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Article

Gnotobiotic rearing and controlled infection with gut symbionts improve adult fitness in transgenic diamondback moth, *Plutella xylostella*

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Abstract: Mass insect rearing can have a range of applications, for example in biological control of insects. Since the performance of released biological control agents determines efficacy, the competitive fitness of insects post release is a key variable. Here, we tested whether inoculation with a gut symbiont, *Enterobacter cloacae*, and gnotobiotic rearing of larvae could improve insect growth and male competitive fitness of a transgenic diamondback moth, which has shown variation in fitness when reared in different insectaries. All larvae were readily infected with the focal symbiont. Under gnotobiotic rearing pupal weights were reduced and there was a marginal reduction in larval survival. However, gnotobiotic rearing substantially improved the fitness of transgenic males. In addition, in gnotobiotic conditions, inoculation with the gut symbiont increased pupal weights and male fitness, increasing the proportion of transgenic progeny from 20 to 30% relative to symbiont-free insects. Gnotobiotic conditions may improve the fitness of transgenic males by excluding microbial contaminants, while symbiont inoculation could further improve fitness by providing additional protection against infections, or by normalizing insect physiology. The simple innovation of incorporating antibiotic into diet, and inoculating insects with symbiotic bacteria that are resistant to that antibiotic, could provide a readily transferable tool for other insect rearing systems.

Keywords: genetically modified insects; symbiosis; microbiome; transgenic; self-limiting; insect rearing; mutualism

1. Introduction

The environmental impact of chemical insecticides and widespread resistance has meant that biological and biotechnological approaches to pest management are more important than ever [1,2]. Many biological control approaches rely on effecient rearing of insect pests or natural enemies. This is especially true of sterile insect technique (SIT) and inundative biological control methods that rely on mass production and release of populations that are unable to support themselves in the environment [3,4,5], while some biological control pathogens such as baculoviruses can only be produced *in vivo* [6]. There is also increasing interest in the mass production and rearing of insects for food and feed, based on the efficiency with which insects can produce fat and protein from low quality diets [7]. In all these entomological applications, ensuring the health and fitness of insects is vital, and this is arguably particularly important for biological control programs where artificially reared insects must compete with wild insects for access to mates [8].

In the sterile insect technique, for example, mass-produced male pest insects are irradiated with gamma rays, leading to chromosomal irregularities at meiosis and the inability to produce healthy

gametes. Mass release of these sterile males ensures that mating occurs with wild females, who are unable to produce viable offspring [9]. This technique was first developed to eradicate New World screw-worm (*Cochliomyia hominivorax*), a highly damaging cattle parasite [4]. SIT eradicated the screw-worm from the United States and Central America, and has been used to suppress or eradicate other pest insects such as the Mediterranean fruit fly [5]. Although successful, SIT has some limitations. Large insect production facilities can be the source of accidental releases of wild type insects, so insect production factories are best sited far from eradication zones. The fitness of the irradiated males can be very low, necessitating the release of very large numbers of males, while not all insect species can tolerate high levels of irradiation. Mass release in itself imposes technical challenges, including the difficulty of sex-separating males from females [5].

An analogous approach to the SIT addresses some of these issues. Genetically engineered insects carrying transgenes under the control of the 'Tet-off' genetic switch [10,11], can be configured to regulate conditional sex-specific expression of a given gene such that females will not survive beyond early instars [12,13]. Since these transgenes are dominant lethals, they impose strong fitness costs, meaning these transgenes are rapidly lost from populations after release. These transgenes are therefore 'self-limiting', in the sense that these costs drive a rapid decline in transgene frequency, post-release. Female-specific self-limiting transgenes ensure sex-separation by the elimination of the females, while supplementing larval feed with tetracycline (or suitable analogues) represses the lethal phenotype, allowing rearing in the laboratory [11]. Self-limiting transgenic males are typically marked with fluorescent protein genes [14], making it straightforward to monitor of self-limiting genes in experimental or wild populations. Field-tests on the mosquito *Aedes aegypti* have shown that this self-limiting transgene technology can locally reduce populations by 95% [15,16,17].

Although self-limiting transgenic technologies have advantages over SIT [18], transgenes still impose fitness costs: male fitness can be reduced relative to that of wild type insects, potentially because of insertional effects or low rates of transcription of transgenic elements in males [19]. Critically for this study, we have previously observed variation in the fitness of self-limiting diamondback moth (DBM, *Plutella xylostella*) between laboratories, even when using standard insect stocks reared on carefully developed protocols that have yielded strong-performing males in other insectaries [13,20].

Diet can be an important factor in determining the fitness of laboratory-reared insects [21]. Many insects in laboratory studies are typically reared on a combination of antibiotics, commonly tetracycline and streptomycin, among other antimicrobials [11]. Although tetracycline is necessary to suppress dominant lethal transgenes [11], antibiotics will further reduce the microbial diversity within insects [22,23]. Variability in insect quality with the self-limiting DBM, when moved off tetracycline diet, maybe be related to the female larvae that die as young instars within rearing containers, and these cadavers may provide a source of microbial contamination on the larval feed. In addition to the use of antimicrobials, the consumption of artificial diet *per se* can reduce microbial diversity within insects [22,24]. Reduced microbial diversity may increase the vulnerability of insects to some pathogens [25,26], while insect gut symbionts have a range of potentially beneficial roles that could impact male fitness. For instance, gut microbes may improve nutrient assimilation [27]; aid in the production of mating pheromones [28]; or play a general role in nutrition and regulation of host metabolism [29].

Here, we aimed to test two hypotheses using the self-limiting DBM as a model insect. First, whether gnotobiotic rearing is able to prevent potentially compromised insect fitness by reducing opportunities for contamination of artificial diet, and second, whether the addition of microbial gut symbionts can further increase the fitness of these transgenic insects reared in the laboratory. We selected *Enterobacter cloacae* as our focal gut symbiont as this species can form persistent associations with the Lepidopteran gut [30] and because *Enterobacter* spp. are a common component of the gut microbiota in a variety of insects [30,31]. Following from previous work we used a population of DBM carrying a female-specific self-limiting gene developed by Oxitec Ltd, a strain carrying a fluorescent marker that allows efficient calculation of mating success [13].

2. Materials and Methods

Development of the self-limiting DBM (OX4319L, Oxitec Ltd) has been described previously [13]. In brief, the self-limiting system was implemented using sequences from the *doublesex* (*dsx*) gene of pink bollworm [13]. Sex-alternate splicing of this *dsx* sequence allows the development of a female-specific lethal genetic system that is repressible by the provision of tetracycline, or suitable analogues, in the larval feed [13]. The self-limiting (SL) strain was constructed in the Vero Beach genetic background. Wildtype (WT) insects in this study were the population VLSS, a stock produced by out-crossing Vero Beach with the diet-adapted NO-QA strain, as described previously [20]. The gut microbe *Enterobacter cloacae* (isolate JJBC), forms a persistent association with the gut of Lepidoptera and was recovered from larvae of DBM feeding on Chinese cabbage, *Brassica pekinensis*, in the insectary in the Department of Zoology, University of Oxford. The *E. cloacae* strain used in these experiments was JJBC 11.1B Strep^{R}, which is a spontaneous mutant that is able to grow in the presence of streptomycin. This enabled us to combine the use of antibiotic with the addition of a symbiont and therefore specifically test the benefit of symbiont inoculation in addition to gnotobiotic rearing.

Standard rearing conditions followed published protocols [32] with some minor modifications. In brief, insects in standard conditions were reared in non-sterile 100mm plastic tubs with a depth of 45mm. Diet (F9221B, Frontier Agricultural Sciences) was autoclaved prior to pouring; vitamins (Vanderzant's 4g l^{-1}, Ascorbic Acid 3.6 4g l^{-1}) were added after diet had reached a temperature of ≈ 60°C. All *P. xylostella* eggs were surface- sterilised (2% sodium hypochlorite solution, and three washes of autoclaved water) and counted before being added to rearing containers.

Gnotobiotic rearing conditions were adapted from previously established aseptic protocols using insects that are the offspring of parents reared on antibiotics [33] with some modifications. Here, insects were reared in 90mm Petri dishes on artificial diet (as above). Filter-sterilized vitamins (concentrations as above) and antibiotic (streptomycin 0.125g l^{-1}) were added to diet before pouring 20ml into each dish in a class 2 microbiological safety cabinet. All manipulation of diet and Petri dishes took place inside a safety cabinet. In both rearing treatments insects were reared on quarter sections of diet and fresh diet added as older diet became dehydrated or consumed. Insect egg densities were controlled to ≈ 120 SL eggs per container.

For both gnotobiotic and standard conditions, three treatment groups were established; SL without *E. cloacae* (SL-Ec) and SL with *E. cloacae* (SL+Ec); while wild type insects without *E. cloacae* (WT-Ec) were included as a positive control and to assess fitness costs of transgenes relative to a standard outbred stock. Details of inoculation of insects with *E. cloacae* gut bacteria are given below. Treatment groups were incubated at 24+/- 1°C. At the fourth instar males and females from the WT-Ec treatment groups were separated and replaced on fresh diet; pupal weights were recorded for males only. Mean pupal weights were recorded for each rearing container, to avoid pseudo-replication. Surviving SL pupae from all treatment groups in both standard and gnotobiotic conditions were counted and compared to initial egg counts.

Emerging neonate larvae were inoculated with *E. cloacae* (*Ec*) using 1000-fold dilutions of overnight culture (5ml L-Broth with 50µg ml^{-1} streptomycin, 30°C, 150rpm). Bacteria were diluted in sterile saline (0.85% w/v NaCl). Insects were inoculated by coating quarter sections of diet with diluted *Ec* (600µl in standard conditions; 400µl in gnotobiotic conditions); diet was dried in a class 2 microbiological safety cabinet, before adding surface sterilized eggs on Parafilm strips just before egg hatch. In order to enumerate bacterial infections, fourth instar larvae (1-2 per rearing container) were homogenized in 135µl saline, serially diluted, and 15µl of the homogenate was plated out using a dilution range of 10^{-1} to 10^{-4} onto 2% LB agar.

Mating competition experiments took place in 30x15x15cm mating cages using a release ratio of 10 SL males to each WT male and a total of 10 WT males and 10 females in each cage. Bacterial treatments, rearing with *E. cloacae* (+Ec) or without *E. cloacae* (-Ec) were replicated five times in the gnotobiotic regime and four times in the standard rearing conditions.

Mating occurred over a three day period at 24+/- 1°C once pupae had emerged as moths. Eggs collected over this mating period were reared on standard conditions, as above. Again when larvae

had reached fourth instar, females were removed from all diet tubs. As the self-limiting construct contains a dominant heritable, fluorescent DsRed2 protein marker [13], pupae were sorted using a binocular microscope with Nightsea™ light source (excitation 510–540 nm) and 600-nm filter, enabling us to score the proportion of WT and SL male progeny for each cage.

Statistical analysis was carried out in R (http://www.r-project.com) using analysis of variance, and generalized linear models (glms), or with simple chi-square tests. Bacterial counts were $\log_{10}$ transformed (after adding 1 to all count) prior to analysis. Proportional data (survival, proportion of transgenic progeny) were analysed in glms with quasi-binomial error distributions to compensate for over-dispersion. All model assumptions were checked with graphical analysis of error distribution assumptions.

3. Results

3.1 Bacteria

To test for the efficacy of our bacterial inoculation, and to investigate the presence or absence of contaminating bacteria in experimental treatments, whole insect larvae were homogenized and plated out to test for the presence of culturable bacteria. Inoculation with gut bacteria led to the effective colonization of insects, while uninoculated insects lacked any culturable gut microbes ($t = 6.53$, $p<0.0001$; Figure 1). Out of 96 larvae sampled in the SL+Ec treatment group, only two did not contain *E. cloacae*, while one larva contained another bacterial morphotype. In standard conditions, a sub-sample of larvae ($n = 8$) showed 100% inoculation and there were clear differences in bacterial densities between inoculated and uninoculated insects ($t = -4.16$, $p=0.001$).

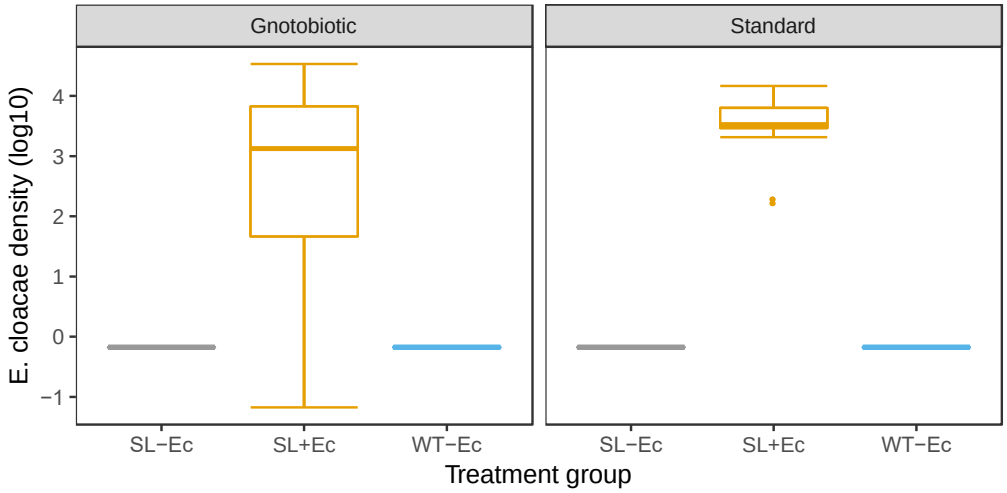

Figure 1. Culturable bacterial densities in inoculated (SL+Ec) and symbiont free treatment groups (-Ec) under gnotobiotic and standard conditions. Bacterial densities were assessed in self-limiting transgenic larva (SL) that had *E. cloacae* added to their diet (SL+Ec) and compared to SL larva without *E.* cloacae in their diet (SL-Ec) and wild type (WT) larva without *E.* cloacae in their diet (WT-Ec). In gnotobiotic conditions, in the SL+Ec treatment, 94 out of 96 larvae were successfully inoculated with JJBC. In standard conditions, all SL+Ec (n=8) contained the focal symbiont. In both standard and gnotobiotic conditions, treatments that were not inoculated with *Ec* had bacterial densities of 0 c.f.u/µl (WT-Ec (gnotobiotic: n=36, standard n=7) and SL-Ec (gnotobiotic n=60, standard n=8). Bacteria in the inoculated larvae were streptomycin resistant and confirmed in morphology to our focal *E. cloacae* JJBC 11.1B strain.

3.2 Development and weight gain

Survival from the egg to pupae stage was recorded for all self-limiting genotypes. Here there was a marginally non-significant trend with slightly elevated survival in the standard rearing conditions in comparison to gnotobiotic rearing ($F_{1,77} = 3.73$, $p = 0.057$; Figure 2). Inoculation with *Ec* did not have a consistent effect on larval survival ($F_{1,76} = 3.37$, $p = 0.070$; Figure 2) and these treatments

did not interact ($F_{1,75}$ = 0.047,p = 0.83). Note that because the self-limiting gene was not repressed under these conditions, all female larvae should die, meaning that survival, on average, was not expected to exceed 50%.

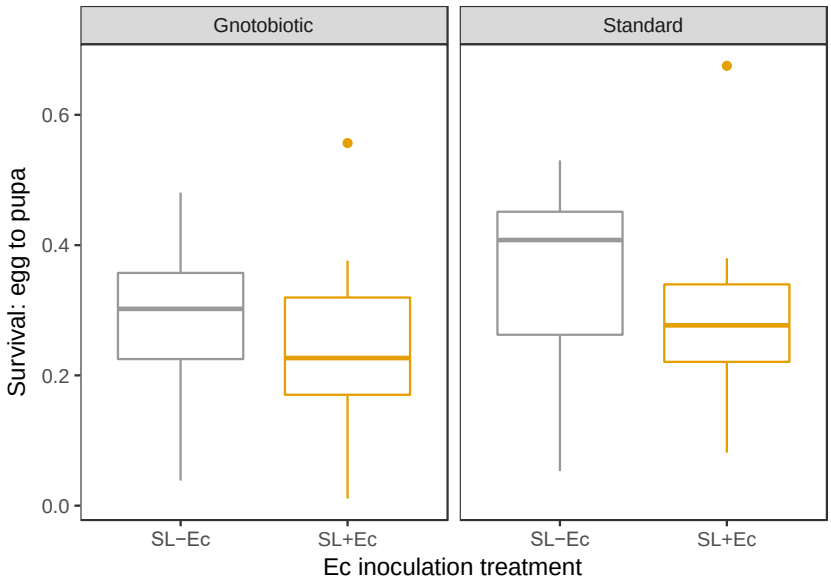

Figure 2. Survival egg to pupa for self-limiting insects in both gnotobiotic and standard conditions, and with and without *E. cloacae* inoculation (+Ec –Ec). Eggs were counted on Parafilm strips before being added to rearing containers, and the numbers of healthy pupae recorded after 7 days. In the gnotobiotic experiment, survival was recorded in 25-26 Petri dishes in each inoculation treatment, while under standard condition survival was assessed in 11-12 tubs per treatment.

To test the hypothesis that *E.cloacae* would affect SL male growth, pupal weights were scored for inoculated SL insect (SL+Ec), uninoculated SL insects (SL-Ec) and uninoculated wildtype insects (WT-Ec) in gnotobiotic and standard conditions. Insect genotype and inoculation treatment significantly affected pupal weight, with WT pupae having the greatest weight in both rearing regimes ($F_{2,83}$ = 80.6, p < 0.0001). Rearing regime also affected weight; on average insects reared in standard conditions produced heavier pupae ($F_{1,82}$ = 36.4, p < 0.0001; Figure 3). The effect of *Ec* inoculation, however, depended on rearing regime ($F_{2,80}$ = 3.51, p < 0.03; Figure 3). Under gnotobiotic conditions the gut bacteria marginally increased weight (*post-hoc* contrast, t = 2.34, p = 0.0217), while under standard conditions the gut bacteria appeared to be slightly parasitic and decreased pupal weight (post-hoc contrast, t = 2.28, p = 0.025). Overall, we confirmed a significant impact of *Ec* inoculation through model simplification; collapsing the *Ec* treatment groups for the self-limiting insects had a significant effect on pupal weight ($F_{2,82}$=3.38, p < 0.038).

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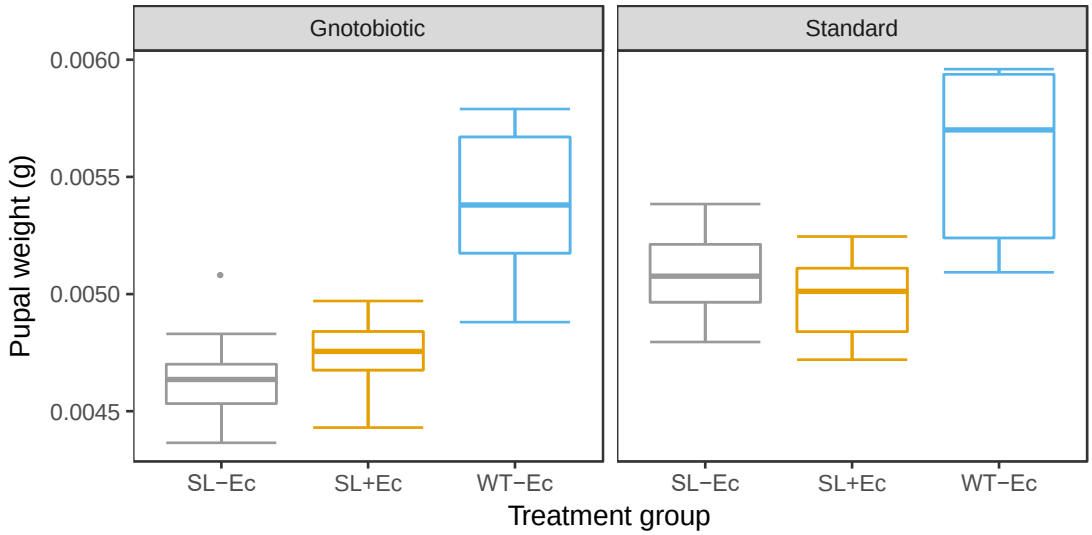

Figure 3. The effect of rearing regime, genotype and inoculation with gut bacteria (*E.* cloacae) on the distribution of male pupal weights. 'SL-Ec' represents self-limiting transgenic *P. xylostella* pupae without *E. cloacae*, 'SL+Ec' represents self- limiting transgenic *P. xylostella* pupae with *E. cloacae* and 'WT-Ec' represents wild type *P. xylostella* pupae without *E. cloacae*. For gnotobiotic conditions, male pupae were weighed from 35 Petri dishes in each SL treatment group (700 pupae in all) and 12 Petri dishes in the WT-Ec treatment group (110 pupae). Standard rearing conditions used 11-12 replicate tubs (674 SL pupae, and 85 WT pupae).

3.3 Fitness in mate competition experiments

Here, the hypotheses that rearing conditions and *E. cloacae* inoculation would affect SL male mating success was tested by comparing the proportion of male progeny sired by transgenic and WT males. The presence of a semi-dominant GFP fluorescent marker in OX4319L males means that heterozygous WT / SL progeny can be readily identified, allowing us to make accurate measures of mate competitiveness [13]. The strongest effect was the increased fitness of SL males in the gnotobiotic rearing regime versus those reared in standard conditions ($F_{1,14}$ = 105, $p < 0.0001$; Figure 4). However, inoculation with *Ec* also improved the ability of males to compete for mates ($F_{1,13}$ = 5.58, $p = 0.036$; Figure 4). Although the raw data suggest that this effect was greatest in the gnotobiotic regimen, there was no formal statistical support for this rearing*inoculation interaction ($F_{1,12}$ = 0.014, $p = 0.9$). However inspection of model fits indicated that there was more heterogeneity in the data for the standard rearing treatment and the low proportions in this treatment mean we have low power to resolve the symbiont inoculation treatment in standard rearing conditions. A conservative analysis shows a clear and strong effect of *Ec* inoculation using the data from the gnotobiotic regime only ($F_{1,16}$ = 5.1, $p = 0.0033$; Figure 4).

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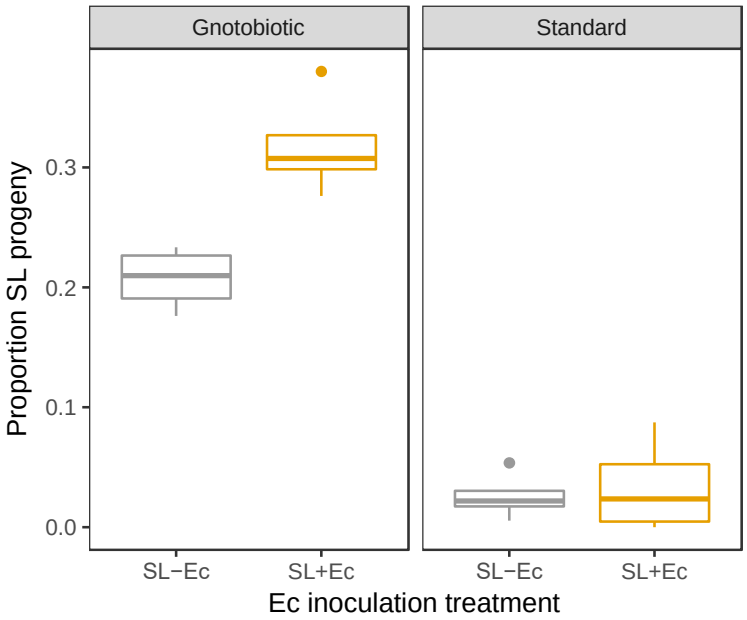

Figure 4. The impact of rearing regime and inoculation with *E. cloacae* gut bacteria on male fitness in mating competition experiments. Here, fitness is measured from the proportion of transgenic fluorescent male SL progeny in cages with 10:1 SL: wild type release ratio and 120 insects per cage. In the gnotobiotic regime, we scored a minimum of 100 progeny per mating cage; in the standard treatment, 200-400 progeny were scored per cage.

4. Discussion

We tested whether exerting greater control over the microbiome of insects in larval culture could improve their fitness as adults. Excluding potential microbial contaminants by rearing larvae in near-aseptic conditions substantially increased adult fitness; inoculating larvae with a known enteric symbiont provided further improvements in male pupal weight and competitive fitness. The standard larval rearing conditions for *P. xylostella* have had a long period of optimization, and it was beyond the scope of this study to fully optimize rearing conditions in our gnotobiotic set-up. Unsurprisingly there was evidence that larval rearing conditions were not ideal in the gnotobiotic set up: pupal weights were lower and average survival of larvae marginally reduced (albeit non-significantly). Clearly the gnotobiotic rearing could be improved either by designing custom plastic-ware, or experimentally assessing the optimal egg load per container. Nevertheless, improvements in adult fitness occurred in the gnotobiotic regime despite these sub-optimal larval conditions. Moreover, the results indicate that 'probiotic' manipulation of the larval microbiome had a positive effect on the fitness of adult DBM, over and above that of near-aseptic rearing conditions. These data are in contrast to previous studies with *Enterobacter* and transgenic medfly, in which gut bacteria enhanced larval survival but did not improve male mating fitness [34]. More broadly, our results suggest that the lepidopteran microbiome, despite its low diversity and the prevalence of many transient species [35], can have significant impacts on fitness.

In standard conditions, *E. cloacae* exhibited slight parasitism in terms of reduced pupal weight in the presence of the *E. cloacae*. The overall results suggest a condition-dependent mutualism between *E. cloacae* and SL males. In insects, host-bacteria interactions are well-documented [25,26] and more specifically, symbioses can flip from parasitic to mutualistic according to environmental conditions, such as diet quality [36,37]. In this study, diet was initially sterile and consisted of the same components in gnotobiotic and standard conditions. Even in our near aseptic rearing conditions (Figure 1), the presence of non-culturable bacteria can still occur, although it is relatively straightforward to exclude culturable microbes [33,38]. While we did not characterize the non-culturable community in these experiments, variation in abundance or community composition is a plausible cause of the impact of rearing conditions on adult fitness. The additional benefit of

Ec inoculation could therefore arise from additional benefits in reducing the presence of pathogens or parasites [39].

There are alternative explanations for the improved growth and fitness of *E. cloacae* inoculated males. Gut bacteria can improve efficiency of digestion or provide essential nutrients [25]. There is also a link between gut bacteria and nutrient uptake, particularly with respect to nitrogen [27]. *Enterobacter* populations can contribute to the production of dinitrogen reductase in insects [40], an important enzyme involved in nitrogen fixation [41]. Enhancement of sexual signalling by symbionts also occurs in several species [28,42,43]. In locusts, the gut microbiota (containing *Enterobacter* species) increase pheromone production, which in turn increase aggregations and mating success [42]. In *P. xylostella,* males secrete chemical signals in the presence of females, from a specialised hair-pin gland found on their abdomens, which has a putative role in sexual signalling during courtship [44]. However, since the benefits of *E. cloacae* inoculation depend on rearing conditions in this study, nutritional or signalling explanations for these results are not the most parsimonious, since *E. cloacae* densities were indistinguishable in both standard and gnotobiotic rearing conditions. Benefits of *Enterobacter* infection, for instance, would have to depend on the presence of other elements of the microbiota. Alternatively, since the removal of gut microbes can perturb insect metabolism [29], one explanation for the condition-dependent benefit of *E. cloacae* is that gut microbes normalize insect physiology and offset some of the potential side effects of rearing in near-aseptic conditions. Overall, further research is needed in order to depict the exact mechanisms underlying the benefits of the host-bacteria symbiosis investigated here.

5. Conclusions

While gnotobiotic rearing methods and gut microbiota inoculation are not essential for the high fitness of transgenic insects [45]; inclusion of these extra controls may make rearing methods more robust and less variable between laboratories [20]. For large scale insect releases, rolling out robust rearing regimes across a number of sites is likely to be operationally important. The simple innovation of incorporating antibiotic into diet, and inoculating insects with symbiotic bacteria that are resistant to that antibiotic, provides a readily transferable tool for other insect rearing systems. If incorporated into transgenic pest insects under the right abiotic conditions, gut bacteria could potentially contribute to the enhanced controlling of pest populations at lower costs, which in turn could contribute to the reduction of crop destruction or disease transmission [15,34].

Supplementary Materials:

Author Contributions: conceptualization, B.R. and L.Z.; methodology, B.R. and L.Z.; formal analysis, J.S. and B.R.; investigation, J.S. and L.Z. ; data curation, J.S. and B.R. ; writing—original draft preparation, J.S.; writing—review and editing, all authors.; funding acquisition, B.R.

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