



# Intracellular symbionts drive sex ratio in the whitefly by facilitating fertilization and provisioning of B vitamins

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## Abstract

Symbionts can regulate animal reproduction in multiple ways, but the underlying physiological and biochemical mechanisms remain largely unknown. The presence of multiple lineages of maternally inherited, intracellular symbionts (the primary and secondary symbionts) in terrestrial arthropods is widespread in nature. However, the biological, metabolic, and evolutionary role of co-resident secondary symbionts for hosts is poorly understood. The bacterial symbionts *Hamiltonella* and *Arsenophonus* have very high prevalence in two globally important pests, the whiteflies *Bemisia tabaci* and *Trialetrodes vaporariorum*, respectively. Both symbionts coexist with the primary symbiont *Portiera* in the same host cell (bacteriocyte) and are maternally transmitted. We found that elimination of both *Hamiltonella* and *Arsenophonus* by antibiotic treatment reduced the percentage of female offspring in whiteflies. Microsatellite genotyping and cytogenetic analysis revealed that symbiont deficiency inhibited fertilization in whiteflies, leading to more haploid males with one maternal allele, which is consistent with distorted sex ratio in whiteflies. Quantification of essential amino acids and B vitamins in whiteflies indicated that symbiont deficiency reduced B vitamin levels, and dietary B vitamin supplementation rescued fitness of whiteflies. This study, for the first time, conclusively demonstrates that these two intracellular symbionts affect sex ratios in their whitefly hosts by regulating fertilization and supplying B vitamins. Our results reveal that both symbionts have the convergent function of regulating reproduction in phylogenetically-distant whitefly species. The 100% frequency, the inability of whiteflies to develop normally without their symbiont, and rescue with B vitamins suggests that both symbionts may be better considered co-primary symbionts.

## Introduction

Inherited symbionts impact animal biology in a multitude of ways [1–3]. Symbionts play a key role in regulating animal reproduction [4–6]. Some symbionts are designated as reproductive manipulators and their acquisition can lead to feminization, parthenogenesis, male-killing, or cytoplasmic incompatibility of their animal hosts [4–9]. Mechanistically this is accomplished by disruption of mitosis or meiosis essential for gamete production or production of a male-killing toxin. In addition, several symbionts are able to influence the fecundity of their hosts by altering the host's nutritional status; these studies span diverse interactions including bedbug and tsetse fly feeding on animal blood and planthoppers feeding on plant sap [10–14]. However, the physiological and biochemical mechanisms underlying reproduction manipulation of phytophagous animals by symbionts remain largely unknown.

The presence of multiple lineages of maternally inherited, intracellular symbionts in animals is widespread in

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nature [15–18]. These intracellular symbionts include primary symbionts, which are required by the host and one or more secondary symbionts, which are not essential to hosts but may function under specific conditions [3]. The location of primary and secondary symbionts within phloem-feeding insects varies. Some secondary symbionts coexist with the primary symbiont in the same host cell (bacteriocyte) of whiteflies [17, 19] and mealybugs [20, 21]; while in aphids [22] and psyllids [23], the primary and secondary symbionts reside in different bacteriocytes. Although genome analyses suggest nutritional functions of symbionts for insects [18, 24–27], the biological, metabolic, and evolutionary roles of co-resident secondary symbionts are elusive.

There are more than 1900 species of whiteflies [28]. Among them, the whiteflies *Bemisia tabaci* MEAM1 and *Trialeurodes vaporariorum* are globally important pests in the agriculture. Their reputation as notorious invasive pests is documented by their invasions into China in the 1990s and 1970s, respectively [29, 30]. In all whitefly species, at least one secondary symbiont cohabits with the primary symbiont “*Candidatus* *Portiera aleyrodidarum*” (hereafter *Portiera*) in the same bacteriocyte [17, 19]. Additionally, whiteflies may harbor up to four secondary symbiont lineages out of seven bacterial genera that have been detected and represent naturally occurring microbial communities [17, 19].

Whitefly symbiosis is a valuable model system to study the function of secondary symbionts, which co-exist with the primary symbionts [31]. Previous studies showed that the secondary symbiont “*Candidatus* *Hamiltonella defensa*” (hereafter *Hamiltonella*) has a very high prevalence in the whitefly *B. tabaci* MEAM1 and MED, and *Arsenophonus* sp. in the whitefly *T. vaporariorum* and other *B. tabaci* species [17, 19, 31]. Additionally, *Hamiltonella* and *Arsenophonus* in *B. tabaci* MEAM1 and *T. vaporariorum*, respectively, can be vertically transmitted with *Portiera* via the whitefly’s bacteriocytes [17, 19, 32, 33]. Recently, we found *Hamiltonella* influences the sex ratio in the whitefly *B. tabaci* MEAM1 [34]. However, whether the effect of secondary symbionts on the sex ratio is ubiquitous in whiteflies is not clear. *Arsenophonus* retains most genes involved in synthesis of B vitamins in its genome as *Hamiltonella* [27, 35]; thus in *T. vaporariorum*, *Arsenophonus* may have similar functions as *Hamiltonella* has in *B. tabaci* MEAM1. Elucidating the mechanisms underlying the effect of these secondary symbionts on whitefly reproductive biology will advance our understanding of the evolution and function of co-habitant symbionts in animals as well as provide new avenues for future pest control. Here, we investigated the physiological and biochemical mechanisms of *Hamiltonella* and *Arsenophonus* impact on whitefly reproductive biology. Our results reveal the evolutionary convergence in function of these intracellular symbionts in whitefly symbiosis.

## Materials and methods

### Geographic survey on the whitefly and associated symbionts

The whiteflies *T. vaporariorum* were collected from six locations in China (Supplementary Table 1). The mtCOI gene sequence for each whitefly population and the infection frequency of *Portiera* and seven secondary symbionts in whiteflies was determined by PCR and Sanger sequencing, using the primers as described previously [36] and shown in Supplementary Table 2. For more details, see the Supplementary Text.

### Insect rearing and plants

*B. tabaci* MEAM1 colony (mtCOI GenBank accession no. GQ332577) was collected in Zhejiang Province, China in 2008 [37]; the colony was maintained on cotton plants (*Gossypium hirsutum* cv. Shiyuan 321). The *B. tabaci* colony harbors *Portiera*, *Hamiltonella* and *Rickettsia* sp. (hereafter *Rickettsia*) as identified previously [34]. The *T. vaporariorum* colony was established with 50 females and 50 males collected from field populations on tobacco (*Nicotiana tabacum*) in Shenyang of Liaoning Province, China. This colony was maintained on the tobacco *N. tabacum* cv. NC89 as described previously [38] and its genotype corresponds to the mtCOI GenBank accession no. MH422959. The *T. vaporariorum* colony harbors *Portiera* and *Arsenophonus*. The genotype of the whitefly colonies was monitored every three to five generations.

Both cotton and tobacco plants were grown in potting mix (Pindstrup, Denmark) supplemented with Miracle-Gro Water Soluble All Purpose Plant Food every 2–3 days. The cotton and tobacco plants were grown singly in 1.5 L pots to the six-to-seven true-leaf stage for the experiments described below. All whitefly colonies and plants were maintained in separate climate-controlled chambers, at  $26 \pm 2$  °C with 14 h light:10 h dark regime and 60–80% relative humidity (RH). The LED fluorescent lights were used and light intensity in the walk-in chamber was approximately  $400 \mu\text{mol}/\text{m}^2 \text{ s}$ .

### PCR and quantitative PCR (qPCR)

Individual insects were homogenized and DNA was extracted by the Nonidet-P40-based protocol as described previously [33]. The symbionts were quantified by qPCR using the CFX96 Real-Time PCR Detection System (Bio-Rad) with SYBR-Green (2×SYBR Green master Mix, Bimake). *B. tabaci*’s symbionts (*Portiera*, *Hamiltonella* and *Rickettsia*) and *T. vaporariorum*’s symbionts (*Portiera* and *Arsenophonus*) were quantified using the primers as shown

in Supplementary Table 2. The *B. tabaci*  $\beta$ -actin gene and *T. vaporariorum* NADH gene serve as internal standard for normalization. Three technical replicates were performed for each of 12 biological replicates for *B. tabaci* and six biological replicates for *T. vaporariorum* as described below. The relative symbiont density was calculated using the  $2^{-\Delta Ct}$  method [39]. For more details, see the Supplementary Text.

### Fluorescence in situ hybridization (FISH)

The localization of *Portiera* and *Arsenophonus* in eggs, 4th instar nymphs, and female adults of *T. vaporariorum* and *Portiera*, *Hamiltonella*, and *Rickettsia* in the female adult of *B. tabaci* was investigated using FISH following the protocol as described previously [40]. Briefly, the insect material was fixed, digested, decolorized, and hybridized in hybridization buffer with fluorescent probes. Stained samples were viewed under a FV3000 confocal microscope (Olympus, Japan). For more details, see the Supplementary Text.

### Low and high symbiont titer whitefly lines established by antibiotic treatment

*Hamiltonella* was specifically eliminated by treating *B. tabaci* adults with antibiotics. Hundreds of adult whiteflies of *B. tabaci* (F0, 0–7 days after emergence) were released into each feeding chamber and fed on 25% sucrose solution (w/v) supplemented with the antibiotics ampicillin, gentamycin and cefotaxime (BBI Life Sciences, Shanghai, China), each at 500  $\mu$ g/mL for 4 days. The artificial diets with antibiotics were renewed every two days as described previously [34]. Hundreds of adult whiteflies of *T. vaporariorum* (F0, 0–7 days after emergence) were released into each feeding chamber and fed on 30% sucrose solution (w/v) supplemented with the antibiotic Zhongshengmycin (Hubei Jiuzhoukangda Technology Co., Ltd, Zaoyang, China) at 150  $\mu$ g/mL for two days. Control insects were administered sucrose solution not supplemented with antibiotics. Following the diet regimens, *B. tabaci* and *T. vaporariorum* were transferred to cotton and tobacco plants, respectively. Recently emerged F1 female and male adults (within 1 week after eclosion) were collected and used for symbiont quantification by qPCR. The DNA was extracted from 12 and six female adults of *B. tabaci* and *T. vaporariorum*, respectively, and used for symbiont quantification by qPCR. The F1 *B. tabaci* with reduced *Hamiltonella* titers (–HBt) and *T. vaporariorum* with reduced *Arsenophonus* titers (–ATv), which were obtained by antibiotic treatment, and control F1 *B. tabaci* (+HBt) and *T. vaporariorum* (+ATv), which were obtained by feeding sucrose solution not supplemented with antibiotics, were identified and used for the experiments unless specified elsewhere.

### Whitefly performance experiment

Single pupae were cut from different leaves of multiple cotton and tobacco plants and transferred to individual glass tubes (0.4  $\times$  4 cm) until they developed to adulthood as described previously [33]. Ten matings with one pair of virgin whiteflies (+HBt $\varphi$  $\times$ +HBt $\sigma$  and –HBt $\varphi$  $\times$ –HBt $\sigma$ ) were made. Similarly, nine matings were made for +ATv $\varphi$  $\times$ +ATv $\sigma$  and –ATv $\varphi$  $\times$ –ATv $\sigma$ . All adults were collected at 2 h post emergence. Insect pairs were released into a clip-cage that was secured to the abaxial surface of a cotton (HBt matings) or tobacco (ATv matings) leaf. The third-fifth leaves from the top of both plant species were used for the experiments. Adult whiteflies were removed after 5 days. After emergence of next generation, the sex ratio was recorded.

### Genotyping

One pair of virgin female and male adults of –HBt whiteflies (2 h post emergence) were released into a clip-cage that was secured to the abaxial surface of a cotton leaf and allowed to oviposit for five days. Three biological replicates were conducted. Insects from each of three F0 mating pairs were collected and heads were dissected for microsatellite determination. Once the F1 insects emerged, adult male whiteflies were collected and heads were dissected for microsatellite determination as for the F0 insects. Three microsatellite markers developed for *B. tabaci* [41] were amplified by PCR multiplexing with QIAGEN Multiplex PCR Plus Kit (Qiagen, Hilden, Germany) using primers listed in Supplementary Table 3. The PCR products were analyzed on a capillary sequencer, ABI 3730  $\times$  1 DNA Analyzers (Applied Biosystems) as described previously [33, 38]. The alleles were analyzed using the software Genemarker (SoftGenetics LLC., USA) following the user manual. Finally, the head microsatellite profiles of F0 females, F0 males and F1 males were compared.

### Cytogenetics

To examine whether infection of *Hamiltonella* and *Arsenophonus* impacts fertilization and to synchronize the age of adults for the cytogenetic analyses, approximately 800 pairs of female and male adult whiteflies (2 h post emergence) were collected from *B. tabaci* +HBt and –HBt lines and released onto different cotton plants; this experiment was replicated three times. Similarly, whitefly pairs from each of *T. vaporariorum* +ATv and –ATv lines were released onto different tobacco plants; this experiment was repeated four times. After feeding and mating for five days, 20 pairs of female and male adult whiteflies for each line were released into each clip-cage attached to the leaves

(third–fifth leaf from the top of both plant species), and allowed to lay eggs for less than 1 h and eggs were collected immediately. There were 40 clip-cages for each of the whitefly lines (+HBt, –HBt, +ATv, and –ATv) in each replicate experiment. Eggs were dechorionated by 3% hypochlorite in PBS for 5 min, washed with PBS twice, fixed with 4% paraformaldehyde at room temperature for 30 min, and then permeabilized with 0.1% Triton X-100 in PBS for 30 min referring to the method described previously [42]. The time between oviposition and egg fixation was less than 1 h. The 1-h egg samples were incubated with DAPI (4'-6-diamidino-2-phenylindole) (0.1 µg/mL in PBS; Sigma, St. Louis, MO USA) for 30 min at room temperature. Between 18–25 eggs and 12–22 eggs were observed in the three replicates of +HBt and –HBt matings, respectively. While 8–11 eggs and 12–17 eggs were observed in the four replicates of +ATv and –ATv matings, respectively. Images were collected and analyzed on a FV3000 confocal microscope (Olympus, Japan). We distinguished egg pronucleus from sperm pronucleus based on their shape, size and localization. For eggs within 1 h of deposition in our lab conditions, fusion of egg pronucleus and sperm pronucleus had not yet occurred. Therefore, the fertilization rate is based on an early stage of fertilization (i.e., presence of a sperm within the egg).

### Amino acid measurement

Three to four days post emergence, 50 adult whiteflies (1:1 of female/male ratio) from +HBt and –HBt lines were collected for each of six replicates. Similarly, at 3–4 days post-emergence, 50 adult female whiteflies of +ATv and –ATv lines were collected for each of three replicates. Samples were homogenized for amino acid analysis by Ultra Performance Liquid Chromatography (UPLC) using the protocol as described previously [43]. Samples were injected into Agilent UPLC with PDA detector and AccQ-Tag Ultra 2.1 × 100 mm column. Amino acids are determined by comparing their retention time with standards, protein-amino acids/µl (Waters amino acid hydrolysate standard #088122, supplemented with asparagine, tryptophan, and glutamine) and quantified with standard curves. Proteins were quantified using Lowry Protein Assay Kit (Sangon, Biotech, China) following manufacturer's instructions using bovine serum albumin (BSA) as a standard. Amounts of individual amino acids were normalized to total protein content. For more details, see the Supplementary Text.

### Vitamin measurement

Totally, 50 male and 50 female adult whiteflies (3–4 days postemergence) were collected from each of +HBt, –HBt, +ATv, and –ATv lines and flash-frozen in liquid N<sub>2</sub>. Three

biological replications of each line were performed. In an experiment, insects from each line were pooled, weighed, and homogenized in 100 µL citrate buffer for riboflavin, NAD(P)H, pyridoxine and biotin determinations as described previously [44]. For folate determinations, 0.1 M K<sub>2</sub>HPO<sub>4</sub>–KH<sub>2</sub>PO<sub>4</sub> buffer with 1% ascorbic acid (pH 6.3) was used as described previously [13]. A MP FastPrep-24 homogenizer (MP Biomedicals LLC, USA) was used for all samples. Vitamins were quantified using a standard microbiological assay as described previously with slight modifications [13, 44]; for each assay, vitamin levels are quantified by measuring the relative growth of a vitamin-deficient bacterium. The microorganisms, media, and standard compounds used in the vitamin assay were provided in Supplementary Table 4. Absorbance at 630 nm was measured using a spectrometer (Molecular Devices, USA). Three technical replicates were performed for each of three biological replicates. The vitamins were quantified using the standard curves and normalized to the weight of insects included in the homogenate.

### Vitamin supplementation experiment

Adult whiteflies (2 h postemergence) were collected from the –HBt and –ATv lines. For the vitamin supplementation treatments, each feeding chamber had 100 males and 100 females (–HBt line) or 30 males and 30 females (–ATv line). Vitamin-treated adults fed on artificial diets with 30% sucrose and B vitamin mix (the concentration of each B vitamin was provided in Supplementary Table 5) based on previous studies [10, 45–47]. Control adults were fed on 30% sucrose. The diets were renewed every 2 days. After feeding on diets for 4 days, one female adult and one male adult whitefly were removed from each treatment; there were 11 individual replicates of each treatment per whitefly line. Adults of the whitefly *B. tabaci* and *T. vaporariorum* were transferred to individual clip-cage attached to the cotton and tobacco plants, respectively. Insects were removed after 5 days. The sex ratio of the offspring was recorded.

### Statistical analyses

For effect of B vitamin supplementation on whitefly performance, data were evaluated using one-way ANOVA at a 0.05 level followed by LSD tests. For the symbiont titer, percentage female in offspring, fertilization rate, amino acid quantities, and vitamin quantities, statistical significance was evaluated between low symbiont titer lines and high symbiont titer lines using one-way ANOVA at a 0.05 level. Data in percentages were transformed by arcsine square root before analysis. All data analyses were conducted using the software STATISTICA 6.1 (StatSoft, Inc., Tulsa, USA).



## Results

### ***Arsenophonus* is fixed in whitefly populations and localized in bacteriocytes**

*T. vaporariorum* were collected from seven plant species from six locations with temperate and subtropical climates across five provinces in northeast, north and southwest of China (Supplementary Table 1). Only *Portiera* and *Arsenophonus* were detected in field populations of *T. vaporariorum*. Interestingly, *Arsenophonus* has very high prevalence in the whitefly populations with infection frequency of 100% in most locations (Supplementary Table 1). These data indicate *Arsenophonus* has been fixed in whitefly populations in China after the *T. vaporariorum* invasion.

As with *Portiera* and *Hamiltonella* in *B. tabaci* [32], *Portiera* and *Arsenophonus* cohabited in the bacteriocytes of eggs, nymphs and adults in *T. vaporariorum* (Fig. 1a), suggesting that two symbionts are maternally transmitted via bacteriocytes in the whitefly. Confocal microscopy observation confirmed that *Portiera* and *Hamiltonella* were localized in bacteriocytes, while *Rickettsia* was distributed in the whole body cavity of the female adult of the whitefly *B. tabaci* (Fig. 1b). Our observation also indicated that *Portiera* and *Arsenophonus* in *T. vaporariorum* as well as *Portiera* and *Hamiltonella* in *B. tabaci* cohabit but they seem to be some “clustering” (seen as small points of fluorescence). High prevalence and maternal transmission of *Arsenophonus* and *Hamiltonella* suggests they may have specific function for hosts.

### **Deficiency of *Hamiltonella* and *Arsenophonus* distorts the sex ratio in *B. tabaci* and *T. vaporariorum***

We generated low *Hamiltonella* titer line (–HBt) by treatment with an antibiotic cocktail. The *Hamiltonella* titer was reduced by 90% in –HBt whiteflies compared to +HBt whiteflies (Fig. 1c;  $F_{1,22} = 14.68$ ,  $p = 0.00091$ ), while the titer of *Portiera* and *Rickettsia* remained unchanged (Fig. 1c;  $F_{1,22} = 0.39$ ,  $p = 0.54$  for *Portiera* and  $F_{1,22} = 1.51$ ,  $p = 0.23$  for *Rickettsia*). This study and our previous work [34] show that our method to specifically eliminate *Hamiltonella* is very stable, reliable, and repeatable. The percentage of female offspring was reduced by 78% in –HBt whiteflies compared to the +HBt control (Fig. 1d;  $F_{1,18} = 31.39$ ,  $p = 0.000026$ ).

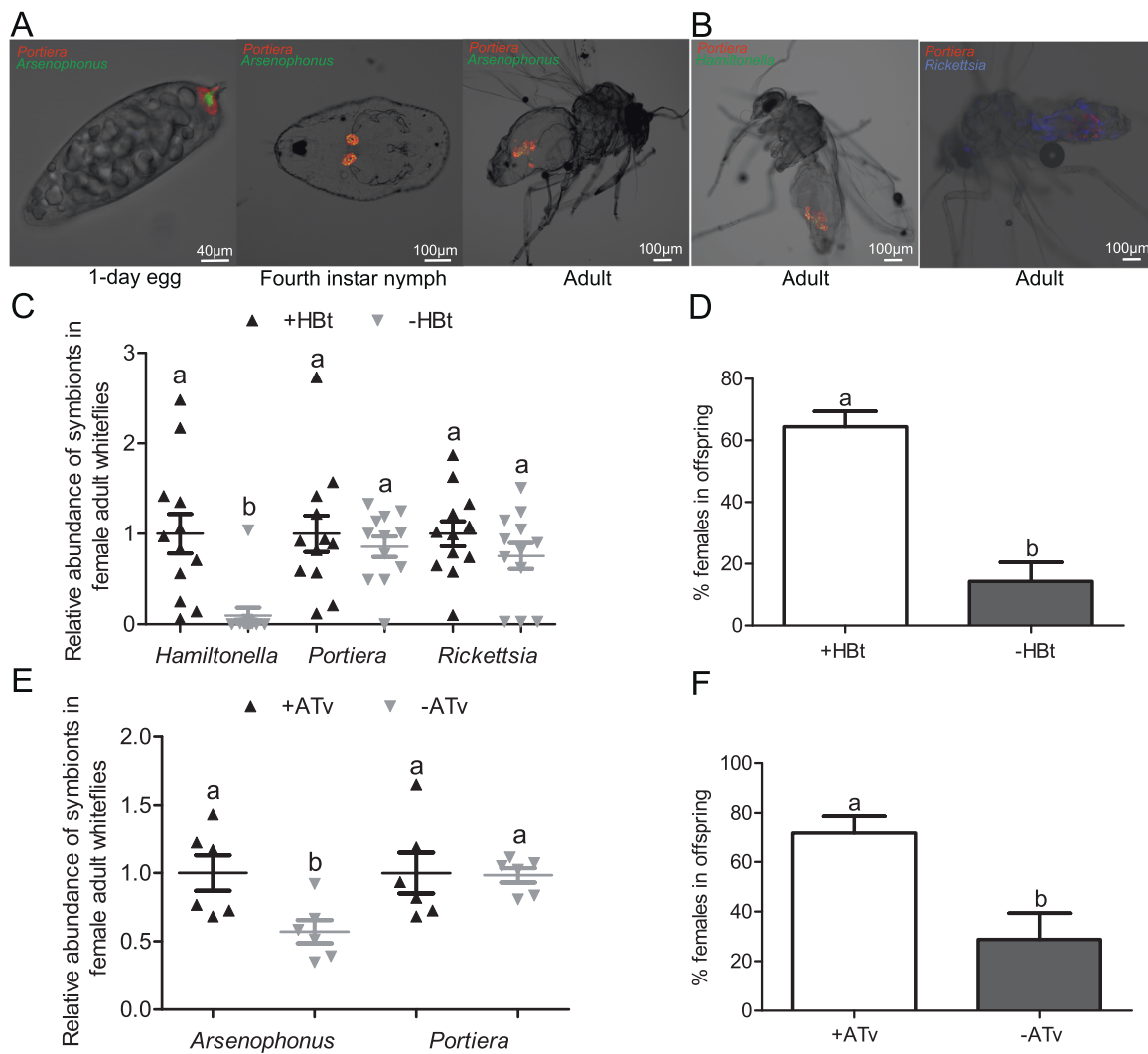
To determine if sex ratios are also influenced by other secondary symbionts, the interactions of *Arsenophonus* and *T. vaporariorum* were examined. We generated low *Arsenophonus* titer line (–ATv line) via antibiotic treatment. The *Arsenophonus* titer was reduced by 43% in –ATv whiteflies compared to +ATv whiteflies, while the *Portiera*

titer remained unchanged (Fig. 1e;  $F_{1,10} = 7.75$ ,  $p = 0.019$  for *Arsenophonus* and  $F_{1,10} = 0.011$ ,  $p = 0.92$  for *Portiera*). We found the percentage of female offspring was significantly reduced in –ATv compared to +ATv whiteflies (Fig. 1f;  $F_{1,16} = 11.12$ ,  $p = 0.0042$ ). Taken together, our data demonstrated that low titers of *Hamiltonella* or *Arsenophonus* are correlated with a decline in the percentage of female offspring in *B. tabaci* and *T. vaporariorum*, respectively, suggesting their equivalent function in whiteflies.

### **Symbiont deficiency inhibits fertilization in the whitefly**

Whiteflies utilize a haplo-diploid genetic system; fertilized eggs develop into female offspring and unfertilized eggs produce male offspring [48]. Our hypothesis is that the reduction in females in low titer symbiont lines is due to a reduction in egg fertilization. However, in some cases, unanticipated genetic outcomes have been observed after elimination of parthenogenesis-inducing endosymbionts. For example, for haplodiploid wasps that have low titers of parthenogenesis-inducing endosymbionts, diploid male wasps are produced [49, 50]. Therefore, to determine if the decrease in the female:male ratio in *Hamiltonella*-deficient lines is due to lack of production of females or the conversion of diploid zygotes to males, we determined the genotypes of males in the F0 and F1 generations. To this end, mating pairs using –HBt whiteflies were set up and the microsatellite profile of the heads of the parents (F0) and the male offspring (F1) was determined. We found that male heads had one maternal allele in every male offspring tested in F1 of the whitefly *B. tabaci* (Fig. 2a–f; Supplementary Dataset 1). Therefore, the possibility that male offspring produced by *Hamiltonella*-deficient lines are diploid is ruled out. As transmission of the two female microsatellite alleles was unimpaired to male progeny, these data suggest that F1 males of –HBt whiteflies are haploid, and the decline in female progeny may be due to a defect in fertilization.

To determine if the low-titer symbiont lines have a defect in fertilization, we determined if a change in the fertilization rate was the basis for the distorted sex ratio. The primary sex ratio, which reflects the fertilization rate of eggs, was determined for eggs within 1 h of deposition in low-titer and high-titer symbiont lines; at these times, both egg and sperm pronuclei are visible [42]. For both *B. tabaci* and *T. vaporariorum*, the egg pronucleus, which has a rounder shape (with the diameter of 3–4  $\mu\text{m}$ ), was located at the center of each egg. Whereas, the sperm pronucleus is long and thin (with the length of 6–15  $\mu\text{m}$ ) and was visible as a bright streak near the apex of the female egg (Fig. 3a, b). Sperm staining clearly showed that the fertilization rate in low symbiont titer lines including –HBt and –ATv



**Fig. 1** Deficiency of *Hamiltonella* and *Arsenophonus* influences the sex ratio of *B. tabaci* MEAM1 and *T. vaporariorum*. **a** Localization identified by FISH of symbiotic bacteria *Arsenophonus* (green) and *Portiera* (red) in the egg, nymph and female adult of +ATv whiteflies. **b** Localization identified by FISH of symbiotic bacteria *Hamiltonella* (green), *Rickettsia* (blue) and *Portiera* (red) in the female adult of +HBt whiteflies. **c** Relative abundance of *Hamiltonella*, *Portiera*, and

*Rickettsia* in female adults of +HBt and –HBt whiteflies ( $n = 12$ ). **d** Percentage of female offspring in +HBt and –HBt lines ( $n = 10$ ). **e** Relative abundance of *Arsenophonus* and *Portiera* in female adults of +ATv and –ATv whiteflies ( $n = 6$ ). **f** Percentage of female offspring in +ATv and –ATv whiteflies ( $n = 9$ ). Error bars represent one standard error. Different letters above the bars indicate significant statistical differences at  $p < 0.05$ .

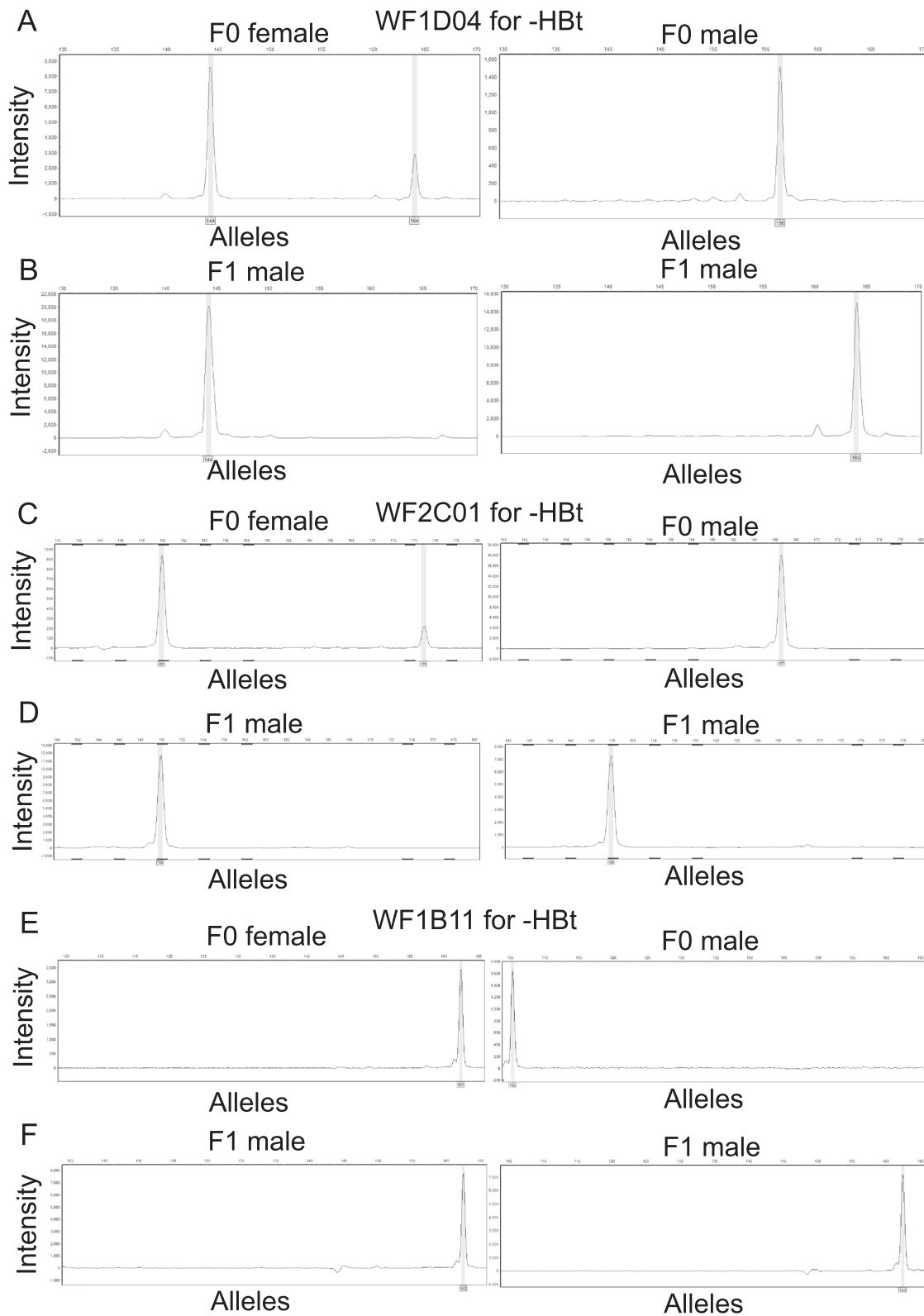
whiteflies was significantly lower compared to +HBt and +ATv whiteflies, respectively (Fig. 3c;  $F_{1,4} = 58.4$ ,  $p = 0.0016$  for *B. tabaci*; Fig. 3d;  $F_{1,6} = 20.45$ ,  $p = 0.004$  for *T. vaporariorum*). These results reveal that the low titers of *Hamiltonella* and *Arsenophonus* impede fertilization of eggs at the early stage of fertilization and thereby decreases the percentage of female offspring in whiteflies.

### Symbiont deficiency reduces the level of B vitamins but not essential amino acids

Whitefly symbionts may contribute to the welfare of their hosts by the synthesis of essential amino acids and B

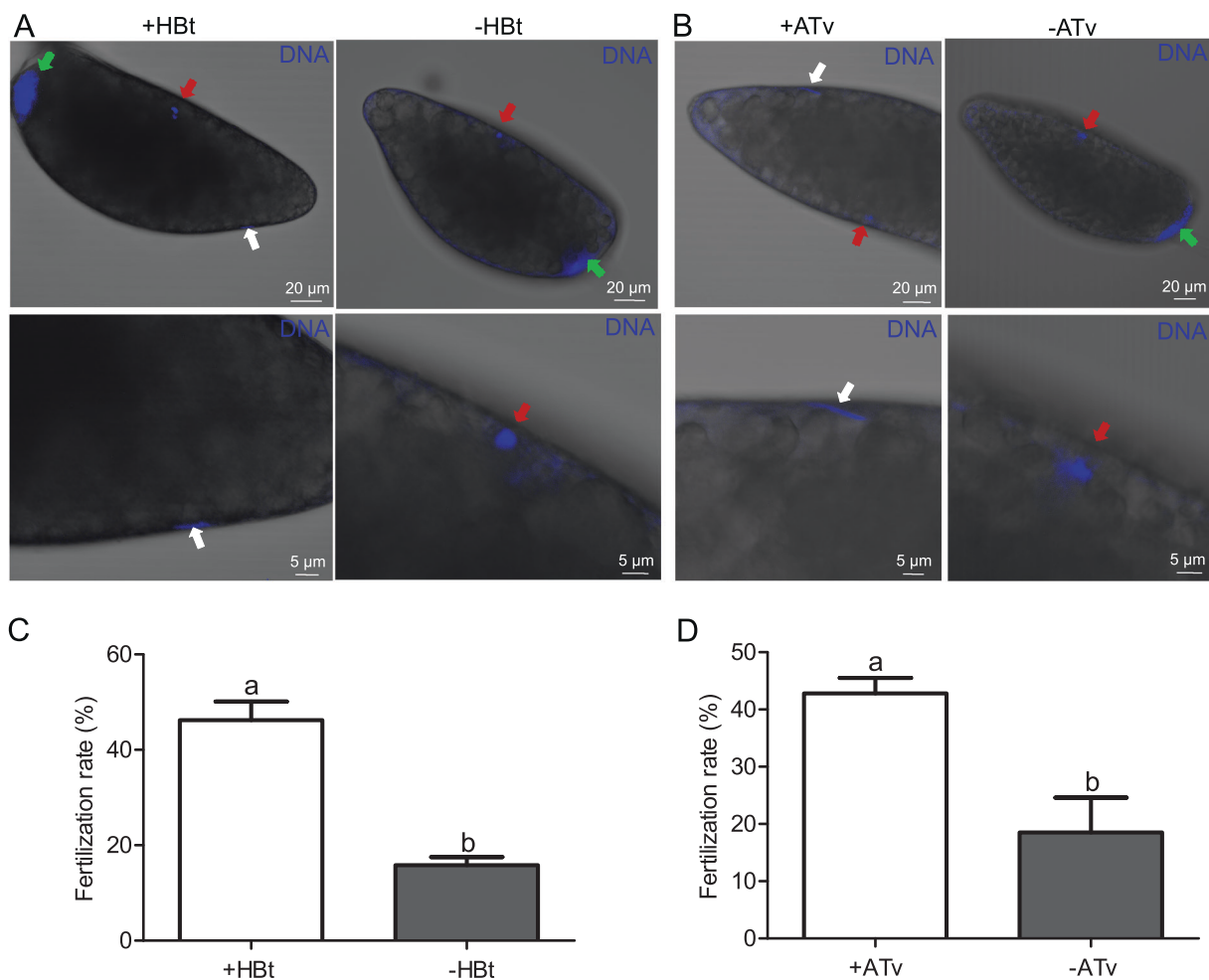
vitamins [27, 35, 51–53]. As the abundance of *Hamiltonella* or *Arsenophonus* were significantly reduced in –HBt and –ATv whiteflies relative to +HBt and +ATv whiteflies, respectively (Fig. 1c, e), it was possible that the altered symbiont abundance might alter one or both of these critical biochemical pathways.

The genome of *B. tabaci*'s and *T. vaporariorum*'s primary symbiont *Portiera* contains most of the genes involved in the synthesis of ten essential amino acids, but only nine amino acids are believed to be essential [3, 51]. While, the genomes of *Hamiltonella* and *Arsenophonus* contain most of genes involved in synthesis of two essential amino acids: phenylalanine (Phe) and lysine (Lys)



**Fig. 2** Microsatellite profiles for the F0 mating pairs and F1 male offspring from symbiont-deficient whiteflies. Microsatellite markers WF1D04, WF2C01, and WF1B11 were followed in a cross of -HBt whiteflies over one generation. Representative microsatellite profiles in

heads of F0 female and F0 male and two F1 males for WF1D04 (a, b), WF2C01 (c, d), and WF1B11 (e, f). The minor peaks with the sizes of 140, 153 and 160 are peak stutter.



**Fig. 3** Deficiency of *Hamiltonella* and *Arsenophonus* inhibits fertilization in whiteflies. **a, b** Egg pronucleus and sperm pronucleus in the egg within 1 h post deposition in +HBt and -HBt whiteflies (**a**) and +ATv and -ATv whiteflies (**b**) with enlarged image for sperm pronucleus and egg pronucleus below. The white arrow denotes the sperm pronucleus, red arrow denotes the egg pronucleus, and green arrow denotes the bacteriocyte nucleus. DNA was stained by DAPI

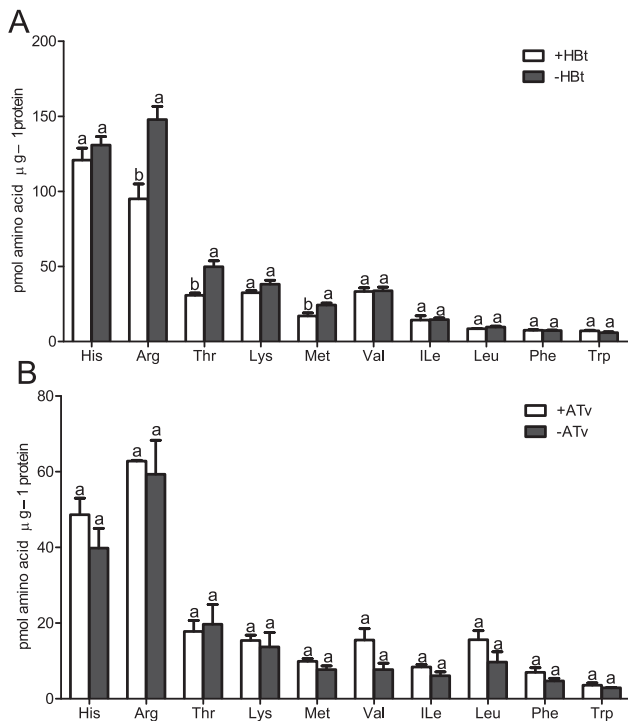
(blue). **c** Fertilization rate of +HBt and -HBt whiteflies ( $n = 3$  with 18–25 eggs and 12–22 eggs observed in each replicate, respectively). **d** Fertilization rate of +ATv and -ATv whiteflies ( $n = 4$  with 8–11 eggs and 12–17 eggs observed in each replicate, respectively). Error bars represent one standard error. Different letters above the bars indicate significant statistical differences at  $p < 0.05$ .

(Supplementary Dataset 2) [27, 35, 52, 53]. To investigate the biochemical impacts of *Hamiltonella* and *Arsenophonus* deficiencies, we determined whether changes of symbiont titer would alter the levels of essential amino acids. For example, a decline in the secondary symbionts may result in a decline of Phe and Lys levels within whiteflies. UPLC analyses showed that Phe and Lys levels were similar in low-symbiont titer lines and high-symbiont titer lines of both *B. tabaci* and *T. vaporariorum* (Fig. 4a, b;  $F_{1,10} = 0.18\text{--}3.88$ ,  $p = 0.077\text{--}0.68$  for Bt;  $F_{1,4} = 0.2\text{--}2.71$ ,  $p = 0.18\text{--}0.68$  for *T. vaporariorum*). Additionally, in *B. tabaci*, Arg, Thr, and Met have elevated levels in -HBt compared to +HBt whiteflies (Fig. 4a;  $F_{1,10} = 8.92\text{--}20.33$ ,  $p < 0.05$ ); however, the other five essential amino acids were present

at similar levels in both -HBt and +HB whiteflies (Fig. 4a;  $F_{1,10} = 0.0074\text{--}3.75$ ,  $p = 0.082\text{--}0.93$ ). For the *T. vaporariorum*, the -ATv and +ATv lines had similar levels of all other eight essential amino acids (Fig. 4b;  $F_{1,4} = 0.11\text{--}5.15$ ,  $p = 0.086\text{--}0.76$ ). These data suggest that reduced titers of either *Hamiltonella* or *Arsenophonus* did not substantially alter the level of the ten essential amino acids in their whitefly hosts.

The genomes of *Hamiltonella* and *Arsenophonus* contain most genes involved in synthesis of B vitamins: B2 riboflavin, B3 NAD(P)H, B6 pyridoxine, B7 biotin, and B9 folate [27, 35, 52, 53] (Supplementary Dataset 3). Therefore, we determined whether a low symbiont titer would influence the levels of B vitamins. Five B vitamins were measured in



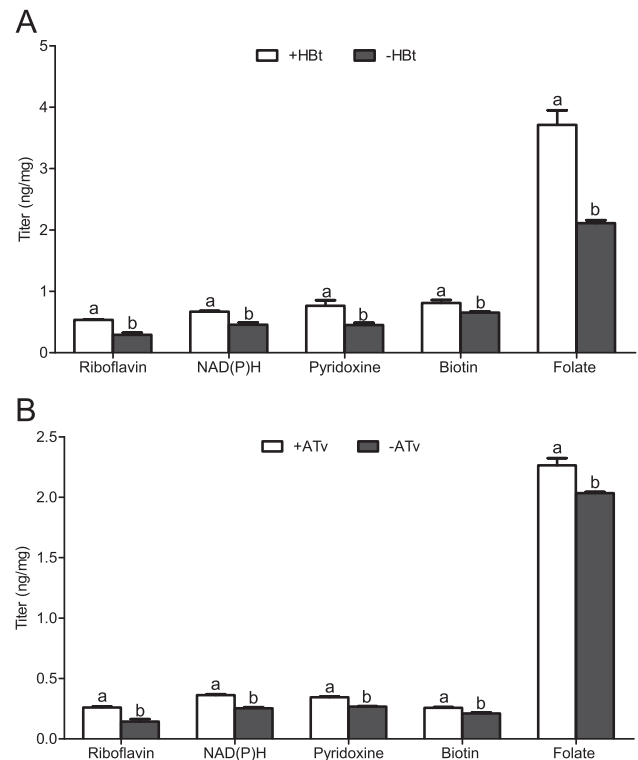


**Fig. 4 Effect of deficiency of *Hamiltonella* and *Arsenophonus* on the titer of amino acids in whiteflies.** Comparison of the titer of amino acids in +HBt and -HBt whiteflies (a) ( $n = 6$ ) and in +ATv and -ATv lines (b) ( $n = 3$ ) by UPLC. Error bars represent one standard error. Different letters above the bars indicate significant statistical differences at  $p < 0.05$ .

low-symbiont titer lines and high-symbiont titer lines. All five B vitamins (riboflavin, NAD(P)H, pyridoxine, biotin and folate) had significantly lower levels in -HBt compared to +HBt whiteflies (Fig. 5a;  $F_{1,4} = 9.05\text{--}50.84$ ,  $p = 0.0021\text{--}0.04$ ). Similarly, all five B vitamins had significantly lower levels in -ATv compared to +ATv whiteflies (Fig. 5b;  $F_{1,4} = 14.27\text{--}112.17$ ,  $p = 0.00045\text{--}0.02$ ).

### B vitamin supplementation restores fitness of the whitefly lacking symbionts

Given the substantial alteration of B vitamin levels in lines with secondary symbiont deficiencies, we determined whether vitamin supplementation would rescue whitefly fitness. Consistent with data from feeding on host plants (Fig. 1), the percentage of female offspring was lower in -HBt compared to +HBt whiteflies that fed on a 30% sucrose artificial diet (Fig. 6a). Supplementation of the 30% sucrose diet with B vitamins restored the percentage of female offspring produced by -HBt whiteflies to levels observed in +HBt whiteflies (Fig. 6a;  $F_{2,30} = 12.35$ ,  $p = 0.00012$ ). Likewise, a B vitamin-supplemented diet restored female fertility in -ATv whiteflies to levels observed in +ATv whiteflies (Fig. 6b;  $F_{2,30} = 5.85$ ,  $p = 0.0072$ ).

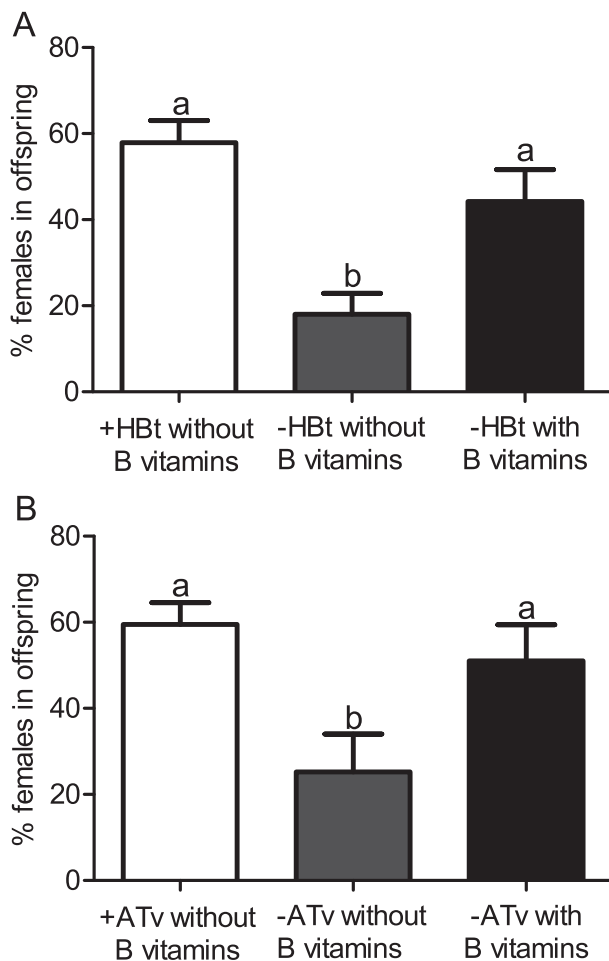


**Fig. 5 Deficiency of *Hamiltonella* and *Arsenophonus* reduces the titer of B vitamins in whiteflies.** Comparison of the titer of B vitamins in +HBt and -HBt whiteflies (a) ( $n = 3$ ) as well as in +ATv and -ATv lines (b) ( $n = 3$ ). Error bars represent one standard error. Different letters above the bars indicate significant statistical differences at  $p < 0.05$ .

## Discussion

Roles of bacteriocyte-associated secondary symbionts in supplying B vitamins for plant-feeding hemipteran insects are mostly inferred from genomic data and have rarely been investigated using experimental approaches. *Hamiltonella* are found in the invasive whiteflies *B. tabaci* MEAM1 and MED, as well as in aphids and psyllids [24, 54–56]. *Arsenophonus* is widespread in whiteflies and other insects [56–59]. Here we take advantage of *Hamiltonella*-*B. tabaci* MEAM1 and *Arsenophonus*-*T. vaporariorum* as study systems. Using a multidisciplinary approach, for the first time, we demonstrated that intracellular secondary symbionts impact the sex ratio of insects by facilitating fertilization and supplying B vitamins. This study reveals the convergent function of two different lineages of secondary symbionts in the regulation of reproduction for phylogenetically-distant whitefly species. Our findings will promote our understanding of the function and evolution of secondary symbionts, which co-habit with the primary symbiont in the host cell of animals.

The “secondary symbionts” *Hamiltonella* and *Arsenophonus* are able to provision B vitamins to their phloem-feeding whitefly hosts, as do the primary symbionts



**Fig. 6 Supplementation of B vitamins improves insect fitness.** Effect of supplementation of B vitamins on the percentage female in offspring of -HBt (a) ( $n = 11$ ), and -ATv (b) whiteflies ( $n = 11$ ). Error bars represent one standard error. Different letters above the bars indicate significant statistical differences at  $p < 0.05$ .

*Wolbachia*, *Wigglesworthia*, and *Francisella* for the bed bug, tsetse fly and tick, which feed on vertebrate blood [10–13, 46, 60]. Likewise, *Hamiltonella* of *Cinara* aphids has drastic genome reductions but has retained genes involved in synthesis of several B vitamins [55]. In addition, the symbionts *Baumannia*, *Hodgkinia*, and yeast-like fungal symbionts, which possess genes involved in B vitamin synthesis, are inferred to be the primary symbionts, as they coinhabit bacteriomes with additional primary symbionts in the xylem-feeding sharpshooter and cicada [61–64]. Previous work and our study have shown that both *Hamiltonella* and *Arsenophonus* are fixed in the whitefly populations of *B. tabaci* MEAM1 worldwide and *T. vaporariorum* in China, respectively [17, 19, 31]. A worldwide *T. vaporariorum* population study shows that *Arsenophonus* was almost fixed in all populations [65]. Collectively, these data suggest that bacteriocyte-associated “secondary symbionts” of plant-phloem feeding insects are

evolving towards primary symbionts. Furthermore, the primary symbiont *Portiera* in *B. tabaci* MEAM1 and *T. vaporariorum* retains genes involved in synthesis of pantothenate [66]. Overall, although bacteriocyte-associated symbionts in insects feeding on phloem, xylem, and animal blood are under various evolutionary processes, they appear to be under equivalent selection for B vitamin synthesis.

*Hamiltonella* and *Arsenophonus* increase the female sex ratio in whiteflies by provisioning riboflavin, NAD(P)H, pyridoxine, biotin, and folate. Folate and pyridoxine are pivotal for sexual maturation and reproduction in tsetse flies [11, 13]. So, the beneficial effect of symbionts on the female sex ratio in whiteflies could be caused by folate and pyridoxine or the combination of all five B vitamins. In contrast, in planthoppers, *Wolbachia*, which is not bacteriocyte-associated symbiont, increases host fecundity by producing riboflavin and biotin [14]. Both whiteflies and planthoppers are phloem-feeding Hemipteran insects. But the former (Hemiptera: Aleyrodidae) is a representative of the Sternorrhyncha and the latter (Hemiptera: Delphacidae) is a representative of the Auchenorrhyncha. Taken together, the evidence demonstrates that symbiont species and their provisioned B vitamins influence the specific reproductive biology of certain species of insects feeding on plant phloem. It appears that this biochemical enhancement of hemipteran fitness has evolved multiple times and is dependent on the co-evolution of different combinations of insects and symbionts.

Nutrition is a central regulator of fertility. Insects need to acquire adequate C and N to produce the large amounts of lipids and protein required for oogenesis, spermatogenesis, copulation, and embryogenesis [67, 68]. B vitamins function as coenzymes in multiple key reactions including synthesis and metabolism of protein, lipids and more [66]. Thus, *Hamiltonella* and *Arsenophonus*-provided B vitamins likely influence the quality of eggs and sperm or early embryogenesis events in whiteflies to mediate egg fertilization, which ultimately impacts the sex ratio in haplodiploid organisms. The molecular mechanisms underlying how B vitamins influence the success of fertilization in whiteflies are still unclear. It will be very valuable to investigate it in the future.

Maternally inherited symbionts regulate host reproduction in multiple ways [4–13, 69]. Whiteflies are haplodiploid insects; fertilized eggs produce female adults and unfertilized eggs develop into male adults. Our results clearly show that deficiency of *Hamiltonella* or *Arsenophonus* impacts an early step in fertilization, which is consistent with the distorted sex ratio in adult whiteflies. We further present evidence that *Hamiltonella* and *Arsenophonus* manipulate host sex ratio by provisioning nutrients. Overall, whitefly acquisition of the symbionts *Hamiltonella* or *Arsenophonus* determines the percentage of female offspring. This interaction explains why at least one symbiont coexists with the

primary symbiont *Portiera* in the bacteriocytes of whiteflies. It will be very interesting to examine whether other coexisting secondary symbionts impact insect reproduction and, if they do, will similar or distinct mechanisms be employed. Elucidating the mechanisms underlying the effect of these intracellular symbionts on insect reproductive biology should also provide new avenues for pest control.

## Data availability

All relevant data supporting the findings of this study are included within the article and its Supplementary Information files or available on request.

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**Author contributions** J.B.L. conceived the study and wrote the first draft of the manuscript. Y.B.W. conducted ecology experiments. N.N.L. conducted genotyping experiments. Y.L.Y. and X.S. carried out cytogenetic experiments. F.R.R., Y.L.Y., Y.B.W., X.S., B.B., and X.Y.B. performed nutritional physiology experiments. X.S. and X.R.X. carried out FISH experiments. L.L.W. contributed to writing the manuscript. All authors edited and approved the paper.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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