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Disruption of termite gut-microbiota and its prolonged fitness consequences†

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ABSTRACT

The disruption of host-symbiont interactions through the use of antibiotics can help elucidate microbial functions that go beyond short-term nutritional value. Termite gut symbionts have been studied extensively, but little is known about their impact on the termite's reproductive output. Here we describe the effect that the antibiotic rifampin has not only on the gut microbial diversity, but also on the longevity, fecundity, and weight of two termite species - *Zootermopsis angusticollis* and *Reticulitermes flavipes*. We report three key findings: (i) the antibiotic rifampin, when fed to primary reproductives during the incipient stages of colony foundation, causes a permanent reduction in the diversity of gut bacteria, and a transitory effect on the density of the protozoan community, (ii) rifampin treatment reduces oviposition rates of queens, translating into delayed colony growth and ultimately reduced colony fitness and (iii) the initial dosages of rifampin on reproduction and colony fitness had severe long-term fitness effects on *Z. angusticollis* survivorship and colony size. Taken together, our findings demonstrate that the antibiotic-induced perturbation of the microbial community associates with prolonged reductions in longevity and fecundity. A causal relationship between these changes in the gut microbial population structures and fitness is suggested by the acquisition of opportunistic pathogens and incompetence of the termites to restore a pre-treatment, native microbiota. Our results indicate that antibiotic treatment significantly alters the termite's microbiota,

48 reproduction, colony establishment and ultimately, colony growth and development. We discuss the implications for antimicrobials as a new
50 application to the control of termite pest species.

INTRODUCTION

The long-standing associations between termites and their prokaryotic and eukaryotic microorganisms have been crucial to the evolutionary and ecological success of this social insect group. The presence of cellulolytic microorganisms in the hindguts of termites is one of the key events that allowed termites to thrive on nitrogenous deficient food resources (49, 63). Fossil records (80) and the similarity in gut flora and other microbial endosymbionts with those of their roach relatives (59) support the hypothesis that these associations existed in the termite ancestor (3, 50, 59). Termite gut symbionts reside in the lumen or are attached to the wall of the hindgut region and can represent more than 40% of the termite's weight (6). They are horizontally transmitted through coprophagy, a common behavior in termites. Indeed, the need for transfaunation of hindgut symbionts has been proposed as one of the main factors favoring group-living (44) and specifically, favoring the evolution and maintenance of termite sociality (18). Yet, little is known about the impact that termite gut symbionts have beyond their role in cellulose degradation and host nutrition.

Here we report on the impact that antibiotic treatment has on the reproductive survival and fecundity of the dampwood termite *Zootermopsis angusticollis* and the Eastern subterranean termite *Reticulitermes flavipes*. Although previous experiments demonstrated that antibiotics compromise and/or eradicate the gut microbiota (protozoa and/or bacteria) of termites

(11, 26, 53), no studies have yet characterized the short- and long-term fitness costs associated with antibiotics in these social insects, nor their impact on colony growth and development. Our findings suggest that rifampin disrupts one or more mutualistic interactions essential for normal termite reproduction and longevity.

MATERIALS AND METHODS

Collection and maintenance of termites. Two mature colonies of *Z. angusticollis* were collected from Huddart Park, San Mateo, CA. and maintained as described in Rosengaus (57). Reproductives of *R. flavipes*, an important structural pest in the USA, were collected from two stock colonies from West Roxbury, Massachusetts.

Establishment of incipient colonies. Incipient colonies were bred in the laboratory from virgin alates (winged adult dispersal forms). These fully pigmented individuals were collected, sexed and paired only if their wings could be removed easily when folded anteriorly along the humeral suture (57). These selection criteria guaranteed that only ready to disperse virgin females and males were used in our studies. To prevent mating prior to colony establishment, the de-winged reproductives were housed in same sex/same colony containers (18 x12 x 8 cm) lined with moist paper towels and some nest material. Within seven days of removal from the parental nest, reproductives pairs were placed inside Petri dishes (60 x 15 mm) lined

with filter paper (Whatman qualitative #1) and approximately 5.0 g of decayed birch wood. Subsequently, the filter paper was moistened with either distilled water (controls) or rifampin (Sandoz Inc, Princeton, NJ; 300 mg capsules; see below for details). Rifampin is bacteriostatic or bactericidal depending on dosage and acts by specifically inhibiting DNA-dependent RNA polymerase activity in Eubacterial cells (27). It is a broad-spectrum compound active against a variety of gram-positive and gram-negative organisms (8, 16, 55, 76). The dishes, stacked in covered plastic boxes (30 x 23 x 10 cm), were maintained at 22°C.

Effects of antibiotic ingestion on *Z. angusticollis* gut microbiota. To determine if rifampin affected the composition of the termite's gut microbial community, *Z. angusticollis* reproductive pairs were established in incipient colonies as described earlier. The diet of four incipient colonies was supplemented with 300 µL of a 0.5% suspension of rifampin on the day of pairing and 14 and 34 days after the initial dose. Three corresponding control colonies were similarly established but received distilled water instead. Subsequently, these colonies were left undisturbed until day 85 post-pairing when control and rifampin-fed females were surface sterilized with 2% NaClO and then their guts dissected in sterile PBS buffer and preserved in 70% molecular grade ethanol. This time frame was chosen because it was approximately at this time that the initial differences in oviposition rates became evident. Each sample was then centrifuged at 12,000X G, and the ethanol was decanted. The DNA of the

guts was extracted using the QUIAGEN DNeasy Blood & Tissue kit per the manufacturer's instructions for "Purification of Total DNA from Animal Tissues". All samples were homogenized and treated with proteinase K for 3 hr at 55°C before the column extraction procedure. Aliquots of the resulting DNA samples were then pooled and stored at 4°C until PCR, cloning and sequencing (see Supplemental material for detailed protocol). Extraction controls of sterile water were treated identically to samples and carried through all subsequent procedures. Negative water controls, as expected, showed no PCR amplification and did not yield clones containing an insert.

Effects of antibiotic ingestion by *Z. angusticollis* on abundance of eukaryotic microbes. To assess the effect of rifampin on the abundance of eukaryotic symbionts, we quantified protozoa in the guts of *Z. angusticollis* nymphs (given the unavailability of reproductives since they are produced only once a year). To control for the possible effect of termite density on gut symbionts and simulate social conditions between the two reproductives, pairs of control (N = 26) and 0.5% rifampin-fed (N = 30) nymphs were established and hindgut protozoa density was estimated on the third, eighth and 14th day post-treatment. These nymphs were first surface sterilized by submersion in 5% hypochlorite solution for 60 seconds followed by two consecutive one-minute washes in sterile water. Subsequently, their entire gut was dissected. The gut, placed inside a 1.5 mL sterile microcentrifuge tube containing 1000 µL of U solution (73), was

homogenized with a sterile pestle. To quantify the protists, 10 µL of the suspension was immediately transferred to a hemocytometer and the number of intact and active protozoa was recorded. These estimates likely represent an underestimate of the total eukaryotic microbial community as the possibility of lysis of the anaerobic protozoa existed during this procedure. Given that both the control and experimental animals were treated in an identical manner, our quantification allows for a relative measure of the impact that rifampin had on gut protozoa between the treatments rather than providing an absolute density of such microbes.

Survival of *Z. angusticollis* and *R. flavipes* reproductives and colony fitness. Incipient colonies were established as described above to examine the effect of rifampin on termite survival and fitness. These colonies ensured the monitoring of complete families throughout colony ontogeny by performing periodic censuses. The filter paper was initially moistened with either 300 µL of distilled water (controls) or 300 µL of a 0.5% rifampin solution (dissolved in sterile water) on the day of pairing and then again on the third (50 µL of water or rifampin) and seventh day post-establishment (100 µL of distilled water or rifampin). Hence, the filter paper upon which experimental termites fed was impregnated with a total of 2.2 mg of rifampin throughout the entire length of the experiment. *Z. angusticollis* colonies were followed throughout the first 730 days post-pairing while the survival and fitness parameters for *R. flavipes* colonies were monitored for 150 days post-pairing. For *Z. angusticollis*, a total of 87

(*N* = 49 control and 38 experimental replicates) and 132 (*N* = 65 control and 67 experimental replicates) nestmate pairs were initially established from each of the two stock colonies, BDTK19 and BDTK17, respectively. In addition, 29 incipient colonies were established by pairing non-nestmate male and female reproductives from these same two stock colonies (i.e. non-sibling pairs, *N* = 14 control and 15 experimental replicates). For *R. flavipes*, control (*N* = 49) and experimental nestmate pairs (*N* = 49) were treated in an identical manner as the *Z. angusticollis* incipient colonies.

Incipient colonies of *Z. angusticollis* and *R. flavipes* underwent censuses every third day for the first 50 days post-pairing. During these frequent initial censuses, when the incipient colonies were housed in Petri dishes (Fig. 1), we recorded survival of the reproductives, the time elapsed till first oviposition and first hatching. Subsequently, colonies were censused approximately on day 150 following initial pairing for both termite species. For *Z. angusticollis*, the entire colony was then transferred to a larger covered plastic container (15 x 10 x 6 cm) lined with moist paper towels and decayed birch (~12 x 6 x 6 cm wood block) to allow colony expansion. They were left undisturbed until the 465 and 730 day census except for the addition of wood and water when needed (Fig. 1).

Effect of antibiotic ingestion on termite mass. To test if rifampin supplementation during the initial stages of *Z. angusticollis* colony foundation negatively impacted the reproductive's nutritional health and thus, their survival and fitness, we recorded on day 50 and 465 post-

establishment the mass of each surviving reproductive as an indirect measure of nutritional status. For *R. flavipes*, the mass of the surviving reproductives was determined immediately after the 150 day census. No differences in the rates of wood and filter paper consumption were observed between control and antibiotic-fed reproductives for either species.

RESULTS

Effects of antibiotic ingestion on *Z. angusticollis* gut microbiota. Diets of *Z. angusticollis* reproductives were supplemented with a low dose of rifampin antibiotic suspension (0.005 grams of rifampin in 1ml of sterile deionized water) on days 0, 14, and 34 after pairing. Sampling of the gut bacterial diversity by cloning and sequencing of 16S rRNA gene amplicons at day 85 indicated there was a significant difference in the bacterial population structures between the control and rifampin-treated termites of *Z. angusticollis* ($P = 0.01$, UniFrac; Table 1). As expected, rifampin treatment reduced the 16S rRNA gene bacterial diversity (Table 1). Of the 87 clones sequenced from the control termite 16S rRNA gene library, 17 operational taxanomic units (OTUs) were represented based on a 97% identity cutoff (mean Chao1 = 23 ± 6 OTUs, mean ACE = 21). However, among the 85 clone sequences in the rifampin-treated termites, only six OTUs were represented (mean Chao1 = 6 ± 1 OTUs, mean ACE = 6), amounting to a 64% reduction in bacterial diversity. The rarefaction

analyses of the two libraries also showed that despite similar sequencing efforts in each treatment, the control termite library was less exhaustively sampled than the antibiotic-treated termites (Fig. S1 Supplemetal Material), indicating a greater diversity in the untreated termites. The species richness and diversity indices confirmed that there was an unequal distribution of the bacterial OTUs in both treatments (reciprocal Simpson's evenness = 6, control; 3, rifampin).

Of the six OTUs in the rifampin-treated guts, three were shared with the control group and two of these were maintained at the same relative proportion among the treatments (Table 1). These three bacteria include a *Treponema* sp.; the endomicrobia termite symbiont, Termite Group 1; and a *Desulfovibrio* sp. These bacteria are known inhabitants of termite guts (35, 40, 48). The other three OTUs in the treated group were unique and included *Serratia*, an uncultred *Enterococcus*, and an uncultured Epsilonproteobacterium that was the dominant bacteria in the rifampin-treated guts (Table 1). Given that resistance to rifampicin is easily attained by single random mutations of the bacterial RNA polymerase (16), it was necessary to establish whether the recorded alterations in gut microbial communities were influenced by a build-up of antibiotic-resistant species. After cross referencing our microbial diversity data with the expected species that contain these resistance mutations (Table S1 Supplemental Material), we found that the frequency of strains with rifampin resistance was low and equivalent between pre- and post-

treatments (P = 0.6, Fisher's exact test). Thus, we conclude that the antibiotic treatment did not select for rifampin resistance.

Ingestion of rifampin also had a significant short-term negative impact on the number of gut protozoa per gram of termite. In a separate experiment, nymphs fed rifampin for three days had a significantly lower median number of protozoa (± interquartile range) in their gut relative to the controls (median rifampin = $9.7 \times 10^6 \pm 3.4 \times 10^6$ vs. median control = $2 \times 10^7 \pm 9.4 \times 10^6$, P = 0.01; medians are reported given that the frequency with which gut protozoa were recorded was not normally distributed). However, in subsequent dissections on days eight and 14 post-feeding, the number of protozoa per gram of termite did not differ significantly between the two treatments (median control = $1.2 \times 10^7 + 9.3 \times 10^6$ vs. median rifampin = $8.7 \times 10^6 + 7.6 \times 10^6$, P = 0.5 on day eight; median control = $9.8 \times 10^6 \pm 1 \times 10^7$ vs. median rifampin = $7 \times 10^6 \pm 9.3 \times 10^6$; P = 0.5 on day 14). Thus, although rifampin temporarily affected the number of protozoa in termite guts, it did not destroy them completely. Collectively, our results indicate that rifampin has only a moderate and transitory effect on the density of the culturable, protozoa gut community, and a prolonged effect on the diversity of bacteria in termite guts.

Survival of *Z. angusticollis* reproductives and colony fitness. The effects of the antibiotic on survival was evaluated throughout the first two years of colony life. A Cox proportional regression model with the variables "colony of origin" (either BDTK17 or BDTK19), "gender", "sibship"

(nestmate or non-nestmate pairs) and "treatment" (controls or antibiotic-
264 fed) revealed that colony of origin (WS = 7.3, d.f. = 1, $P = 0.007$) and
treatment (WS = 25.1, d.f. = 1, $P < 0.0001$) had significant effects. First,
266 reproductives from colony BDTK17 had 1.3 times the hazard ratio of death
in comparison to reproductives from colony BDTK19, after controlling for
268 the effect of treatment (Table 2). Second, rifampin-fed reproductives after
two years post-pairing were 1.7 times as likely to suffer premature
270 mortality compared to untreated individuals, even after controlling for the
effect of colony of origin (Fig. 2; Table 2).

272 The time course of survival for the reproductives did not differ
significantly between the rifampin and control treatments (Breslow X^2 =
274 2.8, d.f. = 1, $P = 0.09$ for BDTK17 and Breslow $X^2 = 0.1$, d.f. = 1, $P = 0.7$
for BDTK19; Fig. 2), until after day 150 (Fig. 2). By 465 days, the survival
276 distributions and percent survival were significantly different between the
control and antibiotic treatments for each of the stock colonies (Fig. 3).
278 These differences were pronounced by 730 days. At this time, 50% of the
original control reproductives had died. In contrast, the rifampin-fed
280 reproductives reached 50% mortality by the 465 day census (Fig. 2; Table
2). Thus, on average, the control termites lived approximately 265
282 additional days before reaching the 50% mortality mark (LT_{50} estimate,
Table 2). These findings indicate that the effects of rifampin treatment
284 significantly affect survivorship of reproductives from both stock colonies,
with mortality differences being most prominent between 465 and 730 days
286 (Fig. 2; Table 2).

Rifampin-fed reproductives originating from both colonies had consistently fewer offspring than their corresponding untreated controls. Because none of the reproductive output metrics differed significantly between BDTK17 and BDTK19 (Mann-Whitney U tests), statistical analyses were carried out by combining all colonies within a treatment. Given the longitudinal nature of this study, we present a detailed description of the effects of antibiotic treatment on colony fitness at each of the census dates.

150 days post-pairing census. The addition of low dosages of rifampin during the initial stages of colony foundation in *Z. angusticollis* resulted in a significant reduction in fecundity. Fig. 3 shows a significant disparity between the frequency distribution of offspring number between surviving control and rifampin-treated colonies. The percentage of surviving control colonies with eggs, larvae and soldiers on day 150 post-establishment was higher than that of rifampin-treated colonies; furthermore, a higher percentage of control colonies produced the highest number of eggs, larvae and soldiers (Fig. 3). One hundred fifty days post-pairing, surviving control colonies also had a significantly higher median number of eggs, larvae and soldiers than their rifampin counterparts (Fig. 4). Furthermore, the effect of the antibiotic on *Z. angusticollis* reproductive output appeared to be immediate, since it significantly delayed first oviposition by approximately 47 days (MW = 870, z = -5.5, $P < 0.0001$;

310 Fig. 5a) and had a tendency to delay first hatching by roughly 33 days (MW = 1220, z = -1.8, P = 0.06; Fig. 5b) relative to controls.

312 *465 days post-pairing census.* The antibiotic continued to have a long-term negative effect on colony reproduction. All fitness parameters of 314 surviving rifampin-fed reproductives were significantly reduced relative to controls (Fig. 5).

316 *730 days post-pairing census.* Two years post-pairing, the negative effect of rifampin on colony fitness persisted despite the antibiotic 318 treatment being provided only during the initial stages of colony foundation (Fig. 4). After controlling for the effects of mass (see below), sibship and 320 colony of origin, treatment significantly influenced colony fitness (t = -2.9, P = 0.004 for eggs, t = -3.8, P < 0.0001 for larvae and t = -4.1, P < 0.0001 322 for soldiers; by multivariate linear regression (SPSS, 57). By the last census, 69.8% of the 128 originally established control colonies had 324 oviposited at least one egg while only 38.6% of the 120 original rifampin-treated colonies had done so (Pearson's X^2 = 24.0, d.f. = 1, P < 0.0001). 326 Moreover, 63.5% of the original control colonies produced at least one larva whereas only 28.6% of the original rifampin-treated colonies did 328 (Pearson's X^2 = 30.0, d.f. = 1, P < 0.0001).

330 **Survival of *Reticulitermes. flavipes* primary reproductives and colony fitness.** *R. flavipes* reproductives treated with antibiotic had a 332 comparable survival rate to the controls for the first 5 months of colony life. A Cox proportional regression indicated that neither colony of origin,

sibship, gender nor treatment were significant and independent predictors of termite survival (Wald Statistic (WS) = 0.2, 1.5, 0.002 and 0.07, d.f. = 1, $P > 0.2$, respectively). In regards to fecundity, after 5 months of colony formation 69.4% of the original control established colonies oviposited at least one egg relative to 53.3% of the original rifampin-treated colonies (Pearson's $X^2 = 2.5$, d.f.= 1, $P > 0.05$). Approximately 45% and 44% of the original control and rifampin-treated colonies hatched at least one larva, respectively (Pearson's $X^2 = 0.002$, d.f.= 1, $P > 0.05$). After 150 days post-establishment, no soldiers had differentiated. Although these proportions were not statistically significant, several additional reproductive parameters of the rifampin-treated reproductives were negatively impacted relative to controls. Rifampin-fed *R. flavipes* reproductives had fewer maximum number of eggs (MW = 242.5, $z = -2.7$, $P = 0.007$), fewer maximum number of larvae (MW = 159.0, $z = -2.8$, $P = 0.005$) as well as fewer number of larvae on day 150 post-pairing (Mann Whitney U test (MW) = 112.0, $z = -3.5$, $P < 0.0001$; Fig. 6). Although some additional reproductive parameters were reduced for rifampin-fed reproductives, they were not statistically different. Larger sample sizes and longer surveys past the first 150 days post-establishment are needed to elucidate if rifampin has similar long-term effects on *R. flavipes* reproduction as it had in *Z. angusticollis*.

Taken together, these results indicate that small amounts of rifampin provided during the incipient stages of colony foundation alter the reproductive output of both termite species for the long term.

Termite mass. The mass of each surviving *Z. angusticollis* reproductive was recorded on day 50 and 465 post-establishment (Table 2). Our results show that by day 50, control reproductives were no more than 0.005 grams heavier than their rifampin-fed counterparts. These differences, although small, were significant (Table 2). On day 465 post-pairing, the differences in mass of the surviving reproductives were reversed and now rifampin-fed reproductives were heavier than their respective controls (Table 2). The reversal in the weight differences from day 50 to day 465 was apparently due to accelerated weight loss in the controls for both BDTK17 (0.060 g vs. 0.052 g, $t = 6.0$, d.f. = 180, $P < 0.001$) and BDTK19 (0.063 g vs. 0.057 g, $t = 4.1$, d.f. = 164, $P < 0.0001$), rather than significant weight gain in the antibiotically-treated termites of BDTK17 (0.055 g vs. 0.053 g, $t = 0.96$, d.f. = 131, $P = 0.3$) and BDTK19 (0.058 g vs. 0.060 g, $t = -1.6$, d.f. = 113, $P = 0.1$). The significant weight loss of controls could be due to a higher investment of their energetic reserves in reproduction than that of the antibiotic treated reproductives, which consistently had lower reproductive output.

On day 150 post-pairing, the mass of control and rifampin-treated *R. flavipes* male and female reproductives were not significantly different (males: average ± S.D.= 0.0042 ± 0.0007 vs. 0.0038 ± 0.0006 respectively, $t = 1.9$, d.f. = 39, $P > 0.05$; females: 0.0046 ± 0.0007 vs. 0.0042 ± 0.0007 respectively, $t = 1.7$, d.f. = 39, $P > 0.05$) and therefore, we conclude that the addition of rifampin to the diet of *R. flavipes* reproductives did not cause malnutrition, starvation or higher mortality relative to controls.

DISCUSSION

This investigation demonstrates that the addition of the antibiotic rifampin to the diet of *Z. angusticollis* and *R. flavipes* during colony establishment reduces bacterial diversity in the reproductive's guts, as well as colony fitness. Relative to controls, rifampin-treated *Z. angusticollis* reproductives had reduced survival and lower reproductive success. They exhibited a delayed first oviposition and significantly lower production of eggs, larvae and soldiers throughout the 730 days of colony life (Figs. 4, 5). Similarly, rifampin treatment in *R. flavipes* showed a reduction in the total number of eggs and larvae during the first 150 days of colony foundation (Fig. 6). How does rifampin treatment mediate the fitness costs on reproduction in these termite species? We propose two possible explanations.

First, the antibiotic could influence reproductive success of reproductives indirectly by compromising the nutritional health of the royal pair, causing reduced weight gain and reproductive output. Rifampin could have caused defaunation of the eukaryotic hindgut microbes resulting in malnutrition and/or starvation. The elimination of wood-digesting protozoan symbionts through the use of antibiotics has previously been demonstrated (11, 26, 53). However, in this study, rifampin-treated termites had numerous protozoa (median number = $7 \times 10^6 \pm 9.3 \times 10^6$ protozoa per gram of termite) 14 days post-treatment, and it is the gut protozoa that are

primarily responsible for cellulase activity in the digestive tract of primitive
406 “lower” termites (13, 37). Rifampin does not have a prolonged negative
effect on the cellulolytic gut protozoa of *Z. angusticollis*, most likely
408 because this antibiotic specifically inhibits the bacterial RNA polymerase
(32). Moreover, the most abundant bacteria in the termite’s hindgut, the
410 spirochetes, play an important role in the digestion process and are highly
resistant to rifampin (12). Hence, the facts that (i) rifampin did not
412 eradicate protozoan symbionts of *Z. angusticollis*, (ii) body mass of *Z.*
angusticollis reproductives was transiently affected (Table 2) and was
414 unaffected in *R. flavipes* and (iii) the experimental replicates survived up to
the 465 and 730 day (for *Zootermopsis*) and 50 day (for *Reticulitermes*)
416 census while continuing to show a reproductive output biased against
rifampin treatment, do not support antibiotic toxicity, malnutrition and/or
418 starvation as factors reducing fitness. Furthermore, endogenous production
of cellulases has been reported in this insect order and hence, termite
420 nutrition may not be completely dependent on their protozoa communities
(7, 25, 71, 72, 77).

422 Similar studies using antibiotics in the phylogenetically-related
roach *Periplaneta americana* resulted in poor growth and reduced
424 reproductive output (54). These effects were attributed to the elimination of
Blattabacterium which mobilizes nitrogen from urate waste deposits within
426 the fat tissue. They also provide vitamins, proteins and essential amino
acids to the roach (3, 4, 54, 59). Although *Z. angusticollis* lacks an
428 association with *Blattabacterium* (59), other bacteria, including the

rifampin-eliminated Bacteroidetes and *Treponema*, are similarly involved in nitrogen fixation (11, 13, 36, 46) and/or the production of NH_3 from uric acid (52, 59, 66). The absence of these taxonomic groups may have irreversibly restricted nitrogen availability in female reproductives. Given that dietary nitrogen supplementation is known to significantly increase ovariole number and fecundity in *Z. angusticollis* neotenics and other insects (5, 10), the loss of the Bacteroidetes and Mollicutes may have compromised nitrogen reserves and/or the essential amino acids required for oogenesis. However, some Epsilon- and Gammaproteobacteria, two classes that were overly-represented in the treated guts, may perform ammonification, denitrification and nitrogen fixation (38, 46). Thus, further work is required to associate the fitness cost in treated termites with a shift in the ability to use nitrogen.

A second possible explanation for the long-term fitness costs associated with antibiotic treatment is that rifampin disrupted one or more mutualistic bacterial partnerships within the termite hosts. Specifically, a partnership(s) that goes beyond the breakdown of cellulose. Given the long co-evolutionary history between the gut symbionts and termites, it is likely that these social insects accrue additional benefits from their micobiota that are unrelated to cellulolytic activity. Microbes can play other important roles within their termite hosts including detoxification (17), mediation of disease resistance and immune function (15, 23, 31, 51, 58, 60, Schultheis et al., in preparation), production of volatile compounds that are co-opted to function as aggregation or kin recognition pheromones and defensive

secretions (2, 24, 28, 39, 45, 47), as well as performing atmospheric
454 nitrogen fixation (5, 11, 36). Results from this work suggest that the microbial communities of *Z. angusticollis* and *R. flavipes* may also
456 contribute to the fecundity of reproductives and ultimately, to the successful establishment of colonies. One such candidate for affecting
458 reproduction is *Wolbachia pipientis*, a widespread intracellular bacterium known to infect *Z. angusticollis* (9). However, based on PCR surveys of the
460 *Wolbachia wsp* gene from antibiotic-treated and untreated reproductives, *Wolbachia* was not involved in influencing colony fitness since all
462 reproductives, nymphs and eggs from both the experimental and control colonies harbored *Wolbachia* regardless of treatment and colony of origin.

464 The bacteria identified in our control animals have previously been associated with termite guts, either as normal symbionts (34, 35, 40, 48, 70)
466 or as opportunistic pathogens (75; Table 1). The long-term fitness costs likely resulted from perturbations in the termite gut symbionts in treated
468 termites. Rifampin is a bactericidal antibiotic that preferentially targets gram-positive bacteria (78, 81). The consequence of employing this
470 antibiotic is that it shifted the gut microbial community largely towards gram-negative microorganisms including known termite symbionts;
472 Termite group 1, *Desulfovibrio* sp., and *Trepanema* sp. (Table 1).

The most striking change was the abundance of an
474 Epsilonproteobacterium that was not represented in the control termite library. This bacterium's 16S sequence is 98% similar to a rare symbiont of
476 the termite luminal lining (35) and appears to have increased its

proportional representation in the gut microbiota. At least two potential reasons for this shift in the dominant bacteria exist. First, the decline in gram-positive bacteria may have allowed rare members of the community such as the gram-negative Epsilonproteobacterium to exploit the new, unoccupied niche space of the gut. Members of the rare biosphere potentially offer an unlimited source of microbial diversity that flourishes upon ecological perturbations (64). By altering the normal microbiota of the gut with antibiotics, rare but relatively fast growing microaerophilic species (i.e. some proteobacteria and *Serratia*) not susceptible to the antibiotic may now exploit the host niche as well as the levels of available oxygen, ultimately overgrowing and becoming dominant in the gut (14, 27, 41, 58, 79 and references therein). Second, the appearance of rare or non-native bacterial members in the rifampin-treated guts may be impacted by interactions with other bacteria. For example, the *Serratia marcescens* 16S rRNA gene sequence identified in our study is 99.9% identical to an opportunistic pathogen of termites that has been hypothesized to induce replication of normal termite gut bacteria by suppressing the host immunity, changing available oxygen, and producing bacterial growth promoting enzymes like carboxymethylcellulase (1, 19, 68, 75). A *Serratia*-induced proliferation of the symbiotic community could cause septicemia that can result in early termite mortality (75). *S. marcescens* is present in the rifampin gut library, but not in the control termite gut library. Thus, its appearance in the treated termites may have directly or indirectly led to the proliferation of the rare Epsilonproteobacterium symbiont of the

luminal lining (48). Yet, it is important to keep in mind that not all
502 associations with *Serratia* are necessarily pathogenic. *Serratia grimesii*, for example, has been implicated as a source of folate compounds important to
504 the maintenance of a functional hindgut microbiota of *Z. angusticollis* (29). The shift in gut bacterial population structure is strikingly prolonged since
506 termites were not fed antibiotics for ~50 days prior to dissections. The inability to return to a pre-treatment microbial homeostasis (70), coupled
508 with the acquisition of putative, opportunistic pathogens and the slow growth rates of many of these termite gut micoorganisms (27, 30, 41, 42,
510 62), may help explain the prolonged effects that the antibiotic had on longevity and fecundity.

512 This study provides the first report of the long-term fitness consequences of disrupting the normal gut microbiota of termites. The long
514 coevolutionary history of termites and their associated microbiota, coupled with the environmentally stable conditions inside their nests, lends itself to
516 study the nature and dynamics of symbiotic interactions (33). The mutualistic gut partnerships of social insects may impact not only the
518 fitness of individuals but also have significant repercussions at the colony level. Symbionts, whether parasitic, commensal or mutualistic, pose
520 important selective pressures on their hosts. These host-microbial interactions likely influence the evolution of multiple host life history traits
522 including longevity, behavior, reproductive biology, immunity and the evolution and maintenance of sociality (20, 33, 56, 61, 69,74 and
524 references therein). Furthermore, the use of rifampin and/or other

antibiotics has potential applicability for biological control of social insect pests. By disrupting the mutualistic interaction between termite hosts and their symbionts, better management practices of these social insect pests may be achieved without the environmental and ecological drawbacks typically associated with the use of other toxic chemicals.

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FIGURE LEGENDS

FIG. 1. Diagramatic representation of the protocol. Incipient colonies of *Z. angusticollis* were established by paring dealates inside Petri dishes lined with filter paper and wood on day zero. Arrows indicate the days at which rifampin was added to the experimental colonies. Control colonies received distilled water only on these same days. In subsequent census, colonies were sprayed with distilled water as needed. Pd indicates that incipient colonies were housed in Petri dishes. Q and K denote queen and king, respectively. See text for details.

FIG. 2. Survival distributions of control (solid line) and rifampin-treated (dashed line) male and female *Z. angusticollis* reproductives originating from colony BDTK17 (a) and BDTK19 (b) during the first two years of colony life. Filled and open circles represent the percent survival of control and rifampin-treated individuals at each of the major census dates (indicated by the arrows), respectively. These percentages differed significantly on days 465 and 730 post-establishment (Pearson's X^2, $P < 0.004$). * and NS above the arrows represent significant and insignificant differences in the median survival time at each of the census dates, respectively (MW test; see text). Additional survival parameters are shown in Table 2.

FIG.3. Percent number of established control (□) and rifampin-treated (▨) colonies in relation to the number of eggs (a), larvae (b) and soldiers (c) produced 150 days post-establishment. Kolmogorov-Smirnov tests and their associated Z score were used to test for differences in the location and shape of the distributions and whether the two treatments had equal distributions. Note that a higher percentage of control colonies produced the highest number of eggs, larvae and soldiers.

FIG. 4. Number of eggs (a), larvae (b) and soldiers (c) produced by control (□) and rifampin-treated (▨) *Z. angusticollis* reproductives at each of the major census days. Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper edge of the box. The numbers below each of the boxplots represents the number of colonies. Reproductive parameters between treatments within each census day were compared by MW test.

FIG. 5. Number of days elapsed to first oviposition (a) and first hatching (b) for *Z. angusticollis* colonies headed by untreated (□) and rifampin-treated reproductives (▨). Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper edge of the box.

Reproductive parameters between treatments were compared using MW test.

FIG. 6. Maximum number of eggs, maximum number of larvae and number of larvae recorded on day 150 post-colony establishment produced by control (□) and rifampin-treated (▨) *R. flavipes* reproductives. Each boxplot shows the median value and interquartile range. The numbers below each of the boxplots represents the number of colonies. Numbers below each of the boxplots represents the number of colonies. Reproductive parameters between treatments were compared using a non-parametric Mann-Whitney U test.

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TABLE 1. Number of 16S rRNA OTUs in *Zootermposis*control and treated guts. Refer to Fig S1 in supplemental material to see corresponding rarefaction curve.

Class	Bacteria genus	Control Gut	Refampacin Gut	References
Endomicrobia	Termight Group 1	19	0	Hongoh et al., (2003); Nakajima et al., (2005); Kudo (2009)
Bacteroidetes	Bacteroides	22	7	Nakajima et al., (2005); Kudo (2009)
Betaproteobacteria	Propionibacter	1	4	
Deferribacteres	Lincoln Park 3'	1	0	Kudo (2009)
Acintobacteria	Treponema	19	0	Kudo (2009)
Verrucomicrobia	Verrucomicrobia	6	0	Hongoh et al., (2005)
Clostridia	Clostridiales	7	0	Hongoh et al., (2005); Nakajima et al., (2005); Kudo (2009)
Clostridia	Uncult Rumen bacterium (<95%)	2	0	Hongoh et al., (2005); Nakajima et al., (2005); Kudo (2009)
Betaproteobacteria	Uncult Sludge (<95%)	2	0	
Verrucomicrobia	Opitutaceae	1	0	Hongoh et al., (2005); Nakajima et al., (2005); Kudo (2009)
Epsilonproteobacteria	Sulfurospirillum	1	0	Hongoh et al., (2003)
Betaproteobacteria	Uncult Beta-proteo	3	0	
Gammaproteobacteria	Uncult Gamma-proteo (<95%)	1	0	
Deltaproteobacteria	Uncult Desulfovibrionales (<95%)	3	0	Hongoh et al., (2005); Nakajima et al., (2005); Kudo (2009)
Gammaproteobacteria	Pseudomonas	0	13	Veivers et al., (1982); Devi and Kothamasi (2009)
Bacilli	Enterococcus	0	3	Thongaram et al., (2005)
Gammaproteobacteria	Providencia	0	1	Veivers et al., (1982)
Betaproteobacteria	Oxalobacteraceae	0	1	
Alphaproteobacteria	Methylobacterium	0	11	
Alphaproteobacteria	Afipia	0	20	
Acintobacteria	Arthrobacter	0	13	Hongoh et al., (2003); Kudo (2009)
Flavobacteriales	Cytophaga	0	4	
Betaproteobacteria	Ralstonia	0	4	

Bacteroidetes	Uncult bacterium (<95%)	3	2	Nakajima et al., (2005); Kudo (2009)
Bacilli	Streptococcus	0	1	Thongaram et al., (2005)
Alphaproteobacteria	Sphingomonas	0	2	
Total number of clones		**91**	**86**	

TABLE 2. Survival parameters and mass estimates of control and rifampin-treated *Z. angusticollis* primary reproductives originating from two parental colonies across the first two years post-establishment.

	BDTK17			**BDTK19**		
	Control	**Rifampin**	***P*†**	**Control**	**Rifampin**	***P*†**
LT_{50} on day 730	730 ± 31	465 ± 39	*P* < 0.0001 BS = 1 8.6	730 ± 29	465 ± 46	*P* = 0.008 BS = 7.0
% survival on day 730	26.8	4.1		27.3	11.1	
Hazard ratio of death	Reference	1.7X	*P* < 0.0001 WS = 19.0 d.f. = 1	Reference	1.4 X	*P* = 0.07 WS = 5.5 d.f. = 1
Mass (in grams) on day 50 post-pairing	0.0604 ± 0.01 (*N* = 100)	0.0549 ± 0.009 (*N* = 92)	*t* = 3.8 d.f. = 190 *P*§ < 0.0001	0.0626 ± 0.008 (*N* = 95)	0.0577 ± 0.009 (*N* = 74)	*t* = 3.6 d.f. = 167 *P*§ < 0.0001
Mass (in grams) on day 465 post-pairing	0.0518 ± 0.008 (*N* = 79)	0.0530 ± 0.01 (*N* = 40)	*t* = -0.7 d.f. = 117 *P*§ = 0.4	0.0567 ± 0.009 (*N* = 71)	0.060 ± 0.009 (*N* = 41)	*t* = -2.0 d.f. = 110 *P*§ = 0.04

† indicate differences in the survival distributions between control and rifampin treated reproductives. These distributions are depicted in Figs. 2a,b. BS=Breslow statistic (Survival analysis), WS=Wald Statistic (Cox proportional regression). § denotes differences in the average

mass between control and rifampin treated reproductives within each parental stock colony (*t* test,

888 SPSS). The numbers in parentheses indicate the number of surviving individuals on which mass averages were based upon.

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892 Fig. 1.

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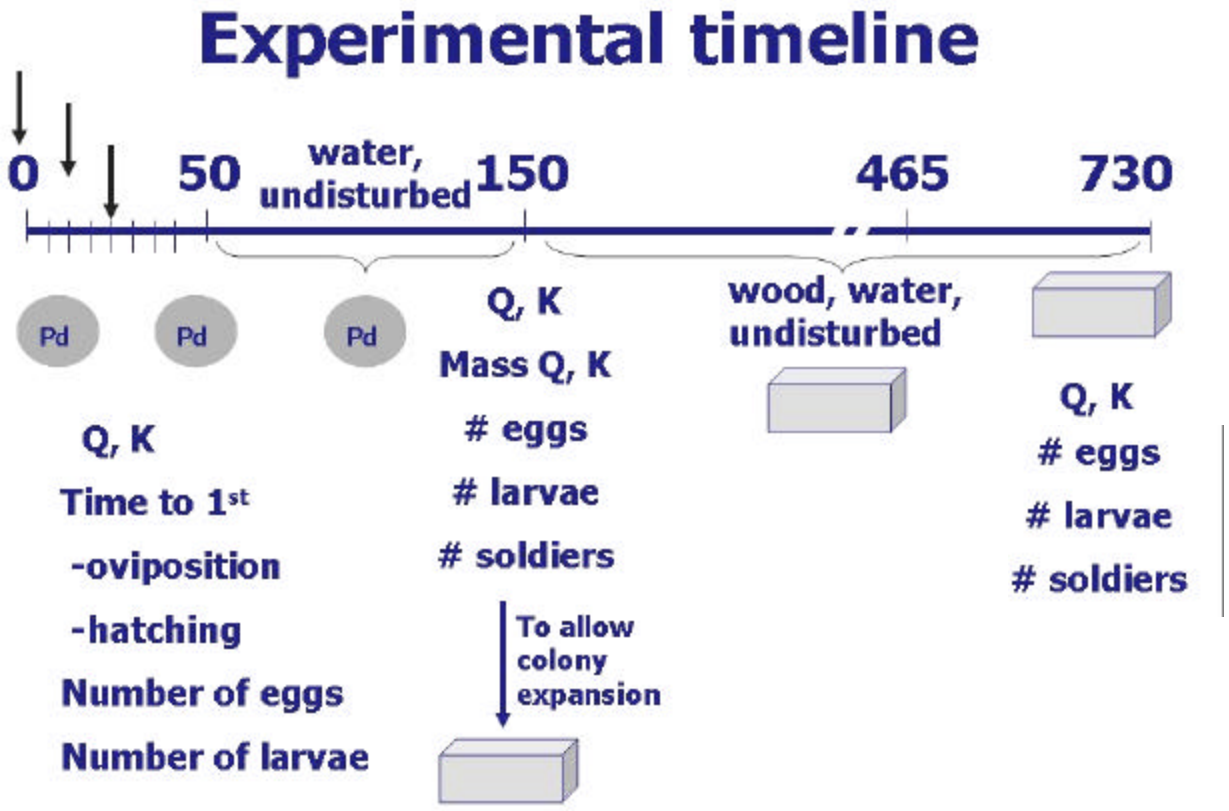

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906 Fig. 2.

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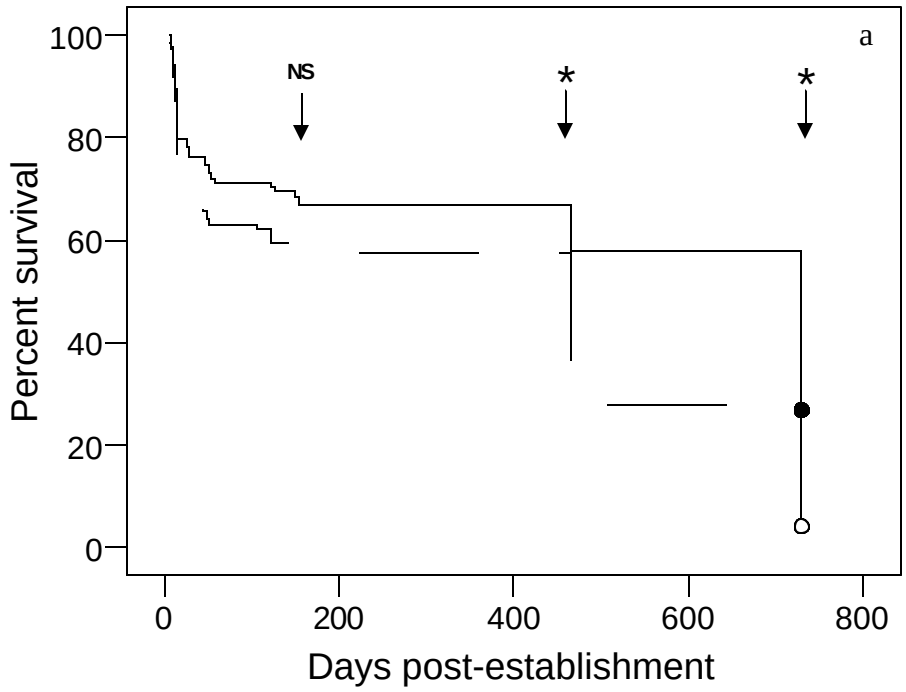

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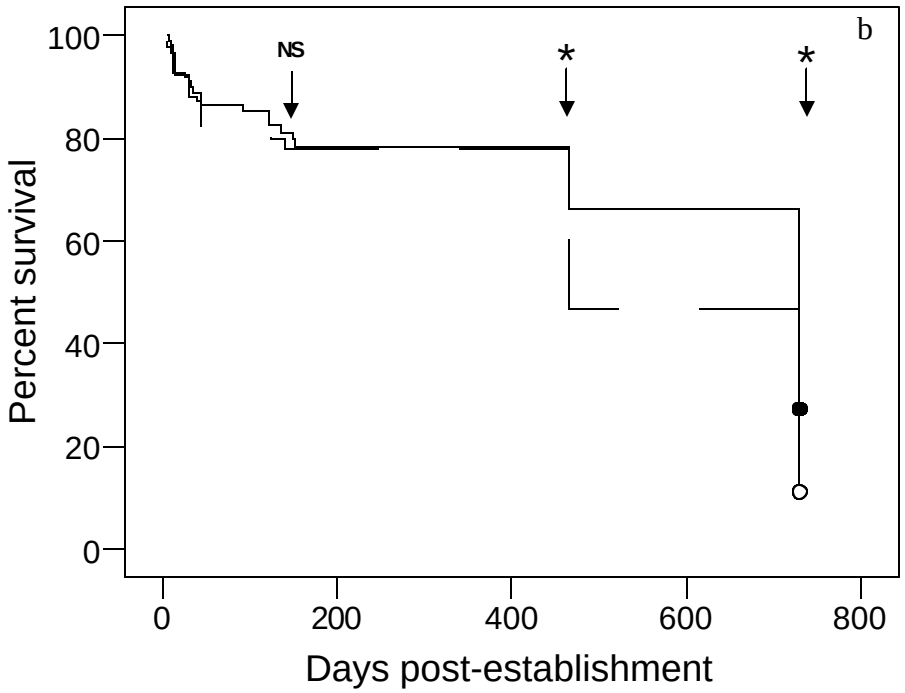

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Fig. 3.

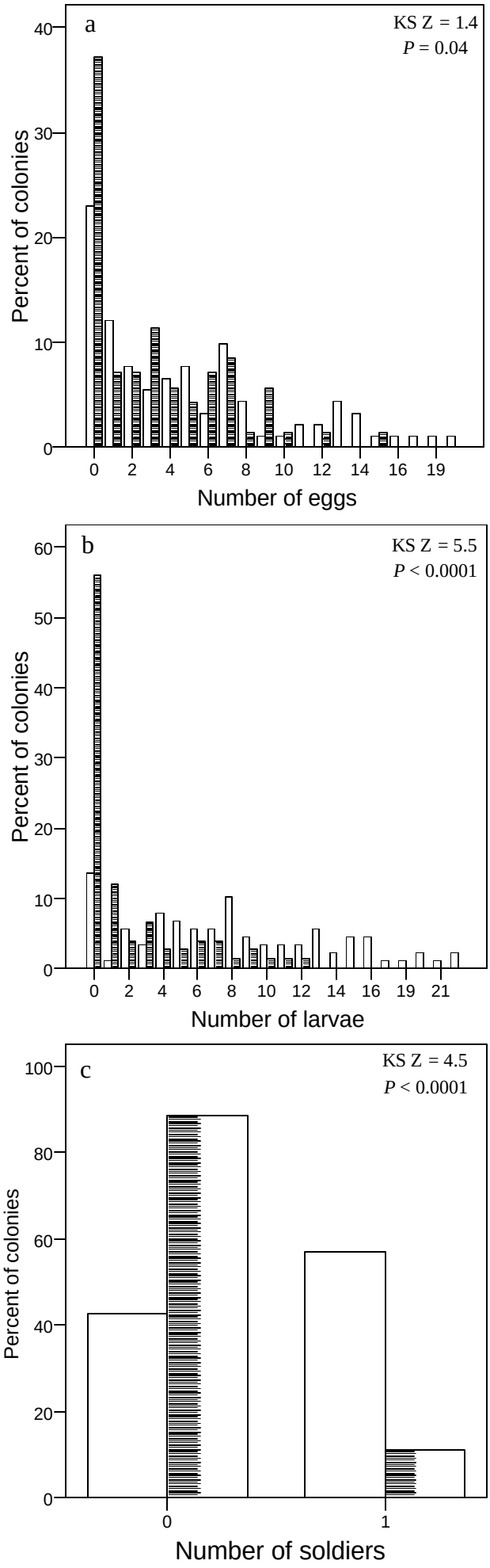

a
KS Z = 1.4
P = 0.04
Percent of colonies
Number of eggs
b
KS Z = 5.5
P < 0.0001
Percent of colonies
Number of larvae
c
KS Z = 4.5
P < 0.0001
Percent of colonies
Number of soldiers

Fig. 4

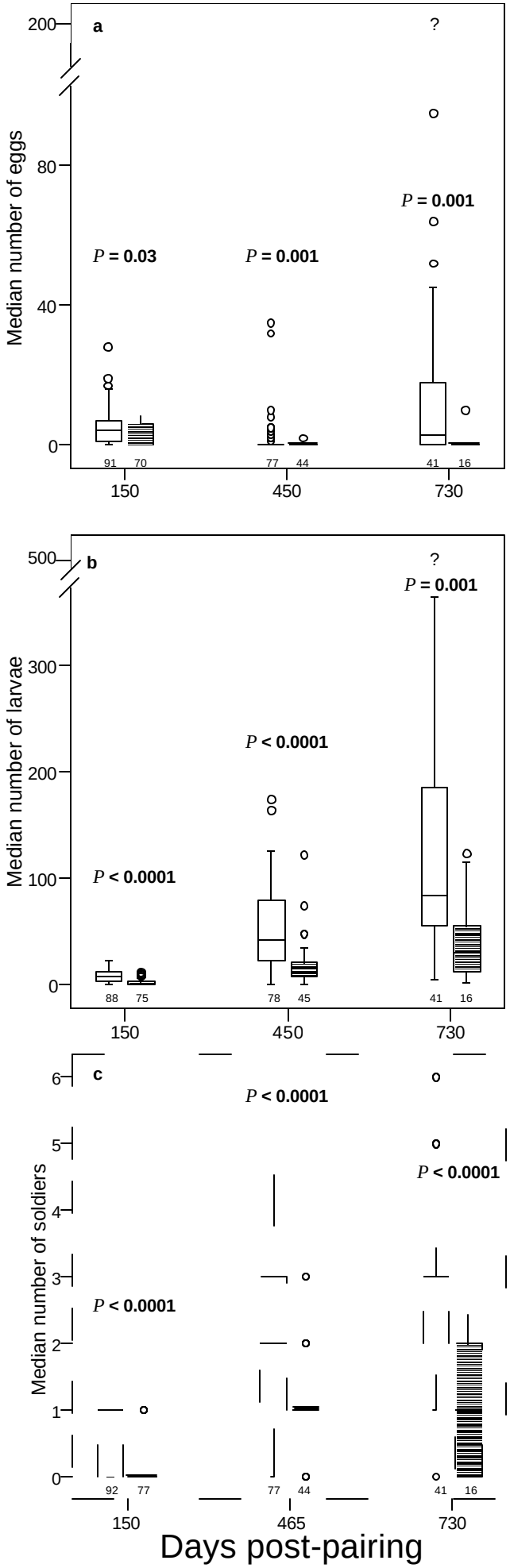

a
Median number of eggs
200
80
40
0
?
P = 0.001
P = 0.03
P = 0.001
91 70
77 44
41 16
150
450
730
b
Median number of larvae
500
300
200
100
0
?
P = 0.001
P < 0.0001
P < 0.0001
88 75
78 45
41 16
150
450
730
c
Median number of soldiers
6
5
4
3
2
1
0
P < 0.0001
P < 0.0001
P < 0.0001
92 77
77 44
41 16
150
465
730
Days post-pairing

Fig. 5.

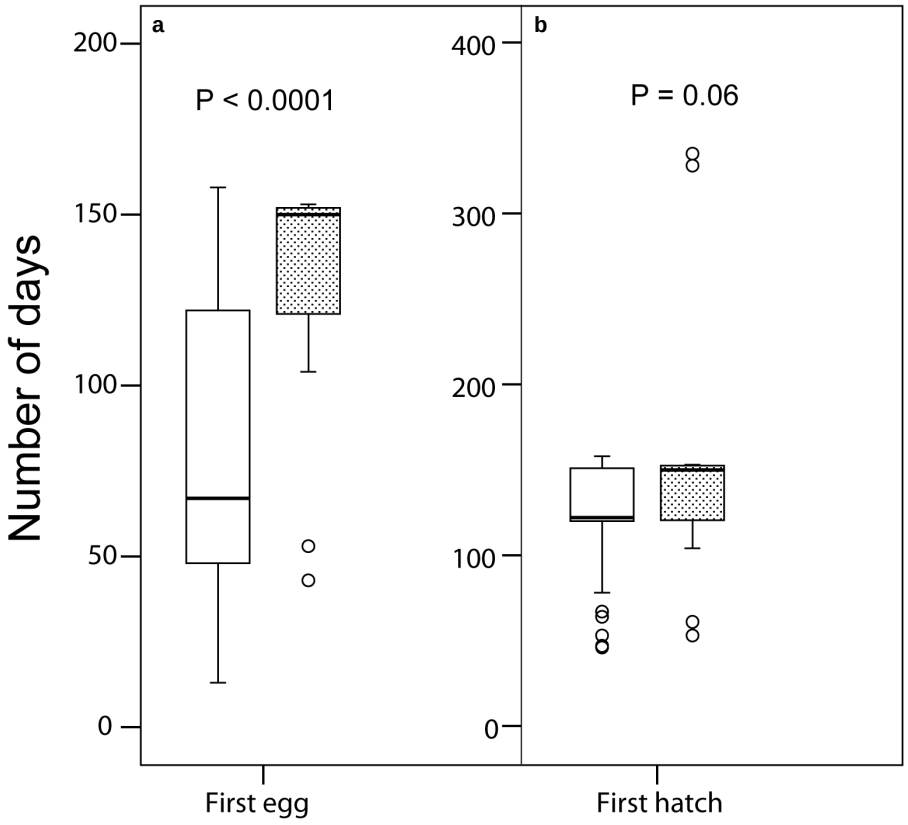
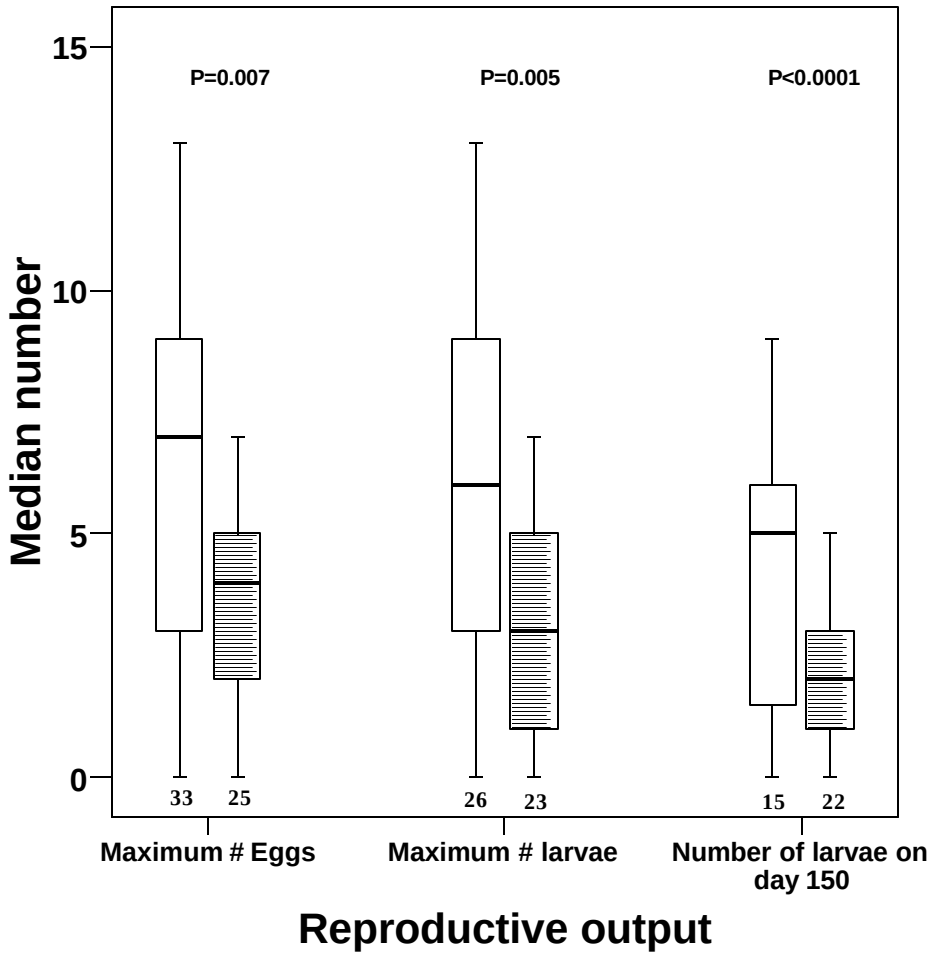

a
b
P < 0.0001
P = 0.06
Number of days
200
150
100
50
0
400
300
200
100
0
First egg
First hatch

Figure 6


15
10
5
0
Median number
P=0.007
P=0.005
P<0.0001
33
25
26
23
15
22
Maximum # Eggs
Maximum # larvae
Number of larvae on day 150
Reproductive output

Supplemental Material

Disruption of termite gut-microbiota and its prolonged fitness consequences

Rebeca B. Rosengaus[1*], Courtney N. Zecher[2], Kelley F. Schultheis[1], Robert M. Brucker[3], and Seth R. Bordenstein[2, 3]

METHODS

PCR, Cloning, and Sequencing. To prepare samples for cloning, PCR amplification of the bacterial 16S rRNA gene was performed using 5 µl of the DNA samples as template. This template was combined into a 50 µl reaction using 15.8 µl of H_2O,10 µl of 5x Buffer (Promega, Madison WI), 5 µl of 2.5 mM DNTP's (Invitrogen, Carlsbad, CA), 6 µl of 25mM MgCl2 (Promega), 0.2 µl GoTaq® Flexi (Promega), 4 µl of 5uM forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-'3, Sigma-Aldrich), and 4 µl of 5uM reverse primer 1492R (5'-ACGGCTACCTTGTTACGACTT-'3, Sigma-Aldrich; Suzuki and Giovannoni, 1996). The thermalcycling program was set up as follows: 94°C (5 min), then 35°C repeats of 94°C (1 min) 55°C (45 sec) 72°C (2 min), followed by 72°C (15 min). 5 µl of the resulting amplicon products were run on a 1% agarose gel (Fisher Scientific), stained with the nucleic acid stain GelRed (Biotium, Hayward, CA), and imaged for the proper band size under UV illumination. The remaining amplicon product is run on a 1% low melting point agaraose gel (USB Scientific, Cleveland, OH) and similarly stained. A Dark Reader® transilluminator (Clare Chemical Research) was used to image the gel and excise bands for purification with the Wizard® SV Gel and PCR

Product Purification Kit (Promega). The resulting gel-purified product was then used for cloning and sequencing. The amplicon product was ligated using the Invitrogen Topo TA cloning Kit for Sequencing (4-TOPO V2, vector) and the resulting vector was used to transform One Shot® Top 10 Chemically Competent transformation cells (Invitrogen), per the manufacture's recommended procedure. Plasmid inserts were undirectionally sequenced at Genewiz® (South Plainfield, NJ) using rolling circle amplification off the TOPO vector with inserted amplicon. For the rifampin and control groups, 94 clones were sequenced.

Clone Library and Sequence Processing. Sequences were trimmed and sorted in Geneious® v4.8. Sequences were then aligned using the GreenGenes online workbench (http://greengenes.lbl.gov) using a 97% identity cutoff, DeSantis *et al*., 2006). The resulting alignment was used to remove any chimeric sequences with the Bellerophon Chimera Checker (Ribosomal Database Project v10). Genera and class were assigned to each sequence using the GreenGenes Comparison algorithom that searches across three nucleic databases (NCBI, RDB, and Hugenholtz). To compare the two libraries (rifampin and untreated control) in UniFrac, a representative sequence for each genera in each sample type was aligned using a MUSCLE alignment (500 iterations) with all gaps removed. A PhyML tree was then generated with a Jukes-Cantor substitution model and branch lengths calculated (Guindon and Gascuel, 2003). In total, 171 high-quality, non-chimeric, bacterial ribosomal sequences were obtained from the two libraries. The control group had a total of 86 assignable sequences and the rifampin group had a total of 85 sequences, with an average sequence length of 802bp for each library.

Statistics

(i) Effects of antibiotic ingestion on termite gut microbiota. Rarefaction analysis on the gut microbiota was conducted using the Analytic Rarefaction v2.0 software distributed by Hunt Mountain Software. UniFrac was used to test for differences between clone libraries with 100 permutations. To estimate the microbial diversity, species richness and diversity indices were calculated using Estimates 8.2 (50 runs, randomized with replacement, using the classic Chao1, ACE, and Simpson's reciprocal formulas; Colwell *et al*., 2008). To determine if occurrences of shared outstanding taxonomic units (OTUs) were significantly different between the two treatments, a Fisher's exact test was conducted. The median number of cultured gut bacteria and protozoa between control and experimental animals were analyzed with MW. Because mass of the reproductives was normally distributed, differences between treatments were analyzed with t-tests.

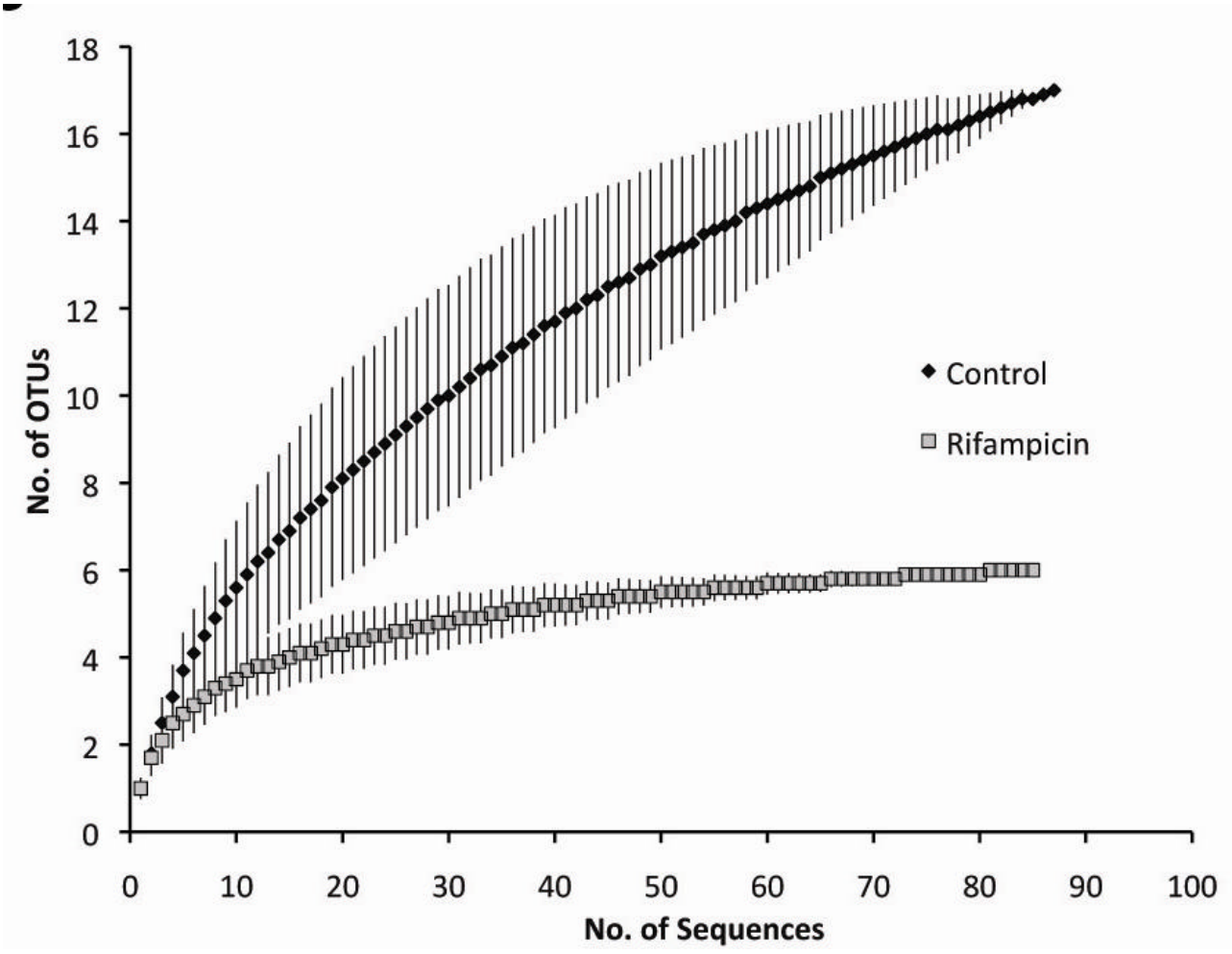
(ii) Survival of reproductives and colony fitness. Because all colonies were not established or did not undergo a census on the same day, results were standardized by analyzing survival and reproduction data based on the time elapsed between pairing and each of the subsequent censuses. Survival data was analyzed using both a Cox proportional regression and Survival analyses (SPSS, 1990). While the former analysis helps identify which variables are significant and independent predictors of death, the latter allows the estimation of several parameters including the number of days elapsed since pairing until 50% of the individuals died (median survival time; LT_{50}), percent survival at the end of the census period and the time course of survival (or survival distributions). In addition, we calculated the likelihood with which animals in the experimental treatment died relative to control termites (or relative hazard ratios of death). These hazard functions therefore, characterize the instantaneous rate of death at a particular time, given that the individual survived up to that point, while controlling for

the effect of other significant variables on survival (Cox regression model; SPSS, 1990; Rosengaus *et al*., 2000).

Reproductive output was analyzed by comparing the frequencies of distributions with which eggs, larvae and soldiers were produced by the control and rifampin treated reproductives on day 150 post-pairing [Kolmogorov-Smirnov Z test (KS), SPSS, 1990]. In addition, differences in the number of colonies with eggs, larvae and soldiers as a function of treatment two years post-pairing were analyzed with 2x2 Pearson's X^2 tests. The number of offspring produced by 50% of the colonies in each of the control and rifampin treatments (median number of eggs, larvae and soldiers) was compared for each of the census dates with non parametric Mann-Whitney U tests (MW), as well as differences in the number of days elapsed since pairing until 50% of the colonies/treatment oviposited their first egg and hatched their first larva.

FIGURE LEGEND

Fig. S1 Supplemental . Rarefaction analysis of OTUs from control and rifampin-treated hindguts. The arc of the curve indicates the likelihood of sequencing a new bacterial OTU if more clones were sampled. The curve is generated based on the occurrences of OTUs within the clone libraries.



Supplemental TABLE 1. 16S rRNA gene homology of *Zootermposis* gut bacteria to species with known mutations for rifampicin resistance

	Species with rifampin resistance gene		
Bacterial genus in termite gut	**Bacterial OTU with known rifampin resistance**	**NCBI GI**	**16S % Pairwise id**
Arthrobacter	Arthrobacter sp. FB24	116668568	95.0%
	Arthrobacter aurescens TC1	119960487	92.7%
	Arthrobacter arilaitensis Re117	308175814	92.7%
Cytophaga	Cytophaga hutchinsonii ATCC 33406	110279108	81.4%
Enterococcus	Enterococcus faecium strain DSM 10663	42560437	96.9%
Pseudomonas	Pseudomonas fluorescens SBW25	229359445	96.3%
	Pseudomonas aeruginosa strain MYL-21	321159400	94.5%
Treponema	Treponema sp. ZAS-1	4235383	97.8%
	Treponema phagedenis strain YG3903R	219551879	91.7%
	Treponema medium strain G7201	310975273	91.4%
	Treponema socranskii subsp. Socranskii	2653628	90.6%
	Treponema denticola ATCC 35405	41821838	91.3%
	Treponema pallidum	176249	90.1%
Verrucomicrobium	Verrucomicrobium spinosum DSM 4136	219846674	78.3%

An analysis was conducted to determine whether the frequency of rifampin-resisant bacteria are more frequent post-treatment vs. pre-treatment, as would be expected if treatment selected for rifampin resistant bacteria. The results indicate that there are relatively few OTUs observed in the termite gut (before or after rifampin treatment) that are closely related to known rifampin resistant bacteria. Further, if we assume that if a strain in our dataset is related to a resistant strain, then they themselves are resistant, we still observe that the frequency of strains with rifampin resistance is low and the same between pre- and post-treatments ($P = 0.656$, Fisher's exact test). Thus, we have not selected for rifampin resistance by treating the termites with antibiotic. OTU denoted operational taxonomic unit.

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