Supplementary Material from "The inherited bacterial symbiont *Hamiltonella* influences the sex ratio of an insect host"

Materials and Methods

The codes for the sections (a) to (g) below correspond to the same codes under Materials and Methods in the main text.

(a) Whitefly cultures plants

No further details are required to be given here.

(b) Effects of heat treatments on the symbionts and host reproduction

We first investigated the removal of symbionts by heat stress. The abundance of the three symbionts in the insect was determined by qPCR with 8-10 replicates each having 10 adult females collected from the experimental and control cultures at 0, 5, 10, 15 days (F0) and 30 days (F1) after 3 days of heat-treatment. To investigate effect of *Hamiltonella* depletion on host reproduction, the abundance of symbionts (F1-F4) and the sex ratio (F2-F5) of heat-treated and control lines were monitored over four generations. To obtain F2 whiteflies for observation, F1 adults were used in four treatments of mating: (1) CK♀×CK♂, where control females mated with control males; (2) CK♀×HT♂, where control females mated with heat-treated males; (3) HT♀×CK♂, where heat-treated females mated with control males; and (4) HT♀×HT♂, where heat-treated females mated with heat-treated males, with 10 replicates per treatment. In each replicate, 10 virgin females and 10 virgin males were reared together to mate and oviposit for 10 days. The offspring (F2) of these crosses were reared to 25-30 days, by which time they had developed to adults whose sex ratio was assessed. For F3-F5 generations, two mating treatments were conducted: (1) CK♀×CK♂, and (2) HT♀×HT♂. Since very few female adults emerged in F2 and subsequent generations of the heat-treated line, 1-10 pairs of adults per replicate and 4-8 replicates were used in each of the two treatments. The number of pairs of adults per replicate and the number of replicates in each treatment were restricted by the number of adults available at a given generation. Symbiont abundance was quantified by qPCR with 10 females per replicate in F1, a single female in F2-F4, and 10 males in all four generations. The mating experiments used newly-emerged virgin adults in "Lock & Lock" rearing units following the procedure of Wang *et al*. [1].

To observe the effect of a milder heat stress on the symbionts and host reproduction, the duration of the treatment at 40 °C was reduced from three days to two days (2dHT), and their offspring (F1) were used for mating experiments (2dHT and CK) as described above. The symbiont density of whiteflies in F1 was analyzed and sex ratio in F2 was recorded.

The abundance of symbionts was assessed using quantitative PCR (qPCR). Whiteflies were firstly sexed under microscope, and then female and male adults were separated for DNA extraction. The insects were ground in 0.2 ml PCR tube with 50 µl Lysis buffer by a

Tissuelyser-48 grinding miller (Shanghai Jingxin Industrial Development Co. Ltd, Shanghai, China) for 300 s at 70 Hertz. Extractions were incubated at 65 ºC for 2 h, and then 100 ºC for 10 min. After a brief centrifugation, the extractions were kept at -20ºC for assaying symbionts later. Lysis buffer contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 % gelatin, 0.45 % Nonidet P-40, 0.45 % Tween 20, and 60 μg/ml proteinase K. Abundance of symbionts of the insects was assessed using qPCR with the SYBR® Premix Ex Taq™ (Takara, Dalian, China) and Bio-Rad CFX96™ Real-Time System (Bio-Rad, CA, USA). The symbionts of the three whitefly populations were determined using 16S rRNA gene or gltA gene (electronic supplementary material, table S2 for the primer sequences). The relative abundance of symbionts was calculated using the comparative CT method (2-ΔΔCt), and β-actin gene (nuclear gene) of whiteflies was measured in parallel for normalization.

(c) Effect of antibiotic treatment on symbionts and host reproduction

The offspring (F1) of the MEAM1 whiteflies which naturally harbored *Hamiltonella* in all individuals (CK) and those whiteflies that were depleted of *Hamiltonella* by antibiotics in F1 (AT) were used in four treatments of mating: CK♀×CK♂, CK♀×AT♂, AT♀×CK♂, and AT♀×AT♂. In each replicate of a given treatment, a single virgin female and a single virgin male were allowed to mate and oviposit for 7 days in a leaf-clip cage attached to a leaf of cotton plants. Then the mated pair was collected for assessing the presence and abundance of symbionts by qPCR. The qPCR protocol was the same as that in (b) above. For any replicate of which one of the adults was found to be dead during the collection, the replicate was discarded. The adult offspring (F2) collected at 18^{th}, 23^{rd}, 28^{th} and 33^{rd} day were combined to count female and male adults for calculation of total number and sex ratio.

The Asia 1 and Asia II 1 species of *B. tabaci* complex, which are naturally free of *Hamiltonella,* were used as a control to examine the direct effect of antibiotic treatment on host reproduction, and a mating experiment of two treatments (CK♀×CK♂ and AT♀×AT♂) was conducted as above. The symbionts of F1 female adults were assessed by qPCR, and sex ratio and number of F2 adults were assessed.

(d) Survivorship of immature stages

The survivorship of *Hamiltonella*-cured and control MEAM1 whiteflies was observed in the following four treatments: (1) Unmated (CK), a single virgin female of CK whitefly; (2) CK, a female and a male from CK whiteflies; (3) HT, a female and a male of F1 from the heat-treated line; and (4) AT, a female and a male of F1 from the antibiotic-treated line. Previous observation has shown that adults in the treatments (2) to (4) would all have mated soon after emergence, usually within one day. Adult whiteflies in the various treatments were introduced in leaf-clip cages attached to cotton plant leaves and left there to mate and oviposit for 7 days. The adults were then discarded and the number of eggs in each replicate was counted. After a further 10 days, the number of the nymphs in each replicate was observed to calculate egg hatch rate, and then female and male adults were collected at 18^{th}, 23^{rd}, 28^{th} and 33^{rd} day to

calculate emergence rate and sex ratio.

(e) Observation of mating events

No more details need to be given here.

(f) Sperm detection in spermathecae and eggs

The spermathecae were dissected from 7-day-old, mated female adults of F1 from each of the three treatments including CK, HT and AT. The dissected tissues were fixed in 4% paraformaldehyde in PBS for 1 h, permeabilized with 0.1% Triton X-100 for 1 h, and then stained with the Fluoroshield mounting medium containing DAPI (2 μg/ml) (Abcam, Cambridge, UK) for 30 min. For eggs, the F1 adults from each of the three treatments were allowed to oviposit for 30 min, and then the eggs were immediately collected and fixed in 4% paraformaldehyde in PBS for 1 h, bleached in 6% hydrogen peroxide for 2-3 h, permeabilized in 0.1% Triton X-100 overnight, and then stained with DAPI. Stained sperm heads in spermathecae and eggs were viewed under a Zeiss LSM780 confocal microscope (Zeiss, Jena, Germany).

(g) Localization of symbionts

Two methods were used including Fluorescence *in situ* hybridization (FISH) and Transmission electron microscope (TEM).

FISH: The body cavity and ovaries of females and the testes of males were dissected to examine the presence/absence of bacteriocytes in these organs, which were dissected in phosphate-buffered saline (PBS) at pH 7.4 from 7-day-old F1 female and male adults respectively, then the tissues were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, and then were permeabilized with 0.1% Triton X-100 for 30 min, and hybridized overnight in hybridization buffer (20 mM Tris-HCl (pH 8.0), 0.9 M NaCl, 0.01% sodium dodecyl sulfate, and 30% formamide) with 10 pmol/ml of fluorescent probes BTP1-Cy3(5'-Cy3-TGTCAGTGTCAGCCCAGAAG-3') for *Portiera* and BTH-Cy5 (5'-Cy5-CCAGATTCCCAGACTTTACTCA-3') for *Hamiltonella* [2]. Finally, samples were stained with DAPI for 30 min at room temperature and were viewed under a Zeiss LSM780 confocal microscope.

TEM: The abdomen of F1 female adults were dissected in PBS at pH 7.4 and fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.0) overnight, and postfixed with 1% OsO_4 in PBS for 1-2 h. After dehydration by a graded series of ethanol and absolute acetone, the samples were embedded in Spurr resin, then sectioned with a LEICA EM UC7 ultratome (Leica, Wetzlar, Germany) and stained by uranyl acetate and alkaline lead citrate. Finally, the sections that contain bacteriocyte of body cavity were observed in Hitachi Model H-7650 TEM (Hitachi, Tokyo, Japan).

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Table S1 Primers used for PCR

Organism	Target gene	Primer name	Primer sequences (5'→3')	Length (bp)	Reference
Portiera	16S rRNA	Por-F	GGAAACGTACGCTAATAC	~900	[3]
		Por-R	TGACGACAGCCATGCAGCAC		
Hamiltonella	16S rRNA	Ham-F	TGAGTAAAGTCTGGGAATCTGG	~750	[4]
		Ham-R	AGTTCAAGACCGCAACCTC		
Rickettsia	16S rRNA	Ric-F	GCTCAGAACGAACGCTATC	~900	[5]
		Ric-R	GAAGGAAAGCATCTCTGC		
Wolbachia	16S rRNA	Wol-F	TTGTAGCCTGCTATGGTATAACT	~900	[6]
		Wol-R	GAATAGGTATGATTTTCATGT		
Cardinium	16S rRNA	CFB-F	GCGGTGTAAAATGAGCGTG	~450	[7]
		CFB-R	ACCTMTTCTTAACTCAAGCCT		
Arsenophonus	23S rRNA	Ars23S-1	CGTTTGATGAATTCATAGTCAAA	~600	[8]
		Ars23S-2	GGTCCTCCAGTTAGTGTTACCCAAC		
Fritschea	23S rRNA	U23F	GATGCCTTGGCATTGATAGGCGATGAAGGA	~600	[9]
		23SIGR	TGGCTCATCATGCAAAAGGCA		
Hemipteriphilus[a]	16S rRNA	Ric-F	GCTCAGAACGAACGCTATC	~900	[5]
		Ric-R	GAAGGAAAGCATCTCTGC		

[a]*Hemipteriphilus* were detected by the same primers as *Rickettsia* because the primers were universal for these two bacteria, and then they were distinguished by the sequences.

Table S2 Primers used for qPCR

Organism	Target gene	Primer name	Primer sequences (5'→3')	Length (bp)	Reference
Portiera	16S rRNA	Port73-F	GTGGGGAATAACGTACGG	~200	[10]
		Port266-R	CTCAGTCCCAGTGTGGCTG		
Hamiltonella	16S rRNA	H-16S-Fis	GCATCGAGTGAGCACAGTTT	~240	[11]
		H-16S-Ris	TATCCTCTCAGACCCGCTAGA		
Rickettsia	*gltA*	glt375-F	TGGTATTGCATCGCTTTGGG	~200	[10]
		glt574-R	TTTCTTTAAGCACTGCAGCACG		
Arsenophonus	16S rRNA	Ars-16S-F	AGCCTGATGCAGCCATGCCG	~200	This study
		Ars-16S-R	TGCGTGCCCTTTACGCCCAG		
Wolbachia	16S rRNA	Wol-16S-F	CGCCCTTTACGCCCAATAAT	~150	This study
		Wol-16S-R	AGTGAAGAAGGCCTTTGGGT		
Hemipteriphilus	16S rRNA	OLO-16S-F	TAGTGGCAAACGGGTGAGTA	~120	[12]
		OLO-16S-R	GCTCATCCATCAGCGATAAA		
B. tabaci	*β-actin*	Actin-F	TCTTCCAGCCATCCTTCTTG	~200	[13]
		Actin-R	CGGTGATTTCCTTCTGCATT		

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14.

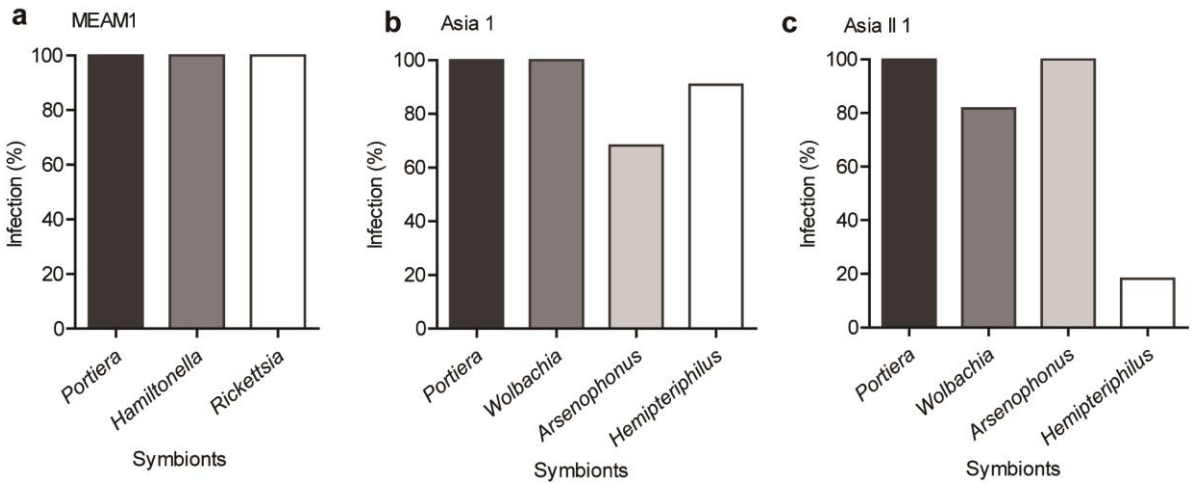

Figure S1. Frequencies of symbiont infection in the cultures of the three species of whiteflies MEAM1, Asia 1 and Asia II 1. Each of the three whitefly cultures was examined for the presence/absence of the eight symbionts reported so far for whiteflies in the *B. tabaci* complex, only the data on the symbionts that were detected in each of the three cultures are presented in this figure, i.e. for the data on those symbionts that were undetected are omitted from the diagrams.

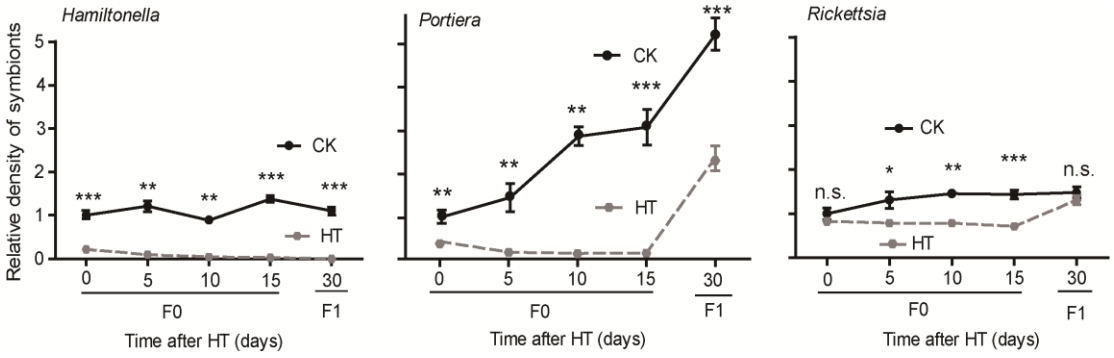

Figure S2. The dynamic abundance of *Hamiltonella*, *Portiera* and *Rickettsia* in adult female MEAM1 whitefly following heat treatment. HT: exposure F0 MEAM1 whitefly to 40 °C for 3 days. Note: the data of F1 is the same as that shown in figure 1*a*. The data are mean ± SEM, and the asterisks indicate significant differences between heat treatments (HT) and control (CK) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not statistically significant).

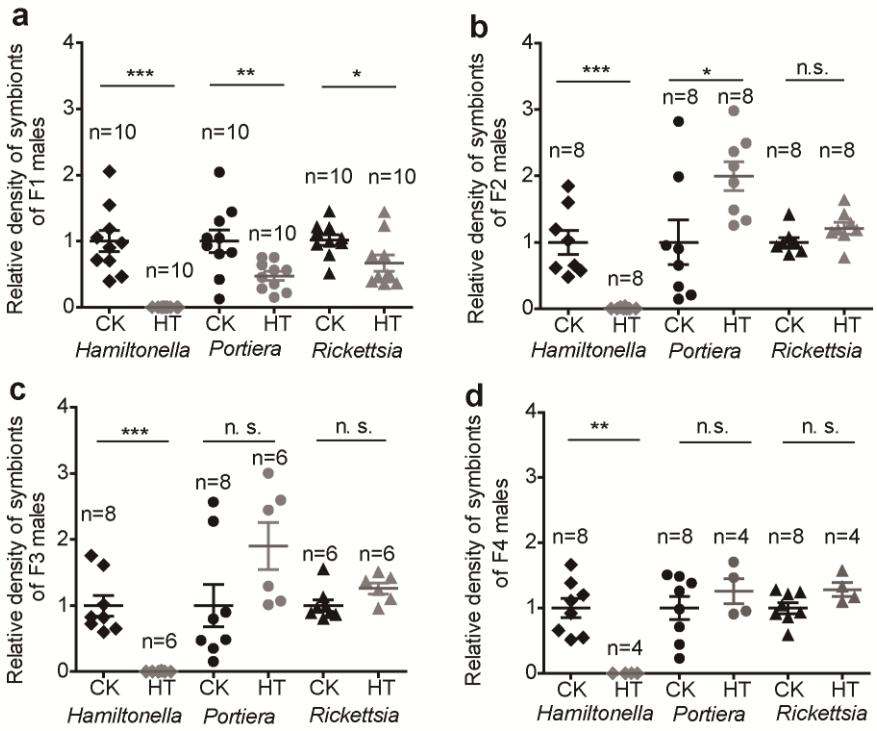

Figure S3. Relative densities of symbionts in male adults of F1-F4 (**a-d)** after exposure of F0 MEAM1 whitefly to 40°C for 3 days. The data are mean ± SEM, and the asterisks indicate significant differences between heat treatments (HT) and control (CK) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, n.s., not statistically significant).

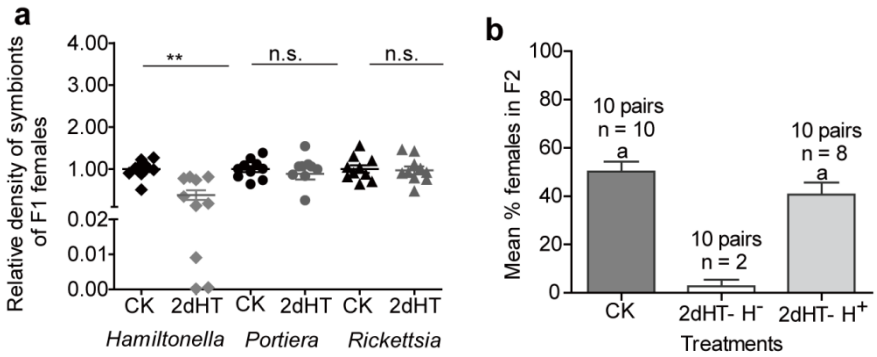

Figure S4. Relative densities of symbionts in F1 female adults and sex ratio of their offspring after exposure of F0 MEAM1 adults to 40 °C for 2 days. Ten replicates were assayed in each treatment. CK: Control; 2dHT: F0 adults exposed to 40°C for 2 days; 2dHT-H^-: F1 adults without *Hamiltonella* after heat stress at F0; 2dHT- H^+ : F1 adults with *Hamiltonella* after heat stress at F0. In panel (**a**) data are mean ± SEM, ** $P < 0.01$ compared to control; in panel (**b**) data are mean ± SEM and different letters above the data columns indicate significant difference at $P < 0.05$. Note that the data of 2dHT-H^- was excluded from the statistical analysis because of the low number of replicates.

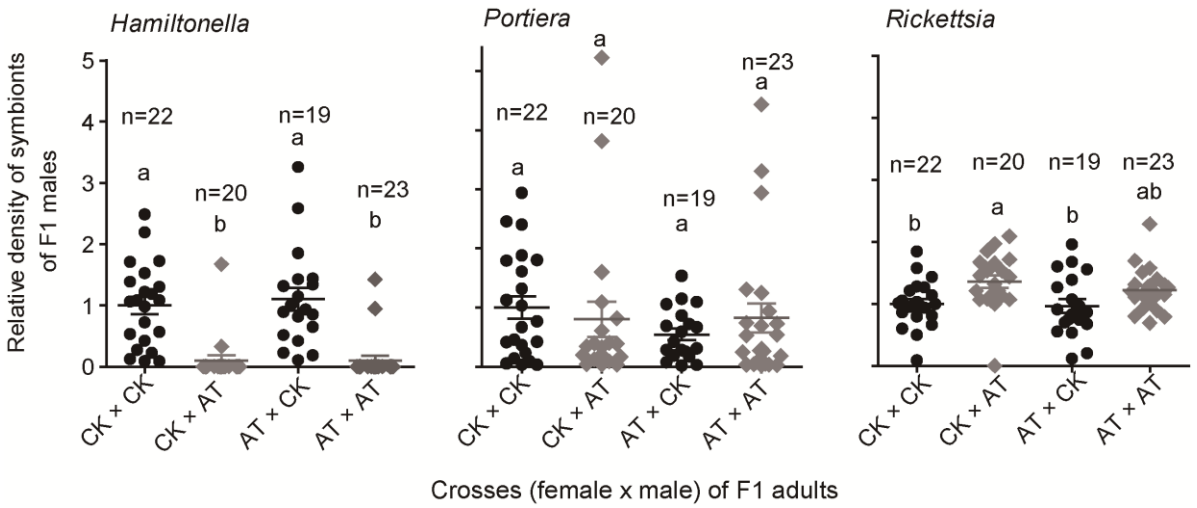

Figure S5. Relative densities of symbionts in F1 male parent for crosses between CK (control) and AT (Antibiotic treatment) in MEAM1 whitefly. In the cross of CK♀×CK♂ and AT♀×CK♂, the F1 male adults were derived from control whiteflies, and in the CK♀×AT♂ and AT♀×AT♂, the F1 male adults were derived from AT whiteflies. The data are mean ± SEM, and the different letters above the data columns indicate significant difference at $P < 0.05$.

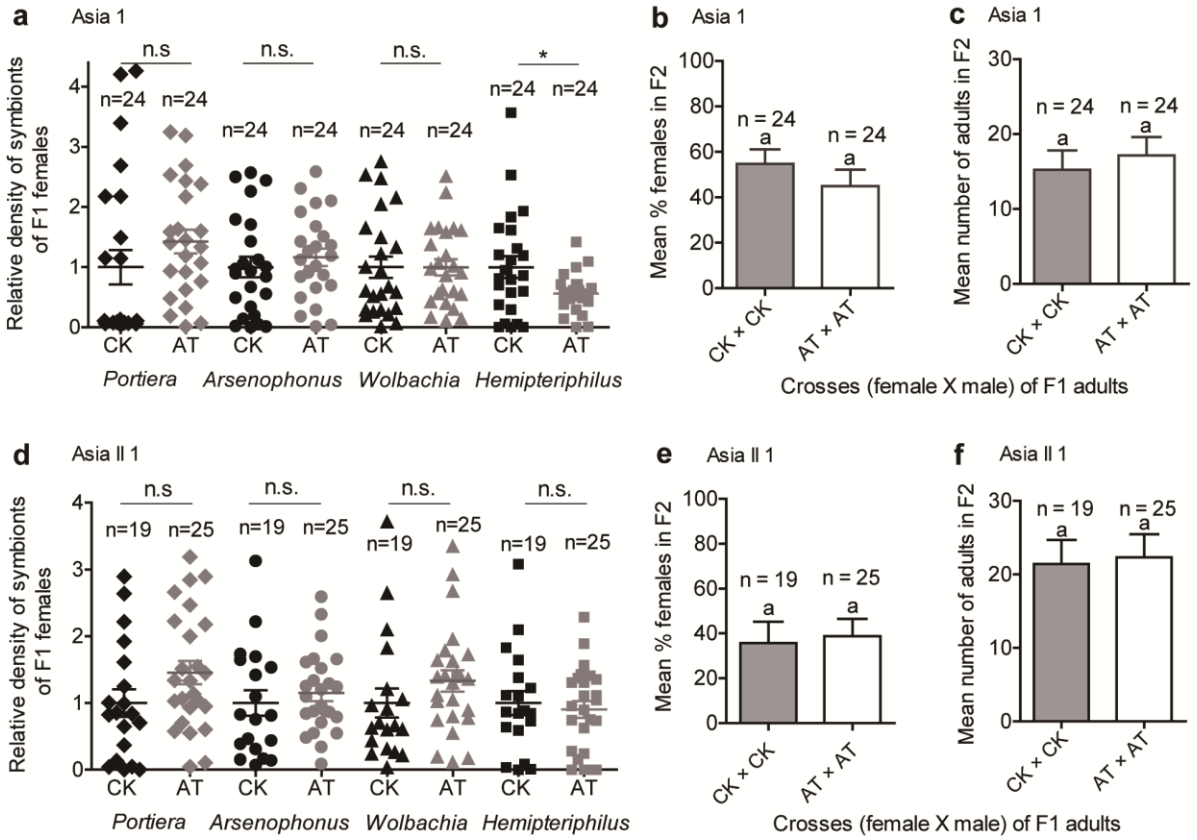

Figure S6. Relative densities of symbionts in F1 female adults, and sex ratio and number of F2 adults in Asia1 and Asia II 1 when F0 adults were treated with antibiotics. (**a**) Relative density of symbionts in F1 female adults of Asia1 that were used as parents for the crosses between CK

(control) and AT (Antibiotics treatments). (**b-c**) Female ratios (**b**) and number of F2 adults (**c**) in Asia 1. (**d**) Relative density of symbionts in F1 female adults of Asia II1 that were used as parents for the crosses between CK and AT. (**e-f**) Female ratios (**e**) and number of F2 adults (**f**) in Asia II1.The data are mean ± SEM, and the different letters above the data columns indicate significant difference at $P < 0.05$.

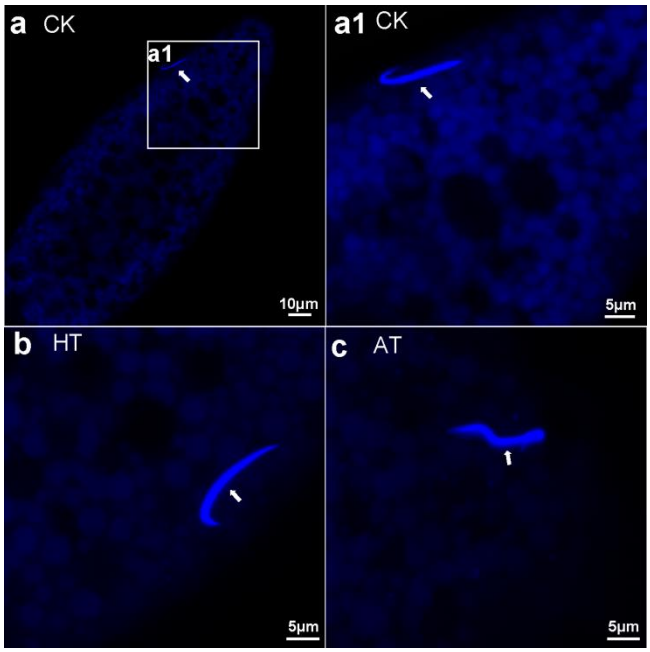

Figure S7. Location of sperm on eggs produced by F1 adults that were depleted of *Hamiltonella* by heat or antibiotic treatment. (**a-c**) A sperm was located in an egg that was produced by a CK mated F1 female (CK, **a**), a HT mated F1 female (HT, **b**) or a AT mated F1 female (AT, **c**). (**a1**) Higher magnification of box in **a**. Newly deposited eggs (0-30min after oviposition) were used for sperm observation. Thick arrow: elongate nucleus of sperm.