the pair of fighting males and stored them in a tube of 80% ethanol. We then collected all the other males in the fig and stored them in a second tube, such that each fig studied yielded (i) one pair of fighting males and (ii) a collection of all the other males in the fig. Female numbers were not recorded as we focused on observing male behaviour and allowed females to emerge, mate, and disperse from experimental figs for mate competition to proceed as normally as possible.

Molecular methods

We extracted DNA from the abdomen of each male in a 100- μ l 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 then centrifuged them for 5 min at 3500 g in a standard benchtop microcentrifuge. We amplified nine microsatellite loci (see Table S2) as described in Bouteiller-Reuter *et al.* (2009). We then analysed the samples on an ABI3700 machine and scored fragment sizes using Genescan 3.5 and Genotyper 2.5 software (ABI).

Molecular data analysis

We first used our microsatellite data to identify each male to species and then to identify which conspecific males were brothers. We then combined these two levels of genetic information with our observations of which males had fought and which other males were in the same fig. We first used Structure (Pritchard *et al.*, 2000), which identified three distinct genetic clusters, to assign wasps to species. We then used Colony (Wang, 2004) to identify which males were brothers. Colony places individuals into families (matrilines) using a group-likelihood approach that allows for typing errors in marker data. Genotyping errors can bias 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 certain alleles. Class II errors are stochastic typing errors that can stem from one or more of: mutations, false alleles, miscalling of genotypes, contaminant DNA, and data entry (Wang, 2004).

Fighting analysis

The role of genetic relatedness on fighting was tested at two levels – species and brothers. First, randomisations were used to compare the observed number of fights with heterospecific males with the number expected if rival males were drawn at random (from any of the three species) within each fig. The null distribution (100 000 replicates) was created in R by drawing pairs of males at random from each fig. Second, we performed a similar randomisation test comparing the observed and expected numbers of fights between brothers if rival males were chosen at random within a species. This test was applied only to figs in which (i) two or more brothers co-occurred with at least one

Table 1. Species level fighting patterns across 58 figs.

Species	Males	Figs present	Males/fig	Fights with A	Fights with B	Fights with C
A	178	50	3.56	35	8	1
B	75	28	2.68	8	4	3
C	58	23	2.52	1	3	8

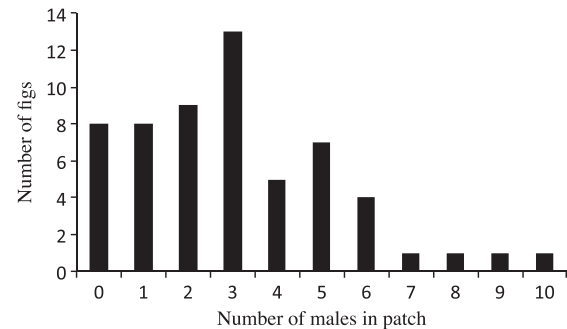


Fig. 1. Variation in the number of *Sycoscapter* A males across figs.

Table 2. Wasp species occurrence across 58 figs.

Species	A	B	C	AB	AC	BC	ABC	Total
Figs	22	1	1	12	7	6	9	58

unrelated male, and (ii) there was a fight between two of these conspecific males.

Results

Species composition and frequency of heterospecific fights

There were 311 *Sycoscapter* males distributed across the 58 figs (Tables 1 and S1). Species A was both the most prevalent (found in 50/58 figs) and most abundant with a mean of 3.56 males per fig (Table 1) and a range of 1–10 (Fig. 1). The mean numbers of males per fig were low for all species, but most figs (34/58) contained two or more species and 9 of these contained all three (Table 2).

Overall, we recorded 59 fights in the 58 figs, because in one fig two fights started simultaneously. Most of these fights (47/59) occurred between conspecifics, despite the fact that most patches contained males of at least two *Sycoscapter* species (Table 2). However, there were still 12 fights between males of different species (Table 1). In eight of these cases, one or more of the males lacked conspecific rivals in the same fig (Table S1).

The overall number of heterospecific fights was significantly less than expected by chance based on our randomisation tests (Table 3). However, when this is broken down to focus on each species individually, the result is highly significant for species C, but not significant for A or B (Table 3). This suggests that the three species differ in their tendencies to engage in fights with

Table 3. Comparing observed and expected numbers of heterospecific fights, based on 100 000 randomisations of the observed data (see text for details).

Focal species	Actual number	Bootstrap mean	<i>P</i>
A	9	13.35	0.124
B	11	11.48	0.498
C	4	13.28	0.001
Pooled	12	19.05	0.021

P shows probability of the actual number or fewer heterospecific fights in the randomisation.

Table 4. Male co-occurrence with brothers and with both brothers and non-siblings (choice).

Species	Total males	Males with brothers	Males with choice
A	178	33	33
B	75	17	17
C	58	15	13

heterospecifics, with C the most and B the least discriminating (Table 3).

Frequency of fights between brothers

Although most fights occurred between conspecific males, we recorded no fights between brothers. A key finding was that only about 21% of males (19%, 23%, and 26% in species A, B, and C) had a brother in the same fig (Table 4). Nearly all of these males also encountered unrelated males, providing a choice of rivals.

In the best-sampled species (A), males had a choice of a brother and an unrelated conspecific rival in 10/35 figs where the fight involved two species A males (an AA fight). In all 10 cases, the AA fight was between unrelated males. The sample size was much lower in the other two species, but the two relevant BB fights and the three relevant CC fights were between unrelated males. Overall, then, there are 15 cases where males had a choice of brother(s) and unrelated male(s) and the observed fight was always between unrelated males (Table S1). In addition, in three more figs, brothers were present but the fight was between heterospecific males (AB, AB, and BC). However, we cannot reject the hypothesis that males fight without regard to relatedness, because simulations show that if males choose rivals randomly, it is still likely that all observed fights will be between unrelated males (Fig. 2). This is true if we restrict the analysis to the 10 cases of AA fights ($P = 0.33$), or pool across species and also analyse the 2 BB and 3 CC cases ($P = 0.17$).

Discussion

We applied genetic markers for the first time in fighting fig wasps to determine the relatedness of pairs of fighting males in three co-habiting *Sycoscapter* species. Overall, most figs contained males from at least two species (Table 2), but typically only

two or three males of any given species (Table S2). Most fights occurred between conspecific males, but a substantial minority of fights (12/59) were between heterospecific males. We found that males of species C were significantly more likely to fight conspecifics than heterospecifics. However, the number of males of species A and B involved in heterospecific fights did not differ significantly from the null expectation based on no species discrimination (Table 3).

The occurrence of escalated mate competition fights between members of different (though closely related) species is surprising, and appears maladaptive. However, existing evidence suggests that *Sycoscapter* A males also make an only limited phenotypic assessment of their conspecific rivals before fighting (Moore *et al.*, 2008). A previous study found that the decision to stop fighting was correlated only with a male's own condition, and not with the morphology or behaviour of its rival (Moore *et al.*, 2008). While it might seem obviously beneficial to first assess a potential rival's species identity (or fighting ability), this depends on the details of fighting behaviour. If a rival can attack and kill a male with relative ease while he is assessing the antagonist, then assessment is costly and may not be favoured.

Most of the heterospecific fights recorded occurred in figs where at least one of the fighters had no conspecific males present (Table S2). Overall, male behaviour appears to be relatively 'hard-wired' (at least in A and B) towards fighting rivals for the very limited mating opportunities in the natal patch. Heterospecific fights could result from mechanistic constraints on accurate recognition of closely related species. Alternatively, if a male can sometimes remove a heterospecific 'rival' with relative impunity, he may benefit from a reduced risk of mating interference or subsequent attack (Reinhold, 2003).

Overall, only about 20% of males occurred in figs with a brother, but nearly all of these also competed with at least one unrelated (conspecific) male. We recorded only the first fight per fig, which was sometimes between heterospecific males, or between conspecific males of another species. Consequently, the final dataset provided only 10 (species A only) or 15 (all species) cases to test whether males avoided fighting brothers. All 15 fights were indeed between unrelated males, consistent with the avoidance of siblicide. However, this result ($P = 0.17$) is also consistent with males choosing rivals randomly (Fig. 2), because most of these figs contained only two brothers but a larger number (up to 8) of unrelated males.

Our results reveal limited power to test if males avoid siblicide (Greeff, 1997; Reinhold, 2003) and this question remains open. However, they also suggest limited scope for kin selection to oppose fatal fighting, because mate competition is largely between unrelated males (Greeff, 1997). Consequently, even if kin selection does oppose siblicide, it may not lead to precise adaptation [see (Herre, 1987) for an analogous case involving sex ratios]. Interestingly, an analogous study of *Melittobia* parasitoid wasps, which have quite similar life histories to *Sycoscapter*, occupying enclosed mating arenas (inside host cocoons) with local mate competition and fighting males, also failed to find evidence of kin discrimination (Innocent *et al.*, 2011). In contrast, a previous (non-genetic) study of some African fig-pollinating wasps suggested that males do fight with brothers (Greeff *et al.*, 2003). However, the fig-pollinating wasps

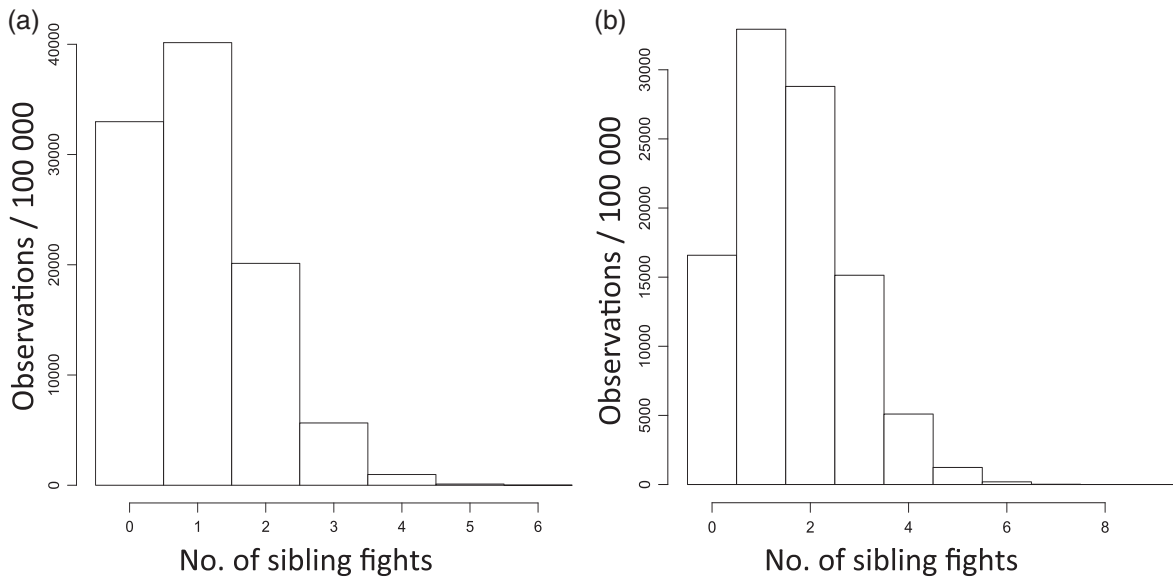


Fig. 2. The number of fights between brothers in simulations of random choice of rivals in figs with both brothers and unrelated males in (a) species A only and (b) in all three species.

(only distantly related to *Sycoscapter*) in that study engage in non-lethal combat that rarely results in injury.

The patterns revealed by our genetic analyses probably reflect a female strategy of laying one son per fig, although developmental mortality could remove co-occurring brothers in some cases (Hardy & Cook, 1995). Mothers might be selected to lay only one son per patch owing to sex ratio selection, with local mate competition (LMC) favouring a female-biased sex ratio and mothers laying few eggs per fig (Greeff, 1997). Indeed, studies of some parasitoid wasps have found that mothers almost always lay only one son, but a larger number of daughters, per insect host (Green *et al.*, 1982; Morgan & Cook, 1994). However, such cases tend to involve species in which most patches contain the offspring of only a single mother (foundress), whereas our data show that most figs contain the sons of multiple mothers. Additional females laying eggs in a patch generally selects for a less female-biased sex ratio (Hamilton, 1967; Herre, 1985), but this could still translate to a single male when clutches are small.

Fatal fighting occurs in many NPFWs (Hamilton, 1979; Murray, 1990; West *et al.*, 2001; Cook, 2005; Pereira & do Prado, 2008), but siblicide is largely avoided if females typically lay only one son per patch. Greeff (1997) argued that this result would be expected if females are selected to optimise both clutch size and sex ratio, and that lack of relatedness between males could facilitate the evolution of fatal fighting. Thus, 'peaceful' LMC and females laying one son per fig might have preceded the evolution of fatal fighting. Across fig-pollinating wasp species, Nelson & Greeff (2009) found that high sex ratios, male dispersal, and fighting were all positively correlated. They concluded that it is unclear which trait has driven these correlations, but favoured the idea that an increase in sex ratio facilitated the subsequent evolution of both male dispersal and fighting.

Comparative studies can provide statistical power to test theory, but often rely on 'secondhand data' that are sub-optimal because the original studies were conducted for other reasons. Previous studies of fig wasp fighting have all used proxies (mainly sex ratio) for relatedness, but clearly these have their limitations (Nelson & Greeff, 2009). In addition, the situation is made worse by the growing number of cases where genetic studies show that what was thought to be one fig wasp species is actually two or more (e.g. Molbo *et al.*, 2003; Darwell *et al.*, 2014). Both sources of error argue for more widespread application of genetic markers.

As only male *Sycoscapter* wasps fight, mothers could minimise siblicide by placing sons in different patches, without doing the same for daughters, and further studies could assess the number of daughters laid by a female in a patch. Another interesting issue is the total number and distribution across figs of offspring from a single mother. Our study was not designed to test this, and it would be challenging using an unmanipulated survey approach, as even a single tree might have hundreds of figs into which a female could lay eggs, and we do not know how much dispersal between trees occurs. Our data do reveal cases where offspring from an individual female were found in up to four different figs (Table S1), but true maxima (or means) could be much higher than this.

Fatal fighting occurs in many *Sycoscapter* species (Bean & Cook, 2001; West *et al.*, 2001; Cook, 2005; Moore *et al.*, 2008), but not all, and future work could also test whether brothers occur in the same patch in related species without fatal fighting. In addition, fatal fighting has evolved independently in other subfamilies of fig wasps (Hamilton, 1979; Pereira & do Prado, 2005) and their patterns of offspring allocation and fighting behaviour deserve further study. In all these cases, molecular markers will be needed to describe natural patterns accurately.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference:

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Table S1. The number and species of wasps found in each of 58 figs, with identification of fighting pairs.

Table S2. Microsatellite genotypes of individual wasps.

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