Assessing the cost of parasitism to *Ficus* trees and their pollinators

Background

Mutualism is a broad category of ecological interactions between individuals of different species in which all participants benefit. Mutualisms are extremely widespread and, therefore, have great ecological and evolutionary significance. Species that have evolved to exploit or cheat on mutualisms are quite common as well (for examples, see Porter and Simms 2014, Zhang et al 2014), and these exploiters can have important ecological impacts. They can directly harm one or more partners in the mutualism by draining resources without giving anything in return, potentially exerting selection pressure on the organism to exclude them. They may also successfully compete with an organism for the resources it obtains from the mutualism, since exploiters can take resources without having to pay the fitness cost of giving resources or services in return (Porter and Simms 2014, Zhang et al 2014). The relationship between angiosperms and their animal pollinators is one of the most well-known examples of mutualism. Like other mutualisms, plant-pollinator relationships can be exploited, as is the case in the *Ficus*-wasp system.

Overview of the Ficus-wasp mutualism

The *Ficus*-wasp system is an example of an obligate mutualism, in which both participants depend on each other for survival and reproduction. *Ficus* plants are woody angiosperms (Moraceae, genus *Ficus*) that are found in a diverse range of habitats throughout the tropics (Janzen 1979, Berg 1990, Herre et al 2008). Their unique enclosed inflorescences, called synconia, are only accessible by a small, bract-lined opening called the ostiole. These highly specialized structures reflect the specificity of the relationship between *Ficus* species and their pollinators, small agaonid wasps (Hymenoptera: Chalcidoidea: Agaonidae) (Janzen 1979, Rasplus 1996, Weiblen 2002, Herre et al 2008). A female wasp enters through the ostiole when

the syconium is receptive, that is, when it is in female phase (Galil and Eisikowitch 1968, Janzen 1979, Herre et al 2008). The wasp pollinates the female flowers and oviposits into some of them, usually those with shorter styles and ovules closer to the center of the syconium (Jousselin et al 2001). The wasp larvae hatch inside the ovules and secrete chemicals that cause the ovules to form galls. The larvae live in these galls and feed on plant tissue until the male fig flowers mature, preventing the ovules from developing into seeds (Weiblen 2002, Cook and Rasplus 2003, Herre et al 2008). In most species, the male wasps emerge from their galls first and mate with females through the wall of their gall. After mating, the male wasps chew an exit hole for the females, either by re-opening and enlarging the ostiole or chewing through the wall of the syconium (Galil and Eisikowitch 1968, Weiblen 2002, Cook and Rasplus 2003). The female wasps gather pollen either passively or actively, exit the fig, and fly to a receptive fig on another tree to begin the cycle again (Janzen 1979, Weiblen 2002, Herre et al 2008).

Non-pollinating fig wasps

In addition to pollinating fig wasps, there is a diverse group of non-pollinating fig wasps (Hymenoptera: Chalcidoidea: Agaonidae, Torymidae, Pteromalidae, Eurytomidae, Ormyridae) that also lay their eggs in figs (Bronstein 1991, Weiblen 2002, van Noort and van Harten 2006). Most oviposit externally by drilling their long specialized ovipositors through the wall of the syconium and depositing their eggs in ovules, but several species, thought to be close relatives of the pollinators who eventually evolved to become "cheaters," enter through the ostiole to lay their eggs (Kerdelhué and Rasplus 1996, Herre et al 2008). These wasps pollinate either not at all (in the case of wasps that oviposit externally) or very minimally (in the case of wasps that enter the syconium), and they can be considered parasites or exploiters of the *Ficus*-wasp mutualism (Kerdelhué and Rasplus 1996, Dunn et al 2008, Herre et al 2008). They seem to "get

away" with this strategy because they are attracted to the same volatile cues that figs use to attract pollinators, and *Ficus* species seem unable to exclude non-pollinating wasps without also excluding their pollinators (Bronstein 1991, West and Herre 1994).

The group of insects referred to as parasitic or non-pollinating fig wasps encompasses a diverse set of wasps with a wide range of larval behaviors and diets. Some oviposit directly into ovules already containing the egg or larva of a pollinator. The non-pollinating wasp larvae then live either as parasitoids of the pollinators or as inquilines, living in galls made by the pollinator, feeding on plant tissue, and either killing the pollinator directly or out-competing it. Other non-pollinating wasps oviposit into ovules without pollinator larvae, and their larvae are capable of making their own galls in which they live and feed until it is time to emerge. These galls can be extremely large or close to the same size as pollinator galls (Dunn et al 2008, Herre et al 2008).

Non-pollinating wasps are somewhat under-studied compared to pollinating fig wasps. Associated non-pollinating wasp species have been identified and catalogued for a variety of *Ficus* species, though in many cases all that is known about each wasp is a species name and perhaps a general categorization as a parasitoid, inquiline, gall-maker, etc (Bouček et al 1981, Bronstein 1991). Various studies (West and Herre 1994, Kerdelhué and Rasplus 1996, Dunn et al 2008) have also explored the effects of non-pollinating wasps on *Ficus* plants and their pollinators. Dunn et al (2008) suggest that non-pollinating fig wasps may play a role in stabilizing the *Ficus*-pollinator mutualism, because non-pollinators tend to oviposit in ovules closer to the syconium wall. This makes pollinator oviposition into the longer-styled flowers (ovules closer to the syconium wall) less likely to result in a successful larva and, thus, not worth the time in the short life of the pollinator. This prevents the pollinator from laying an egg in every ovule. However, this study is somewhat problematic because it lumps together several

kinds of non-pollinating wasps with different larval diets and therefore the potential for very different effects on the mutualism.

A 1994 study by West and Herre more rigorously examined the effects of non-pollinating wasps, with attention paid to the diversity of larval diets. They studied non-pollinating wasps in the genus *Idarnes* associated with several *Ficus* species and observed a negative relationship between the presence of non-pollinators and the fitness of *Ficus* and/or the pollinator. Specifically, they found that when *Idarnes* wasps (gall-makers) were present, there were fewer surviving pollinators but no difference in seed number. In a similar study, Kerdelhué and Rasplus (1996) found that the number of non-pollinators assumed to be parasitoids (*Apocrypta spp., Sycoscapter spp.*, and *Sycophila spp*) emerging from synconia of *Ficus sur* and *Ficus vallis-choudae* was negatively correlated with the number of pollinators emerging. Interestingly, they also found that the number of gall-makers in a syconium was negatively correlated with seed number in *Ficus sur* but had no significant impact on seed number in *Ficus vallis-choudae*.

These studies provide observational evidence of the effects of different kinds of nonpollinators and provide a reasonable starting point for predictions about the effects of nonpollinators associated with other as-yet-unstudied *Ficus* species. However, they either completely neglect larval diet and behavior or make somewhat broad assumptions about the larval diet and behavior of the non-pollinating wasps studied. Additionally, to my knowledge, these studies in which figs are simply collected and examined have not been supplemented by controlled experiments in which known combinations of wasps are introduced. Finally, West and Herre's (1994) results suggest that there are important differences between *Ficus* species. with a given *Ficus* species and then using this understanding to conduct controlled experiments will be a valuable and necessary contribution to the study of the *Ficus*-wasp mutualism.

Study Questions

The overall aim of this study is to experimentally examine the effects of non-pollinating wasps on both *Ficus* trees and their pollinators, and to determine how those effects differ based on the behavior and diet of the non-pollinator. The specific questions that will be explored are as follows: 1) How does the presence of non-pollinating wasps affect the fitness of *Ficus* trees and their pollinators? 2) Do parasitoid, inquiline, small gall-forming, and large gall-forming non-pollinators have different fitness consequences for *Ficus* and pollinator? 3) What is the outcome of competition or antagonism between pollinating and non-pollinating fig wasp larvae hatching from eggs laid in the same ovule? 4) If pollinators are ever able to kill or out-compete non-pollinators in the same ovule, are their chances of success affected by the oviposition timing of the non-pollinating wasp?

I expect that the effects of non-pollinators on *Ficus* and pollinator fitness will depend on the larval behavior of the non-pollinator. In particular, parasitoids and inquilines should have a negative impact on the number of surviving pollinator offspring while having little effect on seed number. Gall-makers should generally have the opposite effect, decreasing *Ficus* female fitness while having little effect on pollinator survival. If pollinators are able to out-compete nonpollinators, I expect this phenomenon to be somewhat rare, and I expect that the success of the non-pollinating wasp will be affected by the developmental stage of the pollinator at the time of non-pollinator oviposition.

Experimental

Study species and site

Three *Ficus* species and their associated wasp fauna will be considered in this study: *Ficus lutea, Ficus cyathistipula,* and *Ficus ovata. Ficus lutea* is a fast-growing hemi-epiphytic tree up to 20 m tall found in forests and near rivers throughout West Africa, usually pollinated by *Allotriozoon heterandromorphum* (Berg and Hijman 1989, Rasplus 1996, Sonibare et al 2008). *Ficus cyathistipula,* pollinated by *Agaon fasciatum,* can be terrestrial or hemi-epiphytic and can grow up to 8 m tall. It is found in forests in West Africa, often near rivers or lakes (Berg and Hijman 1989, van Noort and van Harten 2006). *Ficus ovata,* like the other two *Ficus* species, is found in forests and next to rivers in West Africa, though it may also be found in grasslands. It is a hemi-epiphytic strangler that can grow up to 10 m tall and is pollinated by *Courtella hamifera* (Berg and Hijman 1989, Berg 1990, van Noort and van Harten 2006).

Field work will be conducted in Sapo National Park in Sinoe County, Liberia, as the ranges of all three *Ficus* species include Liberia, and the Sinoe River passes through the park, creating an ideal habitat for the two riverine *Ficus* species (Jongkind 2003, Voorhoeve and Jongkind). The specific study site will be a 1000 ha plot adjacent to the Sinoe River.

1. Observation/survey phase

The purpose of this portion of the study is to gain familiarity with the particular location and species being studied, especially the non-pollinating wasp fauna associated with each *Ficus* species, as the three *Ficus* species considered here are not as well-studied as some others.

Survey of the study area

The study area will first be surveyed in 5 m x 500 m line transects (Tuomisto et al 2014) to determine the local distribution and density of each *Ficus* species. This will be helpful in

planning the later stages of the study that involve bagging figs and introducing wasps in the field. Surveying will begin along the river and move away until ~30 individuals of each species are located, with additional surveying conducted later in the study if any more individuals are needed. All individuals will be marked on a GPS map to facilitate their location for use in later parts of the study.

Observation of non-pollinating wasp fauna

Individuals of each *Ficus* species will be observed to determine which species of nonpollinating wasps are most commonly found ovipositing into synconia. Five trees of each species bearing receptive figs will be selected randomly, and two yellow sticky traps will be placed on randomly selected branches of each tree to catch wasps arriving at the tree (Bronstein 1991). Receptive figs are easily identifiable by the loosening of bracts around the ostiole (Galil and Eisikowitch 1968). Each day, traps will be collected, and all wasps will be identified morphologically, tallied, and removed from the traps (Bronstein 1991). This procedure will be repeated every day for a month to ensure that wasps arriving at different stages of fig maturity are accounted for (Galil and Eisikowitch 1968, Weiblen and Bush 2002).

Assessing gall-forming ability of non-pollinating wasps

It is likely that larval habits will be unknown for many of the wasps counted in this portion of the study, and some may even be as-yet-undescribed species. Therefore, it will be necessary to determine which of the unknown non-pollinating wasps are parasitoids, which are inquilines, and which are gall-makers. Wasps will first be tested for gall-making ability by introducing them individually to receptive figs. In order to access figs on trees, especially *Ficus cyathistipula*, it will be necessary to have some tree climbing expertise and equipment. For each *Ficus* species, 5 trees bearing pre-receptive figs, which are small and enclosed in bracts (Galil

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and Eisikowitch 1968), will be selected at random. Figs on four randomly selected branches per tree (or enough to sample 15-20 figs per tree) will be covered with mesh bags to exclude wasps (Jousselin et al 2001, Wang et al 2012), and the branches will be covered with chicken wire to deter frugivores. These trees will function as acceptor trees and will receive wasps later in the experiment. One additional tree will then be chosen as a donor tree, from which adult wasps will be captured (Jousselin et al 2001). This donor tree will be the first tree encountered that has male figs at the same time that the acceptor trees are pre-receptive. Figs on several branches



Figure 1. Schematic diagram of experimental setup for one *Ficus* individual. This example is shown with 6 species of non-pollinators, but the actual number will likely differ for each *Ficus* species. Here each outer circle represents 2-3 figs. This treatment will be repeated on five trees of each species.

from this tree will be bagged when wasps are about to emerge (figs soft but lacking an exit hole; see Kerdelhué and Rasplus 1996). Wasps, both pollinating and non-pollinating, will be trapped in the bag as they emerge. Pollinators will be separated out and released, and nonwill immediately pollinators be introduced to receptive figs. Bags will be removed from figs on acceptor trees one at a time, and one non-pollinator individual will be introduced before rebagging. The wasp species introduced to

each fig will be noted by labeling the bag. There will also be a group of figs that remain bagged and receive no wasps to test for efficacy of bagging. There should be 60-100 bagged figs of each species, depending on possible frugivore damage (which should be minimized by the chicken wire) and the number of figs initially bagged per tree. For each *Ficus* species, the total number of figs will be divided by the number of associated non-pollinating wasp species to obtain the number of trials that will be conducted with each wasps species. For instance, if there are 100 figs bagged and 10 non-pollinating wasp species, 10 individuals of each wasp species will be introduced to figs, ideally spread out between the five acceptor trees to account for any differences among *Ficus* individuals (see Figure 1).

The five trees receiving wasps may or may not all be bagged at the same time, depending on how many trees at similar points in their flowering cycles can be located at one time. Thus the procedure described above may be repeated several times per species with different donor trees and smaller groups of receptive trees. However, since figs mature asynchronously between trees, it is reasonable to assume that a donor tree and a receptive tree or trees will be available at the same time (Janzen 1979). The fact that figs mature asynchronously also means that there is a fairly continuous supply of receptive *Ficus* trees (Janzen 1979, Herre et al 2008), so bagging of figs can take place at any point during the year.

Once all wasps have been introduced, figs will be allowed to mature for about a month, after which they will be removed from trees and preserved in ethanol. All figs will be brought back to the lab, cut into eighths, and examined for the presence of galls. Wasps introduced to figs that contain galls about the size of a pollinator gall after a month will be classified as small gall-makers, and wasps introduced to figs that contain galls noticeably larger than pollinator galls will be classified as large gall-makers ("large" galls are obviously visually different from pollinator galls and are immediately noticeable upon opening the syconium; see Dunn et al 2008). Wasps introduced to figs that contain no galls will be assumed to be parasitoids or inquilines, which require the presence of pollinator larvae to survive.

Stable isotope dietary analysis

The wasps that fail to form their own galls will be tested using stable isotope analysis to determine if they are parasitoids or inquilines. Because the relative abundances of ¹³C and ¹²C in an organism's body are influenced in predictable ways by the isotope composition of the organisms it eats, it is possible to use stable isotope analysis to determine an animal's diet (DeNiro and Epstein 1987, Peterson and Fry 1987, Källgren et al 2015). Pollinating fig wasps and fig tissue will be treated as the two potential diet sources for each unknown non-pollinator. In order to establish a baseline for comparison, tissue samples taken from the ovules of each *Ficus* species and the entire bodies of individuals of each pollinator species will be prepared, combusted, and analyzed using mass spectrometry, following the procedure used by DeNiro and Epstein (1987). This procedure will then be repeated using non-pollinating wasps, and the isotope composition of each non-pollinator species will be compared to the isotope compositions of the pollinator and Ficus species with which it is associated. If the non-pollinator is a parasitoid and consumes pollinator larvae, its stable isotope composition should resemble that of the pollinator. If it is an inquiline and feeds on plant tissue, its stable isotope composition should more closely resemble that of the fig tissue. The most common non-pollinating wasps species associated with each *Ficus* species will be analyzed first, followed by the next most common, until one parasitoid and one inquiline non-pollinator is found associated with each *Ficus* species.

When these procedures are completed, I will have identified the most common parasitoid, inquiline, small gall-maker, and large gall-maker associated with each *Ficus* species. These non-pollinators will be used as representatives of their groups in later parts of the study. Several adult females of each "representative" species will be captured with sticky traps following the

procedure outlined above and brought back to the lab. Their ovipositors will be measured to determine an average ovipositor length for each species.

2. Effects of non-pollinating wasps on fitness of Ficus trees and pollinators (study questions 1 and 2)

This portion of the study is a controlled experiment in which wasps will be selectively introduced to figs in different numbers and combinations in order to separate the fitness effects that each type of non-pollinating wasp has on the *Ficus* tree and the pollinator.

Experimental introduction of wasps

For each *Ficus* species, five acceptor trees bearing pre-receptive figs and one donor tree bearing male figs will be selected and bagged as described above in Part 1. As in the observational phase, wasps will be trapped in the bags as they emerge from figs on the donor trees. Pollinators will be kept for introduction to figs on acceptor trees, along with individuals of the most common species of parasitoid, inquiline, small gall-maker, and large gall-maker associated with each Ficus species. All other wasps will be excluded from this portion of the study, and the terms "parasitoid," "inquiline," "small gall-maker," and "large gall-maker" as used below should be understood to refer to the chosen representative species. Ideally, there will be at least one parasitoid, one inquiline, one small gall-maker, and one large gall-maker per *Ficus* species. This is not an unreasonable assumption, as *Ficus* species for which the wasp fauna have been well-catalogued are associated with a very high number and diversity of nonpollinating wasps (Bouček et al 1981, Kerdelhué and Rasplus 1996, Cook and Rasplus 2003). Even if some or all of the three *Ficus* species turn out not to be associated with at least one wasp in each of the above broad categories, it is highly unlikely that any one kind of wasp will be completely unrepresented in the study when all three Ficus species are taken into account.



Figure 2. Schematic diagram of experimental setup for one *Ficus* individual. Each outer circle represents 2-3 figs. This treatment will be applied to 5 trees of each *Ficus* species.

Wasps will be introduced to figs on acceptor trees in the following combinations: 1) no wasps (control to check for efficacy of bagging), 2) 1 pollinator individual (to obtain a baseline number of offspring produced by a pollinator), 3) 1 pollinator individual + 1 parasitoid individual, 4) 1 pollinator individual + 1 inquiline individual,

5) 1 pollinator individual + 1 small gall-maker individual, 6) 1 pollinator individual + 1 large gall-maker individual, or 7) 2 pollinator individuals (to control for effects of increased foundress number). These treatments will be split up between the figs on each tree so that 2-3 figs on each tree receive the same treatment (see Figure 2).

After figs are exposed to these treatments, bags will be replaced and figs will be allowed to grow normally for about a month, or until figs enter the male phase (when they will be soft but not yet have an exit hole; see Kerdelhué and Rasplus 1996), to give larvae time to hatch and mature (Galil and Eisikowitch 1968, Janzen 1979). Figs will then be harvested, preserved in ethanol, and taken back to the lab for analysis.

Analysis of fig contents

Each fig will be cut into eight sections and its contents examined under a dissecting microscope. Two sections will be selected at random (Wang et al 2012), and their contents will be counted and categorized as follows: 1) number of seeds, 2) number of pollinators, and 3)

number and type of non-pollinator. Wasps will be identified by morphospecies, and the accuracy of these identifications will be confirmed genetically by sequencing the mitochondrial cytochrome c oxidase I gene, following the protocol used by Zhou et al (2012).

The data collected will be used to quantify the fitness costs to the pollinator and the tree as a result of the presence of each type of non-pollinating wasp. Pollinator fitness will be measured as the number of pollinators, *Ficus* female fitness will be measured as the number of viable seeds, and *Ficus* male fitness will be measured as the number of pollinators.

Expected results

I expect the larval diet and behavior of non-pollinating wasps to affect their impacts on pollinator and *Ficus* fitness, in accordance with past research (West and Herre 1994, Kerdelhué and Rasplus 1996). Parasitoids and inquilines should have significantly negative effects on pollinator fitness and therefore *Ficus* male fitness. Parasitoids eat pollinator larvae, and inquilines either directly kill pollinator larvae or out-compete them by consuming plant tissue that would otherwise be eaten by the pollinator. Thus, the natural history of these non-pollinator larva. This prediction is supported by previous work by Kerdelhué and Rasplus (1996), discussed above, which found that the presence of parasitoids or inquilines to have much, if any, effect on *Ficus* female fitness, because in most cases they must lay their eggs in ovules already containing a pollinator larva or egg. Therefore, these non-pollinators should not cost the tree any seeds that have not already been lost to a pollinator larva.

Gall-forming non-pollinators may have a negative impact on pollinator fitness and *Ficus* male fitness, especially large gall-makers, whose galls are so large that they can reduce the total

space inside a syconium that is available for pollinator galls (West and Herre 1994, Dunn et al 2008). However, I expect the cost to be less than that inflicted by parasitoids or inquilines, which generally kill the pollinator larvae. Gall-forming non-pollinators should also have a much larger negative impact on *Ficus* female fitness than either parasitoids or inquilines. This is because they result in the destruction of an ovule that would not have otherwise contained a pollinator, and which therefore could have developed into a seed. I expect this effect to be especially pronounced for large gall-makers, as the production of these large galls presumably costs the tree more energy that it could otherwise spend producing seeds (Herre et al 2008). However, it would not be completely surprising if in at least some cases small gall-makers did not significantly impact seed number, given the diversity of fitness effects reported by West and Herre (1994) and Kerdelhué and Rasplus (1996) mentioned above.

3. Outcomes of competition and antagonism between pollinating and non-pollinating wasp larvae (study questions 3 and 4)

The purpose of this portion of the study is to indirectly examine competition between pollinators and non-pollinators for the same ovules. Both parasitoid and inquiline non-pollinating wasps must lay their eggs in ovules that already contain a pollinator, and it is generally assumed that the non-pollinating larva is then successful in killing the pollinator (Janzen 1979, Kerdelhué and Rasplus 1996, Dunn et al 2008, Herre et al 2008). However, to my knowledge no work has been done to determine whether or how often the pollinator larva is able to survive despite the presence of a non-pollinator egg or larva. This is especially interesting in the case of non-pollinating wasps that do not directly kill the pollinator but instead simply outcompete it for resources. Knowing how often, if ever, the pollinator larva triumphs in this competition would have important implications for thinking about the magnitude of selection

pressures exerted on *Ficus* and pollinator by parasitoid and inquiline non-pollinators. To explore this question, DNA will be extracted from ovules containing wasps and sequenced to determine if there is evidence of the prior presence of a wasp of a different species, suggesting that the wasp present was successful in killing or out-competing another wasp.

Collection of figs

For each of the same three *Ficus* species studied above, ~ 10 male phase figs will be collected from each of 3 randomly selected trees within the study area, preserved in ethanol, and brought back to the lab for analysis.

DNA extraction and analysis

Figs will be opened and cut into eight sections, similar to the procedure outlined above in Part 2. However, all sections of each fig will be examined as this portion of the study is investigating what is likely to be a somewhat rare phenomenon.

DNA will be extracted from galls containing pollinators using QIAamp DNA micro kit (*QIAGEN*, Hilden, Germany). PCR and sequencing will be carried out using primers for the mitochondrial cytochrome c oxidase I gene, following the protocol of Zhou et al (2012). The presence of the DNA of a non-pollinating wasp in an ovule containing a pollinator would suggest that a non-pollinator was present at one time in the gall but was out-competed by the pollinator.

As a test for the efficacy of these methods, DNA will also be extracted and sequenced from ovules containing non-pollinators. I expect to find DNA of pollinators in galls containing parasitoid and inquiline non-pollinators, and the presence of this DNA will provide support for the assumption that finding non-pollinator DNA in a gall containing a pollinator is evidence of the prior presence of a non-pollinator. Furthermore, if no non-pollinator DNA is found in galls containing pollinators, but pollinator DNA is found in galls containing non-pollinators, I will be able to conclude that the absence of non-pollinator DNA is not a result of ineffective methods but rather of either 1) the fact that pollinators simply never out-compete non-pollinators, or 2) the fact that pollinators out-compete non-pollinators so rarely that my sample size was too small to detect it.

Follow-up study

If non-pollinator DNA is found in galls containing pollinators, suggesting that pollinators are capable of out-competing non-pollinators, I will conduct a follow-up study to explore the effects of time between pollinator oviposition and non-pollinator oviposition on the outcome of the eventual competition between the two larvae.

For each *Ficus* species, pre-receptive figs and figs on a donor tree will be bagged following the procedure outlined above in Parts 1 and 2. Pollinators, parasitoids, and inquilines will be collected from the donor trees ("parasitoids" and "inquilines" still refer to the most common representative species chosen above). Pollinators will be introduced to previously bagged figs when they become receptive, and either an inquiline or a parasitoid will be introduced at one of the following time intervals after introduction of the pollinator: 1) 0 hours (essentially simultaneous), 2) 6 hours (most likely while the pollinator is still alive and eggs are being laid; see Wang et al 2009), 3) 24 hours (likely soon after the end of the pollinator's life; see Wang et al 2009), 4) 4 days (well after the pollinator has finished laying eggs and died; see Wang et al 2009), 5) 1 week, 6) 2 weeks, or 7) 3 weeks (1, 2, and 3 week treatments are intended to test success of non-pollinator larvae at different points in pollinator offspring development and fig maturity; see Galil and Eisikowitch 1968).

Following wasp introductions, figs will be re-bagged and allowed to mature to male phase (Phase D, Galil and Eisikowitch 1968), when they will be harvested. DNA will again be extracted and sequenced following the procedure described above, and number of pollinatorcontaining galls showing evidence of prior presence of a non-pollinator will be tallied for each treatment category.

Expected results

Work by Weiblen and Bush (2002) suggests that the oviposition timing (relative to fig development) of externally ovipositing non-pollinating wasps is correlated with ovipositor length, with species with longer ovipositors laying their eggs later in fig development, when syconium diameter and wall thickness are both greater. An interesting implication of this finding is that each parasitoid and inquiline wasp species normally oviposits during a specific stage of pollinator development. They may therefore have adaptations to attack pollinators at a certain point in their development. With this in mind, I would expect the offspring of wasps with shorter ovipositors (who presumably oviposit very early in pollinator development, possibly before the pollinator hatches; see Galil and Eisikowitch 1968, Weiblen and Bush 2002) to become increasingly worse competitors compared with non-pollinators with longer ovipositors as time between pollinator oviposition and non-pollinator oviposition increases. This would make sense if wasps with shorter ovipositors are adapted to oviposit when the syconium wall is thin and/or to oviposit before the pollinator hatches. Ovipositing later, when the pollinator has hatched and begun to develop, would then presumably result in the non-pollinator attempting to kill a pollinator that is older, more mobile, and potentially better equipped to defend itself. Likewise, non-pollinators that normally oviposit when the pollinator is a larva or when it is pupating may be less successful if forced to oviposit earlier or later if they have specific adaptations to attack a larva or a pupa.

Proposed timeline

Part 1 should take about 14 months. This will consist of the initial 1-2 month observation of fig distribution and wasp fauna, followed by a year for dietary analysis and wasp introductions to allow for the possibility of not being able to conduct all wasp introductions simultaneously. Part 2 should also take about a year, again allowing time for multiple repetitions if there are not enough *Ficus* trees at the necessary points in their flowering cycles at one time. Finally, Part 3 should take about a month not including the follow-up study. If the results leading to the follow-up study are obtained, an additional year will be needed for more wasp introductions.

Conclusion

There is currently a relatively small amount known about non-pollinating fig wasps, and much more study is needed to fully understand and appreciate their diversity and their effects on the *Ficus*-pollinator mutualism. For many *Ficus* species, the non-pollinating wasp fauna is only partially known, and in many cases nothing is known about the behavior and diet of non-pollinating wasp larvae. This study will contribute valuable information about the larval behavior and diet of some of the wasps associated with *Ficus cyathistipula, Ficus lutea,* and *Ficus ovata*, which will be useful background information for future studies. It may also identify previously unknown species of non-pollinating wasps. This study will also provide experimental evidence separating the effects of each major class of non-pollinator on *Ficus* and fig wasp fitness, which will supplement and strengthen observations presented in previous studies. Finally, it will look for evidence of the outcomes of antagonisms between non-pollinators and pollinators hatching from eggs laid in the same ovule, a phenomenon that has received little, if any, study. The information gathered in this study will contribute to a greater understanding not only of the *Ficus*-wasp system, but also of mutualism in general. It will be especially important

for understanding the exploitation of mutualisms, which is relevant both for studying specific systems that involve a cheating or exploitative species, and for thinking more generally about the evolution of mutualism from exploitation and vice versa. Finally, my results will contribute to a body of knowledge about the rich variety of biotic interactions that characterize the tropics.

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