Adaptive and maladaptive sex ratios in a pollinating fig wasp

by

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Submitted in partial fulfilment of the requirements for the degree

MSc Genetics

In the Faculty of Natural & Agricultural Sciences

University of Pretoria

Pretoria

January 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Natural and Agricultural Sciences

Declaration

I, Karina Pentz declare that the thesis, which I hereby submit for the degree MSc. Genetics at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Summary

When a trait enhances fitness and arose as a result of natural selection, it is termed an adaptation. The optimization strategy employs selection thinking which makes the explicit assumption that the globally best trait will be fixed. Organisms, do not always respond optimally across all environments encountered as the strength of selection is finite. An excellent example of optimality is skewed sex ratios predicted by Hamilton's local mate competition (LMC) theory. It occurs when brothers compete in isolated groups for matings. Hamilton showed mathematically that in such groups, the unbeatable sex ratio is female-biased and this bias increase as the number of mothers that contribute to isolated patches decrease. One taxon that has been used to test LMC is the fig wasps.

Fig wasps are haplodiploid, with unfertilized haploid eggs developing into males and fertilized diploid eggs into females. One or a few mothers crawl into a fig and lay all their eggs within, resulting in extreme local mate competition and sib mating. Sibmating increases relatedness of the daughter to the mother as genes from the father are identical by descent to the mother. Relatedness to sons is unaffected. This relatedness asymmetry selects for even more female-biased ratios. Thus, an extra daughter instead of a son will decrease local mate competition, and an extra daughter provides a higher fitness to the mother due to relatedness. There are two mechanisms that can explain how fig wasps sex ratios are adjusted to the number of mothers in a patch. First, a byproduct of a constraint on clutch size, where mothers lay all or most of their sons first, followed by daughters. Mothers do not change their behaviour, but limited oviposition space prevents them from laying all their eggs destined to be daughters. Second, a facultative strategy where mothers sense other ovipositing females and adjust the number of sons they lay.

We studied sex ratio adjustments in *Ceratosolen galili*, the cuckoo wasp that oviposits in *Ficus sycomorus,* which is pollinated by *C. arabicus*. Different numbers of *C. galili* foundresses and a mix of these species were entered into figs to study the mechanism of sex ratio adjustment. We found that

mothers adjust their sex ratios facultatively when another foundress is present, and there is no constraint on clutch size. However, if the fig becomes saturated with eggs from three foundresses, the mothers' clutches are constrained, and sex ratios are also adjusted as a result of a byproduct. Since these foundress numbers are frequently observed in nature, selection effectively solved this problem. However, *C. galili* mothers often share figs with *C. arabicus* and *C. galili* should not adjust their sex ratios in their presence of *C. arabicus*. However, *C. galili* erroneously adjust their sex ratio facultatively in the presence of *C. arabicus*. Erroneous adjustment can be considered a maladaptation. Interestingly, the lack of pollination behaviour of *C. galili* is not sanctioned by reduced offspring survival.

Chapter 1 Literature Review

Natural selection

Darwin (1895) envisioned natural selection as a slow process of gradual improvement of individuals. Even though a large amount of modification needs to accumulate in one direction with successive generations, the process starts with small individual differences arising in nature, however small. These individual differences, when heritable, are the variation on which natural selection works (Darwin 1859).

Halliburton (2004) pointed out that   "the rate of increase in fitness of any organism at any time and is equal to its genetic variance in fitness at that time". The process causes an observable change in the means, variances and covariances of phenotypic distributions (Williams 1966, Arnold and Wade 1984b). While selection intensity can be described in terms of phenotypes, the resultant evolutionary response depends on the traits' heritability (Arnold and Wade 1984b). Thus, for natural selection to occur, there must be a statistical bias that causes differences in survival or reproduction, among individuals (Halliburton 2004). There may be correlated responses within an organism and selection will work on the net effect. Natural selection functions in producing an immediate improvement within a system; it does not anticipate future events and will not take steps to avoid a detrimental effect (Williams 1966). It can, however, be predictable if the fitness constraints are well understood (Williams 1966, Charnov 1982).

Traits that are fixed in population due to their effect on fitness are called adaptations (Williams 1966). Social behaviour performed between individuals has fitness consequences for parties involved and could be adaptations as well (West *et al.*2011). Whether this behaviour is beneficial or detrimental is based on the lifetime fitness pay-offs of the behaviour and the individual fitness relative to the population fitness. Note, however, that if an individual benefit from an activity, then it is not necessarily an adaptation, a benefit could be coincidental in which case it is known as an exaptation (Gould and Vrba 1982).

The additive effect of individual adaptations can lead to the entire group benefiting. However, if the group benefits, it is not necessarily group selection. However, if each individual is specialized in a collective function, it can form an adaptively organized entity, potentially favoured by group selection (Williams 1966). Group selection is not as strong as individual selection, but if group survival is favoured, then selection at the population level may counter selection at the individual level (Williams 1966). The selection efficiency depends on the population size, degree of isolation and the strength of selective forces (Williams 1957). When looking at adaptation, one should note the level of adaptation that the facts necessitate. Sex ratio skew due to local mate competition, a phenomenon I will return to below, is an example where both group and individual selection plays a role (Frank 1985, Frank 1986).

Adaptation, optimality and sexual selection

The fitness of an organism is defined as the contribution (contingent on viability, fecundity, mating ability and gamete competition) to the next generation (Halliburton 2004). A trait that enhances the fitness of an organism and arose as a result of natural selection is termed an adaptation for a specific function. Adaptations appear to be designed to maximize an individual's fitness (West *et al.* 2011). However, we should not claim that adaptation is perfect. Instead, we should look at it through specific examples in terms of selective forces, historical and developmental constraints that are in play. In understanding this, we can use models for each case and given a set of assumptions that we can expect. If biological observations fit the assumptions of the model, then it will add to a more accurate interpretation.

If social interactions affect fitness, two additional factors need to be considered. 1) If interacting individuals are related, the inclusive fitness (Hamilton 1964a, Hamilton 1964b) is the quantity that is optimized. 2) If fitness depends on what other individuals do, then evolutionary games theory must be used to find an evolutionary stable strategy (Maynard Smith 1982). The basic optimization approach assumes that the strategy that results in the highest fitness is fixed (Orzack and Sober 2001). When other individuals' strategies affect fitness, an ESS must be sought; the optimal strategy is if the entire population adopts it, then there is no better strategy (Parker and Maynard Smith 1990). The optimization criterion is often an indirect measure of the fitness of an organism (Parker and Maynard Smith 1990). Models can be constructed to give the maximum return per investment in order to identify the critical factors that shape selection for a specific trait or even to study adaptation itself (Parker and Maynard Smith 1990). Organisms do not always respond optimally across all environments they encounter (Herre 1985, Herre 1987), because the strength of selection is finite.

Often natural selection is said to be the same as sexual selection. However, both of these selection forms may work in opposite directions (Arnold and Wade 1984a). Sexual selection pertains to the struggle for mating opportunities and not for existence. An unsuccessful competitor does not necessarily die but will have fewer or no offspring as opposed to the victor (Darwin 1859, Seger and Stubblefield 2002). Sexual selection acts on both the primary and secondary sexual characteristics (Darwin 1888, Seger and Stubblefield 2002). The two sexes can differ in traits, when these traits are indispensable for one sex, such as locomotion used to find the other sex, then it would be an act of natural selection. If, however, the trait is different for only specific individuals so that it gives the individual an advantage over another, then it is sexual selection acting on that trait. These traits depend significantly on the choices and rivalries of individuals of the two sexes (Darwin 1888). Sexual selection does not necessarily have a limit to the amount of advantageous modifications that can be fixed. However natural selection will prevent certain characters being acquired as some might be injurious to the individual, expending too much energy or exposes them to danger (Darwin 1888).

Selection thinking

Natural selection leads to selection thinking which is our understanding of nature structured in terms of the ultimate causes (why questions) rather than the proximate mechanisms (how questions)(Charnov 1982). We can use selection to understand biological problems, and selection thinking helps to emphasize that there is a lot more to the exercise than a simplistic notion of "well-designed organisms" (Charnov 1982). An excellent example of how the simplistic notion differs from selection thinking is social behaviour. Social behaviour can show this transition as a conflict of interest among participants. For instance, with birds, the parents can only allocate so many resources to offspring. If there are too many hatchlings, it requires more resources from the parents than they can give, leading to death due to starvation (Charnov 1982). On the other hand, if there are too few offspring, then the probability of any chick surviving to the reproductive age decreases since there are fewer to start with. Adjusting the clutch size to an intermediate value in a way that would maximize the number of surviving offspring. The behaviour of the bird thus has an impact on its reproductive fitness (Trivers 1974, Charnov 1982). Another useful application of selection thinking is in the theory of sex ratios (West 2009), to which I turn now.

Sex ratio

Darwin (1888) pointed out that since sex ratios do not affect the number of offspring, it is unclear how it affects fitness. It turns out that it affects the number of grandchildren and sex allocation theory is used to study adaptation as it offers predictions about natural selection acting on a trait with a direct effect on fitness (Darwin 1888, Herre 1987, Burton-Chellew *et al.* 2008). According to Fisher's (1930) equal allocation theory, selection would favour unbiased sex ratios in a panmictic population. Males and females contribute equal genetic quantities to the next generation.

Two scenarios that can lead to skewed sex ratios are the Trivers-Willard effect and local mate competition (LMC), and I discuss these next. Trivers and Willard (1973) theorized that not all mothers are of equal quality and offspring of one sex can benefit more from being larger/ better nourished. If sons benefit more from extra investment than daughters, then selection for genes that allow larger mothers to have more sons and vice versa will be favoured (Trivers and Willard 1973). Therefore parents who can modify their investment into sons and daughters would be favoured by natural selection as it is a way to maximize the fitness of the parent in regards to environmental conditions (Komdeur 2012). For instance, in humans, mothers in good condition will invest in males (Cameron and Dalerum 2009) whilst those in adverse environmental conditions during pregnancy will invest in females.

Hamilton (1967) recognized that local mating could alter optimal sex ratio predictions and the sex ratio behaviour of organisms substantially. Specifically, he proposed that a phenomenon known as local mate competition (LMC), can result in female-biased sex ratios (Flanagan *et al.* 1998, Burton-Chellew *et al.* 2008). Hamilton (1967) showed that when n mothers lay their eggs in a patch and the males remain on that patch so that all matings are between the offspring of the n mothers; then the unbeatable sex ratio is c=(n-1)/2n (Figure 1.1). When a foundress lays a daughter instead of a son, then the daughter will increase the mating success of males and fewer sons will reduce male reproductive competition (Taylor 1981). A single foundress only needs to produce enough males to fertilize all females and so reduces local mate competition between brothers. The sex ratio is thus skewed towards a female-biased sex ratio (Pereira and Prado 2006). If more foundresses start ovipositing in a host, then the value of males increase. This is because sons can potentially mate with the daughters of the other foundresses skewing the sex ratio to a less female-biased ratio (Hamilton 1967, Herre 1985, Hu *et al.* 2013).

These local mating conditions increase relatedness of daughters to haplodiploid mothers but not sons and Hamilton (1979) adjusted his theory for relatedness in haplodiploids, which result in an even bigger skew in the sex ratios. His new formula enables the evaluation of the unbeatable sex ratio for any given values of inbreeding and mean foundress numbers. In haplodiploid organisms, haploid males (unfertilized eggs) receive all their genetic content from mothers whilst diploid females (fertilized eggs) receive half of the genetic content from their mothers and a half from their fathers. Sib mating increases the relatedness of the daughter to the mother because the father donates genes that are identical by descent with the mother’s (Hamilton 1967, Herre 1985, Herre and West 1997).

Subsequently, Herre (1985) improved this model to allow variable numbers of mothers and obtained with some more assumptions the equation $p = (1-m)(2n_h-1)/(4n_h-1)$ where m is the fraction of a mother's clutch, and n_h is the harmonic mean foundress number (Figure 1.1).

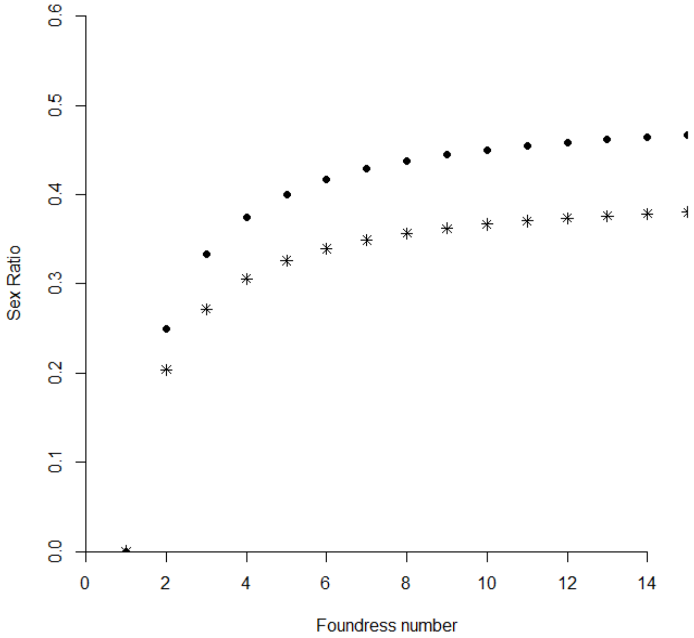

Figure 1.1. Optimal sex ratios for specific numbers of mothers with Hamilton's equation $c = (n - 1)/2n$ (filled circles) and with Herre's equation $p = (1-m)(2n_h-1)/(4n_h-1)$ when the harmonic mean number of mothers is 1.6 (asterisks).

Fig wasps and sex ratios

Fig wasps turn out to be an excellent taxon to test Hamilton's ideas. First, wasps are haplodiploid and can control sex ratios by either fertilizing the egg or not during oviposition. This sex ratio control means there is a proximate mechanism to achieve sex ratio adjustments in line with LMC (Kinoshita *et al.* 2002). Second, the fig wasp life history agrees closely with Hamilton's (1967) biofacies for LMC (Pereira and Prado 2006). For instance, one to a few mothers enter a fig to oviposit their eggs simultaneously (but see Greeff and Compton 1996). In fig wasps, we call these females, foundresses. Males typically do not disperse before mating (but see Greeff *et al*. 2003). However, Kathuria *et al.* (1999) pointed out that all the model assumptions are frequently not entirely correct. For instance, they showed that *Eupristina belagaumensis* foundresses from multi-foundress figs do not all contribute the same number of eggs. This may be due to competition, but also because the first female may lay her entire clutch before the second female enters the fig (Greeff and Compton 1996). As a result of sequential entry, clutch sizes will not be roughly equal, and the optimization problem is somewhat different (Greeff and Compton 1996). This being said, they are useful taxa to compare to theoretical predictions as long as assumptions are scrutinized. Herre *et al.* (1997) compiled data from six studies in 24 pollinator species that relates brood sex ratio with foundress number. Local mate competition predictions and inbreeding theory were at least qualitatively supported in these pollinators.

Fig tree morphology

The fig tree is part of the family Moraceae and occurs in a variety of habitats ranging from tropical forests to savannahs. This wide range of habitats allows Moraceae to have a wide variety of physiological adaptations, such as some of the highest photosynthetic rates in nature (Zotz *et al.* 1995). Moraceae has characteristic parenchymatous tissue with milky latex and unisexual flowers. The growth forms include free-standing trees, root climbers, epiphytes and hemi-epiphytes (Jousselin *et al.* 2003, Datwyler and Weiblen 2004). All fig trees fall in the genus *Ficus*, these trees usually are evergreen, but some species are deciduous such as those endemic to higher elevations and other non-tropic areas (Berg 2005). There are over 750 species of fig trees, and 30 species occur in the southern part of Africa (Nefdt 1992). They have fruit that ranges from 3mm up to 10cm in diameter. During development, figs may appear red, green or yellowish and during ripening the figs enlarge and may turn an intense bright colour such as orange, red, purple or remain yellow or green (Verkerke 1989, Herre *et al.* 2008). The leaves have waxy glandular spots (or phenolic glands based on histochemical tests by Pennisi *et al*. (1999) on the leaf blades (lamina) making them unique in the Moraceae family (Berg 2005).

Another unique feature of the fig trees is that the fig is a closed receptacle; thus, all the flowers are situated inside the urn-shaped syconia or inflorescence (Jousselin *et al.* 2003). The syconium is thus inaccessible for pollinators such as bees, hoverflies or butterflies. The only option is a very specific pollination system where only a particular seed galler-pollinator (the fig wasp) can enter the unique syconia and pollinate the flowers (Harrison 2005). The ostiole of the fig is the only entrance to the syconia and thus plays a vital role as a barrier to undesirable wasps. The ostiole forms when the outer bracts of the fig interlock during development. The direction the bracts interlock in is the determining factor for the different ostiole types (Verkerke 1989). The first type is the helicoidal passage, where all the bracts interlock forming a helicoid towards the syconia cavity. The second type is tunnel-shaped, where the upper ostiole bracts interlock, but the lower bracts remain loose and point downwards toward the syconia. The third type is bilabiate, where the bracts do not interlock but hang down, forming a bilabiate split (Verkerke 1989).

The inside of the fig is hollow with all the flowers arranged on the inside wall forming a lumen, and flowers are attached to the wall by pedicles of variant lengths. The flowers are urn-shaped and have a style facing the lumen, the style length is negatively correlated with pedicles and thus also vary

(Nefdt and Compton 1996, Van Noort and Compton 1996, Dunn *et al.* 2008a, Dunn *et al.* 2008b). The genus *Ficus* has two breeding systems, one being monoecious whilst the other is dioecious. The monoecious breeding system entails pollinator offspring, pollen and viable seeds all developing within one fig (inflorescence or syconia). The dioecious system consists of two types of trees: a gall or male tree and seed or female tree (Kjellberg *et al.* 1987). The male tree is where pollinator offspring develop, and the male flowers produce pollen which spreads to other areas via the offspring when they exit the fig. The female tree is where viable seeds develop due to pollinators entering and pollinating the flowers' stigmas, but the female wasps are not able to oviposit as the flowers have long styles preventing the wasp's ovipositor reaching the ovule (Verkerke 1989, Weiblen 2002). The monoecious fig and the dioecious male fig have both female and male flowers within the syconia whilst the dioecious female fig consists of only female flowers.

Passive pollination occurs in about a third of *Ficus* species. Abundant pollen is shed at the time of wasp emergence, dusting the wasp thoroughly. Even though the wasps clean themselves before departing, some pollen gets trapped in the abdominal pleura (Kjellberg *et al.* 2005b). Other *Ficus* species have active pollination. The anthers in these species are few, and the pollen is not shed. Instead, female wasps pick pollen from the anthers with their forelegs and put it inside pollen pockets before departing (Kjellberg *et al.* 2005b). Apart from *Ficus,* there are only a few species that have active pollination in nature; the Yucca and Senita cactus are two examples (Kjellberg *et al.* 2005b).

Fig wasp morphology

Pollinating fig wasps are part of the order Hymenoptera and the Agaonidae family which consists of subfamilies; Agaoninae, Kradibiinae, Sycophaginae and Tetrapusiinae (Van Noort and Rasplus 2019). Sycophaginae does not pollinate figs but was included in the family due to their phylogenetic affiliation. The tree requires the wasp for pollination, and thus reproduction whilst the wasp utilises the tree's resources during larvae development and reproduction (Dunn *et al.* 2008a). The wasp's morphology is uniquely adapted to fit the fig's morphology. The female wasp's head is flattened dorso-ventrally and elongated to fit through the ostiole. The shape of the head also relates to the size of the fig and the ostiole type (Liu *et al.* 2011). Big figs have long and thick ostiole walls in comparison to smaller figs. The long ostiole applies more pressure on the head of the wasp, and by elongating it, it relieves some pressure. The elongation also helps force more bracts open at the same time. There are rows or plates of backwards-pointing teeth on the front tibia and mandible of the female wasp. The teeth enable the female to manoeuvre through the bracts by pulling herself forward and prevents backwards sliding (Van Noort and Compton 1996). As the female enters the fig, she will use the spine on the third segment of the antennae to hook and pull the outer ostiole brackets open (Weiblen 2002).

The antennae of the wasp have several multiporous plate sensilla that occur along the length. The sensilla are elongated and allow the receptor surface area to be increased, thus intensifying host detection (Ware *et al.* 1992). The sensilla are the primary receptor for olfactory cues produced by receptive trees. Volatiles and olfactory cues can thus attribute to host specificity (Van Noort and Compton 1996).

The antennae of fig wasps have multiple functions. In the wasp *Eupristina sp.* for example, there are seven types of sensilla present: Elongated multiporous placoid sensilla (MPS-E), rounded multiporous placoid sensilla (MPS-R), sensilla trichodea (ST), basiconic sensilla (BS), chaetica sensilla (ChS), coeloconic sensilla (CoC) and sensillum obscurum (SO). Generally, chemoreceptors are innervated with dendrites or sensory neurons, and Li *et al.* (2014) found that MPS-E, MPS-R, BS, and CoS were indeed innervated and had multiple pores. These sensilla are associated with olfaction, thus enabling wasps to detect fig volatiles from long- and short-range distances. The aporous Chs, ST and SO were not innervated by sensory neurons, and it is believed they function as mechanoreceptors thus aiding with overcoming the physical barriers of the ostiole bracts (Li *et al.* 2014).

Female wasps that move through the fig can inadvertently deposit pollen on the stigmas, and this is termed passive pollination. Active pollination, on the other hand, is where females purposefully pollinate the flower's stigma. These females have pollen sacs that are located on the metathorax and the fore coxae's corbiculae (Compton *et al.* 2010, Weiblen 2002). The corbiculae have long setae which function as a pollen basket. The pockets have a narrow opening which is shielded by a row of thick setae. Females use their hairy legs to remove pollen from the pocket and deposit it on the stigma of the female flowers (Kjellberg *et al.* 2001, Weiblen 2002).

Most male fig wasps remain within the natal fig (Greeff *et al.* 2003) and thus, sexual dimorphism is present. The males lack wings (apterous) and have vestigial eyes (Weiblen 2002). The rows of cerated teeth that are present in females are not present on the mandibles or the front tibia of the males. The mandibles are small but well developed for chewing through galls and the fig wall (Nefdt 1992). Apart from a few species (Greeff *et al.* 2003) pollinating male fig wasps have a hooked shaped abdomen. Hook shaped wasps have elongated and narrow heads as well as moderately long thoraxes (Murray 1990). The elongated head and armature on the foretibia, enlarged femora and retractable antennae facilitate the male whilst burrowing an opening through the fig wall. The abdomen curves forward so that the extendible tip that is used for copulation is extended anteriorly to the head. By curling, the abdomen segments underneath the body enables easier copulation with females still in their galls (Murray 1990, Weiblen 2002).

The mutualism

The adult female wasp (foundress) enters the fig through the ostiole to enter the lumen of the spherical fig (Nefdt and Compton 1996, Van Noort and Compton 1996, Dunn *et al.* 2008a, Dunn *et al.* 2008b). Once inside the foundress will probe the female flowers with the ovipositor and lay an egg per flower whilst simultaneously pollinating the other flowers. This phase of the fig is called phase B (Galil and Eisikowitch 1968) The flowers with eggs starts to gall and develop endosperm for larvae to feed on whilst the pollinated flowers without eggs develop into seeds, known as phase C (Galil and Eisikowitch 1968). The male flowers will be mature at the same time the larvae are. Male wasps emerge first; they locate and chew through the galls containing females and copulation follows soon after. Phase D starts when males chew an opening through the fig wall for the pro-ovigenic females to leave. However, the females from actively pollinating species will first collect pollen from male flowers to eventually pollinate the receptive figs tree whose figs it will enter (active pollination) (Santinelo Pereira *et al.* 2007, Valdeyron and Lloyd 1979).

The pollinating wasps and fig tree thus have a mutualistic relationship where the tree is pollinated, and the wasps have oviposition sites (Cook and Rasplus 2003, Dunn *et al.* 2008a, Herre *et al.* 2008). However, there is also a cost involved for both partners in the mutualistic relationship; the tree sacrifices potential seeds for wasp development whilst the wasp wastes energy and time on pollination, reducing its already short lifespan (Herre and West 1997, West *et al.* 2011). If a partner can gain all the benefits and avoid the costs by cheating, then the other partner would be exploited, and this poses a risk to the breakdown or destabilization of the relationship.

There are several mechanisms involved in maintaining mutualisms, such as rewarding co-operators or punishing cheaters (West *et al.* 2011). Punishment is an additional cost to the actor, and we would not expect it to be favoured by selection unless the pay-off is higher than this cost. This enforcing behaviour can lead to direct or indirect benefits such as dismissing interactions with uncooperative partners, allowing more focus on the cooperative partners. Punishment could also change the behaviour of the cheater, thus ensuring future cooperation (West *et al.* 2011).

In the case of fig trees, several mechanisms have been suggested to maintain its stability. One important mechanism is sanctioning. The host or fig tree in this scenario will abort the figs if they remain unpollinated (Douglas 2008) or reduce nutrients to the individual galls. This is indeed the case in some fig wasps (Jander and Herre 2010). Since we will study the oviposition behaviour of a wasp that do not pollinate, we need to understand the potential role of sanctions.

The fig tree-fig wasp relationship provides evidence that a mutualistic relationship can be stable for a long time (Cook and Rasplus 2003, West *et al.* 2007). The age of this relationship has been documented by numerous authors. One of the most substantial cophylogenetic analyses recently was by Cruaud *et al*. (2012) and showed that this was an extreme case of plant–Insect codiversification. Rønsted *et al*. (2006) combined molecular phylogenetic trees with fossil data and provided an example of plant-insect co-divergence spanning at least 60 million years. Machado *et al*. (2001) found this mutualism to have arisen 87 million years ago by taking DNA sequence divergences and standardised it with a 25 million-year-old fig wasp fossil (Genus *Pegoscapus*) (Cook and Rasplus 2003). Compton *et al*. (2010) found that the fig wasp and fig tree's key co-adaptive features were already established 34 mya based on ancient fig wasps, which already had pollen pockets thus actively pollinating fig trees.

Selective regime

Sex ratios of fig wasps fit theoretical predictions closest in conditions they encounter most frequently (West *et al.* 2000). Females can adjust their sex ratios easier if they encounter a more variable number of females that enter a fig than when they only encounter a single foundress. The deviations from the optimal sex ratio are most considerable in wasp species that encounter a given number of foundresses less frequently (Herre 1985, Herre *et al.* 2001).

For instance, foundresses of species that mostly oviposit alone adjust their sex ratio less in variable environments than wasps that commonly experience ovipositing in unpredictable foundress number situations (Herre *et al.* 2001). Also, species that encounter both situations more frequently have the most significant shift in sex ratio from one to two foundresses (Herre *et al.* 2001). If a species is subjected to more variable selective regimes, then the adaptive behavioural plasticity is more developed (Herre *et al.* 2001).

Evidence for two sex ratio adjustment mechanisms

One question is, how do fig wasps adjust their sex ratio in response to the environment? Two proximal mechanisms have been proposed for adjusting the sex ratios. By laying most or all of their sons first followed by daughters, a fortuitous byproduct is that their sex ratio is increased, this is also known as the constant male hypothesis (CM). This is due to a constraint on the foundress's clutch size, and the alteration is a byproduct (Chung *et al.* 2019, Kinoshita *et al.* 2002, Kjellberg *et al.* 2005a), an exaptation. Alternatively, foundresses may sense other foundresses and adjust the number of males they lay this is known as a facultative strategy or facultative male adjustment (FMA) (Fellowes *et al.* 1999, Frank 1985, Herre 1985, Herre 1987, Moore *et al*. 2002, West and Herre 1998a, West *et al*. 2000). Now I will look at the support for each mechanism.

With the CM hypothesis if more foundresses enter a fig the number of eggs a foundress can lay based on the space available can be less. As clutch size increases, the sex ratio tends to decrease (Kinoshita *et al.* 2002, Kjellberg *et al.* 2005a). This will contribute to a less female-biased sex ratio at higher foundress numbers as fewer females are laid in comparison to the males. Thus, clutch size could be a constraint and inadvertently change the sex ratio of a foundress. Kjellberg *et al.* (2005a) set out to test these predictions using species with large brood sizes of up to 400 offspring. The six pollinating wasp species used were: *Pegoscapus tristani*, *Courtella gabonensis*, *Alfonsiella fimbriata*, *Alfonsiella pipithiensis.*, *Allotriozoon heterandromorphum* and *Kradibia tentacularis*. They found that with decreasing clutch size, there was a higher proportion of males being produced. Also, when foundresses compete with one another, the brood size decreases. This could be the explanation for why the sex ratio responds to an increase in foundress density.

Another case for the where sex ratio adjustments are best understood as a fortuitous byproduct was described by Moore *et al.* (2005). They investigated the sex ratios of *Lipporrhopalum tentacularis* (now called *Kradibia tentacularis*) using microsatellite markers. They experimentally introduced two foundresses into a fig (in increments of half an hour starting at 0.5 and up to 1.5 hours). The clutch sizes of the two foundresses were then compared to single foundress clutches from Moore *et al.* (2002). The two foundress broods showed that each foundress's contribution to the brood is unequal. Also, there was a negative relationship between sex ratio and clutch size (Generalised linear mixed model was used). They found that the foundresses use clutch size as a cue rather than foundress density. The increase in brood sex ratio with density is not a direct response to other foundresses but is rather due to the accompanying average clutch size that decreases. It seems that the sex ratios are generally more female-biased than predicted by the theory of local mate competition. The authors developed a model where clutch size is the only cue used. The model is in qualitative agreement with their data. For instance, large clutches occur mostly in low-density broods and have high brood contributions which implies high LMC.

Another study that looked at clutch size as a cue was done by Raja *et al.* (2008) using *Kradibia tentacularis*. They also took a closer look at the clutch size composition to see whether male eggs were produced first as this will affect the sex ratio solely due to oviposition site limitations. Since a wasp that re-emerges after oviposition in its first fig will be able to enter and oviposit a second fig. Experiments were performed by inserting one, two or three foundresses within a receptive fig for foundresses depositing their first clutch. The experiment was repeated by introducing only foundresses that were about to deposit their second clutch (re-emerged). They found that females only increase their sex ratio if oviposition sites are limited and not as a direct response to foundress density. Foundresses that re-emerged had no site limitation in multi-foundress fig and did not increase their sex ratio. In contrast, foundresses depositing their first clutch was limited by the number of oviposition sites in a multi-foundress fig and had increased sex ratios. To determine the sequence of egg deposition, insecticide was used periodically to terminate oviposition. This was performed on foundresses depositing their first clutch as well as those depositing their second clutch. Male eggs were produced predominantly at the start of bouts, followed by mainly females interspersed by some males.

The constant male hypothesis was shown by Ramirez-Benavides *et al.* (2009) using two fig wasp pollinator species: *Pegoscapus silvestrii* and *Pegoscapus tonduzi*. Their sample size consisted of 25 or more syconia and two trees per species. The male number per foundress did not differ between one, two, three and four foundresses per in *P. silvestrii*. The same applied for *P. tonduzi* apart from syconia with unusually high numbers of foundresses. In *P. tonduzi*, five foundresses and above produced almost all-male broods. Overall the number of males increased with the number of foundresses. The number of females, on the other hand, decreased. This is because both wasp species lay male eggs first independently of clutch size and the number of foundresses. The results show that the wasps do not adjust their sex ratios by laying more male eggs when multiple foundresses occur. The study provides evidence of the constant number of males hypothesis and not foundresses using cues such as another female's sex ratio, presence, clutch size or other environmental conditions (Ramirez-Benavides *et al.* 2009).

Although Kjellberg *et al.* (2005a) did not look at the adjustment of sex ratios, they found clear evidence in six pollinating species that sex ratios decrease as clutch size increases.

Finally, Chung *et al.* (2019) developed a model for passive allocation and tested this against seven pollinators. Their model made predictions and fitted observed sex ratios of single foundresses very well. One species, *C. arabicus*, was the exception, and since I look at *C. galili,* which also oviposits in *F. sycomorus*, this study will throw some light on this finding.

I will now present support for the facultative mechanism. An earlier study on the same species used by Ramirez-Benavides *et al.* (2009); *Pegoscapus tonduzi*, was studied by Pereira and Prado (2006). They focused on the offspring sex ratio in relation to foundress density and compared their data to theoretical values. They found a positive correlation between sex ratio and foundress number.

There was also a positive correlation between sex ratio and brood size, but this relationship was weaker than with foundress density. Since the correlation was stronger for foundress density, it suggests that brood size is not necessarily the determining factor for the change in sex ratio, but instead foundress density was. This is in contrast to the study by Ramirez-Benavides *et al.* (2009). It is possible that the wasp utilises both strategies, but further investigation would be required to decide. Their data, however, was more female-biased than predicted. This could be because models assume that all foundresses deposit the same number of eggs. Since *Pegoscapus* females frequently fight (Dunn *et al.* 2014), fighting could result in unequal clutches and lower sex ratios. Another factor might be that the experimentally introduced number of foundresses differ from foundresses' selective regime (Pereira and Prado 2006). The MEN (male egg number) model of Chung *et al*. (2019) could also explain this observation.

In *K. tentacularis* Moore *et al.* (2002) looked at the mechanism involved with sex ratio control. They found that their results were not consistent with just one hypothesis but that both the FMA and CM hypotheses were supported by the data. Specifically, there is a positive relationship between the number of males and the number of females in a brood at each foundress density. This is an indication that the male number is adjusted facultatively and that the two variables are not independent of one another, as is the case with the CM hypothesis. There was a negative relationship between brood size and sex ratio, which is indicative of the CM hypothesis as the sex ratio should be independent of clutch size for a facultative strategy.

Kinoshita *et al.* (2002) studied the pollinator *Blastophaga nipponica* where one female was fertile, and another was rendered sterile. Sterility was achieved using gamma rays to irradiate the female, causing her to oviposit normally, but the eggs do not develop. The sequence of entry and time intervals for entering was manipulated. They found that each foundress contributed a different number of offspring and sex ratios to the total brood. They introduced foundresses sequentially but with different time intervals (0.5, 4 and 24 hours). They also changed the sequence of entry by introducing an irradiated female first followed by a normal female and vice versa. They compared this to two normally ovipositing foundresses introduced in the same way and a single foundress. This allowed them to observe how each foundress behaves in different scenarios. Two normal foundresses compared to each other and a single foundress produced sex ratios that are similar between different intervals and higher than the lone foundress's sex ratio. At half an hour when two foundresses are present, the clutch size is twice that of a lone foundress whilst with longer interval periods the clutch size starts to decrease. The four-hour interval showed that the sequence of entry and clutch sizes had a significant effect on the sex ratio. After 24 hours, the only factor affecting the sex ratio was the clutch size. A mother adjusts her sex ratio facultatively if two females enter within 0.5 hours from each other. However, after 24 hours, when there is no contact between foundresses, the sex ratio is only influenced by the clutch size, where the second female lays a smaller clutch (Kinoshita *et al.* 2002).

Another study that supports the notion for both strategies was by Hu *et al.* (2013) using the wasp *Ceratosolen solmsi*. Hu *et al*. (2013) stopped oviposition using warm water. The number of males and females both increased for each treatment with the majority of males laid at the start of the bout, followed by mostly females. To test the effect of foundress density on sex ratio, they used multiple foundress figs (1-5 foundresses). To ensure oviposition sites are not limited, they ceased oviposition for one and three foundress figs. The sex ratios for the different treatments were significantly different and increased with foundress density. The ceasing of oviposition provided one and multi-foundress wasps with similar clutch sizes. They could thus confirm that sex ratio increased with density regardless of the clutch size. The wasp *C. solmsi* will adjust its sex ratio to the foundress density by using the information of other foundresses instead of clutch size when oviposition sites are not limited. However, when oviposition sites are limited, their sex ratio will be adjusted automatically due to a reduced clutch size (Hu *et al.* 2013).

Although there are many papers on sex ratio, they have not actively compared these two mechanisms and cannot explicitly claim that their data support either mechanism. However, it seems as if most species have a constraint on their clutch size, and as a byproduct, their sex ratios are altered, but that sometimes an active or facultative strategy will also be used (Chung *et al.* 2019).

Too many females

As noted earlier, it is common that sex ratio data are more female-biased than theory predicts (Kinoshita *et al.* 1998, Moore *et al.* 2005, Pereira and Prado 2006). There are several optimality models that have good qualitative predictions but might lack quantitative accuracy (Greeff and Newman 2010). One potential reason is that the assumptions underlying the equations are unrealistic. For instance, unequal clutch sizes will increase inbreeding (Frank 1985). Alternatively, multiple species can co-occur and be treated as one (Molbo 2003). Or fighting may reduce the effective number of mothers (Dunn *et al.* 2014). Some females may enter after the first is dead, resulting in a very skewed sex ratio by the first female, which will require a minor adjustment by the second (Greeff and Compton 1996). Although this would affect the predicted sex ratio, it would not affect the general trend of a decrease or increase.

The MEN model developed by Chung *et al.* (2019) that considers only the CM byproduct adjustment predicts sex ratios at lower foundress numbers that are too female-biased. A general trend of automatic sex ratio adjustment through CM could thus explain why sex ratios are more female-biased than models that assume exact sex ratio adjustments.

It should be noted that several factors can result in less female-biased sex ratios – in naturally entered figs, some foundresses may have left after oviposition. If figs with more foundresses produce more females per fig, inbreeding will be less than calculated in Herre (1985), and the ratio would be less skewed.

Alternatives to LMC

Alternative mechanisms can also result in skewed sex ratios. Although these do not predict less bias with an increasing foundress number, for instance, cytoplasmic elements are only inherited via females, and organisms such as *Wolbachia* can then be expected to bias sex ratios towards daughters. However, Ahmed *et al.* (2013) found no link between female bias and the presence of *Wolbachia* in 17 fig wasp species.

Males only pass their DNA to daughters and would thus benefit if they can cause females to skew their sex ratio towards males. Greeff (pers. comm.) did not find any evidence for male effects in *P. awekei* as the number of times females were mated had no effect on the sex ratio. On the other hand, Peng *et al.* (2005) found that sex ratios are lower when females were mated multiple times.

Although still driven by LMC, sequential oviposition can also influence the outcome (Kathuria *et al.* 1999, Moore *et al.* 2002). Sequential oviposition results in unequal clutch sizes and a sequential, rather than a simultaneous game that has a less biased solution (Greeff and Compton 1996, Kathuria *et al.* 1999, Moore *et al.* 2002). Thus, females that arrive later will be limited by oviposition sites and produce smaller clutches that are less female-biased. Also, the first foundress to enter will not have knowledge of foundresses to follow and thus will have a more female-biased clutch (Greeff and Compton 1996, Moore *et al.* 2002).

Realized (adult) sex ratios do not necessarily reflect the primary sex ratio as mortality rates and double oviposition can modify them. It is assumed that wasps avoid laying in ovules that already contain eggs. However, there are few empirical data to confirm this due to limited counts of eggs in flowers (Jousselin *et al.* 2001). If double oviposition were to take place, then there is possibly intense competition between developing larvae as only a single adult wasp usually emerges. Bladders are empty galls that are common in figs; these empty galls are presumed to be eggs that did not survive, and thus the adult sex ratio does not reflect the primary sex ratio. The wasp *Kradibia tentacularis* was used by Ghana *et al.* (2012) to compare oviposition and wasp emergence frequencies in the absence of parasitoids. This showed that most galls that failed to produce wasps had eggs within. Few ovules contained two eggs (0.63%); thus, double oviposition is not responsible for the difference in primary and realized sex ratios. It is also irrespective of whether it is her egg or another female's egg. The number of flowers that contained eggs were about 90% and of these about a third of the larvae died in the absence of parasitoids.

Ficus sycomorus model

Some *Ficus* species present novel opportunities to test ideas. One example is *Ficus sycomorus* which is associated with three species from the family Agaonidae that all enter the fig and oviposit from within the fig. One of these, the seed galler *Sycophaga sycomori*, is in the subfamily Sycophaginae, whereas the other two are in the subfamily Kradibiinae (Compton *et al.* 1991). The one, *Ceratosolen arabicus*, is the pollinator, whilst the other, *C. galili*, is a cuckoo wasp. *Ceratosolen galili* has pollen pockets but does not show any of the associated pollinating behaviour. Both wasps are common in the southern part of Africa, especially in humid conditions on the eastern side. The wasp *C. arabicus* is light brown and approximately, 2.5mm long. The female has a depressed head with a thick first antennal segment and a hook on the third segment (Galil and Eisikowitch 1968). The ovipositor is relatively short, averaging at 0.9mm (Nefdt 1992). The male is light brown and wingless with vestigial eyes. The head is oval with strong mandibles, and the abdomen is tube-like. The female wasp actively pollinates the tree by using its front legs to pick up pollen from the male flowers and putting it inside their pollen pockets before leaving their natal figs. After each oviposition event, the female unloads the pollen before pulling out the ovipositor from the flower (Galil and Eisikowitch 1974). The female *C. galili* is similar in size and morphology to *C. arabicus* with the main difference being the colour which is dark brown or almost black (Galil and Eisikowitch 1968, Wiebes 1968). The male is very similar to *C.*

arabicus and difficult to distinguish from it. The dorsal views of wasps can be used to distinguish between males. The pronotum of *C. arabicus* has striations on the anterior lateral side whilst *C. galili* has a smooth pronotum. The head shape of *C. arabicus* is more oval than that of *C. galili*, which narrows towards the front (almost flask-shaped) (Simon Van Noort personal communication).

Uncooperative pollinating wasps can be sanctioned by the tree by having unpollinated figs aborted (Jander and Herre 2010), or higher larval mortalities (Jansen-González *et al.* 2012). Compton *et al*. (1991) found no evidence that *C. galili* only entered pollinated figs, nor that *F. sycomorus* reduced offspring survival. Compton *et al*. (1991) found that *C. galili* is smaller than *C. arabicus,* and this may be a result of sanctions. They also found that the sex ratio of *C. galili* is similar to that of *C. arabicus,* with approximately 70% of offspring being female. Given that *C. galili* has the biofacies for LMC (Hamilton 1967) we expect a similar sex ratio adjustment as in pollinating wasps.

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Chapter 2. Adaptive and maladaptive sex ratios in a pollinating fig wasp

This chapter is based on introductions and collections that were made by Marié Warren in the Kruger National Park. I dissected the figs, counted the wasps, analysed the data and wrote the first draft. Since it is written as a manuscript, there is some overlap with the literature review.

Abstract

Local mate competition selects for female-biased sex ratios. Fig wasps are known to adjust their sex ratios with surprising accuracy in line with optimal predictions. The wasps seem to rely on one or both of two proximal mechanisms to adjust sex ratios in line with LMC predictions. Females may fortuitously adjust their sex ratio by laying all or most of their sons first followed by daughters. If the clutch size is a constraint for the mother, her sex ratio will be adjusted without her having changed her behaviour. Alternatively, mothers may sense the number of mothers and actively change the fraction of eggs they fertilize, a so-called facultative adjustment. We studied sex ratio adjustments by the cuckoo wasp, *Ceratosolen galili* that oviposit its eggs in the ovules of *Ficus sycomorus*, which is pollinated by *Ceratosolen arabicus*. In order to determine whether *C. galili* uses a facultative strategy or whether adjustment is just a byproduct and whether optimality is compromised by multiple species entering figs for oviposition, we quantified sex ratios of *C. galili* in controlled experiments with one, two or three foundresses within a fig as well as for figs with both *C. galili* and *C. arabicus.* Clutch sizes and foundress counts were quantified to understand the role that the two proximal mechanisms play and the selective regime of the species. The foundresses facultatively adjusted their sex ratios when another female is present. This is the only source of adjustment when there are only two foundresses, as they do not saturate the fig with eggs. However, as soon as there is a third female, the mothers' clutches are constrained, and sex ratio adjustment due to the fortuitous effect biases the sex ratio further. Even though *C. galili* mothers often share figs with *C. arabicus*, they erroneously make a facultative adjustment to their sex ratios in the presence of *C. arabicus*.

Introduction

Fisher stated that in a large randomly mating population, both sexes would have an equal parental investment (Fisher 1930). Hamilton (1967) noticed that when a population is not panmictic, the sex ratio may be skewed towards investing more into one sex. Specifically, the model developed by Hamilton considered a fixed number of inseminated females that colonized a patch, mating occurs within the patch, and the newly inseminated females disperse to other patches whilst the males remain stationary (Taylor and Bulmer 1980, Pereira and Prado 2006). In this scenario, a female-biased sex ratio is favoured by selection (Hamilton 1967).

Fig wasps are an excellent model system to observe skewed sex ratios as the males remain within the natal fig whilst the females disperse to new figs (Herre 1987). These haplodiploid species can control their sex ratio by either fertilizing an egg or not, using sperm that is stored in the spermatheca (Flanders 1956). In addition to conforming to the requirements of local mating, pollinating fig wasps can thus also potentially adjust their sex ratios. Furthermore, controlled experiments can be conducted on wild populations, and there are multiple species to study (Frank 1985).

Hamilton (1967) arrived at the unbeatable sex ratio of $c = (n - 1)/2n$, with n being the number of foundress females in the patch. Females that crawl into the fig to lay their eggs are called foundresses and theory predicts female-biased sex ratios (Figure 2.1; Hamilton 1967). With $n = 1$, a sex ratio of zero is predicted which in fact means that the mother should lay just enough sons to fertilize all her daughters and create an exit tunnel (Galil and Eisikowitch 1968, Hamilton 1967, Hamilton 1979). As the number of foundresses increases, the predicted sex ratio approaches 0.5 asymptotically (Figure 2.1; (Werren 1983). The ultimate reason for the female bias when few foundresses are present is that when a son is exchanged for a daughter, the sons will compete less and her remaining sons will have more expected matings (Taylor 1981). This is termed local mate competition (LMC) (Hamilton 1967).

Hamilton's (1967) model has been supported by numerous field studies and experiments, in many organisms, including fig wasps (Herre *et al.* 2001).

Hamilton (1979) adjusted his theory for the relatedness skew in haplodiploids where inbreeding occurs. His new formula enables the evaluation of the unbeatable sex ratio for any given values of inbreeding and mean foundress number.

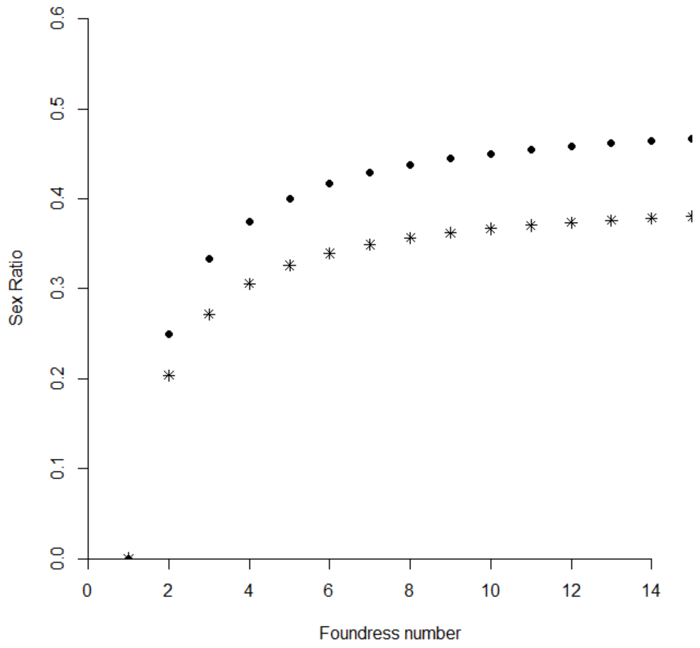

Figure 2.1. Predicted sex ratios for various foundress densities with Hamilton's original equation (filled circles) and with Herre's equation when the harmonic mean number of foundresses is 1.6 (asterisks; Equation 1 explained below).

Using a *Blastophaga* species where foundress number can be determined by counting foundress females' remains, Hamilton (1979) confirmed that offspring sex ratio correlated with foundress number. Even though the observed sex ratio was more female-biased than predicted, it increased with increasing foundress number (Hamilton 1979). A potential reason for the difference between predicted and observed sex ratios is the "earliness" of entering or female aggression which can cause unequal broods and thus a higher relatedness in the progeny. The two *Blastophaga* species studied both are now called *Pegoscapus,* and these pollinators are known for severe fighting and killing of other foundresses (External examiner pers. comm.). This leads to an overestimate of the actual number of foundresses that laid clutches. For example, in a fig that contains three dead foundresses, only two may have oviposited. To calculate the optimal sex ratio, Herre (1985) derived the formula:

$$p= (1-m)(2n_h-1)/(4n_h-1) \qquad \text{(equation 1),}$$

where n_h is the harmonic mean number of foundresses and m is the proportion of one mother's offspring contribution to total brood size (the inverse of the number of foundresses within a fig; Figure 2.1)(Herre 1985). If n is the foundress number for a specific fig and all females lay clutches of equal size, then equation 1 can be written as $p = [(n-1)/n][(2n_h-1)/(4n_h-1)]$. If there is no inbreeding, the second square bracket is equal to ½, simplifying to Hamilton's (1967) result. Equation 1 allows for different intensities of LMC for a given level of inbreeding as well as different levels of inbreeding for a given LMC intensity to be calculated (Herre 1985).

The female wasp relies on cues around her to assess the environmental conditions and depending on the environment will use these cues to adjust her behaviour in order to gain maximum fitness (Burton-Chellew *et al.* 2008). Using density as a cue in the fig the female can evaluate the potential local mate competition within the fig and adjust her sex ratio accordingly to produce the optimal sex ratio (Moore *et al.* 2005).

This raises a question around the mechanism used to adjust the sex ratio, and currently, there is a debate as to which mechanism is correct. Females may simply lay all or most of their future sons first followed by females and as a byproduct, can alter the sex ratio if there is a constraint on clutch size (Kinoshita *et al.* 2002). If individual clutch sizes are smaller as more mothers enter a fig, the sex ratio will, with this simple rule, adjust automatically to become less female-biased as foundress number increases (Kjellberg *et al.* 2005a, Raja *et al.* 2008). The clutch size is thus a constraint for the wasp causing the female to "inadvertently" change the sex ratio of her clutch. This fortuitous byproduct relies on the same number of males being laid regardless of the total number of offspring (Moore *et al.* 2002) and is an exaptation (Gould and Vrba 1982). Alternatively, females may sense the number of females and facultatively produce a larger fraction of sons if more females are present (Fellowes *et al.* 1999, Frank 1985, Herre 1985, Herre 1987, West and Herre 1998a, West *et al.* 2000). In this case the sex ratio should be independent of the brood size (Moore *et al.* 2002).

In figs, the fortuitous effect will not work in cases where more than one species enter the fig and compete with one another for oviposition strategies. While this situation is not found in the new world, it occurs regularly in African fig wasps. For instance, the majority of *Galoglychia* figs in Africa have sycoecines that enter and oviposit within the figs. African *Ficus* in the subgenus *Sycomorus* are frequently entered by *Sycophaga* species, but it is not the case in the Asian *Sycomorus* (Van Noort 2019). In addition, many figs host cryptic pollinator species (Molbo 2003) that will also result in an erroneous adjustment.

Here we aimed to see if the fig wasp, *Ceratosolen galili*, that compete with other species while ovipositing inside figs uses a facultative strategy to adjust their sex ratios or whether it is a byproduct of clutch size constraint. We test whether optimality is compromised by having foundresses from multiple species. We confirmed that the species commonly co-occur in figs in natural conditions. We quantified the sex ratios of *C. galili* mothers in controlled experiments with one, two or three foundresses within a fig as well as for figs with both *C. galili* and *C. arabicus.*

Materials and Methods

Predictions

We expect that if sex ratio adjustment is only a byproduct, those sex ratios will only change once oviposition sites become limiting. Before oviposition sites become limiting, mothers will produce the same number of sons and daughters irrespective of the number of foundresses. Further, if sex ratios are adjusted as a byproduct, then the species identity of other mothers will be irrelevant, and adjustments will occur as soon as mothers' combined clutch sizes exceed the oviposition sites in a fig. If a facultative strategy is used, the sex ratio should be adjusted in cases of single species even if the combined clutch sizes of females do not fill all available oviposition sites. Furthermore, we expect that the number of sons per mother will increase and the number of daughters per mother will decrease if space is a constraint. In contrast, if it was a fortuitous byproduct, we expect that the number of sons per mother will remain the same, but that the number of daughters per mother will decrease as soon as space becomes a constraint. When more than one species enters a fig, we expected no change in the number of males per mother for both strategies, but if it was just a byproduct, then it will result in a decrease in daughters as soon as the fig's carrying capacity is exceeded.

Study species

We worked on the wasps associated with *Ficus sycomorus*, a monoecious fig tree. *Ficus sycomorus* occurs in tropical and subtropical Africa stretching from the south (South Africa) up to the north (Sudan and Ethiopia) and the southwestern part of the Arabian peninsula (Berg and Wiebes 1992). In the Kruger National Park in South Africa, it occurs naturally along the rivers in the park and is close to the southern distributional limit of the species (Warren *et al.* 2010). It is associated with three species from the family Agaonidae that all enter the fig and oviposit from within the fig. One of these, the seed galler *Sycophaga sycomori*, is in the subfamily Sycophaginae, whereas the other two are in the subfamily Kradibiinae (Van Noort and Rasplus 2019). The one Kradibiinid wasp, *Ceratosolen arabicus*, has golden coloured females and is the bona fide pollinator, whilst the other Kradibiinid, *C. galili*, is darker in colour and is one of two cuckoo wasps in *Ficus* (Galil and Eisikowitch 1968, Wiebes 1968). *Ceratosolen galili* is known as a cuckoo because, even though it is a pollinator in terms of its phylogenetic position (Kerdelhue *et al.* 1999) and have pollen pockets, it does not collect nor disseminate pollen (Compton *et al.* 1991). Both *Ceratosolen* wasps are common in the southern part of Africa, especially in humid conditions on the eastern side (Wiebes 1968). *Ceratosolen galili* is not dependent on a fig that already contains *C. arabicus* and seems to avoid figs containing *C. arabicus*, rather than prefer such figs (Rasplus 1996). *Ceratosolen galili* and *C. arabicus* fly at different times of the day (Warren *et al.* 2010), and as a result, they share figs less frequently than would be expected at random (Compton *et al.* 1991). Although the two species avoid each other, it is still common for the two species to co-occur (Compton *et al.* 1991) and the byproduct of a constrained clutch size will result in an erroneous adjustment. Since *C. galili* shows all the hallmarks of a species that have local mate competition (Hamilton 1967) it should produce female-biased sex ratios.

Typical wasp densities

We estimated female wasp densities at two time points, September to October 2005 (19 trees) and January to February 2006 (11 trees), from two rivers, the Sabie (17 trees) and Nwatsitsontso (13 trees) in the Kruger National Park, South Africa. From each of the 30 trees, we collected 50 early C-phase figs. Early C-phase figs are figs where the foundresses are already dead inside the fig, but they entered the figs recently (Galil & Eisikowitch, 1968). Figs were halved and the number of *C. galili*, *C. arabicus* and *S. sycomori* foundresses were counted under a dissecting microscope.

To see if *C. galili* prefers or avoid entering figs with either of the other two wasp species we compared the numbers of figs containing either or both of those species in samples with *C. galili* to the respective frequencies without *C. galili* using a χ^2-test. All statistical analyses were done using R version 3.1.3 (2015-03-09).

To test more specifically if *C. galili* and *C. arabicus* co-occur less frequently than expected, we calculated the fraction of times each species occurred (f_{Ca}, f_{Cg} and f_{Ss} respectively) in the 1288 scored figs. Figs with none of the species will either abort or receive wasps during a subsequent time interval. Therefore, the expected random associated frequency was calculated as the product of each separate scenario divided by $(1 - (1-f_{Ca})(1-f_{Cg})(1-f_{Ss}))$. For instance, if wasps settled randomly, then figs containing *C. gallili* and *C. arabicus*, but not *S. sycomori*, can be expected to have a frequency of $(f_{Ca}f_{Cg}(1 - f_{Ss}))/(1 - (1 - f_{Ca})(1 - f_{Cg})(1 - f_{Ss}))$. To calculate how frequently deviations larger than the observed should be seen, the occupancy was randomized 10^6 times with respect to each other, and all occurrences equal to or greater to our observations were recorded.

Sex ratios

We used a single *F. sycomorus* tree in Skukuza Rest Camp in the Kruger National Park for the female wasp introductions. We bagged early stage A figs on 8 January 2008 in netted mesh bags, after checking that the figs were not yet receptive by attempting to enter female wasps. Fig receptivity was checked from 8-15 January 2008. We collected live female wasps from stage D figs from two trees in Skukuza Staff Camp from 13 January to 25 January 2008 and allowed them to enter the bagged receptive figs in four different combinations (16-20 January 2008).

In three treatments, we entered either one, two or three *C. galili* per fig to test if and how foundresses adjust their sex ratios. In a fourth treatment, we entered one *C. galili* foundress and one *C. arabicus* foundress per fig to test if and how *C. galili* foundresses adjust their sex ratios in this scenario.

We used between 18 and 25 figs per treatment on two to four branches each (2 - 12 figs per branch used, minimum of two branches per combination used). Three percent of the figs aborted before harvesting, which reduced the sample size per combination. We allowed the figs to develop to maturity and collected live wasps at stage D figs from 18 February 2008 to 21 February 2008. We collected figs from which wasps were ready to emerge and placed them in individual sampling jars (we tied small bags over individual figs, and in a few cases some wasps had already emerged into mini-bags at the time of collection, these were collected and added to the sampling jar). We took samples back to the field station and placed them into individual plastic jars with gauze secured with an elastic band at 27°C. We allowed the wasps to emerge; thereafter, the samples were frozen for later processing.

The figs were dried out by the time counting started (January 2015). A few drops of 10x phosphate-buffered saline solution (PBS) were added to the fig halves and when soft enough was cut further into quarters. PBS allowed fig tissue to become hydrated, swollen and soft without being tough, enabling easy removal of plant tissue. A dissecting microscope was used for identifying, counting and sexing the wasps. The total number of males and females of each species were counted outside and a few still inside galls.

We distinguished between females based on morphology, focusing mainly on the colour of the body. The females of *C. arabicus* are yellow or light brown whilst those of *C. galili* are dark brown or almost black (Galil and Eisikowitch 1968). Males of the two species are tricky to distinguish (Compton et al. 1991) but Dr Simon van Noort provided us with diagnostic features to separate the two species. The pronotum of *C. arabicus* has striations on the anterior lateral side whilst *C. galili* has a smooth pronotum. The head shape of *C. arabicus* is more oval than that of *C. galili* which narrows towards the front (almost flask-shaped).

Wasps that cannot fertilize their eggs, due to too little sperm, for example, will produce an excess of males known as constrained allocation (West 2009). For this reason, figs with uncharacteristically high sex ratios were excluded from further analyses (although their number was recorded). To see if sex ratios increased as foundress number increased, a Generalized Linear Model was fitted explaining sex ratios as a factor of foundress number. We used a GLM with binomial errors, but due to over-dispersion, quasibinomial errors were specified, and the drop1 function with an F-test was used (Crawley 2007, Hardy and Wilson 2002). The overdispersion might be due to larvae mortality (Ghana *et al.* 2012). Since the overall GLM was significant (see below), we also compared the sex ratio of the one and two foundress treatments with one another as well as those of the two and three foundress treatments. To see if sex ratios were adjusted when a single *C. galili* and *C. arabicus* shared a fig, we fitted a GLM with all previous modifications comparing the sex ratio of single *C. galili* females with and without *C. arabicus*. The optimal sex ratio was calculated using equation 1 and the 95% confidence interval for one, two and three foundresses were calculated using the binom.test command in R.

In figs with more than one female, we can only count the brood size (sum of clutch sizes of all foundresses). To test if the clutch sizes differed, we performed an ANOVA with foundress number as the independent variable. We compared the average clutch sizes of females by dividing brood sizes by the number of foundresses. Since average clutch size was significantly affected by foundress number, we compared the clutch size of single foundress figs to the two foundress figs and the two foundress figs' clutch sizes with the three foundress figs using a Welch two-sample t-test. We also compared the clutch size of a lone *C. galili* foundress and a *C. galili* foundress with a *C. arabicus* female.

To determine if the sex ratio was increased due to an increase in the number of sons or a decrease in the number of daughters, or both, we used a Welch two-sample t-test. If there was a significant difference, we performed a one-tailed t-test specifying "less" or "greater" as an alternative hypothesis.

Results

Typical wasp densities

We were able to reliably count foundresses in 1288 figs, 331 of which contained a total of 963 *C. galili* females. About 50% of *C. galili* females share figs with at least one other species, and the majority of these are with *C. arabicus* (Table 1). The figs containing *C. galili* had almost identical occupancy ratios of only *S. sycomori* (0.27 vs 0.24), only *C. arabicus* (0.61 vs 0.62), or both species (0.14 vs 0.13) to those not containing *C. galili* (Table 2.1; χ^2-test: $\chi^2 = 0.63$, df = 2, P = 0.73), suggesting that *C.galili* does not seem to avoid or associate with either of these species. However, randomizations showed that the observed incidence of *C. arabicus* and *C. galili* being the only species were significantly overrepresented, whereas the frequency of their co-occurrence was significantly underrepresented (Excluding *S. sycomori*: $p < 1 \times 10^{-6}$; Including cases with and without *S. sycomori*: $p < 1 \times 10^{-6}$; Table 2.2). The lower than expected co-occurrence of the two species may be the result of an active avoidance but could also result from a temporal (the wasps fly at different times of the day (Warren *et al.* 2010) or spatial (densities vary between locations and between trees, pers. obs. J. M. Greeff) structuring in the population.

Table 2.1. A comparison of the frequencies of wasp abundance as numbers of figs and number of wasps for figs with and without *C.galili*.

C. arabicus or *S. sycomori* present	*C. galili* present					*C. galili* absent	
	number of figs	% of figs	% excluding neither	number of wasps	% of wasps	number of figs	% of figs
Neither	158	48		492	51	-	-
C. arabicus	105	32	62	267	28	594	62
S. sycomori	46	14	26	129	13	231	24
Both	22	6	12	76	8	132	14
Total	331			963		957	

Table 2.2. The percentages of expected and observed sharing of figs by wasps if wasps settled at random. Presence indicated by 1 and absence by 0. Numbers in brackets are the counts. All numbers were rounded to the nearest unit.

Species present			Expected	Observed	More/less frequent
Ca	Cg	Ss			
1	0	0	39 (506)	46 (594)	more
0	1	0	7 (89)	12 (158)	more
0	0	1	20 (258)	18 (231)	less
1	1	0	14 (175)	8 (105)	less
1	0	1	20 (255)	10 (132)	less
0	1	1	3 (45)	8 (105)	more
1	1	1	7 (88)	2 (22)	less

The harmonic mean number of *C. galili* foundresses for the 331 figs that contained them was 1.62, and the mean number of foundresses was 2.9. Although one fig contained 23 *C. galili* females, the majority of figs contained fewer foundresses (Table 2.3). Even so, a substantial number of figs, 56%, had more than 2 foundresses (Table 2.3). Only 19% of *C. galili* foundresses were in figs containing less than 3 foundresses (Table 2.4).

Table 2.3. The number of 263 figs that did not contain *S. sycomori* that contain a specific number of foundresses of each *Ceratosolen* species. Percentages rounded to nearest 1 below numbers in italics; left open in case it equals 0. Bold type indicates figs with less than three foundresses. The total percentage of figs containing a certain number of *C. galili* females are given in the final row.

Number of foundresses															
	C. galili														
C. arabicus	1	2	3	4	5	6	7	8	9	10	11	12	17	19	23
0	**65**	**22**	25	16	8	5	4	1	4	5	1	0	1	0	1
	25	***8***	*10*	*6*	*3*	*2*	*2*		*2*	*2*					
1	**30**	14	6	3	3	4	1	1	0	0	0	0	0	0	0
	11	*5*	*2*	*1*	*1*	*2*									
2	13	3	1	5	0	1	0	0	0	1	0	1	0	0	0
	5	*1*		*2*											
3	7	1	0	0	0	1	0	1	0	0	0	0	0	0	0
	3														
4	2	0	1	0	0	0	0	0	0	0	0	0	0	1	0
	1														
5	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total % of *C. galili*	46	16	12	10	4	5	2	0	2	2	0	0	0	0	0

Table 2.4. The number of 758 *C. galili* females found in 263 figs without *S. sycomori* that contain a specific number of foundresses of each *Ceratosolen* species. Percentages rounded to nearest 1 below numbers in italics; left open in case it equals 0. Bold type indicates figs with less than three foundresses. The total percentage of females in figs containing a certain number of *C. galili* females are given in the final row.

	Number of foundresses														
	C. galili														
C. arabicus	1	2	3	4	5	6	7	8	9	10	11	12	17	19	23
0	**65**	**44**	75	64	40	30	28	8	36	50	11	0	17	0	23
	9	*6*	*10*	*8*	*5*	*4*	*4*	*1*	*5*	*7*	*1*		*2*		*3*
1	**30**	28	18	12	15	24	7	8	0	0	0	0	0	0	0
	4	*4*	*2*	*2*	*2*	*3*	*1*	*1*							
2	13	6	3	20	0	6	0	0	0	10	0	12	0	0	0
	2	*1*		*3*		*1*				*1*		*2*			
3	7	2	0	0	0	6	0	8	0	0	0	0	0	0	0
	1					*1*		*1*							
4	2	0	3	0	0	0	0	0	0	0	0	0	0	19	0
														3	
5	1	2	0	4	0	0	0	0	0	0	0	0	0	0	0
				1											
6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total % of *C. galili* females	16	11	13	13	7	9	5	3	5	8	1	2	2	3	3

Sex ratios

Of the 34 figs that were counted three had high sex ratios and were removed (1: 0.63 in two *C. galili* foundress treatment, 2: 0.89 in mixed-species treatment and 3. 0.54 in mixed-species treatment). The 31 figs included in the final analyses, were ten figs single foundress, and six for the two and three foundress treatments, and nine figs for the mixed-species treatment.

We found a significant increase in sex ratio when foundress numbers were increased (GLM: F=21.17, df=3, p = 2.93×10^{-7} for all treatments, GLM: F=25.28, df=2, p=4.43×10^{-6} for same species treatments; Figure 2.2; Table 2.5; Table 2.6).To understand sex ratio adjustments, one needs to look at it in the light of clutch size and composition. There was a significant difference in the clutch sizes of the different treatments (Overall species: ANOVA: $F_{3, 27}$ =3.40, p=0.03; Excluding mixed-species clutches: ANOVA: $F_{2, 19}$ =5.85, p=0.01; Figure 2.3). Sex ratio changes may thus be a byproduct of a constraint on clutch size.

Table 2.5. Predictors with corresponding coefficients and significance values in the GLM explaining sex ratios of *C. galili* treatments as well as *C. galili* and *C. arabicus* treatment. Back transformed sex ratios were calculated using $y=e^a/1+e^a$ with "a" being the coefficient.

Predictor	Coefficients	Back transformed sex ratio
Single foundress/ Intercept	-1.86	0.13
2 Foundresses	-1.86+0.36	0.18
3 Foundresses	-1.86+0.81	0.26
Mixed species	-1.86+0.65	0.23

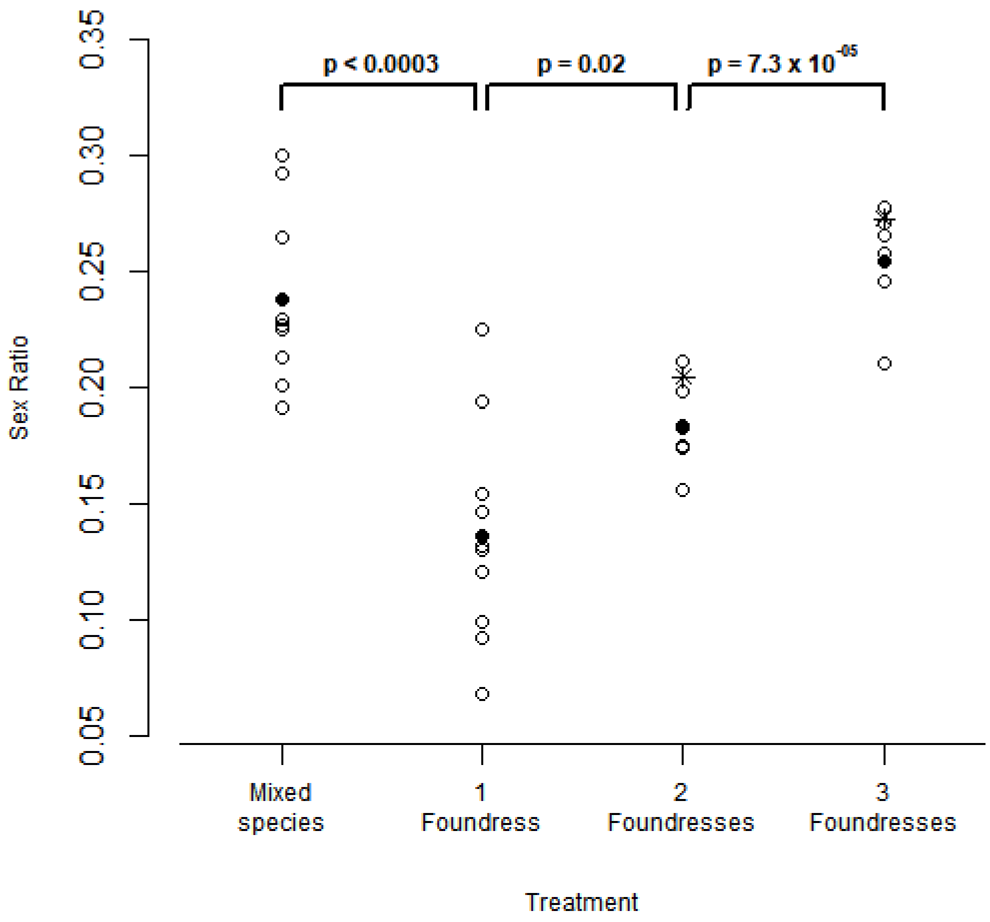

Figure 2.2. The mean sex ratio (total number of males divided by brood size) of *C. galili* for each treatment and significant values in bold. Open circles = sex ratio per fig, solid circles = mean sex ratio and asterisks = optimal sex ratio.

Single foundresses produced significantly more female-biased sex ratios than two foundresses (GLM: F=6.89, df=1, p=0.02; Figure 2.2; Table 2.5; Table 2.6). Single foundresses produced clutches that are roughly the same size as average two foundress clutches (t= -1.59, df=12,41; p=0.14; Figure 2.3a; Table 2.6). However, the number of sons was not the same within these clutches as the lone foundress had significantly less (t= -3.59, df=13.56, “less” p<1.54 x 10^{-3}; Figure 2.3b; Table 2.6) sons but the number of daughters were approximately the same (t= -0.89, df=12.48, p=0.39; Figure 2.3c;

Table 2.6). A lack of change in clutch size and an increase in the number of sons suggest that the sex ratio adjustment from one to two females is facultative and not a just a byproduct of the constraint on clutch size.

Table 2.6. Mean and median values for each *C. galili* treatment's sex ratio, clutch size, number of daughters and number of sons. Also shown for the treatment of *C. galili* and *C. arabicus*.

Treatment	Sex ratio mean	Sex ratio median	Clutch size mean	Clutch size median	Daughters mean	Daughters median	Sons mean	Sons median
1 Foundress	0.14	0.13	202.7	210	175.4	177	27.30	24.50
2 Foundresses	0.18	0.18	232.6	226.5	190.3	188	42.25	42.75
3 Foundresses	0.26	0.26	153.3	168	113.55	124	39.72	44
Mixed species	0.23	0.23	184.4	189	142.1	151	42.33	43

Three foundresses produced higher sex ratios than two foundresses (GLM: F = 41.65, df = 1, $p = 7.3 \times 10^{-5}$; Figure 2.2; Table 2.5; Table 2.6). Two-foundress clutches were significantly larger than three-foundress clutches (t=3.38, df=9.06, $p< 8.03 \times 10^{-3}$; Figure 2.3a; Table 2.6). The number of sons was however not the reason for the changed clutch size, as it was approximately the same (t=0.40, df=6.29, p=0.70 Figure 2.3b; Table 2.6), rather, each mother in a two-foundress fig had significantly more daughters than each mother in a three-foundress fig (t=4.28, df=9.93, "greater" $p<8.15 \times 10^{-4}$; Figure 2.3c; Table 2.6). This suggests that the sex ratio adjustment between two and three foundresses is a fortuitous byproduct rather than facultative. It is important to note that the variance increase in the number of sons in three foundress treatment could be due to larvae mortality (Ghana *et al.* 2012).

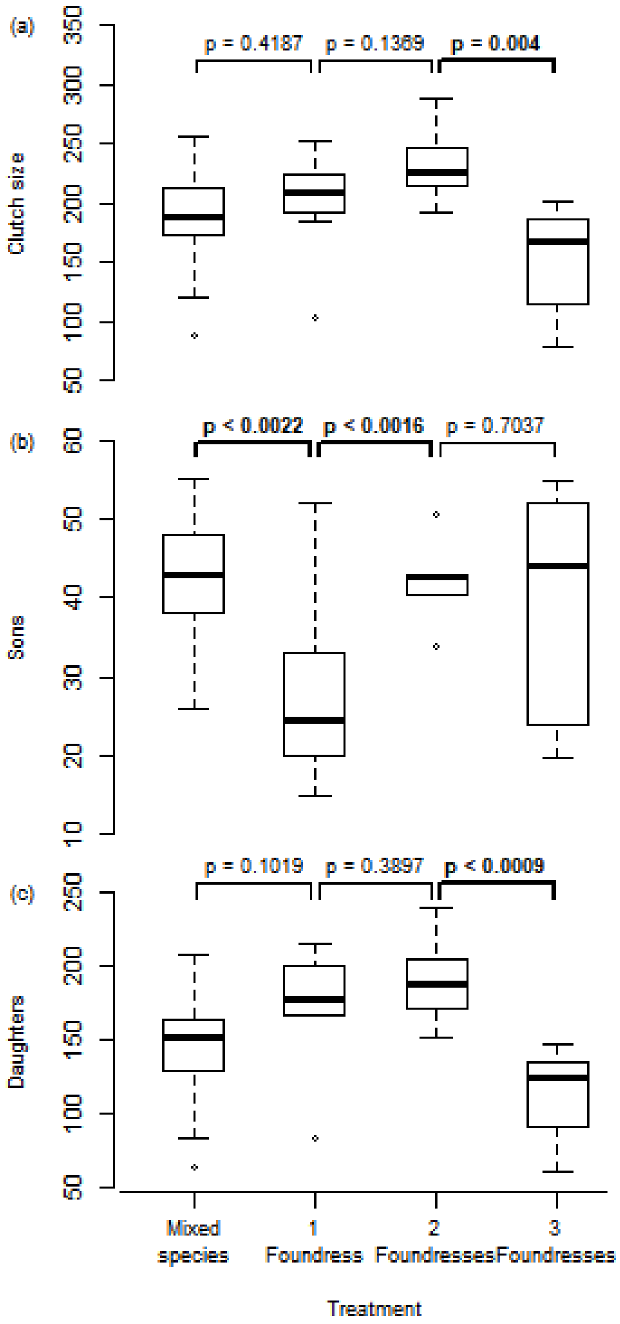

Figure 2.3. Boxplots of the (a) clutch size, (b) number of sons and (c) daughters for all *C. galili* treatments and the *C. galili* and *C.arabicus* treatment. Brood sizes were divided by the number of foundresses to give the average number per male. The black lines are the median, and boxes are the Range (Quartile 1 at the bottom and top is Quartile 3). Whiskers indicate the highest point within Q3+1.5R and the lowest point above Q1−1.5R. Significant values indicated in bold. Circles are outliers.

The *C. galili* females ovipositing in the presence of a *C. arabicus* females produced a higher sex ratio than the lone *C. galili* females (GLM: F=21.83, df=1, $p<2.19 \times 10^{-4}$; Figure 2.2; Table 2.5; Table 2.6). This sex ratio difference was not a byproduct as the clutch sizes did not differ significantly (t=0.83, df=14.97, p=0.42; Figure 2.3a; Table 2.6). Rather, the *C. galili* females ovipositing in the presence of *C. arabicus* produced more sons (t= -3.30, df=16.57, $p<2.16 \times 10^{-3}$; Figure 2.3b; Table 2.6) but a similar number of daughters (t=1.74, df=15.33, p=0.10; Figure 2.3c; Table 2.6). This indicates that the sex ratio adjustment was facultative rather than a byproduct. It also shows that the tree does not sanction the cuckoo wasp's number of offspring.

While the optimal sex ratio of the three foundresses fell within the 95% confidence interval of the observed sex ratios, two foundress figs' 95% confidence interval was just slightly more female-biased than the optimal sex ratio (Figure 2.2, Table 2.7). Although the model predicts a sex ratio of zero for a single foundress, the models assume that all females are mated which would require sufficient sons to fertilize and release the mothers' daughters (Hamilton 1967).

Table 2.7. Predicted and observed mean sex ratios for one, two and three foundress treatments of *C. galili*, along with their 95% confidence intervals.

Foundress number	Predicted	Observed	95% CI
1	-	0.14	0.12-0.15
2	0.21	0.18	0.17-0.20
3	0.27	0.26	0.24-0.28

Discussion

The cuckoo wasp *C. galili* seems to utilise a facultative strategy to adjust its sex ratios when going from one to more foundresses, a situation that presents itself in 54% of all figs and 84% of females. With the occurrence of more than two foundresses, a situation that arises in 38% of figs and which affects 73% of all females, the sex ratio is also altered as a fortuitous byproduct. Clutch sizes were not constrained in one and two-foundress figs but were constrained in three-foundress figs. Sex ratios should thus not be erroneously adjusted due to a byproduct if one *C. arabicus* foundress is added to a *C. galili* foundress. However, the sex ratio was erroneously adjusted through a facultative strategy. Even though these two species co-occur significantly less than expected (Table 2.2), 36% of *C. galili* females share a fig with a *C. arabicus* female (Table 2.1). Adjustment as a byproduct should thus be seen as an exaptation rather than an adaptation, and erroneous adjustment to another species should be considered a maladaptation.

Our findings are very similar to that of Hu *et al*. (2013), that found that the pollinating wasp, *C. solmsi*, also use both these mechanisms. The use of a facultative strategy in some species may explain why a purely byproduct model failed to explain sex ratio variation in some species (Chung *et al.* 2019). The reality of the byproduct adjustment is that it is unavoidable if wasps lay most of their male eggs first, as is the case for pollinating fig wasps (Nefdt and Compton 1996, Wang *et al.* 2015, West and Herre 1998b). Indeed sex ratios typically decrease as clutch size increase (Kjellberg *et al.* 2005a). As a result, sex ratios at low foundress densities will undershoot the optimal ratio to reduce the cost of overshooting it at higher foundress numbers. The male-biased sex ratios observed under unnaturally high foundress numbers in *C. fusciceps*, *C. solmsi*, *C. gravelyi* and *Eupristina koningsbergeri* (Wang *et al.* 2015), is the result of this unavoidable overshoot. This phenomenon could explain why the sex ratio of two foundress figs was too female-biased. However, this explanation cannot account for why *Blastophaga nipponica* produce too many daughters as the ratio remains low even at higher numbers and few foundresses were more common (average of 1.5 (Kinoshita *et al.* 1998)).

Another potential reason for the too female-biased sex ratio in two-foundress figs is that our underlying assumptions are unrealistic. For instance, if all females do not have equal clutches, the inbreeding coefficient can be higher, and the degree of LMC increased for the female with a bigger clutch which should result in a more female-biased ratio (Frank 1985, Kinoshita *et al.* 1998, Moore *et al.* 2002, Moore *et al.* 2005).

Our observations are consistent with findings of Kjellberg *et al.* (2005a) where the sex ratio is influenced by the clutch size, which acts as a constraint. Foundresses deposit a fixed number of males first followed by females and some intermittent males later. In multi-foundress figs several foundresses cause a reduction in clutch size due to a limited number of galling flowers, forcing the female to inadvertently adjust her sex ratio by laying fewer females (Raja *et al.* 2008, Wang *et al.* 2015). This has been seen in several wasps such as *Pegoscapus silvestrii* and *P. tonduzi* (Ramírez-Benavides *et al.* 2009), *Kradibia tentacularis* (Raja *et al.* 2008), *Ceratosolen solmsi* (Hu *et al.* 2013), *Blastophaga nipponica* and parasitoid wasps *Teleonomus remus*, *Telenomus heliothidis* and *Trichogramma evanescens* (Moore *et al.* 2005). Another factor that can influence the clutch size of a female is lifespan. If a female has a short lifespan, then she might not be able to lay all her eggs mimicking the lack of oviposition sites if the majority of males are laid first (Wang *et al.* 2015).

In addition to this byproduct of a constraint on clutch size, our and several other studies such as Kinoshita *et al*. (2002), Greeff & Newman (2010) and Hu *et al*. (2013) found evidence for facultative adjustment. Wang *et al*. (2015) performed experiments where foundresses were killed with ether an hour after starting oviposition or allowed the foundresses to continue until they are finished. All five species; *Ceratosolen fusciceps*, *C. solmsi*, *Eupristina koningsbergeri*, *C. gravelyi* and *C. emarginatus* deposited most of their male eggs within the first hour (66.1-70.1%). With the continuation of egg deposition, the number of sons decreases and the overall number of sons per foundress is 14.0% to 21.0%. This shows that sons can be added after the first bout and that mothers can facultatively adjust their sex ratios by choosing when to lay male eggs (Wang *et al.* 2015). Mothers of *P. awekei* use knowledge of their clutch size as well as the other foundresses' clutch size to adjust the sex ratio (Greeff and Newman 2010). The knowledge of using both strategies contributes to a facultative strategy to adjust sex ratios. The wasp *C. solmsi* will adjust its sex ratio facultatively to the foundress density by using the information of other foundresses instead of clutch size when oviposition sites are not limited (Hu *et al.* 2013).

From studies on other species it seems that foundresses can only make the facultative adjustment if the other female is alive (Greeff and Compton 1996, Wang *et al.* 2015) and possibly only if another female is present early on in the oviposition sequence (Kinoshita *et al.* 2002). Similarly, Wang *et al*. (2015) found a lower sex ratio when females enter after the first foundress died.

For the two-foundress case, the sex ratios are less female-biased than the one-foundress case but slightly less than predicted. This slight misfit may be the trade-off to avoid too high sex ratios at higher foundress numbers. Seeing as 11% of females find themselves in two-foundress figs whereas 73% of females are in figs containing more than two foundresses, such a trade-off seems expected. For the three-foundress treatment, the predicted value fell into the data's 95% CI. The observed and predicted values thus have a close fit. How should this fit be interpreted? It seems that when males are laid first, and the sex ratio is altered as a byproduct of constraint on clutch size, it is an exaptation. On the other hand, increasing the number of sons in line with LMC theory could be an adaptation. The wasps managed a high accuracy of adjustment. This is to be expected as the foundress densities tested are typical for the wasp. Our study reflected the selective regime of the wasp.

Given that two foundresses do not saturate all the oviposition sites in a fig, we expected that a *C. galili* sharing with a *C. arabicus*, should not adjust her sex ratio as this would be maladaptive. Nevertheless, they adjusted their sex ratio facultatively as if the *C. arabicus* was a conspecific. The cost of this facultative adjustment is that the foundress will have fewer mated daughters. This could have been expected if the wasps did not share figs commonly in nature, yet here 36% of *C. galili* females share a fig with a *C. arabicus* female. Given that this situation is more common than lone, two and three-foundress figs where selection has optimized wasp behaviour, it suggests that optimality is constrained by an inability to distinguish between the situations. This is surprising given the high degree to which females actively adjust their sex ratios. For instance, in the pollinator *Platyscapa awekei*, females adjust their sex ratios to other females' expected clutch sizes (Greeff and Newman 2010). This adjustment in *P. awekei* increases fitness by one extra egg, a tiny fraction of fitness that's successfully optimized.

If this scenario of two species only originated recently, there may not have been sufficient time for the correct mutants to have occurred. It is, however, not clear how long both species have co-existed on *F. sycomorus*. *Ceratosolen galili*, must have transferred horizontally to *F. sycomorus* as *C.arabicus* and *C. galili* are not sister species (Kerdelhue *et al.* 1999). In fact, these two species have probably been separate lineages for 40 Mya (Cruaud *et al*. 2012). While their distributions and microhabitat requirements are slightly different, they co-occur over the majority of their ranges (Warren *et al.* 2010). This observation should thus be considered a true maladaptation. One other example of reproductive interference of sex ratios is known from brine shrimp (Lievens *et al.* 2016), but there may be an alternative adaptive explanation in this case as Alpedrinha and Magalhães (2016) pointed out "lack of discrimination between conspecific and heterospecific females by brine shrimp males".

The frequency with which a particular situation occurs is called selective regime (Orzack and Sober 2001). It is well established that the selective regime is a crucial part of the optimality approach (Herre *et al.* 1997, Herre *et al.* 2001). For instance, it is no surprise that fig wasps produce suboptimal male-biased ratios when the foundress number is experimentally pushed way beyond the normal range (Wang *et al.* 2015). Similarly, when females fail to adjust their sex ratio to the relatedness of co-foundresses (Hu *et al.* 2010), it may be that this situation is not encountered frequently enough. It is clear that the selective regime is essential in fig wasp sex ratios as well (Herre *et al.* 2001). For instance, if the selective regime is more variable, then the adaptive behavioural plasticity is more developed in that species (Herre 1987). Thus foundresses that encounter variable selective regimes (single and multiple foundresses frequently) are more able to adjust their mean sex ratio to variable LMC as opposed to wasps that are used to a specific selective regime (West and Herre 1998b).

Compton *et al.* (1991) found an average foundress number of 5.66 in figs only containing *C. galili*. Here we observed an average of 3.11 for figs only containing *C. galili* and 2.83 overall. This may indicate temporal fluctuations in abundance of the two species over a long time but could also be the result of temporal and spatial fluctuations in abundance patterns over much smaller scales (JM Greeff pers. obs.). The selective regime in terms of average foundress numbers may vary, resulting in generally more plastic strategies, or slight maladaptations. For instance, since *C. galili* cannot suppress a facultative response in the presence of *C. arabicus* its strategy should be a slightly more female-biased compromise between actual two-foundress figs and one-foundress figs that happen also to have the congeneric species.

Zhang *et al*. (2004) argued that because females of rarer species should have lower foundress numbers, they should produce more female-biased sex ratios. This implies that rarer species will produce more daughters that in turn, implies that rarer species will have higher growth rates. Zhang *et al*. (2004) suggested that this creates a situation of frequency-dependent selection where rarer species may be protected from extinction by a mothers' sex ratio strategies. This may explain why so many *Ficus* species seem to support more than one pollinating wasp species, despite the wasps being in direct competition with each other. In this light, our observation of maladaptive sex ratio adjustment in the presence of a very distantly related species, suggest that frequency-dependent selection may not be a general explanation for pollinator co-existence on the same *Ficus* species.

Although Compton *et al*. (1991) found a suggestion that the two species compete with each other, this was not significant. Because we only looked at mixed species with two foundresses, the figs' carrying capacity was not exceeded, and our experiments were not able to determine if the two species compete with one another.

Since we did not weigh females, we cannot test if *F. sycomorus* sanctions *C. galili's* larvae as was suggested by Compton *et al.* (1991). However, we can confirm that with experiments where the foundress number has been controlled, *C. galili* mothers did not have larger clutches in the presence of *C. arabicus*. Thus, *C. galili* larvae's survival is not sanctioned by the trees because mothers do not pollinate.

In sex ratio models, a simplifying assumption that is made is that the number of females that leave a fig is independent of the number of foundresses (Herre 1985). In other words, a fig with two foundresses produces the same number of females as a fig with one foundress. This is not at all the case in our sample as mothers from two foundress figs produced the same number of females as one-foundress figs. Foundresses from three foundress figs produced about 60% the number of loan mothers (not 33%). The result of this deviation is that the calculated inbreeding will be more than it actually is. As a result, current predictions are thus more female-biased than they should be. If these inconsistencies were corrected, the current situation where observed sex ratio is too female-biased will be exacerbated.

Using a combination of observations and experimental introductions, we were able to show that sex ratio adjustment in line with LMC predictions is a result of a facultative strategy as well as a fortuitous byproduct. We also showed that it erroneously adjusts its sex ratio in the presence of another species in a situation that it encounters frequently. This should be considered a maladaptation. The information on clutch sizes indicates that even though *C. galili* does not pollinate, offspring number is not sanctioned by *F. sycomorus*.

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