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Nematode–bacteria mutualism: Selection within the mutualism supersedes selection outside of the mutualism

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The coevolution of interacting species can lead to codependent mutualists. Little is known about the effect of selection on partners within verses apart from the association. Here, we determined the effect of selection on bacteria (*Xenorhabdus nematophila*) both within and apart from its mutualistic partner (a nematode, *Steinernema carpocapsae*). In nature, the two species cooperatively infect and kill arthropods. We passaged the bacteria either together with (M+), or isolated from (M−), nematodes under two different selection regimes: random selection (S−) and selection for increased virulence against arthropod hosts (S+). We found that the isolated bacteria evolved greater virulence under selection for greater virulence (M−S+) than under random selection (M−S−). In addition, the response to selection in the isolated bacteria (M−S+) caused a breakdown of the mutualism following reintroduction to the nematode. Finally, selection for greater virulence did not alter the evolutionary trajectories of bacteria passaged within the mutualism (M+S+ = M+S−), indicating that selection for the maintenance of the mutualism was stronger than selection for increased virulence. The results show that selection on isolated mutualists can rapidly breakdown beneficial interactions between species, but that selection within a mutualism can supersede external selection, potentially generating codependence over time.

KEY WORDS: **Adaptation, coevolution, experimental selection, symbiosis.**

Reciprocal natural selection imposed by species interactions (coevolution) is the driving force behind a large proportion of adaptive evolution in nature (Thompson 1982, 1994). In particular, selection generated by antagonistic interactions is capable of dominating the evolutionary trajectories of species (Decaestecker et al. 2007; Wade 2007; Brockhurst and Koskella 2013; Brockhurst et al. 2014; Lively and Morran 2014). Presumably, selection generated within mutualisms is also a strong evolutionary force. Mutualistic interactions likely played a role in shaping the evolutionary trajectories of chloroplasts and mitochondria, leading to the complete loss of their free-living states (Margulis 1970; Margulis and Sagan 2002; Wernegreen 2012). Such extreme interdependence may result from restricted evolutionary trajectories imposed upon symbiont species by selection and drift within mutualisms (Moran 1996; Herre et al. 1999; Wernegreen 2002; Regus et al. 2014). As a beneficial interaction becomes a major determinant of fitness for symbionts, selection to maintain a successful mutualism can be quite strong (Axelrod and Hamilton 1981; Bull and Rice 1991; Douglas 1998; Rispe and Moran 2000; Tamas et al. 2002; Regus et al. 2014; Murfin et al. 2015).

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Recent studies have suggested that intergenomic epistasis (i.e., genotype-by-genotype interactions between symbionts; Wade 2007) can determine the benefits of a mutualistic interactions and the fitness of the symbionts (Parker 1995; Heath and Tiffin 2007; Heath 2010; Heath et al. 2010). Further, experimental evolution studies have demonstrated that selection within a mutualism can be vital to maintaining beneficial interactions between symbionts. Symbiotic bacteria evolving in the absence of their hosts can rapidly become poor symbionts, as evidenced by the complete or partial loss of beneficial interactions upon reintroduction to their respective hosts (Sachs et al. 2011; Chapuis et al. 2012). This work indicates that selection within mutualisms may act to maintain advantageous associations between partners. However, the extent to which selection within a mutualism can alter the evolutionary trajectories of symbionts remains unclear.

Here, we conducted experimental evolution to determine the effects of selection within a mutualism on the evolutionary trajectories of symbiotic bacterial populations. We used the Gram-negative bacterium *Xenorhabdus nematophila* and its symbiotic mutualist nematode *Steinernema carpocapsae*, because these species can be disassociated and reared independently in the laboratory. In nature, the nematode and bacteria are mutualistic partners that together parasitize the larvae of multiple arthropod species. The nematode houses the bacteria in a gut receptacle (Martens et al. 2003; Martens and Goodrich-Blair 2005; Martens et al. 2005; Synder et al. 2007). The nematode also facilitates dispersal and gains entry into arthropod hosts. Upon entry, the nematodes release the bacteria; and, together, the nematodes and bacteria kill the host. The bacteria then digest the host and facilitate the growth and reproduction of the nematodes within the host cadaver (Goodrich-Blair 2007; Richards and Goodrich-Blair 2009). As resources are depleted, the bacteria reassociate with juvenile nematodes; one or two bacterial cells colonize a specialized receptacle in the nematode, growing to a population of ~200 cells (Martens et al. 2003, 2005; Chaston et al. 2013). The juvenile nematodes (called "I.J.s" for "infective juveniles") then emerge from the insect cadaver and search for a new host. Importantly, *X. nematophila* can be maintained independently of its nematode partner in the laboratory and then reintroduced (Sicard et al. 2004; Chapuis et al. 2011).

We tested the relative strength of selection within the mutualism versus selection apart from the mutualism. We disassociated the bacteria–nematode partners, and passaged the bacteria apart from the nematode in one treatment (M−), and together with the nematode in another treatment (M+; Fig. 1). During each passage, we also imposed selection for greater virulence (faster host killing; S+) of the insect host, *Galleria mellonella*, combined with controls (S−) in which selection was random. After 20 rounds of selection, we conducted assays measuring changes in bacterial virulence and symbiotic function to determine the relative strength of selection imposed by evolution within the mutualism.

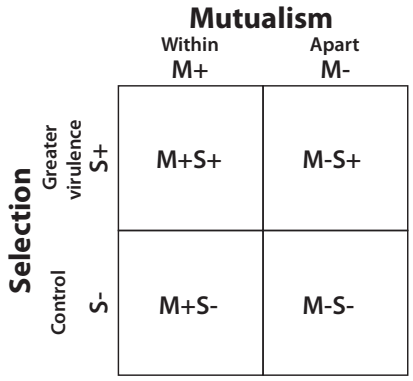

Figure 1. Bacterial experimental evolution treatments. Replicate populations of bacteria derived from a single ancestral colony were exposed to four different treatment combinations of differing mutualism and selection conditions. Bacterial populations were passaged within nematodes (M+) or apart from nematodes (M−). Further, the populations were either exposed to selection for greater virulence against the *Galleria mellonella* hosts (S+) or passaged under random selection (S−). Four replicate populations were passaged under each mutualism-by-selection treatment combination (M+S+, M+S−, M−S+, and M−S−) for 20 rounds of selection.

Materials and Methods

The ancestral strain of *X. nematophila* used here was extracted from the R8-1 line of *S. carpocapsae* (Vigneux et al. 2008) and grown on nutrient agar supplemented with 0.004% (w / v) triphenyltetrazolium chloride and 0.0025% (w / v) bromothymol blue (NBTA) agar containing ampicillin (50 μg/mL). Bacteria were extracted as described by Sicard et al. (2003 and 2004). A single colony was grown overnight at 28°C in Luria-Bertani broth (LB) and used to create all replicate populations used in this experiment. Samples of the ancestral population were frozen at −80°C.

The *S. carpocapsae* used in this experiment were also derived from the R8-1 line (Vigneux et al. 2008). Infective juveniles were axenically reared, as described by Sicard et al. (2005). Axenic nematodes were then reassociated with the ancestral *X. nematophila* by inoculating *G. mellonella* larvae (Vanderhorst Wholesale, St. Mary's, OH) with approximately 1×10^4 colony-forming unit (CFU) and then infecting the host larvae with approximately 100 axenic nematodes. Nematodes and bacteria then reassociated within the hosts and emerged from the host carcass in accordance with their normal life cycle. Samples of emerging *S. carpocapsae* were crushed as described above to confirm bacterial colonization, and then used to found the M+ experimental populations (Fig. 1). All M+ experimental populations were derived from the same stock of newly reassociated nematodes and bacteria.

EXPERIMENTAL EVOLUTION

Four different treatments were used on the *X. nematophila* bacteria in this experiment: Mutualism + Selection+ (M+S+), Mutualism + Selection− (M+S−), Mutualism− Selection+ (M−S+), and Mutualism−Selection− (M−S−; Fig. 1). "Mutualism+" indicates passage within the mutualism with *S. carpocapsae* with potential for coevolution; and "Mutualism - " indicates passage apart from the mutualism with potential for independent evolution. "Selection+" indicates selection for increased virulence against the *G. mellonella* host. "Selection−" (control) indicates no selection for virulence. There were four replicate populations of each treatment and, therefore, 16 total experimental populations. Populations were maintained at 28°C throughout experimental evolution.

Experimental evolution was performed for 20 host passages, totaling at least 240 bacterial generations and 40 nematode generations in the M+ populations, and at least 320 bacterial generations in the M− populations. M+ passages were performed by pipetting 40 I.J.s onto each *G. mellonella* host. Overall, nematodes have approximately a 50% success rate when infecting *G. mellonella* larvae under these conditions (Gaugler 2002; Bashey and Lively 2009). Because each individual nematode carries a very small population of bacteria, the within host bacterial genetic diversity was small (Martens et al. 2003). We estimated that a maximum of 20 unique bacterial genotypes, a maximum of one unique bacterial genotype per successful nematode infection, and 4×10^3 CFUs per *G. mellonella* host were transmitted with the nematodes, each nematode carries approximately 200 CFUs (Martens et al. 2003, 2005; Chaston et al. 2013). We infected 15 larval *G. mellonella* hosts per replicate population during each round of selection. The exact number of bacterial generations per round of selection is unclear. Using previous calculations of within *G. mellonella* (Vivas and Goodrich-Blair 2001; Sicard et al. 2004) and within *S. carpocapsae* growth rates (Martens et al. 2003; Chaston et al. 2013), we determined that bacteria in this treatment underwent at least 12 generations per round of selection, at least seven generations in *G. mellonella* and five to 10 generations within the nematode. However, that number is likely an underestimate, given that bacterial growth within the nematode has been detected outside of the receptacle (Chaston et al. 2013) and within-host and within-nematode growth rates were determined by measuring CFUs, which underestimates bacterial replication in the absence of population growth.

For the M− treatment, needles (26 gauge, ½ inch) were used to passage the bacterial populations by directly infecting *G. mellonella* hosts. Passages were performed by picking 20 unique *X. nematophila* colonies, mixing these colonies in 1 mL of phosphate-buffered saline (PBS), then introducing them to *G. mellonella* hosts by pushing a needle dipped in the *X. nematophila* slurry through the host skin into the hemolymph. Fifteen hosts were infected per replicate population per passage, each with the same slurry made from 20 colonies. Each host was jabbed four times, introducing approximately 4×10^3 total CFUs per host. *Xenorhabdus nematophila* cells extracted from dead *G. mellonella* larvae (that died of bacterial infection) were serially diluted with PBS and grown separately from their nematode symbionts on NBTA agar containing ampicillin (50 μg/ml) at 28°C for approximately 36 h then the infection process was repeated. Based on *X. nematophila* growth rates within *G. mellonella* (Vivas and Goodrich-Blair 2001; Sicard et al. 2004), and estimated growth rates of our ancestral bacterial strain on NBTA-ampicillin (50 μg/ml) agar ($1.63 \times 10^3 \pm 241$ total CFUs per colony under this experimental evolution protocol), we estimated that bacterial populations in the M− treatment underwent at least 16 generations per round of selection, at least seven generations in *G. mellonella* and nine to 14 generations on NBTA-ampicillin plates. Again, this measurement is based on CFU counts, which can underestimate bacterial generations.

Selection for higher virulence was performed in the S+ treatments by monitoring infected *G. mellonella* hosts, beginning 10 h postinfection, and identifying the first host (of 15) to die in each replicate population. In the M+S+ treatment, this host was placed in a white trap (Bashey et al. 2007); emerging nematodes were then collected from the host, and passaged to the next group of hosts. We transferred nematodes from the second host to die in the event that the first dead *G. mellonella* did not produce I.J.'s in the M+S+ treatment. In the M+S− treatment, nematodes were collected from a randomly chosen dead *G. mellonella* host (rather than the first one to die) and passaged to a new group of 15 hosts in the same way as for the M+S+ treatment. Dice were used to randomly select hosts.

In the M−S+ treatment, the first *G. mellonella* host to die from each replicate population was crushed to allow for the extraction of *X. nematophila*. These bacteria were then plated on NBTA-ampicillin (50 μg/ml) agar and passaged as described above. Bacteria were also isolated from the second host to die in the event that extract from the first host was unsuccessful.

Samples from all bacterial populations were frozen from a slurry of 20 colonies prior to selection (the ancestor). Similar samples were also taken after 10 and 20 host passages. M+ bacterial populations were obtained by crushing nematodes as described above, then frozen. The 20 colony slurries were grown overnight at 28°C in LB, preserved in a 20% glycerol solution, and frozen at −80°C in 1.5 mL aliquots.

BACTERIAL VIRULENCE ASSAY AFTER 10 ROUNDS OF SELECTION

Bacterial populations in the M− treatment were assayed after 10 rounds of passage, along with the ancestor and a PBS control. Samples of frozen bacterial populations were streaked onto

NBTA-ampicillin (50 μg/ml) plates and grown at 28°C for 36 h. Twenty unique colonies were picked from agar plates, and then mixed together in 0.25 mL of PBS. The mixture was used to infect *G. mellonella* hosts via three jabs per host with 26 gauge needles. Fifteen *G. mellonella* hosts were infected per replicate bacterial population. Each host was infected with approximately 1×10^4 CFUs and then incubated at 28°C. Host survival was monitored for 40 h. The ancestral population and one control group of hosts (jabbed with PBS alone) were also monitored. No hosts in the (PBS) control died during this assay.

Statistical analyses were performed in JMP-10, (SAS Institute, Cary, NC). A nonparametric Kruskal–Wallis test was used to test for a treatment effect on the mean time to host death in experimental populations (S+ vs. S−).

BACTERIAL VIRULENCE ASSAY AFTER 20 ROUNDS OF SELECTION

Replicate populations for each of the four treatments (Fig. 1) were assayed after 20 host passages, along with the ancestral population. Samples of the frozen bacterial populations were streaked onto NBTA-ampicillin (50 μg/mL) plates, and infection carried out as described above. Thirty hosts were infected with bacteria from each replicate population. Only one host of 30 total hosts in the control group died over the course of the assay.

Statistical analyses were performed in JMP-10. An ANOVA was performed on mean time to *G. mellonella* death for all experimental populations. We tested the main effects of the mutualism treatment, the selection treatment, and the interaction between the mutualism and selection treatments. All effects were treated as fixed. Comparisons between specific treatment means were performed using least squared means contrast tests within the ANOVA framework. The nonparametric Kruskal–Wallis test was used to compare the experimental populations to the ancestral population.

Hosts in this assay exhibited increased mean times to host death, relative to the assay carried out after 10 passages. The assays testing virulence after 10 and 20 passages were conducted at different times and on different batches of hosts. Previous results have shown that host susceptibility can vary between batches, but qualitative differences between bacterial populations are repeatable across host batches (Bashey et al. 2007). Therefore, differences in mean values between the assays after 10 and 20 passages likely reflect differences among host batches.

MUTUALISM VIRULENCE ASSAY

We assessed virulence in the nematode–bacteria partners from the M+ treatment after 20 rounds of selection. We also measured virulence in the ancestral pairing of the nematode and bacterial populations. We did this by infecting 15 *G. mellonella* hosts with 40 nematodes from each replicate population; we then monitored the time to host death at 28°C over the next 40 h. The ancestral mutualism used in this assay was not the original founding population of the experimentally evolved populations, but was derived from the original population. *Steinernema carpocapsae* cannot be reliably resuscitated from frozen stocks, therefore the original population was passaged through *G. mellonella* hosts on two separate occasions, to maintain viability, and then stored at 4°C in between passages.

Statistical analyses were performed in JMP-10. A nonparametric Kruskal–Wallis test was used to test for a treatment effect (S+ vs. S−) on the mean time to host death in the experimental populations. We also used the Kruskal–Wallis test to compare both of the treatment combinations to the ancestral population.

MUTUALISM REASSOCIATION ASSAY

We reintroduced all four replicate populations of M+S+, M+S−, M−S+, and M−,S− to nematodes after 20 rounds of selection and storage at -80°C. As previously stated, the M+ populations were isolated from nematodes after selection and prior to freezing. Each replicate bacterial population was introduced to R8-1 axenic nematodes. First, we injected twenty *G. mellonella* hosts per replicate population with 1×10^4 CFUs for each experimental bacterial population and the ancestral population. Then, we exposed 40 axenic nematodes to each *G. mellonella* host and stored the hosts at 28°C. All hosts died within 48 h of injection and exposure and were moved to White Traps maintained at 28°C. We monitored each *G. mellonella* host for nematode emergence. Successful re-association and production of a viable mutualism required the emergence of nematodes harboring the bacteria. All emerging nematodes were collected and a sample of approximately 1000 nematodes from each replicate population or the ancestor was crushed to confirm the presence of the bacteria. All sampled replicate populations produced nematodes that carried the bacteria. However, some *G. mellonella* carcasses failed to produce emerging nematodes. Therefore, the viability of the restored mutualisms was measured as the proportion of *G. mellonella* hosts infected that produced nematodes harboring *X. nematophila*.

Statistical analyses were performed in JMP-10. Nematode emergence was treated as binomial data for each infected host (emergence or no emergence). A generalized linear model (GLM) assuming a binomial distribution and logit link function was used to test for mutualism treatment effects (M+ vs. M−, with replicate population nested within mutualism treatment) on emergence in the experimental populations. An additional GLM with binomial distribution and logit link function was used to test the effect of selection treatment within the M− populations (M−S+ vs. M−S−, with replicate population nested within selection treatment). Lastly, a GLM with binomial distribution and logit link function was used to compare the ancestral population to the M+ populations. Bonferroni corrections were applied to correct for multiple tests, $P < 0.0166$.

REASSOCIATED MUTUALISM VIRULENCE ASSAY

After each bacterial experimental replicate population successfully colonized nematodes, we conducted a virulence assay on the reconstituted mutualisms. We infected 24 *G. mellonella* hosts with 40 nematodes each from each replicate population. *G. mellonella* death was monitored over time (40 h) in all replicate populations, as well as the ancestral population. Five hosts infected by nematodes harboring bacteria from the M−S+ population no. 3 did not die. These data points were excluded from the analysis, but further emphasize the reduced virulence exhibited by the M−S+ populations.

Statistical analyses were performed in JMP-10. Time-to-host death measurements were square root transformed to meet assumptions of normality. An ANOVA was performed testing the main effects of mutualism treatment, selection treatment, and the interaction between mutualism and selection treatments. All effects were treated as fixed. A least squared means contrast test was used to compare the M−S+ mean to the other treatment means.

Results

BACTERIAL VIRULENCE

We found that the experimental bacterial populations in both the M+ and M− treatments evolved greater virulence (faster killing rate) relative to the ancestral population over the course of the experiment (Fig. 2A; $\chi^2_1 = 19.72$, $P < 0.001$). More interestingly, we found that the relative increase in virulence differed between treatments. After 10 passages, the bacterial populations maintained apart from the mutualism and under selection (M−S+) exhibited significantly greater virulence than the controls (M−S−; Fig. S1; $\chi^2_1 = 4.08$, $P = 0.043$). After 20 passages, the M−S+ populations continued to exhibit greater virulence than the M−S− populations (Table S1; Fig. 2A; $F_{1,12} = 21.52$, $P = 0.0006$). Further, the M−S+ populations also evolved greater virulence than the bacterial populations evolved within hosts for 20 passages, both those under selection (M+S+; Table S1; Fig. 2A; $F_{1,12} = 5.382$, $P = 0.0388$) and controls (M+S−; Table S1; Fig. 2A; $F_{1,12} = 7.437$, $P = 0.0184$). Conversely, we observed no effect of selection for greater virulence in the bacterial populations passaged with the nematodes (M+S+ vs. M+S−; (Table S1; Fig. 2A; $F_{1,12} = 0.166$, $P = 0.691$). Therefore, virulence evolved most rapidly in the bacterial populations evolved under selection and apart from the mutualism, and selection within the mutualism did not alter the rate of virulence evolution.

MUTUALISM VIRULENCE

Selection within the mutualism did not result in greater bacterial virulence (Fig. 2A), but may have produced a more virulent nematode–bacteria mutualism. We tested for the effect of selection within the mutualism more broadly by assaying virulence

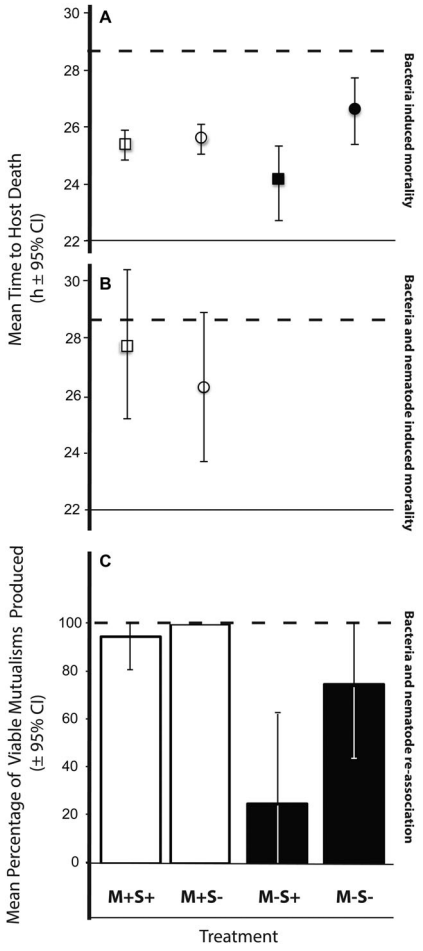

Figure 2. Evolution within the mutualism alters the response to selection. (A) Experimental and ancestral bacterial populations were injected into *Galleria mellonella* hosts, virulence was measured as mean time to host death. Experimental populations evolved greater virulence relative to ancestor, but populations evolved apart from the mutualism and under selection for greater virulence (M−S+) evolved the greatest degree of virulence. (B) Experimental and ancestral bacterial populations associated with their respective nematode populations were exposed to *G. mellonella* hosts, and mean time to host death measured for each population. Selection for greater virulence within the mutualism did not alter levels of virulence of the whole mutualism relative to the controls, and virulence did not increase relative to the ancestral population. (C) Experimental and ancestral bacterial populations were reassociated with the same population of aposymbiotic nematodes. The mean percentage of viable mutualisms produced was determined for each bacterial population. Mutualism viability required bacterial colonization and survival of both the nematode and bacteria. Bacterial populations evolved apart from the nematodes (M−) produced fewer viable mutualisms upon reassociation with the nematode relative to populations that evolved within the mutualism (M+) and the ancestral population. Further, bacterial populations that evolved apart from the mutualism under selection for greater virulence (M−S+) exhibited significantly lower viability than those that did not evolve under selection (M−S−). Dashed lines indicate the ancestral mean.

evolution in nematodes carrying the bacteria. We found no significant difference in virulence between the nematodes and bacteria that we subjected to selection, M+S+, versus those that did not experience selection for greater virulence, M+S− (Fig. 2B; $\chi^2_1 = 2.108$, $P = 0.147$). In accordance with the bacterial virulence assay results, selection within the mutualism also did not alter the rate of virulence evolution in the mutualism as a whole.

MUTUALISM REASSOCIATION

We tested for the ability of each bacterial population to successfully reassociate with ancestral nematodes after experimental evolution. We found that the M+ bacterial populations produced viable mutualisms at the same rate as the ancestral bacteria (Fig. 2C; $\chi^2_1 = 0.477$, $P = 0.489$). However, the bacterial populations that evolved apart from the mutualism (M−) produced fewer associations upon reintroduction to the nematode than those that evolved within the mutualism (M+; Table S2, Fig. 2C; $\chi^2_7 = 63.94$, $P < 0.001$). Moreover, the bacterial populations that evolved apart from the mutualism and under selection (M−S+) exhibited significantly lower frequencies of successful associations than those that evolved in the absence of selection (M−S−; Table S3; Fig. 2C; $\chi^2_7 = 37.5$, $P < 0.001$). Therefore, passage apart from the nematode, coupled with selection for greater bacterial virulence, produced bacterial populations with significantly reduced fitness in the context of the mutualism.

REASSOCIATED MUTUALISM VIRULENCE

We then examined the functionality of each bacterial population within the reconstituted mutualism by testing the virulence of the nematode–bacteria pairs as a whole. We found that nematodes harboring bacterial populations that evolved apart from the mutualism and under selection (M−S+) induced significantly lower rates of host death than nematodes carrying all other bacterial populations (Table S4; Fig. 3; $F_{1,12} = 12.06$, $P = 0.005$). Thus, the most virulent bacterial populations (M−S+) prior to reassociation with nematodes caused significantly lower mortality in the larval host following reintroduction to nematodes, relative to bacteria from all other treatments.

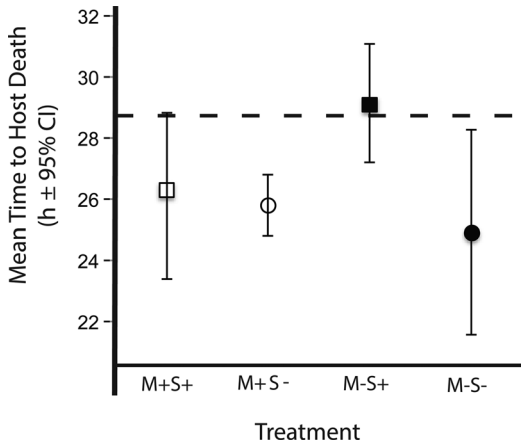

Figure 3. Bacterial virulence apart from the nematode is not an indicator of virulence within the mutualism. Virulence was measured in nematodes after experimental and ancestral bacterial populations were reassociated with nematodes. *Galleria mellonella* hosts were exposed to bacteria-carrying nematodes, mean time to host death was determined for each bacterial population. Bacterial populations that evolved apart from the mutualism and under selection for greater virulence (M−S+) exhibited significantly reduced virulence when infecting insect hosts as part of the mutualism. The dashed line indicates the ancestral mean.

Discussion

Overall, selection within the *X. nematophila/S. carpocapsae* mutualism precluded a response to selection for greater virulence in *X. nematophila*. The bacterial populations that evolved within the mutualism and under selection (M+S+) evolved significantly lower virulence than the populations that evolved apart from the mutualism and under selection (M−S+; Fig. 2A; Table S1). In fact, the populations that evolved under selection within the mutualism (M+S+) did not exhibit a response to selection relative to their controls (M+S−; Fig. 2A, B; and Table S1). Rather, the populations that evolved within the mutualism (M+) maintained a greater level of fitness as mutualist partners relative to the populations evolved apart from the mutualism (M−), and particularly the populations that evolved apart from the mutualism and under selection (M−S+; Figs. 2C and 3; and Tables S2–S4). Therefore, populations of *X. nematophila* were capable of responding to selection outside the mutualism, but the response to selection for increased virulence was coupled with reduced fitness within the mutualism. Ultimately, selection applied by the mutualism was the more dominant force because evolution within the mutualism constrained the populations' response to external selection.

Despite the lack of response to direct selection for increased virulence, the bacterial populations evolved within the mutualism, and all other experimental populations evolved greater virulence relative to the ancestral bacteria (Fig. 2A). This result was likely due to adaptation to the rearing temperature of 28°C that was used throughout the study. The ancestral population was previously maintained at 26°C (Vigneux et al. 2008), and *X. nematophila* populations can evolve greater virulence during serial passage at 28°C as a consequence of evolving increased growth rates (Chapuis et al. 2011). Therefore, bacterial evolution within the mutualism was not completely constrained. Rather, bacterial evolution within the mutualism was likely limited by any negative consequences of evolutionary change on the fitness of the mutualism as a whole.

Bacterial evolution within the mutualism may have also been affected by reduced effective population sizes and longer

generation times imposed by the nematode. The *S. carpocapsae–X. nematophila* mutualism permits partner choice by the nematode (Chaston et al. 2013). Partner choice can reduce symbiont effective population sizes, as the partner may choose to associate with only the most beneficial symbiont genotypes (Kiers et al. 2003). In the context of our experiment, this may have depleted genetic diversity in the M+ bacterial populations and decreased the efficacy of selection. Conversely, such partner choice may have also maintained the mutualistic interaction by selecting for beneficial symbionts rather than the most virulent symbionts (Fig. 2C). In addition to effective population size, the nematode likely also constrained the number of bacterial generations per passage. Bacterial growth within the nematode is quite limited after colonization (Martens et al. 2003, 2005; Chaston et al. 2013), particularly relative to the bacterial populations passaged apart from the nematode in our experiment. This difference in generations per passage may have altered the evolutionary trajectories of populations with regards to the state of the mutualism.

Constraints on symbiont effective population sizes and growth rates may be a common consequence of evolution within a mutualism in nature (Thompson 2005) and could be viewed as part of the mutualism treatment effect within the context of our experiment. However, it is important to note that the lack of response to selection for increased virulence in the M+ populations after 20 passages was not driven by an insufficient amount of genetic diversity nor a sufficient number of generations to facilitate evolutionary change. Indeed, the M+ populations evolved greater virulence relative to the ancestral bacteria (Fig. 2A). Instead the populations that evolved within the mutualism did not respond to selection that ultimately favored the evolution of a poor symbiont phenotype in the M−S+ populations. Thus, selection within the mutualism maintained beneficial bacterial symbionts.

The altered evolutionary dynamics that we observed as a result of evolution apart from the mutualism may be a product of the genetic interactions that facilitate the association between the bacteria and the nematode. Several *X. nematophila* genes have pleiotropic functions that facilitate mutualistic interactions between the bacteria and its nematode host, while also facilitating virulence against the arthropod host (Cowles et al. 2007; Herbert et al. 2007; Richards and Goodrich-Blair 2009; Tran and Goodrich-Blair 2009). Improper signaling between the nematode host and the bacteria can disrupt the mutualism, reducing nematode fecundity and delaying reproduction (Richards and Goodrich-Blair 2010). In the absence of selection to maintain the mutualism, the bacterial genome was free to evolve along different evolutionary trajectory, one dominated by the bacteria's virulence function against the insect host. In contrast, selection for beneficial interactions within the mutualism likely selected against alleles that disrupted signaling between the partners, likely slowing the response to selection for greater virulence. However, this does not necessarily imply that selection within a mutualism will persistently limit evolutionary change in symbiont populations. Selection within a mutualism should limit certain trajectories, specifically those that reduce the efficacy of the interaction. But, evolution within the host may also provide novel evolutionary pathways for coevolving symbionts (Gibson et al. 2015). Selection for the maintenance of mutualistic interactions may also drive rapid symbiont adaptation when host populations undergo evolutionary change. Such host evolution may have been limited in our experiment due to the potential strength of genetic drift relative to selection in the nematode populations. Nonetheless, the evolutionary trajectories of the bacterial populations were altered as a result of the evolution within the mutualism.

Consistent with previous experimental evolution studies on symbiotic bacteria (Sachs et al. 2011; Chapuis et al. 2012), we found that selection imposed by the mutualism was important in maintaining the symbiotic functions of the bacteria. Additionally, our work implies that selection within a mutualism can be a dominant force that persistently reinforces the mutualistic interaction. Species that rely heavily on intergenomic epistasis (Heath 2010; Heath et al. 2012) may experience similar evolutionary dynamics with regard to natural selection when selection acts to maintain specific beneficial allelic combinations within the mutualism (Wade 2007). Although most mutualisms remain understudied, such genetic interactions are thought to be a feature of many symbiotic interactions and are known to contribute to the overall fitness and genetic architecture of both mutualistic partners (Marchetti et al. 2010; Heath and Stinchcombe 2014).

Given the widespread prevalence of mutualistic interactions in nature, it is possible that the evolutionary trajectories of many species are altered in one way or another as a result of selection imposed by mutualistic relationships. Our study demonstrates that selection imposed by mutualistic interactions can be a dominant force. Such strong selection to maintain mutualistic integrity, when continuously imposed over many generations, could contribute to the evolution of closely associated mutualisms and potentially give rise to endosymbiosis.

DATA ARCHIVING

The doi for our data is doi:10.5061/dryad.p3hr1.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Bacterial virulence ANOVA table.
Table S2. Mutualism reconstitution GLM table.
Table S3. Free-living bacteria reconstitution GLM table.
Table S4. Reconstituted mutualism virulence ANOVA table.
Figure S1. Virulence evolution apart from the mutualism after 10 rounds of selection.