Strong environment X genotype interactions determine the fitness costs of antibiotic resistance *in vitro* and in an insect model of infection

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In vivo and *in vitro* resistance costs uncorrelated

Abstract

The acquisition of antibiotic resistance commonly imposes fitness costs, a reduction in the fitness of bacteria in the absence of drugs. These costs have been primarily quantified using *in vitro* experiments and a small number of *in vivo* studies in mice, and it is commonly assumed that these diverse methods are consistent. Here, we used an insect model of infection to compare the fitness costs of antibiotic resistance *in vivo* relative to *in vitro* conditions. Experiments explored diverse mechanisms of resistance in a Gram-positive pathogen, *Bacillus thuringiensis,* and a Gram-negative intestinal symbiont, *Enterobacter cloacae.* Rifampicin resistance in *B. thuringiensis* showed fitness costs that were typically elevated *in vivo,* although these were modulated by genotype-environment interactions. In contrast, resistance to cefotaxime via de-repression of AmpC β-lactamase in *E. cloacae* resulted in undetectable costs *in vivo* or *in vitro*, while spontaneous resistance to nalidixic acid, and carriage of the IncP plasmid RP4, imposed costs that increased *in vivo*. Overall, fitness costs *in vitro* were a poor predictor of fitness costs *in vivo* because of strong genotype environment interactions throughout this study. Insect infections provide a cheap and accessible means of assessing fitness consequences of resistance mutations, data that is important to understand the evolution and spread of resistance. This study emphasizes that the fitness costs imposed by particular mutations or different modes of resistance are extremely variable, and that only a subset of these mutations are likely to be prevalent outside of the laboratory.

Keywords: antibiotic resistance, antimicrobial stewardship, antimicrobial susceptibility, *B. thuringiensis, E. cloacae*, fitness cost, insect models, pleiotropic cost

Introduction

Antibiotics target enzymes with essential cell functions, such as RNA polymerase, DNA gyrase and the 30S or 50S subunits of the ribosome, while resistance mutations in binding sites can impair essential biochemical functions such as transcription (1, 2). Though mutations in drug targets confer a fitness advantage in the presence of antibiotic over some range of doses, they also typically reduce fitness in the absence of antibiotics (3-5). This detrimental consequence of resistance is commonly described as a fitness cost (3-5). While target site mutations are obvious candidates for imposing high fitness costs, the acquisition of plasmids bearing antibiotic resistance genes can also reduce fitness (6-8). Moreover, while the individual cost of each additional gene encoding for resistance can be small (4, 9, 10), the cumulative effect of increasing numbers of resistance genes is associated with impaired growth (11).

A greater understanding of the evolutionary biology of resistance, including fitness costs, can inform efforts to control the spread of resistance (3, 12-14). A range of approaches to antimicrobial stewardship increase the heterogeneity of selection pressure by prescribing different drugs to different parts of the population ('mixing'), or by using preferred drugs for short periods of time ('cycling'). Under cycling strategies in particular, the fitness costs of resistance (12, 15) and patient turnover (16) are expected to be the main factors driving down the frequency of resistance when drugs are withdrawn. Modelling studies have argued that there are insufficient data to estimate some of these key parameters (16, 17) or to predict which antibiotic stewardship interventions would be most effective (18).

Importantly, antibiotic resistance mechanisms are diverse and not all will necessarily impose a cost: there will be a distribution of fitness effects (5). Most commonly, fitness costs are assessed *in vitro* and results are extrapolated to costs *in vivo* (4). While some have argued that fitness costs are broadly similar *in vitro* and *in vivo* (10), this is not universally accepted (4). Some resistance mutations in a range of microbes show enhanced or altered costs *in*

vivo (4, 19-21). In addition, resistance mutations seen in clinical contexts often represent a low-cost subset of those detectable *in vitro* (20, 22, 23), the implication being that selection based on fitness costs might be particularly efficient *in vivo.* Arguably, *in vivo* conditions are likely to be harsher than *in vitro* and environmental stress can increase fitness costs in a wide range of organisms (24-26). Although this pattern is by no means universal (1), environmental conditions could substantially affect antibiotic resistance fitness costs (1, 4, 25) and there are still limited comparative data on costs *in vivo* and *in vitro* (10).

Previous experimental studies of *in vivo* costs are almost entirely restricted to mouse models (10). In this study, we wanted to explore the practicality of a cheaper, higher-throughput insect model. Secondly, previous *in vivo* studies have focused on spontaneous target site modifications in housekeeping genes, where pleiotropic fitness costs are likely to be high (4, 10). Here, we compare spontaneous target site resistance with metabolic resistance based on constitutive up-regulation of β-lactamases. Finally, we incorporated a measure of the cost of carriage of a typical antibiotic resistance plasmid because there are limited data on the costs of plasmid carriage *in vivo* (10),

To examine the difference in antibiotic resistance fitness costs *in vitro* and *in vivo*, this study used an insect model of infection and two different microbes, a Gram-positive pathogen *Bacillus thuringiensis*, and a Gram-negative intestinal symbiont, *Enterobacter cloacae.* Wax moth, *Galleria melonella,* are a commonly used insect model for bacterial infections partly because they can be reared at 37°C (27). Insects also have a well-developed innate immune system that shares several features with vertebrates (27, 28). However, wax moth larvae have weak immunity relative to many insects, with reduced expression of antimicrobial peptides (29) and weak cellular based immunity to macro-parasites such as nematodes (30, 31). Their status as permissive hosts for infection generally means that they are widely used as traps for the isolation of new insect parasites (32, 33).

Weak immunity may make wax moth a questionable host for assessing *in vitro* fitness costs of antibiotics and studying antibiotic resistance. There is abundant circumstantial evidence suggesting immunity is important for the fitness of resistant microbes. For instance, repressed immunity may facilitate the persistence of high cost resistance mechanisms (34, 35) and active immunity is thought to be important for shaping the fitness of drug resistance mutation in HIV(36). Since interactions with the immune system may be an important factor for fitness costs this study used larvae (caterpillars) of an alternative host, diamondback moth, *Plutella xylostella,* which is a well-established infection model for both *B. thuringiensis* and *E. cloacae* (37-40). While this insect cannot be reared at 37°C, it has high fecundity and a short generation time (ca. 14 days at 25°C) can be reared gnotobiotically on artificial diet in Petri dishes (41), making it a valuable alternative model host.

Results

Characterization of mutants

Sequencing of the rifampicin-binding pocket of *rpoB* in the *B. thruringiensis* mutants gave 37 sequences that could be aligned with the wild type template. This region is 216 base pairs long, starting at base 1,054 in the template *rpoB* gene. All the mutants contained one of five SNPs, each a single base pair substitution that resulted in a single amino acid change (Table 1). Amino acid alignments for these mutants are presented in supplementary figure S1. The Ser-Tyr mutants (9a) could not be reliably cultured and these were excluded from fitness experiments; one mutant from each of the remaining genotypes was used in subsequent experiments. All mutants had minimal inhibitory concentrations (MICs) at least 100x greater than that of the wild type (Table 1).

After 48h of incubation on cefotaxime-supplemented agar (4 µg/ml), two distinct phenotypes of resistant *E. cloacae* mutants were observed: satellite colony-forming and non-satellite colony forming mutants (see Supplementary Fig. S2). Satellite colonies were susceptible

117 cells, which were able to grow on agar plates containing 4μg/ml cefotaxime in the zone
118 around a resistant mutant colony. Satellite colonies were phenotypically susceptible and not
119 able to grow on cefotaxime in the absence of a mutant colony. Satellite colonies are
120 produced when β-lactamases detoxify solid media and facilitate the growth of susceptible
121 cells coming out of persister states (6).

122 Eight mutants of each resistant phenotype were sequenced at two potential target genes:
123 *ampD* and *ampR*. Sequences were assembled and analyzed using a wild type positive
124 control and the annotated reference genome of *E. cloacae* KU6334, Sequence ID:
125 AY789446 (42) and wild type ancestor of experimental strains *E. cloacae* jjbc. No genetic
126 changes were observed in sequences of the *ampD* gene. In contrast, sequence variation
127 was found in the *ampC* transcriptional activator gene *ampR*, resulting a range of genetic
128 variants, three of which were used in experiments (see Table 2). Two genotypes produced
129 satellite colonies (cef D-A and cef D-Y); the third (cef ins) did not. Genotypes cef D-A and cef
130 D-Y had non-synonymous single base pair mutations on the same codon, at positions 410
131 and 409, respectively. Fitness experiments were conducted with one mutant for each of
132 these genotypes. Two additional mutants with substitutions affecting the same residue,
133 resulting in a change to Glycine or Valine, were isolated but not used further. All three
134 experimental genotypes had MICs at least 100-fold greater than the wild type, although the
135 cef-ins genotype, which did not produce satellite colonies, had the lowest MIC of all strains
136 used (Table 2).

137 Nalidixic acid resistant mutants of *E. cloacae*, capable of growth at 30ug/ml could be readily
138 produced. However, all characterized clones had wild type sequences in both *gyrA* and *parC.*
139 MIC values (<0.1mg/ml) were modest compared to those of clinical fluoroquinolone-resistant
140 isolates of *E. cloacae,* which are commonly >100mg/ml nalidixic acid (43, 44). We selected a
141 single mutant (11.1B) for fitness experiments to act as a comparison for the cefotaxime
142 resistant mutants; this genotype was also the host for the RP4 transconjugants.

143 *Relative fitness*

144 For the rifampicin resistant mutants of *B. thuringiensis* we observed strong
145 environment*genotype interaction effects on competitive fitness in the absence of antibiotics
146 ($F_{3,206}$ = 26.0, P < 0.0001; Fig 1); and strong differences between genotypes ($F_{3,201}$ = 59.6, P
147 < 0.0001). As hypothesized we saw lower mutant fitness overall in larvae, and therefore
148 enhanced fitness costs for the *in vivo* infections ($F_{1,209}$ = 25.9, P < 0.0001; Fig 1). In broth *B.*
149 *thuringiensis* typically underwent 11.9 doublings (se 0.35), and our estimate of the typical
150 number generations *in vivo* was 13.0 (se 0.08), although this assumes an initial infection
151 bottleneck of 50 cells.

152 In *E. cloacae,* the fitness cost imposed by resistance depended on the particular resistance
153 mechanism ($F_{4,139}$ = 72.4, P < 0.0001, Fig 2A). As hypothesized, the fitness costs of
154 resistance could be enhanced *in vivo,* but this was only true for nalidixic acid resistance and
155 carriage of RP4 plasmid (genotype*treatment interaction $F_{4,135}$ = 41.1, P < 0.0001, Fig 2A).
156 Carriage of RP4 was the only resistance trait that imposed a detectable cost *in vitro* (Fig 2A).
157 The enhanced fitness cost of RP4 *in vivo* was not as clear in our supplementary analysis on
158 the estimates of Malthusian parameters (Fig S3). Note that both these analyses excludes 9
159 replicates in which relative fitness could not be calculated because plasmid carrying cells
160 could not be detected in live hosts but which support a picture of poor fitness in that
161 environment. In the *E. cloacae* experiments there was greater disparity in the number of
162 generations in different environments- with 11.1 generations in broth (se 0.05) and
163 approximately 17.5 generations in larvae (se 0.17).

164 The three cefotaxime resistant genotypes of *E. cloacae* behaved similarly and for these three
165 genotypes there no was no evidence that environment affected fitness (treatment*genotype
166 interaction $F_{1,93}$ = 1.21, P = 0.3, Fig 2A) nor was there a significant impact of genotype ($F_{1,95}$
167 = 1.17, P = 0.31). There was some evidence that the competitive fitness of resistant mutants
168 in larvae was actually slighter greater than the wild type (test for fitness > 0, mean 0.186, SE

0.08, t = 2.306, P = 0.0232); for the cef D-Y mutant in particular the mean fitness had confidence intervals (0.55,0.15) that did not overlap with zero, indicating a fitness benefit for this mutation.

Using data from both *E. cloacae* and *B. thuringiensis* we also assessed whether fitness costs in broth culture were correlated with costs *in vivo,* as investigated previously (10). We found that fitness costs *in vitro* were a poor predictor of costs *in vivo,* based on a simple linear model of mean log-transformed fitness values (Rsq = 0.035, $F_{1,7}$ = 0.25, P = 0.63; Figure 3).

While growth rates or relative fitness in direct competition are the most commonly reported measures of fitness, there are other fitness components that likely impact on pathogen transmission, such as the total population size of infectious propagules within a host, sometimes termed yield (4). For the three cefotaxime resistant mutants we had sufficient data to conduct a yield analysis. An important biological factor for naturalistic *in vivo* infection is the existence of a considerable bottleneck during establishment (45). The infection bottleneck in *E. cloacae* meant that 50 larvae carried infections consisting of single genotypes (either the wild type or resistant mutant). With these infections competitive fitness cannot be calculated and yield offers an important complementary fitness parameter. The high number of zero yield counts for one partner or another (Fig 2B) meant that data were not normally distributed. However, a non-parametric analysis indicated that resistant mutants reached higher population size than wild type bacteria within larvae (paired Wilcoxon rank test of yield, V = 1783, P < 0.0001, Fig 2B). The slight excess of infections in which the wild type strain failed to establish (37 wild type out of the 50 single genotype infections) contributed to this pattern (Fig 2B). Again, there was no good evidence for differences in yield in infections between the three cefotaxime resistant mutants (Kruskal one way ANOVA χ = 3.2, df = 2, P = 0.2).

Discussion

194 The fitness costs of antibiotic resistance are well established. However, the literature directly
195 comparing such costs *in vitro* and *in vivo* is very limited (10), potentially because conducting
196 *in vivo* studies in mice is both costly and laborious. As a result, tractable model systems
197 present a valuable opportunity to close this knowledge gap and potentially inform antibiotic
198 stewardship. We investigated genotype-environment interactions on the fitness costs of
199 rifampicin resistance in *B. thuringiensis* because of the clinical importance of rifampicin
200 resistance in other Gram-positive organisms (23) but also because rifampicin has been used
201 to develop a well-established model of the genetics of resistance (1, 4, 5, 46, 47).
202 Comparisons of data from the insect model to prior studies can give us some idea of the
203 value of this system. Encouragingly, we found that distinct mutations in the rifampicin
204 binding pocket produced a distribution of fitness costs, as in prior studies, although the range
205 of mutations isolated is reduced relative to those seen in *Pseudomonas aeruginosa* or UV-
206 irradiated *Bacillus anthracis* (5, 48). Here, we were also able to demonstrate that fitness
207 costs were consistently higher *in vivo,* although this effect varied with genotype. This result is
208 also consistent with the apparent efficiency of selection on limiting the diversity of mutations
209 seen in clinical studies (23).

210 Intestinal infection of *E. cloacae* in moth larvae also constituted an experimentally tractable
211 model for the spontaneous evolution of resistance to third generation cephalosporins. For
212 instance, it was possible to readily isolate a number of independent mutations that are
213 consistent with the constitutive up-regulation of AmpC β-lactamases, as seen in clinical
214 Enterobacteriaceae (42, 49, 50). In contrast to all other resistance mechanisms in this study,
215 AmpC up-regulation imposed undetectable fitness costs in media. This result is consistent
216 with previous *in vitro* work on the low costs of plasmid-encoded extended spectrum β-
217 lactamases (9) and AmpC β-lactamases (51). Comparative studies suggest that some
218 classes of β-lactamase impose costs, but the dominant mechanisms found in the clinic
219 appear to be the classes that impose the lowest fitness costs (52, 53).

In this study, we found some evidence that AmpC up-regulation conferred a slight fitness benefit *in vivo*, both in terms of competitive fitness and population size. This is difficult to explain and should be treated with caution, although a range of resistance mutations can provide context dependent fitness benefits (54). Methods of calculating relative fitness here are not without bias or limitations, particularly since wild type susceptible bacteria lack independent markers. Nevertheless, similar biases should pertain to *in vivo* and *in vitro* treatments and both treatments used identical methods for scoring genotypes and common inoculation methods. The production of β-lactams by resident microbes is possible, but unlikely, given than insects are reared in near aseptic conditions (41). Finally, β-lactam production by arthropods has been described but this is thought to be extremely rare and must be considered a very remote possibility (55). Of speculative interest is the fact that the insect pathogen *B. thuringiensis* carries multiple β-lactamase genes (56) while its close relative, *B. anthracis,* is typically β-lactam sensitive and is a mammalian pathogen (57), suggesting that β-lactamases may have some fitness benefits in insect infections.

The insect infection model yielded valuable fitness cost data for both Gram-negative and Gram-positive bacteria. Not all drug classes or resistance modes were equally amenable to study. We believed it would be easy to isolate fluoroquinolone resistance conferring mutations in *gyrA* and *parC*, but these proved elusive and may be much rarer than we anticipated, or not easily recoverable in this genetic background. Alternative sites for fluoroquinolone resistance are efflux pumps or *MarR*, which are known to be an important source of mutations in other Enterobactericeae (58, 59), and these could account for the resistance phenotypes here. In addition, our isolate of insect-associated *E. cloacae* appears to be poorly adapted for the carriage of a classic IncP plasmid: the plasmid appears to be non-conjugative when in this background (60) and this study shows that it imposes high fitness costs. IncP plasmids can conjugate into a broad range of bacteria (61). Nevertheless, this does not mean these plasmids are able to persist and be transmitted in all recipient

genetic backgrounds (62), a fact which can explain the restricted distribution of plasmids in the field (63).

Overall, there are several possible mechanisms that could account for elevated fitness costs *in vivo.* Since growth in host requires expression of a range of virulence factors not required in media, one hypothesis is that resistance mechanisms affecting gene expression impose greater costs *in vivo* (19). Since the costs of plasmid carriage commonly manifest through disrupted gene expression (64, 65), this is one possible explanation for elevated costs of plasmid carriage *in vivo* that merits further study. In addition to the fundamental differences in nutrient supply between *in vitro* and *in vivo* conditions that can alter fitness costs (54) host immunity may have an important role in altering or increasing fitness costs. For instance, interactions with macrophages can shape environment dependent fitness costs of rifampicin resistance (66) while all growth rate based fitness costs could confer increased susceptibility to immune cells. Moreover, a range of spontaneous resistance mutations can confer increased sensitivity to antimicrobial peptides, especially for mutations that affect the make-up of the cell wall (67, 68). One possibility that is unlikely to be important here is the presence of competing microbial species, since experiments used gnotobiotic insects; other studies have shown that a community context can be important for shaping the evolution of resistance (69).

One consequence of the experimental techniques used here is that naturalistic oral infections lead to strong infection bottlenecks, which will increase the variation in measures of competitive fitness and produce a proportion of single genotype or clonal infections (45). This can be thought of as methodological benefit, or a drawback. Bottlenecks are commonly produced during the process of infection, and so hosts are frequently colonized by single genotypes (70-72). With a single genotype per host, selection on competitive fitness will be weak as within-host competition occurs infrequently, so parameter estimates of competitive fitness may not reflect the transmission potential of resistant mutants. Instead, competition between hosts and selection on traits such as population size and efficiency of transmission

will be more critical (73). In this case, experiments that incorporate establishment of infection and can measure population size are valuable. If, on the other hand, it is important to measure competitive fitness precisely, experiments with insects can use injection rather than oral feeding to overcome bottlenecks (38), or can simply increase sample sizes to cope with noise (74).

We have previously argued that fitness costs of resistance are not necessarily a reliable basis for antimicrobial stewardship (13). This is partly because compensatory mutations that reduce fitness can occur quickly and readily (64, 65). This study emphasizes that the fitness burden of different mechanisms or mutations may be magnified *in vivo* but also that fitness costs vary considerably with genotype. This does not necessarily improve our prospects for exploiting fitness costs. Instead, one consequence is that elevated costs *in vivo* shape mutation supply, by determining which mutations or resistance mechanisms are able to persist effectively and establish in clinical contexts (14, 21, 23, 52). Similarly, while the horizontal mobility of resistance plasmids is often emphasized, clinically resistant microbes are often dominated by the clonal expansion of successful lineages with effective compensation in place. The success of the *Escherichia coli* lineage ST131, an unholy alliance of plasmid-borne β-lactamases, chromosomal fluoroquinolone resistance and plasmid compensation, is a well-known example (75). If high cost resistance is transient or exceptional (14) then it will be a poor basis for antibiotic resistance management. Antibiotic stewardship efforts may be better served by studying in detail the fitness of resistance lineages that are the targets of antibiotic stewardship (12), rather than on a general assumption of high fitness cost.

Materials and Methods

Bacterial isolates

Bacillus thuringiensis kurstaki 7.1.o is a recently isolated wild type strain (*Bt* WT) which is highly pathogenic to many Lepidoptera (41). This isolate has sequence type 8, placing it within an abundant and successful clonal expansion in this species (76). *Enterobacter cloacae* jjbc was recently isolated from the midgut of diamondback moth *Plutella xylostella* larvae and forms a persistent symbiotic association with this host (38).

Independent resistant mutants were produced via a modified fluctuation test based on (5). In brief, overnight cultures were diluted into LB in 24 well plates such that each well contained 1ml of broth inoculated with approximately 10^2 cells. Plates were cultured overnight at 30°C and the resulting cultures centrifuged (5000*g,* 8mins) and re-suspended in 200µl of saline (0.85% NaCl). Re-suspended cells were spread over LB agar plates containing either 10µg/ml rifampicin (*B. thuringiensis* only), 4µg/ml cefotaxime or 30µg/ml nalidixic acid. Colonies with strong growth were re-streaked at least twice on media containing antibiotics before sequencing and storage of glycerol stocks. RP4 transconjugants of *E. cloacae* were produced by co-streaking *Escherichia coli* MG1655 carrying the Inc P plasmid RP4 (provided by Tatiana Dimitriu) with a nalidixic acid resistant mutant (11.1B Nal^R) of *E. cloacae* on agar plates. Transconjugants were identified by re-plating cells on nalidixic acid and tetracyline. The RP4 plasmid is essentially non-conjugative in this system (60). For experiments antibiotic resistant mutants were cultivated by streaking wildtype ancestors from glycerol stock onto selective antibiotic plates followed by growth overnight in Lysogeny Broth (LB) at 30°C.

Characterization of antibiotic resistant mutants

MICs of wild type and resistant colonies were determined by broth dilution methods in microtiter plates using a two-fold dilution series of antibacterial agents, as per EUCAST recommendations (77). MICs were defined as the lowest concentrations of drug that completely inhibited visible growth of the inoculum after incubation for 18h at 30°C.

PCR and sequencing

B. thuringiensis clones were then sequenced to identify mutations in *rpoB.* The primers used to amplify the *rpoB* gene in *B. thuringiensis* were RIF-F1 (5' CGTGAGAGAATGTCGATCC) and RIF-R1 (5' CGCGAACGAAGATAATGA) for the cluster I region β-subunit in RNA polymerase (48). The *ampD* and *ampR* genes of cefotaxime resistant *E. cloacae* clones were targeted for PCR using primers from M. Hilty et al. (78) or designed in house: *ampR* EcAmpR_74F (5'-TGTGCCTGACAAACGGTTAA-3') and EcAmpR_1112R (5'-AGCGGTAAAGGGGTCTTCTA-3'); ampD_F (5'-TATTAATACGTTCCAGAAGC-3'); and ampD_R (5'-CATGGTAAACAACGTCATGT-3').

For the nalidixic acid resistant mutants of *E. cloacae,* we amplified gene fragments at two loci typically associated with fluouroquinolone resistance in Enterobacteriaceae: *gyrase A* and the ParC subunit of topoisomerase IV (*parC*) (43, 44). PCRs used the following primers: EcGyrA_1734F (5'- CGCATACCGTCTTTGTCAGA 3'); EcGryA_2672 (5'-TGCGAGAGAAATTACACCGG-3'); ParC_970F (5'-CAGAATCGCCTGAAGCTGAT-3'), ParC_2088R (5'- GCCAAGTTCAAGAAATCCG-3').

All PCR reactions used 25µl volume reactions with 0.4µl 0.2mM dNTPs, 0.4µl of each primer required at 25nM, 0.1µl 0.05U AmpliTaq DNA polymerase, and 1.5µl of the template DNA (overnight culture mixed with equal volumes of Tris-HCl ph8.0, boiled for 10mins). PCR conditions for *rpoB* amplification used: initial denaturation at 95°C for 5mins, thereafter 35 cycles of denaturation at 95°C for 30s, annealing at 52°C for 30s, extension at 72°C for 30s, and final extension at 72°C for 7mins. Amplification of ampR used similar conditions, but with an annealing temperature of 54°C, while all other PCRs (a*mpD, gyrA,parC)* used an annealing temperature of 56°C. PCR reactions were subject to ExoSAP cleanups using 0.2µl Exonuclease I (NEB, 20,000 U/ml) 0.1µl Antarctic phosphatase (NEB) and 1.5µl ExoI buffer per 10µl of PCR product. Products were incubated in a thermocycler for 30mins at 37°C and deactivated for 15mins at 80°C before being Sanger sequenced in both directions by Eurofins.

Competition experiments

All mutants were competed against the wild type ancestor *in vitro* and *in vivo,* with the exception of the RP4 transconjugant, which was competed against the relevant plasmid null strain, the nalidixic acid resistant *E. cloacae* mutant 11.1B. All comparisons of fitness *in vitro* and *in vivo* were assessed using a common inoculum master mix of wild type and resistant bacteria. *B. thuringiensis* inocula used spores, while the *E. cloacae* experiment used stationary phase cells. Each inoculum was plated on selective LB agar containing antibiotics and on antibiotic-free LB agar and incubated overnight at 30°C to establish initial and final ratios of resistant bacteria. Spores of *B. thuringiensis* were produced, pasteurized and enumerated via colony forming counts as described previously (39), stocks of spores for each genotype were prepared at 500cfu/µl. Inoculants for *in vitro* treatments were prepared by mixing stocks in 1:1 ratio with the wild type ancestors, and 10µl of each inoculant was added to 1ml LB in 24 well plates. Mixtures of wild type and resistant *E. cloacae* mutants were prepared from overnight cultures in LB (30°C, shaking at 180rpm). Cultures were diluted ten-fold in sterile saline (0.85% NaCl) and, after adjusting for differences in optical density at 600nm, were mixed with their ancestors in 1:1 ratio. *E. cloacae* cultures were also inoculated with 10µl of each mixture, the final dilutions being equivalent to a 1,000x dilution of overnight cultures. All experiments used a minimum of six replicates, each culture plate used six wells with uninoculated broth as checks for contamination, and *in vitro* competitions were run for 18h at 30°C and repeated twice.

For *in vivo* competition treatments *P. xylostella* larvae, from the population VLSS, were raised in gnotobiotic conditions on sterile diet as described previously (79). Ten third instar larvae were inoculated in 50mm Petri dishes using 100µl of each inoculant; all inoculation took place at 25°C. Inocula were added to the surface of a quarter of sterile caterpillar diet and allowed to dry in a class 2 safety cabinet. *B. thuringiensis* stocks were used at the total dose of 1000cfu/ µl, as above; this dose confers approximately 100% mortality with this

pathogen (39). *E. cloacae* mixtures were diluted a further 100-fold to give a final inoculum concentration equivalent to 1000x dilution of overnight cultures. Previous work has shown that this dose of *E. cloacae* ensures effective colonization of midgut but minimizes pathogenic effects (38).

Cadavers killed by *B. thuringiensis* were recovered after three to four days and final ratios of resistant and wild type spores assessed in homogenized cadavers as described previously (74). In brief, cadavers were incubated at 30°C for five days to ensure complete sporulation before being pasteurized, homogenized in a bead-beater (Qiagen Tissue Lyzer II) and dilution plated. Inoculation methods were adjusted for the gut symbiont *E. cloacae*. Approximately 50 eggs (just prior to hatching) were placed in Petri dishes containing inoculated diet, emergent larvae were allowed to feed for 48h before being transferred to fresh uninoculated diet for another 48-72h and then homogenized for bacterial enumeration. This ensures that experimental larvae are carrying persistent gut infections, not simply the initial inocula, and are approximately the same size as *B. thuringiensis*-killed insects (early third instars). Each master mix was inoculated into at least 32 larvae, although not all insects yielded data suitable for analysis of competitive fitness.

In vitro & *in vivo* fitness

The proportions of each strain and the ancestral competitor were calculated using total colonies on LB agar and the resistant colonies on selective agar plates. If the number of colonies on antibiotic plates was equal to, or exceeded, the LB agar counts, the proportion of wild type bacteria was inferred to be zero. Initial and final proportions on both LB and antibiotic-supplemented LB plates were used to calculate relative fitness V, where $V = x_2(1 - x_1)/x_1(1 - x_2)$, x_1 being the initial proportion of resistant mutants in inoculum and x_2 their final proportion (80). Fitness analyses were also conducted using the ratio of the Malthusian parameters, *W.* This method, however, relies on indirect estimation of the initial infection bottleneck in insects, while *in vitro* initial densities are calculated directly. We preferred to use

a parameter that was less subject to this potential bias, although analyses using *W* produced qualitatively similar results (Supplementary Fig S3).

Data availability.

The Genbank accession for the genome of the wild type *B. thuringiensis* 7.1.o is PRJNA395643 (76). A draft genome of *E. cloaceae* jjbc is available through the JGI Genome Portal, with the reference tag Anc C2. Raw experimental data for this project are publicly available from the University of Exeter institutional repository (unique doi to be allocated following acceptance).

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Table 1. Characterization of spontaneous rifampicin resistant colonies of *B. thuringiensis* with mutations in *rpoB*. All genotypes bar 9a were used in competition experiments. The MIC for the *B. thuringiensis* wild type was 0.2 μg / ml.

Mutant ID	**MIC** μg / ml	**Position of mutation** (bp)	**Amino acid change**	**Number of mutants isolated**
11c	100	18	Glu → Lys	1
4b	200	18	Glu → Arg	5
2d	100	31	His → Asp	10
10a	100	36	Ser → Phe	17
9a	100	36	Ser → Tyr	4

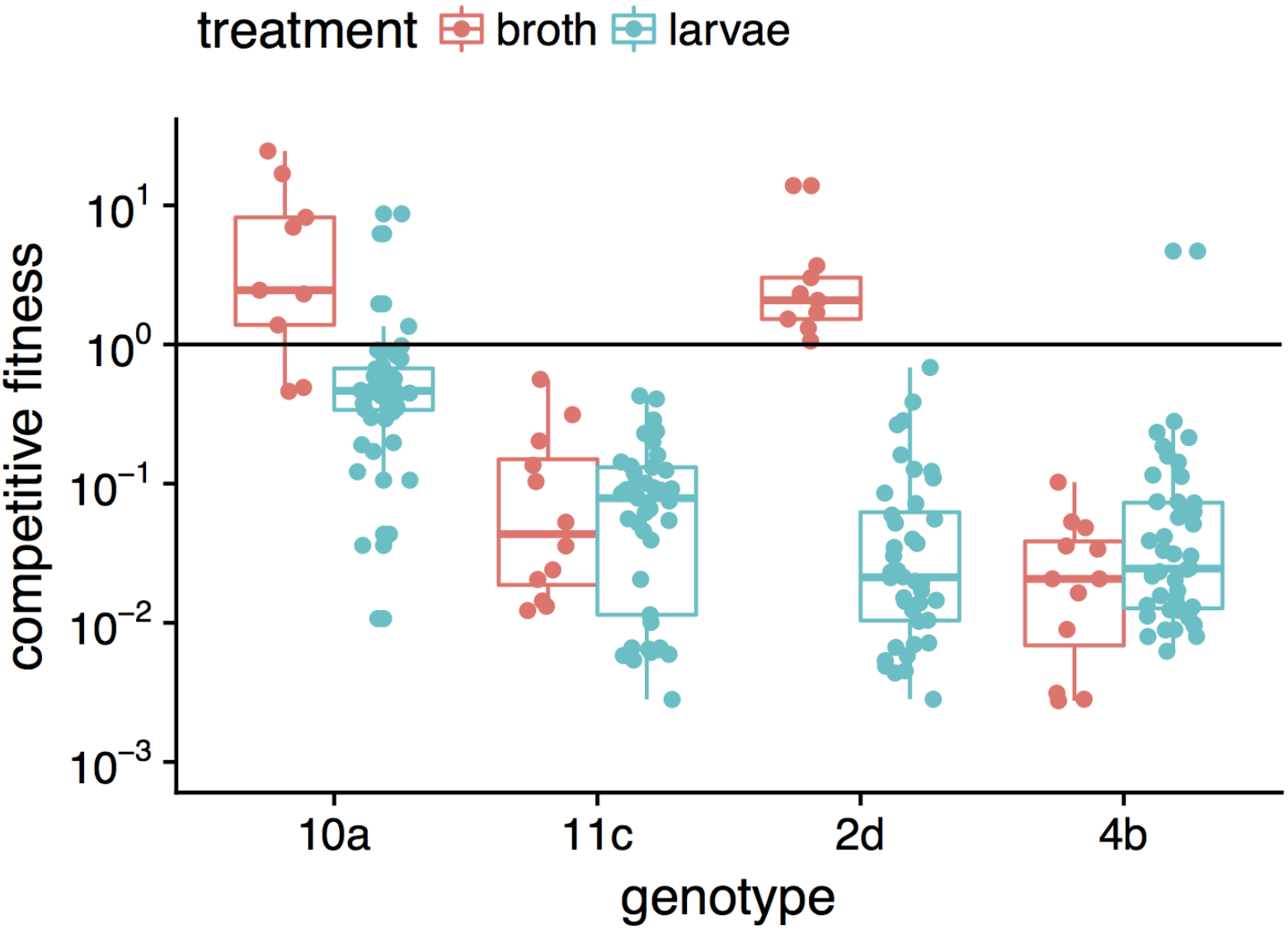
Table 2. Characterization of spontaneous cefotaxime resistant colonies of *E. cloacae* with mutations in the transcriptional regulator gene *ampR*. *These mutants produced satellite colonies on LB agar plates containing 4 μg/ml cefotaxime. The MIC for the wild type *E. cloacae* was 0.25 μg/ml.

Mutant ID	**MIC** μg / ml	**Mutation**	**Position of mutation** (bp)	**Amino Acid Change**	**Number of mutants isolated**
cef D-A*	256	A to C	410	Asp → Ala	2
cef D-Y*	256	G to T	409	Asp→ Tyr	2
cef ins	64	25 bp insertion	Starts at 370	+ Asp, Leu, Gln, Leu, Ser, Thr, His, Asn	1

Figure 1. The relative fitness of four rifampicin resistant mutants of *B. thuringiensis* in pairwise competition assays against the WT ancestor *in vitro* (LB) and in oral *in vivo* infections in larvae of diamondback moth, *P. xylostella.* Boxplots shows medians and interquartile distances, data points are independent infections of insects or replicated broth culture. Fitness was calculated according to the change in proportion of resistant and wild type genotypes from initial inoculum to end of culture/infection.

Figure 2. A. The relative fitness of diverse antibiotic resistant mutants of *E. cloacae* in pairwise competition assays against the WT ancestor *in vitro* (LB) and in oral *in vivo* infections in larvae of diamondback moth, *P. xylostella.* Fitness was calculated according to the change in proportion of resistant and wild type genotypes from initial inoculum to end of culture/infection. **B.** The total population size of cefotaxime resistant mutants and wild type *E. cloacae* in live intestinal larval infections. Zero counts reflect infections in which single genotypes were recovered from larvae. Boxplots shows medians and interquartile distances; data points are independent infections of insects or replicated broth culture. On the x- axis cefotaxime resistant genotypes (with the prefix cef) are listed in Table 2, nal and RP4 refer to a spontaneous nalidixic acid resistant mutant and a carrier of the IncP plasmid RP4, respectively.

Figure 3. Competitive fitness *in vitro* was a poor predictor of the fitness costs of resistance *in vivo* during live larval infections. Data are mean $\log_{10}$ transformed fitness values for the different resistance genotypes of both *E. cloacae* and *B. thuringiensis* in this study; the line represents the fitted linear model with a 95% confidence interval.


treatment
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10^{1}
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11c
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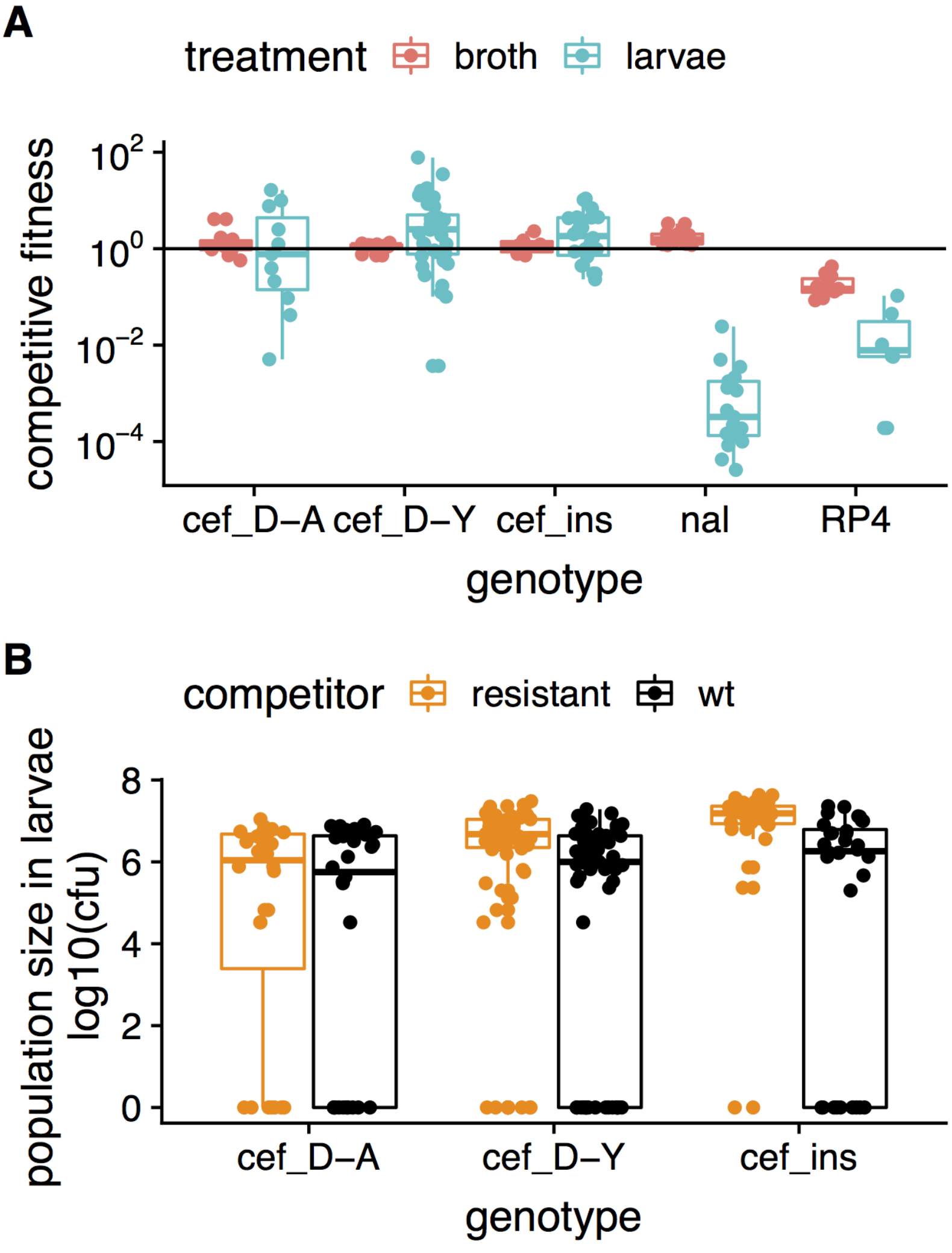
A
treatment
broth
larvae
competitive fitness
10^2
10^0
10^{-2}
10^{-4}
cef_D–A
cef_D–Y
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nal
RP4
genotype
B
competitor
resistant
wt
population size in larvae
log10(cfu)
8
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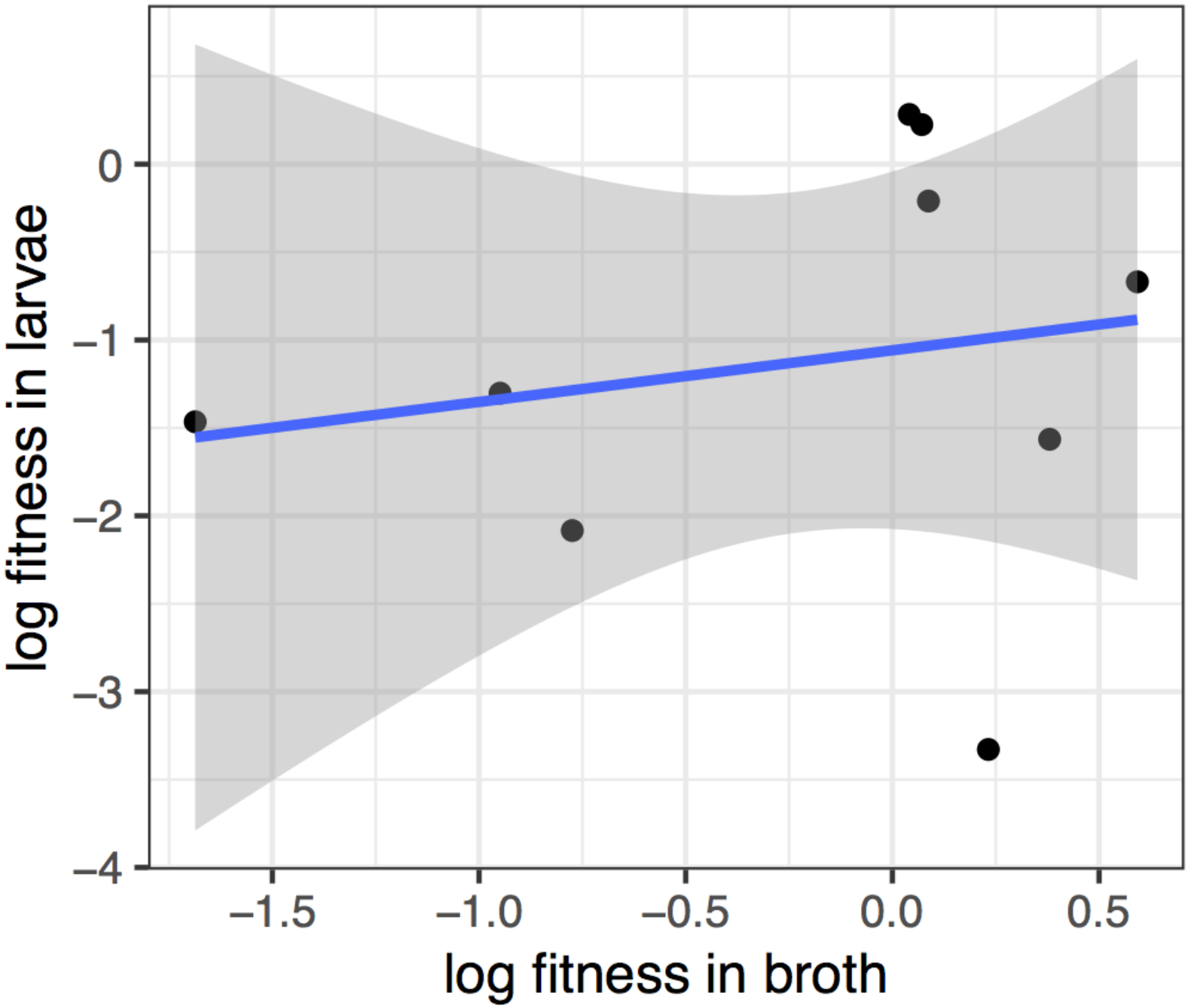
cef_D–A
cef_D–Y
cef_ins
genotype


log fitness in larvae
0
−1
−2
−3
−4
−1.5
−1.0
−0.5
0.0
0.5
log fitness in broth