SEX RATIOS IN HAPLODIPLOID HERBIVORES (ALEYRODIDAE AND
THYSANOPTERA): A REVIEW AND TOOLS FOR STUDY

by

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- **Abstract**

Studies confirming adaptive sex allocation, or a flexible relative investment in males and females, have been extensive in Hymenoptera, and have been used to predict the quality of biological control agents, to theorize how eusociality is maintained, and to predict population dynamics. Most hymenopterans are haplodiploid, in which females are derived from fertilized eggs and males from unfertilized eggs, so haplodiploid mothers may have a greater ability to adaptively adjust sex allocation. Thysanoptera and Aleyrodidae are also haplodiploid organisms, many of them being important agricultural pests, but less comprehensive studies on sex allocation have been performed with these taxa. Both taxa are also mostly herbivorous, drawing in new elements that may affect sex allocation patterns. This review is a summary of all studies of Aleyrodidae and Thysanoptera sex ratios as affected by temperature, host plant and nutrition, conspecifics, competitors, bacterial and viral endosymbionts, predators, parasitoids and other pathogens. Sex ratio influences were found in studies testing effects of temperature, host plant, conspecifics, competitors, bacterial endosymbionts and a fungal pathogen. Viruses, predators and parasitoids were not found to affect sex ratios in Aleyrodidae and Thysanoptera, although not many studies have collected the data to assess these potential factors. Because sex ratio studies in Thysanoptera and Aleyrodidae have only recorded secondary, adult or operational sex ratios instead of primary sex ratios, differential developmental mortality is left as a confounding variable, and it is difficult to determine whether sex ratio changes are due to sex allocation. To conclude whether sex allocation of mothers is taking place, the primary sex ratios need to be recorded, so we have also included methods for tools, including cytogenetics and survival assays, to find primary sex ratios or confirm differential developmental mortality in these haplodiploid

organisms. Future discoveries of sex allocation in Aleyrodidae and Thysanoptera may lead to better predictions of pest populations.

- **Key definitions**

-Sex allocation: reproductive investment to male and female functions or offspring.

-Sex ratio: The proportion of all offspring that are males.

-Primary sex ratio: The proportion of males after egg fertilization (Majerus 2003), when sex allocation by haplodiploid parents may occur via selective fertilization.

-Secondary sex ratio: The proportion of males after egg eclosion (Terry and Kelly 1993, Majerus 2003).

-Adult sex ratio: The proportion of males after pupal eclosion or sexual maturity. It also may be referred to as tertiary sex ratio (Terry and Kelly 1993, Majerus 2003) or secondary sex ratio (if there is no way to identify proportion of males after egg eclosion). Here we use "adult sex ratio" exclusively.

-Operational sex ratio: The proportion of sexually active/receptive males (relative to all adults) in a population (Emlen 1976, Shuster 2016).

-Even/ Fisherian sex ratio: 50% male.

-Female/male bias: Statistically significantly greater or less than 50% male.

-Arrhenotoky and thelytoky are two forms of parthenogenesis or reproduction without fertilization.

Thelytoky: A form of parthenogenesis in which diploid females are produced from unfertilized haploid eggs after doubling of the chromosome complement.

Arrhenotoky: synonymous with the haplodiploid genetic system: males are produced from unfertilized, haploid eggs, while females are produced from fertilized, diploid eggs.

- **Relevant sex allocation theories**

-**Fisher's principle:** First proposed by Darwin (Darwin 1871, Edwards 1998) but clearly articulated, and largely attributed to Fisher (1930). In a randomly mating population with equal costs to producing males and females, the sex ratio should converge on 50%. This is because, if a bias exists, then the rarer sex will have more mating opportunities and selection will favor individuals that produce the rarer sex and the bias will then decrease. A Fisherian sex ratio of 50% is most commonly found in sexually reproducing species, and is considered an evolutionarily stable strategy (ESS, Maynard-Smith and Price 1973).

-Hamilton's (1967) **local mate competition (LMC)** predicts female bias when resources are patchy, males mate on the natal patch and do not disperse, and females typically mate once. Under these conditions, female bias will reduce competition among males that may be brothers. In the extreme case of a single foundress, a mother's best strategy would be to produce a single male, given that he is able to mate with all of his sisters. More male offspring will occur with greater number of female foundresses. The inbreeding that results also favors female-biased sex ratios in haplodiploid organisms since it increases the average relatedness between mothers and their diploid daughters (Frank 1986, Keller et al. 2011). Although Hamilton referred to a study on the thrips *Limothrips denticornis* as a candidate for LMC (Pussard-Radulesco 1930, Hamilton 1967), studies of LMC have mostly been supported by empirical study in mites and parasitoid wasp species that sib-mate and attack patchily distributed hosts (Werren 1980, Charnov 1982, Godfray 1994).

-Partial local mate competition: When males disperse and have mating opportunities beyond the natal patch, their reproductive value is increased, and the expected sex ratio is less female-biased (Werren and Simbolotti 1989).

-Clark's (1978) **local resource competition (LRC)**: is similar to local mate competition in that it predicts mothers to bias offspring sex ratio towards the sex that will compete less with each other or with their mothers, but in this case for limited resources (other than mates) such as food or territory. Under these conditions, greater allocation is predicted for the sex that disperses from the natal patch. In many insect species, a male-bias is expected in sexually dimorphic insects where males are winged and disperse but females do not. In addition, female insects are usually larger than males and therefore will compete more with each other for resources for development, especially in depleted environments. Mealybugs are one example of insects with large females and dispersing males, and in which LRC has been investigated (Varndell and Godfray 1996, Ross et al. 2010).

- Clark's (1978) **local resource enhancement (LRE)**: occurs when a particular sex increases the fitness of its mother, for example by helping the mother care for offspring. Mothers are expected to bias production to the beneficial sex, but once the benefit has been optimized, the bias may decrease as the mother starts to produce the other sex. Researchers argue that sex ratios in primitively social or eusocial Hymenoptera are guided by LRE (Schwarz 1988, Gardner and Ross 2013).

-The **Trivers-Willard hypothesis (TWH)** (1973): depends on three conditions: (1) An individual's physiological condition correlates with that of its mother; (2) this endures into adulthood; (3) the reproductive success of one sex relies relatively more on its individual physiological condition. The original Trivers-Willard assumption stated that males will benefit relatively more with better maternal condition, especially in environments with dominance hierarchies (Trivers and Willard 1973). Mothers with better health should invest more in the sex that will benefit more from her condition. A famous example applying the Trivers-Willard

hypothesis is found in high ranking red deer females investing more in sons than daughters (Clutton-Brock et al. 1984).

Charnov et al. (1981) focused on the third condition of the Trivers-Willard hypothesis that the sexes may not benefit equally from greater physiological condition. Charnov reasoned that when resource quality predicts offspring quality, mothers will lay the sex that benefits the most from greater physiological condition. For instance, if a parasitoid wasp is exposed to hosts of different sizes, she will oviposit female eggs in relatively larger hosts and male eggs in relatively smaller hosts. Wasps that develop in larger hosts will have a larger body size, and generally larger female size translates into greater numbers of offspring, while the same linear relationship is not true for males. For instance, larger female *Itoplectis naranyae* (Ueno 1998) and *Pimpla nipponica* (Ueno 1999) parasitoid wasps lived longer than smaller females, but larger males did not live longer than smaller males. In fact, smaller males of both species mated successfully with all females, whereas larger males only mated successfully with larger females (Ueno 1998, 1999). In general, a mother should allocate each sex to the environments that benefit them the most.

- **Introduction**

The study of sex allocation in arthropods has focused extensively on the insect order Hymenoptera, in large part due to the flexible nature of the haplodiploid sex-determination system, which allows females to allocate reproduction toward female (diploid, fertilized eggs) or male (haploid, unfertilized eggs) offspring through selective fertilization. Rich theory in sex ratio has been solidified through empirical study using hymenopterans, including local mate competition (LMC; Hamilton 1967), the Trivers-Willard hypothesis (Charnov et al. 1981), and theories on the origins of eusociality (Schwarz 1988, Gardner and Ross 2013). The

hymenopteran species chosen for study have been primarily parasitoid wasps, fig wasps, and eusocial ants, bees and wasps. This research has served as a cornerstone in the field of behavioral ecology, as qualitative and even quantitative predictions of sex ratios have been developed and then tested in different environments based on adaptive hypotheses (Charnov 1982, Godfray 1994, Bourke and Franks 1995). Sex ratios also are an important component of population dynamics. An understanding of factors that influence sex ratios also have applications in the use of parasitoid wasps in biological control (Chow and Heinz 2005), as even or female biased sex ratios are considered beneficial for pest management and evidence of high quality biological control agents (Heimpel and Lundgren 2000, van Lenteren et al. 2003).

Given the pressing need to predict dynamics of herbivorous pests, it is perhaps surprising that hypotheses regarding sex ratios have been hardly developed outside of Hymenoptera, especially since two groups of economically important insect pests are haplodiploid and may exhibit labile sex ratios. These are the important hemipteran sap-feeding family of whiteflies, Aleyrodidae, and the entire order of thrips, Thysanoptera. Whiteflies are generally specialized, tree-feeding sap-feeders, but include a handful of notorious polyphagous forb-feeding species that have broad host ranges, attack many crop plants, and vector a hundred or more viruses (Jones 2003, Oliveira et al. 2013, Gilbertson et al. 2015). Thrips are mostly herbivorous cell-feeders, and some pest species can also vector viruses (Jones 2005, Mound 2005, Gilbertson et al. 2015). We might expect resource use (plants rather than other insects), life history, and other environmental pressures to affect the sex ratios of these insects differently than in parasitic or predatory Hymenoptera. However, while many life history studies have been performed on these taxa, often measuring adult sex ratios along with other demographic parameters such as development time and fecundity, there are relatively few studies that interpret sex ratios within a conceptual

framework. Here, I will review what is known about sex ratios of herbivorous, haplodiploid taxa as they may be influenced by host plants, conspecifics, competitors, predators, parasitoids, pathogens, endosymbionts, viruses, and temperature. Although selfish genetic elements may also play a crucial role in altering sex ratio, there is little recorded for these in whiteflies and thrips, so they will not be discussed in this review.

Sex ratios in most studies of thrips and whiteflies to date have been limited by the data collected; investigators commonly record adult sex ratios, or occasionally operational sex ratios from field studies. However, comparing the influence of treatment effects on sex allocation is at best very approximate when adult or operational sex ratios are measured. Adult sex ratios may be hard to interpret because primary sex ratios and differential mortality of the sexes during development may vary simultaneously. Operational sex ratios are even farther from primary sex ratios and can include confounding variables such as differential timing of emergence, longevity of the sexes, differential dispersal and different microhabitat choices that may influence what adults are collected in a sample. We present tools for directly investigating or inferring the primary sex ratios of whiteflies. We argue that these techniques, borrowed from the study of hymenopteran parasitoids, can improve our data collection and ultimately our understanding of the cause of variable sex ratios of haplodiploid herbivores, and help to put the study of sex ratio of these groups on a more rigorous footing. We introduce a cytogenetic technique to capture the primary sex ratio, developed for the sweetpotato whitefly, *Bemisia tabaci*, that can potentially aid in further sex ratio study of *B. tabaci* and other Aleyrodidae. A survival assay that can help identify differential mortality of male and female whitefly or thrips nymphs is presented as well.

- **Effects of temperature on sex ratio**

Temperature may differentially affect the sexes and adult sex ratios of both Aleyrodidae and Thysanoptera. In the species complex that is collectively known as *B. tabaci*, short exposure to extremely high temperatures increases the proportion of males in the *B. tabaci* species known as MED as well as in MEAM1 (Cui et al. 2008, Lü et al. 2014, Xiao et al. 2016). Researchers that reared *B. tabaci* MEAM1 from egg to adult under temperatures of 15-37°C found no alterations in adult sex ratios, but did observe female-biased offspring at all temperatures (Wagner 1995, Wang and Tsai 1996), or sex ratios that approximated 50% (Guo et al. 2013). To find effects of exposure to high temperatures, Cui et al. (2008) and Lü et al. (2014) heat-shocked parents for one hour at temperatures ranging from 37-45°C, and then reared offspring at 26°C to record survival and sex. Cui et al. (2008) found that sex ratio became significantly more male-biased when MEAM1 parents were exposed to 43-45°C, while Lü et al. (2014) also found even or male-biased sex ratios after exposing MEAM1 parents to 41-45°C. In both cases, the offspring were female-biased at lower temperatures of parental heat-shock, including at the control temperature of 26°C. Xiao et al. (2016) heat-shocked MED and MEAM1 parents at 41°C for two hours, and found male-biased offspring (38-46% female) in comparison to the control group exposed to 26°C (55-62% female). In all cases, alterations of sex ratio corresponded to decreases in developmental survival, suggesting that differential mortality of females might explain these patterns. In contrast, Elbaz et al. (2011) heat-shocked MED and MEAM1 parents for 1.5 hours at 42°C and found no significant difference in adult sex ratio of the offspring compared with the 27°C control. These authors did find declines in adult survival after 43°C heat-shock in MEAM1 females or after 45°C in MED females, suggesting that these higher temperatures approach lethal limits for these species. Cui et al. (2008) found declines in parental adult survival at or above

41°C, egg survival at or above 43°C, and in egg to adult survival after 41°C. Lü et al. (2014) did not track offspring survival, but in separate heat-shock experiments on adults, found significant declines in survival after one hour of exposure to 43-45°C. Xiao et al. (2016) found marginal to significant declines in egg survival and significant declines in egg to adult survival after a two-hour 41°C parental heat shock. Although these studies in aggregate show mixed results, in general it appears that lethal temperature limits of *B. tabaci* are reached near 41°C, with greater impacts on developmental survivorship of females, leading to increases in sex ratio.

Reports of lower temperature effects on *B. tabaci* sex ratio are inconsistent. Sharaf and Batta (1985) found an increase in proportion of females by rearing *B. tabaci* (unknown species) from egg to adult at cold temperatures. Whiteflies reared at 14°C were 76% female while those reared at 25°C were 64% female. The developmental survivorship was not reported. In contrast, Xiao et al. (2016) cold-shocked *B. tabaci* MED and MEAM1 adults for two hours at 4°C, but did not find effects on offspring sex ratio. Egg survival also was unaffected by 4°C of parental cold shock (Xiao et al. 2016).

Temperature has not been reported to influence sex ratios in the greenhouse whitefly, *Trialeurodes vaporariorum*. In addition to *B. tabaci*, Cui et al. (2008) also heat-shocked *T. vaporariorum* parents from 37-45°C for one hour. Offspring sex ratios remained close to 50% for all treatments, with no offspring produced at 43-45°C (Cui et al. 2008). The results suggest that *B. tabaci* and *T. vaporariorum* have two responses when lethal temperatures are approached: more male offspring will result in *B. tabaci* as developmental survivorship declines, while an even sex ratio in *T. vaporariorum* is maintained until their thermal lethal limit is reached.

In the thrips *Ceratothripoides claratris*, Premachandra et al. (2004) found a positive correlation between temperature and the proportion of females. Male-biased offspring emerged

at the rearing temperature of 25°C, while female-biased offspring emerged at 30 and 35°C (Premachandra et al. 2004). Interestingly, reproductive success, as judged by pre-adult survival, fecundity and sex ratio, peaked at 30°C; female-bias in this case may approximately represent sex allocation strategy (Premachandra et al. 2004). In *Frankliniella occidentalis*, Kumm and Moritz (2010) found a similar pattern. Kumm and Moritz (2010) reared *F. occidentalis* at three temperatures (17°, 23°, and 32°C), and found a linear decrease in adult sex ratio with temperature, ranging from 55% male at 17°C to 33% male at 32°C. The change in sex ratio could be a result of mothers altering the primary sex ratio, or higher male mortality with higher temperatures, as survival was not measured. Surprisingly, the authors also found that 0.5% of offspring from virgin females were female, even though *F. occidentalis* is known to be arrhenotokous. To test whether the apparent thelytoky was due to parthenogenesis-inducing bacteria, the authors surveyed the population for *Wolbachia*, but not other bacteria now known to also cause parthenogenesis (e.g. *Cardinium*, *Rickettsia*). The phenomenon remained unexplained as *Wolbachia* was undetected in the studied population (Kumm and Moritz 2010). At a higher temperature of 41°C, Wang et al. (2014) found opposing results. Wang et al. (2014) heat-shocked *F. occidentalis* adults at 41°C for five time-intervals of between 2-36 hours and reared and sexed offspring at 25°C. Although all treatments produced female-biased offspring, the adults that were heat-shocked for longer time periods produced a relatively higher proportion of male offspring.

As for Kelly's citrus thrips, *Pezothrips kellyanus*, no clear pattern in sex ratio was associated with temperature (Varikou et al. 2012). At rearing temperatures of 15 and 25°C, female-biased offspring were found, while male-biased offspring were found in the 20°C and 30°C treatments (Varikou et al. 2012).

Overall, there appears to be some association between high temperatures and increased proportions of male offspring in the few Aleyrodidae and Thysanoptera studied. These temperatures are also correlated with declines in developmental as well as adult survival. Further, the absence of any direct measure of primary sex ratios make it impossible to determine the cause of the pattern of increasing sex ratio with temperature. High temperature exposures of parents could reduce sperm viability or the ability of females to fertilize eggs, leading to a lower primary sex ratio. The reduced developmental survivorship observed in many studies could also explain changes in adult sex ratios if females were more susceptible to heat during development. These hypotheses should be confirmed with mechanistic studies that measure the primary sex ratio of newly laid eggs.

- **Other abiotic effects on sex ratio**

Few studies have tested abiotic effects on Aleyrodidae and Thysanoptera sex ratios outside of temperature. To our knowledge, no studies have examined the influence of humidity on sex ratios of whitefly or thrips greenhouse populations, but this would be valuable to investigate.

- **Host plant and nutritional effects on sex ratio**

More than any other environmental factor, host plants mediate the life history of herbivorous insects. Plants provide varying levels of nutrition and have evolved a wide array of physical and chemical defenses against their herbivores (Bernays and Chapman 1994, Price et al. 2011). For whiteflies and thrips, there have been several studies determining the relative host quality of crops and other plants as assessed by insect life history parameters, with high quality host plants supporting shorter development times to adulthood, higher survival during development, increased adult longevity and greater fecundity. How does sex ratio fit into this collection of work? Can haplodiploid insects adjust offspring sex ratio according to host plant quality, and is

there a certain strategy that enhances fitness? More fundamentally, what relationship is observed between host plant quality and sex ratios? In a high-quality environment, resources are less limited, so sexes may become equal in costs to produce, trending toward even sex ratios in accordance with Fisher's principle. On the other hand, the Trivers-Willard and Charnov hypotheses may come into play, in which females may benefit relatively more than males with better maternal or environmental condition, leading to female-biased sex ratios on high quality host plants. This would suggest the high-quality plants are a limited resource, however, and when the plant is not limiting, even sex ratios may be adaptive. Studies in some herbivorous insects such as sawflies and aphids suggest that females are favored on higher quality host plants (Craig et al. 1992, Barker and Maczka 1996, Purdy and Miller 2013). For example, Barker and Maczka (1996) found that the dry mass of *Pachynematus clitellatus* sawfly females was significantly greater on preferred host plants, whereas there was no difference in male dry mass between host plants of differing quality. Relatively higher fitness gains may have led to the greater female bias on the preferred host plant (Barker and Maczka 1996).

Similar results have been found in the castor whitefly, *Trialeurodes ricini* (Shishehbor and Brennan 1996). Shishehbor and Brennan (1996) determined eggplant and cotton to be the better hosts for *T. ricini* due to higher fecundity, shorter development time and higher survivorship to adulthood. On these host plants, sex ratios were even, whereas sex ratios on poorer host plants were male-biased. The data for this species is inconsistent, however. Huang et al. (2014) also found that the best host plant resulted in even sex ratios for *T. ricini*. However, poorer host plants (including cotton and eggplant, in contrast to Shishehbor and Brennan 1996) had sex ratios that varied from 43-73% female. The difference in results could have been due to many environmental variables as well as to the specific plant or *T. ricini* strain. Overall, *T. ricini*

appears to abide by Fisher's principle on high quality host plants, but has variable sex ratios on host plants of lower quality.

As for the greenhouse whitefly, *T. vaporariorum*, Hosseini et al. (2015) artificially created host plants of varying quality by providing different dosages of Nitrogen to tomato plants, a common limiting nutrient in phloem-feeding insects. Increases of Nitrogen levels in tomato plants increased growth rate of *T. vaporariorum*, but did not alter sex ratio, which was close to 50% in all treatments (Hosseini et al. 2015).

Other studies also do not find any associations between host plant quality and sex ratio. Greenberg et al. (2009) found no relationship for species of *B. tabaci* MEAM1, *Trialeurodes abutiloneus* and *T. vaporariorum* while Fekrat and Shishehbor (2007) found no associations in *B. tabaci* MEAM1. Studies of Thysanoptera have also been unable to associate host plant quality with predictable changes in sex ratio (Zhang et al. 2007, Tang et al. 2015, and Cao et al. 2018).

Currently then, published studies do not show any consistent relationship between sex ratios and host plant quality in Aleyrodidae and Thysanoptera. This is clearly an area that would benefit from a more rigorous examination of the sex allocation patterns that lead to adult sex ratios. Given potentially large differences in host plant quality, do females allocate sex similarly on hosts of a given quality? And does host quality differentially influence male and female developmental success?

Although mycophagous insects have different life histories than herbivorous ones, varying levels of resource quality may prompt sex allocation in the same way. Sex ratios in one mycophagous thrips, *Elaphrothrips tuberculatus*, appears to conform to the Trivers-Willard hypothesis. Crespi (1988) did not need to rule out differential mortality to determine sex allocation, because mothers are flexible in producing all-male broods viviparously, or all-female

broods oviparously. Therefore, one may only need to look at the reproductive mode of mothers to verify the current population sex allocation (Crespi 1988). Crespi (1988) found a high correlation between viviparity and good maternal condition, measured by either a larger body size in early summer, or when mothers had access to denser fungal fruiting bodies on leaves in spring. Crespi (1988) quantitatively confirmed the Trivers-Willard hypothesis, in which larger males gained relatively higher fitness benefits in contrast to larger females. In this case, the relationship of male size and their ability to mate-guard was stronger than the relationship of larger females and their fecundity: after standardizing male and female body sizes separately and then plotting against relative fitness, males had a significantly steeper slope than females (Crespi 1988). A mother should therefore become viviparous during times where she can produce larger offspring, and oviparous at all other times.

- **Effects of conspecifics on sex allocation**

Among Hymenoptera, conspecific strategies drive sex allocation. The responses in sex allocation to LMC, LRC and LRE have been documented across multiple taxa and sex determination systems, and these responses have generally been larger in effect size in comparison with the influence of other environmental cues (West et al. 2005).

In Thysanoptera, LMC is expected to affect gall-inducing thrips, especially in cases where galls are closed, males do not disperse, and there is one founding female, or the founders are sisters (Crespi 1993). Fisherian sex ratios are expected for free-living thrips under random mating, as they are not constrained to mate on their natal patches (Crespi 1993, Higgins and Myers 1992). As expected, most gall-inducing thrips comply to LMC, or partial LMC (Crespi 1997, Tsuchida and Ohguchi 1998, Willis et al. 2004, Tree and Walter 2009). *Akainothrips francisi*, a thrips species that resides in the nest of domicile-building thrips as an inquiline, may

also have female-biased sex ratios due to LMC (Gilbert et al. 2012). Surprisingly, female-biased sex ratios have been recorded in free-living thrips as well (Higgins and Myers 1992, Crespi 1993, Gerin et al. 1994, van Rijn 1995, Rugman-Jones et al. 2012, Kanara and Acharya 2014). Researchers suggest that this may be due to inbreeding of brothers and sisters before dispersal, as chances of finding a mate are very small considering the size of the thrips and uncertainty of where the wind will take them, but this needs confirmation (Higgins and Myers 1992, Kranz et al. 2001). Crespi (1993) suggested that the female-biased sex ratios may be due to a systematic sampling error, because mostly adult or operational sex ratios have been recorded. Male thrips have been shown to have higher nymphal mortality (Higgins and Myers 1992), shorter adult life spans (Higgins and Myers 1992, Kranz et al. 2001), and differential dispersal and distribution compared to females (Terry and Kelly 1993), making it difficult to determine if the common female bias is due to differential mortality during development, differential longevity, incomplete sampling or to female sex allocation.

LMC is expected to be reduced or absent for most whiteflies. Feeding sites are not generally patchily distributed for whiteflies as they are for gall-enclosed thrips or parasitoid wasps, and male whiteflies are winged and can disperse. Perhaps unusually, then, Hu et al. (2010) invoked partial LMC in the spiralling whitefly *Aleurodicus dispersus*. Strongly female biased adult sex ratios were recorded from single egg spirals laid by isolated females, and sex ratios increased linearly to 50% on leaves with many egg spirals, evidence of multiple females (Hu et al. 2010).

Examples of LRC have not been reported in Aleyrodidae, possibly because both sexes can disperse, and whiteflies such as *B. tabaci* are extremely polyphagous, providing more opportunities to switch host plants when the current host plants are exhausted. Kranz et al. (1999, 2001) studied potential LRC and LMC adjustments to sex ratios in eusocial galling thrips.

Eusocial galling thrips have alternating generations; after a queen establishes a gall, she rears a generation of wingless male and female soldiers that protect the nest from invaders. The soldiers and the queen will then lay eggs to produce a second generation of winged dispersers. LMC and LRC may both occur in these tight quarters, with single foundresses, sib mating, and limited dispersal of soldiers (LMC) as well as food limitation and the potential differential use of gall tissue by the sexes, especially in the second generation (LRC). Perhaps unsurprisingly, given the complexity, Kranz et al. (1999, 2001) found results in *Kladothrips hamiltoni*, *Oncothrips waterhousei* and *O. habrus* that were difficult to interpret. Even sex ratios were found in *K. hamiltoni* soldiers, and dispersers of *O. waterhousei* and *O. habrus*. Meanwhile female-biased sex ratios were found in *K. hamiltoni* dispersers, and soldiers of *O. waterhousei* and *O. habrus*. Although LMC and LRC could have played roles in these thrips species, it is difficult to conclude whether sex allocation played any role in the observed adult sex ratios.

Studies have not shown clear LRE in Aleyrodidae or Thysanoptera, but it may possibly contribute to the high female bias found in *Dunatothrips aneurae*, a social thrips in which females have more-armed forelegs than males and thus may be more helpful in defense of the colony (Bono and Crespi 2008). Overall, the studies above support the possibility of sex allocation by Aleyrodidae and Thysanoptera in response to conspecific behavior, but measurement of primary sex ratios would help confirm these patterns.

- **Effects of interspecific mating interference on sex ratio**

Mating interference may influence the outcome of interspecific competition, as has been shown in a few examples in the cryptic whitefly species complex collectively known as *Bemisia tabaci* (De Barro et al. 2011, Liu et al. 2012). In haplodiploid organisms, interspecific copulations can result in unsuccessful fertilization and more male offspring. Male-biased

offspring may thus signify the loser in these interactions. The Middle East Asia Minor 1 (MEAM1) and Mediterranean (MED) species are the two most invasive *B. tabaci* whiteflies worldwide, and interactions between the two have been well documented. The MED species is more tolerant to pesticides, so the MED species may outcompete the MEAM1 species in locations of widespread pesticide use (Tsueda and Tsuchida 2011, Sun et al. 2014). In the absence of heavy pesticide use, MEAM1 can eventually exclude the MED population after a starting population frequency higher than 10% (Sun et al. 2014). There is evidence that the MEAM1 takeover is partly due to asymmetrical mating interference; in comparison to MEAM1 or MED populations by themselves, MED whiteflies show male-biased sex ratios in mixed populations, whereas MEAM1 whiteflies showed the same sex ratios as the control group (Pascal and Callejas 2004, Crowder et al. 2010b, Tsueda and Tsuchida 2011, Sun et al. 2014). In mating behavior studies, MEAM1 males were more successful at interrupting mating events of a MED pair than vice versa (Sun et al. 2014). Further, MEAM1 females increased copulation events with MEAM1 males in the presence of MED males, in contrast to when MEAM1 females were only exposed to MEAM1 males (Crowder et al. 2010b). Overall, MEAM1 males and females can discriminate more effectively than MED males and females (Crowder 2010a).

Behavioral asymmetries were also found between MED and native Asia II-1 (formally known as ZHJ2 biotype) whiteflies in China (Wang et al. 2012), and between MEAM1 whiteflies and native Australian or Asia II-3 (formally known as ZHJ1 biotype) whiteflies (Liu et al. 2007). In both studies, the MED or MEAM1 females increased copulation events in the presence of more males, regardless of the male species identity (Liu et al. 2007, Wang et al. 2012). MED and MEAM1 females also had greater proportions of female offspring in mixed cohorts, in comparison to single cohorts (Liu et al. 2007, Wang et al. 2012). Native females, on the other

hand, decreased copulation events in the presence of MED or MEAM1 males, and produced male-biased offspring (Liu et al. 2007, Wang et al. 2012). In general, MED or MEAM1 males had a greater capacity to interfere in mating events than native whiteflies. These studies provide evidence for asymmetrical mating interference helping to drive displacement of native *B. tabaci* by *B. tabaci* MED and MEAM1.

T. vaporariorum may also be asymmetrically affected by *B. tabaci*, but not through significant changes in sex ratio (Zhang et al. 2013). When a tomato leaf was preoccupied by *T. vaporariorum* conspecific eggs or *B. tabaci* eggs, *T. vaporariorum* eggs laid subsequently suffered reduced immature survival (Zhang et al. 2013). On the other hand, the preoccupation of *B. tabaci* conspecific eggs or of *T. vaporariorum* had little or marginal consequences on the fitness of *B. tabaci* nymphs hatched from eggs laid subsequently (Zhang et al. 2013).

- **Effect of symbionts on host sex ratio**

-Bacterial endosymbionts

Intracellular endosymbionts that are strictly vertically transmitted can generally spread only through egg cytoplasm, as sperm is usually too small to transfer bacteria to the next generation of hosts. Therefore, transmission of endosymbionts depends on host females, and spread either by increasing the fitness of infected mothers, or by manipulating the reproduction of the host to produce more, or higher quality females (Bull 1983, O'Neill et al. 1997). Many bacterial endosymbionts metabolize essential nutrients for their hosts (Moran et al. 2008, Douglas 2009, 2015), or defend hosts against pathogens and parasitism (Oliver et al. 2003, Scarborough et al. 2005, Douglas 2015). There are also four well-known reproductive manipulations.

Parthenogenesis inducing (PI) endosymbionts act on haplodiploid organisms to convert haploid males into diploid females (Ch. 4 in O'Neill et al. 1997, Cordaux et al. 2011, Ma et al. 2014, Ma

and Schwander 2017). Feminizing endosymbionts transform genetic males into fully functional females (Cordaux et al. 2011, Ma et al. 2014). Male-killing endosymbionts cause death to male embryos or later larval/pupal stages. Male-killing may result in enhanced fitness of females by decreasing resource competition among female survivors, providing more resources to cannibalistic females, and decreasing inbreeding (Hurst and Majerus 1993). Cytoplasmic incompatibility (CI) is a mating incompatibility observed, at its simplest, between bacteria-infected males and uninfected females. In this cross, many if not all of the fertilized eggs are inviable, or behave as if unfertilized. In haplodiploid systems, since unfertilized, uninfected eggs are incipient males, then uninfected females that only mate with infected males may have only male offspring. In haplodiploid organisms then, we expect a short-term sex ratio skew towards males as the CI-causing symbiont sweeps through the population, driven by the relative fitness cost of uninfected females in CI crosses compared to infected females. Only *Wolbachia* has been found to induce all four reproductive manipulations.

In whiteflies, there is not yet any evidence that symbionts cause parthenogenesis, feminization, male-killing or CI, although female bias has been associated with facultative symbionts in a few instances. All whiteflies examined to date, including *B. tabaci*, contain *Portiera*, an obligate nutritional bacterial endosymbiont not shown to alter sex ratio. Cryptic species in this complex also contain different combinations of facultative bacterial endosymbionts: *Wolbachia*, *Cardinium*, *Rickettsia*, *Hamiltonella*, *Arsenophonus*, *Fritschea* and *Hemipteriphilus*. Intracellular symbionts are difficult to study in this host family, as they cannot be cultured outside of their hosts, and antibiotic curing or heat shock treatments can lower titers of the primary symbiont *Portiera* or incompletely remove the desired bacteria.

The most comprehensive studies on the effects of facultative symbionts in *B. tabaci* to date have been on *Rickettsia* and *Cardinium*. Himler et al. (2011) found a rapid spread of *Rickettsia*-infected *B. tabaci* MEAM1 populations in the Southwestern USA in a six-year period. After introgression of naturally infected and uninfected lines to homogenize nuclear genetic differences, they found that *Rickettsia*-infected whiteflies developed faster, had higher survival from egg to adult, higher fecundity, and 70-85% female offspring compared to ~40-60% female offspring in the uninfected line (Himler et al. 2011). Other studies showed that host genotypes varied in the phenotype of the *Rickettsia*-host interaction (Cass et al. 2016), and that host nuclear genotype was more influential than *Rickettsia* or other maternally-inherited symbionts in affecting the influence of *Rickettsia* (Hunter et al. 2017). The importance of host genotype may explain why Chiel et al. (2009), examining the same whitefly and a highly similar if not identical *Rickettsia* in Israel (Cass et al. 2015), found that *Rickettsia* decreased development time but did not affect any other fitness parameters. Parrella et al. (2013) also showed an association between *Rickettsia* and female biased sex ratios in *B. tabaci* MED in Italy. One mitochondrial type of the whitefly, Q1, contained no *Rickettsia* and exhibited even sex ratios, whereas the mitochondrial type Q2 was nearly fixed for *Rickettsia* and had a strong female bias (71% female). One cannot separate *Rickettsia* effects and host genetic effects in this study, but they are suggestive of a possible symbiont effect.

In another study of whitefly symbionts, Fang et al. (2014) observed that only 7.6-17.3% of *B. tabaci* MED whiteflies were infected with *Cardinium* in Shandong, China. After establishing infected and uninfected lines in the laboratory (all fixed for *Portiera* and *Hamiltonella*), Fang et al. (2014) found that uninfected whiteflies greatly outnumber infected individuals after a few generations, and have higher growth rates, indicating that *Cardinium* may be detrimental to its

host. However, the authors did not control for host genotype, which makes it difficult to distinguish how much of the difference seen is due to *Cardinium* relative to host differences coincidentally associated with *Cardinium* infection. *Cardinium* was not shown to manipulate reproduction or sex ratios in this study (Fang et al. 2014).

Laboratory manipulations have also been conducted to study the effects of *Arsenophonus*, *Wolbachia* and *Hamiltonella*. *Arsenophonus* was cured via tetracycline in the *B. tabaci* Asia II-1 species, and no changes in sex ratio were observed, but the cured whiteflies had increases in fecundity, longevity, and egg to adult survival (Raina et al. 2015), suggesting that *Arsenophonus* is costly to *B. tabaci*, at least in the laboratory environment (Raina et al. 2015). However, *Rickettsia* and *Wolbachia* were also found to infect 55-60% of the tested population, and maintenance of *Portiera* was only verified through diagnostic PCR, leaving the possibility that partial curing of other symbionts, at least, may have confounded these results. *Wolbachia* has not been shown to skew *B. tabaci* sex ratio. In a survey in China, Ji et al. (2015) found that MED and MEAM1 invasive species appeared to be female biased (the operational sex ratio in the field for each was 74% female). When Ji et al. (2015) reared *Wolbachia*-infected and uninfected populations in the lab, the *Wolbachia*-infection was associated with reduced female bias compared to uninfected females, suggesting that *Wolbachia* was not the cause. As for *Hamiltonella*, Ruan et al. (2006) cured *B. tabaci* MEAM1 of *Hamiltonella* in three treatments using different antibiotics, and found the offspring were male-biased; however, the sample sizes were too small for analysis of the sex ratio results. Due to the small sample size and lack of qPCR to verify if titers of *Portiera* fully recovered, these results should be interpreted as preliminary.

More studies on the roles of bacterial endosymbionts on reproduction and performance of *B. tabaci* would be valuable. The endosymbiont complex and the species limits of the *B. tabaci* complex itself are still being determined, and methods to manipulate the symbiont complex (antibiotic treatment, introgression) are still in progress. Outside of *B. tabaci*, studies of endosymbionts of Aleyrodidae are rare, but it would be interesting to know if endosymbionts are involved with thelytokous parthenogenesis in *Parabemisia myricae* (Rose et al. 1981) or *Aleurotrachelus atratus* (Borowiec et al. 2010).

In Thysanoptera, *Wolbachia* PI has been shown in *Frankliniopsis vespiformis* (Arakaki et al. 2001) and *Hercinothrips femoralis* (Kumm and Moritz 2008), where male offspring appear after antibiotic and/or heat treatment. In many other cases, *Wolbachia* was not found in parthenogenetic thrips, or is found in arrhenotokous thrips (Nault et al. 2006, Kumm and Moritz 2008, van der Kooi et al. 2014, Nguyen et al. 2015). Van der Kooi et al. (2014) warn that the presence of male offspring after antibiotic treatment should not be conclusive evidence for *Wolbachia* PI. After treating *Aptinothrips rufus* with antibiotics, van der Kooi et al. (2014) observed male offspring, but then pointed out that less than 70% of their sampled, parthenogenetic *A. rufus* populations were infected with *Wolbachia*. The thrips may be parthenogenetic due to another unidentified bacterium (van der Kooi et al. 2014); both *Cardinium* and *Rickettsia* have been shown to cause PI (Zchori-Fein et al. 2004, Hagimori et al. 2006, Giorgini et al. 2010), and there may well be others.

Recently, Nguyen et al. (2017) found the first-recorded CI in Thysanoptera, caused by *Wolbachia* and *Cardinium* coinfections in *Pezothrips kellyanus*. In haplodiploid organisms, CI can cause female mortality (FM) or a male development (MD) phenotype (Vavre et al. 2001, Nguyen et al. 2017). In FM, all fertilized eggs in a CI cross fail to develop, while in MD,

fertilized eggs survive but as haploid males (Vavre et al. 2001, Nguyen et al. 2017). Uninfected females do not lose as many offspring in a CI cross of the MD phenotype, but will still suffer fitness costs by a sex ratio skew up to 100% male. To differentiate between the phenotypes, Nguyen et al. (2017) counted male offspring of CI crosses and compared it with the number of males in a control cross of uninfected thrips. Because the number of males was significantly greater in the CI cross compared to the control cross, Nguyen et al. (2017) concluded that *Cardinium* causes a CI phenotype intermediate between FM and MD. In contrast, *Wolbachia* CI causes FM (Nguyen et al. 2017).

-Viral endosymbionts

Few studies have shown a viral influence on arthropod sex ratios (exceptions include Juchault et al. 1991, Nakanishi et al. 2008, Wang et al. 2017). Few viruses of Aleyrodidae or Thysanoptera have been described. Báo et al. (1996), Costa et al. (1996), and Hunter et al. (2001) provided morphological evidence of one or more *B. tabaci* viruses but there is no evidence that this/these viruses influence sex ratios. In comparison, there are many studies of plant virus effects on whitefly and thrips vectors. Plant viruses appear to have relationships with insect vectors that span mutualism to parasitism (Colvin et al. 2006, Jiu et al. 2007, Nogia et al. 2014), likely dependent on viral replication in the vector (Sinisterra et al. 2005) and whether the relationship is general or special (Colvin et al. 2016). Two studies formally tracked how plant viruses affected the adult sex ratios of their Aleyrodidae vectors, both directly, when infected vectors fed on clean plants, and indirectly, when uninfected vectors fed on virus-infected plants. Thompson (2002) found that the *East Africa cassava mosaic virus* (Ugandan variant) did not affect the sex ratio of *B. tabaci* collected in Uganda in either instance. Maluta et al. (2014) also did not find any changes in sex ratio for *B. tabaci* MED infected with *Tomato yellow leaf curl*

virus (TYLCV). Zheng et al. (2014) tested the indirect effects of *Tomato zonate spot virus* (TZSV) on *F. occidentalis* feeding on tomato or pepper, and also did not find significant viral effects on sex ratio, although there was a trend towards more female offspring from TZSV-infected plants.

Although there are no current studies in Aleyrodidae or Thysanoptera that show viruses affecting sex ratio, *Tomato spotted wilt virus* (TSWV) has been shown to differentially change the behavior and other fitness parameters of female and male *F. occidentalis* thrips (Stafford et al. 2011). TSWV-infected males increased both feeding and probing events up to three-fold, while TSWV-infected females increased predation (Stafford et al. 2011, Stafford-Banks 2013). These changes are most likely a vector response to the virus' propagative nature, replicating within its vector (Stafford-Banks 2013). *Franklinella occidentalis* males appear to be better transmitters of TSWV, because the virus needs viable plant cells to replicate, and male probing is innocuous relative to female probing that will often lead to greater plant cell death (Stafford-Banks 2013). Indeed, Rotenberg et al. (2009) found that male *F. occidentalis* were better than females at transmitting TSWV multiple times. If a virus depends disproportionately on one sex to be transmitted from vector to plant, it would be in the virus evolutionary interest to alter not only the behavior but also the sex ratio of its vector, altering the host plant to produce an environment more favorable to the sex that is more reliable for transmission. For example, when *F. occidentalis* fed on TSWV-infected foliage at 18.3 °C, one of two tested strains of TSWV significantly decreased the development time of males but not of females (Stumpf and Kennedy 2007).

- **Sex ratio effects from biological control: interactions with predators, parasitoids, and pathogens**

The literature is replete with documentation of predator and parasitoid sex ratios on different hosts, in part to determine whether certain predators or parasitoids are feasible biological control agents (Heimpel and Lundgren 2000, van Lenteren et al. 2003). Unfortunately, we know little about whether these natural enemies influence the sex ratio of their prey or hosts. This missing data may be an overlooked factor in determining effective biological control agent effectiveness, especially those that have high parasitism/predation rates: biological control agents that induce a male bias in their host/ prey will be more effective at decreasing population growth. A male bias in pests may also decrease crop damage absolutely if females cause more feeding damage as adults, such as in the western flower thrips *F. occidentalis* (Higgins and Myers 1992, Stafford-Banks 2013).

Although there is a lack of study in this area, it would be interesting to determine if biological control agents of Aleyrodidae cause male-bias in their hosts. In whiteflies, female nymphs, especially at later instar stages, are significantly larger than male nymphs (Hora et al. 1995, Baig et al. 2015, 2016). Parasitoids targeting these stages could consistently prefer larger, female hosts, especially when only female eggs are laid in whiteflies, such as in parthenogenetic wasps such as *Encarsia formosa*, or autoparasitoid *Encarsia* in which males are hyperparasitoids and eggs are not laid in whiteflies but in developing wasp pupae (Hunter and Woolley 2001). One laboratory study, however, has shown no preference in sex/size of its host, as *E. formosa* mothers appear to be more time-limited than egg-limited (Hora et al. 1995). Alternatively, parasitoid wasps may selectively oviposit female offspring in relatively larger hosts and males in smaller hosts (Clausen 1939, Charnov 1981, Werren 1984), and larger hosts might be differentially

targeted when wasp sex allocation is female biased. Similarly, if a whitefly predator preferentially consumes larger whitefly instars, a male-bias in the whiteflies could emerge.

Whether preference for larger hosts occurs is, however, unknown, and complicating the picture is the fact that cohorts of whitefly immatures are rarely synchronous, so parasitoids and predators may be most often presented with a range of stages and sizes, such that the relationship between relative size at a particular time and whitefly sex may not be very strong.

Many insect prey, including thrips and whiteflies, alter their behavior in the presence of predators and parasitoids. Through non-consumptive effects, natural enemies cause changes in feeding behavior (Schmitz et al. 2003, Jandricic et al. 2016), landing and settling behavior (Lee et al. 2014, Meng et al. 2006), oviposition behavior and in some cases reproductive allocation (Deas and Hunter 2012). For example, Jandricic et al. (2016) found that second instars of *F. occidentalis* are too large to be eaten by the predatory mite *Neoseiulus cucumeris*, but can still be attacked. Second instar thrips exposed to the mites decreased feeding activity, most likely to avoid attacks, and had significantly decreased survival to adulthood in comparison to thrips raised without mites. *Bemisia tabaci* will also exhibit avoidance behavior, increasing settling time on enclosed leaf discs or moving to upper leaves of a cucumber plant in the presence of the predatory beetle *Delphastus catalinae* (Lee et al. 2014). If insects alter behavior when enemies are present, it would not be far-fetched to imagine that these herbivores may alter the sex ratio of their offspring as well, perhaps biasing offspring sex towards the sex that disperses the greatest distance.

As for pathogens, the exposure of *F. occidentalis* to an entomopathogenic fungus, *Beauveria bassiana*, resulted in male-biased offspring, compared to an even sex ratio found in unexposed thrips (Zhang et al. 2015). No significant differences in fecundity or survival to adulthood were

recorded, implying that exposed thrips fertilized less eggs, possibly due to *B. bassiana* interfering with sperm production in males, egg fertilization by females (Zhang et al. 2015), or sex allocation. Overall, the ability of potential biological control agents to skew host/prey sex ratio should be considered in the assessment of their quality.

- **Tools for connecting sex ratios to sex allocation**

Many have studied the life histories of Aleyrodidae and Thysanoptera, including the measurement of adult sex ratios. However, it has been difficult to interpret the results in light of sex allocation. If a mother allocates more resources to a certain sex, it is best observed through the primary sex ratio, or the eggs. All studies to date of Thysanoptera and Aleyrodidae have recorded changes in sex ratio, which may be a result of changes in sex allocation, but this remains to be clarified. Knowledge of sex allocation in these taxa may be helpful in predicting future pest population dynamics. For instance, global climate change or higher pest densities may initiate unforeseen sex allocation patterns. To find primary sex ratio, we present a cytogenetic protocol and handout in the supplementary material for finding the primary sex ratio in *B. tabaci*. We also present an old but reliable method to determine differential mortality during development. This technique is a quicker, albeit indirect method of determining whether adult sex ratios are equivalent to primary sex ratios, i.e. sex allocation by the female.

The primary sex ratio of *B. tabaci* can be found after a simple dechoriation of eggs with bleach, a series of fixation steps, and application of the general DNA fluorescent stain, DAPI, to bind to female and male pronuclei (modified from Vavre et al. 2004, fig. 1). As one example of an application, *Rickettsia* bacteria caused a female bias in our laboratory lines of *B. tabaci* MEAM1 (Himler et al. 2011, Cass et al. 2015). We tested a laboratory line (“MAC1”) in which introgression had been used to homogenize the genetic background of both R⁺ and R⁻ sublines

Figure 1: *Bemisia tabaci* MEAM1 fertilized, incipient female (a) and unfertilized, incipient male (b) eggs fixed at less than one hour after oviposition and stained with DAPI. The base of each egg is fluorescing due to bacterial DNA in the bacteriome progenitor cell (*Portiera*, *Hamiltonella*), which is included in the laid egg (Luan et al. 2018). In each egg, the female pronucleus is near the center of the egg, and in the female egg only, the sperm is visible as a bright streak near the apex of the egg near what is presumably an autofluorescing micropyle. Photos were taken with a confocal microscope.

(Himler et al. 2011), and tested whether *Rickettsia*-infected (R^+) females fertilized more eggs than uninfected (R^-) females. Although adult R^+ sex ratios were female-biased (63% R^+ females compared to 55% R^- females, fig. 2), they were not significantly different. The primary sex ratios of both R^- and R^+ whiteflies were close to equivalent, providing no evidence for greater fertilization rates of R^+ females (fig. 2).

If there are no protocols to directly find the primary sex ratio, and the cytogenetics approach is unfeasible, one can determine differential developmental mortality in haplodiploid organisms through survival assays. Haplodiploid virgin mothers only produce male offspring, whereas mated females produce both male and female offspring. Therefore, comparing the developmental mortality between offspring of virgin and mated females can determine whether differential mortality among males and females is occurring. If offspring mortality is different between

Figure 2: Box and whisker plot of primary sex ratio in black (% fertilized eggs or % female) compared to adult sex ratio in red (pooled % female due to high variation and low block sizes) of *Rickettsia*-uninfected (R⁻) and infected (R⁺) *B. tabaci* MEAM1 (using a MAC1 lab line). Pooled primary sex ratios were 56.7% R⁻ females (n=90) and 51.2% R⁺ females (n=82). Pooled adult sex ratios were 55% R⁻ females (n=60) and 63.2% R⁺ females (n=95). Multiple logistic regression was performed in R to determine significant differences in primary sex ratio; whether *Rickettsia*-infected *B. tabaci* MEAM1 females fertilized more eggs than uninfected females. The explanatory variables were block and *Rickettsia*-infection status, while the response variable was the proportion of fertilized eggs. No significant effects were found for block (p=0.66) or *Rickettsia* infection (p=0.94). Multiple logistic regression was also used to see if the adult sex ratios were significantly different, with the response variable as the proportion of adult females and the explanatory variables as block and *Rickettsia*-infection status. Again, no significant effects were found for block (p=0.56) or *Rickettsia* infection (p=0.99).

virgin and mated females, then we can infer that females either have a lower or higher developmental mortality than males. This method has been practiced with many parasitoid wasps (Sandlan 1979, King 1988, Seidl and King 1993, Pandey and Singh 1999, West 2009), but to our knowledge has not been applied to haplodiploid herbivores.

Returning to our previous example, R⁺ whiteflies also had higher egg to adult survival than R⁻ whiteflies (Himler et al. 2011), so we next hypothesized that the mechanism of female bias was differential developmental mortality between males and females. We predicted that R⁺ nymphal females would have greater egg to adult survival, relative to R⁺ males, R⁻ females or R⁻ males. We predicted that there would be no differential survival between the offspring of R⁻ virgin or R⁻

mated females, but higher survival of the offspring of R^+ mated females than of R^+ virgin females. Our hypothesis was rejected, as no greater survival was detected in the offspring of R^+ mated females (fig. 3). No significant effects were associated with *Rickettsia* infection status

Figure 3: Box and whisker plot of a survival assay between offspring of virgin and mated female *B. tabaci* MEAM1 (using lab line MAC1), and also between *Rickettsia*-infected (R^+) and uninfected (R^-) *B. tabaci*. Multiple logistic regression was performed in R to determine significant differences in survival between all treatments, using survival as the response variable and *Rickettsia*-infection status, mated status and block as the explanatory variables. No significant effects were found for *Rickettsia*-infection status ($p=0.99$), mated status ($p=.91$), or block ($p=0.9$).

or mated status, disagreeing with previous results. Taken together, the two experiments did not explain R^+ offspring female bias. In fact, although female bias was generally a consistent feature of *Rickettsia* infection (Cass et al. 2015, Hunter et al. 2016), neither primary or adult sex ratios were significantly different in the fertilization rate experiment where they were measured directly. Nonetheless, this experiment allowed, for the first time, a direct test of this hypothesis.

- **Conclusions and future directions**

In summary, sex ratios of some species of Aleyrodidae and Thysanoptera are influenced by temperature, host plant, conspecifics, competitors, bacterial endosymbionts and fungal pathogens. Extremely high temperatures appear to cause male-biased offspring in whitefly *B. tabaci* (Cui et al. 2008, Lü et al. 2014, Xiao et al. 2016) and thrips *F. occidentalis* (Wang et al. 2014), while a positive correlation between temperature and the proportion of female offspring is found in both thrips *C. claratris* (Premachandra et al. 2004) and *F. occidentalis* (Kumm and Moritz 2010) at less extreme temperatures. Higher quality host plants may be associated with even sex ratios in the castor whitefly, *T. ricini* (Shishehbor and Brennan 1996, Huang et al. 2014), but the results in the current literature appear inconsistent. LMC has been invoked in a convincing manner to explain the sex ratios of certain galling thrips (Crespi 1993, Tsuchida and Ohguchi 1998, Willis et al. 2004, Tree and Walter 2009), inquiline thrips (Gilbert et al. 2012) and the spiralling whitefly, *A. dispersus* (Hu et al. 2010). Otherwise, LMC has not been found in pest species such as *B. tabaci* and *F. occidentalis*, and there are no clear examples of LRC or LRE in these taxa. The Trivers-Willard hypothesis most likely explains reproductive allocation toward male broods in times of greater fungal-resources in the fungus-feeding *Elaphrothrips tuberculatus* (Crespi 1988).

There is evidence that the *B. tabaci* species complex undergoes interspecific mating interference, in which *B. tabaci* MEAM1 causes a male bias in *B. tabaci* MED through asymmetric mating interference (Pascal and Callejas 2004, Crowder et al. 2010b, Tsueda and Tsuchida 2011, Sun et al. 2014). The presence of both *B. tabaci* MED and MEAM1 trigger the production of male-biased offspring in endemic *B. tabaci* species, and female-biased offspring themselves (Liu et al. 2007, Wang et al. 2012). Bacterial endosymbionts may also influence sex

ratios. *Rickettsia* may cause a female bias in *B. tabaci* (Himler et al. 2011, Parrella et al. 2013, Cass et al. 2016), *Wolbachia* causes parthenogenesis in the thrips *F. vespiformis* (Arakaki et al. 2001) and *H. femoralis* (Kumm and Moritz 2008), and *Wolbachia* and *Cardinium* simultaneously cause male-bias as result of cytoplasmic incompatibility in *P. kellyanus* (Nguyen et al. 2017). Finally, a *Beauveria bassiana* fungus causes male-biased offspring in exposed *F. occidentalis* (Zhang et al. 2015). No effects of sex ratio by viruses, predators or parasitoids have been yet recorded.

The various life histories of Aleyrodidae and Thysanoptera have been thoroughly documented, especially from the “supervectors” *B. tabaci* and *F. occidentalis* (Gilbertson et al. 2015). Although changes in adult sex ratio may be sufficient to predict future populations of Aleyrodidae and Thysanoptera, discovering sex allocation in haplodiploid herbivores would further enhance our predictions and fundamental understanding of these important and interesting insects. If sex allocation that departs from even sex ratios occurs in these taxa, it may also elucidate patterns in other herbivorous insects with less obvious sex allocation. The tools presented in this study provide methods to find primary sex ratios in Aleyrodidae or differential developmental mortality in whiteflies and thrips.

- **Supplementary material**

-DAPI staining *Bemisia tabaci* eggs to determine sex (modified from Vavre et al. 2004)

-Egg collection

Prepare a microscope slide by wounding a stretched piece of parafilm around one end. Alternatively, a liquid-repellent slide marker pen can be used to keep a drop from spreading on a slide. Allow female whiteflies to oviposit on clean leaves. After 30-60 minutes, collect several eggs with a fine probe for immediate fixation. During preliminary experiments, eggs that were

fixed at three hours post-oviposition were too old, as karyogamy had already occurred, and it was not possible to easily distinguish between haploid and diploid nuclei. Therefore, limiting egg fixation to within one hour is a conservative time to ensure observation of the sperm prior to paternal chromatin remodeling. Unharvested eggs can be reared to adulthood to record adult sex ratio with which to compare with the primary sex ratio as determined by fertilization rate. Before removing eggs, place drops of bleach (0.83% Sodium hypochlorite) on the parafilm. If removing the eggs proves to be a timely process, place drops of PBS on the parafilm, and then replace with bleach when all eggs are collected. The bleach will dechorionate the eggs after 10-15 minutes.

After removing the bleach, fix the eggs with glacial acetic acid for 3 minutes, Clarke's solution [3:1 of absolute ethanol:glacial acetic acid] for 10 minutes and then 70% ethanol for 10 minutes (Vavre et al. 2004). After most of the ethanol evaporates, place drops of 1x PBS buffer on the eggs for 30 minutes to ensure that the pH is 7.0. Insert the slide into a humidity chamber, such as a pipette tip box with a wet paper towel, to prevent PBS evaporation. After removing the PBS, add drops of 0.1 μ g /mL DAPI to the eggs. Place the slide into a dark humidity chamber for 15 minutes. After the DAPI stain and removal, wash the eggs three times with 1x TBST for 5 minutes each. Then carefully pipette the eggs from the parafilm onto the uncovered area of the microscope slide. Add a 20 μ L drop of mounting media (80% glycerol and 20% TBST with 2% n-propyl-galate), and carefully place a clean cover slide onto the drop. Seal the slide with clear nail polish if the slide will be stored for later use. The slide can be viewed with a fluorescence microscope, or stored in the dark at 2° C for later viewing. If a lozenge-shaped paternal pronucleus is at or close to the micropyle of the egg, the egg is fertilized and presumed female (Figure 1). A pamphlet of these methods with photos and extra tips can be found in supplementary material.

-*Bemisia tabaci* Cytogenetics Protocol Handout with Tips**Oviposition set-up**

1. Allow female whiteflies to oviposit on clean leaves. During preliminary experiments, eggs that were fixed at three hours post-oviposition were too old, as female pronuclei already multiplied and no sperm tails were present. Therefore, limiting female oviposition time to 30-60 minutes is a conservative time to ensure observation of any sperm tails in eggs. If there are abundant unharvested eggs, they can be reared to adulthood to record adult sex ratio.

Microscope slide set-up

2. Before or during oviposition, prepare microscope slide by cleaning with soap and water, and then stretching a piece of parafilm on the side, making sure the parafilm surface will not break.

-Microscope slide with parafilm and drops of bleach

Dechoriation

3. With Pasteur pipette *, add drops of bleach solution (0.83% Sodium hypochlorite) to parafilm (or PBS if slow at collecting eggs). It is easier to keep track of eggs if only 2-3 eggs are in each drop

4. Collect eggs with a probe and drop into bleach/PBS (if using PBS; remove PBS once all eggs are collected, then add bleach)

Tip: Slowly lift egg from bottom until the pedicel is removed from the leaf. The pedicel is sticky and the egg should be stuck to the probe tip until it is dipped in bleach

-Probe, fashioned with heat, from pipette tip and minuten pin:

5. Wait 10-15 min. Older eggs require longer exposure to bleach for dechoriation

Fixation (taken from Vavre et al. 2004)

6. Remove bleach. Add glacial acetic acid and wait 3 min.
7. Remove glacial acetic and add Clarke's solution (3:1 of absolute ethanol: glacial acetic acid). Wait until majority of solution evaporates (or 10 min. max)
8. Add 70% ethanol to eggs and wait until most ethanol evaporates (or 10 min. max)

Staining

9. Add 1XPBS to eggs to get pH around 7.0 (pH needed for DAPI to specific-stain). Wait 30 min. Set slide in humidity chamber to prevent desiccation

-Humidity chamber fashioned from an empty pipette tip box and wet paper towel

10. Remove PBS and add 0.1 μg /mL DAPI. Set slide in a dark, humid area and wait at least 15 min.

Washing

11. Remove DAPI. Wash at least 3X with 1XTBST for 5 min. each wash.

Mounting

12. After final wash, carefully pipette all eggs from the parafilm onto a clean part of the microscope slide. Remove excess TBST, add 20 μL of mounting media (80% glycerol and 20% TBST with 2% n-propyl-galate), and place clean cover slide on top of eggs
13. Seal cover slide with clear nail polish if storing, and then either store in the dark at 2° C or view under fluorescent microscope

* It is recommended to use Pasteur pipettes for all other reagents as well, and possibly even modify with heat to make the tips smaller and less capable of accidentally aspirating whitefly eggs. To prevent residue and contamination, be sure to clean pipette with DI water after every use, or allocate a pipette for each solution.

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