LETTER

Precision of host sanctions in the fig tree–fig wasp mutualism: consequences for uncooperative symbionts

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Abstract

Host sanctions that reduce the relative fitness of uncooperative symbionts provide a mechanism that can limit cheating and thus stabilise mutualisms over evolutionary timescales. Sanctions have been demonstrated empirically in several mutualisms. However, if multiple individual symbionts interact with each host, the precision with which individual cheating symbionts are targeted by host sanctions is critical to their short- and long-term effectiveness. No previous empirical study has directly addressed this issue. Here, we report the precision of host sanctions in the mutualism between fig trees and their pollinating wasps. Using field experiments and molecular parentage analyses, we show that sanctions in *Ficus nymphaeifolia* act at the level of entire figs (syconia), not at the level of the individual flowers within. Such fig-level sanctions allow uncooperative wasps, which do not bring pollen, to avoid sanctions in figs to which other wasps bring pollen. We discuss the relevance of sanction precision to other mutualisms.

Keywords

Cheating, cooperation, exploitation, *Ficus*, mutualism, resource allocation, sanctions.

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INTRODUCTION

Mutualisms, relationships between two species that benefit both partners, are ubiquitous and of fundamental ecological and evolutionary importance. Nonetheless, the maintenance of mutualisms is not well understood, particularly when cooperation is costly and not merely a cost-free by-product (Trivers 1971; Axelrod & Hamilton 1981; Bull & Rice 1991; Herre *et al.* 1999; Sachs *et al.* 2004; West *et al.* 2007). What prevents beneficial symbionts from becoming parasitic? Individual symbionts that reap the benefits from an interaction without paying the costs are expected to gain a relative fitness advantage, increase in frequency and, over time, break down the mutualism. Therefore, mutualisms with costly cooperation likely require mechanisms that limit cheating (Axelrod & Hamilton 1981; Bull & Rice 1991).

Host sanctions that reduce the relative fitness of less-beneficial symbionts provide one mechanism that can limit cheating. Host sanctions have now been documented in several plant–insect and plant–microbe mutualisms. For example, both yuccas and *Glochidion* can selectively abort fruits that are overexploited by their pollinating moths (Pellmyr & Huth 1994; Goto *et al.* 2010), legumes can selectively withhold resources from those nodules that contain the least productive nitrogen-fixing bacteria (Kiers *et al.* 2003; Simms *et al.* 2006), some host plants selectively allocate less resources to less-beneficial strains of mycorrhizae (Bever *et al.* 2009; Kiers *et al.* 2011b) and fig trees can reduce the reproductive success of fig wasps that do not pollinate their host (Nefdt 1989; Jousselin *et al.* 2003; Tarachai *et al.* 2008; Jandér & Herre 2010). Thus, although the exact physiological mechanisms underlying plant sanctions require further detailed study, host resources seem to be selectively allocated to the tissues and symbiont(s) that most benefit the host. As a by-product, such selective allocation also decreases the relative fitness of uncooperative symbionts and thereby helps prevent their spread through the population (Denison 2000; West *et al.* 2002; but see Akçay & Simms 2011).

The effectiveness of sanctions will greatly depend on how symbionts, and the benefits that they provide, are distributed in time and space, and whether the host can distinguish between, and respond to, the actions of individual symbionts (Bull & Rice 1991; Denison 2000; Simms & Taylor 2002; Bever *et al.* 2009; Friesen & Mathias 2010; Jandér & Herre 2010). Although many theoretical approaches have assumed one-on-one interactions (Trivers 1971; Axelrod & Hamilton 1981; Weyl *et al.* 2010), most well-known mutualisms consist of a relatively large host that interacts with multiple smaller symbionts simultaneously (Kiers *et al.* 2011a). In many cases, the interaction between host and symbionts occur in modules (sensu Weyl *et al.* 2011), such as flowers (yuccas, *Glochidion*), inflorescences (figs) or nodules (legumes). In the simplest case, each host module is occupied by only one symbiont, and modular sanctions will equal individualised sanctions (Fig. 1a). This situation promotes the greatest precision and efficiency of host sanctions. However, if multiple symbionts occupy a host module, and sanctions act at the modular level, then cheating symbionts might partly avoid sanctions by free-riding on other, more beneficial symbionts within the module (Fig. 1b) (Denison 2000; Simms & Taylor 2002; Jandér & Herre 2010). Therefore, when multiple symbionts occupy a host module, sanctions that can target individual symbionts within a host module (Fig. 1b) will be more effective at maintaining cooperation (Friesen & Mathias 2010).

The biology of figs and their pollinating wasps has provided both an inspiration for developing mutualism theory (Axelrod & Hamil-

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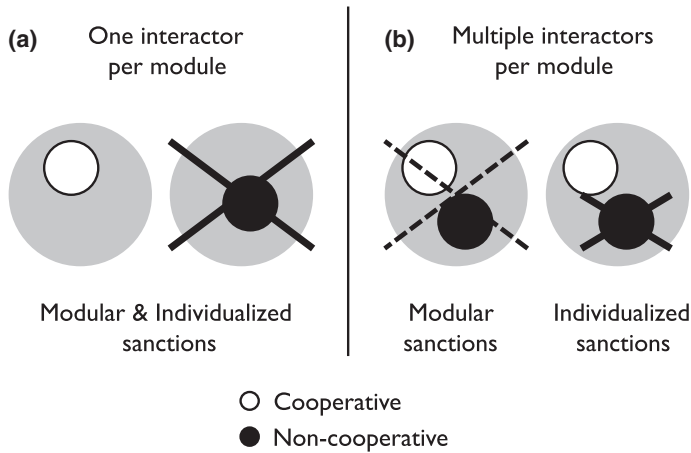
Figure 1 Large grey circles represent host modules (e.g. fig inflorescences or legume nodules), smaller circles represent cooperative (white) or non-cooperative (black) symbiont individuals or strains interacting with each module. The cross represents resource reduction due to host sanctions. (a) When there is only one interactor per module, modular sanctions will always also be individualised sanctions. (b) When there are multiple interactors per module, sanctions can be modular, or individualised.

ton 1981; Bull & Rice 1991) and a useful system for testing it (reviewed in Herre *et al.* 2008; Jandér & Herre 2010). We here investigated the precision of sanctions in the mutualism between fig trees and their pollinating fig wasps, a mutualism in which the lifetime fitness can be quantified for the wasps and wasp cooperation level can be manipulated (Herre 1989; Jandér & Herre 2010). We set up field experiments on *Ficus nymphaeifolia* (Miller 1768; Berg & Villavicencio 2004) to assess the precision of fig sanctions. We introduced either two pollen-free (P−) wasps, two pollen-carrying wasps (P+) or one P− and one P+, into each receptive fig (Fig. 2), then counted and measured the size of the wasp offspring. With these data, we aimed to distinguish among several hypothesised sanction scenarios:
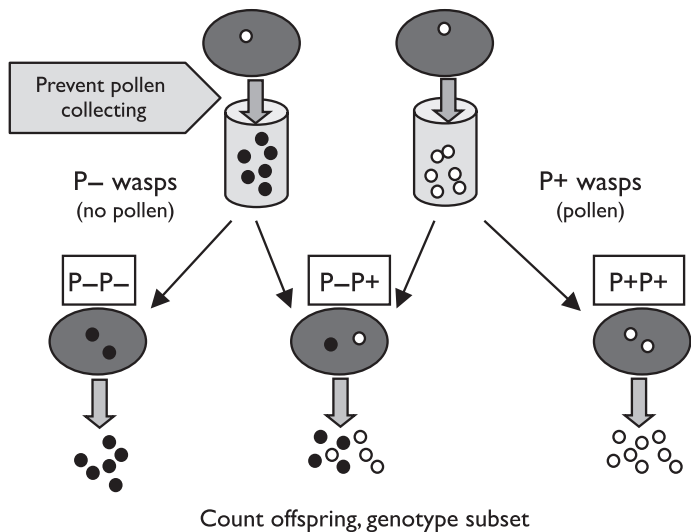
Figure 2 Diagram of the experimental setup. Wasp lineages originated from single-foundress figs, were subjected to either the pollen-free (P−) or control (P+) treatment, and transferred to vials. Two foundresses were introduced into each experimental fig, and the resulting offspring collected when figs had matured.

1A Fig-level sanctions; 1P+ sufficient. Pollination by one wasp ensures sufficient resources for *all* developing wasp larvae in the fig.
1B Fig-level sanctions; pollen-dependent. Resource allocation to the fig increases linearly with pollination level.
2A Flower & nearby level. Sanctions act on the flower level, but without a sharp distinction between pollinated and unpollinated flowers – benefits from pollinated flowers 'leak' to nearby flowers.
2B Flower-level sanctions only. For example, resources might be strictly allocated to pollinated flowers only. From a different perspective, this pattern would also be seen if fertilised flowers (endosperm) provide better larval nourishment than unfertilised flowers (Verkerke 1989).

The four hypotheses generate distinct sets of predictions, detailed in Table 1. Our experiments revealed that fig tree sanctions in *Ficus nymphaeifolia* act on the modular level of entire fig inflorescences, rather than on individual flowers within each fig inflorescence, but individual trees differed in how allocation to a fig varied with pollination level.

METHODS AND STUDY SYSTEM

Study system

Fig trees produce hundreds of tiny flowers inside each hollow inflorescence (formally syconium, hereafter 'fig'). One or several female wasps (foundresses) enter each fig and pollinate and lay eggs in the flowers; each flower can yield either a single wasp or a seed. The fig species that we study here are actively pollinated: wasps actively collect pollen from their natal fig, store it in specialised pockets and deposit it using their front legs (Frank 1984; Jandér & Herre 2010). Although fig wasps of the pollinating species typically carry pollen (P+), a small fraction of individuals in natural populations do not carry pollen (P−) (Jandér & Herre 2010). It is currently unclear if P− wasps derive any benefit from not carrying pollen (Jandér & Herre 2010), but from the tree's perspective these P− wasps clearly are uncooperative. We can manipulate wasps to be artificially pollen free (Jousselin *et al.* 2003; Jandér & Herre 2010).

Fig trees can abort figs or reduce wasp offspring numbers in figs within which wasps have oviposited but not pollinated (Nefdt 1989;

Table 1 Predictions based on the four hypotheses regarding the precision of sanctions. For example, under hypothesis 1A, averaged over many figs, P− and P+ wasps would produce equal numbers of offspring in a P−P+ fig, and on average, a P− wasp would produce more offspring in a P−P+ fig than would a P− wasp in a P−P− fig (assuming that each P− wasp in a P−P− fig produces half the offspring of that fig). The same hypothesis further predicts that, on average, a P+ wasp in a P−P+ fig would produce just as many offspring as a P+ wasp in a P+P+ fig (assuming that each P+ in a P+P+ fig produces half the offspring of that fig)

Hypothesis	Predictions		
	Within P−P+ figs	P− in P−P+ figs	P+ in P−P+ figs
1A Fig level; 1P+ sufficient	P− = P+	> ½ P−P−	= ½ P+P+
1B Fig level; pollen dependent	P− = P+	> ½ P−P−	< ½ P+P+
2A Flower & nearby level	P− < P+	> ½ P−P−	< ½ P+P+
2B Flower level only	P− < P+	= ½ P−P−	= ½ P+P+

Jousselin *et al.* 2003; Tarachai *et al.* 2008; Jandér & Herre 2010). Whereas fig abortions clearly act on the modular (fig) level, offspring reductions might reflect sanctioning of individual flowers and thereby offspring of individual wasps. Reduction of offspring numbers is a significant component of sanctions, responsible for, on average, 52% of the total fitness reduction by sanctions in the studied Panamanian fig species (Jandér & Herre 2010) and 93% in the eight studied Old World fig species (Herre *et al.* 2008; Tarachai *et al.* 2008). Reduction of wasp offspring size might also affect wasp fitness as larger females are more likely to reach receptive figs (Herre 1989).

The studied trees and wasps belong to natural populations in the Barro Colorado Nature Monument, near the Panama Canal, Panama. *Ficus nymphaeifolia* is here pollinated only by *Pegoscapus piceipes*, an active pollinator (Wiebes 1995; Molbo *et al.* 2003). We chose to work with *F. nymphaeifolia* because it 1) has a sufficiently high proportion of single-foundress figs (41%) to produce the P+ and P− wasps needed for the experiments, and 2) shows a sufficiently strong reduction of offspring numbers for us to detect a difference between P+ and P− foundresses in our experiments should sanctions be on the flower level (Jandér & Herre 2010).

Field experiments

Following the methods described in Jandér & Herre (2010), we matched a receptive (flowering) tree with a wasp-producing tree. We collected wasps for the experiments at a stage when females were mated, but still inside their galls. To create P− females, we removed the pollen from half of these figs; the rest of the figs were untouched and females (P+) were allowed to collect pollen naturally (see Appendix S1 in Supporting Information). The manipulation to create P− wasps does not reduce the number of eggs a wasp can carry or lay (Jandér & Herre 2010). Experimental wasps emerging from the same natal fig were collected into a single vial. We then searched each empty fig for the old bodies of wasp foundresses to ensure that experimental wasps originated from figs with a single foundress. Thus, each vial of experimental wasps, either P+ or P−, represented a single maternal lineage, which greatly facilitated the later parentage analyses.

We prevented uncontrolled pollination of pre-receptive experimental figs by enclosing branches in mesh bags. When experimental figs became receptive, each was randomly assigned to one of three wasp treatments (Fig. 2): (1) two P− wasps, (2) one P− and one P+ wasp or (3) two P+ wasps. There are typically one to three foundresses per fig in this species, so introducing two experimental foundresses is well within the normal range (Herre 1989). All experimental figs were standardised for size. The second wasp was introduced 5–10 min after the first one had completely disappeared through the ostiole. In the P−P+ figs, we alternated which type of wasp was the first to enter. Each vial of experimental wasps was used for setting up a maximum of two figs of each type, and the remaining wasps (full sisters of the experimental foundresses) were stored in 70% ethanol to later reconstruct and confirm maternal lineages. After wasp introductions, we re-bagged the branches and monitored the experimental figs for abortions. We replicated the experiment on two different trees, separated in time by a week, and in space by about 1 km; on one of the trees (BCI#1), the sample size was small as only a few of the figs were accessible.

At the end of the experiments, we collected the experimental figs just before wasps emerged, and allowed wasps to emerge within enclosed Petri dishes (Appendix S1). We immediately placed a subset (> 50 when possible) of male and female wasp offspring in ethanol, and froze the rest of the fig contents for later dissections to determine seed number and the total number and sex of wasps emerging from each fig. Very few experimental figs aborted; only figs that matured and were successfully treated were included in this study (Table S1, Appendix S1). Some figs ripened earlier than expected, allowing some wasps to leave before collection; in these, we quantified the total number of wasp offspring by counting the number of empty wasp galls, but could determine neither the sex nor maternal lineage of the offspring that had escaped.

We sorted wasp offspring from P−P+ figs into their maternal lineages using molecular markers (see below). In each of the 28 figs for which the P−P+ treatment was successful, we aimed to genotype 20 female offspring and 20 male offspring. In a few of these figs, we could not reach this goal, either because wasps had emerged early (three figs), or because the fig contained fewer than 20 males (seven figs). Before DNA extractions, we measured each wasp under a dissecting scope with an ocular micrometer to the nearest micrometre; we measured femur length of the front leg of females and thorax length of males. We calculated the mean femur and thorax length for each maternal lineage in each fig, and compared the means of P− and P+ offspring in each fig using paired t-tests, with a separate test for each sex.

Molecular sorting of offspring into maternal lineages

We first sequenced up to 886 base pairs of the cytochrome oxidase subunit I (COI) mitochondrial gene on full sisters of the experimental foundresses to identify the mitochondrial haplotype of the maternal lineage in each vial. DNA extractions followed Molbo *et al.* (2002). Primer and PCR details are given in Appendix S1. Sequences were compared using Sequencher® software (Gene Codes Corporation, Ann Arbor, MI, USA) and haplotypes assigned to each maternal lineage. Mitochondrial COI haplotypes of the two foundresses differed in 19 of the 28 P−P+ figs. From each of these 19 figs, we attempted to assign 20 randomly chosen female and 20 randomly chosen male offspring to maternal lineage by sequencing the COI gene, as described above. Sequences were successfully obtained for 339 female offspring from 19 figs, with 20 females from each of all but three figs. All sequenced females could be unambiguously assigned to one of the two maternal lineages in each fig. Sequence quality from the male offspring was frequently poor, and could not be used to assign males to maternal lineages.

To assign male offspring to maternal lineages, and to distinguish between maternal lineages within each of the figs whose foundresses shared COI haplotypes (9 of 28 figs), we used three microsatellite loci that previously had been developed for this species: Pe77, Pe91 and Pe99 (Molbo *et al.* 2002). We determined fragment sizes using LIZ600® (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 96 capillary 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at UC Berkeley sequencing facilities, and scored the peaks using Peakscanner® software (Applied Biosystems, Foster City, CA, USA). We obtained 16 scorable alleles at the Pe77 locus, 11 at the Pe91 locus and 19 at the Pe99 locus. The family produced by a singly mated haplodiploid foundress can receive up to three different alleles at each locus (two from her and one from her haploid mate). We identified the alleles belonging to each maternal lineage by genotyping either five of each foundress's

female offspring (when they could be identified by their COI haplotypes) or six of her sisters (when foundresses had identical haplotypes). Within each fig, private wasp alleles were available for at least one of the microsatellite loci, often two, which made manually sorting the offspring into maternal lineages straightforward. Only two males, from two different figs, could not be assigned to a specific maternal lineage; in those two figs, we based our analyses on the remaining 19 males.

Calculations and statistical methods

For each P−P+ fig, we used the 40 genotyped offspring to estimate what proportion of the female and male offspring had originated from each foundress (P− vs. P+). We used this information to estimate the total number of offspring produced by each foundress in the P−P+ figs. For each P−P+ fig in which all offspring had been counted and sexed, we estimated the overall number of offspring produced by the P− foundress: $P-_{P-P+} = r_F \times F + r_M \times M$ (equation 1), where, for each fig, F = the total number of female offspring, M = the total number of male offspring, r_F = the proportion of female offspring belonging to the P− foundress in the genotyped subset and r_M = the proportion of male offspring belonging to the P− foundress in the genotyped subset. We assumed that the genotyped subset accurately represented the total offspring population of each sex. We then compared the average of this value ($P-_{P-P+}$) with the average number of offspring a P− foundress produced in a P−P− fig ($P-_{P-P-} = 0.5 \times F + 0.5 \times M$). The complement of the progeny in each P−P+ fig was produced by the P+ foundress ($P+_{P-P+}$); we compared the average of this value with the average number of offspring a P+ foundress produced in a P+P+ fig ($P+_{P+P+} = 0.5 \times F + 0.5 \times M$). We here assumed that each of the two foundresses in P−P− and P+P+ figs contributed half of the total offspring in each fig. To estimate the progeny sex ratios in the P−P+ figs that ripened early and for which we only had counts of empty galls, we used different equations described in Appendix S1.

To test whether the proportion of offspring from P− foundresses in P−P+ figs was different from that originating from the P+ foundresses in those figs, we used R version 2.11 (http://CRAN.R-project.org/, Vienna, Austria) to perform randomisation tests. Male and female offspring were analysed separately. For each tree, the proportion of males or females originating from the P− foundress was randomly switched with that originating from the P+ foundress in the same fig, and iterated 10 000 times to create a distribution as would be seen if there had been no difference between P− and P+ foundresses in their contribution to the brood. For each tree, the mean proportion from the original data was then compared with the created distribution to obtain a P-value. For comparison, one-sample t-tests comparing the proportion P− males or females against the value of 0.5 gave almost identical results, but are not ideal to use as the data are proportions. All other statistical analyses were performed using SPSS 17.0 (IBM, Armonk, NY, USA).

RESULTS

The effect of treatment on seed number

As expected, the number of seeds varied across the experimental groups (Fig. 3). The included P−P− figs on both trees did not contain any seeds, except two figs that contained three and one seeds
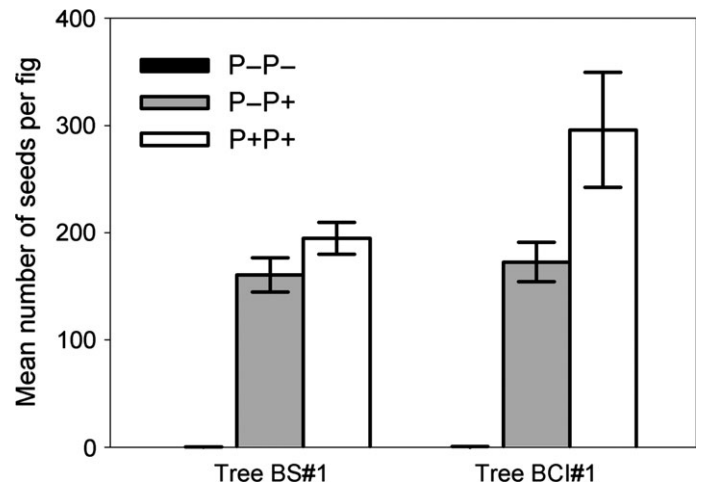

Figure 3 Mean number of seeds in P−P−, P−P+ and P+P+ figs from trees BS#1 and BCI#1. P−P− bars are very close to zero. Error bars represent 1 SEM.

respectively (tree BS#1: $n = 21$, mean = 0.14 seeds, SEM = 0.14; tree BCI#1: $n = 3$, mean = 0.33, SEM = 0.33; Fig. 3); excluding these two figs did not change the results. As expected, adding one pollinator raised seed number dramatically and adding a second pollinator further increased seed number (Fig. 3). The P+P+ figs contained significantly more seeds than P−P+ figs on tree BCI#1 (P+P+: $n = 4$; P−P+: $n = 5$; t-test, $t_7 = -2.39$, $P = 0.048$), with a non-significant trend in the same direction on tree BS#1 (P+P+: $n = 17$; P−P+: $n = 23$; t-test, $t_{38} = -1.52$, $P = 0.14$).

Partition of offspring within P−P+ figs

On average, at least half the offspring within P−P+ figs originated from the P− foundress (Fig. 4). On neither tree did P− and P+ foundresses differ significantly in the proportion of offspring they contributed to each brood (randomisation tests: Tree BS#1 female offspring: $P = 0.98$, male offspring: $P = 0.99$; Tree BCI#1 female offspring: $P = 0.12$; male offspring: $P = 0.50$). Furthermore, P−
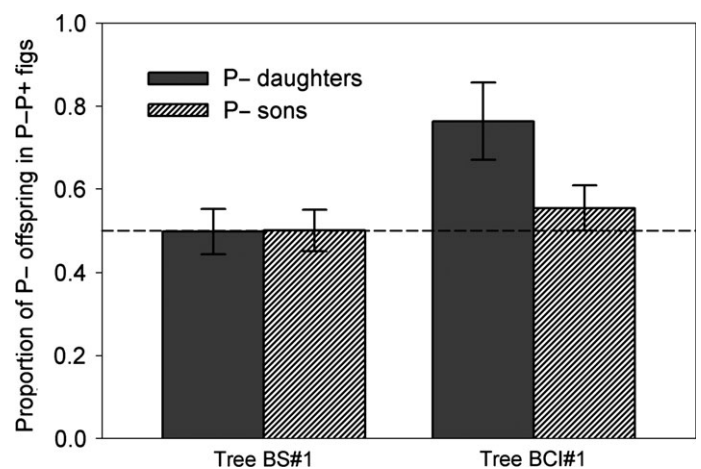

Figure 4 The mean proportion of female and male offspring that belonged to the P− foundress in P−P+ figs did not significantly differ from 0.5 (dotted reference line) on the two experimental trees. Error bars represent 1 SEM.

offspring were not significantly smaller than were P+ offspring from the same fig. Both P− and P+ females had a mean femur length of 2.15 mm, SEM 0.02 (paired t-test, $t_{24} = −0.34$, $P = 0.74$). Mean thorax length of P− males was 6.30 mm (SEM 0.03) as compared to 6.37 mm (SEM 0.04) on P+ males (paired t-test, $t_{25} = −1.8$, $P = 0.08$).

Offspring production per foundress depending on which kinds of foundresses share the fig

On each experimental tree, P− foundresses produced significantly more offspring when sharing a fig with a P+ foundress than when sharing a fig with another P− foundress (Tree BS#1: unequal variances t-test, $t_{30.3} = −4.78$, $P = 4.2 \times 10^{-5}$; Tree BCI#1: t-test, $t_6 = −3.23$, $P = 0.018$; Fig. 5). Furthermore, on tree BCI#1, P+ foundresses produced significantly more offspring when sharing a fig with another P+ foundress, than when sharing a fig with a P− foundress (t-test, $t_7 = −2.94$, $P = 0.022$; Fig. 5b). However, on tree BS#1, P+ foundresses did not produce significantly more offspring in P+P+ figs than in P−P+ figs (t-test, $t_{38} = −0.59$, $P = 0.56$; Fig. 5a). Results did not change if we instead used t-tests paired by maternal linage to compare the mean offspring number (for P− and P+ foundresses respectively) in figs shared with a P− or P+ foundress.
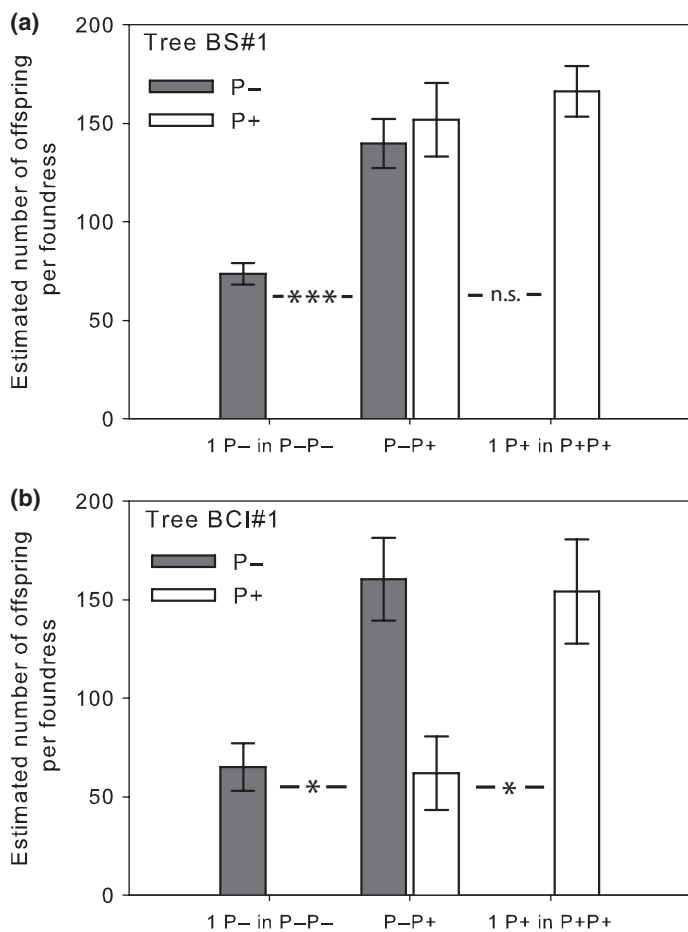
Figure 5 The mean estimated number of offspring that a foundress produced in the different types of figs on trees (a) BS#1 and (b) BCI#1. Error bars represent 1 SEM. *$P < 0.05$; ***$P < 0.001$.

DISCUSSION

To our knowledge, this study is the first to unambiguously determine the precision of host sanctions in a mutualism. We found that sanctions in *Ficus nymphaeifolia* act on the level of entire figs rather than on individual flowers inside each fig. Such modular sanctions are less precise than individualised sanctions, and enable a pollen-free wasp to free ride on cohabiting pollinating foundresses. The pollen-free wasp can enjoy the benefits of pollination by the pollinating foundress(es) without herself paying any possible cost of pollination. This finding is consistent with the observation that there is a low, but persistent number of pollen-free wasp individuals naturally occurring in the Panama populations of *Pegoscapus piceipes* that we studied (Jandér & Herre 2010).

Modular sanctions in figs, and their consequences

There was no support for the hypothesis that sanctions act on the flower level (hypotheses 2A & 2B, Table 1), which predicts fewer or smaller offspring from P− wasps than from P+ wasps in P−P+ figs. Instead, within the P−P+ figs, P− wasps produced equal (tree BS#1) or a tendency to more (tree BCI#1) offspring than did P+ wasps. The most likely explanation for this result is that sanctions act on the fig level. We do not consider lateral growth of pollen tubes (Jousselin & Kjellberg 2001) or preferential P− oviposition in already pollinated flowers likely explanations for the observed results, because either mechanism would restrict the number of flowers available for P− wasps and thereby make them unlikely to contribute 50% or more of the offspring.

Previous experiments with only a single P− or P+ wasp in each fig (Galil & Eisikowitch 1971; Nefdt 1989; Jousselin & Kjellberg 2001; Jousselin *et al.* 2003; Tarachai *et al.* 2008; Jandér & Herre 2010) have been unable to distinguish between the hypotheses that the reduced offspring numbers in unpollinated figs are due to (i) unfertilised flowers being poorer food for growing wasp larvae (lack of endosperm) (Verkerke 1989), (ii) unfertilised flowers are less likely to successfully transform into wasp galls (Jousselin *et al.* 2003) or (iii) figs without seeds receive less resources from the tree, and thereby less resources for the growing wasp larvae. By showing that offspring-reducing sanctions do not act at the flower level, our results contradict for this species both the hypothesis that fig wasp larvae would receive better nutrition from endosperm (fertilised flower structures) than from unfertilised flowers, and the hypothesis that fertilisation increases the likelihood of successful gall formation.

Consistent with the outcome when single P− or P+ wasps were introduced into figs (Jandér & Herre 2010), foundresses in figs with some pollination (P−P+ and P+P+) produced many more offspring than foundresses in completely unpollinated figs. The most likely reason for this observation is that resources were allocated to entire figs based on the overall pollination level, and the developing offspring in pollinated figs therefore had access to a larger resource pool than did those in P−P− figs. In the larger experiment, on tree BS#1, P+ wasps in P+P+ figs did not produce significantly more offspring than P+ wasps in P−P+ figs, which suggests that one pollinator inside a fig is sufficient to avoid sanctions (hypothesis 1A). However, on this experimental tree, the average number of seeds in a P+P+ fig was marginally but not significantly larger than that in a P−P+ fig. Therefore, even if hypothesis 1B were true, we could not necessarily expect P+ foundresses in P+P+ figs to have significantly

more offspring than would P+ foundresses in P−P+ figs on this tree. The smaller experiment, on tree BCI#1, showed a different pattern, which was partly driven by two P−P+ figs that had very high proportions of P− female offspring (95% and 100% respectively). This does not seem to be a maternal lineage effect, as these lineages each produced less-skewed offspring ratios in other experimental figs on the same tree. Although naturally occurring P− foundresses could be expected to obtain some benefit from not collecting, carrying and depositing pollen [saving energy or time; these wasps are typically not limited by egg numbers and use all their live time inside a fig ovipositing and pollinating (Jandér unpublished)], most of those benefits would not be realised in artificially produced P− wasps such as were used in these experiments. With the two trees showing somewhat different patterns, we cannot distinguish between hypotheses 1A and 1B with the current dataset. Nonetheless, a reasonable interpretation is that the pollination accomplished by one foundress might not be sufficient to completely avoid sanctions in this species, but only slightly more resources are added in response to the pollination efforts of a second P+ wasp. Different fig species are likely to activate sanctions at different thresholds, which might depend on factors such as fig size, the relative cost of mistakenly aborting a beneficial fig and resource availability (Herre 1989, 1996; Jandér & Herre 2010; see also Grman 2012).

We propose that the absence of flower-level sanctions by *F. nymphaeifolia* can be extrapolated to other closely related fig species. All studied Panamanian fig species in the actively pollinated Urostigma group show the same type of sanctions, differing only by degree: they all abort a proportion of unpollinated figs and reduce wasp offspring numbers in unpollinated figs that mature (Jandér & Herre 2010). To pick a specific example that usefully illustrates the consequences of modular-level sanctions, we choose the closely related *F. nymphaeifolia* and *F. popenoei* (Machado *et al.* 2005; Jackson *et al.* 2008). In *F. nymphaeifolia*, naturally occurring pollen-free wasps (NP-) are very rare, comprising only 0.3% of the population. Although 76% of wasps associated with *F. nymphaeifolia* end up in figs with more than one foundress, 24% of wasps occupy single-foundress figs where wasps that do not pollinate suffer strong sanctions (Jandér & Herre 2010). In contrast, in *F. popenoei,* only 7% of wasps end up in single foundress figs and sanctions against wasps that do not pollinate are milder (Jandér & Herre 2010). The remaining 93% of wasps end up in figs with more than one foundress. Thus, although NP- wasps are considerably more common in *F. popenoei* (5% of the wasp population), they would frequently escape modular sanctions. If carrying pollen and pollinating actively entails fitness costs, free riders might spread in the population until the benefits of not pollinating are balanced by the costs of sanctions [the likelihood of experiencing sanctions increases as the proportion of pollen-free wasps in the population increases, (Jandér unpublished)].

The precision of sanctions in other mutualisms

Although host sanctions have been described in other mutualistic systems (Pellmyr & Huth 1994; Jousselin *et al.* 2003; Kiers *et al.* 2003; Simms *et al.* 2006; Tarachai *et al.* 2008; Bever *et al.* 2009; Goto *et al.* 2010; Jandér & Herre 2010; Sachs *et al.* 2010), few have attempted to study the precision of sanctions. In the majority of fig species, more than one female wasp routinely enter each fig (Herre 1989; also inferred from flower numbers in Kjellberg *et al.* 2001), and more than one moth commonly oviposits in each individual

flower in yuccas and *Glochidion* (Pellmyr & Leebens-Mack 2000; Kato *et al.* 2003). In legumes, multiple bacterial strains per nodule occur in 7–74% of nodules under laboratory conditions (Denison 2000; Gage 2002; Sachs *et al.* 2010). Therefore, the precision of sanctions is an important question in many well-studied mutualisms, and probably also for many less-studied mutualisms.

In mutualisms between plants and nutrient-supplying root symbionts such as mycorrhizae and nitrogen-fixing rhizobia, plants would theoretically benefit from being able to impose individualised sanctions (Denison 2000; Bever *et al.* 2009; Friesen & Mathias 2010). Although legumes are known to have modular nodule-level sanctioning (Kiers *et al.* 2003; Simms *et al.* 2006; Sachs *et al.* 2010), a single nodule can be infected by multiple rhizobial strains (Denison 2000; Gage 2002; Sachs *et al.* 2010), rendering nodule-level sanctioning less effective (Denison 2000; Friesen & Mathias 2010). A recent empirical study (Sachs *et al.* 2010) used techniques that could distinguish between nodular and individualised sanctions in legumes, but did not present data in a way that clearly addressed this question. Two experimental studies that examined the scale of plant sanctions in response to infection by different mycorrhizal fungi yielded different results. In one study, *Allium* plants could selectively allocate resources to more beneficial mycorrhizal fungi when fungal populations were clearly separated within the root system but not when fungal populations were well mixed (Bever *et al.* 2009). Plants in that study therefore could not control resource allocation on the scale of individual arbuscules, but it is not clear whether plants allocated resources on the scale of either whole roots or rootlets (Bever *et al.* 2009). In contrast, another experiment found that *Medicago* plants selectively allocated carbon to the more beneficial mycorrhizal strain, even when strains were well mixed in the soil, suggesting allocation on a very fine scale (Kiers *et al.* 2011b). Because these studies compared allocation by a plant to different symbiont species that might differ in many traits other than benefit to host, it is hard to tease apart inherent reasons for differing symbiont growth (for example differing competitive abilities) from those caused by selective plant allocation. This problem could be circumvented by experimentally manipulating individuals of a single symbiont genotype or species to be either cooperative or uncooperative, as was done in this study and by Kiers *et al.* (2003), but this method is not easily available in all systems.

An interesting situation is found in the more complex behavioural mutualism between reef fish clients and cleaner fish. Cleaners of the species *Labroides dimidiatus* often work in pairs, and although it is theoretically possible that a cheated client could punish (chase) cheating cleaners individually, in practice a cheated client simply leaves the cleaning station (equivalent of modular sanctions) (Bshary *et al.* 2008). Interestingly, pairs of cleaners cheat less often than cleaners that work alone, seemingly because the male in the pair often punishes the female if she cheats (Bshary *et al.* 2008). Thus, in this system, the fellow cleaner, rather than the host, imposes the equivalent of individualised sanctions.

It seems likely that hosts in different mutualistic partnerships would differ in the precision of their sanctions, balancing the costs associated with more precise sanctions against their benefits. In mutualisms where hosts interact with multiple partners simultaneously, the host will theoretically always benefit from more precise sanctions (Friesen & Mathias 2010). However, if increasing sanctioning precision is costly, important factors affecting its evolution will likely include the frequency of mixed interactions, the frequency

and magnitude of cheating and the relative cost of less precise (modular) sanctioning alternatives. Our study determined the precision of host sanctions in a fig species, which has direct implications for the fitness costs experienced by uncooperative pollinators and therefore on the effectiveness of fig sanctions. Determining the precision of sanctions in other mutualistic systems would allow better understanding of sanction effectiveness and therefore of mutualism maintenance.

AUTHOR CONTRIBUTION

KCJ conceived and designed the study, performed field experiments and laboratory work, analysed data and was lead author. EAH provided primers and advice. ELS advised on design and molecular work. All authors contributed to revisions.

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Appendix S1

Methods

Field experiment: P- and P+ wasps
To create P- wasps, we collected figs at the stage when male wasps had emerged from their galls, and were mating with the females that were still inside their galls; all wasps were still inside the fig. Figs that were assigned to the P- treatment were divided into quarters with a scalpel, taking care not to damage any wasp galls. Male flowers (containing pollen) were quickly removed with fine forceps under a dissecting scope, and the fig quarters placed in a vial. Thus, when P- females emerged from their galls, there was no pollen to collect. Figs that were assigned to the P+ treatment were placed into vials untouched, consequently P+ females collected pollen from their fig as they normally would. We have previously shown that the P- treatment does not negatively affect the wasps' ability to lay eggs (Jandér and Herre 2010).

Field experiment: collecting experimental figs
We visited the experimental trees regularly during the duration of the experiment to collect any aborted figs (Table S1). As figs were approaching maturation, we visited the trees once or twice daily to check the maturity level of figs. We collected figs when males were out of their galls and mating with the females, typically 12-24 hours before wasps would exit the fig naturally. In a few cases we underestimated the maturity level of the figs, so some wasps had already left the fig when we collected it; see details in the next paragraph for the calculations we did to retain these figs in the study.

Estimating males and females in figs where we only had total offspring numbers
In the P-P+ figs we needed to know the offspring sex ratio to be able to estimate how many offspring belonged to each foundress. To estimate the progeny sex ratios in the P-P+ figs for which we only had counts of empty galls (Table S1), we averaged the proportions observed in the 59 figs from which all offspring had been sexed, 0.87 (s.e.m. = 0.007) female and 0.13 (s.e.m. = 0.007) male. We then used this average sex ratio to estimate how many offspring were produced by the P- and P+ foundresses of these early-ripened figs: $\text{P-}_{\text{P-P+}} = 0.87 \times T \times r_F + 0.13 \times T \times r_M$ (equation 2), and $\text{P+}_{\text{P-P+}} = T - \text{P-}_{\text{P-P+}}$ (equation 3), where, for each fig, T = the total number of offspring (quantified as the number of empty galls), r_F = the proportion of female offspring belonging to the P- foundress in the genotyped subset, and r_M = the proportion of male offspring belonging to the P- foundress in the genotyped subset. When equation 2 was applied to figs for which the exact numbers of male and female offspring were known, it produced very similar estimates of total number of offspring per fig as did equation 1, differing on average 0.1% and at most 3%. Excluding these early-ripened P-P+ figs from the analyses produced very similar results for tree BS#1, and results in the same direction, but non-significant due to the then very small sample size, for tree BCI#1.

Table S1. Initial and final sample size on the two experimental trees.

Tree	Treatment	Initial sample size	Excluded from analyses					Included in analyses
			Pollen treatment failure[1]	Foundress number failure[2]	Aborted due to herbivory[3]	Successful treatment		
						Aborted[4]	Matured[5]	
BCI#1	P-P-	4	0	0	0	1	3 (1)	
	P-P+	6	0	1	0	0	5 (3)	
	P+P+	4	0	0	0	0	4	
BS#1	P-P-	28	3	2	2	0	21 (4)	
	P-P+	35	2	5	3	2	23 (2)	
	P+P+	21	1	1	1	1	17 (4)	

[1]Pollen treatment failure here means that there were more than a few seeds in P-P- figs, or no seeds in P-P+ or P+P+ figs. We included in the analyses two P-P- figs that contained very few seeds (1 and 3 seeds respectively; less than 2% of the mean number of seeds in the P-P+ treatment); excluding these two figs from the analyses did not change the results. The three P-P- figs that had more than a few seeds were excluded from the study. A P-P- fig might have seeds because an unintended pollen-carrying wasp entered the fig without us noting it, or because the pollen-removal treatment had been incomplete in that wasp lineage. As a conservative test, we excluded all P-P+ figs that originated from the same P- wasp lineages as the failed P-P- figs, but, as this did not change the results, we kept these P-P+ figs in the study.

[2]Upon maturation, the number of foundresses was checked in all figs; only figs with two foundresses inside were included in the analyses. Failure here meant that there were one or three foundresses inside the fig lumen. When only one foundress was inside the lumen, the other was often stuck facing inwards in the entrance (ostiole). In the P-P+ figs that were included in the study and thus analyzed genetically, all offspring were of the expected two genetic lineages (based on full sisters of the introduced foundresses), so we are confident that the analyzed P-P+ figs contained only the intended wasps.

[3]Some figs were attacked by herbivorous caterpillars and aborted.

[4]These are abortions where there were two foundresses inside the fig, but no herbivory or other fig damage. We interpret the relative absence of such abortions in P-P- figs (compared to previous experiments on these very trees, in which up to 60% of single foundress P- figs aborted) to suggest that the presence of wasp larvae helps prevent abortions; a study of this hypothesis is underway.

[5]These are the sample sizes that went into the analyses reported here. The number in the parenthesis is the number of figs in which we counted empty wasp galls rather than wasps, either because these figs ripened early, or because some wasps, but not figs, were damaged in frozen international transit. Equations 2 and 3 were used to estimate counts of wasp progeny in these P-P+ figs; analyses of P-P- and P+P+ figs were not affected.

DNA extractions, sequencing primers and PCR

We extracted DNA from individual wasps using the Puregene® DNA isolation kit (Gentra), with the modified *Drosophila* protocol described in (Molbo *et al.* 2002). We used the previously designed primer pair Jerry (5'-CAA CAT TTA TTT TGA TTT TTT GG-3') (Simon *et al.* 1994) and Georgina (5'-CGD GGT ATH CCD GCT AAW CCT A-3') (Machado, C. pers. com.), product size 493 basepairs, and also developed a new primer pair, Alphonse (5'-TGG GTG CTG TTT ATG CAA TTT-3') and Maisy (5'-AAA ACC TCC ACC AGG AAC AG-3'), product size 432 basepairs, overlapping 133 bp with the previous product. The PCR mix consisted of 5.6µl DNAse-free H_2O, 1µl 10x buffer, 0.5µl dNTPs, 0.3µl 50 mM $MgCl_2$, 1.25µl BSA, 0.125µl 10µM forward primer, 0.125µl 10µM reverse primer, 0.1µl Platinum® Taq

DNA polymerase, and 1µl DNA, for a total of 10µl per sample. The PCR protocol for Jerry-Georgina was 94 °C for 2 min, 10 cycles of denaturation (94 °C, 30 s), annealing (46 °C, 30 sec), and extension (72 °C, 30 s), then 30 cycles of denaturation (94 °C, 30 s), annealing (48 °C, 30 sec), and extension (72 °C, 30 s), followed by a final extension (72 °C, 10 min). The PCR protocol for Alphonse - Maisy was 94 °C for 2 min, 30 cycles of denaturation (94 °C, 30 s), annealing (53 °C, 40 sec), and extension (72 °C, 40 s), followed by a final extension (72 °C, 10 min). PCR fragments were sequenced in both directions using an Applied Biosystems 96 capillary 3730xl DNA Analyzer at UC Berkeley sequencing facilities.

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