Endosymbionts mediate the effects of antibiotic exposure in the tramp ant *Cardiocondyla obscurior*

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**Abstract.**

1. Bacterial endosymbionts play a fundamental role in insect ecology. Ants host a large diversity of bacterial symbionts, but comparatively little is known about how the loss or reduction of symbionts affects ant fitness.

2. We investigated the effects of the rifampicin, a commonly used antibiotic, on colonies from several populations of the globally distributed tramp ant *Cardiocondyla obscurior*, which differ in their endosymbiont communities.

3. We found that rifampicin treatment negatively affected queen fecundity and colony productivity, even when there was a delay of 3 months between treatment and productivity assessment. In addition, the viability of sperm from males produced in rifampicin-treated colonies was significantly reduced, pointing towards a trans-generational effect of antibiotics on male ant fitness. As expected, rifampicin treatment also led to a significant decrease in the titres of *Candidatus Westeberhardia cardiocondylae* and *Wolbachia* sp., the main bacterial endosymbionts of this ant.

4. The negative effects of antibiotic exposure on ant and symbiont fitness were modulated by the presence and strain of symbiotic bacteria, revealing a complex relationship between the microbiome and ant fitness.

**Key words.** Bacteria, *Candidatus Westeberhardia cardiocondylae*, fitness, rifampicin, social insects, symbiosis, *Wolbachia* sp.

**Introduction**

Bacterial endosymbionts can play fundamental roles in the ecology of their insect hosts, for instance by supplementing poor food with essential nutrients or by aiding in the defence against pathogens (Feldhaar & Gross, 2009; Feldhaar, 2011). Symbionts can also have negative impacts on insect fitness, especially when infection reduces fecundity (Fry et al., 2004) or symbionts manipulate host reproduction (Stouthamer et al., 1999; Werren et al., 2008). To expose the effects of symbionts on host biology, hosts need to be cured of their bacteria. In experimental settings, this is done with antibiotics, typically in the context of symbiont-induced manipulation of host reproduction, with the aim of rescuing the wild-type reproductive phenotype (Shropshire et al., 2020). In cases where bacterial endosymbionts are beneficial for hosts, the reduction or loss of symbionts as a result of antibiotic treatment can drastically decrease host fitness (Dedeine et al., 2001; Koga et al., 2007; Miller et al., 2010). When insects are infected with more than one endosymbiont, negative effects can be dampened or further strengthened by interactions between bacteria (Moran et al., 2005; Vorburger & Gouskov, 2011; McLean et al., 2018). Experimental antibiotic treatment has also been shown to influence other fitness-related traits across a range of insect taxa, including offspring sex ratios and mate discrimination behaviour (Rosengaus et al., 2011; Engl et al., 2018; Schneider et al., 2019). As antibiotics may cause damage to the mitochondria of eukaryotic cells with potentially detrimental consequences for fitness (Ballard & Melvin, 2007; Moullan et al., 2015), assessing the contributions of direct damage to host mitochondria and indirect effects of symbiont reduction or removal remains challenging (Ridley et al., 2013).

Ants are often ecologically dominant in terrestrial habitats (Lach et al., 2010), where they provide such essential services as seed dispersal, nutrient recycling, and soil aeration (Ohashi et al., 2007; Wardle et al., 2011; Frouz et al., 2016), and act as prey for other organisms (Robinson et al., 2016). However, in contrast to pollinating social insects such as honeybees, little is known about how the loss of bacterial endosymbionts affects ant fitness. Experimental studies of ant-endosymbiont dynamics have mainly been conducted in associations between
Camponotus carpenter ants and their obligate gut-associated symbiont Blochmannia, which supplements host diet with essential amino acids (Feldhaar et al., 2007). In this system, reduction of Blochmannia levels via antibiotic treatment negatively affected pupae production when whole colonies were treated (Zientz et al., 2006). This effect disappeared when either brood or workers remained untreated provided colonies were fed diets containing essential amino acids (Zientz et al., 2006; Feldhaar et al., 2007), confirming the role of the bacteria in nutritional upgrading. In terms of the effects of antibiotic treatment on reproductive phenotype of queens and males, and on colony fitness in general, things are less clear. While workers cleared of Blochmannia via treatment with the antibiotics tetracycline and rifampicin did not experience reduced survival (Sauer et al., 2002), long-term treatment with rifampicin resulted in lower colony growth (De Souza et al., 2009), and a study of Blochmannia titres across development mentioned unpublished data on developmental arrest in offspring produced by antibiotic-treated queens (Wolschin et al., 2004).

The ant Cardiocondyla obscurior is a cosmopolitan tramp species, which is infected with two main bacterial endosymbionts: Candidatus Westeberhardia cardiocondylae (Klein et al., 2016) and Wolbachia sp. (Klein, 2015; Ün et al., 2021). Cand. Westeberhardia reside in bacteriocytes, specialised cells that house endosymbionts, as well as in ovaries in queens, from where they are transmitted vertically to offspring. The bacterium has an eroded genome; together, these traits are typical of obligate symbionts (McCutcheon & Moran, 2012), and functional genome analysis suggests that the symbiont provides the ant host with a tyrosine pre-cursor molecule used in cuticle development (Klein et al., 2016). Surprisingly however, among samples collected from introduced populations worldwide, all colonies from Brazil are infected with Cand. Westeberhardia, while most but not all colonies from Japan are infected. The Brazilian and Japanese populations also carry distinct Wolbachia strains, which differ in their infection densities and ability to induce cytoplasmic incompatibility (Ün et al., 2021), and exhibit divergence in genotype and behaviour (Schrader et al., 2014; Erbbi et al., 2021). With its naturally occurring diversity in endosymbiont strains, pathogenicity, and infection rates, C. obscurior represents a good system for understanding the interplay between antibiotic exposure, symbiont infection, and ant fitness.

Here, we attempted to disentangle the direct effects of antibiotic treatment from indirect effects following endosymbiont loss by investigating how treatment with the antibiotic rifampicin affected the phenotype of C. obscurior colonies which differed in their Cand. Westeberhardia infection status and Wolbachia strains. We assessed how rifampicin treatment affected the densities of the two main endosymbionts and documented effects of rifampicin on the fecundity of queens and on overall colony productivity, as well as on the sperm traits of males produced by treated queens. Based on results from other studies of antibiotic treatment in insects, we predicted that rifampicin would have direct negative effects on ant fitness, particularly regarding queen fecundity, irrespective of symbiont infection or strain. In colonies infected with Cand. Westeberhardia, we predicted rifampicin to decrease symbiont densities, leading to additional negative effects on host fitness as a result of the loss of symbiont-provided metabolites. Rifampicin is known to decrease Wolbachia densities in ants from the Japanese population (Ün et al., 2021), and we expected similar effects in the Brazilian population. As Wolbachia can play a role in insect development (e.g. Dedeine et al., 2001), its loss can be predicted to exacerbate negative effects on ant fitness, albeit to varying degrees depending on the strain’s susceptibility to rifampicin, and potential interactions with Cand. Westeberhardia. As expected, rifampicin drastically reduced endosymbiont densities and host fitness, and the degree to which treatment affected the fitness of the ant and densities of its symbionts varied with Cand. Westeberhardia infection status and Wolbachia strain, highlighting a complex relationship between the bacterial symbionts and their ant host.

**Material and methods**

**Ant colonies**

Based on the geographical distribution of early-branching species in the genus Cardiocondyla, C. obscurior presumably originates from Southeast Asia (Oettler et al., 2010; Heinze, 2017), but has been spread to disturbed habitats such as fruit tree plantations and city parks throughout the tropics and subtropics by human activities (Oettler, 2020). Typically, C. obscurior colonies contain a few dozen or hundred workers, one or several queens, and a single wingless ‘ergatoid’ male. Ergatoid males are an evolutionary novelty (Oettler et al., 2010) and, unlike other social Hymenopteran males, they are relatively long-lived and exhibit life-long spermatogenesis (Heinze & Hölldobler, 1993). C. obscurior ants have short development (~30 days from egg to adult) and generation times (~14 days from hatching of a new queen to production of first egg) and mating occurs among siblings within the nest (Kinomura & Yamauchi, 1987), making this ant an excellent lab model.

In this study, we evaluated colonies from two populations that were collected in Brazil and Japan (Schrader et al., 2014). The Brazilian colonies were collected from aborted coconuts in plantations in Ilhéus, Bahia in 2009 (lineage BR). The Japanese colonies were collected from two coral trees in Onoyama city park in Naha, Okinawa in 2011 (lineage JP<sub>ee</sub>; Cand. Westeberhardia infected; lineage JP<sub>we</sub>; Cand. Westeberhardia uninfected). For simplicity, we use the term lineage to refer to the three colony types in the remainder of the text. We kept the colonies in artificial nests with plaster floors and plastic nest inserts under a 12h/12h light/dark and 28°C/23°C temperature cycle with humidity constant at 70%. These stock colonies were provided with honey, water, and pieces of insects (cockroaches or fruit flies) twice a week. Animal treatment guidelines applicable to ants under international and German law were followed throughout the study.

**Antibiotic treatment**

Rifampicin is a widely used antibiotic that inhibits prokaryotic transcription by binding to DNA-dependent RNA polymerase (Campbell et al., 2001). Prior to antibiotic treatment several
large stock colonies from each lineage were split into two equal halves and moved into new nests. From each pair, one colony fragment served as a control while the other colony fragment was treated with rifampicin (BR: 10 colonies, BR<sup>rid+</sup>; 13, JP<sub>we</sub><sup>rid+</sup>; 13, JP<sub>we</sub>; 8). We treated the ants with antibiotics by feeding colonies with a rifampicin-honey solution. To this end, we weighed 0.0025 g of the solid antibiotic (Sigma-Aldrich, St. Louis, Missouri, USA) on a fine-scale (AX224, Sartorius, Göttingen, Germany) and diluted this amount in 500 µl of a 1:1 honey–water solution for a final antibiotic concentration of 5 µg·µl<sup>−1</sup>. This concentration was chosen because it may effectively remove endosymbionts from arthropod hosts (Chiel et al., 2009; Li et al., 2014). The colonies were fed with this solution twice per week every other week for a total period of 10 weeks. On the days after antibiotic treatment, the antibiotic solution was removed from the nest. In the weeks between antibiotic treatments, the treated colonies were provided with water and fed with honey and autoclaved pieces of cockroaches (to prevent re-infection with Wolbachia) twice per week. The control colonies were provided with water and fed with honey and pieces of cockroaches twice per week. After termination of rifampicin treatment, all colonies were provided with water and fed with honey and pieces of cockroaches twice per week.

**Cand. Westeberhardia and Wolbachia infection titres**

*Cand. Westeberhardia* and Wolbachia titres were assessed using qPCR. One month after termination of rifampicin treatment, we collected brown worker pupae from control colonies (BR = 29 pupae from 9 colonies, JP<sub>we</sub> = 21 pupae from 7 colonies, JP<sub>we</sub><sup>rid+</sup> = 21 pupae from 7 colonies) and rifampicin-treated colonies (BR<sup>rid+</sup> = 30 pupae from 9 colonies, JP<sub>we</sub><sup>rid+</sup> = 21 pupae from 7 colonies, JP<sub>we</sub><sup>rid+</sup> = 21 pupae from 7 colonies) in individual Eppendorf tubes. DNA was extracted from individual samples using a standard CTAB DNA extraction protocol. We performed qPCR using specific primers for the ribonucleoside diphosphate reductase 1 subunit beta gene of *Cand. Westeberhardia* (nrDB) (Klein et al. 2016) and the cytochrome c oxidase subunit 1 gene of Wolbachia (*coxA*) (Un et al., 2021). The *C. obscursior* gene elongation factor 1-alpha 1 was used as a housekeeper (EF1) (Klein et al. 2016). Reactions were run with 5 µl KAPA SYBR FAST Universal (Peqlab), 2 µl sterile water, 1 µl each of forward and reverse primer (2 µM), and 1 µl template DNA in a real-time PCR detection system (BioRad) under the following conditions: 95°C for 3 min followed by 39 cycles of 95°C for 5 s, 60°C for 20 s, and 95°C for 10 s, followed by melt curve analysis with a 0.5°C stepwise increase from 65 to 95°C. For each sample, three technical replicates were analysed, and single-amplicon production was confirmed with melt curve analyses. The 2<sup>−ΔΔCT</sup> method was used to calculate relative quantities of *Wolbachia* and *Cand. Westeberhardia* (Schmittgen & Livak, 2008).

**Queen fecundity and colony productivity**

To measure queen fecundity and colony productivity, we set up experimental fragments three months after termination of rifampicin treatment with individuals from control and rifampicin-treated colonies from each lineage. Each experimental fragment was set up by transferring six adult workers and three mated queens from a single control or rifampicin-treated colony to a new nest (BR = 9, BR<sup>rid+</sup> = 10, JP<sub>we</sub> = 11, JP<sub>we</sub><sup>rid+</sup> = 9, JP<sub>we</sub> = 8, JP<sub>we</sub><sup>rid+</sup> = 8). We set up fewer experimental fragments than initially planned because four (23%) of the original 13 JP<sub>we</sub><sup>rid+</sup> colonies succumbed to the treatment and died. In addition, two control colonies from the JP<sub>we</sub> and one control colony from the BR lineage were accidentally dropped and thus had to be removed from the experiment. We monitored the fragments once per week for seven weeks and counted all eggs and pupae (queens, workers, and males). All pupae were removed from the fragments after counting. The number of workers and mated queens in each fragment was standardised once per week by removing or adding individuals from the maternal rifampicin-treated or control colonies.

**Queen reproductive tissue**

Between one to three months after termination of rifampicin treatment, queens were collected from rifampicin-treated and control colonies (BR = 10 queens from 10 colonies, BR<sup>rid+</sup> = 16 queens from 7 colonies, JP<sub>we</sub> = 10 queens from 10 colonies, JP<sub>we</sub><sup>rid+</sup> = 27 queens from 10 colonies) and their reproductive tissues were removed to verify mating status and assess fecundity. Each queen was placed on a microscope slide in a drop of distilled water and the abdomen was opened using a pair of forceps under a dissection microscope (Stemi 12000 C, Zeiss, Germany). Reproductive tissue was removed by pulling on the sting and transferred to a new microscope slide in a drop of water where the spermathecae and the ovarioles were separated from other tissues. The spermathecae and the ovarioles were photographed with 10× magnification using a digital camera (Motica 58, Motic, China) connected to a microscope (Primo start, Zeiss, Germany). To verify mating status, the spermatheca of each queen was checked for the presence of sperm. To assess fecundity, the total number of mature oocytes in the ovarioles of each queen was documented.

**Sperm traits**

The sperm quality of JP lineage males collected from control and rifampicin-treated colonies was assessed 2 months after termination of the treatment. Prior to assessment of sperm quality, each male was mated once to a queen from the BR lineage. We measured sperm viability (JP<sub>we</sub> = 9, JP<sub>we</sub><sup>rid+</sup> = 6) and sperm length (JP<sub>we</sub> = 5, JP<sub>we</sub><sup>rid+</sup> = 5) of individual males. Samples were randomised prior to measurements and measurements conducted blindly by an independent observer.

**Sperm viability.** Each male was dissected on a microscope slide in a drop of Beadle solution (128.3 mM NaCl, 4.7 mM KCl, 2.3 mM CaCl<sub>2</sub>) and seminal vesicles transferred to 15 µl of fresh Beadle solution. Sperm was then released from seminal vesicles and mixed carefully with clean forceps. The position of...
the sperm mass was marked on the bottom of the slide. Sperm viability was assessed with the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, Oregon, USA) following the manufacturer’s protocol. In short, after the addition of 5 μl of a SYBR 14 working solution (SYBR stock solution diluted 1:50 in Beadle solution) to the sperm mass, the slide was incubated in the dark in a box lined with humid tissue paper for 10 min. We then added 2 μl of propidium iodide to the sperm mass and incubated the slide under the same conditions for another 7 minutes. Live and dead sperm cells were determined with fluorescent microscopy at 20× magnification (Axiophot, Zeiss, Germany). Each sample was divided into five equally sized partitions, which were photographed and evaluated using the cell counter in ImageJ (http://rsbweb.nih.gov/ij/, NIH, USA). For each sample, live (green) and dead (red) sperm cells were counted in all five photos and the overall proportion of live sperm was calculated.

Sperm length. After sperm viability measurements, the slides were fixed with 70% ethanol and allowed to dry overnight. Each sample was then divided into five equally sized partitions and each partition photographed under a microscope at 100× magnification (Axiophot). After appropriate scaling, the length of all sperm in each partition was measured by tracing from head to tail using the measurement tool in ImageJ (http://rsbweb.nih.gov/ij/, NIH, USA). For each male, the lengths of 8–199 (median ± SD: 35 ± 31) sperm cells were measured.

Statistical analyses

R version 3.6.3 (R Team, 2016) was used to perform all statistical analyses. To test for an effect of treatment on Cand. Westeberhardia and Wolbachia titres in worker pupae, Kruskal-Wallis rank sum tests followed by pairwise Mann–Whitney U Tests with Bonferroni-Holm correction of P-values were used. For productivity data (total eggs per week, total pupae per week, oocyte numbers), we were interested in the effects of treatment (control, rifampicin), the effects of lineage (BR, JPwe+, JPwe−), as well as the interaction between treatment and lineage. Total egg numbers were log-transformed prior to analysis, and analysed by fitting a linear mixed effects model with random intercept to account for natural variation between stock colonies, i.e., the original lab colonies that were split to create rifampicin-treated and untreated fragments (function lmer, package lme4) (Bates et al., 2015): total eggs ~ treatment × lineage + (1 | stock colony). Total pupae number was analysed by fitting a generalised linear mixed effects model with log-link function and random intercept to account for natural variation between stock colonies (function glmer, package lme4): pupae number ~ treatment × lineage + (1 | stock colony). Oocyte number was analysed by fitting a generalised linear model with log-link function (function glm): oocyte number ~ treatment × lineage. To test for an effect of treatment on sperm viability, a generalised linear model with logit-link function for a binomial response variable was fit (function glm): (live sperm, dead sperm) ~ treatment. To test for an effect of treatment on sperm length, a linear mixed effects model was fit, with a random intercept to account for non-independence of sperm length measurements obtained from a single male (function lmer): sperm length ~ treatment + (1 | male identity).

For all models, residual diagnostics were performed using plot and test functions implemented in the DHARMa package (Hartig, 2020). Overall effects of categorical variables were estimated with Wald χ² tests (function ANOVA, package car) (Fox & Weisberg, 2019). For categorical variables with more than two levels and for interaction terms, pairwise differences were tested using contrasts among estimated marginal means with Tukey-corrected P-values for multiple comparisons (function pairs, package emmeans) (Lenth et al., 2020).

Results

Cand. Westeberhardia and Wolbachia infection titres

Rifampicin treatment led to a significant reduction of Cand. Westeberhardia titres in the BR and JPwe+ lineages but had no effect on titres in the naturally uninfected JPwe− lineage (Fig. 1a, Kruskal Wallis rank sum test, χ² = 87.30, d.f. = 5, P < 0.001, see Table S1 for Bonferroni-Holm corrected pairwise P-values). In the control treatment, Cand. Westeberhardia titres were similar in JPwe+ and BR colonies. After rifampicin treatment, pupae from JPwe+ colonies exhibited lower Cand. Westeberhardia titres than pupae from BR colonies.

Rifampicin treatment also reduced Wolbachia titres in each of the three lineages (Fig. 1b, Kruskal Wallis rank sum test, χ² = 92.43, d.f. = 5, P < 0.001, see Table S2 for Bonferroni-Holm corrected pairwise P-values). Wolbachia titres were overall lower in the BR lineage compared to the JP lineages, and rifampicin treatment had a much stronger negative effect in the BR lineage. There was no difference in Wolbachia titres between the two JP lineages before or after rifampicin treatment.

Colony productivity

In all lineages, the total number of eggs produced by queens from rifampicin-treated fragments over the course of 7 weeks was significantly lower than that produced by queens from control fragments (Fig. 2a) (Table 1, and see Table S3 for Tukey-corrected pairwise P-values). There was no difference between the three lineages regarding the total egg numbers produced by queens from control fragments, but rifampicin-treated JPwe+ and JPwe− fragments produced significantly more eggs than rifampicin-treated BR fragments. In addition, rifampicin-treated fragments produced significantly fewer pupae than control colonies in all lineages (Fig. 2b, Table 1, and see Table S4 for Tukey-corrected pairwise P-values). In the control treatment, there was no difference in the total number of pupae produced by the three lineages. However, among rifampicin-treated fragments higher numbers of pupae were observed in JPwe+ and JPwe− fragments compared to BR fragments, and JPwe− fragments produced the most pupae of all three lineages. The sex and caste ratios of pupae could not be compared because rifampicin-treated fragments produced very few off-spring overall.

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**Fig. 1.** Effects of rifampicin on endosymbiont titres in the ant *Cardiocondyla obscurior*. (a) Relative *Candidatus Westeberhardia* cardiocondyla titres in worker pupae collected from control and rifampicin-treated colonies. (b) Relative *Wolbachia* titres in worker pupae collected from control and rifampicin-treated colonies. Differences between groups were assessed with pairwise Mann–Whitney-U-tests followed by P-value correction according to Bonferroni-Holm. Letters show significant differences between groups (P < 0.05). Rifampicin treated (rif+) and untreated Brazilian (BR) and Japanese (JP) colonies were used. JP colonies were infected (JPwe+) or uninfected with *Candidatus Westeberhardia* cardiocondyla (JPwe−).

**Fig. 2.** Productivity of control and rifampicin-treated fragments from three lineages of the ant *Cardiocondyla obscurior*. (a) Total eggs produced over the course of 7 weeks. (b) Total pupae produced over the course of 7 weeks. Data were analysed with a linear mixed effects model (total eggs) and a generalised linear mixed effects model (total pupae). Letters show significant differences between groups based on pairwise contrasts of estimated marginal means (P < 0.05). Rifampicin treated (rif+) and untreated Brazilian (BR) and Japanese (JP) colonies were used. JP colonies were infected (JPwe+) or uninfected with *Candidatus Westeberhardia* cardiocondyla (JPwe−).

**Queen reproductive tissue**

All dissected queens had successfully mated and had sperm in their spermathecae. However, the reproductive tissues of queens from rifampicin-treated colonies appeared degenerated (Fig. 3a). Queens from rifampicin-treated colonies also had fewer oocytes in their ovaries than queens from control colonies (Fig. 3b, Table 1, and see Table S5 for Tukey-corrected pairwise P-values). There was no difference between the lineages regarding the total number of oocytes in ovaries of queens, neither when control nor when rifampicin-treated colonies were compared (Fig. 3b, Table 1).

**Sperm traits**

The proportion of live sperm in JPwe+ male testes differed between males collected from control and rifampicin-treated colonies (Fig. 4a). Specifically, males produced in rifampicin-treated colonies had significantly fewer live sperm.
Table 1. Statistical analysis of fitness-related traits of *Cardiocondyla obscurior* ants following rifampicin treatment.

| Trait         | Analysis                              | Fixed effects |                           |                           | Random effects |
|---------------|---------------------------------------|---------------|---------------------------|---------------------------|---------------|
|               | Treatment                              | Population    | Treatment × population    | Stock colony              |               |
|               | $N$ | $\chi^2$ | d.f. | $P$ | $\chi^2$ | d.f. | $P$ | $\chi^2$ | d.f. | $P$ | Variance | Standard deviation | $n$ |
| Total eggs    | Linear mixed effects                   | 55            | 447.36                    | 1 | $<0.001$ | 3.73 | 2 | 0.155 | 33.387 | 2 | $<0.001$ | 0.043 | 0.208 | 27 |
| Total pupae   | Generalised linear mixed effects model | 55            | 245.51                    | 1 | $<0.001$ | 0.48 | 2 | 0.785 | 59.690 | 2 | $<0.001$ | 0.486 | 0.697 | 27 |
| Number of oocytes | Generalised linear model | 63            | 84.01                     | 1 | $<0.001$ | 0.10 | 1 | 0.748 | 0.161 | 1 | 0.688 | – | – | – |
| Sperm viability | Generalised linear model | 15            | 176.81                    | 1 | $<0.001$ | – | – | – | – | – | – | – | – |
| Sperm length  | Linear mixed effects                   | 452           | 17.65                     | 1 | 0.184 | – | – | – | – | – | – | 0.555 | 0.745 | 10 |

Fig. 3. Effect of rifampicin on oocyte numbers in ovaries of control and rifampicin-treated queens from two lineages of the ant *Cardiocondyla obscurior*. (a) Reproductive tissue of queens from BR (top) and JP<sub>we+</sub> (bottom) lineages. Green arrows show mature oocytes in the ovary. Black arrows show the location of the spermatheca. (b) Total number of oocytes in ovaries of queens from control and rifampicin-treated colonies from BR and JP<sub>we+</sub> lineages. Differences between groups were tested with a generalised linear model and letters show differences between the groups ($P < 0.05$). Reproductive tissues were photographed with 10x magnification and brightness, contrast and background colour of pictures was adjusted using Adobe Photoshop elements 2021. Samples were collected from rifampicin-treated (rif<sup>+</sup>) and untreated Brazilian (BR) and Japanese (JP) colonies. All JP colonies were infected with *Cand*. Westeberhardia cardiocondylae (JP<sub>we+</sub>).

Discussion

Host–symbiont interactions are a driving force in ecology and evolution (Moran, 2006), and ants are no exception to this rule (Russell et al., 2017; Moreau, 2020; Rafiqi et al., 2020). However, functional studies of ant–symbiont interactions have mostly been conducted in the context of obligate relationships with gut-associated bacteria (e.g. in *Camponotus* carpenter ants (Sauer et al., 2000; Sinotte et al., 2018) and *Cephalotes* turtle ants (Jaffe et al., 2001; Hu et al., 2018)), and few assessments exist of the general effects of antibiotics on ant fitness. Treating colonies of the ant *C. obscurior* with the antibiotic rifampicin resulted in strong negative effects on colony productivity, which were visible even when three months had passed between treatment and productivity assessment and occurred in all lineages irrespective of bacterial infection. Ovaries of queens collected more than males produced in control colonies (Table 1). Sperm length was similar in both treatments (Fig. 4b, Table 1).
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from rifampicin-treated colonies were highly degenerated, a result that is in accordance with studies of other insects (Jayaraj et al., 1967; Stevens & Wade, 1988; Dickel et al., 2016). For both productivity and ovary assessment, queens of unknown age were collected three months after termination of antibiotic treatment. As C. obscurior queens live ~6 months on average (Oettler & Schrempf, 2016), some of these queens may have been produced by antibiotic-treated colonies while others received the treatment directly. Thus, the data suggest that rifampicin treatment may have trans-generational effects on queen reproductive traits. In zebrafish, antibiotics are transferred from mothers to eggs, with offspring of antibiotic-treated females exhibiting reduced survival and potential metabolic deficits (Qiu et al., 2020). In insects, maternal trans-generational effects have been described in the context of immune response (e.g. Freitak et al., 2009) but the maternal trans-generational effects of antibiotic treatment have not been investigated. In addition to producing fewer eggs, treated colonies also produced fewer pupae, with some colonies producing no pupae at all. This points towards additional negative effects of rifampicin on development, similar to what has been found in the nematode Caenorhabditis elegans following tetracycline treatment (Moullan et al., 2015).

Rifampicin treatment also strongly reduced sperm viability in males. C. obscurior males are unique among ants, being relatively long-lived (up to several months) (Metzler et al., 2016) and exhibiting life-long spermatogenesis (Heinze & Hölldobler, 1993). Much like in queens, both direct effects on males receiving the treatment and maternal trans-generational effects may explain the changes to reproductive phenotype. On a cellular level, bactericidal antibiotics induce the production of reactive oxygen species (Dwyer et al., 2014; Piccaro et al., 2014). This causes oxidative stress, which can negatively affect sperm number, viability, motility, and morphology (Mahfouz et al., 2010; Takeshima et al., 2018). For example, tetracycline–treated pseudoscorpion males and their sons suffered from reduced sperm viability (Zeh et al., 2012) and in Drosophila melanogaster, tetracycline treatment reduced the number of progeny produced by males (O’Shea & Singh, 2015). In C. obscurior, lower sperm viability of males from rifampicin-treated colonies should manifest in the process of egg fertilisation. As ants exhibit haplodiploid sex determination, with males arising from unfertilised eggs and females arising from fertilised eggs, this should only affect the production of new queens and workers. Since rifampicin-treated

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fragments produced only few pupae overall, we could not compare the sex ratios of pupae produced in the experiment. However, the reduced sperm viability of males collected from rifampicin-treated JP colonies was sufficient to rescue colony productivity and female production in crosses between queens and males from the BR and JP lineages, which otherwise suffer from cytoplasmic incompatibility due to Wolbachia infection (Ün et al., 2021). Keeping in mind the strong negative effects of rifampicin treatment on queen ovary phenotype found here, this suggests that sperm viability may only have limited influence on the fitness of C. obscurior colonies.

As expected, rifampicin treatment clearly impacted the fitness of the bacterial symbionts Cand. Westeberhardia and Wolbachia. Colonies from the JPwC lineage did not carry Cand. Westeberhardia, thus rifampicin treatment could not affect densities in this lineage. Densities were similarly high in untreated colonies from the other two lineages. After treatment, densities decreased significantly in both lineages, and worker pupae from JPwC colonies exhibited even lower Cand. Westeberhardia densities than pupae from BR colonies. Together with the discovery of Cand. Westeberhardia naturally-uninfected colonies in the Japanese but not Brazilian lineages (Klein et al., 2016), this suggests that colonies from the Japanese population are more susceptible to losing the symbiont. We did not find differences in the number of eggs or pupae produced by untreated JPwC and JPwF fragments; however, JPwC colonies produced more pupae after rifampicin treatment than the two other lineages, even though egg numbers were similar. Antibiotic treatment must therefore affect development differently depending on the presence of the bacteria. Cand. Westeberhardia, like bacteriocyte-colonising symbionts in other insects (e.g. beetles (Anbutsu et al., 2017)), carries a gene that codes for a precursor for tyrosine, an amino acid that is important for cuticle formation (Klein et al., 2016). One possibility is that developing individuals in infected colonies depend on the bacteria to provide the gene product and pupation fails more often when Cand. Westeberhardia have been experimentally removed. Individuals from naturally-uninfected colonies may be better able to compensate for a lack of symbiont-provided molecules, especially under ad libitum access to high quality food in the lab. Future studies will elucidate how Cand. Westeberhardia affects cuticle formation and quality and reveal other benefits or costs of infection for the ant host.

In contrast to Cand. Westeberhardia, which has thus far only been described in the genus Cardiocondyla, Wolbachia is a generalist bacterium that infects ~40% of insect species (Werren & Windsor, 2000), with roughly similar infection rates reported for ants (Russell, 2012). Wolbachia are best known for their role as reproductive manipulators (Stouthamer et al., 1999), but infection can also have positive effects on host fitness (Zug & Hammerstein, 2015), for instance by providing resistance against parasites (Hansen et al., 2012). C. obscurior populations from Brazil and Japan are infected with distinct Wolbachia strains (wCobs BR, wCobs JP), both of which belong to the Wolbachia superclade A that typically contains reproductive manipulators (Werren et al., 2008; Dohna et al., 2018); however, only the Japanese strain induces cytoplasmic incompatibility (Ün et al., 2021). In addition, workers and queens infected with the Japanese strain exhibited significantly higher densities than individuals infected with the Brazilian strain (Ün et al., 2021), a result confirmed in the present study. Although treating colonies with rifampicin led to a significant reduction of Wolbachia in all three lineages, densities in the Japanese lineages dropped only marginally while densities in the Brazilian lineage decreased fivefold. Along with the putative origin of C. obscurior in Southeast Asia and the presence of the Japanese strain in a second Asian population from Taiwan, this suggests that the Japanese Wolbachia strain is ancestral and more tightly integrated into the host’s biology than the Brazilian strain, which was presumably acquired horizontally in the New World (Ün et al., 2021). In addition, the two strains may differ in their natural resistance to antibiotics, perhaps due to differences in outer membrane permeability (Miller, 2016; Ghai & Ghai, 2018; May & Grabowicz, 2018) or resistance-conferring mutations (Drencourt & Raoult, 1999). The degree to which rifampicin treatment affected Wolbachia levels in the two populations correlated with a significantly stronger decrease in the number of eggs produced by Brazilian rifampicin-treated colonies, even though oocyte numbers in queen ovaries were comparable between the populations. The presence of Wolbachia may thus affect egg viability in C. obscurior, similar to what has been found in parasitoid flies (Puttaraju & Prakash, 2009). As we were not able to cure ants of Wolbachia completely in any of the three lineages, we cannot definitively say whether oogenesis fails without the bacteria (as is the case in a wasp (Dedeine et al., 2001)). Recovery of Wolbachia titres to pre-treatment levels approximately 6 months after termination of rifampicin feeding, however, support the idea that the bacteria play a fundamental role in the biology of this ant (Ün et al., 2021).

Antibiotics inhibit bacterial growth and proliferation with minimal harm to the host. As a result, the global use of antibiotics in medicine and agriculture has steadily increased (Sayadi et al., 2010; Mehdi et al., 2018; Lulijwa et al., 2020), leading to the contamination of the environment with antibiotic residues and the spread of resistant bacteria as a result of improper use (Martinez, 2009; Economou & Gousia, 2015; Olesen et al., 2018). In both cases, the dynamics of ecosystems are estimated to be highly affected (Grenn et al., 2015), and studies have identified adverse effects on both animals and plants (Wang et al., 2015; Minden et al., 2017). C. obscurior ants may be exposed to residual antibiotics in the environment via direct contact, for instance following spraying of fruit tree plantations (McKenna, 2019), or indirectly via their prey. Ingestion of comparably high amounts of rifampicin had a strong negative influence on reproductive phenotype and colony productivity in three different lineages of this ant. The severity of these effects was modulated by the presence and strain identity of the ants’ symbionts, with Cand. Westeberhardia potentially influencing larval and/or pupal development while Wolbachia infection titres correlated with egg production. Although further study is needed to understand the link between Wolbachia and egg production, data from an experiment in which rearing temperature was increased showed that colonies continued producing eggs at high temperatures (with BR colonies producing fewer and JPwC colonies producing more eggs at high temperatures),
even though Wolbachia densities in the two lineages decreased at similar rates as following antibiotic treatment (Schultner, unpublished). Together, these results can best be explained by a combination of direct damage to host physiology (although the influence of rifampicin on mitochondrial function has only been quantified in mammals (Li et al., 2019)), and indirect effects following interference with the host’s microbiome, highlighting the importance of taking a holobiont approach to the study of ecology and evolution (Simon et al., 2019). In the future, testing ecologically relevant doses of antibiotics will help assess whether such effects occur in natural environments, and hopefully inform policy decisions on conservation and agriculture management.

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Supporting Information

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

Table S1. Corrected P-values for pairwise comparisons of Cand. Westeberhardia titers

Table S2. Corrected P-values for pairwise comparisons of Wolbachia titers

Table S3. Corrected P-values for pairwise comparisons of total egg numbers

Table S4. Corrected P-values for pairwise comparisons of total pupae numbers

Table S5. Corrected P-values for pairwise comparisons of total oocytes

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