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Original article

Relative investment in egg load and poison sac in fig wasps: Implications for physiological mechanisms underlying seed and wasp production in figs

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ABSTRACT

Fig pollinating wasps and most non-pollinator wasps apply secretions from their poison sacs into oviposited flowers that appear necessary to the formation of the galls that their developing offspring consume. Thus, both eggs and poison sac secretions appear to be essential for wasp reproduction, but the relative investment in each is unknown. We measured relative investment in poison sac and egg production in pollinating and non-pollinating wasps associated with seven species of monoecious Panamanian figs representing both active and passive pollination syndromes. We then collected similar data for four fig hosts in China, where some wasp species in the genus *Eupristina* have lost the ability to pollinate (“cheaters”). All wasps examined possessed large poison sacs, and we found a strong positive correlation between poison sac size and absolute egg production. In the Panamanian species, the relative poison sac to egg investment was highest in the externally ovipositing non-pollinator wasps, followed by active pollinators, then by passive pollinators. Further, pollinator wasps of fig species with demonstrated host sanctions against “cheating” wasps showed higher investment in the poison sac than wasps of species without sanctions. In the Chinese samples, relative investment in the poison sac was indistinguishable between pollinators and “cheaters” associated with the same fig species. We suggest that higher relative investment in poison sac across fig wasp species reflects higher relative difficulty in initiating formation of galls and subsequently obtaining resources from the fig. We discuss the implications for the stability of the fig–wasp mutualism, and for the ability of non-pollinators to exploit this mutualism.

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1. Introduction

For over 60 million years, fig trees (*Ficus*, Moraceae) and the fig wasps (Agaonidae Chalcidoidea, Hymenoptera) that pollinate them have constituted one of the most complex and interdependent insect–plant mutualisms known (Corner, 1958; Ramirez, 1974; Wiebes, 1979; Berg, 1989; Machado et al., 2001a; Herre et al., 2008; Cruaud et al., 2011; Xu et al., 2011). All *Ficus* species are taxonomically united by their distinctive, enclosed inflorescence, known as a syconium, that ultimately develops into the fig fruit. Nonetheless, figs are taxonomically and functionally diverse, with over 750 species (Harrison, 2005; Cruaud et al., 2011). Reproductively, figs can be either functionally monoecious or functionally dioecious, and the pollination syndrome can be either active or passive (Kjellberg et al., 2001; Machado et al., 2001b; Jousselin et al., 2003; Harrison, 2005; Herre et al., 2008; Jandér and Herre, 2010).

Within a receptive syconium, tens to hundreds of uniovulate flowers are pollinated by female wasps (foundresses). Generally, one or a few species of wasps pollinate any fig species (Michaloud et al., 1996; Molbo et al., 2003; Cornille et al., 2012; Cruaud et al., 2012). When a foundress wasp enters a receptive fig, she inserts her ovipositor into the style of the flower and attempts to deposit an egg between the inner integument of the flower’s ovule and the

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maternally derived diploid nucellus (Verkerke, 1986, 1989; Jansen-González et al., 2012). The foundress also deposits several drops of a maternal secretion that is produced and stored in the wasp's poison sac (see Fig. 1). This secretion, possibly in concert with larval secretions, appears to be essential in transforming the oviposited flowers into galls that, in turn, are essential for the wasp larvae to feed and develop (Verkerke, 1986, 1989; Jansen-González et al., 2012). The maternal secretion is associated with gall growth because only inflorescences that receive the maternal secretion develop into galls and the gall tissue rapidly grows after the drops are delivered; often several hours or days before the wasp's egg hatches (Jansen-González et al., 2012). Thus, both eggs and poison sac secretions seem to be essential for pollinator wasp reproduction. Pollinating wasp species exhibit large poison sacs relative to overall body size (Grandi, 1938), consistent with the importance of poison sac function.

Importantly, without pollen-bearing foundress wasps, fig inflorescences cannot produce fertile seeds under natural circumstances. Without the flowers within fig inflorescences, the pollinator wasps cannot reproduce, and the pollinators' female offspring are essential for a fig to disperse its pollen to other receptive figs (Wiebes, 1979; Herre, 1989, 1996). Individual fig flowers within a syconium can support the development of either an intact viable seed, or the development of a single adult wasp (Herre, 1996; Jandér and Herre, 2010). Generally, seeds develop in flowers with longer styles in which the ovules are located closer to the syconium wall and the galls containing the wasp offspring develop from shorter styled flowers in which the ovules are located toward the interior of the syconium (see references in Herre et al., 2008; Wang et al., 2012). Figs benefit reproductively from both seed production and female wasp production; wasps, however, only benefit directly from the production of more wasps (Herre, 1989). This underlies a conflict of interest between the figs and their wasps that would appear to threaten the stability of mutualism (Herre and West, 1997; Herre et al., 2008; references within).

Fig trees also host non-pollinating wasps that belong to at least six subfamilies within the superfamily Chalcidoidea (Boucek, 1993; Rasplus et al., 1998; Jousselin et al., 2008; Cruaud et al., 2011). Many of these species oviposit from the outside of the syconium, thereby initiating gall growth (Ghara and Borges, 2010) but without providing pollination services (also see Van Noort and Compton, 1996). Although less well studied than the pollinators, a wealth of detailed work on the ecology of these wasps indicates that they are generally parasitic on some aspect of the fig–pollinator mutualism or on other non-pollinator wasp species (West and Herre, 1994; West et al., 1996; Pereira and do Prado, 2005; Pereira et al., 2007; Herre et al., 2008). The most common ecological roles are: small, pollinator sized gall-forming wasps that function ecologically as competitors to the pollinators (Compton and van Noort, 1992; West and Herre, 1994; Elias et al., 2008, 2012); large gall-forming wasps that are physically much larger than the pollinators (West et al., 1996); parasitoids of pollinators, small or large non-pollinators (Compton et al., 1994; West et al., 1996; Compton et al., 2000; Dunn et al., 2008; Ghara and Borges, 2010).

As with the pollinators, non-pollinating wasps possess a poison sac and many of them induce galls that appear similar to those induced by pollinating wasps and utilize the same flower tissue. Interestingly, several non-pollinator wasps, like the New World competitors of pollinators (of the genera *Idarnes* and *Critogaster*) also utilize a similar set of short-styled flowers as the pollinators, despite the fact that they oviposit from the exterior of the fig and thus are farther away from the interior ovules (West and Herre, 1994). Non-pollinating wasps appear to cost the fig tree not only by initiating gall growth without providing pollination services, but also by directly competing with the pollinating wasps for potential gall flowers, reducing the number of pollinators that develop (Compton and van Noort, 1992; Compton et al., 2000; West and Herre, 1994; West et al., 1996; and see references in Herre et al., 2008).

Directly or indirectly, all pollinator and most non-pollinator wasp species exploit fig flowers. However, there are many still-unresolved questions (reviewed in Herre et al., 2008; Jandér and Herre, 2010; Jandér et al., 2012; Wang et al., 2012). Concerning fig–pollinator interactions: How does the fig prevent pollinator wasps from galling all of the flowers to rear their offspring? Why are flowers with shorter styles the ones that are predominately galled by pollinators? Concerning non-pollinators there are also many unresolved questions: Why does the fig not prevent gall formation of non-pollinating wasps? Why do the non-pollinator species that most directly compete with the pollinators also predominately gall short-styled flowers? More broadly, what limits the ability of these non-pollinators to exploit and undermine the mutualism?

Despite great progress (reviewed in Herre et al., 2008; also see West and Herre, 1994; Jousselin et al., 2003; Elias et al., 2012; Jandér et al., 2012; Jansen-González et al., 2012; Wang et al.,

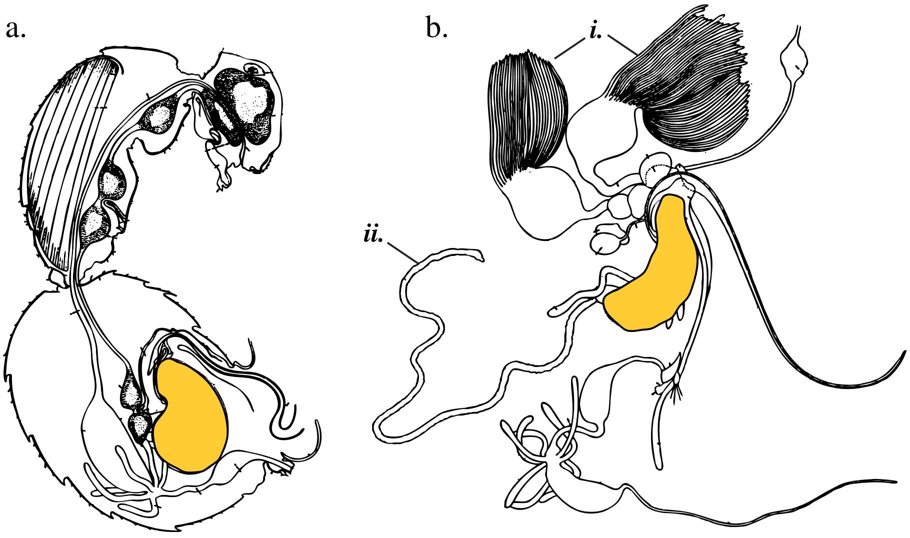

Fig. 1. The poison sac (yellow) (A) *in situ* in the fig wasp abdomen, and (B) when dissected out, attached to other abdominal organs (i. ovaries, ii. acid gland). Modified from Grandi (1938).

2012), we suggest that these questions cannot be answered adequately until the mechanisms underlying gall initiation by pollinator and non-pollinator wasps are understood. All available observations suggest that without the fluids secreted from the wasp poison sac gall formation cannot occur, which is essential for offspring development in the vast majority of fig-associated wasp species. Nonetheless, given their complementary and non-overlapping functions, the relative investment in eggs or poison sac has never been addressed directly. Motivated by these considerations, this study provides the first attempt to estimate relative investment in poison sac and egg production across fig-associated wasps that represent different ecological and evolutionary relationships with their host fig.

2. Methods

2.1. Study species

Mature figs from seven *Ficus* species including two passively and five actively pollinated fig species, were collected at the Barro Colorado National Monument, Panama between January and April 2010. Passively and actively pollinated species of figs and their associated wasps diverged between 60 and 80 million years ago and evidence suggests that the associated lineages of wasps have been exposed to and selected by the same pollination syndromes for millions of years (Machado et al., 2001a). *Ficus maxima* and *Ficus insipida* are passively pollinated Panamanian species classified in the *Ficus* subgenus Pharmacosycea, which available evidence suggests is basal to all other fig subgenera (Berg, 1989; Rønsted, 2005; Cruaud et al 2011). The primary pollinators of these two species in central Panama have been identified as *Tetrapus americanus*, and *Tetrapus costaricensis* (see Molbo et al., 2003; Jandér and Herre, 2010)

The five actively pollinated Panamanian species (*Ficus citrifolia*, *Ficus obtusifolia*, *Ficus popenoei*, *Ficus* near *trigonata*, and *Ficus costaricana*) are classified in the *Ficus* subgenus Urostigma, section Americana. The primary pollinators of these species in central Panama have been identified, respectively, as *Pegoscapus tonduzi*, *Pegoscapus hoffmeyeri*, *Pegoscapus gemellus*, *Pegoscapus lopesi*, *Pegoscapus* spp. (see Jandér and Herre, 2010). Previous research on the Panamanian species indicates that there is not always a perfect one to one correspondence of species of wasps to host trees (Molbo et al., 2003; Machado et al., 2005; Jackson et al., 2008; Jandér and Herre, 2010). Externally ovipositing non-pollinator Panamanian gall-forming wasp species were also collected from *F. maxima* (*Critogaster* sp.) and *F. popenoei* (*Idarnes* (*flavicollis* group) sp.) (West et al., 1996;Machado et al., 2007; Marussich and Machado, 2007).

Mature figs from four *Ficus* species classified within the subgenus Urostigma section Conosycea were collected at the Xishuangbanna Tropical Botanical Garden, People's Republic of China between July and September 2012. Although these are closely related fig species they included species exhibiting both active (*Ficus benjamina*, *Ficus microcarpa*, and *Ficus altissima*) and passive (*Ficus curtipes*) pollination syndromes. Further, in addition to their proper active pollinators (*Eupristina altissima* and *Eupristina verticillata*), *F. altissima* and *F. microcarpa* also harbor congeneric "cheater" species that have ceased providing pollination services to their hosts (Peng et al., 2008, 2010). In the case of *F. altissima*, the "cheater" (*Eupristina* sp., collected from *F. altissima*) appears to be the sister species of the pollinator (Peng et al., 2008). The phylogenetic association of the "cheater" associated with *F. microcarpa* has yet to be determined.

2.2. Collection and measurements

Fruits were collected after male wasps had emerged from their galls and started to mate with female wasps, and females were allowed to emerge in the lab. For each Panamanian fig species, 20–28 wasps were examined from 2 to 6 syconia (inflorescences that develop into fruits) collected from the same tree. For each Chinese wasp species, three wasps were sampled from four different fruits per tree, totaling 12 wasps per species. Newly emerged wasps were dissected with microforceps under a dissecting microscope. Measurements were taken either on digital photographs or with an eyepiece graticule mounted on a binocular microscope to the nearest 0.01 mm.

Thorax area, poison sac area, and number of eggs were estimated for each individual. Thorax measurements were chosen as surrogates for body size because they are easy to measure (being significantly larger than for example head length or femur length), and thorax length correlates well with both wasp dry mass and wet mass (KCJ unpublished). Both measurements were collected with the wasp on its side; thorax length was measured along a line through the coxae, and thorax height was measured as the dorso-ventral distance at mid-thorax. For wasp individuals where the thorax height measurement was unavailable, thorax height was estimated from a species-specific ratio between thorax length and thorax height. Thorax area was calculated as thorax length × thorax height. Poison sac area was calculated as the elliptical area using the measured length (L) and width (W), $A = \pi LW/4$, which corresponds well with the natural shape of the poison sac. Poison sac size was corrected for body size by dividing the area of the sac by the thorax area of the wasp. Ovaries were removed, allowed to soak in water for two minutes, and gently spread with a cover slip. Eggs in each ovary were photographed and counted under a microscope.

2.3. Statistical analyses

Species of wasps were grouped as actively pollinating, passively pollinating, cheaters, or externally ovipositing non-pollinating gall-forming wasps (see Tables 1 and 2). The body-size corrected sac area per egg [(sac area/egg number)/thorax area] of active, passive, cheater, and non-pollinators was compared with either a Kruskal–Wallis test or an ANOVA followed by planned comparisons. We also included more conservative analyses comparing only species means where possible. The relationship between the mean poison sac area and egg number per species was compared using correlation. We corrected for phylogenetic relationship and body size using the method recommended by Garland et al. (1992): we first calculated independent contrasts (details below) for each of the variables egg number, poison sac area and thorax area (using the mean for each species), then regressed the contrasts (linearly; through the origin) for egg number on thorax area, and sac number on thorax area. The resulting residuals were used in the final linear regression through the origin, testing whether poison sac size increases with egg number when taking both phylogenetic relationship and body size into account (Garland et al., 1992; Midford et al., 2005). We corrected for body size only using a similar method (partial regression plots; Fox, 2008): regressing egg number on thorax area and sac area on thorax area, then regressing the resulting residuals; the result is identical to a multiple regression with sac area as the dependent variable and thorax area and egg number as independent variables. There was one potential outlier in the Panamanian contrast regressions to get the residuals, but excluding that data point affected the final regression only minimally, so it was retained in the analyses. There were no other outliers in any of the regressions or correlations. Assumptions were tested and transformations or non-parametric tests used where appropriate. All tests were two-tailed unless otherwise stated. Statistics were performed in JMP v. 8.0.1 (www.jmp.com) and SPSS 19.0 (IBM, Armonk, NY, USA).

Table 1
Characteristics of fig wasp species collected from seven species of *Ficus* in lowland Panama. Columns indicate the host *Ficus* species; the number of individuals collected; the averaged area of the poison sac; the poison sac area corrected for body size by thorax area, and the average number of eggs for each species. Standard error is listed in parentheses.

Wasp species	Fig host	Individuals	Poison sac area (μm^2)	Poison sac/thorax area	Average egg
Active pollinator					
Pegoscapus tonduzi	*F. citrifolia*	28	29.7 (1.4)	0.286 (0.015)	131.0 (2.6)
Pegoscapus estherae	*F. costaricana*	25	25.3 (1.0)	0.250 (0.010)	119.9 (3.8)
Pegoscapus hoffmeyerii	*F. obtusifolia*	22	52.8 (1.8)	0.240 (0.012)	218.5 (6.2)
Pegoscapus gemellus	*F. popenoei*	27	20.0 (0.8)	0.229 (0.009)	104.0 (4.1)
Pegoscapus lopesi	*F. triangle*	27	34.1 (1.1)	0.305 (0.012)	206.3 (8.1)
Passive pollinator					
Tetrapus costaricanus	*F. insipida*	26	36.3 (0.9)	0.162 (0.004)	187.1 (3.8)
Tetrapus americanus	*F. maxima*	28	44.6 (1.5)	0.190 (0.011)	232.4 (4.2)
Non-pollinator					
Idarnes sp.	*F. popenoei*	24	16.4 (0.8)	0.139 (0.009)	56.4 (2.0)
Critogaster sp.	*F. maxima*	19	23.3 (2.1)	0.118 (0.016)	61.7 (5.1)

2.4. Phylogenetically independent contrasts

A tree for independent contrasts of the Panamanian wasp species was constructed from a 1.5 kb sequence of the cytochrome oxidase subunit I (COI) gene from each wasp species (Machado, 2001; Molbo et al., 2003; Marussich and Machado, 2007). The sequences were aligned with MUSCLE (Edgar, 2004) with further manual adjustments in MacClade (Maddison and Maddison, 2005). Phylogenetic relationships for members of each class were inferred using Bayesian Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses. Modeltest 3.7 (Posada, 2006) was used to select the GTR + I + G model using the Akaike information criterion. Analyses were executed in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) for two runs of up to 4.6 million generations each, sampling every 1000th generation. Likelihoods converged to a stable range for each data set, and all trees prior to convergence were discarded as burn-in. Independent contrasts were determined using molecular branch lengths in PDAP:PDTREE module of Mesquite (Midford et al., 2005; Maddison and Maddison, 2009).

3. Results

3.1. Panamanian species: correlation sac area–egg number

The nine species of wasps surveyed had a greater than three-fold difference in the total size of the poison sac (Table 1). *Idarnes* (*flavicollis*-group) sp., a non-pollinator from *F. popenoei*, had the smallest poison sac. The largest poison sac was found in the active pollinator of *F. obtusifolia*, *Pegoscapus hoffmeyerii*. The average number of eggs a wasp carried ranged from 56.4 (non-pollinator of *F. popenoei*, *Idarnes* sp.) to 232.4 eggs (passive pollinator of *F. maxima*, *T. americanus*) (Table 1). Across the Panamanian species, the area of the poison sac was positively correlated with the number of eggs a wasp carried (Pearson correlation, $n = 9$, $r = 0.91$, $p = 0.0008$; Kendall tau non-parametric correlation, $r = 0.83$, $p = 0.002$; Fig. 2A). This relationship remained significant when corrected for only body size (linear regression of residuals, $n = 9$, $r^2 = 0.81$, $p = 0.001$), and when correcting for both body size and phylogenetic relationship (see Methods; regression through the origin of standardized contrasts for egg number vs. thorax area: $n = 8$, $r^2 = 0.46$, $p = 0.045$, saving residuals; regression through the origin of standardized contrasts for sac area vs. thorax area: $n = 8$, $r^2 = 0.80$, $p = 0.001$, saving residuals; regression of the saved residuals, $n = 8$, $r^2 = 0.72$, $p = 0.004$).

3.2. Panamanian species: comparison across ecological groups of wasps

Wasp species were categorized into ecological groups based on their pollination activity (active pollinator, passive pollinator and non-pollinator). The body-size corrected sac area per egg varied across the three ecological groups (Kruskal–Wallis, test statistic 140.0, df = 8, $p < 0.001$; Fig. 3A). Non-pollinators had a significantly larger body-size corrected sac area per egg than did actively pollinating fig wasps (sequential Bonferroni corrected Mann–Whitney *U* test, $U = 2929$, $p = 0.011$). Passively pollinating wasps

Table 2
Characteristics of fig wasp species collected from six species of *Ficus* in People's Republic of China. Columns indicate the host *Ficus* species; the number of individuals collected; the averaged area of the poison sac; the poison sac area corrected for body size by thorax area, and the average number of eggs for each species. Standard error is listed in parentheses.

Wasp species	Fig host	Individuals	Poison sac area (μm^2)	Poison sac/thorax area	Average egg
Active pollinator					
Eupristina altissima	*F. altissima*	6	42.2 (2.3)	0.259 (0.011)	258.0 (4.5)
Eupristina verticillata	*F. microcarpa*	12	24.1 (1.1))	0.304 (0.020)	95.0 (4.6)
Eupristina koningsbe rgeri	*F. benjamina*	12	38.7 (1.3)	0.167 (0.0095)	258.0 (7.2)
Passive pollinator					
Eupristina sp.	*F. curtipes*	12	42.8 (1.2)	0.327 (0.025)	110.0 (3.1)
Cheater wasps					
Eupristina sp.	*F. altissima*	12	47.2 (2.2)	0.241 (0.009)	236.7 (4.5)
Eupristina sp.	*F. microcarpa*	11	22.6 (1.4)	0.319 (0.019)	88.5 (4.1)

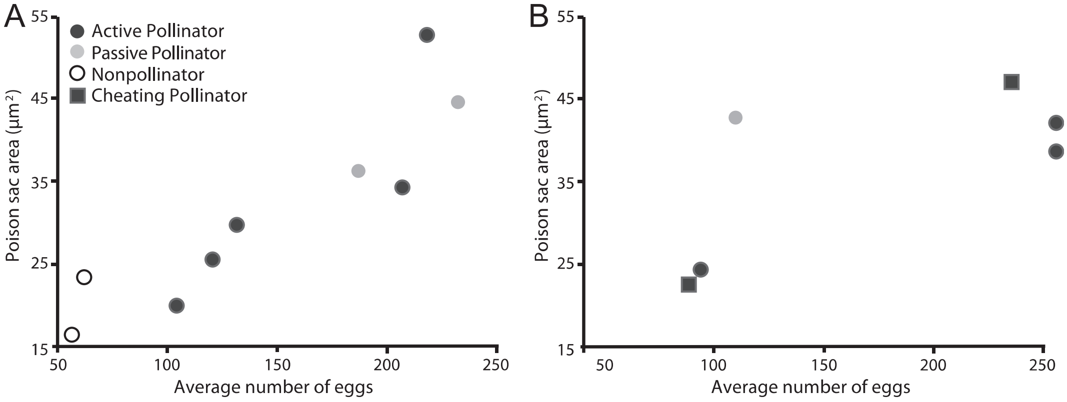

Fig. 2. The poison sac area was positively correlated with the average number of eggs per female wasp across both (A) Panamanian and (B) Chinese wasp species. See text for corrections related to body size and phylogenetic relationship.

had a significantly smaller body-size corrected sac area than did actively pollinating wasps (sequential Bonferroni corrected Mann–Whitney *U* test, $U = 6208$, $p < 0.001$). When comparing only species means, the body-size corrected sac area per egg still varied across the three ecological groups in the same way (ANOVA on log-transformed data, $F_{2,8} = 6.89$, $p = 0.028$), with non-pollinators having a significantly larger sac area per egg than pollinators (planned contrast $t_6 = -2.5$, $p = 0.046$), and active pollinators having a significantly larger sac area per egg than passive pollinators (planned contrast $t_6 = 3.2$, $p = 0.018$).

In two *Ficus* species we had sampled both the associated non-pollinators and the pollinators. In both cases, non-pollinating wasps had larger body-size corrected sac area per egg than the pollinating wasp of the same host species, but only significantly so in *F. maxima* (*F. maxima*: sequential Bonferroni corrected Mann–Whitney *U* test, $n = 41$, $U = 333$, $p < 0.001$; *F. popenoei*: sequential Bonferroni corrected *t*-test, $t_{50} = -1.34$, $p = 0.19$; Fig. 3A).

3.3. Chinese species: correlation sac area–egg number

The six species of Chinese wasps surveyed had a two-fold difference in poison sac area, and a three-fold difference in egg numbers (Table 2). Across the Chinese species, the area of the poison sac was positively correlated with the number of eggs that a wasp carried, although not significantly so (Pearson correlation, $n = 6$, $r = 0.709$, $p = 0.12$; Kendall tau non-parametric correlation, $r = 0.41$, $p = 0.25$; Fig. 2B). However, this relationship was explained by differences in wasp size, because when we corrected for body size, the relationship disappeared (linear regression of residuals, $n = 6$, $r^2 = 3.4$ E−4, $p = 0.97$). We are currently unable to correct for phylogeny in these species.

3.4. Chinese species: comparison across ecological groups of wasps

In contrast to the Panamanian species, Chinese wasps associated with the passively pollinated *F. curtipes* exhibited a significantly larger body-size corrected sac area per egg than wasps associated with the three actively pollinated fig species (ANOVA on log-transformed data, $F_{5,59} = 90.67$, $p = 2.2$ E−26; planned contrast active vs. passive $t_{59} = -8.06$, $p = 4.3$ E−11; Fig. 3B). Comparing species means only is not possible here due to only having data from one passive species. Further, in each of the two cases studied, there was no difference in body-size corrected sac area per egg between the legitimate pollinator and the “cheater” wasp species associated with the same host fig species (*F. altissima:* *t*-test, $t_{16} = 0.20$, $p = 0.85$; *F. microcarpa*: *t*-test, $t_{21} = -0.59$, $p = 0.56$; Fig. 3B).

3.5. Sac area and sanction strength

In five of the Panamanian fig species, and one of the Chinese, host sanction strength, the fitness cost for pollinator wasps that do

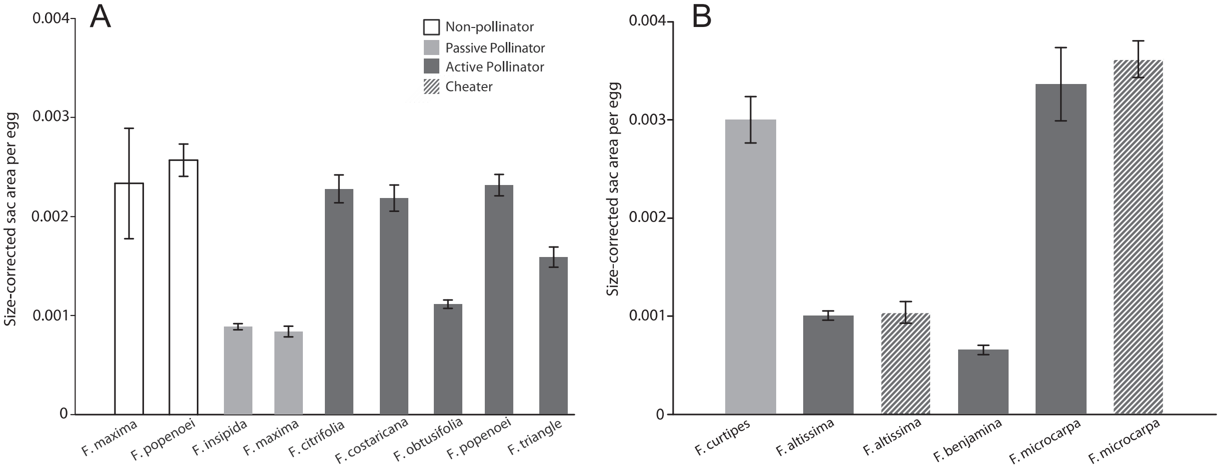

Fig. 3. The relative investment in the poison sac varied across the ecological groups of wasps in (A) Panama and (B) China.

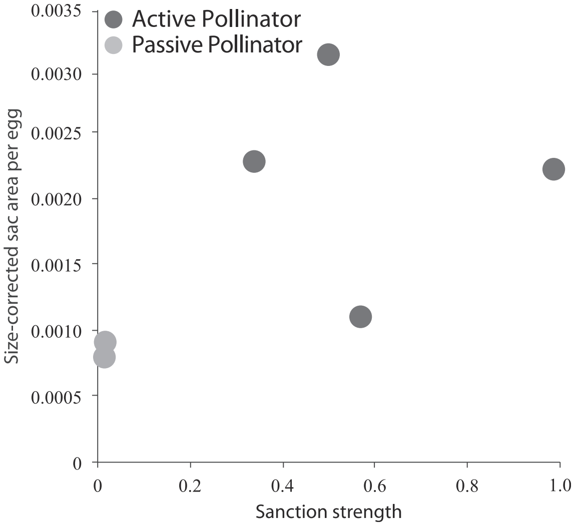

Fig. 4. Across pollinator species, there was a non-significant positive relationship between the relative investment in the poison sac and the sanction strength of the host.

not pollinate, has previously been measured (sanction strength calculated as $1 - W_R$, where W_R is the species-specific relative fitness of a single experimentally pollen-free foundress compared to a pollen-carrying foundress; Jousselin et al., 2003; Jandér and Herre, 2010). Across these species, the body-size corrected sac area per egg was larger for pollinating wasps associated with fig species with sanctions compared to those without (ANOVA on log-transformed data, $F_{5,139} = 102.3$, $p = 5.3$ E−44; planned contrasts sanctions vs. no sanctions $t_{72.9} = 19.7$, $p = 7.6$ E−31). Also when comparing only species means, the body-size corrected sac area per egg is larger for pollinating wasps associated with fig species with sanctions compared to those without (Mann–Whitney *U* test, $n = 6$, $U = 8$, $p = 0.03$ one-sided). The body-size corrected sac area per egg for pollinating wasp species increased with increasing sanction strength, but not significantly so (Kendall tau non-parametric correlation, $n = 6$, $r = 0.41$, $p = 0.25$; Fig. 4).

4. Discussion

Investment in both eggs and maternal secretion stored in the poison sac seem to be essential for the reproductive success of fig-associated wasps that develop in galled fig flowers. Without eggs, no offspring can develop and all available evidence suggests that without the fluids secreted by the poison sac, rapid enlargement of cells in the nucellus and gall formation, which is essential for wasp development, cannot occur (Verkerke, 1986, 1989; Jansen-González et al., 2012). Thus, both the eggs and the poison sac fluid seem to serve essential, complementary reproductive functions for the wasps. Nonetheless, the relative investment in eggs and maternal secretion presents potentially conflicting costs and benefits for wasp reproduction. Both eggs and maternal secretion are costly; neither producing eggs without enough secretion to initiate gall formation, nor producing too much secretion for available eggs is an efficient use of resources. We suggest that the observed relative investment across wasp species likely reflects the specific challenges presented by their different ecological and evolutionary associations with the host fig.

Motivated by these considerations, this study provides the first attempt to estimate relative investment in poison sac and egg production across fig-associated wasps that represent different ecological and evolutionary relationships with their host fig. The species for which we have collected these data include: a) pollinators and externally ovipositing non-pollinators that are found on the same host fig species; b) pollinators that are associated with host figs presenting either active or passive pollination syndromes; and c) pollinators and congeneric wasps that have ceased to pollinate ("cheaters") that co-occur on the same host fig. These wasps represent at least 15 different species from 5 genera associated with 11 fig species from both the New and Old World.

We found that the relative investment in poison sac per egg was less in pollinators than in non-pollinator wasps for the same host species. Here, our available data come from two comparisons associated with two New World fig species. *F. maxima* is a passively pollinated species classified in the subgenus Pharmacosycea (with their associated *Tetrapus* pollinators and *Critogaster* competitor non-pollinators) and *F. popenoei* is an actively pollinated species classified in Urostigma Americana (with their associated *Pegoscapus* pollinators and *Idarnes*, group flavicollis, competitor non-pollinators (Wiebes, 1979; Machado, 2001; Machado et al., 1996; West et al., 1996; Elias et al., 2008, 2012; Cruaud et al., 2011)). Beyond showing higher investment relative to the pollinator of their host, both non-pollinating wasp species showed significantly higher investment in their poison sac compared to all other Panamanian pollinator wasps. We suggest that this relatively high investment observed in the non-pollinators reflects a greater difficulty for them to induce gall formation in the fig. This suggestion is consistent with their ecological role as parasites of the system and that the fig tree is selected to not divert resources to support their development.

Short-lived pollinators (2–3 days) and many gall-forming non-pollinators are almost certainly pro-ovigenic and have their eggs and poison sac fully formed when they leave their natal fig (Copland et al., 1973; Ghara and Borges, 2010). In contrast, the longer-lived non-pollinators and especially parasitoids (~10 days) are almost certainly syn-ovigenic (West et al., 1996; Ghara and Borges, 2010), and can potentially invest more in both eggs and poison sac over their relatively extended lifetimes. Presumably non-pollinators carry sufficient poison sac fluid at any one time to provide for their mature eggs, so as long as the sac size is corrected for the number of eggs carried, the comparison across types of wasps ought to be valid. However, to determine if pollinators and non-pollinators use the same mechanism for gall initiation, additional studies that test the exact function of the poison sac must be conducted across wasp species with different life histories (Ghara and Borges, 2010; Jansen-González et al., 2012). Because it is increasingly understood that different non-pollinator species exploit host figs and pollinator wasps in different ways, these studies should be expanded to include non-pollinator species with different ecological roles on the same host tree species (i.e., flower galler, syconium wall galler, parasitoids of pollinators and non-pollinators) to determine the function of the poison sac in each (Van Noort and Compton, 1996; West et al., 1996; Compton and van Noort, 1992; Pereira et al., 2007; Jousselin et al., 2008; Elias et al., 2008, 2012). Nonetheless, the results here are consistent with the idea that figs are more resistant to gall induction by the non-pollinators, and therefore select for higher relative poison sac investment per egg in gall-forming non-pollinator species.

Among Panamanian pollinators, the relative investment in poison sac was significantly higher in the active pollinators (five species) than in the passive pollinators (two species). This is consistent with higher apparent demands placed on pollinator wasps in active systems (Jousselin et al., 2003; Jandér and Herre, 2010). In contrast, among Chinese pollinators the relative investment in poison sac was higher in wasps (*Eupristina* spp.) associated with the passively pollinated fig species (*F. curtipes*) than in wasps associated with the three actively pollinated species. While

Panamanian species have a 60–80 million year phylogenetic separation between passive and active New World pollinators, the Chinese fig species are associated with the relatively closely related *Eupristina* wasps (Machado, 2001; Cruaud et al., 2011). Therefore the passive pollinators of *F. curtipes* appear to have been active pollinators for the majority of their evolutionary history and only recently switched to passive pollination (unpublished data). While both Old and New World wasps are functionally passive pollinators, their differences in evolutionarily history could possibly explain the differences in poison sac investment.

Clearly, additional studies are required to determine the patterns of poison sac investment in active vs. passive pollination syndrome. Such studies should focus on deeper sampling on at least two phylogenetic levels. First, poison sac and egg investment patterns need to be established among clades that have exhibited one syndrome or the other for extended evolutionary periods (e.g., the Panamanian taxa sampled). Second, extensive sampling is required within clades where both syndromes are observed and there is evidence for recent active–passive transitions in wasps and figs (e.g., the Urostigma Conosycea (Peng et al., 2008) and the Urostigma Malvanthera (Cook et al., 2004; Cruaud et al., 2011)).

Among the Chinese fig species (*F. altissima* and *F. microcarpa*) in which there are both legitimate (active) pollinating wasps and congeneric “cheater” wasps that no longer pollinate (Peng et al., 2008, 2010; Peng, unpublished data), the similarity in poison sac investment in wasps that share the same host is striking, given the variation observed across all *Eupristina* species. This suggests that both legitimate pollinators and cheaters have similar requirements for poison sac investment per egg, possibly dictated by host tree species. This is particularly likely given the close phylogenetic relationships of the wasps (within genus) (Herre et al., 2008; Peng et al., 2008). Currently there are only three fig species known in which there are legitimate pollinators co-occurring with congeneric “cheater” wasps (the two Chinese species as well as *Ficus sycomorus* in Africa (see Compton et al., 1991)), nonetheless, poison sac investment in “cheaters” can be usefully compared within the context of other wasps in the genus (Herre et al., 2008).

Finally, we found significantly higher relative investment in the poison sac in wasp species that are associated with fig species exhibiting significant sanctions against wasps that do not pollinate (Kjellberg et al., 2001; Jousselin et al., 2003; Herre et al., 2008; Jandér and Herre, 2010). This is consistent with the hypothesis that fig hosts that sanction wasps for not pollinating also require more investment in the secretions that initiate gall formation. Nonetheless, we only found a non-significant positive trend between relative poison sac investment and quantitative estimates of sanction strength of the host (Jousselin et al., 2003; Jandér and Herre, 2010). This is qualitatively consistent with the hypothesis that pollinators associated with host figs exhibiting stronger sanctions have greater difficulty inducing gall formation. However, more sanction experiments and poison sac measurements are needed to fully assess this hypothesis.

Up to this point, we have primarily considered the implications for wasps (higher poison sac to egg investment in non-pollinators vs. pollinators; in actively vs. passively pollinated species; in species with relatively stronger sanctions). However there are also implications for the figs – are some fig tissues more sensitive to the poison sac secretions than others? As previously mentioned, there is a conflict of interest to the ratio of seeds and galls within a syconium (Herre, 1989; Herre and West, 1997; Herre et al., 2008). While fig wasps would benefit from the majority of the flowers becoming galls, undescribed mechanisms appear to limit wasp utilization to about half the fig flowers in monoecious figs, specifically to the short-styled flowers in the interior of the syconium (West and Herre, 1994; Herre et al., 2008; Wang et al., 2012). There is some suggestion that the shorter styled flowers are chemically or physically more conducive to supporting wasp offspring (West and Herre, 1994; Wang et al., 2012; Steve Compton, personal communication; E.O. Martinson, unpublished results). In the light of this work we suggest that long and short-styled flowers have different sensitivities to poison sac secretions. Building on observation and experiments (e.g., Herre et al., 2008; Jandér and Herre, 2010; Jansen-González et al., 2012), we suggest that fig flowers resist gall formation and subsequent provisioning unless they have been pollinated (at least at the fig level in actively pollinated species; see Jandér et al., 2012), and if the wasp additionally sends its own signal through the maternal secretion (Verkerke, 1986, 1989; Jansen-González et al., 2012). If longer styled flowers do not or cannot response to the maternal secretion’s signal for gall initiation, it could be a possible mechanism in which the wasp is limited to short-styled flowers (West and Herre, 1994; Herre and West, 1997; Wang et al., 2012).

4.1. Future directions for the study of the function of poison sac secretions

The size of the poison sac and its role in oviposition suggest that it plays an important function in the fig and fig wasp mutualism (Grandi, 1938; Verkerke, 1986). Yet, the exact function of the poison sac remains unknown in both pollinating and non-pollinating wasps (see Jansen-González et al., 2012, and references therein). The poison sac holds secretions produced in the acid gland and is common throughout Hymenoptera and is varied in its function. For example, in honeybees (*Apis mellifera*) the poison sac holds the venom associated with painful stings (Haberman, 1972). In ants it holds communication pheromones (Holldobler, 1971; Vandermeer et al., 1980), and in ichneumonid wasps it stores polydnaviruses that are injected into their prey to compromise their immune system, thus protecting the wasp’s eggs (Espagne et al., 2004). Recent transcriptomic analysis of the poison sac of *Nasonia vitripennis*, a parasitic chalcid wasp within the same superfamily as fig wasps, revealed that at least 79 different venom genes are expressed, of which 24 have no sequence similarity to known proteins (Werren et al., 2010).

In general, many mechanisms behind the varied functions of the poison sac are not well understood. Characterization of genes in model systems will provide some insights, but fig-associated wasp venom genes may have acquired new functions or wasps may have obtained new genes from horizontal transfer. Therefore, a step toward determining the venom’s function in the fig and fig wasp mutualism would be to conduct transcriptomic and proteomic screens of the poison sac from fig-associated wasps in different ecological roles (i.e. flower galler, syconium wall galler, parasitoid of pollinating and non-pollinating wasps; Ghara and Borges, 2010). This approach would provide a comparative framework to identify genes and proteins that are consistent across fig-associated wasps in general, as well as among individual ecological roles. Additionally, the plant’s response is critical for understanding venom function in fig wasps. Analyzing the changes in gene expression in the fig flowers in response to the maternal secretion would elucidate the changes in development and metabolism altered by the presence of the maternal secretion.

The function of the maternal secretion and the sensitivities of different tissues across host fig species to the effects of those secretions could not be directly addressed in this study. Neither could we address the potential function that any larval secretions might have, either in concert with the maternal fluids or by themselves in inducing gall formation or directing resources to the developing larvae. We nonetheless suggest that some fig species are more resistant to the gall inducing effects of the maternal secretion than others. For example, given our results reported here and in previous studies we suggest that flowers of the actively pollinated figs require more pollinator secretion to induce gall formation than flowers of passively pollinated species. We further expect a similar pattern with actively pollinated species characterized by higher sanctions: they would require more secretion than those actively pollinated species exhibiting absent or relatively low sanctions. Moreover, within species we expect that longer styled flowers are more resistant to gall induction than shorter styled flowers (Herre, 1989; West and Herre, 1994; Yu et al., 2004; Jansen-González et al., 2012; Wang et al., 2012; Martinson et al., unpublished results). It is possible that one general mechanism, the sensitivity of the fig tissues to the liquid produced by the poison sac of different pollinating and non-pollinating wasp species, can account for a number of phenomena that play a central role in the success and stability of the fig–wasp mutualism.

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