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Rational engineering of a synthetic insect-bacterial mutualism

--Manuscript Draft--

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Abstract:	Many insects maintain mutualistic associations with bacterial endosymbionts, but little is known about how they originate in nature. In this study, we describe the establishment and manipulation of a synthetic insect-bacterial symbiosis in a weevil host. Following egg injection, the nascent symbiont colonized many tissues, including prototypical somatic and germinal bacteriomes, yielding maternal transmission over many generations. We then engineered the nascent symbiont to overproduce the aromatic amino acids, tyrosine and phenylalanine, that facilitate weevil cuticle strengthening and accelerated larval development, replicating the function of mutualistic symbionts that are widely distributed among weevils and other beetles in nature. Our work provides empirical support for the notion that mutualistic symbioses can be initiated in insects by the acquisition of environmental bacteria. It also shows that certain bacterial genera, including the Sodalis spp. used in our study, are predisposed to developing these associations due to an ability to maintain benign infections and undergo vertical transmission in diverse insect hosts, facilitating the partner fidelity feedback that is critical for the evolution of obligate mutualism. These experimental advances provide a new platform for laboratory studies focusing on the molecular mechanisms and evolutionary processes underlying insect-bacterial symbiosis.
Additional Information:	
Question	**Response**
Standardized datasets A list of datatypes considered standardized under Cell Press policy is available here. Does this manuscript report new standardized datasets?	Yes
Reviewers must have anonymous access to these standardized datasets that is	Raw Illumina sequence data is deposited in the NCBI sequence read archive (SRA) under accession SAMN26947704, SAMN26947705 and SAMN26947706 for the MC1,

free-of-cost. Please provide dataset locations and instructions for access here. If applicable, include accession numbers and reviewer tokens. Please consult these Author's guides for more information: Standardized datatypes, datatype-specific repositories, and general-purpose repositories recommended by Cell Press" and "How standardized datasets and original code accompany Cell Press manuscripts from submission through publication" or email us at current-biology@cell.com. as follow-up to "**Standardized datasets** A list of datatypes considered standardized under Cell Press policy is available here. Does this manuscript report new standardized datasets? "	ΔpheA-tyrA and ΔtyrR strains, respectively.
Original Code Does this manuscript report original code?	No

Dear Christine,

In accordance with our correspondence, here is our revised submission whose main text was condensed to 5000 words via careful editing, without compromising content!

The original cover letter follows:

Please consider our paper “Rational engineering of a synthetic insect-bacterial mutualism” for publication as an article in *Current Biology.* It was originally submitted to Cell and editor Dr. Scott Behie recommended transfer to Current Biology following consultation with Dr. Christine Cosma, who is already familiar with this work. It details the establishment and characterization of a synthetic insect-bacterial symbiosis involving a grain weevil host (*Sitophilus zeamais*) and *Sodalis praecaptivus*, a free-living relative and putative progenitor of the *Sodalis*-allied symbionts that are found in a diverse range of insect hosts where they often perform mutualistic (nutritional) functions.

To our knowledge this is the first description of a synthetic, laboratory engineered, insect-bacterial symbiosis, sustained over many insect generations by maternal (transovarial) transmission. It represents a major technical breakthrough that will facilitate molecular studies of symbiotic processes, advances in paratransgenic insect control and establish a platform for long term study of adaptation and genome degeneration in symbiosis, akin to Richard Lenski’s LTEE. Our work exploits the utility of the system in several important ways, providing answers

to key questions relating to the origin and establishment of these associations and the subsequent evolution of mutualistic functions. The origins of mutualistic symbiosis have long been debated by evolutionary biologists highlighting a causality dilemma, framed by the basic Darwinian notion that free-living organisms operate selfishly and should not surrender their resources or reproductive fate to another organism. Our work addresses this important question by obtaining an understanding of the factors that enable a bacterium to become intimately associated with an insect host and then adapt metabolic processes to facilitate nutrient provisioning.

Importantly, our work provides an empirical validation of the longstanding theory/notion that insect-bacterial symbioses arise as a function of infection by free-living bacteria. It shows that a free-living relative of the widespread *Sodalis*-allied insect symbionts has an intrinsic ability to maintain benign infection in an insect host and propagate in concert with insect developmental processes to achieve transovarial transmission. This leads to the rationalization that transovarially-transmitted symbioses (including many mutualisms) can only arise from bacterial partners that have these special capabilities, which likely arise as a consequence of vectorial relationships. This fits with the observation that certain bacterial genera, including *Sodalis*, are highly represented among mutualistic associates of insects, in spite of the fact that they often have distinct nutritional functions. Their ability to establish transovarially transmitted associations provides a critical foundation for mutualistic functions to evolve under the control of partner-fidelity feedback. In addition, our work also shows that a new symbiont can establish and co-exist along with a long-established native symbiont in grain weevils, exploiting adaptations that exist to facilitate bacterial maintenance. This challenges several observations

published in a paper focusing on the weevil symbiosis in Science in 2011 (https://www.science.org/doi/10.1126/science.1209728), further highlighting the value of this experimental approach in delineating interactions *in vivo*. Moreover, it validates longstanding notions that symbiosis has an autocatalytic quality, explaining observations of symbiont augmentation and replacement events in nature that are known to lead to integrative functionality (e.g. Bublitz et al., 2019, Cell 179, 703–712).

Field Code Changed

Further, we explore the nature of adaptation to mutualism by engineering the *Sodalis* proto-symbiont to modulate its aromatic amino acid (AAA) biosynthetic capabilities. Symbiont AAA provisioning has recently been shown to fuel insect cuticular sclerotization; enhancing strength, desiccation tolerance and resistance to predation and pathogen attack. Its widespread occurrence in two of the most specious and diverse groups of insects on our planet (beetles and ants) indicates that it has played a key role in their extraordinary niche expansion. To date, investigations focusing on this trait have relied on comparative genomic inferences, symbiont elimination and treatment with drugs (e.g. glyphosate) that block the microbial shikimate pathway. Our work, for the first time, employs the "gold standard" of microbial genetics to validate the functionality of this trait in symbiosis, further highlighting the power of this experimental system. It shows that nutrient overproduction can arise from single null mutations in the symbiont, leading to enhancement of fitness on the part of the insect host and yielding what we believe to be the first synthetic inter-kingdom mutualism.

We propose the following reviewers for the paper:

Abdelaziz Heddi, University of Lyon, France – expertise focusing on grain weevil symbiosis

Joel Sachs, UC Riverside, USA – evolutionary genomics and origins of symbiosis

Martin Kaltenpoth, Max Planck Institute for Chemical Ecology, Jena, Germany – ecology and evolution of symbiosis

Gordon Bennett, UC Merced, USA – evolution, genomics and ecology of insect-bacterial symbioses

Naomi Pierce, Harvard University, USA – ecology and evolution of symbiosis

We request reviewer exclusions for **Elad Chiel** (U Haifa, Israel) and **John McCutcheon** (Arizona State University) due to ongoing collaborations.

Thanks for considering our paper!

Crystal Su, Colin Dale and co-authors

Dear Christine and Maddie,

Here is our revised manuscript. The responses to editorial and reviewer comments are as follows:

From an editorial perspective, the paper is in great shape. My colleague Maddie Wilson and I have gone over the paper and we have some items for your attention.

PRODUCTION POINTS

- Please download a copy of our Inclusion and Diversity form, fill it out electronically, and upload the completed form as a submission item along with your final submission. For more information, please see our Author Guidelines and FAQ page. This form will not be included in the combined PDF, but it will come through to us with your submission.

- If you have chosen to publish an Inclusion and Diversity statement, we also ask that any statements selected on the form be included in the manuscript in a section titled “Inclusion and Diversity” following the Declaration of interests section. For more information, please see our Author Guidelines and FAQ page.

Response:
The Inclusion and Diversity statement has been added into the manuscript

- in your supplemental data PDF, please move each item legend to below it’s related item.

Response:
Done

- Please make the page with Table S1 landscape.

Response:
Done.

- If possible, please align all panel letters to the top left of their sub-panels in all figures.

Response:
Done

- There are some unformatted characters on line 1346.

Response:

This was an MS word version incompatibility that only appears on certain word versions. As far as we can tell, it is fixed.
The text should be:
72. Flórez, L.V., Scherlach, K., Gaube, P., Ross, C., Sitte, E., Hermes, C., Rodrigues, A., Hertweck, C., and Kaltenpoth, M. (2017). Antibiotic-producing symbionts dynamically transition between plant pathogenicity and insect-defensive mutualism. Nat. Commun. *8*, 1-9.

STAR METHODS POINTS

- Please review the Data Availability instructions in the STAR Methods guidelines. There are three required statements in this section.

Response:
Added

- misformatted reference on line 686, 857; please check the manuscript thoroughly

Response:
Corrected

-Section on bacterial strains: how were these cultured/maintained?

Response:
Modified to:

"This study involved the use of *Sodalis praecaptivus* strain HS, which is a close relative of insect-associated *Sodalis* spp. symbionts that was isolated from an infected human[24,34], and has been previously deposited in the American Type Culture Collection (ATCC) as product BAA-2554. For all experiments outlined in the study, it was cultured in LB liquid media and plated on LB agar with appropriate antibiotics as outlined in method details, presented below, at 30 °C, under atmospheric air. Strains were preserved at -80 °C in LB media with 15% (w/v) glycerol."

- lines 713, 756, etc. These sections should not be numbered. It's fine to have this level of subheading--just remove the numbers please. Please revise throughout the STAR Methods to remove the numbers from the subheadings

Response:
Corrected as suggested. We also did this for the section on microinjection in STAR method (which you may have missed and had the same problem)

-Similarly, please revise the QUANTIFICATION AND STATISTICAL ANALYSIS section to remove numbered lists.

Response:
Corrected as suggested

KEY RESOURCES TABLE

-When there are more than 10 oligos, we request that they instead be reported in a separate supplemental table. This can be labeled as Table S2 and be included in the supplemental information PDF. In the KRT, then please just write "See Table S2, Oligonucleotides used in this study" (or some such language).

Response:
Corrected as suggested

- for *Sitophilus zeamais*, is there a link that you can include in case someone would like to obtain them or get more information?

Response:
Added the link to KRT:
"https://www.ars.usda.gov/plains-area/mhk/cgahr/"

Responses to review comments:

Reviewer #1: The authors have made a major revision of the manuscript based on the comments of reviewers including mine. I am happy with the revision and would like to recommend the acceptance of the article for publication in Current Biology.

Reviewer #2:

While going through the manuscript (thanks for the line numbers!), I noted a few minor issues in intro and discussion that the authors may want to consider: l. 34: "beetles and weevils": weevils are beetles…

Response:
Changed to "weevils and other beetles"

l. 52: introducing the weevil here seems a bit odd and too early, especially if you place it as the only example here

Response:

Changed to reduce this emphasis:

“Acquisition of nutritional symbionts allows many insects (including the grain weevils, *Sitophilus* spp., highlighted in this study) to persist on diets that are nutritionally imbalanced or incomplete and has facilitated substantial niche expansion in insects, contributing greatly to the ecological success[12]. ”

l. 54-55: in my view, there are substantial issues with the paper that the authors cite here. I strongly suggest toning down the claim. It is well-supported that symbioses allows insects to invade novel ecological niches, but the effect on radiations, while very plausible, remains poorly substantiated by actual data. If you want, you can keep the "ecological success", since this is fuzzier and certainly supported by the tremendous body of literature on insect symbioses. (same in l. 363)

Response:
Changed as shown in the previous response and in line 363:

“Insects have served as important models for study because symbiosis has made an exceptional contribution to their ecological success[50].”

l. 56: Sorry, I just noticed this now, and not in the original version: As stated, this is not correct. There are definitely insect endosymbionts that derive from already host-associated bacteria, e.g. plant or insect pathogens (the latter of which, depending on the definition, you may or may not be including in the term endosymbiont. Regardless of this, there are other routes to insect endosymbiosis than from environmental bacteria). Please specify or narrow down.

Response:
Changed as follows:

“Insect endosymbionts are often derived from environmental progenitors with large gene inventories and capability to synthesize myriad nutrients (e.g. essential amino acids and vitamins) that many eukaryotes cannot synthesize *de novo*[13-15]. ”

l. 81: Given that they are usually considered absent (but I agree with reviewer 3 to be careful here), I suggest to say "Further, these bacteriomes are absent or markedly reduced in size…"

Response:
Corrected as suggested.

l. 440/1: Please streamline to say "Together, this provides a genetic validation of …"

Response:
Corrected as suggested.

l. 443: ref 58 does not seem to fit (is not on beetles at all), and refs 62 and 63 do not provide results that support such a broad claim. Please tone down, as the impact of tyr-provisioning symbioses on beetle and ants radiations remains speculative.

Response:
ref 58 was removed. Statement toned down as follows:

"Together, this provides a genetic validation of the role of symbionts in Tyr/Phe production and cuticular sclerotization, which is thought to have played an important role in the radiation of beetles[62] and ants[63] by enhancing strength, desiccation tolerance and predator/pathogen resistance[64,65]."

Reviewer #3:
The manuscript has been appropriately revised, except for the following minor issues.
L. 318: broader

Response:
Corrected.

L. 443: ref. 58 probably does not apply here.

Response:
Ref 58 is removed.

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The authors declare no competing interests

On behalf of all authors, I declare that I have disclosed all competing interests related to this work. If any exist, they have been included in the "declaration of interests" section of the manuscript.

Name: Colin Dale

Manuscript number (if available):

Graphical abstract

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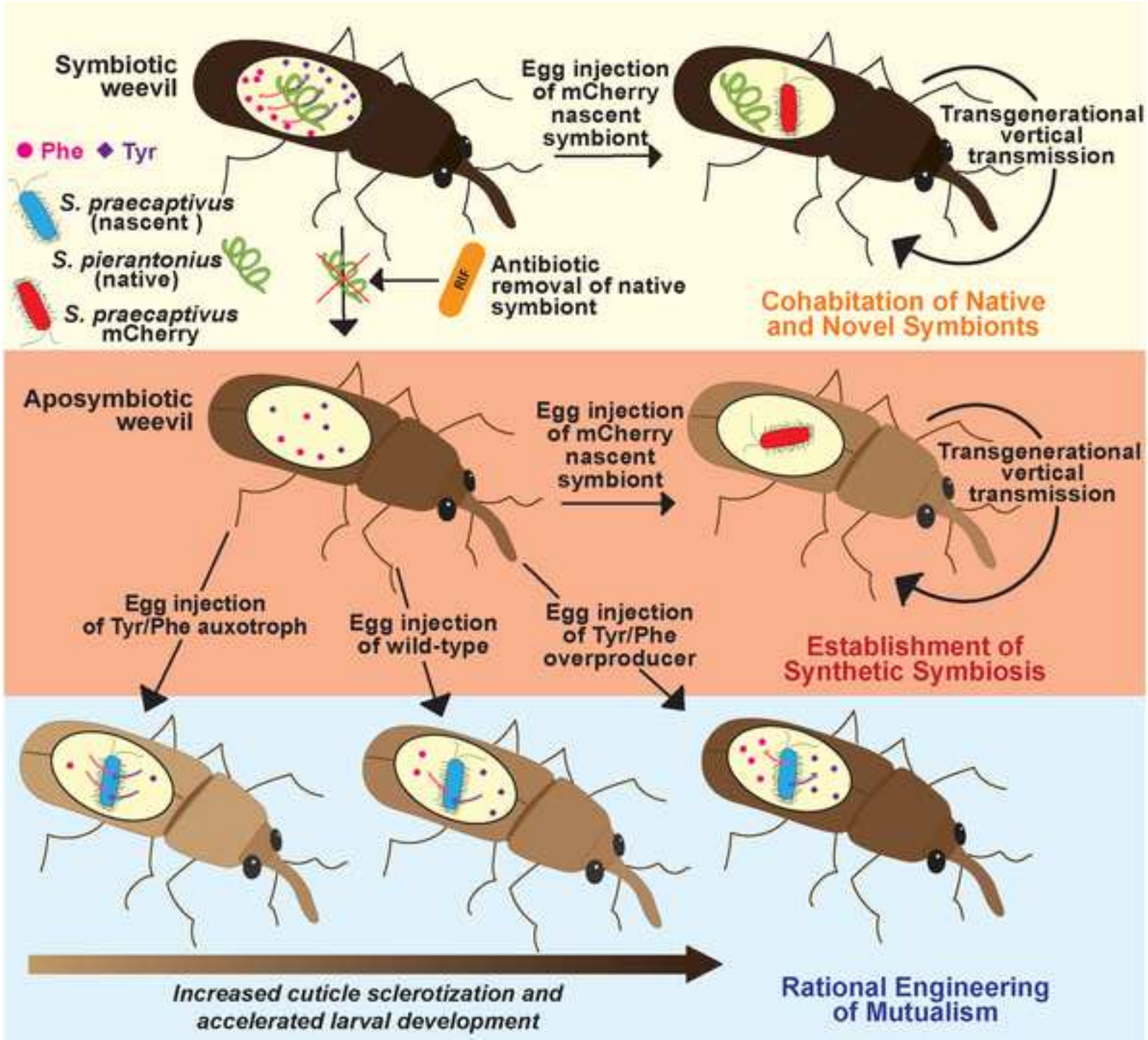

Rational engineering of a synthetic insect-bacterial mutualism

Yinghua Su*, Ho-Chen Lin, Li Szhen Teh, Fabienne Chevance, Ian James, Clara Mayfield, Kent G. Golic, James A. Gagnon, Ofer Rog, Colin Dale[1*]

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SUMMARY

Many insects maintain mutualistic associations with bacterial endosymbionts, but little is known about how they originate in nature. In this study, we describe the establishment and manipulation of a synthetic insect-bacterial symbiosis in a weevil host. Following egg injection, the nascent symbiont colonized many tissues, including prototypical somatic and germinal bacteriomes, yielding maternal transmission over many generations. We then engineered the nascent symbiont to overproduce the aromatic amino acids, tyrosine and phenylalanine, that facilitate weevil cuticle strengthening and accelerated larval development, replicating the function of mutualistic symbionts that are widely distributed among weevils and other beetles in nature. Our work provides empirical support for the notion that mutualistic symbioses can be initiated in insects by the acquisition of environmental bacteria. It also shows that certain bacterial genera, including the *Sodalis* spp. used in our study, are predisposed to developing these associations due to an ability to maintain benign infections and undergo vertical transmission in diverse insect hosts, facilitating the partner fidelity feedback that is critical for the evolution of obligate mutualism. These experimental advances provide a new platform for laboratory studies focusing on the molecular mechanisms and evolutionary processes underlying insect-bacterial symbiosis.

INTRODUCTION

Insects are one of the most successful and diverse groups of animals with 10% of species estimated to harbor obligate mutualistic bacterial endosymbionts[1,2]. Endosymbionts enhance insect fitness by providing essential nutritional supplements[3,4] or protection against enemies[5-8], stress[9] or toxins[10,11]. Acquisition of nutritional symbionts allows many insects (including the grain weevils, *Sitophilus* spp., highlighted in this study) to persist on diets that are nutritionally imbalanced or incomplete and has facilitated substantial niche expansion in insects, contributing greatly to the ecological success[12].

Insect endosymbionts are often derived from environmental progenitors with large gene inventories and capability to synthesize myriad nutrients (e.g. essential amino acids and vitamins) that many eukaryotes cannot synthesize *de novo*[13-15]. Following establishment of mutualistic associations, endosymbionts undergo a degenerative mode of evolution, facilitating (i) loss of metabolic functions shared with the insect host and (ii) retention and potentiation of functions beneficial to the fitness of the association, including nutrient-provisioning pathways. These changes lead to establishment of associations in which partners are obligately co-dependent and metabolically integrated[15-17]. Consequently, hosts often cannot be reared without their symbiotic partners, which often cannot be cultivated outside their hosts (e.g. in laboratory media), constraining experimentation. One essential and defining aspect that remains poorly understood is the transition to stable vertical symbiont transmission, requiring establishment of infection in reproductive tissues and developing

oocytes. Interestingly, certain bacteria (e.g. *Sodalis* spp., *Arsenophonus* spp., *Spiroplasma* spp.) are predisposed to developing relationships with insects[15,18,19], suggesting maintenance of specialized properties that facilitate this outcome.

Grain weevils provide an excellent model to study establishment of symbiosis because it is possible to remove their native bacterial symbiont (*Sodalis pierantonius*) through antibiotic treatment and maintain resulting aposymbiotic (symbiont-free) weevils in the laboratory[20]. Previous studies have shown that *S. pierantonius* supplements its host with vitamins and amino acids[21]. Notably, it secretes tyrosine and phenylalanine during larval and early adult stages to facilitate cuticle strengthening[20]. In addition, it triggers development of bacteriomes, housing symbionts and protecting them from insect innate immunity[22,23]. Further, these bacteriomes are absent or markedly reduced in size in aposymbiotic insects, indicating that symbiotic interactions influence host developmental processes[20].

Interestingly, the symbiosis involving weevils and *S. pierantonius* is recent in origin[15,24,25]. In addition, a diverse range of insects harbor *Sodalis*-allied symbionts that perform distinct nutritional functions (e.g. mealybugs[26], tsetse flies[27], seal lice[28], louse flies[29], stinkbugs[30], lygaeoid bug[31], psyllids[32]). This suggests that free-living *Sodalis* spp. have repeatedly and independently colonized insects inhabiting a wide range of niches[33], catalyzing novel mutualistic relationships with diverse functions. Several studies have exploited the use of a close free-living relative of the *Sodalis*-allied symbionts, named *S. praecaptivus*[24]. This bacterium has a relatively large genome with a high coding

density and few pseudogenes, consistent with the notion that it evolves under strong stabilizing selection in a free-living/opportunistic lifestyle. Comparative studies indicate that related insect symbionts have gene inventories that are subsets of *S. praecaptivus*. They are substantially reduced in coding content, indicating that they have evolved degeneratively, under a relaxed selection pressure facilitating loss of gene functions that lack adaptive value in symbiosis. Because *S. praecaptivus* is amenable to culture[34] and genetic manipulation[35] and yields stable and benign infections in insect hosts that naturally harbor *Sodalis*-allied symbionts[36,37], it has proved useful in studying the mechanistic interactions underpinning symbiosis. These studies are performed by microinjecting adult insects with mutant strains of *S. praecaptivus* and examining their effects. However, in the case of grain weevils, which are oviparous and therefore require bacterial infection of oocytes in female ovaries to facilitate vertical transmission, *S. praecaptivus* is not observed to be maternally transmitted following adult microinjection[36]. Tsetse flies, which are viviparous and nourish developing larvae via milk gland secretions during pregnancy[38], undergo a low frequency of vertical transmission of *S. praecaptivus* following adult microinjection[37], but it is insufficient to facilitate experimentation.

One explanation for the inability of *S. praecaptivus* to achieve vertical transmission in grain weevils following adult microinjection is that bacteria may need to establish infection in germline stem cells. This makes sense considering our understanding of the natural association between grain weevils and *S. pierantonius*, in which adult weevils maintain two populations of symbiotic

bacteria[20]: “germinal” (facilitating maternal transmission) and “somatic” (facilitating nutrient production).

In this study, we describe a protocol for microinjection of *S. praecaptivus* into eggs of the grain weevil *Sitophilus zeamais*, resulting in sustained vertical transmission over multiple insect generations, providing a means for partner-fidelity feedback to facilitate evolution of mutualistic functions. We use this new experimental platform to introduce mutant strains of *S. praecaptivus* with modified tyrosine and phenylalanine biosynthetic capabilities. Notably, these strains significantly impact weevil cuticle sclerotization and larval development time, providing a clear genetic validation of the role of aromatic amino acid production in this symbiosis. This work demonstrates that a *S. praecaptivus* mutant with a single *tyrR* gene knockout can overproduce tyrosine and phenylalanine to impact host cuticle sclerotization and reduce larval development time, signifying that the relationship is mutualistic.

RESULTS

Egg injection establishes a synthetic, insect-bacterial symbiosis

We developed a procedure for microinjection of *S. praecaptivus* into grain weevil eggs to test the hypothesis that egg infection leads to vertical transmission of bacteria. This procedure uses a modified *Drosophila* egg microinjection protocol, followed by transplantation of larvae into grain, facilitating development to adulthood. The procedural efficiency was monitored for a batch of injections

139 performed on 96 aposymbiotic weevil eggs. Herein, 40% (38/96) of eggs incurred
140 lethal damage during isolation and preparation for injection. Out of the remaining
141 58, 50% (29/58) survived and yielded larvae. Following transplantation into
142 maize, 21% (6/29) of larvae completed development and emerged as adults. All
143 six demonstrated mCherry fluorescence, indicative of *S. praecaptivus* MC1
144 infection. First instar larvae maintained 3.74×10^4 bacterial CFU / larva (Mean;
145 SD = 2.88×10^4), increasing to 7.95×10^6 CFU / weevil (Mean; SD = 2.19×10^6)
146 in newly emerged adults.

147 Following injection, weevils were monitored using fluorescence
148 microscopy to track bacteria. Uninjected weevils demonstrated no mCherry
149 fluorescence in egg, larval or adult stages **(Figure S1A-D).** Following injection
150 into the egg posterior pole (**Figure 1A**), bacteria proliferated at the injection site
151 (**Figure 1B**) and then migrated through the embryo, achieving dense infection in
152 the developing gut (**Figure 1C**) and resulting first instar larvae (**Figure 1E**).
153 Following metamorphosis, adults demonstrated widespread mCherry
154 fluorescence (**Figure 1F**), consistent with the presence of *S. praecaptivus* MC1
155 in hemolymph (**Figure S2A**), and other tissues. Adult ovaries harbored *S.*
156 *praecaptivus* MC1 in several regions (**Figure 1I**) including the tropharium apex
157 where the native symbionts of grain weevils are localized[20].

158 To further explore the utility of this technique, we injected a mCherry-
159 expressing *S. praecaptivus* strain lacking *ypeI*, encoding an N-acyl homoserine
160 lactone synthase involved in quorum sensing. This strain kills weevils following
161 microinjection into adults because quorum sensing represses expression of

virulence factors, including insecticidal toxins[36]. Following egg injection, this strain proliferated rapidly in eggs, revealing dense infection after four days (**Figure 1D**). Out of 55 eggs injected with this strain, only one (uninfected) larva emerged, indicating that the Δ*ypeI* strain efficiently kills eggs. This illustrates the utility of the egg microinjection procedure in exploring molecular mechanisms of symbiosis throughout the entire developmental cycle of the host.

Egg injection yields sustained vertical transmission of *S. praecaptivus*

To determine if *S. praecaptivus* MC1 undergoes vertical transmission following egg injection, we tracked ten generations of weevils derived from a single isofemale and isomale aposymbiotic weevil pairing that were successfully infected by egg microinjection. From this line, ten randomly selected F_1 offspring were found to be infected with *S. praecaptivus* MC1, having an average of 2.5×10^6 CFU / weevil (SD = 2.09×10^6). Fluorescence microscopy revealed *S. praecaptivus* MC1 in those F_1 eggs and larvae, confirming that bacteria had been acquired vertically (**Figure 1G-H**). In the F_2 generation, the adult infection frequency declined to 50% (n = 20), with an average number of 1.39×10^6 CFU / weevil (SD = 1.04×10^6), excluding two samples that were considered outliers having very low densities of bacteria. Based on the decline in the F_2 generation, we elected to maintain only weevils showing mCherry fluorescence to serve as parents for the F_3 generation. This selection was repeated at generational intervals throughout the experiment to ensure that sufficient number of weevils maintained *S. praecaptivus* MC1. The rate of vertical transmission and infection

density remained relatively constant in subsequent generations (**Figure 2A**). The high level of transmission from P_0-F_1 stage is likely explained by high numbers of bacterial cells in eggs following injection, yielding a high level of infection in female ovaries, consistent with the observation that bacterial infection densities in adult weevils were higher in P_0 individuals and settled to a lower consistent level in subsequent generations. Nine additional *S. praecaptivus* MC1-injected aposymbiotic isofemale lines were established to assess repeatability of the procedure. While all yielded *S. praecaptivus* MC1-infected offspring, the transmission rate varied from 20% to 100%, with a median of 95% and mean of 78% (**Figure 2B**) and an aggregate average of 1.21×10^6 CFU / weevil (SD = 1.83×10^6). Differences may arise due to variation in the age of the eggs (0-24 h), a factor known to affect *Drosophila* egg microinjection as well[39]. Alternatively, variation in the bacterial inoculum or the precise site of the injection may affect the success of the procedure.

While the majority of our experiments were performed on aposymbiotic weevils, we were also interested to determine how symbiotic weevils, harboring their native symbiont (*Sodalis pierantonius*), responded to introduction of *S. praecaptivus* MC1 into their eggs. Notably, the procedure was also successful with symbiotic weevils, yielding P_0 adults with average infection density of 9.95×10^6 CFU / weevil (SD = 1.25×10^7). However, a substantially lower level of transmission was observed relative to aposymbiotic weevils (Mean = 19%) with only four of nine isofemale lines producing *S. praecaptivus* MC1-infected offspring (with aggregated average of 3.47×10^6 CFU / weevil; SD = 3.91×10^6;

208 **Figure 2B**). This indicates that *S. praecaptivus* and *S. pierantonius* can coexist
209 and be transmitted simultaneously but that *S. praecaptivus* transmission is
210 constrained by the presence of the native symbiont, which is transmitted with
211 ~100% efficiency in our laboratory population.

212 Throughout our experiments, in order to identify weevils infected with *S.*
213 *praecaptivus* MC1, we employed a simple screening method in which live insects
214 were inspected for mCherry fluorescence. However, this detection method could
215 fail to identify weevils that maintain low-density infections. Yet, a low-density
216 infection could be sufficient to lead to transmission of bacteria to offspring,
217 leading to an underestimate of transmission frequency. To evaluate this, 30
218 offspring from 30 non-fluorescent parents (F_6 derivatives) were checked for *S.*
219 *praecaptivus* MC1 by homogenization and plating. Notably, none of those weevil
220 homogenates yielded *S. praecaptivus* MC1 colonies, indicating that absence of
221 fluorescence in parents is strongly correlated with the absence of bacteria in
222 offspring.

223

224 **Dynamics of *S. praecaptivus* transmission**

225 To determine if transmission/maintenance of *S. praecaptivus* MC1 is biased
226 towards offspring sex, we selected 100 random offspring from generation F_6 of
227 the infected aposymbiotic weevils, checked them for mCherry fluorescence and
228 dissected them to determine sex. No significant difference existed between
229 sexes with 29/40 males and 37/60 females harboring infections, X^2 (1, N = 100) =
230 1.255, p > 0.05. To determine if *S. praecaptivus* MC1 is associated with

231 increased development time, 30 mated *S. praecaptivus* MC1-infected F_6 weevils
232 oviposited for three days and their offspring emergence time and infection status
233 were tracked. Kaplan-Meier analysis revealed no significant difference in
234 development time between infected and uninfected weevils (**Figure 2C**; p =
235 0.34). To investigate stability of the *S. praecaptivus* infection throughout
236 development, 20 1st instar larvae and 20 adult offspring were collected from 30
237 infected F_7 parents, for bacterial enumeration. Their infection frequencies
238 demonstrated no significant difference between larvae (40%; 8/20) and adults
239 (45%; 9/20; X^2 (1, N = 40) = 0.102, p > 0.05), indicating robustness over the
240 course of development. To check if transmission of *S. praecaptivus* MC1 is
241 influenced by female reproductive age, six *S. praecaptivus* MC1-infected F_6
242 females in the first 14 days of adulthood were allowed to oviposit for three weeks
243 on fresh maize and adult offspring were collected until no more emerged.
244 Offspring were homogenized and plated to determine infection. We then
245 compared the first seven and last seven offspring from each female, revealing no
246 significant difference in infection frequency (**Figure 2D**; X^2 (1, N = 43) = 8.712, p
247 > 0.05.). Finally, we performed a crossbreeding experiment using unmated F_6
248 weevils to determine sexual dynamics of *S. praecaptivus* transmission. Six pairs
249 were assembled for mating, three of which comprised an uninfected male and
250 infected female and three of which comprised the reciprocal combination.
251 Offspring from each pairing (n = 30) were homogenized and plated to check for
252 infection. All offspring maintaining *S. praecaptivus* MC1 were derived from
253 infected females, indicating exclusively maternal transmission.

Throughout this study, no morphological or behavioral abnormalities were observed in either aposymbiotic or symbiotic weevils at any life cycle stage following establishment of *S. praecaptivus* MC1. Further, following microinjection, adult weevils emerged at a median 45 days for aposymbiotic weevils and 41 days for symbiotic weevils following transfer into grain. This is comparable to the uninfected weevils subjected to identical husbandry lacking only the microinjection (aposymbiotic median 44.5 days; symbiotic median 39 days).

S. praecaptivus colonizes prototypical bacteriomes in grain weevils

The grain weevils native symbiont, *S. pierantonius*, resides in specialized bacteriomes at the anterior of the midgut in larvae, the midgut mesenteric caeca in young adults and ovaries of adult females[20]. To determine if *S. praecaptivus* MC1 infects the same tissues in aposymbiotic weevils, we visualized tissues of F_1 larvae and adults. Both larval and adult bacteriomes that are potentiated in symbiotic weevils are colonized intracellularly by *S. praecaptivus* MC1 in both aposymbiotic and symbiotic weevils, albeit at higher density in the latter (**Figure 3; Figure S2B**). Thus, only *S. pierantonius,* induces larval and gut bacteriome cell proliferation[40,41]. Symbiotic weevils featured fully formed larval and adult bacteriomes, densely infected with both *S. praecaptivus* MC1 and *S. pierantonius* (**Figure 3A** and **3D**), mimicking experimental outcomes observed in aphids[42,43]. Because *S. pierantonius*, has a distinct morphology (**Figure 3B**), microscopy clearly revealed both bacterial species inside the same larval and adult

bacteriome cells (**Figure 3C and 3E**). Bacteriomes from uninjected weevils demonstrated no mCherry fluorescence (**Figure S1E and F**).

S. praecaptivus **infects weevil eggs at early stage of oogenesis**

Figure 1I shows widespread infection of *S. praecaptivus* MC1 in the ovary. For more in depth characterization, we performed confocal microscopy on ovaries from *S. praecaptivus* MC1-infected F_1 females (**Figure 4**). In the telotrophic weevil reproductive system, germ cells are localized in a transition zone between the tropharium and vitellarium. The developing oocytes receive nutrients from nurse cells in the tropharium via nutritive cords[44]. *S. praecaptivus* MC1 was present in tropharium cells in both aposymbiotic (**Figure 4A**) and symbiotic (**Figure 4B**) weevils, suggesting bacteria could be transmitted to developing oocytes from nurse cells. However, *S. praecaptivus* MC1 also infected the zone between the tropharium and vitellarium (**Figure 4C**), containing pro-oocytes, along with central and lateral prefollicular cells[45]. To facilitate oocyte development, pro-oocytes are encapsulated by prefollicular cells in the vitellarium to form egg chambers. Even the most proximal oocytes in the weevil vitellarium maintained *S. praecaptivus* in the oocyte and surrounding follicular cells, indicating that oocytes are infected at a very early stage of development.

In order to confirm that adult injection does not facilitate establishment of *S. praecaptivus* infection that is maternally transmitted, we performed an experiment in which weevils were injected at adult stage with *S. praecaptivus* MC1. Out of 33 weevils, 28 developed mCherry fluorescence, indicating

infection. However, following mating, no offspring displayed mCherry fluorescence or yielded *S. praecaptivus* colonies when their homogenates were plated (n=30), confirming that adult injection does not lead to vertical transmission. Imaging of adult-injected weevil ovaries revealed *S. praecaptivus* MC1 attached to the exterior of the tropharium (**Figure S2C**) and vitellarium (**Figure S2D**), but no infection inside these structures. This was also occurred with midgut mesenteric ceca, which demonstrated only surface colonization with *S. praecaptivus* MC1 following adult microinjection (**Figure S2E**).

Rational engineering of a functional mutualism

Knowing that *S. pierantonius* produces tyrosine and phenylalanine that promotes cuticular sclerotization[20], we engineered strains of *S. praecaptivus* with modified biosynthetic capabilities. These encompass a Tyr/Phe auxotroph (Δ*pheA-tyrA*) and numerous candidate Tyr/Phe overproducing strains that were identified during rational engineering approaches in *E. coli*[46]. While several mutant *S. praecaptivus* strains (Δ*tyrR*, Δ*nuoN,* Δ*csrA*, Δ*zwf* and Δ*mdh*) demonstrated Tyr/Phe cross-feeding (**Figure 5A**; **Figure S3A**), the Δ*tyrR* strain was selected for our experiments because TyrR functions specifically as a repressor for genes of aromatic amino acid biosynthesis[47], whereas the other mutants are anticipated to have broader impacts on metabolic processes, potentially impacting the symbiosis. Tyr/Phe secretion was then confirmed for the Δ*tyrR* strain, using a liquid assay (**Figure 5B**). Δ*pheA-tyrA*, Δ*tyrR* and WT strains were then

321 introduced into aposymbiotic weevil eggs. These strains lacked mCherry, in order
322 to avoid confounding subsequent cuticle color assays.

323 Following injection of Δ*pheA-tyrA*, Δ*tyrR* and WT *S. praecaptivus* into
324 aposymbiotic eggs, two week old adults were collected for imaging along with
325 uninjected aposymbiotic and symbiotic grain weevils of the same age. Following
326 imaging, weevils were homogenized and plated to characterize their infections.
327 Color analysis was performed on a common quadrant of the weevil cuticle under
328 controlled lighting conditions to ensure consistency (**Figure 5C**). Lighter cuticle
329 coloration (increased red pigmentation) indicates decreased cuticular
330 sclerotization and reduced symbiont Tyr/Phe biosynthesis[20]. Accordingly,
331 aposymbiotic grain weevils had cuticles with significantly higher red coloration
332 than symbiotic counterparts ($p < 0.0001$). Among aposymbiotic weevils harboring
333 *S. praecaptivus*, those with auxotrophic Δ*pheA-tyrA* had the reddest cuticles.
334 Weevils with WT *S. praecaptivus* were significantly darker than those with
335 Δ*pheA-tyrA* ($p < 0.01$), but were significantly lighter than uninjected
336 aposymbionts ($p < 0.05$). This suggest that *S. praecaptivus* depletes host
337 Tyr/Phe; an effect that is exacerbated with an auxotrophic strain that cannot
338 synthesize Tyr/Phe *de novo*. Strikingly, weevils harboring the Δ*tyrR* overproducer
339 had cuticles at least as dark as those of aposymbionts (no significant difference).
340 Notably, the WT and Δ*tyrR S. praecaptivus* strains maintained very similar
341 densities in the weevil (*t*-test; $p = 0.84$), indicating that color differences could not
342 be explained as a function of change in the burden of infection.

343 To further assess impact on weevil fitness we compared larval
344 development times of symbiotic and aposymbiotic weevils, including
345 aposymbionts maintaining WT, Δ*pheA-tyrA* or Δ*tyrR* strains. Results (**Figure 5D**)
346 show that symbiotic weevils have the shortest larval development time,
347 consistent with *S. pierantonius* providing the greatest fitness benefit. No
348 significant time differences were observed between uninjected aposymbionts and
349 either (i) aposymbionts injected with WT or (ii) aposymbionts injected with
350 Δ*pheA-tyrA* ($p > 0.05$). However, aposymbionts injected with Δ*tyrR* showed
351 accelerated larval development compared to non-injected aposymbionts ($p <$
352 0.001), indicating that the Δ*tyrR* strain yields a beneficial (mutualistic) outcome,
353 implying that symbiont Tyr/Phe production is also beneficial prior to adulthood.
354
355
356 **DISCUSSION**
357 Mutualistic inter-kingdom interactions involving microorganisms and animals/
358 plants are common and have facilitated many important innovations including
359 aerobic energy generation, photosynthesis and nitrogen fixation[48]. They create
360 new biology from components with exclusive functions, catalyzing exploitation of
361 novel niches, reducing the burden of competition[49]. Insects have served as
362 important models for study because symbiosis has made an exceptional
363 contribution to their ecological success[50]. However, the origin of these
364 associations remains poorly understood[51]. This is partly due to the fact that
365 certain mutualistic adaptations are anticipated to be maladaptive in a free-living

366 state, leading to a causality dilemma. For example, the sharing of nutritional
367 resources in mutualism is contraindicated in the free-living state where
368 individuals must compete to acquire resources for growth. Further, mutualists
369 must overcome natural antagonistic interactions (immunity) to forge an intimate
370 association that mediates partner fidelity feedback necessary for selection to
371 optimize mutualistic functionality[52,53].

372 Here, we established, characterized and engineered a synthetic insect-
373 bacterial symbiosis to gain insight into the nature and complexity of adaptations
374 facilitating mutualism. Following development of a protocol to introduce a nascent
375 candidate symbiont, *S. praecaptivus,* into the eggs of aposymbiotic grain weevils
376 (*Sitophilus zeamais*), we monitored weevils that maintained the association over
377 ten generations through maternal transmission with ~50% efficiency per
378 generation. Our results demonstrate that *S. praecaptivus* undergoes sustained
379 vertical transmission in a novel host, providing a model for long-term study of
380 symbiotic interactions and evolutionary processes in symbiosis. Notably, the
381 association can be maintained by selection of insects that display mCherry
382 fluorescence at generational intervals. Our results show that cyclical vertical
383 transmission mandates introduction of *S. praecaptivus* into eggs, mimicking
384 natural processes of transovarial transmission, as documented for native, *S.*
385 *pierantonius*, in weevils[54]. This accords with *S. praecaptivus* establishing
386 infection in germ and/or stem cells such that subsequent differentiation
387 processes propel infection into mature larval and adult tissues, including
388 ovarioles. Although our experiment focused on introduction of bacteria into eggs,

389 it is possible that establishment could occur later in development when, for
390 example, larvae commence movement/feeding and may encounter injuries that
391 provide opportunities for bacterial entry.

392 Our work also shows that *S. praecaptivus* can establish infection in
393 weevils that maintain their native symbiont, *S. pierantonius*. In those weevils,
394 both bacteria reside in somatic and germinal bacteriomes in the gut and ovary[20].
395 Previous work demonstrated production of specialized antimicrobial peptides
396 (coleoptericins) that (i) prevent growth of symbionts outside of bacteriomes and
397 (ii) control their proliferation inside bacteriomes by inducing bacterial cell
398 filamentation[55]. However, in our study, *S. praecaptivus* was observed infecting a
399 range of weevil tissues, displaying no evidence of filamentation, indicating lack of
400 susceptibility to these effects.

401 We found that *S. praecaptivus* and *S. pierantonius* co-exist in germinal
402 apical bacteriomes, transmitting together, albeit at lower efficiency for *S.*
403 *praecaptivus*. Localization of *S. praecaptivus* in ovarian tissues of aposymbiotic
404 weevils revealed colonization of multiple cell types within ovarioles, including pro-
405 oocytes and prefollicular cells assembling during oogenesis, along with nurse
406 cells that sustain developing oocytes. This provides several, redundant, potential
407 opportunities for transmission, possibly enabling members of the genus *Sodalis*
408 to undergo transmission in insects with diverse (panoistic, polytrophic and
409 telotrophic) reproductive systems. It likely represents another factor explaining
410 the success of *Sodalis* spp. in the board colonization of insects in nature[15].
411 Notably, *S. praecaptivus* is transmitted to eggs at a very early stage of

412 oogenesis, in contrast to several other insect symbionts that are transmitted at
413 later stages. For example, the aphid symbiont (*Buchnera*) is transmitted from
414 maternal bacteriocytes to blastulae in the ovariole tips of pathenogenetically-
415 reproducing aphids at oogenesis stage seven[43], or when eggs reach a size of
416 500μm in aphids reproducing oviparously[56]. Further, *Spiroplasma* enters
417 *Drosophila* oocytes when lipid transport channels open at oogenesis stage ten[57],
418 and *Wolbachia* transmission takes place in the telotrophic planthopper,
419 *Laodelphax striatellus*, during vitellogenin transovarial transportation, which also
420 takes place at a later stage of oogenesis[58].

421 The native symbiont of grain weevils, *S. pierantonius*, produces tyrosine
422 and phenylalanine that facilitate cuticle sclerotization, yielding adult weevils that
423 have a tough, dark exoskeleton[20,59,60]. However, our work shows that wild type *S.*
424 *praecaptivus* does not engage in Tyr/Phe secretion as demonstrated by
425 laboratory cross-feeding assays. Correspondingly, adult weevils injected with WT
426 or auxotrophic strains of *S. praecaptivus* have cuticles that are lighter in color
427 than those of aposymbiotic counterparts, indicating reduced sclerotization,
428 consistent with the notion that host Tyr/Phe levels are depleted by this bacterium.
429 In order to generate a mutualistic strain of *S. praecaptivus*, we employed rational
430 engineering[61] to identify a mutant strain of *S. praecaptivus* (Δ*tyrR*) that
431 overproduces and cross-feeds Tyr/Phe to an auxotroph[46,47]. Introduction of this
432 strain into weevil eggs resulted in the production of adults whose cuticle color
433 was restored to that of uninfected (aposymbiotic) counterparts. Further, weevils
434 maintaining the Δ*tyrR* strain had significantly reduced larval development time

relative to their aposymbiotic (uninfected) counterparts, implying that Tyr/Phe production is also beneficial in the context of larval development and seems to present a more sensitive signal for symbiont Tyr/Phe provisioning in our synthetic system. Of course it is possible that *S. praecaptivus* simply produces more Tyr/Phe in the larval stage or that it provides additional beneficial metabolites that selectively impact larval development. Together, this provides a genetic validation of the role of symbionts in Tyr/Phe production and cuticular sclerotization, which is thought to have played an important role in the radiation of beetles[62] and ants[63] by enhancing strength, desiccation tolerance and predator/pathogen resistance[64,65].

Tyrosine and phenylalanine overproduction and secretion were observed to result from several single gene knockouts in *S. praecaptivus* (Δ*tyrR*, Δ*nuoN,* Δ*csrA*, Δ*zwf* and Δ*mdh*). Since null mutants are anticipated to arise spontaneously in natural populations of bacteria in the environment, this suggests that insects can readily acquire bacterial strains capable of secreting specific nutrients as a consequence of spontaneous mutations. In support of this, many examples of nutrient cross-feeding have been identified in natural microbial communities that increase the collective efficiency of resource utilization[66,67]. Taken together, these results suggest that adaptation to nutrient secretion is not a significant bottleneck in the evolution of mutualistic associations that focus on nutrient provisioning. Further support for this notion was obtained in a recent study showing that mutualism could be established between a stinkbug and an *E. coli* strain that was experimentally evolved to facilitate mutualism in this host[68].

458 However, in the case of this synthetic symbiosis, the *E. coli* are not transmitted
459 transovarially, but are instead inoculated onto the surface of host eggs,
460 facilitating vertical transmission. We reason that bacterial adaptation to
461 transovarial transmission likely requires more complex genetic underpinnings,
462 conferring an ability to infect ovarioles and eggs. Critically, our work shows that a
463 (non-engineered/wild-type) free-living relative of a widely distributed group of
464 insect symbionts has an intrinsic capability to establish and sustain vertical
465 transmission in a novel insect host (albeit one that naturally harbors a *Sodalis*
466 symbiont) with no obvious detrimental effects. However, injection of a quorum-
467 sensing mutant (Δ*ypeI*), demonstrating constitutive expression of virulence
468 factors[36,37] was observed to kill weevil eggs with striking efficiency, highlighting
469 the lability and complexity of interactions facilitating maintenance and vertical
470 transmission of a symbiont.

471 Given that natural selection lacks foresight, it is important to recognize that
472 the ability of *S. praecaptivus* to associate with an insect host might be a function
473 of selection pressures mediated by a biphasic lifestyle comprising a free-living
474 state in addition to host association[69]. Indeed, it has been proposed that *S.*
475 *praecaptivus* might use insects as vectors to facilitate transmission between
476 animal and/or plant hosts in the environment[24]. Alternatively, the ability of *S.*
477 *praecaptivus* to associate with insects might simply be a side effect of its ability to
478 associate with plant and/or mammalian hosts[70-72], although it is notable that *S.*
479 *praecaptivus* maintains virulence factors characterized as insect-specific[36].
480 Interestingly, recent work indicates that free-living *Sodalis spp*. maintain a

481 substantial presence in decaying wood[33] and *S. praecaptivus* was in fact isolated
482 from a human following impalement with a dead tree branch[24]. Since insects are
483 also known to frequently associate with decaying wood, it is possible that free-
484 living members of the genus *Sodalis* use insects as vectors for transmission
485 among decaying trees in the environment[73].

486 Collectively, our work shows that bacterial genera such as *Sodalis*, that
487 frequently develop symbiotic associations with a wide range of insect taxa, have
488 extensive adaptations that facilitate infection, benign persistence and vertical
489 transmission in insect hosts. Vertical transmission, in particular, lays the
490 foundation for the evolution of mutualism by facilitating strong partner-fidelity
491 feedback. Metabolic adaptations leading to nutrient secretion can have relatively
492 simple genetic etiologies that can be honed by subsequent degenerative
493 changes that mimic strategies utilized in microbial rational engineering to
494 eliminate competing metabolic activities, favoring production of selected
495 resources[74]. While our work shows that vertical transmission occurs initially with
496 sub-optimal efficiency, it should be noted that in nature the acquisition of a new
497 biological function often facilitates ecological diversification, providing a unique
498 niche for partners to exploit, replete with strong selection pressure to maintain
499 functionality of the association and thereby increase the efficiency of vertical
500 transmission. Further, our work demonstrates the autocatalytic quality of
501 symbiosis, in which an existing symbiont creates favorable host conditions for the
502 acquisition of a nascent symbiont, leading to functional augmentation, symbiont
503 replacement and metabolic integration[75]. Numerous studies have revealed

evidence of these events in nature, rationalized as a consequence of loss of fitness of an existing symbiont[58,76-78] or acquisition of new functionality in response to environmental change or niche expansion[79,80]. In simple terms, mutualism can be described as a state of coexistence in which the benefits of a partnership outweigh the inherent costs[81]. The ability of *S. praecaptivus* to maintain a benign infection, combined with pre-existing host adaptations that facilitate bacterial maintenance, likely contribute significantly towards a reduction in those costs.

The development of a synthetic, transovarially-transmitted symbiosis provides new opportunities to advance knowledge in symbiosis. First, because *S. praecaptivus* is amenable to culture and manipulation, this system can be used to investigate mechanistic adaptations underlying symbiosis and mutualism, throughout the spectrum of insect development. Second, this system can be maintained for long-term study of host-symbiont adaptation and degenerative evolution. Finally, many insects, including certain disease vectors, are not amenable to germ line genetic modification and symbionts could be used as a platform to express transgenes, either to investigate molecular processes or interfere with processes of disease transmission in natural insect populations[37,82,83].

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AUTHOR CONTRIBUTIONS

Y.S., H.L, K.G.G. and C.D. designed experiments. Y.S., H.L., L.S.T., I.J. F.C., J.G., O.R., C.M. and C.D performed experiments. Y.S. and C.D. analyzed data, wrote the manuscript and prepared figures. All authors read and provided edits for the manuscript and agree to its contents.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community. One or more of the authors of this paper self-identifies as living with a disability.

Figure 1: Establishment of *S. praecaptivus* MC1 in aposymbiotic weevils following egg injection. (**A**) Schematic and micrograph showing microinjection into the egg posterior pole (PP). Subsequent images (B-I) are shown under normal light and under mCherry fluorescence. (**B**) Egg one day post injection (PI) showing infection at PP. (**C**) Egg four days post injection, with infection progressing. (**D**) Egg four days post injection with a Δ*ypeI* mutant, showing extensive pathogenesis. (**E**) First instar larva, immediately following emergence from microinjected egg. (**F**) Adult weevil, injected at egg stage following emergence from grain. (**G**) Egg derived from microinjected parents that acquired *S. praecaptivus* via maternal transmission at five days post deposition (PD). (**H**) First instar larva derived from egg-microinjected parents. (**I**) Ovaries from mated aposymbiotic female derived from microinjected egg, showing extensive colonization. See also Figure S1 and Video S1.

Figure 2: **Dynamics of *S. praecaptivus* MC1 infection following egg injection**. (**A**) Infection frequency and average bacterial density (with error bars showing standard deviation) in adult weevils over ten generations. (**B**) Dynamics of F_1 infection in multiple replicated egg injection experiments involving aposymbiotic (apo) and symbiotic (sym) grain weevils (n=10 for each line). (**C**) Kaplan-Meier analysis of association between infection and developmental status. (**D**) Infection status of the first seven and last seven offspring obtained from six individual aposymbiotic F_6 females infected with *S. praecaptivus* MC1, demonstrating no significant difference.

Figure 3: **Localization of *Sodalis praecaptivus* MC1 expressing mCherry (red) in offspring of aposymbiotic (apo) and symbiotic (sym) weevils infected by egg microinjection**. (**A**) Larval gut with white circle highlighting the bacteriome that develop only in sym weevils, shown under normal (left) and fluorescent (right) light. (**B**) Scanning electron micrograph (SEM) of the weevil symbiont, *S. pierantonius,* isolated from uninjected sym *S. zeamais* bacteriome, showing distinctive spiral morphology. (**C**) Confocal image of larval gut bacteriome from sym weevil, stained with Hoechst 33342 (blue; targeting nucleic acid), showing co-habitation of *S. praecaptivus* MC1 (red) and *S. pierantonius* (blue spirals). (**D**) Adult gut from newly emerged weevils with white circles highlighting cecal bacteriomes that form only in sym weevils. (**E**) Confocal image of cecal bacteriome from sym weevil, stained with Hoechst 33342 (blue; targeting nucleic acid) and CellMask Green (yellow: targeting cell membranes), showing co-habitation of *S. praecaptivus* MC1 (red) and *S. pierantonius* (blue spirals). Inset images in panels C&E are zoomed and enhanced in contrast. See also Figure S1 and S2.

Figure 4: **Low (left) and high (right) magnification confocal images of *S. praecaptivus* MC1 expressing mCherry (red) in ovaries of offspring from aposymbiotic (apo) and symbiotic (sym) weevils following egg microinjection.** Specimens were stained with Hoechst 33342 (blue: targeting nucleic acid) and CellMask Green (yellow: targeting cell membranes). (**A**) Tropharium from adult apo weevil, showing *S. praecaptivus* MC1 inside

tropharium cells. (**B**) Tropharium from adult sym weevil, showing co-existence of *S. praecaptivus* MC1 and *S. pierantonius*. (**C**) Vitellarium from adult apo weevil, with *S. praecaptivus* MC1 in epithelial cells, developing oocytes and the tropharium/vitellarium transition zone containing pro-oocytes.

Figure 5: **Characterization of *S. praecaptivus* strains with modified tyrosine and phenylalanine biosynthesis.** (**A**) Plate-based assay on minimal medium, showing a Δ*tyrR* overproducer cross-feeding Δ*pheA-tyrA* auxotroph. (**B**) Growth of an auxotrophic Δ*pheA-tyrA* strain over seven days in minimal medium alone or in the presence of wild type or Δ*tyrR* strains following inoculation of cells at equal densities. The auxotrophic Δ*pheA-tyrA* strain shows significant growth increase only in the presence of the Δ*tyrR* overproducer, relative to the wild type strain (>10 fold; $p < 0.01$). See additional data presented in **Figure S3B**. (**C**) Thorax cuticular redness of two-week-old sym weevils and their apo derivatives with and without Δ*pheA-tyrA,* WT and Δ*tyrR* strains of *S. praecaptivus* injected at egg stage. Boxes on left show the raw images associated with the highest and the lowest red values in the dataset. (**D**) Larval development time of sym weevils and apo counterparts with and without Δ*pheAtyrA,* WT and Δ*tyrR* strains injected at egg stage. Matrices show results of pairwise statistical analyses (*t*-test) indicating no significant difference and asterisks indicating significance of $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$. See also Figure S3.

STAR METHOD

RESOURCE AVAILIABILITY

Lead contact

Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, Colin Dale (colin.dale@utah.edu)

Materials availability

Mutant strains generated in this study are available upon request from the lead contact, Colin Dale (colin.dale@utah.edu).

Data and code availability

- All sequence reads derived from genomic sequencing were deposited in the NCBI sequence read archive (SRA) under accession SAMN26947704, SAMN26947705 and SAMN26947706 for the MC1, Δ*pheA-tyrA* and Δ*tyrR* strains, respectively.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Insects:

Grain weevils (*Sitophilus zeamais*), originally obtained from USDA, Manhattan, KS, U.S.A, were reared on organic whole yellow maize (Purcell Mountain Farms) in an Darwin insect chamber at 25 °C and 62% relative humidity (RH). Symbiont-free (aposymbiotic) weevils were generated by rearing on rifampicin treated corn

prepared by hydrating dried corn with a 3% (w/v) solution of rifampicin (1 mg/ml)[36]. Following treatment for one generational interval, the resulting aposymbiotic weevils were maintained on untreated grain and are checked periodically to confirm the absence of bacteriomes.

Bacterial strains

This study involved the use of *Sodalis praecaptivus* strain HS, which is a close relative of insect-associated *Sodalis* spp. symbionts that was isolated from an infected human[24,34], and has been previously deposited in the American Type Culture Collection (ATCC) as product BAA-2554. For all experiments outlined in the study, it was cultured in LB liquid media and plated on LB agar with appropriate antibiotics as outlined in method details, presented below, at 30 °C, under atmospheric air. Strains were preserved at -80 °C in LB media with 15% (w/v) glycerol.

All mutant strains of *S. praecaptivus* utilized throughout this study, can be obtained from the lead contact Colin Dale (colin.dale@utah.edu).

METHOD DETAILS

Genetic modification of *Sodalis praecaptivus*

Lambda Red recombineering was utilized to generate recombinant strains of *S. praecaptivus* maintaining plasmid pRed/Gamm (CAT) using methodologies developed and outlined in previous studies[84]. For the work outlined in this study, we engineered a strain that expresses the fluorescent mCherry protein, in order to visualize *S. praecaptivus* in grain weevils. In addition, we engineered *S. praecaptivus* strains that are (1) auxotrophic for phenylalanine and tyrosine and

737 (2) overproduce these aromatic amino acids. The auxotroph was generated by
738 knocking out both the *tyrA* and *pheA* genes, encoding enzymes involved in the
739 terminal steps of tyrosine and phenylalanine biosynthesis, respectively, yielding a
740 strain that is incapable of biosynthesizing either amino acid[85]. Candidate Tyr/Phe
741 overproducing strains were generated in accordance with a rational engineering
742 strategy previously developed to facilitate overproduction of L-DOPA in *E.*
743 *coli*[46,47]. This encompassed generation of *S. praecaptivus* mutants lacking *tyrR*,
744 *nuoN, ppc, ptsHIcrr, csrA, zwf*, *mdh* genes.
745
746 *Preparation of an mCherry-expression cassette*. An mCherry and zeocin
747 resistance cassette (1.5 kbp), codon-optimized for efficient expression in gamma
748 Proteobacteria[86,87], was amplified from bacterial DNA by PCR in a reaction
749 comprising 10 µl of 5X PCR buffer, 4 µl of 25 mM dNTPs, 3 µl of 25 mM $MgCl_2$,
750 1.25 µl of 20 µM forward primer (#2272), 1.25 µl of 20 µM reverse primer
751 (#2273), 0.5 µl of Phusion High Fidelity DNA Polymerase (Thermo Fisher
752 Scientific) and 1 µl of template DNA. The cycling conditions involved initial
753 denaturation at 98 °C for 2 min, followed by 29 cycles of denaturation (98 °C; 30
754 s), annealing (56 °C; 30 s) and extension (72 °C; 1 min), followed by a final
755 extension at 72 °C for 2 min. This yielded a single amplicon of expected size
756 (1.5-kbp), as determined by gel electrophoresis.
757 We elected to insert the mCherry-zeocin cassette into the *lacZ* gene of *S.*
758 *praecaptivus* based on the notion that lactose is not present in insects and
759 therefore, disruption of this gene should not negatively impact the interaction

between *S. praecaptivus* and weevils. Furthermore, insertional inactivation of *lacZ* can be detected by plating bacteria on media with IPTG and X-Gal[88], facilitating selection of recombinants. Three consecutive PCR reactions were employed to generate a construct that could be integrated into the *lacZ* gene of *S. praecaptivus* strain 101[36] using lambda Red recombineering[84]. DNA from wild-type *S. praecaptivus* was isolated from cultured cells by heating at 98°C for 5 min to provide template for PCR reactions. In the first PCR, 212 bp of the 5' end (primer #2286/#2287) and 278 bp of the 3' end (#2289/#2290) of the *lacZ* gene were amplified with a flanking tail using the following a PCR reaction composed of 12.5 µl of 2X Phusion, 6.5 µl of nuclease free water, 2.5 µl of 2.5 µM forward primer, 2.5 µl of 2.5 µM reverse primer and 1 µl of DNA template. The PCR was performed with an initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation (98 °C; 10 s), annealing (58 °C; 30 s) and extension (72 °C; 2 min). The resulting PCR product was then purified using Agencourt AMPure XP magnetic beads, in accordance with manufacturer's protocol. In the second PCR, 4 µl of 5' and 3' *lacZ* PCR products were amplified with 4 µl of the mCherry-zeocin cassette to generate a chimeric product with 12 µl of 2X Taq Polymerase MasterMix (Thermo Fisher Scientific) with initial denaturation at 95°C for 30 s, followed by 10 cycles of denaturation (94 °C; 15 s), annealing (45 °C; 30 s) and extension (72 °C; 1min). The third PCR step was used to amplify the final 2 kbp disruption fragment from the second PCR product using 1 µl each of primers #2287 and #2290, which anneal to the 5' and 3' ends of *lacZ*, respectively. This reaction was conducted with 13 µl of 2X Taq Polymerase MasterMix (Thermo

783 Fisher Scientific), 11 µl of nuclease free water and 24 µl of the 2nd PCR product.
784 The PCR conditions involved an initial denaturation at 95 °C for 30 sec, followed
785 by 35 cycles of denaturation (94 °C; 15 s), annealing (58 °C; 30 s) and extension
786 (72 °C; 1.5 min). The third PCR product was again purified using AMPure XP
787 beads to generate template for recombineering.
788
789 *Preparation of* Δ*pheA-tyrA and* Phe and Tyr overproduction *recombineering*
790 *constructs.* Genetic constructs to generate these mutant strains were prepared
791 using a similar three-step PCR procedure, as detailed above for the mCherry-
792 zeocin cassette, to generate chimeric PCR products with gentamycin,
793 spectinomycin or kanamycin resistance cassettes for Δ*pheA-tyrA* and all
794 candidate gene knockouts yielding Phe and Tyr overproduction (Δ*tyrR*, Δ*nuoN,*
795 Δ*ppc,* Δ*ptsHIcrr,* Δ*csrA,* Δ*zwf* and Δ*mdh*). The PCRs and clean up steps were
796 conducted using reagents and conditions outlined for preparation of the mCherry-
797 zeocin construct, using primers listed in **Table S1**.
798
799 *Lambda Red recombineering.* Wild-type *S. praecaptivus* strain 101 culture
800 maintaining the plasmid pRed/Gamm (CAT)[36] was cultured overnight in 3 ml LB
801 with 30 µg/ml chloramphenicol. The resulting cells were then inoculated into 25
802 ml 2YT medium (20 mg/ml Tryptone, 8 mg/ml Yeast Extract, 10 mg/ml NaCl, pH
803 5.8) with 30 µg/ml chloramphenicol and permitted to grow for 3 hours in a 30 °C
804 shaking incubator (200 rpm). The expression of the lambda Red functions was
805 induced by adding arabinose at 4 mg/ml and the culture was allowed to grow for

another 30 min under the same conditions. *S. praecaptivus* cells were then pelleted by centrifugation at 9000 × g. for 20 min at 4 °C, washed twice in cold sterile de-ionized water and resuspended in a fresh aliquot of 25 ml ice cold sterile de-ionized water. Two additional rounds of washing and resuspension were performed, first using a resuspension volume of 25 ml and second using a resuspension volume of 1 ml. This yields high efficiency electro-competent *S. praecaptivus* cells that can be transformed with recombineering constructs (as outlined here) or plasmids. The prepared PCR products were then combined with 80 µl of competent cells and electroporated at 1600 V/s using an Eppendorf electroporator model 2510. The cells were permitted to recover for 16 hours by plating on L agar without antibiotic selection before replica plating onto L agar with IPTG (100 mM), X-gal (100 mg/ml) and appropriate antibiotic (15 µg/ml zeocin, 40 µg/ml spectinomycin, 5 µg/ml gentamicin or 30 µg/ml kanamycin).

Genetic and phenotypic verification. All resulting transformants were found to be resistant to appropriate antibiotics following recombineering. PCR assays were performed using primers flanking the insertion site in target genes to confirm that the constructs were inserted in the anticipated fashion in the *S. praecaptivus* genome. The amplification of the insertion region (PCR product sizes listed in **Table S1**) was achieved by PCR with a reaction mixture composed of 0.5 µl of 2.5 µM forward and 2.5 µM reverse primer (**Table S1**), 12.5 µl of 2X Taq Polymerase MasterMix (Thermo Fisher Scientific), 10.25 µl of nuclease free water, 1 µl of DNA template and 1.25 µl DMSO. The cycling condition comprised

829 an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation
830 (94 °C; 30 sec), annealing (58 °C; 30 sec) and extension (72 °C; 4 min). In
831 addition, a PCR assay for the presence of the *tam* gene (440 bp) (GenBank:
832 AHF76984.1) was performed to verify that the transformant was *S.*
833 *praecaptivus*[36]. This PCR used 12.5 µl 2X Taq Polymerase MasterMix (Thermo
834 Fisher Scientific), 1 µl of forward (#127) and reverse (#128) primer, 11.5 µl
835 nuclease free water and 1 µl of template DNA from the transformant. The
836 thermocycler conditions included an initial denaturation at 95 °C for 1 min,
837 followed by 25 cycles of denaturation (94 °C; 30 s), annealing (48 °C; 30 s) and
838 extension (72 °C; 3 min).

839 Phenotypic tests were performed to validate the recombinant strains.
840 MCherry fluorescence was confirmed by fluorescent microscopy and the
841 resulting recombinant was designated *S. praecaptivus* strain MC1. The double
842 auxotrophic phenotype of the Δ*pheA-tyrA* strain was confirmed by replica plating
843 on minimal media with and without tyrosine and phenylalanine supplementation.
844 The functions of the candidate Phe and Tyr overproducing strains were assessed
845 using a cross-feeding assay in which putative overproducers and the wild type
846 strain (control) were streaked adjacent to the Δ*pheA-tyrA* auxotroph. In addition,
847 tyrosine production was validated for the Δ*tyrR* mutant during growth of the
848 putative overproducer and wild type strain (control) in minimal media using a
849 colorimetric tyrosine assay (Sigma-Aldrich). These assays were performed in
850 triplicate for the wild type and Δ*tyrR* strains, following 5 days of growth in minimal

media at 30 °C with shaking (200 rpm), according to the manufacture's' protocol and measured using a POLARstar OPTIMA spectrophotometer.

Genome sequencing of recombinant strains. Prior to injection in weevils, *S. praecaptivus* MC1, *ΔpheA-tyrA* and Δ*tyrR* strains were sequenced to confirm that (1) the inserted cassette was integrated into the anticipated genomic location, and (2) the lambda Red recombination event did not induce any extraneous mutations in *S. praecaptivus*. Genomic DNA for each strain was extracted from cells that were isolated as single colonies and cultured on L plates with IPTG (100 mM) and X-gal (100 mg/ml) for 48 hours at 30 °C. Bacterial cells were collected and transferred into 180 µl Buffer ATL, and DNA was extracted using a Qiagen Blood and Tissue Kit protocol (Qiagen, Germany), following the manufacturer's protocol for Gram negative bacteria. The resulting genomic DNA was treated with 1 µl RNaseA for 15 minutes at room temperature and purified with Ampure XP purification beads (Axygen) prior to final elution in 50 µl nuclease-free water.

Library construction was performed using NEBNext Ultra II DNA Library Prep Kit (New England BioLabs, USA) and NovaSeq S4 Reagent Kit v1.5 (2 x150 bp). Whole-genome sequencing was performed on a NovaSeq 600 system (Illumina) at the University of Utah Huntsman Cancer Institute High-Throughput Genomics Core Facility, yielding 34.2 Gb, 39.2 Gb and 41.2 Gb of raw sequence reads, respectively, for the MC1, Δ*pheA-tyrA* and Δ*tyrR* strains. Reads were quality trimmed in BBDuk and aligned back to the *S. praecaptivus* wild type

reference sequence (CP006569) using Geneious Prime 2022.0.2 with default parameters. The resulting alignments were then inspected manually for mismatches. Trimmed reads were also assembled using the SPAdes assembler with default parameters. The resulting contigs were then aligned to sequences comprising the resistance cassettes used for lambda Red recombination to identify those contigs representing regions of the chromosome that were genetically modified. All three mutant strains were confirmed to have the correct genetic modifications with no gene duplications or other rearrangement in the *S. praecaptivus* genome. Further, no extraneous mutations were identified in any of the recombinant strains. All sequence reads derived from genomic sequencing were deposited in the NCBI sequence read archive (SRA) under accession SAMN26947704, SAMN26947705 and SAMN26947706 for the MC1, Δ*pheA-tyrA* and Δ*tyrR* strains, respectively.

Generalized transduction procedure for *S. praecaptivus*

In order to introduce the mCherry allele into a *S. praecaptivus* Δ*ypeI* strain, constructed and validated in a previous study[36], we took advantage of an endogenous phage transduction system. Phage induction was achieved by growing a 1:20 dilution of an overnight culture of *S. praecaptivus* MC1 in LB media at 30 °C for 8 hours with shaking at 200 rpm and then exposing the resulting culture in an open petri dish to UV light from a germicidal lamp in a Labconco model 36208/36209 TYPE A2 laminar flow hood for 30 sec. Following exposure, the culture was maintained at 30 °C for 12 hours. Chloroform was then

added to 1% (v/v) and mixed thoroughly by vortexing. Cells were then pelleted by centrifugation at 8000 rpm for 20 min and the supernatant (containing phage) was stored at 4 °C and plated on LB media to ensure that it did not contain any viable *S. praecaptivus* cells. Transduction was performed by mixing 200 µl of phage suspension with 100 µl of an overnight culture of *S. praecaptivus* Δ*ypeI*[36] and 900 µl of LB media. Following growth for one hour at 30 °C without shaking, the mixture was plated on LB agar with 15 µg/ml zeocin, 40 mg/ml spectinomycin, 100 mM IPTG and 100 mg/ml X-gal, and incubated for 3 days at 30 °C. A single colony demonstrating spectinomycin resistance (indicative of Δ*ypeI*) and zeocin resistance (indicative of mCherry-bleoR presence) was streaked onto a second plate and a single colony was isolated for microinjection into weevil eggs.

Microinjection of *S. praecap*tivus MC1 into aposymbiotic and symbiotic weevil eggs:

Weevil egg isolation. Weevil eggs that were deposited in grain were detected by staining gelatinous egg plugs using acid fuchsin[89,90] and destaining in DI water until only the egg plugs remain stained. The egg plugs were then removed using forceps, and the egg inside the cavity was carefully removed for use in the microinjection procedure. Only eggs that were deposited by weevils within the past 24 hours were used in this study.

Egg preparation for microinjection. Isolated eggs were attached in a consistent polar orientation to a microscope slide with heptane glue to preclude the possibility of movement during the microinjection procedure. Following attachment, eggs were dehydrated for 5 min at 25 °C. Wrinkles on the egg surface were observed to be correlated with a poor outcome of microinjection procedure, perhaps indicating damage incurred during their isolation or excessive dehydration. After dehydration, a 2 µl drop of gas-permeable halocarbon oil 700 (Sigma-Aldrich) was placed on the surface of each egg to achieve complete immersion, inhibiting further dehydration and facilitating gas exchange.

Injection needle preparation. S. praecaptivus MC1 strain was cultured in LB medium overnight in a 30 °C shaking incubator and concentrated to OD_{600nm} = 1 in 0.85% (w/v) NaCl. First, 2 µl of the prepared bacterial culture were drawn into one end of a 3.5'' glass tube (Drummond #2-00-203-G/X) by capillary action. The tube was then pulled on a needle puller (Sutter Instrument Co Model P-97) with settings of heat = 270, pull = 20, velocity = 40, time = 150. Subsequently, a sterilized tweezer was used to break the pulled needle and expose the sharp end for injection.

Microinjection. The prepared needle, replete with bacterial culture was then attached to an empty syringe held by a micromanipulator (Narishige, Model M-152) to facilitate accurate subsequent injection, and adjusted to be perpendicular

to the line of eggs attached to the glass slide. Injections were performed into the posterior poles of eggs under phase contrast microscopic observation following *Drosophila* egg microinjection procedures[91]. Approximately 0.005~0.02 µl of bacterial culture was then injected into each egg. Following injection, the glass slides with injected eggs were maintained in an incubator at 25 °C and 62% RH.

Egg hatching and transfer to grains. After 4-6 days, the first instar larval stage was observed to emerge from microinjected eggs. Immediately following emergence, larvae were transferred back to corn grains to facilitate completion of their larval development. In some cases, eggs that were observed to contain developing larvae failed to hatch, likely due to injury, and were abandoned. Maize grains were soaked in sterile deionized water for 5 min to facilitate weevil transplantation and subsequent survival. Transplantation was achieved by first drilling a 1.5 mm diameter hole into the grain and then carefully implanting the larva. The hole was then gently packed with finely powdered cornmeal and a thin layer of glutinous rice-water cement was used to seal the hole to simulate the tough coating that is found on the grain surface. The glutinous rice-water cement was prepared by combing 0.5 g glutinous rice flour and 2 ml DI water and heated in a 1000 W microwave on full power for 30 sec, providing sufficient cement for 30 larval implantations. The transplanted grains were then maintained under standard conditions (25 °C, 62% RH) for one month to facilitate the completion of weevil development to adulthood.

Microinjection of *S. praecaptivus* MC1 in aposymbiotic (apo) and symbiotic weevil adults

A suspension of *S. praecaptivus* MC1 in 0.85% (w/v) NaCl (OD_{600nm} = 1) was prepared for injection into adult weevils using needles pulled from 3.5'' glass capillary tubes (Drummond #2-00-203-G/X) at settings of heat = 292, pull = 100, velocity = 24, time = 250. Adult aposymbiotic maize weevils (less than 3 weeks following emergence) were then microinjected with *S. praecaptivus* MC1 using an established protocol[35] involving dipping the capillary needle into the bacterial suspension and then piercing the thoracic hemocoel of the adult weevils with the contaminated needle. All of the resulting adult weevils were maintained in the laboratory at 25 °C and 62% RH for 3 weeks to reproduce.

Live staining for confocal imaging

Weevils were processed for confocal microscopic imaging by careful dissection in 0.85% (w/v) NaCl. Dissected tissues (i.e. gut, ovaries) were washed in saline and placed on a freshly made 1.5 mm 0.5% agarose pad on a microscope slide. The tissues were stained with Hoechst 33342 (10 mg/ml) and CellMask Green (Thermo Fisher Scientific, 5 mg/ml) to stain nuclei and membranes, respectively, by adding 1 µl of each dye on to the top of the samples. The slide was then covered with a cover slip (No. 1.5: 0.175 mm +/- 0.015), sealed using Valap (1:1:1 mix of vaseline:lanolin:wax). Twenty minutes later, once the stains penetrated the tissues, confocal imaging was performed on a Zeiss LSM880 microscope equipped with an AiryScan detector, a 20X AIR objective and a 63X

NA1.4 oil immersion objective. Imaging was performed using appropriate excitation and emission filters for Hoechst 33342 and CellMask Green and mCherry, and images were processed in ZEN Blue 2.1 (Zeiss) and Imaris Viewer 9.6.0 (Bitplane). Single plane images selected from the z stacks are presented in this manuscript.

Preparation of *S. pierantonius* for electron microscopy

Fifth instar *Sitophilus zeamais* larvae were isolated from maize grains and subjected to dissection to remove bacteriomes located at the anterior end of the midgut into 0.1 M phosphate buffer (pH 7.2). Bacteriomes were then homogenized in a Dounce glass sub-cellular homogenizer to release bacteria from insect cells. Cellular debris was removed by centrifugation at 500 × g for 1 min. The supernatant was then subjected to three rounds of centrifugation (2000 × g for 5 min) and washing in 0.1 M phosphate buffer (pH 7.2). After the final washing step, the bacterial cell pellets was resuspended in 1% osmium tetroxide for 40 minutes and dehydrated using a graded series of ethanol (30%, 50%, 70%, 90%, 100%) in 5 min steps. Cells were then filtered onto a 0.2 micron polycarbonate filter for critical point drying. Following mounting, specimens were sputter coated with 10 nM gold/platinum and then visualized using a FEI Nova NanoSEM™ scanning microscope.

Measuring weevil cuticle color and larval development time

In order to compare weevil cuticle coloration, we collected aposymbiotic weevils that were injected with Δ*pheA-tyrA*, Δ*tyrR* and WT *S. praecaptivus* individually at egg stage along with non-injected aposymbiotic and symbiotic counterparts that were subject to the same egg isolation and larval implantation procedure. The larval development time for each injected and non-injected group was recorded. This represents the number of days from implantation of a first instar larva into corn to the subsequent emergence of the adult weevil. All weevils were collected at 14 days post adult emergence, based on the results of a pilot experiment demonstrating that the difference in cuticle color between symbiotic and aposymbiotic weevils was highest at that time point. Weevils were first washed in DI water, placed on a white background and a drop of glycerol was added to coat their exoskeleton[20]. Images of each weevil were obtained under consistent lighting conditions with a single light source under a dissection microscope (Leica M205 FCA). A square in the center of the thorax was then cropped with its side length equals to half of the thorax width, and an average red value for the square was then computed using ImageJ software. Weevils were homogenized and plated to verify their infection status following imaging.

QUANTIFICATION AND STATISTICAL ANALYSIS

Chi squared tests were performed manually for the following purposes:

To determine if the adult weevil infection status is biased according to sex; results subsection “Dynamics of *S. praecaptivus* transmission”; n = 100 (number of weevils); significant difference is defined as $p < 0.05$.

To determine the stability of infection in weevil larvae vs. adults; results subsection “Dynamics of *S. praecaptivus* transmission”; n = 40 (number of weevils); significant difference is defined as $p < 0.05$.

To determine if female weevil reproductive age is correlated with offspring infection frequency; results subsection “Dynamics of *S. praecaptivus* transmission” and Figure 2D; n = 43 (number of weevils); significant difference is defined as $p < 0.05$. Two samples were excluded due to an absence of infected offspring.

T tests were performed in Microsoft Excel for the following purposes:

To compare growth of the *S. praecaptivus* Δ*pheA-tyrA* mutant strain during co-culture with other *S. praecaptivus* strains; results subsection “Rational engineering of a functional mutualism” and Figure 5B; n (number of biological replicates) = 4; Mean = 2.04×10^7 CFU/ml (grow with Δ*tyrR*) and 3.84×10^6 CFU/ml (grow with WT); SD = 2.13×10^6 CFU/ml (grow with Δ*tyrR*) and 3.92×10^5 CFU/ml (grow with WT); significant difference is defined as $p < 0.05$.

To compare infection densities in weevils; results subsection “Rational engineering of a functional mutualism” and Figure 5C and D; n (number of weevils) = 16 (for apo+Δ*pheA-tyrA* and apo+WT) and 17 (for apo+Δ*tyrR*); Mean = 2.22×10^6 CFU/weevil (apo+Δ*pheA-tyrA*), 2.84×10^6 CFU/weevil (apo+WT) and 2.67×10^6 CFU/weevil (apo+Δ*tyrR*); SD = 2.36×10^6 CFU/weevil (apo+Δ*pheA-tyrA*), 1.71×10^6 CFU/weevil (apo+WT) and 1.94×10^6 CFU/weevil (apo+Δ*tyrR*); significant difference is defined as $p < 0.05$.

To compare the cuticle color (red value) among weevils; results subsection "Rational engineering of a functional mutualism" and Figure 5C; n (number of weevils) = 16 (for apo, apo+Δ*pheA-tyrA* and apo+WT), 17 (for apo+Δ*tyrR*) and 19 for sym; Mean = 31.6 (apo), 52.9 (apo+Δ*pheA-tyrA*), 40 (apo+WT), 27.3 (apo+Δ*tyrR*) and 11.44 (sym); SD = 10.39 (apo), 13.77 (apo+Δ*pheA-tyrA*), 6.98 (apo+WT), 6.62 (apo+Δ*tyrR*) and 3.7 (sym); significant difference is defined as $p < 0.05$.

To compare the larval development time among weevils; results subsection "Rational engineering of a functional mutualism" and Figure 5D; n (number of weevils) = 16 (for apo, apo+Δ*pheA-tyrA* and apo+WT), 17 (for apo+Δ*tyrR*) and 19 for sym; Mean = 44.41 (apo), 45.35 (apo+Δ*pheA-tyrA*), 43.24 (apo+WT), 40.44 (apo+Δ*tyrR*) and 38.47 (sym); SD = 3.04 (apo), 3.84 (apo+Δ*pheA-tyrA*), 3.44 (apo+WT), 3.05 (apo+Δ*tyrR*) and 2.89 (sym); significant difference is defined as $p < 0.05$.

Kaplan-Meier analysis was performed using the R software package:

To determine if weevil development time is correlated with infection status; results subsection "Dynamics of *S. praecaptivus* transmission" and Figure 2C; n = 29 (number of weevils); significant difference is defined as $p < 0.05$.

Video S1: Live F_{10} descendant of the main *S. praecaptivus* MC1 injected weevil line along with an uninfected control weevil visualized for two seconds under normal light then under mCherry fluorescent light, related to Figure 1.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
S. praecaptivus wild type	ATCC	BAA-2554
S. praecaptivus MC1	This paper	CD 2555
S. praecaptivus 101	[36]	CD 101
S. praecaptivus Δ*pheA-tyrA*	This paper	CD 1663
S. praecaptivus Δ*tyrR*	This paper	CD 1987
S. praecaptivus Δ*nuoN*	This paper	CD 1728
S. praecaptivus Δ*mdh*	This paper	CD 1993
S. praecaptivus Δ*ppc*	This paper	CD 2131
S. praecaptivus Δ*ptsHIcrr*	This paper	CD 1991
S. praecaptivus Δ*csrA*	This paper	CD 708
S. praecaptivus Δ*zwf*	This paper	CD 1989
S. praecaptivus MC1 Δ*ypeI*	This paper	CD 2565
Biological samples		
Sitophilus zeamais	USDA, Manhattan, KS, U.S.A. https://www.ars.usda.gov/plains-area/mhk/cgahr/	N/A
Oligonucleotides		
See Table S2, Oligonucleotides used in this study	N/A	N/A
Recombinant DNA		
Plasmid pRed/Gamm (CAT)	[84]	N/A
Software and Algorithms		
Adobe Illustrator	N/A	https://www.adobe.com/products/illustrator.html
Imaris Viewer	N/A	https://imaris.oxinst.com/imaris-viewer
Geneious Prime 2022.0.2	N/A	https://www.geneious.com
BBDuk	N/A	https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/
SPAdes assembler	N/A	[92]

Image J 1.53a	N/A	https://imagej.nih.gov/ij/

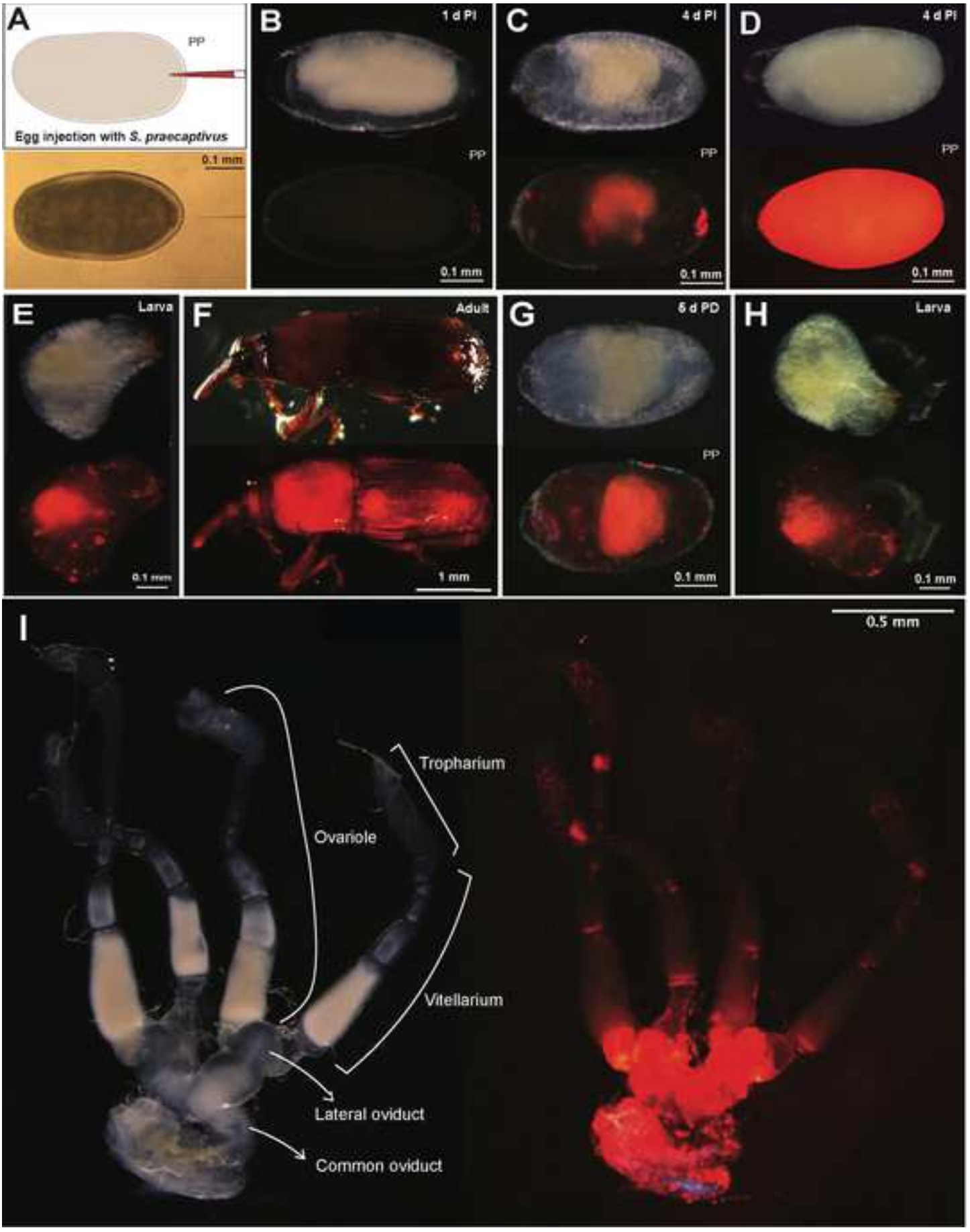

Fig 2

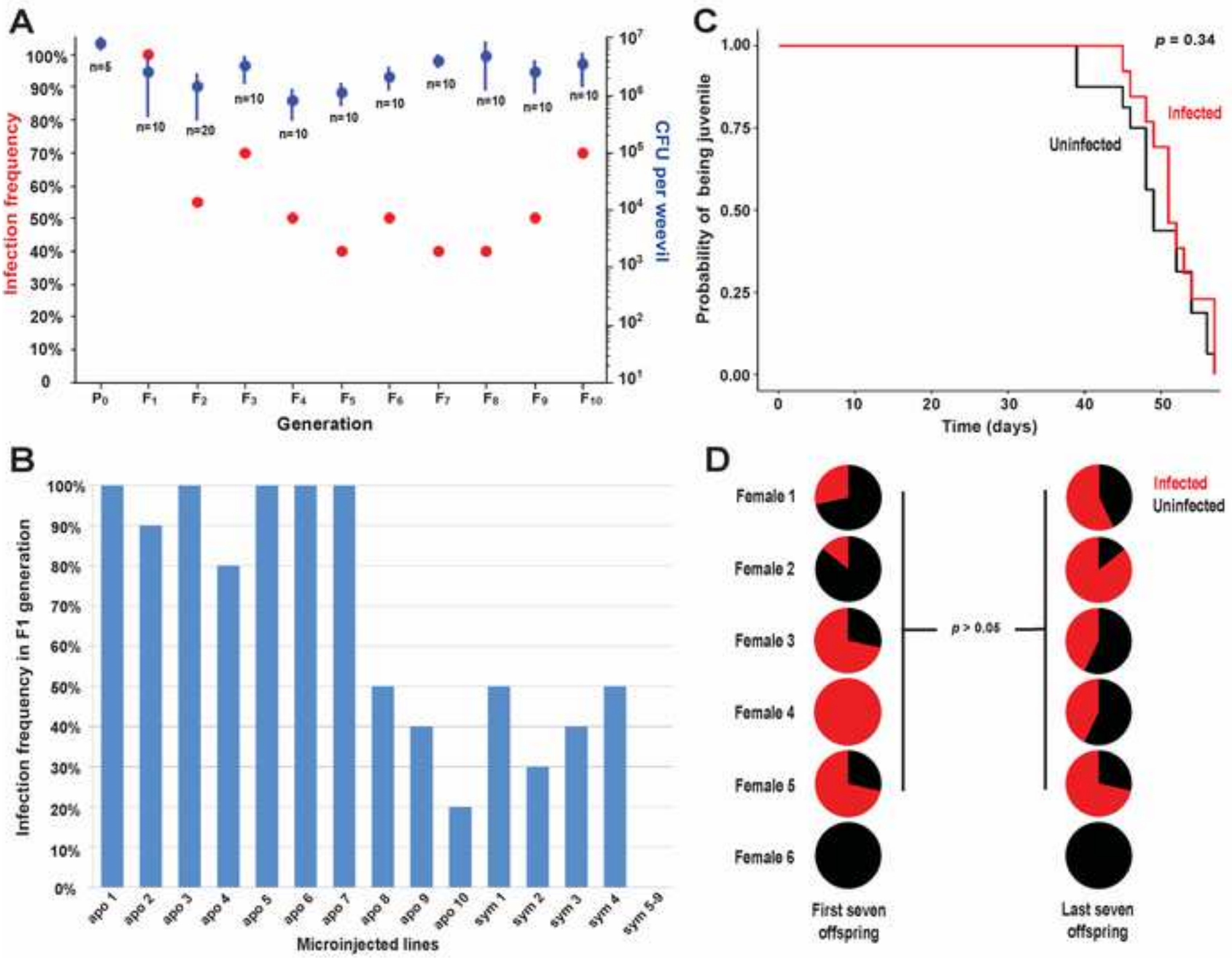

Fig 3

Click here to access/download;Figure;Figure 3.jpg

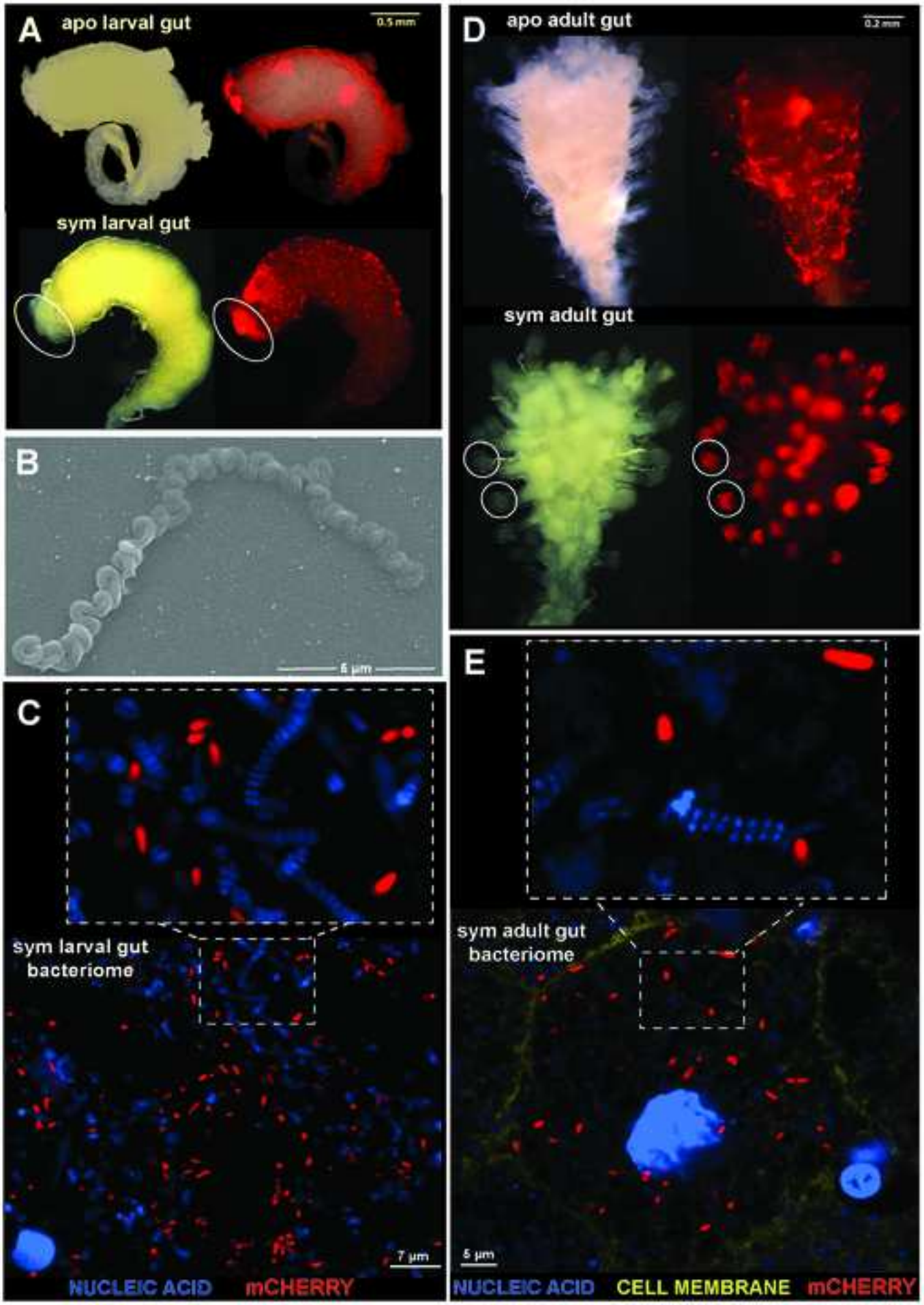

Fig 4

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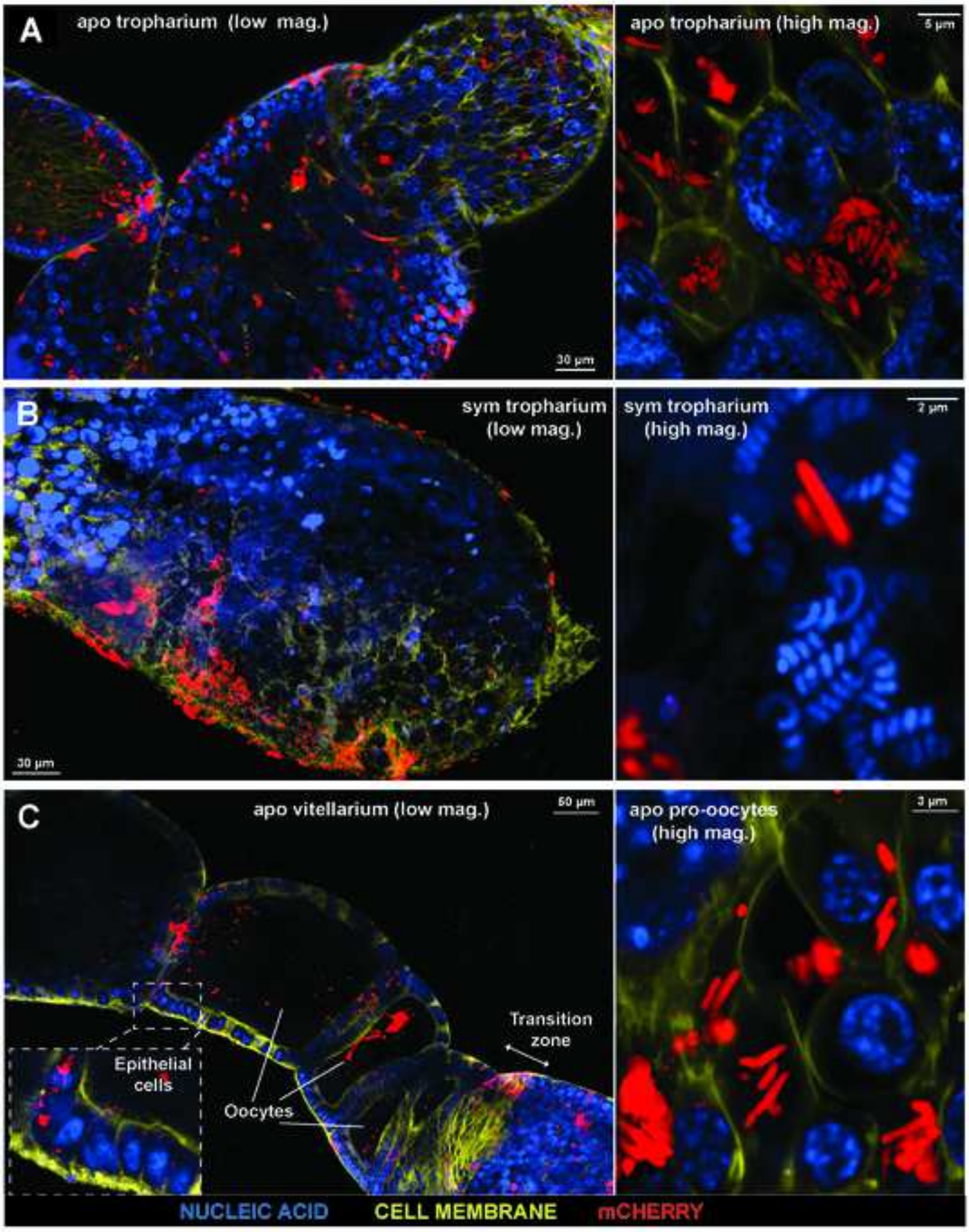

Fig 5

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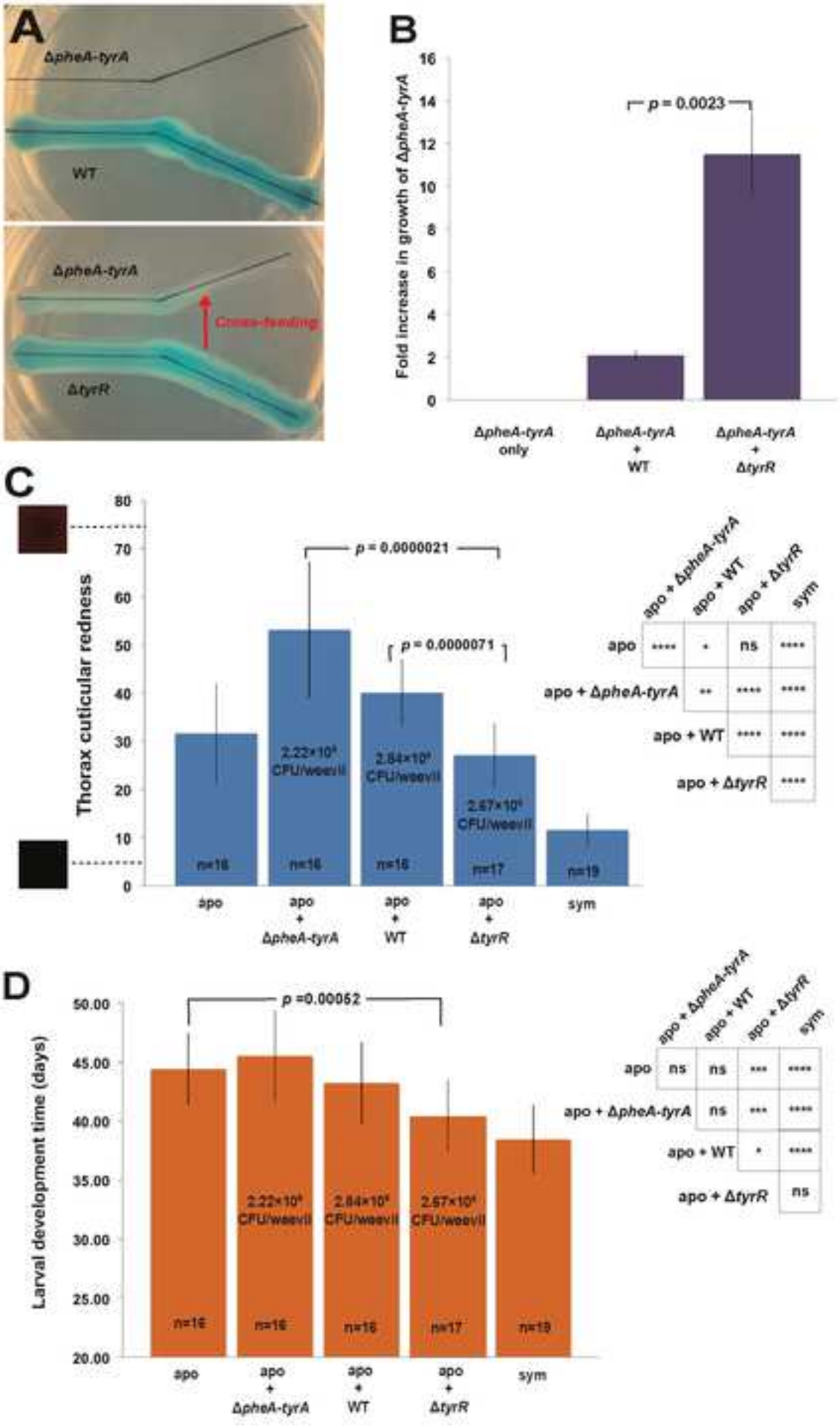


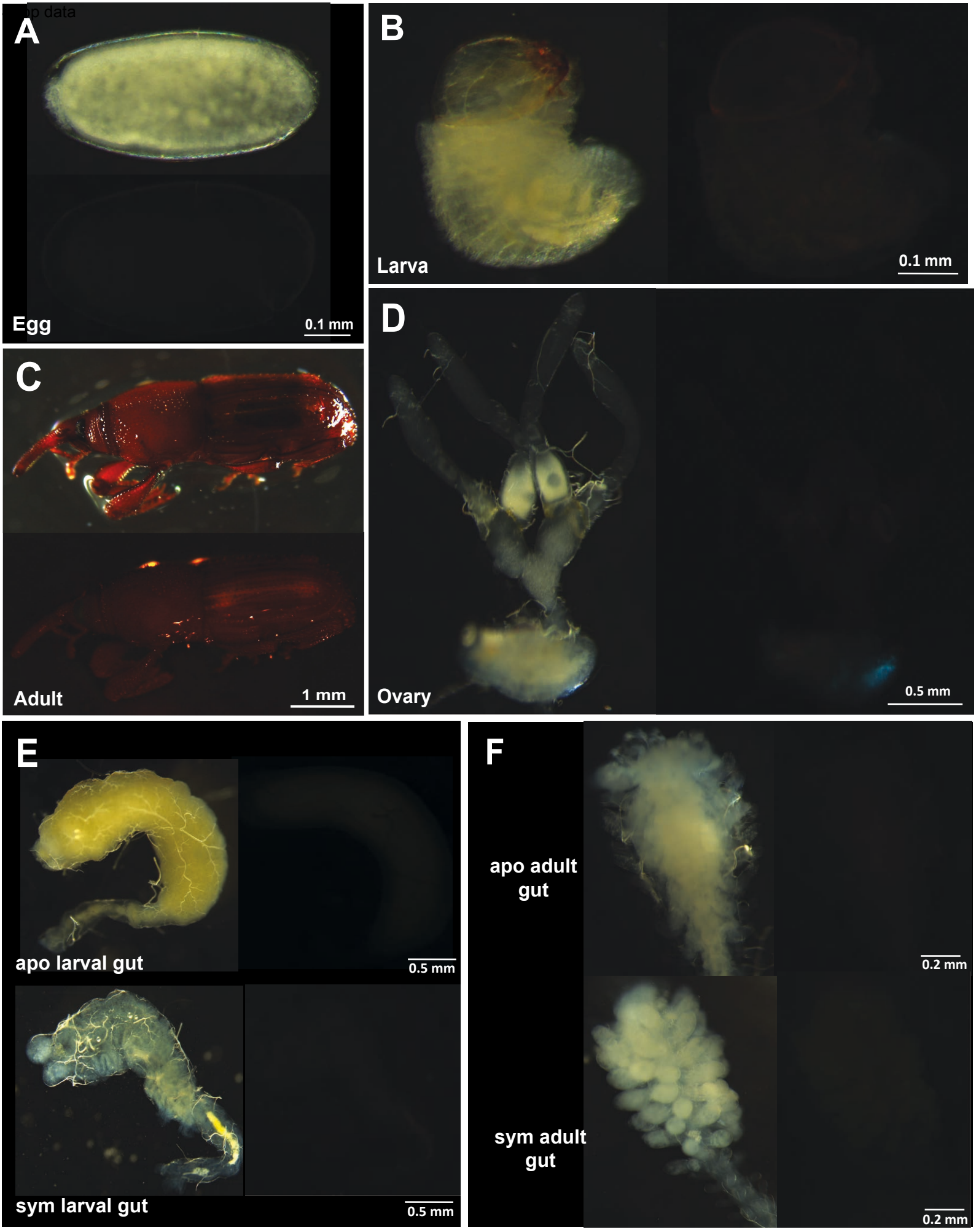

A
Egg
0.1 mm
B
Larva
0.1 mm
C
Adult
1 mm
D
Ovary
0.5 mm
E
apo larval gut
0.5 mm
sym larval gut
0.5 mm
F
apo adult gut
0.2 mm
sym adult gut
0.2 mm

Figure S1: Imaging of uninjected weevils, showing absence of mCherry fluorescence, related to Figure 1 and Figure 3. Panels A-F were captured under normal light (left or upper) and under mCherry fluorescence (right or lower), employing identical light energy and imaging parameters to those used in Figure 1. (**A**) Aposymbiotic weevil egg (1 day old). (**B**) Aposymbiotic weevil 1st instar larva. (**C**) Aposymbiotic weevil adult (**D**) Aposymbiotic weevil ovary (**E**) Aposymbiotic (apo) and symbiotic (sym) larval guts. (**F**) Aposymbiotic (apo) and symbiotic (sym) adult guts.

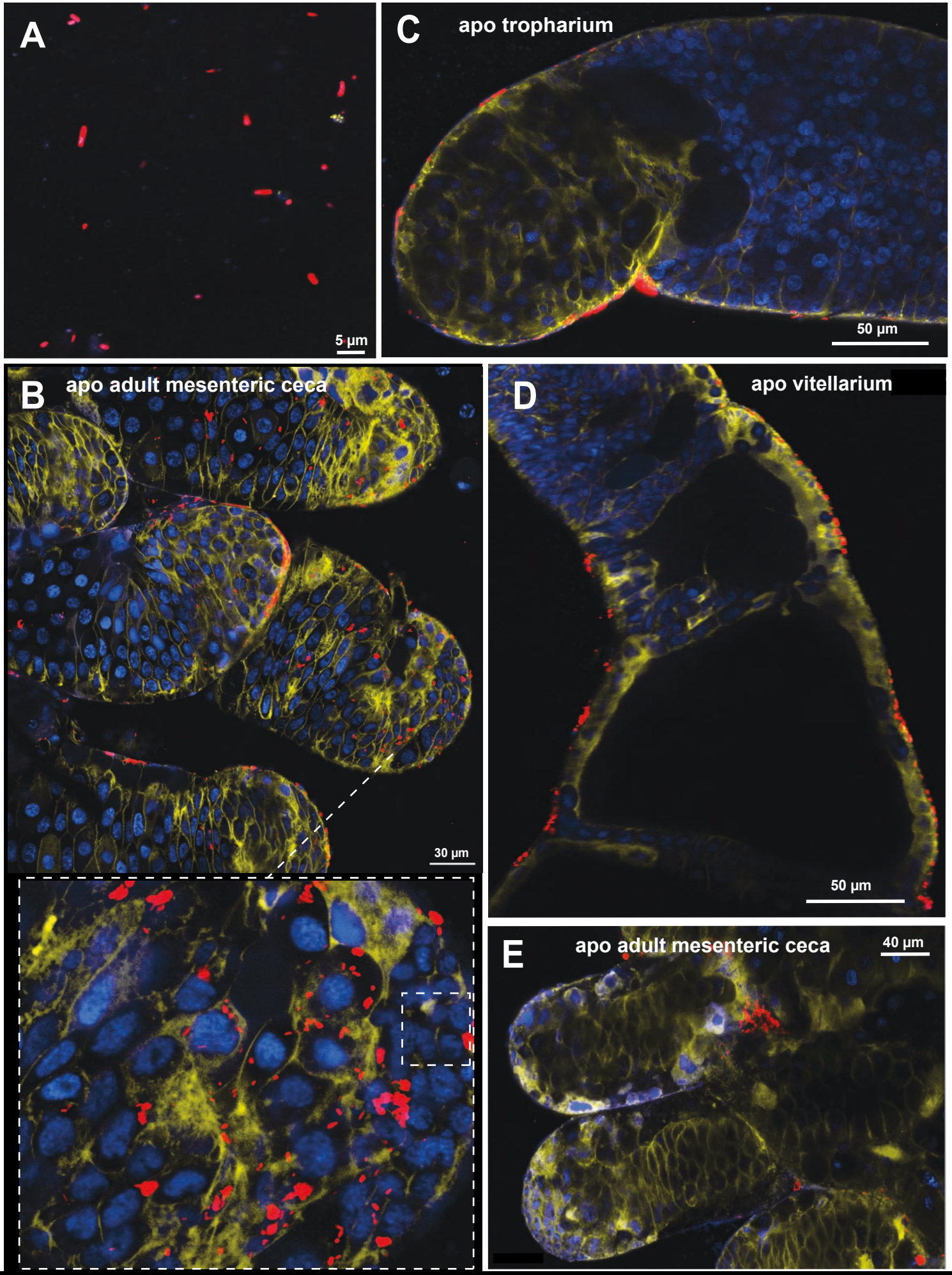
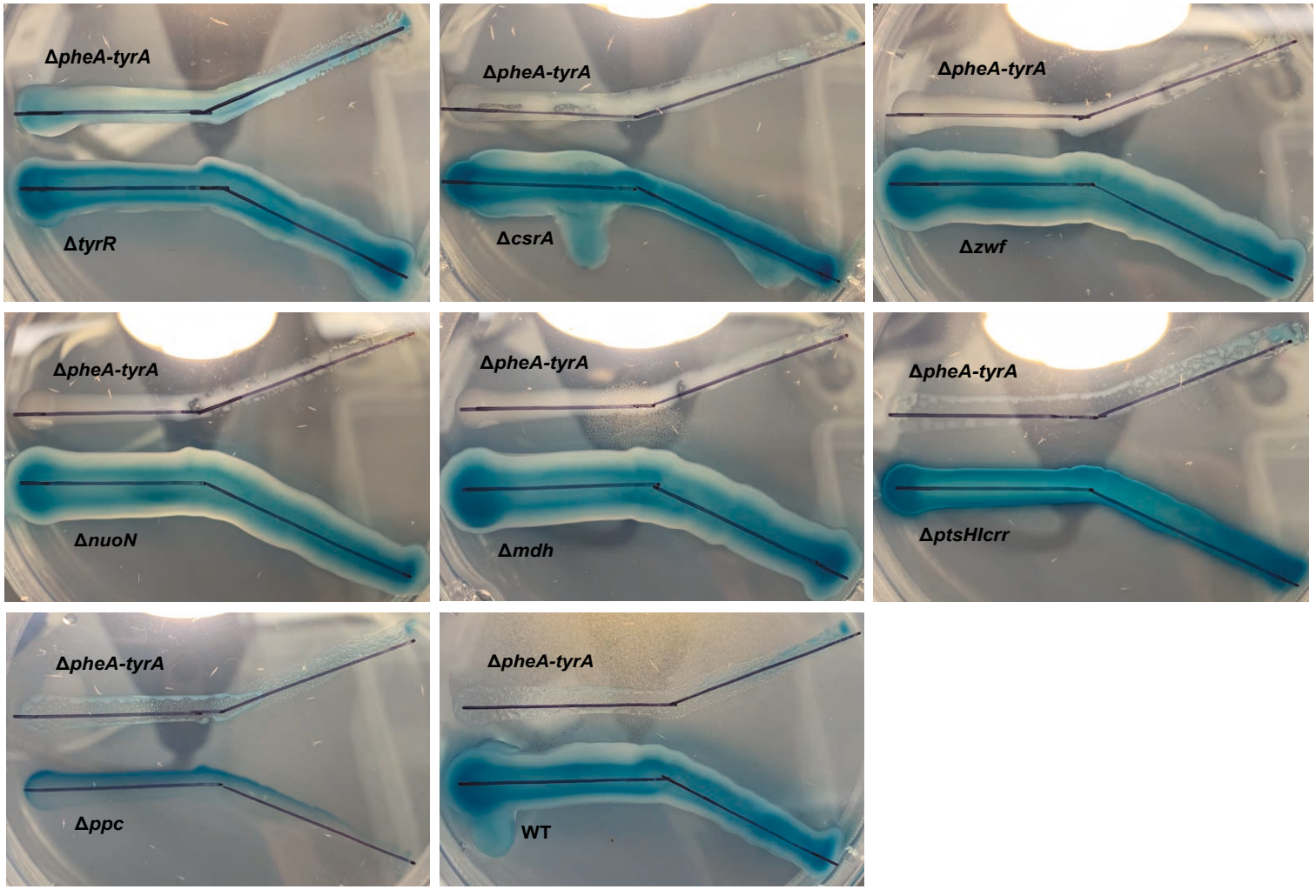

A
5 µm
C
apo tropharium
50 µm
B
apo adult mesenteric ceca
30 µm
D
apo vitellarium
50 µm
E
apo adult mesenteric ceca
40 µm
NUCLEIC ACID
CELL MEMBRANE
mCHERRY

Figure S2: Confocal imaging of *Sodalis praecaptivus* MC1 expressing mCherry (red) in aposymbiotic *Sitophilus* zeamais, related to Figure 3 and Figure 4. (**A**) Hemolymph isolated from adult *S. zeamais* following leg removal. (**B**) Mesenteric ceca from a newly emerged adult weevil obtained from parents injected with *S. praecaptivus* MC1 at egg stage. The inset image (lower) is zoomed and enhanced in contrast. (**C**) Tropharium visualized at 21 days following injection of an adult weevil with *S. praecaptivus* MC1. (**D**) Vitellarium visualized at 21 days following injection of an adult weevil with *S. praecaptivus* MC1. (**E**) Gut mesenteric ceca visualized at 21 days following injection of an adult weevil with *S. praecaptivus* MC1.

A


ΔpheA-tyrA
ΔtyrR
ΔpheA-tyrA
ΔcsrA
ΔpheA-tyrA
Δzwf
ΔpheA-tyrA
ΔnuoN
ΔpheA-tyrA
Δmdh
ΔpheA-tyrA
ΔptsHIcrr
ΔpheA-tyrA
Δppc
ΔpheA-tyrA
WT

B

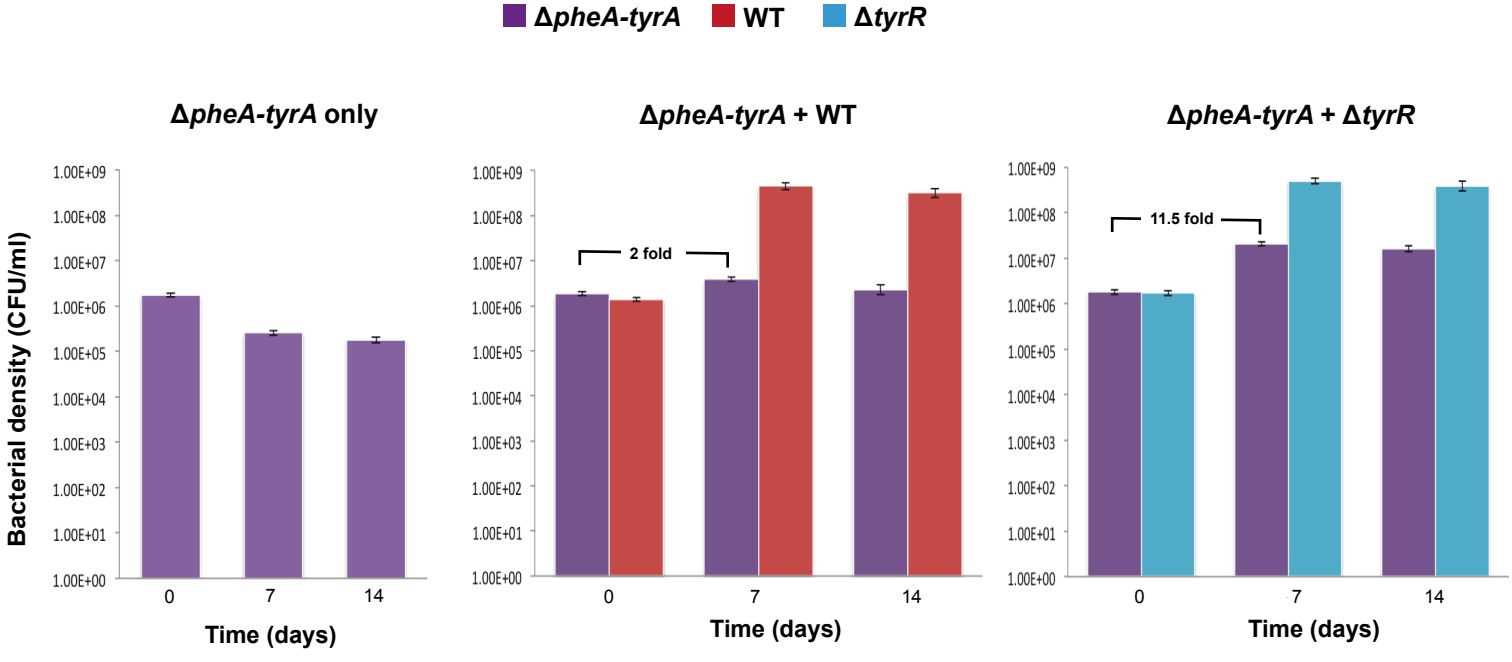

ΔpheA-tyrA
WT
ΔtyrR
ΔpheA-tyrA only
ΔpheA-tyrA + WT
ΔpheA-tyrA + ΔtyrR
Bacterial density (CFU/ml)
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
7
14
Time (days)
2 fold
11.5 fold

Figure S3: Tyr/Phe cross-feeding experiments, related to Figure 5. (**A**) Outcome of cross-feeding assays performed on minimal medium for 12 days with seven candidate Tyr/Phe overproducing *S. praecaptivus* mutant strains (Δ*tyrR*, Δ*nuoN,* Δ*csrA*, Δ*ppc*, Δ*ptsHIcrr*, Δ*zwf* and Δ*mdh*) and a WT control. Each strain was streaked adjacent to a Δ*pheA-tyrA S. praecaptivus* strain that is auxotrophic for Tyr and Phe and requires cross-feeding for growth. (**B**) Pairwise liquid cross-feeding assays in minimal medium. The auxotrophic Δ*pheA-tyrA* strain shows significant growth increase only in the presence of the Δ*tyrR* overproducer.

Modification target	Step 1.1F	Step 1.1R	Step 1.2F	Step 1.2R	Step 3F & Verification PCR F	Step 3R & Verification PCR R	Anticipated size
MC1	#2287: aagtcacacgctcacaccag	#2286: GTTTATAAGGAGACACTTTATGTTTAAGAAGacgtggttgcacgtaaatga	#2289: CTTTTGGAGGGGCAGAAAGATGAATGACTGTCttgaccgagacagctcattg	#2290:tcagcatcgcagtcttcatc	#2287: aagtcacacgctcacaccag	#2290:tcagcatcgcagtcttcatc	2kbp
Δ*pheAtyrA*	#1155:agggcgcgttttatattgaca	#1156: TGTCAAGAATAAACTCCCACATGGATTCGattggttacctttcacgcca	#1593: TGATATCGACCCAAGTACCGCCACCTAAagccttgtcaacctcatcga	#1594:gaaagccatatccatgccgg	#1155:agggcgcgttttatattgaca	#1594:gaaagccatatccatgccgg	1.6kbp
Δ*tyrR*	#1921:ttgctgggccagttaaaatc	#1922: GGGTTCGTGCCTTCATCCGTTTCCACGGTgatcgtacgcgacaccagta	#1925:TTTTATTATTTTTAAGCGTGCATAATAAGaatatggtttgagcggcaag	#1926:gcaggatagacggtggaca	#1921:ttgctgggccagttaaaatc	#1926:gcaggatagacggtggaca	1.1kbp
Δ*zwf*	#1934: gcgcgatatttttgacgttt	#1935:GGGTTCGTGCCTTCATCCGTTTCCACGGTtgatacaacgagggcaacaa	#1937: TTTTATTATTTTTAAGCGTGCATAATAAGggtcgtggaatgagttcgag	#1938: gcgctggttttccagttatt	#1934: gcgcgatatttttgacgttt	#1938: gcgctggttttccagttatt	1.6kbp
Δ*ptsHlcrr*	#1928:aggacgagtatcgcctacga	#1929:GGGTTCGTGCCTTCATCCGTTTCCACGGTcgcttttgccattagaggtc	#1931: CTTTTATTATTTTTAAGCGTGCATAATAAGaacgataacggcgctcaat	#1932: gattacccgcaaagtgctgt	#1928:aggacgagtatcgcctacga	#1932: gattacccgcaaagtgctgt	1.7kbp
Δ*mdh*	#1940: ttaggcgatgcctttatgct	#1941: GGGTTCGTGCCTTCATCCGTTTCCACGGTtacagcgagagttccccatc	#1943:TTTTATTATTTTTAAGCGTGCATAATAAGtgggtatcctcagcgacttc	#1944: ggcggctgtgattataaagg	#1940: ttaggcgatgcctttatgct	#1944: ggcggctgtgattataaagg	1.6kbp
Δ*ppc*	#1946:gttggcaattgacgaacctt	#1947: GGGTTCGTGCCTTCATCCGTTTCCACGGTcccatcgcatctttaatcgt	#1949: CTTTTATTATTTTTAAGCGTGCATAATAAGcgggcatgcgtaacacag	#1950: gacgacacttcatcctgacg	#1946:gttggcaattgacgaacctt	#1950: gacgacacttcatcctgacg	1.6kbp
Δ*nuoN*	#1628: gacgccggttccactatttc	#1629: TGGCAATTCCGGTTCGCTTGCTGTCCATAtgataaagtggttgcgtcgc	#1630: TCTATCGCCTTCTTGACGAGTTCTTCTGAtactgatctcctcgctgctg	#1631: gaggactattctcgccgga	#1628: gacgccggttccactatttc	#1631: gaggactattctcgccgga	1.2kbp
Δ*csrA*	#623:ggccgcaaaactctgagtag	#624: TGTCAAGAATAAACTCCCACATGGATTCGagtcgggtctctcagtttcc	#625: TTGATATCGACCCAAGTACCGCCACCTAAgatccctttcagcgccttg	#626: aacttgcgcagattggcag	#623:ggccgcaaaactctgagtag	#626: aacttgcgcagattggcag	1.5kbp

Note:

Bases presented in lower case are homologous to the target sequences

Bases presented in upper case are homologous to the selective marker sequences

Table S1: Oligonucleotides for the construction of *S. praecaptivus* mutants, related to STAR Methods.

Oligonucleotides	Source	Identifier
#127:gctattggtcgagcgttttacc	[25]	N/A
#128:cggcatcacatggtaatagc	[25]	N/A
#2287: aagtcacacgctcacaccag	This paper	N/A
#2286:gtttataaggagacactttatgtttaagaagacgtggttgcacgtaaatga	This paper	N/A
#2289:cttttggaggggcagaaagatgaatgactgtcttgaccgagacagctcattg	This paper	N/A
#2290:tcagcatcgcagtcttcatc	This paper	N/A
#2272:cttcttaaacataaagtgtctc	This paper	N/A
#2273:gacagtcattcatctttctgc	This paper	N/A
#1155:agggcgcgttttatattgaca	This paper	N/A
#1156:tgtcaagaataaactcccacatggattcgattggttacctttcacgcca	This paper	N/A
#1593:tgatatcgacccaagtaccgccacctaaagccttgtcaacctcatcga	This paper	N/A
#1594:gaaagccatatccatgccgg	This paper	N/A
#1921:ttgctgggccagttaaaatc	This paper	N/A
#1922:gggttcgtgccttcatccgtttccacggtgatcgtacgcgacaccagta	This paper	N/A
#1925:ttttattatttttaagcgtgcataataagaatatggtttgagcggcaag	This paper	N/A
#1926:gcaggatagacggtggaca	This paper	N/A
#1934:gcgcgatatttttgacgttt	This paper	N/A
#1935:gggttcgtgccttcatccgtttccacggttgatacaacgagggcaacaa	This paper	N/A
#1937:ttttattatttttaagcgtgcataataagggtcgtggaatgagttcgag	This paper	N/A
#1938:gcgctggttttccagttatt	This paper	N/A
#1928:aggacgagtatcgcctacga	This paper	N/A
#1929:gggttcgtgccttcatccgtttccacggtcgcttttgccattagaggtc	This paper	N/A
#1931:cttttattatttttaagcgtgcataataagaacgataacggcgctcaat	This paper	N/A
#1932:gattacccgcaaagtgctgt	This paper	N/A
#1940:ttaggcgatgcctttatgct	This paper	N/A
#1941:gggttcgtgccttcatccgtttccacggttacagcgagagttccccatc	This paper	N/A
#1943:ttttattatttttaagcgtgcataataagtgggtatcctcagcgacttc	This paper	N/A
#1944:ggcggctgtgattataaagg	This paper	N/A
#1946:gttggcaattgacgaacctt	This paper	N/A
#1947:gggttcgtgccttcatccgtttccacggtcccatcgcatctttaatcgt	This paper	N/A
#1949:cttttattatttttaagcgtgcataataagcgggcatgcgtaacacag	This paper	N/A
#1950:gacgacacttcatcctgacg	This paper	N/A
#1628:gacgccggttccactatttc	This paper	N/A
#1629:tggcaattccggttcgcttgctgtccatatgataaagtggttgcgtcgc	This paper	N/A
#1630:tctatcgccttcttgacgagttcttctgatactgatctcctcgctgctg	This paper	N/A
#1631:gaggactattctcgccgga	This paper	N/A
#623:ggccgcaaaactctgagtag	This paper	N/A
#624:tgtcaagaataaactcccacatggattcgagtcgggtctctcagtttcc	This paper	N/A
#625:ttgatatcgacccaagtaccgccacctaagatccctttcagcgccttg	This paper	N/A
#626:aacttgcgcagattggcag	This paper	N/A

Table S2: Oligonucleotides used in this study, related to STAR Methods.

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