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ORIGINAL ARTICLE

Conversion of a thelytokous to a stable bisexual line by non-target effect antibiotic elimination of *Rickettsia* in *Anastatus gansuensis*

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Abstract Heritable symbionts are key modulators of host biology, influencing reproduction and fitness. While antibiotic removal of symbionts is common, non-target effects on host fitness are often understudied. This is particularly true for *Rickettsia*, a widespread reproductive manipulator, and a stable, long-term (i.e., >7 generations) cured lineage in Hymenoptera has been lacking. This study aimed to fill this methodological gap by generating a cured bisexual lineage of parasitoid wasp with non-target effects of such treatments. Thus, we investigated not only the efficacy but also the non-target effects of three antibiotics: rifampicin, tetracycline, and sulfadiazine, administered at five concentrations (0.01, 0.05, 0.1, 1, and 10 mg/mL) in *Rickettsia*-infected thelytokous parasitoid *Anastatus gansuensis* (Hymenoptera: Eupelmidae). Survival, parasitism, emergence, and male rate were evaluated to determine the safety of antibiotic treatments, while *Rickettsia* titer reduction was used to assess elimination efficacy. Results showed that at a concentration of 0.01 mg/mL, tetracycline and rifampicin had minimal negative effects on host survival, parasitism, and emergence rates. However, prolonged exposure effectively eliminated *Rickettsia*, leading to the exclusive production of male offspring. Notably, short-term rifampicin feeding (0.01 mg/mL) across multi-generations successfully established a stable *Rickettsia*-cured bisexual line, confirmed via diagnostic PCR, quantitative PCR, and reproductive phenotyping over 10 generations. In contrast, sulfadiazine, previously effective against *Wolbachia*, had minimal impact on *Rickettsia* removal. This study provides a validated protocol for generating genetically stable aposymbiotic lines and a framework for assessing antibiotic specificity and non-target effects, enabling future studies of host adaptation and biological control in *Rickettsia*-cured parasitoids.

Key words antibiotic efficacy; antibiotic side-effects; biological control; haplodiploidy; parasitoid wasp; reproductive manipulation

Introduction

Arthropods constitute the majority of species in the biosphere, leading to complex and diverse reproductive strategies (Sollai *et al.*, 2024). Many arthropod species are associated with one or more symbiotic bacteria,

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which influence host nutrition, reproduction, and overall fitness to varying degrees (Douglas, 2015; Perlmutter & Bordenstein, 2020). A specific group of maternally inherited symbionts, including *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus*, *Spiroplasma*, *Mesenetia*, and *Lariskella*, manipulate host reproduction through mechanisms such as cytoplasmic incompatibility, parthenogenesis, feminization, and male killing (Kageyama *et al.*, 2012; Kaur *et al.*, 2021; Takano *et al.*, 2021; Umanzor *et al.*, 2025). These symbionts often enhance their transmission by altering host reproductive fitness (Hurst & Werren, 2001; Werren, 2011; Dalla Bennetta *et al.*, 2021). To investigate the role of symbiotic bacteria in host biology, researchers typically compare symbiont-associated and aposymbiotic strains with the same genetic background (Tsuchida *et al.*, 2010, 2014; Silva *et al.*, 2024). Thus, eliminating symbionts to establish uninfected lines has been a central focus in symbiosis research (Qazi *et al.*, 2025).

Previous studies have demonstrated that antibiotic treatments have been widely studied to remove reproductive symbionts in order to restore the host's original reproductive patterns (Stouthamer *et al.*, 1990; Pike & Kingcome, 2009). Despite some broad-spectrum antibiotics, such as tetracycline and rifampicin, effectively eliminating *Wolbachia*, *Rickettsia*, and *Cardinium* (Wehrli, 1983; Chopra & Roberts, 2001), their non-target effects are still understudied (Prieto *et al.*, 2025). For instance, these antibiotics have been used to eliminate *Wolbachia* from *Urolepis rufipes* (Hymenoptera: Pteromalidae) (Kyei-poku *et al.*, 2003), *Nasonia vitripenni*s (Hymenoptera: Pteromalidae) (Werren & Loehlin, 2009), *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) (Xiao *et al.*, 2021), and *Encarsia formosa* (Hymenoptera: Aphelinidae) (Wang *et al.*, 2017) with potential non-target effects (Li *et al.*, 2014a). Similarly, *Rickettsia* has been eliminated from *Neochrysocharis formosa* (Hymenoptera: Eulophidae) (Hagimori *et al.*, 2006), *Pnigalio soemius* (Hymenoptera: Eulophidae) (Giorgini *et al.*, 2010), and *Nesidiocoris tenuis* (Hemiptera: Miridae) (Owashi *et al.*, 2024), while *Cardinium* has been successfully removed from *Encarsia suzannae* (Hymenoptera: Aphelinidae) (Doremus *et al.*, 2020) and, *Lariskella* from the leaffooted bug *Leptoglossus zonatus* (Hemiptera: Coreidae) using rifampicin (Umanzor *et al.*, 2025). More recently, sulfadiazine was shown to remove *Wolbachia* from *T. pretiosum* (Xiao *et al.*, 2021; Guo *et al.*, 2023). However, while sulfonamides have demonstrated *in vitro* activity against pathogenic *Rickettsia* species (Rolain *et al.*, 1998), their effectiveness against heritable *Rickettsia* in arthropods remains unclear (Perlman *et al.*, 2006), requiring further investigation.

Furthermore, despite the widespread use of antibiotics to remove symbionts (Li *et al.*, 2014a), their effectiveness varies. Some symbionts are resistant to antibiotics (Gogineni *et al.*, 2020). For example, attempts to eliminate *Rickettsia* in adult whiteflies via antibiotic feeding and injection have failed (Chiel *et al.*, 2009). Similarly, high-dose tetracycline treatment for *Wolbachia*-infected *E. formosa* over five generations did not completely clear the infection (Wang *et al.*, 2017). Even when successful, antibiotic treatments often trigger side-effects, inducing high mortality rates and impairing wasp fitness (Stouthamer *et al.*, 1990; Xiao *et al.*, 2021; Guo *et al.*, 2023). Additionally, antibiotics can negatively impact host biological traits (Ruan *et al.*, 2006; Ridley *et al.*, 2013; Shan *et al.*, 2016). Consequently, distinguishing between direct antibiotic effects and those arising from the removal of symbionts is essential (Ridley *et al.*, 2013). Moreover, the non-target effects of antibiotic treatments reported in previous studies require further investigation. Thus, this study aimed to systematically evaluate the elimination of *Rickettsia* and its non-target effects in *Anastatus gansuensis* (Hymenoptera: Eupelmidae) to establish a bisexual line for further biological control studies. The results obtained here provide valuable insights into symbiont elimination using antibiotics, improving our understanding of *Rickettsia*'s effects on *Anastatus* spp. fitness. Additionally, this study lays the groundwork for evaluating the biocontrol potential of thelytokous and bisexual lines of *A. gansuensis*.

Materials and methods

Parasitoids and host insects

A new species, *A. gansuensis*, exhibiting a high-prevalence *Rickettsia* infection associated with thelytokous parthenogenesis was previously identified (Chen *et al.*, 2019; Gong *et al.*, 2025). The thelytokous population was collected from *Rinaca japonica* (Lepidoptera: Saturniidae) egg masses from Kang County (32.9–33.7°N, 105–106°E), Gansu Province, China, in 2020. The wasps infected with *Rickettsia* were identified through microbial diversity analysis (NCBI database: PRJNA1126341) and gene sequencing (GenBank: PP935349; PP942539; PV768973) (Gong *et al.*, 2025). A colony of the parasitoid was maintained on *Antheraea pernyi* (Lepidoptera: Saturniidae) eggs in the laboratory (Iqbal *et al.*, 2019) at 25 ± 1 °C, 14 h : 10 h L : D photoperiod, and 70% ± 5% RH.

Antibiotic treatment

Antibiotic treatments were prepared by dissolving 20 mg of each antibiotic (sulfadiazine, rifampicin, and tetracycline) in a 1 mol/L fructose to form a stock solution, which was subsequently diluted with the same fructose solvent to generate the five working concentrations (0.01, 0.05, 0.1, 1, and 10 mg/mL). A pure 1 mol/L fructose solution served as the control.

Twenty newly emerged adult female wasps (<6 h) were transferred to a cylindrical transparent plastic jar (diameter × height, 9.0 × 14.0 cm) and fed antibiotic solutions via a fine brush. The wasps were treated with all three antibiotics, each tested across all five concentrations. For each specific antibiotic-concentration combination, wasps were individually fed the solution for a fixed duration (1, 3, 5, 7, 10, 15, or 20 d), and mortality was recorded daily. Then, at the end of the feeding period, each surviving wasp was transferred and provided with 30 *A. pernyi* eggs in a glass tube (diameter × length, 3 × 12 cm) and fed the antibiotic solution for a 24-h parasitization period (15 replicates per treatment). Wasps in the control group were fed the fructose solution without antibiotics, and all other conditions were kept identical to those in the antibiotic groups. After the 24-h parasitization period, the wasps were collected, immediately flash-frozen in liquid nitrogen, and stored individually in 1.5 mL centrifuge tubes at −80 °C until subsequent DNA extraction for *Rickettsia* titer quantification. Finally, the host eggs were incubated in a climate chamber (25 ± 1 °C, 70% ± 5% RH, 14 h : 10 h L : D photoperiod) until offspring emergence. After no wasp emergence for 30 consecutive days, the remaining host eggs were dissected to check for the presence of dead parasitoid under a microscope. The recorded variables were: survival rate, emergence rate, male rate, and number of parasitized eggs (eggs with emergence holes + eggs with dead parasitoids inside).

Acquirement of a bisexual line

Wasps collected within 6 h after emergence were fed a 0.01 mg/mL rifampicin fructose solution (as obtained from the study previously mentioned) for 24 h, and then cultured for successive generations. Each wasp was provided with 30 host eggs for 24 h to parasitize, with 20 replicates per treatment. After parasitism, the female was removed and placed in a −80 °C refrigerator for the detection of *Rickettsia* titers. The eggs were placed in a climate chamber (25 ± 1 °C, 70% ± 5% RH, 14 h : 10 h L : D photoperiod) until emergence. The male rate was recorded for each generation, and then paired males and females were observed to detect their mating behavior. Wasps of every generation were counted and sexed by assessing the offspring (40–60 wasps) of 15 females.

To biologically verify the bisexual line, unmated antibiotic-exposed females were required to produce exclusively male offspring over three consecutive fructose-fed generations, whereas mated control wasps under identical conditions produced both sexes. The stability of the bisexual line was analyzed by statistically evaluating the male rate.

Detection of Rickettsia in A. gansuensis

Genomic DNA was extracted from wasps obtained from both the antibiotic treatment experiments and the bisexual line establishment assays. *Rickettsia* infection status was systematically assessed at post-treatment via PCR amplification of the *gltA* (citrate synthase) gene (CS-239: 5′-GCTCTTCTCATCCTATGGCTATTAT-3′ and CS-1069: 5′-CAGGGTCTTCGTGCATTTCTT-3′; Labruna *et al.*, 2004), using the TIANamp Genomic DNA Kit (Beijing, China) according to the manufacturer's instructions for tissue samples. Samples showing target band amplification (about 800 bp) were considered infected, while the absence of amplification indicated infection-free status. *Rickettsia* infection status was monitored across 17 successive generations (G_0–G_{17}), in three biological replicates (each replicate consisted of five wasps) for each generation.

In particular, PCR was performed in 25 μL reaction volumes containing 12.5 μL Taq DNA polymerase (Promega), 8.5 μL of ddH_2O, 1 μL each of forward and reverse primers, and 2 μL of DNA. The PCR amplification included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 53.7 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Untreated samples of *Rickettsia*-infected *A. gansuensis* were used as a positive control in the PCR.

No-template controls were included in all PCR assays. The identity of the amplification product was confirmed by sequencing from initial positive samples.

Quantifications of Rickettsia

Rickettsia clearance was verified using PCR and absolute quantitative PCR (qPCR) in successive generations. qPCR was used to quantify *Rickettsia* in individual infected wasps with the primer glt375-F: 5′-TGGTATTGCATCGCTTTGGG-3′ and glt574-R: 5′-TTTCTTTAAGCACTGCAGCACG-3′ (Caspi Fluger *et al.*, 2011), by performing PCR to amplify the *gltA* gene (Bing *et al.*, 2013). DNA was extracted as previously described. And quantified with TaKaRa TB Green® Premix Ex Taq™ II (Tli RNaseH Plus)

(Takara Biotechnology, Dalian, Co., Ltd.) according to the manufacturer's protocol. The standard curves were plotted using standard plasmid samples for qPCR.

Then, those samples were disinfected on the body surface (75% alcohol 1 min, 2% NaClO 30 s, ddH_2O 3 times, 3 min each time; all operations were carried out on a clean bench). The ovaries were dissected, and DNA extracted as previously described. After DNA extraction, qPCR was performed to determine the abundance of *Rickettsia* in the ovaries. Three technical replicates were performed for each of the three biological replicates.

After antibiotic treatment, the *Rickettsia* titers in each wasp generation were measured. Following surface disinfection, DNA was extracted from 15 adult female wasps for each generation with three biological replicates (the method was as mentioned above). Then, the qPCR methods were the same as described above.

Statistical analyses

All the statistical analyses were conducted using IBM SPSS Statistics 26. To assess post-antibiotic treatment effects on parasitoid fitness parameters, generalized linear models (GLMs) were applied to feeding duration (7 levels: 1, 3, 5, 7, 10, 15, 20 d), antibiotic type (3 levels: sulfadiazine, rifampicin, tetracycline), and antibiotic concentration (5 levels: 0.01, 0.05, 0.1, 1, 10 mg/mL), and their interaction on the following parameters: the number of parasitized eggs (Poisson distribution), emergence rate (binomial distribution), male proportion (binomial distribution). The log-rank (Mantel–Cox) test compared survival curves across treatment groups. Moreover, GLM was used to analyze the titers of *Rickettsia* after antibiotic treatment under a Gaussian distribution. Comparing the male proportion of thelytokous lines and the bisexual line (mated and unmated) was performed using a binomial distribution GLM. They were also analyzed by a Gaussian distribution GLM to compare the proportion of residual wasps. The data were presented as means ± SEM. The means were differentiated using the Least Significant Difference test (LSD) at $P < 0.05$.

Results

Comparison of different antibiotic treatments on parasitoid survival rate

Compared with the control, high concentrations (1 and 10 mg/mL) of both rifampicin and tetracycline had obvious toxic effects causing wasp mortality ($\chi^2 = 64.14$, df $= 2$, $P < 0.001$; $\chi^2 = 76.86$, df $= 2$, $P < 0.001$), while low concentrations (0.01, 0.05, and 0.1 mg/mL) of those antibiotics had no effect on this parameter ($\chi^2 = 2.52$, df $= 3$, $P = 0.47$; $\chi^2 = 0.52$, df $= 3$, $P = 0.92$; Fig. 1A, B). When 10 mg/mL rifampicin and tetracycline fructose solution were fed separately, all the wasps died on the 7th and 4th day, respectively. The toxicity of 1 mg/mL was slightly lower, and the mortality reached 100% on the 9th and 15th day of feeding for rifampicin and tetracycline fructose solution, respectively (Fig. 1A, B). However, the different concentrations of sulfadiazine had no significant effect on the mortality of female wasps compared with the control ($\chi^2 = 4.99$, df $= 5$, $P = 0.42$; Fig. 1C). Thus, the concentrations of rifampicin and tetracycline at 1 and 10 mg/mL were excluded.

Comparison of different antibiotic treatments on the number of parasitized eggs

Overall, both treated and control groups exhibited a unimodal pattern in parasitism over the 20-d experimental period, characterized by an initial increase followed by a gradual decline. For sulfadiazine-treated cohorts, parasitism counts remained numerically lower than the control throughout all the observations, despite no statistical difference being detected ($P > 0.05$, Fig. 2A). Moreover, there was no concentration-dependent effect among the different concentrations of sulfadiazine ($\chi^2 = 4.254$, df $= 4$, $P = 0.373$). In contrast, rifampicin and tetracycline substantially impaired host parasitic capacity, particularly under prolonged exposure time. Sustained feeding at three concentrations (0.01, 0.05, 0.1 mg/mL) resulted first in an increase and then in a reduction of parasitized eggs. During the first 7 d, parasitism rates at the 0.01 and 0.05 concentrations of both antibiotics showed no significant differences from the control ($P > 0.05$, Fig. 2B, C). By day 10, all antibiotic concentrations induced a pronounced decline in the number of parasitized eggs ($P < 0.001$), which persisted until day 20 (Fig. 2B, C). The number of parasitized eggs in the treatment group was only about half of the control on day 20 ($P < 0.001$). There was no significant difference between the concentrations of tetracycline and rifampicin ($\chi^2 = 2.253$, df $= 2$, $P = 0.324$; $\chi^2 = 4.169$, df $= 2$, $P = 0.124$). However, all three antibiotics exhibited time-dependent efficacy, with longer feeding durations significantly reducing parasitized egg counts ($\chi^2 = 125.94$, df $= 12$, $P < 0.0001$, Table 1).

Effect of different antibiotics on parasitoid emergence

With the increase in antibiotic feeding duration, toxins accumulated in the wasps, leading to a gradual de-

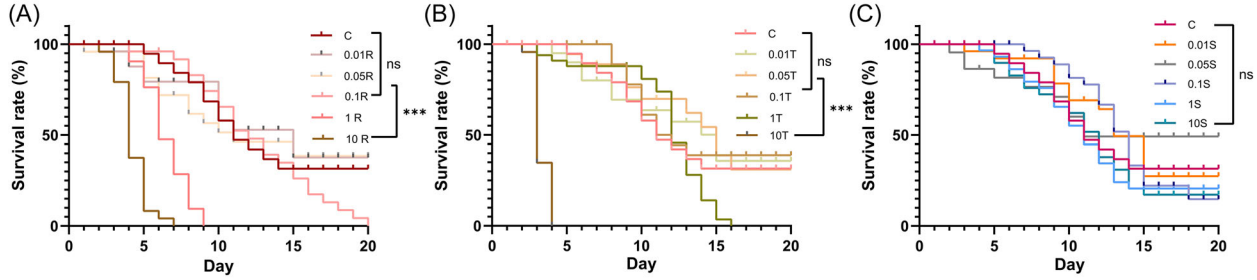

Fig. 1 Survival rate of *A. gansuensis* under different antibiotic treatments. C, control group (fructose solution only); R, rifampicin; T, tetracycline; S, sulfadiazine. Numbers 0.01–10 indicate concentrations in mg/mL. Log-rank (Mantel–Cox) test, ***$P < 0.001$ vs. control, ns, not significant.

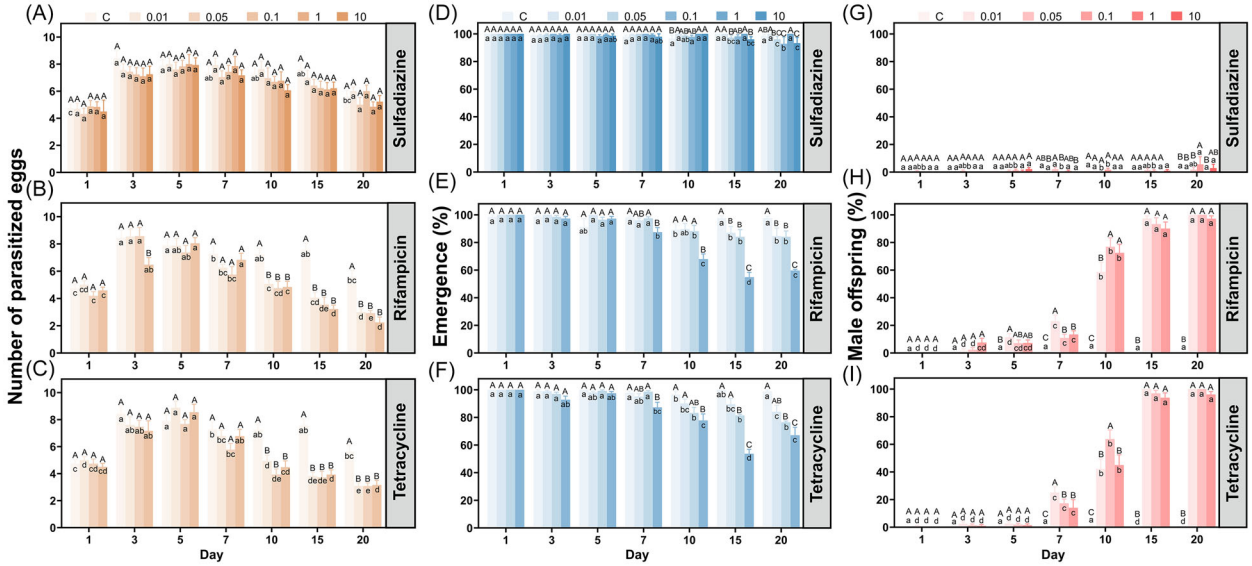

Fig. 2 Number of parasitized eggs, emergence rate, and proportion of male offspring under different antibiotic treatments. C, control group (fructose solution only); S, sulfadiazine; R, rifampicin; T, tetracycline. 0.01–10: 0.01–10 mg/mL. (A–C) Number of parasitized eggs after feeding different concentrations of sulfadiazine, rifampicin, and tetracycline, respectively. (D–F) Emergence rate after feeding different concentrations of sulfadiazine, rifampicin, and tetracycline, respectively. (G–I) Male rate after feeding different concentrations of sulfadiazine, rifampicin, and tetracycline, respectively. Data are means ± SEM. Different uppercase letters above the data columns indicate statistically significant differences ($P < 0.05$) between antibiotic concentrations on the same day of feeding. Lowercase letters indicate statistically significant differences ($P < 0.05$) between different feeding days at the same antibiotic concentrations.

crease in the emergence of offspring. For sulfadiazine, no significant differences in emergence were observed between any concentration (0.01–10 mg/mL) and controls across time points (days 1–20; $\chi^2 = 8.05$, df = 6, $P = 0.24$, Fig. 2D). Conversely, rifampicin and tetracycline treatments exhibited delayed toxicity. During days 1–7, emergence was similar across the three concentrations (0.01, 0.05, 0.1 mg/mL) versus controls ($P > 0.05$). From day 10 onward, all concentrations progressively reduced emergence ($\chi^2 = 30.07$, df = 3, $P < 0.001$; $\chi^2 = 8.34$, df = 3, $P = 0.039$), culminating in 16%–56% reductions by day 20 (Fig. 2E, F). Controls maintained stable emergence rates throughout ($\chi^2 = 10.94$, df = 6, $P = 0.09$), whereas antibiotic-treated groups showed significant temporal declines ($P < 0.001$). Among the dead wasps removed from inside the host eggs through dissection (< 5 per replicate) that could be sexed, males constituted over 50% in the antibiotic-treated group, in contrast to 60% females in the control (Fig. S1).

Effect of different antibiotics on male rate

Sulfadiazine treatment across the five concentrations (0.01–10 mg/mL) for 20 d showed no statistically signif-

Table 1 Results of a generalized linear models (GLMs) analysis on number of parasitized eggs, emergence (%), and male rate (%) of *A. gansuensis* affected by wasps' age, the type and concentration of antibiotics.

Response variable	Variable	df	χ^2	*P*
Number of parasitized eggs	Age	6	516.81	<0.0001
	Type	2	240.40	<0.0001
	Concentration	4	202.17	<0.0001
	Age × Concentration	24	45.92	0.005
	Type × Concentration	8	103.86	<0.0001
	Age × Type	12	125.94	<0.0001
	Age × Type × Concentration	31	57.11	0.003
Emergence rate (%)	Age	6	185.81	<0.0001
	Type	2	345.52	<0.0001
	Concentration	4	501.62	<0.0001
	Age × Concentration	24	89.78	<0.0001
	Type × Concentration	8	220.62	<0.0001
	Age × Type	12	96.21	<0.0001
	Age × Type × Concentration	31	55.4	0.005
Male rate (%)	Age	6	2469.42	<0.0001
	Type	2	836.84	<0.0001
	Concentration	4	80.19	<0.0001
	Age × Concentration	24	284.57	<0.0001
	Type × Concentration	8	115.89	<0.0001
	Age × Type	12	1697.22	<0.0001
	Age × Type × Concentration	31	265.97	<0.0001

icant effects on male rate, with neither concentration ($\chi^2 = 10.44$, df = 5, $P = 0.06$), time interval ($\chi^2 = 9.86$, df = 6, $P = 0.13$), nor their interaction ($\chi^2 = 27.64$, df = 30, $P = 0.59$) reaching significance under a binomial GLM (Fig. 2G). In contrast, rifampicin and tetracycline treatments exhibited time-dependent male-biased sex ratio effects. For both antibiotics, male rate at three concentrations (0.01, 0.05, and 0.1 mg/mL) showed no differences from controls during early post-treatment intervals (days 1, 3, 5; $P > 0.05$) but demonstrated significant increases from day 7 onward ($P < 0.001$, Fig. 2H, I). Notably, while all antibiotic concentrations differed significantly from controls ($\chi^2 = 911.88$, df = 3, $P < 0.001$; $\chi^2 = 823.04$, df = 3, $P < 0.001$), inter-concentration differences within each antibiotic group remained non-significant ($\chi^2 = 0.07$, df = 2, $P = 0.97$; $\chi^2 = 3.85$, df = 2, $P = 0.15$). By the 10th day, the removal effect of the three concentrations of rifampicin (0.01, 0.05, 0.1 mg/mL) reached a maximum of 76.87% (Fig. 2H), while the removal rate of tetracycline was 63.83% (Fig. 2I). By day 20, both concentrations of rifampicin and tetracycline (0.01 and 0.05 mg/mL) eliminated female offspring, resulting in 100% male progeny. The proportion of male offspring with 0.1 mg/mL of the two antibiotics also reached more than 95% (Fig. 2H, I).

Therefore, all concentrations of sulfadiazine were excluded, and 0.01 and 0.05 mg/mL concentrations of rifampicin and tetracycline were selected for further analysis.

Effect of antibiotics on Rickettsia removal and parasitism

Rickettsia titers were analyzed exclusively within a 10-d experimental window to minimize the confounding effects of prolonged host deprivation on parasitoid fitness. As the age of the wasp increased, the number of offspring also gradually increased. The number of parasitized eggs at various times within 10 d for 0.01 mg/mL rifampicin and tetracycline showed no significant differences compared to the control group ($P > 0.05$; Fig. 3A, B). Based on the downward trend of the curve, *Rickettsia* removal was more effective with 0.01 mg/mL rifampicin than with tetracycline. For 0.05 mg/mL rifampicin and tetracycline, there was no significant difference in the number of parasitized eggs compared to the control on the 1st, 3rd, 5th, and 7th days ($P > 0.05$, Fig. 3A, B). On the 10th day, the parasitized eggs with 0.05 mg/mL rifampicin and tetracycline were significantly lower than those of the control group ($\chi^2 = 4.25$, df = 1, $P = 0.04$; $\chi^2 = 10.35$, df = 1, P

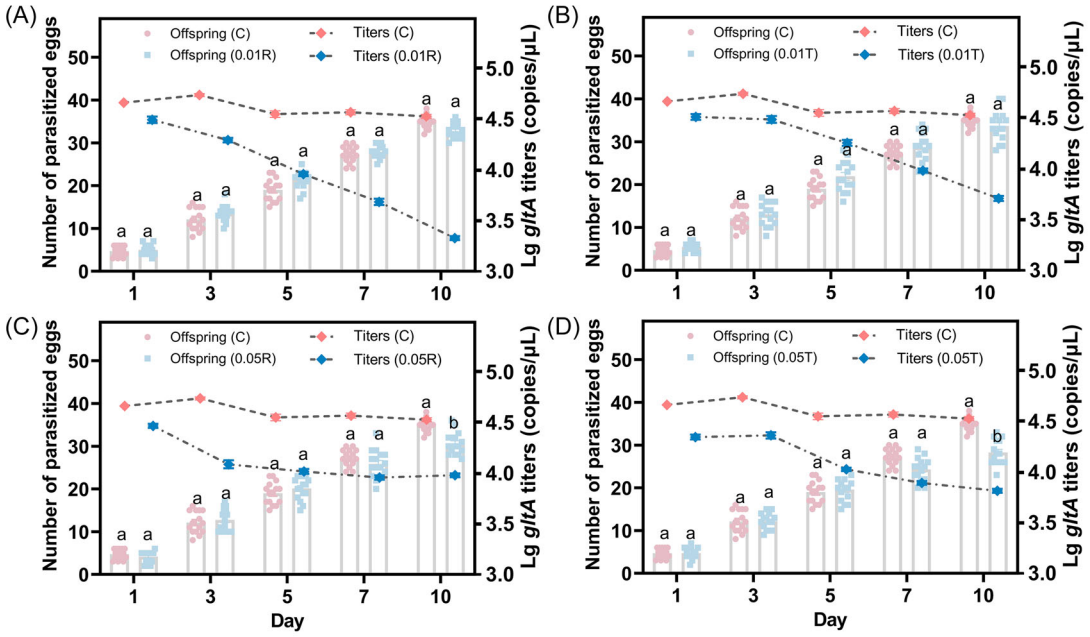

Fig. 3 The relationship between the *Rickettsia* titers of wasps' ovaries and the number of offspring. C, control group (fructose solution only); R, rifampicin; T, tetracycline. (A, B) The wasps were fed 0.01 mg/mL rifampicin and tetracycline fructose solution, respectively. (C, D) The wasps were fed 0.05 mg/mL rifampicin and tetracycline fructose solution, respectively. An independent-samples *t*-test was used to compare the number of offspring between the control and the treatment group. Data are means ± SEM, and different lowercase letters above the data columns indicate significant differences at the 0.05 level.

= 0.001; Fig. 3C, D). The antibiotic treatments were consistently significantly lower than control for the *Rickettsia* titers at various time points ($\chi^2 = 639.15$, df = 4, $P < 0.001$; Fig. 3A–D). While both 0.01 and 0.05 mg/mL rifampicin and tetracycline effectively removed *Rickettsia*, the higher concentration (0.05 mg/mL) showed greater negative impacts on wasp parasitism without a commensurate increase in removal efficiency. Thus, rifampicin and tetracycline at a concentration of 0.01 mg/mL were selected as antibiotics with non-target effects for obtaining the bisexual line.

Establishment of Rickettsia-cured bisexual line

Previous studies have shown that rifampicin effectively eliminates *Wolbachia* without risk of recovery (Wangkeeree *et al.*, 2022). Therefore, 0.01 mg/mL rifampicin was prioritized to establish the bisexual line of *A. gansuensis*.

A resurgence of male-female mating behavior was first documented in the 4th generation under sequential multigenerational antibiotic exposure (0.01 mg/mL rifampicin). As shown in Fig. 4A, bands were detected in G_0 (0.01R), G_1, and G_4 generations, but not in the G_7 samples. The offspring were then continuously fed with antibiotic-free fructose solution, allowing females and males to mate and produce the next generation. No banding was detected in the next 10 generations (Fig. 4A). Furthermore, every PCR run included a no-template control (NTC) to monitor potential contamination, using thelytokous females and bisexual females/males as positive and negative controls, which confirmed that all NTC results were negative (Fig. S2).

To visually assess the removal of *Rickettsia*, the titers' changes across generations were analyzed. When compared with the control (C), the parental generation (G_0) was 100% infected with *Rickettsia*, and the titer of *Rickettsia* gradually decreased across generations. In the fourth generation (G_4), there was a significant difference compared with the first generation (G_1) ($P = 0.035$; Fig. 4B). By the seventh generation (G_7), the *Rickettsia* titer approached 0 gene copies/μL. Subsequently, continuous feeding with antibiotic-free fructose solution maintained the *Rickettsia* titer around 0 gene copies/μL over multiple generations (Fig. 4B, from G_8 to G_{17}).

After antibiotic feeding, the male rate was observed in each generation, and if there were female and male offspring, male and female pairs were made. Video S1 captures the successful mating sequence, commencing with

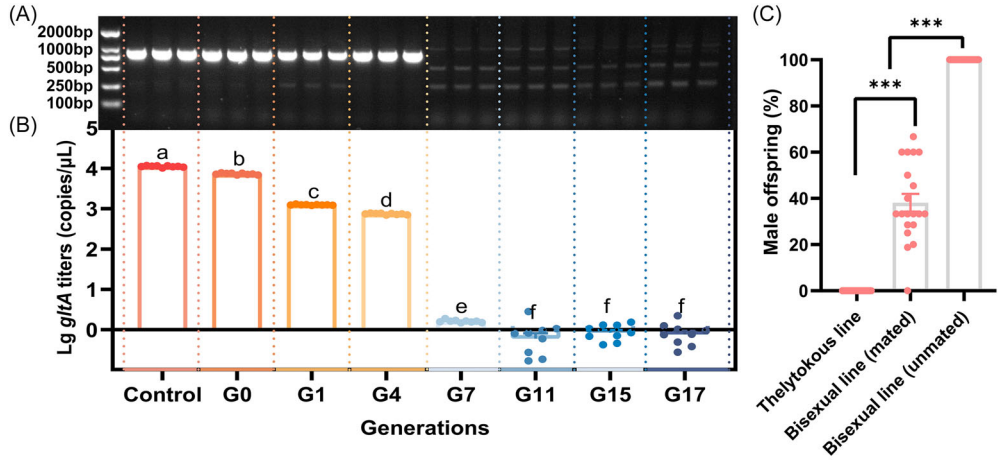

Fig. 4 Verification of a *Rickettsia*-cured line of *A. gansuensis*. (A) Detection of *Rickettsia* after antibiotic treatment in the wasps; each treatment was tested with three biological replicates (each replicate consisted of five wasps). (B) *Rickettsia* titers after antibiotic treatments in wasps. Control, fructose solution; G_0–G_{17}, the wasps were fed 0.01 mg/mL rifampicin fructose solution from the 1st to the 17th generation. Data are means ± SEM, and different letters above the data columns indicate significant differences at the 0.05 level (Gaussian distribution GLM, LSD test). Fifteen wasps for three biological replicates (each replicate consisted of five wasps) were performed, with each including three technical replicates. (C) The proportion of male offspring of the two *A. gansuensis* lines (thelytokous line and bisexual restored line, mated and unmated). Each treatment included 20 wasps. A Poisson distribution GLM was used to compare differences in male rate between the two *A. gansuensis* lines at the 0.05 level, $***P < 0.001$.

male courtship, proceeding to a static copulation phase of about 7 s, and culminating with the male mounting the female in a terminal marking posture. Statistical analysis of male offspring rates in thelytokous and bisexual lines (mated and unmated) showed that the progeny of the thelytokous line had only females (i.e., the male rate was 0). The progeny of the bisexual line (mated) showed both male and female individuals, and the male rate was 40.65%, while in the bisexual line (unmated) there were only male offspring (Fig. 4C). Therefore, the *Rickettsia*-cured bisexual line of *A. gansuensis* was obtained.

Discussion

Anastatus spp. serve as egg parasitoids with significant roles in biological control (e.g., Burks, 1967; Chen *et al.*, 2019). For example, *Anastatus fulloi* has been used to control the lychee stink bug, *Tessaratoma papillosa* (Hemiptera: Tessaratomidae) (Li *et al.*, 2014b), and *Anastatus orientalis* is a dominant egg parasitoid of the spotted lanternfly *Lycorma delicatula* (Hemiptera: Fulgoridae) (Xin *et al.*, 2021). The present study identified rifampicin at 0.01 mg/mL as the antibiotic for the conversion of a thelytokous to a stable bisexual line by non-target effect antibiotic elimination of *Rickettsia* in *A. gansuensis* via targeted antibiotic elimination of symbionts, advancing two key paradigms: (i) symbiont-mediated sex determination, where successful reversal of reproductive mode supports the hypothesis that *Rickettsia* titers regulate host sex allocation; and (ii) fitness trade-offs, where our methodology enables future studies to quantify fitness consequences of symbiont removal in host-parasitoid systems.

As a Gram-negative bacterium in the α-proteobacteria class, *Rickettsia* infects approximately 24% of terrestrial arthropods (Weinert *et al.*, 2015). Although originally identified in hematophagous arthropods as vertebrate pathogens (Roux *et al.*, 1997). *Rickettsia* is also present in non-hematophagous species (Perlman *et al.*, 2006; Weinert *et al.*, 2009), including *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Shan *et al.*, 2021) and *Acyrthosiphon pisum* (Hemiptera: Aphididae) (Chen *et al.*, 2000). *Rickettsia* influences host reproductive processes, causing male embryo mortality in some ladybirds (Werren *et al.*, 1994; von der Schulenburg *et al.*, 2001) and in buprestid leaf-mining beetles *Brachys tessellatus* (Chrysomelidae: Brachys) (Lawson *et al.*, 2001). It also induces parthenogenesis in parasitoid wasps (Hagimori *et al.*, 2006; Giorgini *et al.*, 2010; Nugnes *et al.*, 2015; Xu *et al.*, 2022) and cytoplasmic incompatibility in *N. tenuis* (Owashi *et al.*, 2024). By distorting host offspring sex ratios, *Rickettsia* facilitates its transmission. Consequently, eliminating symbiotic bacteria and establishing non-symbiotic host lines are essential for understanding

symbiont functions. We comprehensively screened antibiotics for non-target effects by assessing *A. gansuensis* survival, parasitism rate, male offspring proportion, and emergence rate. Antibiotic type, concentration, and feeding duration all significantly influenced these parameters, and significant interactions were observed among all factor combinations (two-way and three-way). The pronounced mortality observed under high-dose (1 and 10 mg/mL) tetracycline and rifampicin treatments aligns with their dual toxicity—both as disruptors of essential host cellular processes and as broad-spectrum microbiota eradicators. Rifampicin inhibits bacterial DNA-dependent RNA synthesis (Calvori *et al.*, 1965). Tetracycline suppresses protein synthesis by preventing the binding of aminoacyl-tRNA to the bacterial ribosome (Chopra & Roberts, 2001). Furthermore, antibiotic treatment, particularly at higher concentrations, can disrupt the gut microbiome, potentially reducing microbial diversity and worsening nutritional deficiencies such impaired sterol metabolism, which is essential for insect development. Such disruption may indirectly reduce host fitness by altering nutrient processing or compromising immune homeostasis (Engel & Moran, 2013). Although the present study did not directly quantify microbiome changes, the use of a very low effective concentration of rifampicin (0.01 mg/mL) and the subsequent recovery of key life history traits in the multi-generational cured line suggest that this treatment may have caused only limited disturbance to the gut microbial community. Future work incorporating metagenomic or 16S rRNA sequencing to compare the microbial composition between cured and infected lines, as well as between antibiotic treated and control groups, would allow clear separation of the effects of direct antibiotic toxicity, microbiome disruption, and endosymbiont elimination on host performance.

When excluding the highly lethal concentrations of tetracycline and rifampicin, both control and treatment groups exhibited a unimodal trend in parasitized numbers—characterized by an initial increase followed by a gradual decline—with treatment groups demonstrating a more pronounced reduction. Like most synovigenic parasitoids, *A. gansuensis* requires days to reach peak fecundity as oocytes complete maturation using nutrients from adult feeding (Chen *et al.*, 2024). Prolonged host deprivation fundamentally disrupts parasitoid life-history strategies, leading to accelerated senescence and diminished parasitism efficacy (Heimpel *et al.*, 1997; Fischbein *et al.*, 2016). The number of parasitized eggs in treatment groups was approximately 50% of those in controls, likely attributable to antibiotic-induced reproductive impairment under chronic exposure. Moreover, the emergence rate gradually decreased as the feeding time increased. Further analysis of the non-emerged wasps indicated that the majority were male and had developed into adults or pupae but did not manage to break through their eggshells (Fig. S1). This deformity may be related to the antibiotics removing certain essential symbiotic bacteria from the wasps, leading to a decline in bodily functions. Indiragandhi *et al.* (2011) used lower concentrations of antibiotics and found deformities in larval development. This phenomenon warrants further investigation. There were also some larvae whose sex could not be identified, which may be related to the antibiotics delaying the development of the offspring (Ruan *et al.*, 2006).

The effects of three antibiotics on the removal of symbiotic bacteria in wasps vary, which may be related to the different mechanisms of action of the antibiotics on bacteria. Comparative analysis of male rates revealed that prolonged administration of rifampicin and tetracycline at three concentrations (0.01, 0.05, and 0.1 mg/mL) achieved complete feminization suppression (100% male offspring), with no significant concentration-dependent effects. The reasons may be attributed to a threshold saturation phenomenon in antibiotic efficacy. In contrast, high concentrations of sulfadiazine did not show significant differences in the different indicators of the wasps compared to the control group. Previous studies have shown that sulfadiazine may be superior to tetracycline and rifampicin in inhibiting parthenogenesis-inducing *Wolbachia* (Guo *et al.*, 2023), and bisexual lines can be obtained within three generations. Sulfonamides competitively inhibit bacterial dihydropteroate synthase, interfering with the synthesis of folate, thereby suppressing bacterial growth and proliferation (Castelli *et al.*, 2001). They are generally effective against a wide range of bacteria; however, their efficacy against intracellular symbionts can vary and may be influenced by the restricted tissue tropism of *Rickettsia*, for example, localization within oocytes, which may limited drug accessibility (Gong *et al.*, 2025). Differences in metabolic pathways may also contribute to variation in susceptibility. Sulfonamides act by competitively inhibiting dihydropteroate synthase, yet *Rickettsia* and *Wolbachia* may differ in completeness of this pathway, the affinity of the enzymes for the drug, or the presence of alternative bypass mechanisms. In addition, host specific pharmacokinetics must be considered. Variation in absorption, distribution, metabolism, and excretion can influence the ability of the drug to reach the symbiont within its tissue niche. Factors such as midgut pH, transporter expression, or detoxification enzyme activity may reduce sulfadiazine bioavailability. Future studies designed to test these hypotheses directly would contribute significantly to understanding the mechanistic basis of antibiotic specificity in eliminating heritable endosymbionts.

And there is a need to determine the specificity of sulfadiazine to *Wolbachia*. Targeted removal is particularly significant in species doubly infected by *Wolbachia* and *Rickettsia*.

In general, under the same dose, the removal effect of rifampicin on *Rickettsia* was better than that of tetracycline. This has also been confirmed in *Wolbachia*-infected Leafhopper *Yamatotettix flavovittatus* (Wangkeeree *et al.*, 2022). While 0.01 mg/mL antibiotic maintained parasitization levels comparable to controls over 10 d, 0.05 mg/mL significantly suppressed egg parasitization. This may be associated with the toxic side effects produced by the antibiotics. This finding is consistent with the study by Stouthamer & Mak (2002), who also reported that prolonged treatment with high doses of tetracycline significantly reduced wasp offspring production. Furthermore, the higher antibiotic concentration (0.05 mg/mL) exerted stronger negative impacts on parasitism without a commensurate improvement in *Rickettsia* removal efficiency. This may be attributed to the fact that the lower concentration (0.01 mg/mL) already effectively penetrated host tissues and reached levels sufficient to inhibit or eliminate most *Rickettsia*. Increasing the concentration to 0.05 mg/mL did not yield a linear enhancement in symbiont clearance, indicating a diminishing-returns effect. The 0.01 mg/mL rifampicin fructose solution was screened as the most suitable antibiotic for establishing a bisexual strain. The newly emerged wasps were fed for a short period before oviposition, and successive generations of feeding and parasitism were essential. This is consistent with the method used by Stouthamer to obtain arrhenotokous lines (Stouthamer *et al.*, 1990). When male individuals begin to appear in the offspring of thelytokous wasps after antibiotic treatment, it indicates that the *Rickettsia* has been partially removed. The pairing of male and female individuals signifies a significant advancement in the cultivation of the bisexual line. Research has shown that even if antibiotics can eliminate *Wolbachia* in the parasitoid wasp and male individuals can appear in the offspring, mating behavior is sometimes not observed, which poses a significant obstacle to obtaining bisexual strains (Fein *et al.*, 1992; Stouthamer & Mak, 2002; Wang *et al.*, 2017). This phenomenon may correlate with chemosensory dysfunction in long-term thelytokous females, where relaxed sexual selection pressure has led to degenerative evolution of genes involved in sexual reproduction, impairing their capacity to detect male pheromonal cues (White *et al.*, 2013). It may also be due to symbiont-mediated erosion of male fertilization competence during prolonged coevolution (Zchori-Fein *et al.*, 1992). However, during our process of obtaining bisexual strains, even when the *Rickettsia* has not been completely eliminated, the male and female wasps were still able to mate. While pairing thelytokous female wasps with male wasps after antibiotics (e.g., G_1), the female wasps exhibited an absolute rejection behavior, avoiding the proximity of the males. This raises the question of whether the *Rickettsia* regulates host pheromone synthesis pathways, for instance by altering cuticular hydrocarbon (CHC) profiles, to influence mate acceptance, which warrants further research. Future research should systematically compare the cured line with the original thelytokous line to examine differences in courtship behavior, mating success, female sex pheromone profiles, and the expression of key genes in sex determination pathways (e.g., *doublesex*). Such analysis will help clarify the mechanisms through which *Rickettsia* manipulates reproduction at the behavioral, chemical, and molecular levels, and will provide insights to the evolutionary consequences of symbiont induced changes that may contribute to speciation. Our research shows that the female wasps from the *Rickettsia*-uninfected bisexual strains we obtained produce only male offspring when not mated, while the offspring from mated females include both males and females. The female ratio is approximately 2/3. Comprehensive molecular analyses indicate that we have now acquired a bisexual strain of *A. gansuensis* free from *Rickettsia* infection. Starting from the 8th generation, we ceased administering rifampicin until the 17th generation, and there were still no signs of recovery of the *Rickettsia*. This observation is consistent with findings in other research (e.g., Wangkeeree *et al.*, 2022) and may be attributed to the bactericidal mechanism of rifampicin, which inhibits bacterial DNA-dependent RNA polymerase. Unlike bacteriostatic antibiotics, rifampicin likely achieves complete eradication of *Rickettsia* without leaving residual viable cells, thereby preventing symbiont resurgence after antibiotic cessation. This study enables future comparisons of biological parameters between thelytokous and bisexual lines, and predictive modeling of biocontrol potentials (Stouthamer & Luck, 1993; Stouthamer *et al.*, 1993; Wang *et al.*, 2024). In addition, this study also lays a foundation for further exploration of investigating *Rickettsia*'s multifaceted roles in modulating host mating behavior and sex determination pathways.

In summary, our research indicates that sulfadiazine was ineffective in removing *Rickettsia* in *A. gansuensis*, while high concentrations of tetracycline and rifampicin were toxic to wasps. This study demonstrates an efficient and low impact method for generating a stable *Rickettsia* cured bisexual line using low concentration rifampicin treatment (0.01 mg/mL). This methodological advancement provides a practical framework for evaluating po-

tential non target effects of antibiotics while also generating a valuable biological resource. The cured line established here will support future investigations into the mechanisms through which *Rickettsia* regulates host reproduction and will facilitate the evaluation of its potential applications in biological control.

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Disclosure

The authors declare no competing interests.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Phenotypes of unemerged *A. gansuensis* following antibiotic treatments.

Fig. S2 Verification of PCR specificity and absence of contamination using No-Template Controls (NTCs).

Video S1 Mating video of *Anastatus gansuensis* (cured, i.e., *Rickettsia*-free).