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Establishing a novel beneficial association using a nematode-bacterium model through experimental evolution

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Abstract

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By Kim Hoang

Microbes confer many benefits to their hosts, from nutrient provisioning to protection from enemies. These microbial symbioses are ubiquitous in nature, many of which evolved millions of years ago. However, it is unclear how these associations arise. My dissertation explores the roles of environmental and evolutionary contexts in the evolution of novel beneficial associations. I use the nematode *Caenorhabditis elegans* and the bacterium *Bacillus subtilis* as a model system to investigate these questions by experimentally evolving them under different conditions. Before experimental evolution, I find that *B. subtilis* protects *C. elegans* from heat shock by increasing host fecundity compared to the *C. elegans* standard diet, *E. coli*. To examine how *B. subtilis* influence *C. elegans* adaptation, I evolve nematodes in the presence or absence of ancestral *B. subtilis* with or without heat stress. Hosts evolving with ancestral *B. subtilis* under stress evolve the greatest fitness gain compared to in the absence of heat stress or in the absence of *B. subtilis*. These results show that association with novel beneficial microbes can help hosts adapt to stress under the right context. To determine how microbial evolutionary history affects host fitness, I passage *B. subtilis* through ancestral nematodes, in the absence of nematodes, or through co-passaged nematodes. I find that *B. subtilis* passaged through ancestral hosts evolve to benefit these hosts, but host adaptation is not necessary since bacteria passaged in the absence of hosts also increase host fitness. Finally, because co-passaged bacteria do not improve ancestral host fitness to the same level as bacteria passaged through ancestral hosts, I further investigate co-passaging by examining the fitness of co-passaged hosts. Co-passaged bacteria reduce fitness of their co-passaged hosts but not hosts that evolved with ancestral *B. subtilis*, suggesting that coevolution may impede the establishment of novel symbioses. By leveraging experimental evolution and a tractable model system, this work illuminates how environmental and evolutionary contexts shape the evolution of host-microbe associations.

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CHAPTER I

INTRODUCTION

Many plants and animals receive benefits, such as nutrient supplementation or protection from environmental stresses, by harboring microbes (Douglas, 1998; Russell and Moran, 2006; Vorburger et al., 2010). Such microbe-conferred benefits allow the host to occupy niches that would otherwise be unsuitable for the host alone (Douglas, 2014; McFall-Ngai et al., 2013). In addition, microbes may benefit from living within the host due to more optimal growing conditions than the external environment (Boettcher and Ruby, 1990; Douglas, 1998). Continual association between host and microbe can lead to fitness benefits as well as genomic modifications in both partners (Douglas, 2014; Toft and Andersson, 2010). Symbioses, long-term associations over evolutionary time, are the result of extensive interactions between host and microbial populations. Despite numerous accounts of beneficial associations with microbes, the molecular and evolutionary mechanisms involved in the origins of microbial symbioses remain elusive. The goal of this dissertation is to evaluate the conditions under which a novel beneficial association can be established between a host and a microbe. I examine the roles of environmental and evolutionary contexts in shaping the interaction between an incipient nematode host and a protective bacterium, an association I enforce through experimental evolution. While I did not evolve a symbiosis in terms of a "long-term" association, the findings from this model system will help establish the basis for how novel symbioses evolve.

The origin of beneficial symbioses

While many symbiotic interactions result in net fitness benefits for at least one of the partners

across all contexts (e.g., obligate symbioses), the benefits (and costs) of numerous long-term host-microbe associations are shaped by biotic and abiotic factors, such as the genotypes of the host and microbe, the environment in which they interact, their evolutionary history, and/or the presence of other species (Daskin and Alford, 2012; Polin et al., 2014; Russell and Moran, 2006; Wade, 2007). Several studies have found high genetic variation within host and symbiont populations, suggesting that there are genotype-dependent interactions in which strains of symbionts confer different effects toward a specific host genotype. For example, *Steinernema* spp. nematodes infected with their native *Xenorhabdus bovienii* symbionts have higher fitness than those infected with symbionts isolated from more distantly related hosts, indicating that strain-specificity is important for long-term association between host and microbe (Murfin et al., 2015). In another study, genotype by genotype interactions between populations of the legume, *Medicago truncatula,* and strains of its rhizobium, *Sinorhizobium medicae,* are found to be influenced by environmental factors such as soil nitrogen levels (Heath and Tiffin, 2007). Despite a large body of literature on how associations are maintained between established and often long-term symbioses, we know little about the factors involved in how these symbioses formed initially (Bergstrom et al., 2003; Doebeli and Knowlton, 1998). Do novel beneficial associations evolve under a broad range of conditions, or are they more likely to arise under certain conditions than others?

Adaptation to new environments is critical for population expansion, evolution, and diversification of all organisms. New environments often lack resources that an incipient population may have had in its previous environment, or they may exhibit hostile features (e.g., enemies or harsh temperatures). By themselves, organisms may lack the genetic variation and/or time to adapt, preventing them from proliferating in the new environment. On the other hand, if

individuals in the population harbored microbes that allowed them to utilize resources or protected them from harm in the new environment, the host population would be able to adapt more rapidly. These traits may involve nutrients that the microbes produce to supplement host diet, or increased fitness when exposed to enemies or harsh conditions. Ultimately, microbes serve as a way for hosts to obtain new traits on a much shorter timescale than if hosts were to evolve the traits themselves. These benefits can then facilitate host association with certain microbial partners over an extended period of time, within and across individual host lifespans. As a consequence, association with beneficial microbes can expand host niches and permit them to persist in previously hostile environments (Kitano and Oda, 2006).

To first establish an association with microbes conferring beneficial traits, hosts can take up microbes from the environment, harbor microbes that subsequently evolve the needed trait (the microbes themselves may start out beneficial or parasitic), or obtain microbes from a different host population or species. Because the ancestors of host-associated symbionts are predicted to be free-living (Moran et al., 2008), environmental acquisition is likely the origin of the oldest symbioses. Obtaining microbes from the environment would also allow hosts to obtain a wider range of partners and adapt to more environments (Kitano and Oda, 2006). Even after the association is established, however, there are often costs associated with harboring microbes, even in obligate symbioses (e.g., Chong and Moran, 2016). Taken together, the environmental condition under which hosts and microbes interact is important in determining whether and how much the partners gain from the association.

The role of evolutionary interactions in beneficial host-microbe interactions

Single-partner evolution

Given their importance in established symbioses, evolutionary interactions may be a critical driver in how novel associations evolve. Coevolution is often the explanation provided for the mutualistic benefits between hosts and microbes. However, coevolution is often only inferred and not directly tested (Janzen, 1980; Moran and Sloan, 2015). Changes in one of the partners may be enough for a beneficial association to arise, particularly because microbes do not need to benefit in a symbiosis for the association to persist (Mushegian and Ebert, 2016). Moreover, the host may need to adapt to a novel microbe first for the host to benefit from the association. Because microbes have faster generation times, they can adapt to their host faster, which can subsequently harm the host (e.g., greater microbial abundance has been linked to decreased host fitness (Chong and Moran, 2016; Weldon et al., 2013)). Investigating how hosts evolve in real-time is often difficult, however, because they tend to have long generation times, few offspring, and/or require high maintenance.

By contrast, many studies have examined how microbial passage through hosts influence microbial and host fitness. Microbial passage through non-evolving hosts can lead to evolution towards increased benefits or increased harm to the host (Le Clec'h et al., 2017; King et al., 2016; Matthews et al., 2019; Mikonranta et al., 2015; Sachs and Wilcox, 2006). Compared to free-living microbes found in the external environment, microbes passaged through hosts undergo bottlenecks and reduction in population size. In the case of vertically transmitted symbionts, host passage leads to extremely reduced genomes compared to horizontally transmitted symbionts and free-living relatives (McCutcheon and Moran, 2011; Toft and Andersson, 2010). Hosts have also been found to impose stronger selective pressure than the external environment (Burghardt et al., 2018; Morran et al., 2016). Taken together, these factors

can contribute to the establishment of a novel association with a host, where the evolutionary history of the microbe can shape the evolutionary trajectory of its host.

Coevolution

A large body of work has focused on coevolution as a major driving force behind tight-knit host-microbe symbioses (Bennett and Moran, 2015; Heath et al., 2012; Murfin et al., 2015; Pan et al., 2015; Schultz and Brady, 2008; Wilson and Duncan, 2015). Coevolution may be an important driver in the establishment of beneficial associations because it can create host and microbe genotypes that work well together (Guimarães et al., 2011). For example, host-parasite studies have found that coevolution can maintain genetic diversity in host populations and can lead to local adaptation between host and parasite (Gibson et al., 2015; Kerstes et al., 2012; Lohse et al., 2006; Morran et al., 2011, 2014). Furthermore, experiments testing the role of coevolution have demonstrated that coevolution can lead to positive outcomes for host and microbe, from reduction in virulence to increased benefits (Gibson et al., 2015; Rafaluk-Mohr et al., 2018). However, how coevolution affects the establishment of symbioses remains unclear.

Studies on extant systems have mainly focused on comparative studies (e.g., phylogenetics, genomics) to infer how associations arise. For example, genomic studies of host-associated symbionts often use the closest free-living relative of the symbiont to perform the comparisons (Boscaro et al., 2013; Sabater-Muñoz et al., 2017; Zheng et al., 2015); however, these extant relatives are not true ancestors of the symbiont. Moreover, the factors that maintain the association in present time may not be as important during the initial establishment of the symbiosis, and vice versa. There are many interesting questions that can be answered with comparative approaches, but in order to conduct direct tests of hypotheses related to the origin of

symbiosis, we need a model system where the host and microbe have only begun to associate with one another in the recent past. There is a lack of such models in nature. Experimental evolution, evolving populations under controlled conditions, is an approach that can overcome these limitations—it allows us to look forward and study how these associations evolve on a feasible timescale.

To address how environmental and evolutionary contexts affect the evolution of novel beneficial associations, I experimentally evolved the nematode *Caenorhabditis elegans* and the bacterium *Bacillus subtilis*, a host and a microbe that have had no evolutionary history together in the lab, under different conditions and evaluated the resulting fitness consequences.

Caenorhabditis elegans and *Bacillus subtilis* as a model system to study beneficial associations through experimental evolution

Caenorhabditis elegans has been utilized in numerous experimental evolution studies, particularly those involving host-parasite interactions (Gibson et al., 2015; Gray and Cutter, 2014; Kurz and Ewbank, 2000; Schulte et al., 2011). The nematode has a generation time of about three days and can lay up to 300 eggs in its lifetime, making it an ideal model for experimental evolution. Importantly, it can be cryogenically stored, such that direct comparison of ancestral and evolved strains is possible. *C. elegans* often comes into contact with microorganisms in the soil, and has been shown to be able to distinguish between different classes of microorganisms via behavioral avoidance and immune responses (Engelmann and Pujol, 2010; Schulenburg et al., 2008; Tan and Shapira, 2011; Wong et al., 2007). While *C. elegans* feed on bacteria, some bacteria survive the passage to the intestine and are able to colonize the nematode (Gibson et al., 2015; Portal-Celhay and Blaser, 2012; Vega and Gore,

2017). However, the nematodes do not appear to transmit their microbes vertically to their offspring.

Similar to *C. elegans*, *B. subtilis* is a soil-dwelling bacterium that has been utilized as a model organism to study a range of processes, such as sporulation and biofilm formation (Barbe et al., 2009). For both organisms, annotated genomes are available, as are many tools for genetic analysis and manipulation, making them ideal for identifying the genetic basis of observed traits. Both *C. elegans* and *B. subtilis* are amenable to (co)evolution experiments due to their short generation times, high fecundity, and ease of maintenance in the lab. The organisms can evolve measurable changes within a reasonable experimental timescale, and are conducive to direct tests of (co)evolution under controlled environmental conditions without having to infer the evolutionary history between the interacting species.

A previous study found that wild-type *B. subtilis* protects *C. elegans* from heat stress at 34˚C (normal rearing temperature for *C. elegans* is 12 – 25˚C) (Gusarov et al., 2013). Specifically, nematodes harboring *B. subtilis*, a nitric oxide (NO) producing bacterium, live almost 20% longer following heat stress than those carrying non-NO producing *B. subtilis*. Nitric oxide exposure induces the expression of heat shock proteins in the nematodes, which helps them withstand the heightened temperature. I determined that nematodes and bacteria can reproduce after being heat shocked. This is particularly important from the standpoint of experimental (co)evolution, where we want to passage host individuals to the next generation. My results are also consistent with a previous study that allowed *C. elegans* to recover after being heat shocked at high temperatures (Aprison and Ruvinsky, 2014). Furthermore, I found that nematodes harboring *B. subtilis* produce a greater number of viable offspring than nematodes harboring *E. coli* (the standard lab diet of *C. elegans*) when heat shocked. These

results demonstrate that *B. subtilis* is a beneficial bacterium for *C. elegans* in terms of increased fecundity after heat shock. Therefore, I can use heat shock as a selective pressure to evolve a novel beneficial association between *C. elegans* and *B. subtilis*, where nematodes require live bacteria to survive and reproduce, and only bacteria found inside surviving nematodes will contribute to the next round of passage. This can be accomplished by crushing the nematodes after they reproduce and plating the bacteria within the nematodes. While the starting point of the interaction is beneficial for the host under heat shock, I can determine 1) the conditions that may facilitate an increase in fitness in one or both partners, thus evolution towards a long-term beneficial symbiosis, and 2) the conditions that may reduce the fitness of either partner, such that evolution towards increased benefits is hindered and evolution towards antagonism is possible.

Summary of dissertation chapters

In Chapter II, I review current gaps of knowledge in host-microbe symbioses and how experimental evolution can help address some of these gaps. I discuss the establishment and maintenance of mutualisms, the genomic changes in microbes underlying the transition towards host-association, and the role of the host immune system in symbiont association. I then suggest model systems amenable to studying beneficial interactions using experimental evolution. This work was published in *Frontiers in Microbiology* in 2016 in an article entitled "Experimental evolution as an underutilized tool for studying beneficial animal-microbe interactions."

In Chapter III, I characterize the initial interaction between *C. elegans* and *B. subtilis*. I compare the fitness of nematodes heat shocked on *B. subtilis* or *E. coli*, then determine whether exposure to *B. subtilis* at different times in the host lifespan influences the benefits the nematodes obtain from the bacterium. I show that under the standard rearing temperature,

nematodes produce fewer offspring on *B. subtilis* than on *E. coli*. However, not only does *B. subtilis* increase host fecundity under heat shock compared to *E. coli*, exposure to *B. subtilis* early on in the host lifespan is the most beneficial. These results demonstrate the importance of the environment and timing of the interaction in host-microbe dynamics. This work was published in *Ecology and Evolution* in 2019 in an article entitled "The effects of *Bacillus subtilis* on *Caenorhabditis elegans* after heat stress."

In Chapter IV, I determine how a novel beneficial microbe affects host adaptation to a stressful environment. I evolve *C. elegans* in the presence or absence of *B. subtilis*, with or without heat stress, for 20 generations of selection. I demonstrate that only hosts that evolved with *B. subtilis* under heat stress gain a fitness increase under heat shock compared to the other treatments. Furthermore, these hosts harbor the most *B. subtilis* colonies despite the bacteria not evolving throughout the experiment. These results suggest that environmental context and presence of the beneficial bacterium are important for the evolution of novel associations, and that mutual benefits can evolve from changes that evolve solely in the host. This work is currently under review.

In Chapter V, I examine how microbial passage through hosts affects host fitness and how co-passaging can alter host-microbe dynamics. I passage *B. subtilis* through non-evolving *C. elegans* and *in vitro* under heat shock conditions and show that both treatments increase the ancestral host fitness after 20 passages. I also co-passage host and bacteria, and show that co-passaged bacteria had a tendency to decrease ancestral host fitness. I then determine whether these bacteria were only beneficial when paired with their co-passaged hosts. I demonstrate that co-passaged hosts exhibit decreased fecundity when paired with their co-passaged bacteria

compared to hosts that evolve with non-evolving *B. subtilis* (from Chapter IV). These results suggest that coevolution may impede the evolution of novel beneficial host-microbe associations.

In Chapter VI, I summarize the previous chapters and suggest future directions. I discuss some of the questions that remain pertaining to the evolution of novel symbioses and how the *C. elegans*-*B. subtilis* system can be used to address them. Building on the findings from Chapter V, I plan to perform partner-switching assays between co-passaged host and bacterial populations. I also plan to examine growth of the evolved bacteria by performing assays within and outside hosts to determine how evolution with the host can affect microbial growth and adaptation.

CHAPTER II

EXPERIMENTAL EVOLUTION AS AN UNDERUTILIZED TOOL FOR STUDYING BENEFICIAL HOST-MICROBE INTERACTIONS

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Abstract

Microorganisms play a significant role in the evolution and functioning of the eukaryotes with which they interact. Much of our understanding of beneficial host-microbe interactions stems from studying already established associations; we often infer the genotypic and environmental conditions that led to the existing host-microbe relationships. However, several outstanding questions remain, including understanding how host and microbial (internal) traits, and ecological and evolutionary (external) processes, influence the origin of beneficial host-microbe associations. Experimental evolution has helped address a range of evolutionary and ecological questions across different model systems; however, it has been greatly underutilized as a tool to study beneficial host-microbe associations. In this review, we suggest ways in which experimental evolution can further our understanding of the proximate and ultimate mechanisms shaping mutualistic interactions between eukaryotic hosts and microbes. By tracking beneficial interactions under defined conditions or evolving novel associations among hosts and microbes with little prior evolutionary interaction, we can link specific genotypes to phenotypes that can be directly measured. Moreover, this approach will help address existing puzzles in beneficial symbiosis research: how symbioses evolve, how symbioses are maintained, and how both host and microbe influence their partner's evolutionary trajectories. By bridging theoretical predictions and empirical tests, experimental evolution provides us with another approach to test hypotheses regarding the evolution of beneficial host-microbe associations.

Introduction

Microorganisms inhabit hosts from all branches of life, from bacteria (Bondy-Denomy and Davidson, 2014) to plants (Heijden et al., 2015) and animals (Delsuc et al., 2014; Klepzig et al., 2009), including humans (Cho and Blaser, 2012; Eloe-Fadrosh and Rasko, 2013). Associations with microbes play a pivotal role in the evolution and functioning of possibly all eukaryotes (Douglas, 2014; Eloe-Fadrosh and Rasko, 2013; McFall-Ngai et al., 2013; Rosenberg and Zilber-rosenberg, 2016). Host-microbe interactions can lead to a range of consequences for both the hosts and the microbes. These consequences vary along a continuum of parasitism to mutualism, a spectrum that represents the fitness costs and benefits for both the hosts and the microbes in association (Ewald, 1987; Gerardo, 2015). Host-microbe dynamics may not be static under all contexts (e.g., biotic and abiotic, temporal and spatial), and shifts along the continuum can occur if the net fitness benefit obtained for one partner (particularly the microbe) is higher than previous forms of interactions (Sachs and Simms, 2006).

Numerous microbes confer major benefits to their hosts. These benefits include nutrient provisioning and protection from enemies. Microbes also have essential roles in the development, functioning, and behavior of hosts. Association with microbes allows many hosts to inhabit niches that they normally would not be able to inhabit (Douglas, 2014; McFall-Ngai et al., 2013). Recognition of the importance of microbes in everyday life has heightened interests in understanding how beneficial host-microbe associations evolve, how the partners impact one another, and what are both the proximate and ultimate mechanisms behind these interactions. Important insights have resulted from studies of microbial symbiosis (here, defined as long-term host-microbe associations) (Nyholm and Graf, 2012; Sachs et al., 2011; Wilson et al., 2010); however, there are still many questions that remain, particularly as to how beneficial associations

are initiated between hosts and microbes with little prior interaction or with few benefits exchanged between them initially. Many of the conclusions we have drawn to date come from studies of existing relationships, often after an evolutionarily long period of coevolution between the partners. Factors that maintain associations now may not be the same as those that were present during the initial stages of the association, and the consequences (e.g., fitness costs and benefits, genomic modifications) have mostly been inferred from extant model systems. Here, we propose extending the use of experimental evolution as a way to fill in current knowledge gaps in beneficial host-microbe studies. This approach has helped to answer other fundamental evolutionary and ecological questions, but has still not been used to a great extent to study animal-microbe symbioses. The incorporation of experimental evolution into beneficial host-microbe interaction studies, a combination of approaches that is still in its infancy, will act to directly link proposed hypotheses to phenomena that can occur during the evolution of beneficial associations.

This review will be focused on highlighting the questions that remain unanswered in beneficial animal-microbe associations that are amenable to experimental evolution approaches. We then suggest ways in which experimental evolution can be used to address these questions and animal systems that may be exploited for these experiments moving forward. In this review, we define an interaction as “mutualistic” if a net fitness benefit is obtained by both host and microbe when they interact with each other compared to when they do not, and as a “beneficial association”, a more general term, when it is known that the host benefits (Box 2.1 provides further details on the ambiguity of defining such associations).

Beneficial host-microbe interactions: current questions and challenges

The initial stages of evolution

Past studies of host-microbe associations have focused on models that exhibit long-term, close-knit interactions, or at least where the host and microbe are known to have been in frequent contact with each other over evolutionary time (Douglas, 1998; Nyholm and McFall-Ngai, 2004). However, we have little evidence as to how these beneficial interactions evolve in the first place. One possibility is that these beneficial partnerships arise between hosts and microbes that have had little to no previous contact. For example, an animal might acquire a microbe from the environment that increases its fitness relative to its non-microbe harboring relatives, thus making this trait more prevalent in the population, particularly if eventually that microbe is transmitted vertically. Another possibility is that the initial association is commensal, parasitic, or predatory before selection for a beneficial interaction. Additionally, a situation may arise where this host-microbe pairing encounters a new environment, and it may be selectively beneficial for the microbe to now improve its host's fitness. Discerning the origins of associations is challenging because current associations may be evolutionarily far removed from their pre-partnership ancestors.

Additionally, genetic and environmental conditions underlying already established symbioses may not be the same as when the host and microbe first came into contact. In terms of genetic conditions, this is likely particularly true for vertically transmitted symbioses due to the major genomic changes imposed by the host and microbe on each other as a result of their close-knit association (Bennett and Moran, 2015). Furthermore, studying existing associations does not allow us to directly assess the role that genetic variation had in establishing and maintaining long-term interactions. When there is a high rate of variation being generated through mutation,

recombination, and gene flow during the initial interactions between host and microbe, beneficial associations may more likely evolve because there is more material for selection to act upon. However, it could also prevent the maintenance of a newly formed association because variation-generating mechanisms can disrupt allelic combinations that confer greater fitness (Fisher, 1930). Environmental conditions also likely have a large influence on the maintenance of associations. For example, even though the bacterium *Hamiltonella defensa* protects its pea aphid host from the parasitoid wasp, *Aphidius ervi*, the proportion of aphid hosts infected with the bacterium decreases in the absence of the wasp (Oliver et al., 2008), suggesting a cost in the absence of protection. Thus, fluctuation in wasp presence presumably leads to temporal shifts in the benefits and costs of association. More generally, the relative contributions of genetic and environmental factors and the degree to which these factors exert pressure on the maintenance of the first few generations of established symbioses remain unknown.

Assessing genomic transitions underlying symbiosis evolution

Past studies have shown that microbial lineages associated with a host often contain smaller genomes than their free-living counterparts. For example, in studies examining intracellular bacterial partners, or endosymbionts, genomic reduction is observed with increasing intensity of host association: facultative bacteria have smaller genomes than free-living bacteria, and obligate bacterial symbionts have smaller genomes than facultative bacterial symbionts (McCutcheon and Moran, 2011; Toft and Andersson, 2010). The evolution of endosymbionts, be they commensal, parasitic, or beneficial, has been proposed to involve free-living bacteria transitioning into a host-associated lifestyle. Once within the host, interactions with the host environment may render certain genes redundant (e.g., genes required for coping with external abiotic stressors,

such as UV rays) for the endosymbiont. Combined with bottlenecks that occur when endosymbionts are passed on between hosts, genes necessary for free-living are lost. This leads to reductions in genome sizes and genetic variation in endosymbiont populations (McCutcheon and Moran, 2011; Nilsson et al., 2005; Toft and Andersson, 2010). One consequence is that interactions between host and endosymbiont can lead to complementarity of genomes, where host and endosymbiont produce resources their partner lacks (Wilson et al., 2010). For the symbiont, this could be the genes that they have lost, and for the host, the genes required for them to occupy a new niche in the absence of their microbial partner. Furthermore, microbial genes can be integrated into the host genome through horizontal gene transfer, which has had an important role in eukaryotic evolution (Keeling and Palmer, 2008; McFall-Ngai et al., 2013; Rosenberg and Zilber-rosenberg, 2016).

These insights have all been gained from a comparative approach: genomes of symbionts are compared to those of free-living bacteria (e.g., Zheng et al., 2015) and genome of hosts are compared to animals without intimate symbiotic relationships (e.g., Suen et al., 2011). Often, however, the free-living organisms are not closely related to the symbiotic organisms, so we cannot directly assess the genomic evolution underlying the transition from a free-living to a symbiotic lifestyle. In other words, we lack empirical evidence for inferences drawn from these genomic comparison studies. The challenge is that the ancestors are no longer present to perform direct tests of genomic changes resulting from symbiotic association. For example, a previous study sought to identify the genetic mechanisms involved in genomic reduction in bacteria by serially passaging single colonies over 200 times on supplemented media, effectively implementing strong bottlenecks and no horizontal gene transfer (Nilsson et al., 2005). Though the authors identified important aspects associated with genomic reduction (e.g., that it can

happen in an evolutionarily short period of time), the experiments were done in the absence of any interaction with a host.

The role of the host immune system

The immune system is one of the most important lines of defense for the host; it acts as a way to differentiate between harmful and non-harmful microbes that colonize the host, whether they are environmentally acquired or passed down from the parent generation. Therefore, the immune system serves as a central component of the host that interacts intimately with its microbes. Studies of symbiotic interactions have shown that beneficial microbes have evolved ways to evade or alter host defenses, or have evolved from pathogenic ancestors and have retained their ability to evade or affect host immunity (Nyholm and Graf, 2012; Ruby, 2008). For example, when *Vibrio fischeri* bacteria colonize the light organ of their squid host, the bacteria actually dampen the level of host-produced nitric oxide, a compound involved in eukaryotic innate defense against pathogens (Davidson et al., 2004). Symbionts have also been shown to help regulate the development of normal immune responses and prime the host immune system to fight against pathogens, and the microbiota is required for proper host immune development and functioning in some organisms (Weiss et al., 2012; Yilmaz et al., 2014).

Despite the important role that the immune system has in mediating long-term host-microbe associations, we know little about the role it has in shaping the initial evolutionary stages of beneficial symbiosis. The innate immune system likely has an important role in the initial contact between host and microbe and evolution of a beneficial association due to its ability to shape and be shaped by microbes residing in the host (Chu and Mazmanian, 2013; Nyholm and Graf, 2012; Weiss et al., 2012). Theory suggests that host defenses can influence

establishment of mutualism. For example, a heightened defense may impede the evolution of a symbiosis (Doebeli and Knowlton, 1998). However, most evolutionary theory regarding the establishment of symbiosis lacks direct empirical tests.

The maintenance of mutualisms

Mutualisms are considered an evolutionary dilemma because individuals suffer costs to provide benefits to their partners; therefore, a long-standing question of interest has been to identify factors that stabilize or breakdown mutualisms. Mechanisms that promote shifting along the parasitism-mutualism spectrum and the genetic differences between parasites and symbionts are important factors that should be explored further (Sachs et al., 2011). Another related question is determining the contexts that give rise to cheating partners or endosymbiont reversion to free-living microbes (Jones et al., 2015; Sachs and Simms, 2006). Experimental evolution using tractable model systems provides a powerful way to examine these questions, as it has already been utilized to address related questions, such as the evolution of host-parasite interactions and novel microbe-microbe mutualisms.

An overview of experimental evolution

Experimental evolution refers to evolving populations under controlled conditions to study evolutionary processes (Garland Jr. and Rose, 2009; Kawecki et al., 2012). Precise conditions can be placed upon a population, which can then be tracked throughout its evolution. For instance, the relative strength of genetic drift can be manipulated by modifying population size, and selection can be altered by treatment effects or design. Control treatments can help distinguish between changes caused by the environment versus evolutionary forces.

Interestingly, replicate populations may also give rise to different adaptive genotypes that highlight the role of stochastic forces, like drift and mutation, in the evolutionary process. Richard Lenski's long-term evolution experiment (LTEE), for example, has produced numerous examples of divergent adaptation found across 12 clonal *Escherichia coli* populations over 65,000 generations. While each *E. coli* population began as clones growing in identical environments, they eventually accumulated independent mutations that led to differential fitness gains on alternative sugars, as well as the emergence of one population that can utilize a carbon source, citrate, that *E. coli* normally cannot use (Blount et al., 2008; Travisano et al., 1995). Additionally, computer simulations can be a useful tool for experimental design in conjunction with experimental evolution, such as estimating the power of artificial selection experiments (Kessner and Novembre, 2015). Model systems that have been utilized in experimental evolution studies can be run for tens to thousands of generations, resulting in observation of evolution in real-time. Many organisms utilized for experimental evolution can be cryogenically preserved, allowing for direct comparisons of the ancestral populations against the evolved populations. The tractability of experimental evolution experiments makes them ideal for multispecies interaction studies, where environmental conditions are controlled to tease out the influences of biotic and abiotic factors on the evolution of the traits of interest.

Experimental evolution studies can be setup to test the effects of different variables on evolutionary trajectories. For example, in addition to exploring the adaptive mutations that arise across replicate populations, the LTEE has provided insight into fundamental evolutionary processes, such as diversification of clonal populations, the role of historical contingency in the evolution of novel traits, and the influence of mutation rates during adaptive evolution (Blount et al., 2008; Lenski et al., 1991; de Visser et al., 1999). Furthermore, a major benefit to

experimental evolution is that it can be designed to test specific hypotheses. Biologists can control and isolate specific variables involved in a particular process, facilitating linkage between theory and models to empirical tests. It is important to note that it may be difficult to perfectly represent scenarios proposed by theories in laboratory experiments, which can lead to discrepancies between theories and empirical data (Desai, 2013). Computer simulations may provide a more controlled method to test predictions; however, organisms are more complex than simulations, so the utilization of living organisms in experimental evolution has the potential to reveal unknown biological phenomena, whereas it may not be possible to do so with simulations. For example, if we observed a particular phenotype in an evolved population, we can go back and identify the genes contributing to the particular trait, whereas we would require prior knowledge to perform simulations. More generally, experimental evolution serves as an intermediate between theories and natural populations. By evolving populations under controlled conditions, we can empirically test theoretical predictions, generate new data to parameterize models and simulations, and establish patterns to test in natural populations.

Limitations

First, running experimental evolution under a controlled (often laboratory) environment may lead to simplification of the conditions found in nature, which are often more complex. More specifically, evolutionary processes may function differently in a natural environment where unidentified biotic and abiotic factors are in play, and multiple factors can act in synergy. For example, experimental coevolution of *Pseudomonas fluorescens* and its bacteriophage in rich medium led to directional selection of host-parasite dynamics, whereas coevolution of the same host-parasite pairing in soil microcosms resulted in frequency-dependent selection dynamics

(Brockhurst and Koskella, 2013; Gómez and Buckling, 2011). A remedy to this dilemma may be the use of field experiments or mesocosms, which can more accurately represent the ecology of a particular system (Ebert et al., 2002; Reznick et al., 1997). However, one drawback may be less control over environmental conditions. While the laboratory may not completely encompass natural settings, a larger number of generations can be maintained in a controlled environment relative to those in the field, which is more subject to seasonal variability. Overall, experimental evolution is a useful tool in that it allows us to test what we predict as the most important factors involved in our study of interest and provides us with a starting point with which to test further predictions through field studies, simulations, quantitative and molecular genetics, and subsequent experimental evolution.

Second, in order to observe responses to selection in a set amount of time, extreme conditions are utilized to facilitate the evolutionary process, which may lead to phenotypic and genotypic patterns not observed in nature. For example, laboratory experiments tend to select for strong pleiotropic effects, while selection in nature often involve alleles with weak or no pleiotropy (Kawecki et al., 2012). Additionally, model organisms are restricted to those that have fast generation times if experiments begin with clonal populations (see Box 2.2) because otherwise mutations would not occur fast enough to provide the raw material for selection. This may be a problem because these organisms may not be those of interest for a particular question or may have particular peculiarities that limit generalization to other less rapidly reproducing organisms. This can be remedied, in part, by utilizing methods that increase genetic variation within populations (e.g., mutagenesis for standing genetic variation, or introducing defective DNA repair mechanisms). Despite limitations, experimental evolution studies have contributed significantly to existing areas of studies in ecology and evolution.

Experimental evolution of microbe-microbe mutualisms

Experimental evolution has helped answer a broad range of questions, such as elucidating the role of genetic and environmental variations in adaptation, characterizing life history and reproductive traits, and evaluating the potentials and limits of intra- and inter-species interactions (Morran et al., 2011; Ratcliff et al., 2012; Reznick et al., 1990; de Visser et al., 1999). Microbes have been the model organisms for experimental evolution due to their fast generation time, high fecundity, relatively smaller and more easily manipulated genomes, ease of laboratory rearing, and the ability to be cryogenically preserved (Elena and Lenski, 2003).

Many experimental studies have evolved mutualisms between microbes that are not known to naturally associate with one another. These beneficial behaviors can occur within and across microbial species, as well as across domains of life (as well as in bacteriophages, e.g., Sachs & Bull 2005). A major facilitator of these mutualistic interactions involves the removal of essential nutrients from the environment or genes involved in synthesizing these nutrients (Harcombe, 2010; Hillesland and Stahl, 2010; Shou et al., 2007). For example, Hillesland and Stahl (2010) evolved an obligate mutualism between a sulfate-reducing bacterium, *Desulfovibrio vulgaris*, and an archaeon, *Methanococcus maripaludis*, in the absence of substrates that would otherwise allow them to grow independently of each other. The bacterium produces hydrogen during an energy-producing reaction, while the archaeon feeds on the hydrogen product, keeping the energy reaction going in the bacterium. This interaction allows the bacterium to produce enough energy to grow and provides the only substrate for growth that the archaeon can use. Co-cultures of evolved strains grew faster than co-cultures of the ancestral strains under similar environments, indicating a mutualism had evolved between the species after 300 generations. A

subsequent study determined that several populations of the co-cultured bacteria had lost their ability to reduce sulfate, thus preventing them from proliferating without the archaeon (Hillesland et al., 2014). Such studies demonstrate that microbe-microbe mutualisms can be evolved using experimental evolution. Much less work has been done with eukaryotes.

Experimental evolution of host-pathogen interactions

Eukaryotes have been utilized across a range of different evolutionary experiments (Garland Jr. and Rose, 2009), including in studies of antagonistic host-microbe interactions. Experimental evolution across multiple systems has provided empirical evidence of the principles and mechanisms involved in host-parasite interactions and antagonistic coevolution (Brockhurst et al., 2007; Brockhurst and Koskella, 2013; Ebert and Mangin, 1997; Kawecki et al., 2012; Kerstes et al., 2012; Morran et al., 2011; Schulte et al., 2011). For example, in an experiment between the red flour beetle and its microsporidian parasite, Kerstes et al. (2012) found that populations that coevolve with their parasite exhibited an increase in recombination rate compared to populations without parasites. Another study by Morran et al. (2011) found a higher rate of outcrossing in *C. elegans* populations coevolving with pathogenic *S. marcescens* compared to populations not exposed to parasites or where *S. marcescens* was not evolving alongside *C. elegans.* These studies demonstrate that coevolutionary interactions with parasites can lead to the maintenance of supposedly costly mechanisms in hosts (recombination and outcrossing) because they generate genetic diversity that allows hosts to combat parasites. Coevolutionary experiments also have provided evidence for host-parasite local adaptation, such that specificity evolves between host and parasite populations that coevolve together (Gibson et al., 2015; Koskella and Lively, 2007; Lohse et al., 2006; Morran et al., 2014). For example, coevolution of *Paramecium*

caudatum with its bacterial parasite, *Holospora undulata*, showed that hosts are more resistant against parasites with which they coevolved, but incur a cost when the parasite is absent (Lohse et al., 2006). These studies highlight the importance of coevolutionary interactions in shaping the evolutionary trajectories of both hosts and microbes. Additionally, they show that host populations can evolve measureable phenotypic changes during experiments. In all cases, the experiments were testing specific theoretical predictions relating to host-parasite coevolution and local adaptation. Overall, these studies demonstrate the tractability and rapid evolution of eukaryotic hosts and their microbes, providing further evidence that model systems can be exploited for experimental evolution of beneficial host-microbe interactions. The success of experimental evolution in characterizing relationships between hosts and parasites is evidence that beneficial host-microbe studies would gain from using the approach as well. Despite a large body of work utilizing experimental evolution to study host-parasite interactions, there have been few experimental evolution studies examining beneficial behaviors between eukaryotic hosts and microbes.

Utilizing experimental evolution to study beneficial animal-microbe associations

Reduction in host-microbe antagonism

The first steps in the origin of a beneficial association may be a reduction in antagonism in an existing parasitic or predatory relationship (Degnan et al., 2009; Jeon, 1972). Once conditions (e.g, environmental, genetic) are met such that benefiting the host is better for the microbe and vice versa, the transition from parasitism toward mutualism may take place during a relatively short timescale. For example, Marchetti et al. (2010) evolved *Ralstonia solanacearum,* a plant pathogen, into a potential beneficial symbiont of a legume, *Mimosa pudica*. The authors inserted

a plasmid containing nitrogen-fixation and nodule-forming genes into the pathogen, and allowed the plant to select for bacterial strains that can form nodules, which typically house nitrogen-fixing bacteria. The authors alternated bacterial passages within and outside the plant host, simulating bacterial movement between soil and host plant. They were able to improve the bacterium's nodulating and infecting abilities, as well as their ability to reduce host immune responses (Marchetti et al., 2014). Furthermore, genomic manipulation of these bacterial strains suggest that error-prone mechanisms facilitated evolution toward symbiosis due to temporary increases in genetic diversity (Remigi et al., 2014). Even though the bacteria were not able to fix nitrogen (and confer benefits to the host), these experiments established the initial steps (improved infecting and nodulating capabilities) needed for a mutualistic association to evolve.

In terms of animal models, there are several examples of evolution toward a more beneficial interaction involving the model nematode, *Caenorhabditis elegans.* Gibson et al. (2015) found that 20 generations of coevolution of *C. elegans* with its parasite, *Serratia marcescens,* resulted in higher fecundity in hosts relative to when only the host or parasite population was permitted to evolve in the presence of the other species, leading to a reduction in antagonism in this parasitic association. Another study used *C. elegans* to explore trade-offs to host adaptation in *Burkholderia cenocepacia* (Ellis and Cooper, 2010). *B. cenocepacia* was evolved on onion medium for 1,000 generations before switching to *C. elegans*, where the bacterium exhibited reduced ability to kill the nematode. Finally, perhaps the most direct evidence of a parasitic microbe transitioning into a protective microbe is from a recent study by King et al. (2016). The authors experimentally evolved the bacterium *Enterococcus faecalis* to protect *C. elegans* against the more virulent *Staphylococcus aureus* over 15 host generations, despite the fact that these species were not known to be associated previously, thus establishing a

novel host-microbe association with known evolutionary history and origin over an experimentally tractable time scale. The study also shows that evolution of bacterial protection can be rapid and can occur apart from any significant change in the host. Overall, these studies illustrate the power of experimental evolution to potentiate the transition from parasitism toward a beneficial association. Additional experiments are necessary to determine whether these interactions can be evolved to further increase the fitness of both host and microbe, as well as the stability of the interaction and how it could move toward a long-term beneficial symbiosis.

Evolutionary interactions between host and microbe

Different types of evolutionary interactions between host and microbe likely have an important role in the evolution of beneficial symbioses. Coevolution may be a driving force behind the evolution of mutualistic associations because it can create genotypes that fit well together (Guimarães et al., 2011). However, host and microbe need not be coevolving together in order for a symbiosis to evolve. The microbe may undergo evolutionary changes in the presence of the host without the host evolving itself, or vice versa (Janzen, 1980; Moran and Sloan, 2015). Several experiments tracking the evolution of microbes within non-evolving hosts have provided insight into symbiotic interactions (Barroso-Batista et al., 2014; Kubinak and Potts, 2013; Sachs and Wilcox, 2006; Schuster et al., 2010). For example, Schuster et al. (2010) passaged bioluminescent *V. fischeri* strains that were non-native to *E. scolopes* through the squid host, where it acted as the selective agent for a few hundred bacterial generations. The authors found that these bacteria evolved to be more similar to the *V. fischeri* native to *E. scolopes*, indicating that natural selection can facilitate rapid bacterial adaptation to non-native hosts and potentially in the evolution of close symbiotic relationships.

Although there have been few evolution experiments utilizing mammalian systems to examine gut microbiota, a recent study completed a selection experiment on a non-model mammal—the bank vole—to characterize its microbial composition. Kohl et al. (2016) examined the changes in gut microbial community that occurred after selecting for voles adapted to a high-fiber, herbivorous diet. By comparing the microbiota of selected hosts against that of the control, randomly bred hosts, the authors determined that the herbivorous diet led to a more diverse microbial community. Interestingly, the individuals whose microbiota were sampled had not been exposed to the herbivorous diet themselves (they were offspring of those fed the herbivorous diet), suggesting that the differences in microbial communities were not due to the transient effects of diet and may be due to selection acting on certain microbial members. While mammalian systems are generally more difficult to maintain compared to invertebrates, this study highlights the utilization of experimental evolution of mammals to investigate complex microbial communities, which are often absent in invertebrate models. Moreover, by performing the experiment in a controlled setting, the study contributes empirical support toward the current literature, which traditionally has been mainly comparative studies, on the role the gut microbiota has on herbivore evolution.

From close-knit associations to break down of beneficial symbioses

In many insect symbioses, the insect host harbors secondary symbionts that are part of the host's defense mechanism. Because most of these symbionts are maternally inherited, they depend on host survival to improve their fitness (Oliver et al., 2013). This interaction presents another layer of complexity between the host, its symbiont, and enemies of the host. Because the host now possesses a more dynamic defense system, it places different selective pressures upon enemies of

the host compared to innate host immunity alone. Enemies can also place selective pressure upon the symbiont and innate host immunity, leading to a three-way interaction where each species can evolve in response to the others. A recent study sought to explore this three-way interaction between *Drosophila melanogaster* innate resistance, *Drosophila* C virus, and the fly's *Wolbachia* symbiont known to confer protection against the virus (Martinez et al., 2016). Fly populations where *Wolbachia* was either present or absent were exposed to the virus for nine generations. The authors then quantified the frequency of an allele in the flies known to confer resistance to the virus, where they found the resistant allele to be lower in frequency in populations harboring *Wolbachia* compared to those without *Wolbachia*. This experiment also provides evidence supporting the observation that hosts harboring protective symbionts tend to have a weaker immune system because they do not depend on innate immunity as much as those lacking the symbionts (Gerardo et al., 2010).

Similarly, a few studies have taken advantage of the well-developed aphid models to explore long-term symbiosis in greater depth. Dion et al. (2011) examined the evolution of the pea aphid parasitoid wasp, *A. ervi*, in the presence of the protective bacterium, *H. defensa*, which decreases survivorship of parasitoid eggs laid in the aphid. Parasitoids were exposed to clonal aphid hosts harboring or free of *H. defensa* for 10 generations, after which they were assayed for parasitism ability. The experiment showed that even though *H. defensa* reduced parasitoid offspring number in the first few generations, parasitoids eventually exhibited similar parasitism rate regardless of the presence or absence of *H. defensa*. A later study further evaluated the role of *H. defensa* by experimentally evolving the parasitoid wasp, *Lysiphlebus fabarum*, of the black bean aphid (Rouchet and Vorburger, 2014). By infecting the same aphid clone with either of three different strains of *H. defensa,* the authors directly tested parasitoid adaptation against these

strains while controlling for host genotype over 11 generations. They found that increased success in parasitism to hosts harboring one symbiont strain did not lead to adaptation in the two other strains. Overall, these studies investigated the relative roles of symbiont-conferred protection versus innate host resistance and lent further support to the idea that symbionts are an important source of variation in host defense.

Experimental evolution has also been used to identify factors that break down or stabilize animal-microbe partnerships. For example, Sachs and Wilcox (2006) evolved an algal symbiont of the upside-down jellyfish, which normally provides benefits to its host through the production of photosynthates, into a partner that reduced the fitness of the host by altering the mode of symbiont transmission between host generations. However, more studies would be helpful to assess whether it is difficult to break down associations once host and microbial interests have aligned. For example, to determine if there is a cost to maintaining a mutualistic association under some conditions (i.e., context-dependent mutualism), we can place a mutualistic host-microbe population under different environmental conditions (i.e., in the presence and absence of biotic and abiotic stressors). The host and microbial populations can then be monitored over several generations to determine if the interaction is mutualistic across all contexts, and, if not, what are the consequences for the stability of partnerships when there is environmental contingency in fluctuating environments.

Remaining questions and future directions

While the previous sections have provided several examples of how experimental evolution has been used to study host-microbe associations, more work is needed to create a more thorough understanding of the evolution of beneficial animal-microbe symbioses. Prior studies have set

the stage for much wider investigations into the proximate and ultimate mechanisms shaping the evolution of beneficial animal-microbe associations. For example, evolving microbes that have had little contact with eukaryotic hosts into host-associated microbes could elucidate the mechanisms and consequences resulting from the microbial transition from free-living to endosymbiosis. Sequencing the genomes of ancestral and evolved populations would then provide insight into the initial genomic modifications important for a transition toward adaptation to a host. Likewise, by performing one-sided evolution experiments alongside coevolution experiments, we can identify the traits that have arisen as a result of the presence of a partner or through selection that hosts and microbes impose on each other (an example of such a setup is shown in Figure 2.1). Indeed, studies of coevolution of *de novo* mutualism between a eukaryotic host and its microbes should be considered an important next step in symbiosis research. Experimental evolution can also be used to understand why some symbioses are difficult to break down and the mechanisms involved in maintaining these relationships (Morran et al., 2016). Lastly, there have been few evolutionary studies examining the dynamics between host immunity and beneficial/protective microbes and how they influence microbial and host evolution. To test the importance of host defenses in the initial stages of a beneficial association (Doebeli and Knowlton, 1998), we can evolve hosts differing in immune responses with the same microbial genotype to determine the evolutionary trajectories taken by each host-microbe pairing. Comparison of the immune responses of the ancestral and evolved hosts would provide further insight into the extent that microbes can alter host defenses over time (Kitano and Oda, 2006).

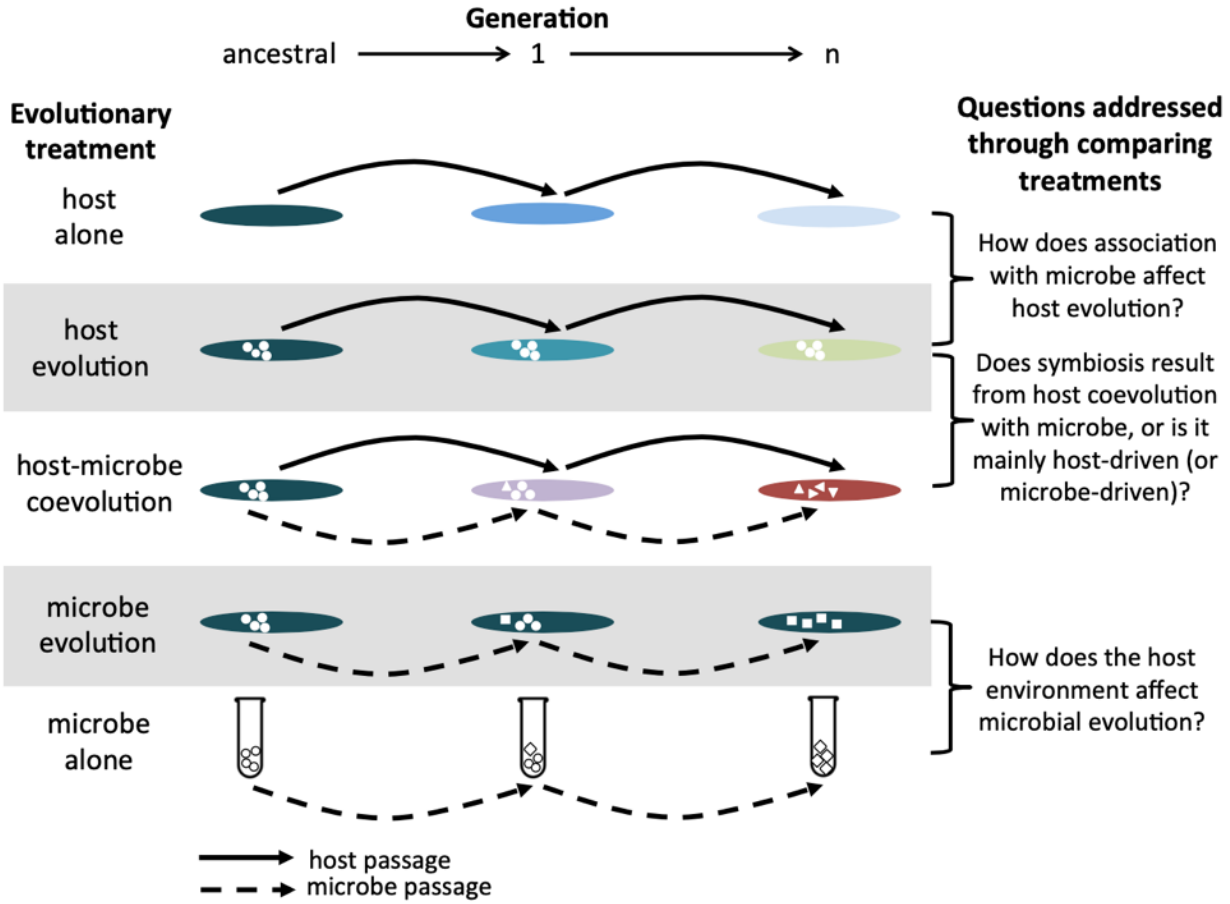

Figure 2.1. Example of host-microbe experimental evolution experiment (a modified setup of host-parasite interactions from Brockhurst & Koskella, 2013).
The host alone treatment consists of passaging the host without the microbe (and vice versa for the microbe alone treatment). The host evolution treatment consists of passaging the host in the presence of a non-evolving microbe (and vice versa for the microbe evolution treatment). The host-microbe coevolution treatment consists of passaging hosts and microbes that have interacted with each other in the previous generation. In combination, these treatments allow researchers of symbioses to assess the effects of partner association and coevolution on the evolution of hosts and microbes. While a single replicate of each treatment is shown for simplicity, replicate populations within each treatment are critical in order to evaluate the relative roles of deterministic and stochastic processes. The greater the contribution from stochastic processes, the greater the degree of divergence between replicate populations.

In general, because evolution experiments control and manipulate environmental conditions and starting population genetics, we can use them to test hypotheses and predictions that have been proposed for the evolution of beneficial association. By using mutants or by

manipulating the environment (such as by removing important dietary substances or imposing selective pressures from an enemy), then measuring the fitness of both hosts and symbionts, we can identify the biotic and abiotic factors that influence the establishment and maintenance of mutualisms. Finally, through replicate populations, we can determine whether deterministic forces (e.g., selection) are more dominant in the evolution of beneficial symbioses compared to stochastic forces (e.g., mutation and genetic drift). Below, we suggest a few animal models for use in evolutionary experiments of beneficial associations.

Animal systems for experimental evolution of beneficial interactions

The ideal model system for experimental evolution of beneficial host-microbe interactions would include several aspects. For the host, a short generation time and easy laboratory maintenance would allow for replicate experiments and observable host evolution. For the microbe, the ability to be cultivated outside of the host would be advantageous for examining the evolution of the microbe in the presence and absence of the host. Additional traits for both hosts and microbes, such as availability of genetic tools and genomic resources, small genomes, and cryogenic storage, would help to link specific genotypes to observed phenotypes. Of course, not every one of these conditions must be met in order to address some questions. For example, even when host populations cannot be studied for a large number of generations, evolving the microbial population within hosts may lend insight into host-microbe dynamics (Barroso-Batista et al., 2014; Kubinak and Potts, 2013; Sachs and Wilcox, 2006; Schuster et al., 2010).

Several animal model systems have potential to be used for experimental evolution of beneficial host-microbe interactions (a subset of systems are highlighted in Figure 2.2). The ubiquity of marine symbioses has made cnidarian-protist associations some of the most widely

studied systems in symbiosis research. A challenge of marine systems has been a lack of tractability of the host and inability to culture the symbiont without the host. Additionally, although protists make up a large proportion of described beneficial microbial associations with marine invertebrates, they have relatively large genomes, making sequencing more difficult than other types of symbionts. However, advances in technology have facilitated genomic and transcriptomic analyses of several cnidarians and their symbionts (Artamonova and Mushegian, 2013; Baumgarten et al., 2015; Bayer et al., 2012; Lehnert et al., 2012; Shinzato et al., 2011). For example, an emerging model for symbiosis establishment is the sea anemone *Aiptasia*, which forms an association with *Symbiodinium*, the algal symbiont of many cnidarians, including corals. Due to its relative ease of laboratory rearing, ability to be maintained without a symbiont, and sequenced genome and transcriptomes, *Aiptasia* is a highly tractable model for studying cnidarian-protist interactions (Baumgarten et al., 2015; Lehnert et al., 2012). Furthermore, induction of spawning is possible in the laboratory, where abundant larvae can be produced when needed (Grawunder et al., 2015).






Animal model	*Aiptasia* sea anemones	Bobtail squid	Pea aphid	*Drosophila* flies	*Caenorhabditis* nematodes	Mice
Symbiont/ microbiota	▪ Can host different types of *Symbiodinium* ▪ Symbiont can be grown *in vitro* ▪ Frequently used as a model to study coral-algal symbiosis	▪ Natural binary system (only one bacterial species present) ▪ Symbiont can be grown *in vitro* ▪ Well-elucidated mechanistic interactions between partners	▪ Simple microbiota ▪ Most known beneficial bacterial symbionts not cultivable ▪ Well-known association with several beneficial microbes of defined benefits	▪ Simple microbiota	▪ Little known about natural associations with microbes ▪ Other nematode systems with natural microbial associations exist, with longer generation times and fewer genetic tools	▪ Complex microbial community ▪ Gut is a natural environment for colonization by many microbes
Ability to control microbial associations	▪ Can rear without known primary symbiont (*Symbiodinium*)	▪ Can rear without known primary symbiont (*V. fischeri*)	▪ Effectively cannot maintain without obligate symbiont (*Buchnera*) ▪ Can rear without facultative bacterial symbionts	▪ Can rear entirely germ-free, though generally difficult to maintain sterile food source and control contact with microbes	▪ Can rear entirely germ-free	▪ Can rear entirely germ-free and control contact with microbes, but costly and labor-intensive
Genetic tools	▪ Sequenced genome and transcriptomes	▪ Sequenced transcriptomes	▪ Sequenced genome and transcriptomes	▪ Extensive	▪ Extensive	▪ Extensive
Generation time	▪ Long ▪ Gametogenesis can be induced	▪ Long	▪ Moderate (clonal) ▪ Long (sexual)	▪ Moderate	▪ Short	▪ Long
Laboratory maintenance	▪ High	▪ High	▪ Moderate	▪ Simple	▪ Simple ▪ Cryogenic storage possible	▪ High

Figure 2.2. Examples of potential animal model systems for experimental evolution of beneficial host-microbe interactions.

Another marine animal that has been studied extensively to investigate beneficial animal-microbe associations is the Hawaiian bobtail squid, Euprymna scolopes. It harbors only one type of bacteria in its light organ—Vibrio fischeri—which it obtains from the environment. The ability to grow the bacteria separately from its host is advantageous because hosts and bacteria can be evolved independently and together. Research on this system has also provided essential findings on the mechanisms involved in partner identification and communication (Davidson et al., 2004; Kremer et al., 2013; Nyholm and McFall-Ngai, 2004). Other advantages and considerations for use of the squid-Vibrio system in experimental evolution are reviewed in Soto & Nishiguchi (2014). Limitations of this system include the generation time of the squid host (first eggs are laid around 60 days post-hatching in the laboratory), and relatively high maintenance of proper environmental conditions (e.g., water quality, lighting) and food sources (Hanlon et al., 1997), which may hamper studying host evolution.

Insects and their symbionts have been widely used models for symbiosis due to their tractability and relatively simple association with microbes (only a few microbes are present in

some insect hosts). Among insect-microbe symbioses, the pea aphid and its bacterial symbionts are among the best characterized. Aphids harbor several beneficial symbionts, including *Buchnera*, an obligate intracellular bacterium that exchanges amino acids with its host. Genomic aspects of host and *Buchnera* interaction are well-defined (Wilson et al., 2010). Aphids also harbor several other symbionts that provide protection against natural enemies (Parker et al., 2013; Scarborough et al., 2005; Vorburger et al., 2010). The aphid-symbiont system is an excellent model for evaluating context-dependent factors involved in beneficial interactions, as well as the three-way interaction between innate host defense, protective symbionts, and natural enemies of the host (Dion et al., 2011; Polin et al., 2014; Rouchet and Vorburger, 2014; Weldon et al., 2013). Although most bacterial symbionts in aphids cannot be grown *in vitro* (an exception is found in Renoz et al., 2015), it is possible to replace natural *Buchnera* strains with *Buchnera* from another aphid lineage or facultative bacterial strains, thus opening the possibility for further study of these tightly-knit associations (Koga et al., 2003; Moran and Yun, 2015). One challenge of studying host evolution in this system is that even though aphids can reproduce sexually and asexually, it is only practical to propagate aphids clonally in the lab, resulting in little genetic variation over experimentally relevant timescales. Although sexual reproduction would increase genetic variation through recombination, this mode of reproduction would take much longer than clonal reproduction.

Other insect systems have been utilized in beneficial host-microbe experiments and experimental evolution studies. Research on the microbiota of *Drosophila* has highlighted the role microbes have in host development and protection from natural enemies, paving the way for studying the influence of a microbial community on host evolution (Hamilton et al., 2015; Mateos et al., 2016; Storelli et al., 2011). The fruit fly has also been used in a wide range of

evolution studies, from research on temperature adaptation to learning abilities (Dunlap and Stephens, 2014; Schou et al., 2014), and in experiments as long as 600 generations (Burke et al., 2010). Additional advantages include sequenced genomes and gene manipulation tools, and elucidated pathways involved in microbe-mediated host development, immune response, behavior, and intestinal activities (Kuraishi et al., 2013; Lee and Brey, 2013). Facilitating comparison of host evolution in the presence and absence of microbe, germ-free organisms can be established, however, it is relatively difficult to maintain a sterile food source and control contact with microbes in *Drosophila*. Similar to *Drosophila*, there is also growing interest in the microbiota of mosquitoes, particularly its influence on human pathogen transmission (Hegde et al., 2015; Jupatanakul et al., 2014). Although a few evolution experiments have been conducted with and within mosquitoes (Legros and Koella, 2010; Vasilakis et al., 2009; Yan et al., 1997), there are many novel approaches in which mosquitoes and their microbes could be exploited to further insight into how host and microbes adapt to one another, and, importantly, how this might influence vectorial capacity of important disease vectors.

Caenorhabditis elegans is an invertebrate system that has been utilized in numerous experimental evolution studies (Gray and Cutter, 2014). Like *D. melanogaster*, *C. elegans* has many genetic tools available and has a very short generation time for a eukaryote. Although not much is known about its natural associations with microbes, the nematode has been used extensively as a model for studying evolution of host-parasite interactions and microbe-mediated immune responses (Couillault and Ewbank, 2002; Dunbar et al., 2012; Ermolaeva and Schumacher, 2014; Irazoqui et al., 2010; Lee et al., 2013; Portal-Celhay and Blaser, 2012). Other nematode systems exhibiting long-standing mutualisms with microbes also exist (Clarke, 2014; Goodrich-Blair, 2007; Murfin et al., 2012). For example, the symbiotic interaction between the

nematode, *Steinernema carpocapsae*, and its bacterial symbiont, *Xenorhabdus nematophila*, is well characterized (Chaston et al., 2013; Cowles et al., 2007; Cowles and Goodrich-Blair, 2008), and the system has been utilized for several experimental evolution studies (Bashey et al., 2007; Bashey and Lively, 2009; Chapuis et al., 2012; Morran et al., 2016; Vigneux et al., 2008). However the system is generally less amenable to experimental evolution relative to *C. elegans*, because *S. carpocapsae* has a longer generation time and fewer available genetic tools. Nonetheless, the *S. carpocapsae* system can be an effective tool for testing hypotheses regarding established mutualisms.

Vertebrates are seldom recognized as models for experimental evolution, partly because they are relatively more difficult to maintain in the laboratory (e.g., expenses and animal care regulations) and have a long generation time. However, the microbiota of vertebrates, particularly mammals, are often much more complex than invertebrates, and thus provide a compelling model to examine host-microbiome evolutionary dynamics. The presence of the adaptive immune system in vertebrates also allows for further elucidation of the interactions between the immune system and the microbiome (Kitano and Oda, 2006). Some vertebrate models, including mice and zebrafish, can be reared to be germ-free until introduction of microbial communities (Ruby, 2008). Several experimental evolution studies have utilized mice as a model, such as artificial selection studies of nest-building and wheel running, and natural selection experiments of mating systems and captivity (Firman et al., 2015; Garland Jr. and Rose, 2009; Lacy et al., 2013). Microbial evolution experiments have been done in mouse models, where microbes are allowed to evolve within the host, providing a better look at how the host environment (e.g., host immunity) and microbial community can affect microbial adaptation (Barroso-Batista et al., 2014; Kubinak and Potts, 2013). Native and introduced microbial

communities have also been extensively examined in mice models (Hasegawa and Inohara, 2014; Laukens et al., 2015). Similarly, zebrafish has great potential for use in host-microbe evolution studies, particularly those addressing alteration of host immune responses by members of the microbial community (Kanther et al., 2011; Rolig et al., 2015).

While no single animal system is ideal in answering every outstanding question in symbiosis research, these highlighted systems are poised to address many of the present questions in beneficial symbioses. By using classic model organisms with fast generation times, high fecundity, and low maintenance (such as *Drosophila* flies and *Caenorhabditis* nematodes), we can determine the fundamental steps necessary for the evolution of mutualism between a eukaryotic host and a microbe. Performing experimental evolution with established symbiotic systems (such as the pea aphid and bobtail squid) can elucidate mechanisms involved in maintaining particular associations and may allow us to retrace the pathways leading to these evolutionary stable associations. While vertebrate-microbe systems may be more difficult to establish, the success of previous experiments with single rodent species (Firman et al., 2015; Garland Jr. and Rose, 2009; Lacy et al., 2013) and microbial evolution within mammalian hosts (Barroso-Batista et al., 2014; Kubinak and Potts, 2013) has shown that host-microbe coevolution experiments with vertebrates is possible.

Conclusion

Our understanding of beneficial host-microbe interactions is based largely on studying established associations. More direct tests are needed to solidify our understanding of how hosts and microbes interact with and affect each other. Experimental evolution, which has succeeded in bridging theory and empirical tests of fundamental evolutionary processes, can provide a way

to examine these interactions, particularly to test those predictions involving the evolution of host-microbe mutualisms. Current model systems in both microbial symbiosis and experimental evolution studies are poised for further explorations of beneficial interactions between animal hosts and their microbes. With more studies of symbioses utilizing experimental evolution, we can then further our understanding of the mechanisms involved in the establishment, maintenance, and short- and long-term consequences of beneficial host-microbe associations for both hosts and microbes.

Box 2.1. Ambiguities in defining "mutualism"

Defining host-microbe mutualisms

Mutualism is most often defined as reciprocally beneficial interactions between species (Bergstrom et al., 2003; Bronstein, 2009). However, the term mutualism is sometimes used to describe associations conferring benefits to the partner of focus, which is usually the host (Ewald, 1987; Moran and Wernegreen, 2000; Perez-Brocal et al., 2011), or when a net benefit is obtained through partner exploitation (Herre et al., 1999). This is in part because it is often difficult to evaluate the consequences of an interaction for all partners involved. In relation to host-microbe symbioses, many studies have shown benefits conferred by microbes to hosts, but few have empirically demonstrated hosts conferring benefits to their symbiotic microbes (Garcia and Gerardo, 2014). Therefore, interactions that are referred to as mutualistic may not be true reciprocal mutualisms, in which both host and microbe fitness is enhanced.

Several considerations are worth noting when defining beneficial host-microbe interactions, making any consensus past a general definition of mutualism difficult to achieve. In facultative interactions, the association may be beneficial only under certain contexts (e.g., a specific environmental condition or presence of an enemy), and being associated with a microbial partner may actually be costly to the host when it does not provide benefits. Identifying the biotic and abiotic factors in context-dependent interactions is important in evaluating the role of genetics and the environment in the evolution and maintenance of mutualisms (Jones et al., 2015). For example, in their discussion of modes of beneficial behaviors, Sachs et al. (2004) introduced the idea of a parasite that can prevent the establishment of a more harmful parasite within a host. The less harmful parasite might be regarded as beneficial because the host benefits from the association with the less harmful parasite when the more harmful parasite is present. In the pea aphid (*Acyrthosiphon pisum*), Weldon et al. (2013)

found that even though the bacterium *Hamiltonella defensa* protects its aphid hosts from parasitoid wasps, the benefits are not conferred when the bacterium is no longer infected by the *A. pisum* secondary endosymbiont phage, and, in the absence of the phage, the host exhibits severe fitness costs when in partnership with the bacterium. Temporal context also should be taken into consideration: an interaction may not have been mutualistic in the initial stages of the association. For example, a microbe may become trapped within a host, where it does not grow as well as in the external environment. Over time, the microbe may evolve to utilize host resources, and eventually proliferate better than it would outside the host. In other words, the beneficial associations we see now may not have had a beneficial beginning.

Mechanistic similarities between beneficial and parasitic symbionts

Both beneficial and parasitic symbionts (microbes that form long-term association with hosts) must be able to overcome host defenses, to acquire and process host resources, and to compete with other microbes. Horizontal gene transfer facilitates successful infection of symbionts through acquisition of genes required for host interaction. These genes can be exchanged between beneficial and parasitic symbionts, suggesting that beneficial symbionts infect their hosts using mechanisms similar to parasitic ones (Hentschel et al., 2000; Perez-Brocal et al., 2011). Many obligate symbionts (those that cannot live in the absence of a host) also undergo genomic reduction as a result of adaptation to a relatively stable host environment (Ochman and Moran, 2001). While the mechanisms are similar, it is the net outcome of the interaction between molecular components of the microbe and host that determines where the interaction lies on the parasitism-mutualism continuum.

Mutualism as an arms race

Coevolution between some hosts and beneficial symbionts has been viewed as an arms race, not unlike between hosts and parasitic symbionts. Theories in host-microbe mutualism suggest that because beneficial symbionts are adapted to their hosts, they should be "evolutionarily static", exhibiting slow rates of evolution and little genetic diversity to remain adapted to their hosts (i.e., any new variant of the symbiont is less likely to be suitable to a host) (Law and Lewis, 1983). However, empirical studies have suggested similar evolutionary trajectories of beneficial and parasitic symbionts (such as rapid evolution and increased recombination), contradicting the previously proposed hypotheses of host-microbe mutualisms (Sachs et al., 2011). Thus, even in obligate symbioses where the fitnesses of the host and beneficial symbiont are the most closely aligned, the host must be able to respond to the rapidly evolving genome of its microbial partner. Because the microbial population is kept at a small size within hosts, genetic drift plays a large role in the genetic structure of the symbiont, leading to gene losses for which the host must compensate (Bennett and Moran, 2015).

Box 2.2: Approaches to experimental evolution

The driving mechanism: natural forces and artificial selection

Classic experimental evolution studies involve studying how evolutionary processes shape populations. They seek to connect evolutionary forces to genotypic and phenotypic changes and to identify the molecular mechanisms involved. These changes are observed and measured before, during, and after specific conditions are set upon experimental populations. By contrast, artificial selection experiments shift the focus from evolutionary processes and toward selecting for certain phenotypes. The consequences of such selection can then be identified and measured after the desired traits are acquired. The most widely known example of artificial selection stems from selective breeding of animals and plants conducted by humans. Studies of domesticated plants and animals have also led to significant insight into the genetic changes resulting from adaptation of these organisms to human practices (Andersson, 2012; Purugganan and Fuller, 2009).

The model system: single species evolution and multiple-species interactions

Fundamental evolutionary questions have been examined using populations of single species systems (Kassen, 2014; Kawecki et al., 2012). By implementing simple environmental conditions and limiting contact with unwanted organisms, this approach has addressed inquiries regarding the very core of evolutionary processes, such as the role of natural selection, gene flow, and genetic drift within and across populations. Multi-species studies have lent insights into processes that cannot be examined with single-species systems (e.g., Brockhurst and Koskella, 2013; Hillesland and Stahl, 2010; Reznick et al., 1990; Rouchet and Vorburger, 2014). For instance, coevolutionary dynamics between *Pseudomonas fluorescens* and its phage were altered when a predator of the bacteria was introduced (Friman and Buckling, 2013). These

experiments have provided insight into how predation, parasitism, and mutualism impact the evolution of the species involved. Multiple-species studies may be more ecologically representative because no species is completely isolated from others. Nonetheless, limiting interactions to a small number of species likely often fails to capture the true ecology in nature.

The starting point: clones and standing genetic variation

Studies solely using microbes tend to start with clonal populations. Because microbes have a short generation time, they can gain enough mutations within a short amount of time for evolutionary forces to act upon. Thus, mutations play a large role in generating genetic diversity in microbial studies. Independent accumulation of mutations can lead to divergence between clonal populations even under identical environments. Biologists can then track evolutionary trajectories taken by populations that begin identical to each other through comparison of ancestral and evolved populations using direct fitness tests. For model systems that have longer generation times (which are most eukaryotes), the starting populations tend to have standing genetic variation, which can be created through natural means (e.g., field collections) or genetic manipulations (e.g., mutagenesis). During the course of the experiment, variation can also be generated through recombination and outcrossing in populations that reproduce sexually. Although not as streamlined as clonal populations, these systems provide insight into the effects that evolutionary forces can have on populations where *de novo* mutation is not the only source of genetic diversity. Whether the populations are clones or contain standing genetic variation, there are usually multiple replicate populations in evolution experiments. This is to ascertain whether populations will converge on similar trajectories (when certain adaptations will arise

predictably), or if the populations will diverge from each other (when rare changes are more important than common and predictable changes).

The environment: laboratory and field experiments

Many experimental evolution studies are run in a laboratory setting. This allows for more control and reproducibility of environmental conditions. The extent to which laboratory conditions represent natural conditions can vary across studies, and the importance of this representation can vary across questions. If a study is focused on broad evolutionary questions that can be applied across many organisms, environments that do not fully represent the natural setting may suffice, as more ecologically sound conditions can be added or modified in studies directed toward specific organisms. Field studies provide a more accurate representation of natural processes, but some conditions can be irreproducible or unidentified, making replications of experimental conditions difficult. Kawecki et al. (2012) suggests evolving populations under laboratory conditions (such as selecting for cold tolerance), and performing field experiments with the evolved populations to determine whether they are well-adapted to the natural environment (such as a cold environment).

Evolution from scratch: synthetic ecology and digital organisms

Questions involving ecology and evolution have mainly involved studies of natural or existing populations and communities (Reznick et al., 1997; Scarborough et al., 2005; Zhen et al., 2012). Using information gained from these natural systems, we can implement another approach to generate hypotheses: creating synthetic or digital populations and communities with defined traits and observing how they evolve. Combined with advances in technology, these approaches

can help us determine the evolutionary forces and mechanisms involved in the adaptation of natural populations throughout time.

Several experimental evolution studies have used constructed mutants as the starting population (Harcombe, 2010; Marchetti et al., 2010; Shou et al., 2007). By evolving populations carrying specific genes, we can identify the proximate mechanisms involved in the evolution of natural populations. Furthermore, the recently emerged field of synthetic ecology focuses on establishing communities composed of different microbial members to examine the consequences of species interaction, particularly for use in biotechnological developments. These experiments are generally composed of engineered mutants or microbes that are not naturally associated with each other (Escalante et al., 2015; Fredrickson, 2015).

Evolution has also been studied using digital organisms, which dwell and replicate inside of computers under user-selected settings (a type of agent-based model, with the most common platform being Avida (Ofria and Wilke, 2004)). The requirements for evolution are simulated through digital equivalents, such as executable codes representing genomes, replication error probabilities representing mutation rates, and limited computer space and energy units for growth (Adami, 2006). Similarly, robots have also been used to study how communication evolves on the individual and colony levels (Floreano et al., 2007). Although there are limitations in using these digital platforms, they have provided support for theories and models in genetics, ecology, and evolution, and can be a powerful tool in combination with studies on living organisms (Adami, 2006; Kawecki et al., 2012).

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CHAPTER III

THE EFFECTS OF *BACILLUS SUBTILIS* ON *CAENORHABDITIS ELEGANS* FITNESS AFTER HEAT STRESS

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Abstract

Microbes can provide their hosts with protection from biotic and abiotic factors. While many studies have examined how certain bacteria can increase host lifespan, fewer studies have examined how host reproduction can be altered. The nematode *Caenorhabditis elegans* has been a particularly useful model system to examine how bacteria affect the fitness of their hosts under different contexts. Here, we examine how the bacterium *Bacillus subtilis,* compared to the standard *C. elegans* lab diet, *Escherichia coli*, affects *C. elegans* survival and reproduction after experiencing a period of intense heat stress. We find that under standard conditions, nematodes reared on *B. subtilis* produce fewer offspring than when reared on *E. coli.* However, despite greater mortality rates on *B. subtilis* after heat shock, young adult nematodes produced more offspring after heat shock when fed *B. subtilis* compared to *E. coli*. Because offspring production is necessary for host population growth and evolution, the reproductive advantage conferred by *B. subtilis* supersedes the survival advantage of *E. coli*. Furthermore, we found that nematodes must be reared on *B. subtilis* (particularly at the early stages of development) and not merely be exposed to the bacterium during heat shock, to obtain the reproductive benefits provided by *B. subtilis*. Taken together, our findings lend insight into the importance of environmental context and interaction timing in shaping the protective benefits conferred by a microbe toward its host.

Introduction

Eukaryotic hosts generally obtain fitness benefits through association with specific microbes. Harboring certain microbes can increase host protection from biotic and abiotic stresses, such as enemies or environmental changes, and can provide hosts with nutrients that they cannot obtain from their diet alone (Douglas, 2009; Feldhaar, 2011; Oliver et al., 2013). Associating with such beneficial microbes can shape host evolution, altering host maintenance of redundant traits (Martinez et al., 2016), and can lead to niche expansion, allowing hosts to occupy environments they normally would not be able to inhabit (Douglas, 2014; McFall-Ngai et al., 2013). Host-microbe associations are often context-dependent such that benefits are associated with harboring microbes only under certain conditions, and costs are revealed under others (Heath and Tiffin, 2007; Russell and Moran, 2006; Weldon et al., 2013). Additionally, different microbial species, or even strains of the same species, can confer different levels of benefits to hosts of the same genotype, and hosts of different genotypes may also differ in the level of benefit that they receive from microbial association (Murfin et al., 2015; Parker et al., 2017). Taken together, the hosts, the microbes, and the environment all shape the nature of these interactions over ecological time, which in turn may shape the evolutionary trajectories of the host and microbial populations. Here, we utilize *Caenorhabditis elegans*, a well-characterized invertebrate model amenable to a range of experimental manipulations, to test the effects of environmentally obtained bacteria on host fitness under stress.

The nematode *C. elegans* has been extensively used as a model system to study host-microbe associations (Clark and Hodgkin, 2014; Kurz and Ewbank, 2000; Zhang et al., 2017). *Caenorhabditis elegans* (Figure 3.1) has a natural interaction with microbes in that it feeds on bacteria and fungi in decomposing plant matter (Frézal and Félix, 2015). The nematode has a

grinder in the pharynx region that crushes microbes that it consumes; however, some microbes survive the grinder and colonize the gut of the nematode (Gibson et al., 2015; Portal-Celhay and Blaser, 2012). Some of these persistent microbes are pathogenic to the host (Couillault and Ewbank, 2002), some are commensal (Clark and Hodgkin, 2014), and some are beneficial (Zhang et al., 2017). Specifically, some microbes have been shown to increase nematode lifespan under environmental stresses (Donato et al., 2017; Grompone et al., 2012; Gusarov et al., 2013; Leroy et al., 2012; Nakagawa et al., 2016), an important finding given that *C. elegans* is a model system to study longevity and ageing (Cabreiro and Gems, 2013; Garigan et al., 2002; Johnson, 2008). Two studies found that the soil bacterium, *Bacillus subtilis*, was able to increase *C. elegans* survivorship after heat shock relative to exposure to the standard lab diet, *Escherichia coli* (Donato et al., 2017; Gusarov et al., 2013). These studies found that *B. subtilis* nitric oxide (NO) production and biofilm formation in the host's gut resulted in elevated host lifespan post-heat stress. For this interaction to impact host-microbe evolution, the bacteria would not only need to increase survival but would also need to increase host reproduction after heat shock. *Caenorhabditis elegans* generally exhibit little to no fecundity after exposure to intense heat stress (Aprison and Ruvinsky, 2014), and it is unclear if interactions with *B. subtilis* could mitigate this substantial fitness loss. In this study, we measure the effects of *C. elegans* interactions with *B. subtilis* on nematode fitness (encompassing both survival and fecundity) after a stressful heat event. Additionally, we determine how exposure to *B. subtilis* at different time points during development affects *C. elegans* fitness.

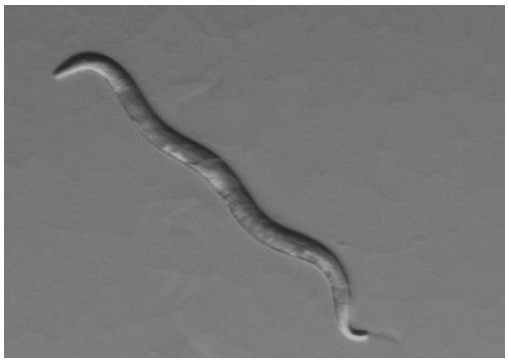

Figure 3.1. An adult *C. elegans*
Photo credit: McKenna Penley

Methods

Nematode and bacterial strains

All nematodes in this study were the *C. elegans* N2 Bristol strain, which were maintained on *E. coli* OP50 prior to experiments. We used *B. subtilis* 168 and *E. coli* OP50 in all experiments. In the first two experiments we also used *B. subtilis* Δ*nos*, which lacks the ability to produce NO. We obtained both *B. subtilis* strains from the study examining the role of NO in *C. elegans* longevity and survivorship post-heat stress (Gusarov et al., 2013). We grew both *B. subtilis* strains and *E. coli* on nematode growth medium (NGM) plus glucose (2%) and arginine (0.5 mM) for all experiments (Gusarov et al., 2013). For experiments involving fecundity, we transferred nematodes to GFP-labeled OP50 (OP50-GFP, grown on NGM) to allow them to produce offspring. We used OP50-GFP to control for the bacterium that nematodes were exposed to during heat shock recovery. GFP-labeled *E. coli* is different from *E. coli* OP50 but is still relatively neutral with respect to *C. elegans* fitness.

Survival of six-day-old nematodes on B. subtilis and E. coli

We first compared the short term survivorship over the six hours post heat stress of nematodes across *B. subtilis* 168, *B. subtilis* Δ*nos*, and *E. coli* by performing the heat shock experiment done

in the previous studies examining the role of *B. subtilis* on host lifespan after heat stress (Donato et al., 2017; Gusarov et al., 2013). We surface sterilized *C. elegans* N2 eggs using an established alkaline hypochlorite protocol (Stiernagle, 2006) and reared L1 larvae on *E. coli* until they reached L4/young adult (on day three). We then transferred nematodes to either *B. subtilis*, *B. subtilis* Δ*nos*, or *E. coli.* When nematodes were five days old, we transferred them to new plates of the appropriate bacteria to prevent mixing of generations. Prior to heat shock, all nematodes were kept at 20˚C. On the next day, when they were six-day-old adults, we heat shocked the nematodes in an incubator set at 34˚C. After three hours, we removed a set of replicate plates for each bacterial treatment from the incubator and scored survival by prodding with a platinum pick to determine signs of movement (Donato et al., 2017; Gusarov et al., 2013; King et al., 2016). After six hours, we removed another set of plates and scored survival. There were three replicate populations per bacterium per time point, each population containing about 20 nematodes.

Survival and fecundity of three-day-old nematodes on B. subtilis and E.coli

Because nematodes cease egg production after about six days (Altun and Hall, 2009), to assess fitness effects of *B. subtilis* association, here, we heat shocked nematodes when they were young adults and still capable of producing offspring. Specifically, we investigated three-day-old nematode survival and fecundity under standard and heat shock conditions on *B. subtilis*, *B. subtilis* Δ*nos,* and *E. coli*. We surface sterilized *C. elegans* N2 eggs and reared L1 larvae on each bacterium for three days at 20˚C until they reached young adulthood. We then placed nematodes in an incubator set at either 20˚C (standard temperature) or set at 34˚C for six hours. We used three replicate populations per bacterium per temperature setting, for a total of 18 populations with approximately 200 nematodes per population. To measure survival, here, and in all

subsequent experiments, we determined the proportion of nematodes that were alive six hours after the heat shock period (based on prodding, as above), not how long they lived after heat shock (i.e., survivorship or lifespan). Afterwards, we washed nematodes from each replicate population with M9 and transferred all adults from each population to another plate seeded with *E. coli* OP50-GFP to produce offspring, where they were maintained at 20˚C. Two days post heat shock, we counted the larvae on each plate. The total number of adults was counted prior to heat shock. Since all plates were transferred using the same protocol, we assumed a similar number of nematodes were transferred to OP50-GFP to produce offspring. We did not count the number of live adults two days post-heat shock because nematodes could have produced offspring and subsequently died before being counted. We calculated the average number of offspring per heat shocked adult to determine relative differences in fecundity between treatments. This measure accounts for both difference in survival and difference in fecundity.

Assaying importance of exposure window, experiment 1

Similar to the survival and fecundity assay described above, we surface sterilized N2 eggs and reared L1 larvae on either *B. subtilis* or *E. coli* for three days, with six replicates per bacterium. We then transferred three *B. subtilis* replicates to *E. coli* and the other three replicates to *B. subtilis*, and similarly transferred the *E. coli* replicates to either *E. coli* or *B. subtilis*, for a total of 12 populations with approximately 100 nematodes per population. We heat shocked nematodes at 34˚C for six hours, scored survival after six hours as above, then transferred them to plates seeded with *E. coli* OP50-GFP to lay eggs, where they were maintained at 20˚C. We quantified larval offspring two days later.

Assaying importance of exposure window, experiment 2

Similar to the first exposure assay, we surface sterilized N2 eggs and transferred about 100 – 200 larvae to a new lawn of bacteria each day as indicated in Figure 3.2 (with four replicates per treatment, for a total of 20 populations). After reaching adulthood, we heat shocked nematodes for six hours at 34˚C, scored survival after six hours as above, then transferred nematodes to plates seeded with *E. coli* OP50-GFP to lay eggs, where they were maintained at 20˚C. We quantified larval offspring two days after.

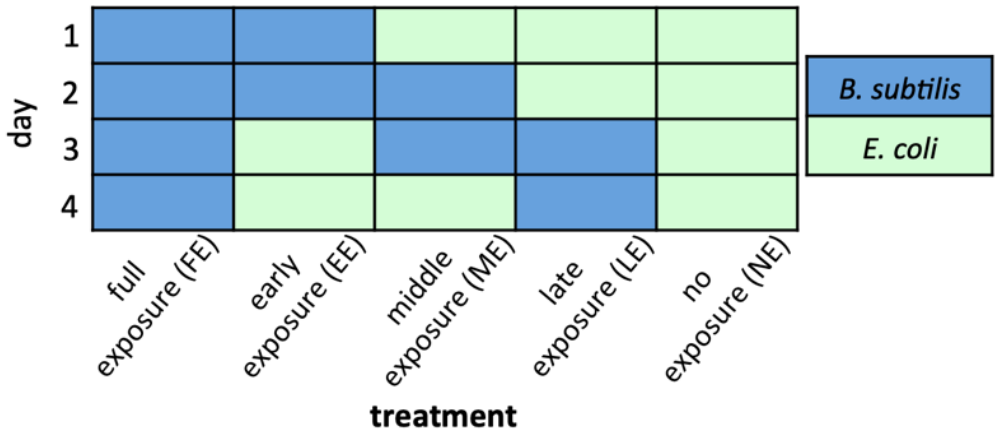

Figure 3.2. Setup of exposure experiment 2

Nematodes were transferred to the indicated bacterium on each day and heat shocked on day 4.

Colonization of day 2 larvae and adult nematodes

Following a modified protocol from Vega and Gore 2017, we determined whether day 2 larvae reared on *B. subtilis* harbored live bacterial cells. Briefly, after surface sterilizing N2 eggs, we transferred roughly 100 larvae to a lawn of *B. subtilis*. The following day (day 2 of Figure 3.2), we washed larvae three times with cold 0.01% Triton X-100 in M9 and incubated them in bleach (1:1000 diluted) for 15 minutes at 4˚C to remove surface bacteria. We then treated larvae with a solution of 0.25% sodium dodecyl sulfate (SDS) + 3% dithiothreitol (DTT) for 20 minutes. After

washing with 0.01% Triton X-100 in M9, we transferred about 10 – 20 larvae to a well of a 96-well plate (five wells total), each well containing a small amount of sterile silicon carbide grit and 0.01% Triton X-100 in M9. We briefly disrupted the samples using a Qiagen TissueLyser II homogenizer. After plating the content onto LB plates, we grew the bacteria for two days before quantifying colony-forming units. For colonization of adults, we reared surfaced-sterilized N2 eggs on *B. subtilis* until adulthood, then heat shocked nematodes for 6 hours at 34˚C. We subsequently washed and homogenized the nematodes using the same protocol as the day 2 larvae, crushing five adults in each of five wells of the 96-well plate.

Statistical analysis

To analyze short term survivorship of six-day-old hosts under different bacterial treatments, we used a Cox proportional-hazards model with the Coxph function of the Survival package in R (Therneau and Lumley, 2015). For subsequent experiments, we analyzed survival using a generalized linear model (GLM) with a binomial distribution and logit link function. For fecundity, we used a GLM with a normal distribution and identity link function. We then performed contrast tests to compare bacterial treatments. We used JMP Pro (v.13) for the GLM analyses.

Results

Bacillus subtilis differentially affects survival of old and young adult hosts, and provides a reproductive benefit in young adult C. elegans

We performed a heat shock experiment, similar to previous studies (Donato et al., 2017; Gusarov et al., 2013), that allowed us to directly compare wild-type *B. subtilis* strain 168, a *B. subtilis*

mutant lacking the ability to produce nitric oxide (NO) (*B. subtilis* Δ*nos*), and *E. coli* strain OP50. Briefly, we reared nematodes on *E. coli* at 20˚C (standard temperature) for three days, then transferred them to one of the three bacterial strains, where they remained for another three days before shifting to 34˚C (heat shock temperature) for six hours. We found that there was a small but non-significant decrease in host survival when nematodes were exposed to *B. subtilis* Δ*nos* compared to hosts exposed to wild-type *B. subtilis* (Figure 3.3; $\chi_1^2 = 3.36$, $p = 0.07$). However, we found a significantly large difference in host survival between wild-type *B. subtilis* and *E. coli* immediately after six hours of heat shock ($\chi_1^2 = 28.05$, $p < 0.001$). Therefore, *B. subtilis* conferred greater host survival following heat shock, but the protective effects of NO can, at most, only account for a portion of the benefits conferred by *B. subtilis*.

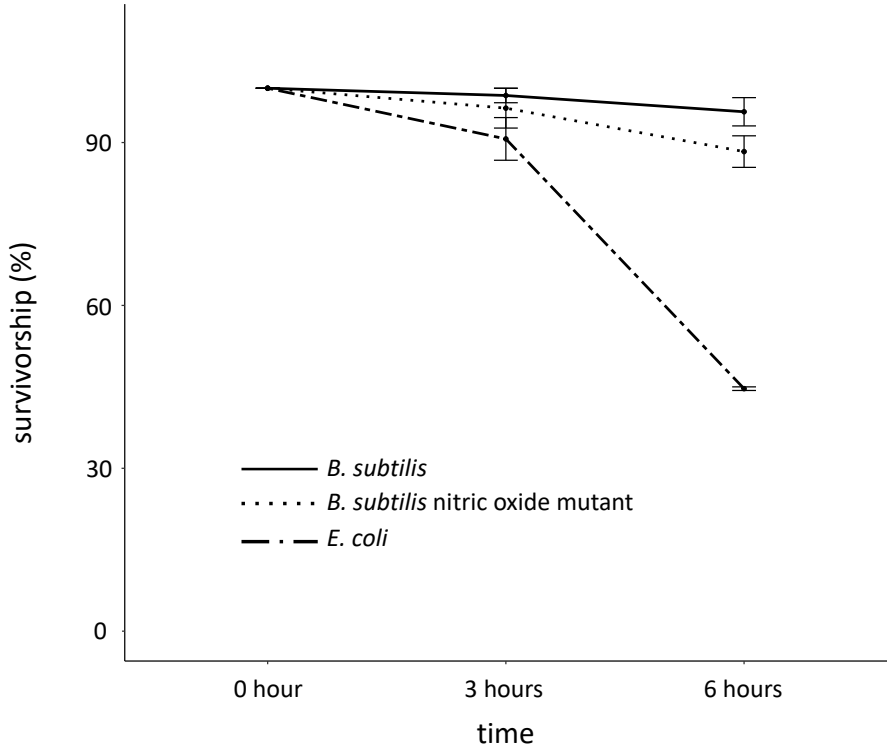

Figure 3.3. Survivorship of older adult (six-day-old) nematodes under heat shock
Nematodes were reared on E. coli *OP50 until L4/young adult stage (at about three days old), then subsequently transferred to the indicated bacterium. They were heat shocked at 34˚C three days later. After three and six hours, replicate plates were removed from the heat and scored for survival. Error bars indicate standard errors. There were three replicate populations per time point per bacterium, each population containing ~20 nematodes.*

Since the nematodes above, as in previous studies, were heat shocked at post-reproductive age (egg laying ceases after about six days from the time nematodes hatch (Altun and Hall, 2009)), we could not determine whether these bacteria affected host fecundity after heat shock. To this end, we examined the survival and fecundity of young adult *C. elegans* when reared on *B. subtilis* 168, *B. subtilis* Δ*nos*, and *E. coli* OP50 under standard and heat shock conditions. Three-day-old nematodes reared on their respective bacterium at 20˚C were either left at the standard temperature or heat shocked. We found no difference in host survival under standard conditions (measured at the same time we scored survival of heat-shocked nematodes) regardless of the hosts' bacterial association (Figure 3.4a). However, nematodes reared on *E. coli* produced more offspring than on either *B. subtilis* strain under standard lab conditions (Figure 3.4b; $\chi_2^2 = 13.04$, $p = 0.0015$). Under heat shock conditions, more nematodes survived on *E. coli* compared to both *B. subtilis* strains (Figure 3.4c; $\chi_1^2 = 611.03$, $p < 0.001$). By contrast, more offspring were produced on both *B. subtilis* strains compared to *E. coli* (Figure 3.4d; $\chi_1^2 = 25.53$, $p < 0.001$). Therefore, *B. subtilis* exposure conferred increased fecundity per adult going into heat shock, but not survival, in young adult nematodes. Further, *B. subtilis* NO production did not increase the survival of heat-shocked, young adult nematodes compared to *B. subtilis* Δ*nos* (Figure 3.4c; $\chi_1^2 = 0.89$, $p = 0.35$) and was not necessary for the increased reproduction conferred by *B. subtilis.* Because we saw no significant differences between the two *B. subtilis* strain treatments, all subsequent experiments used only the wild-type *B. subtilis* 168 strain.

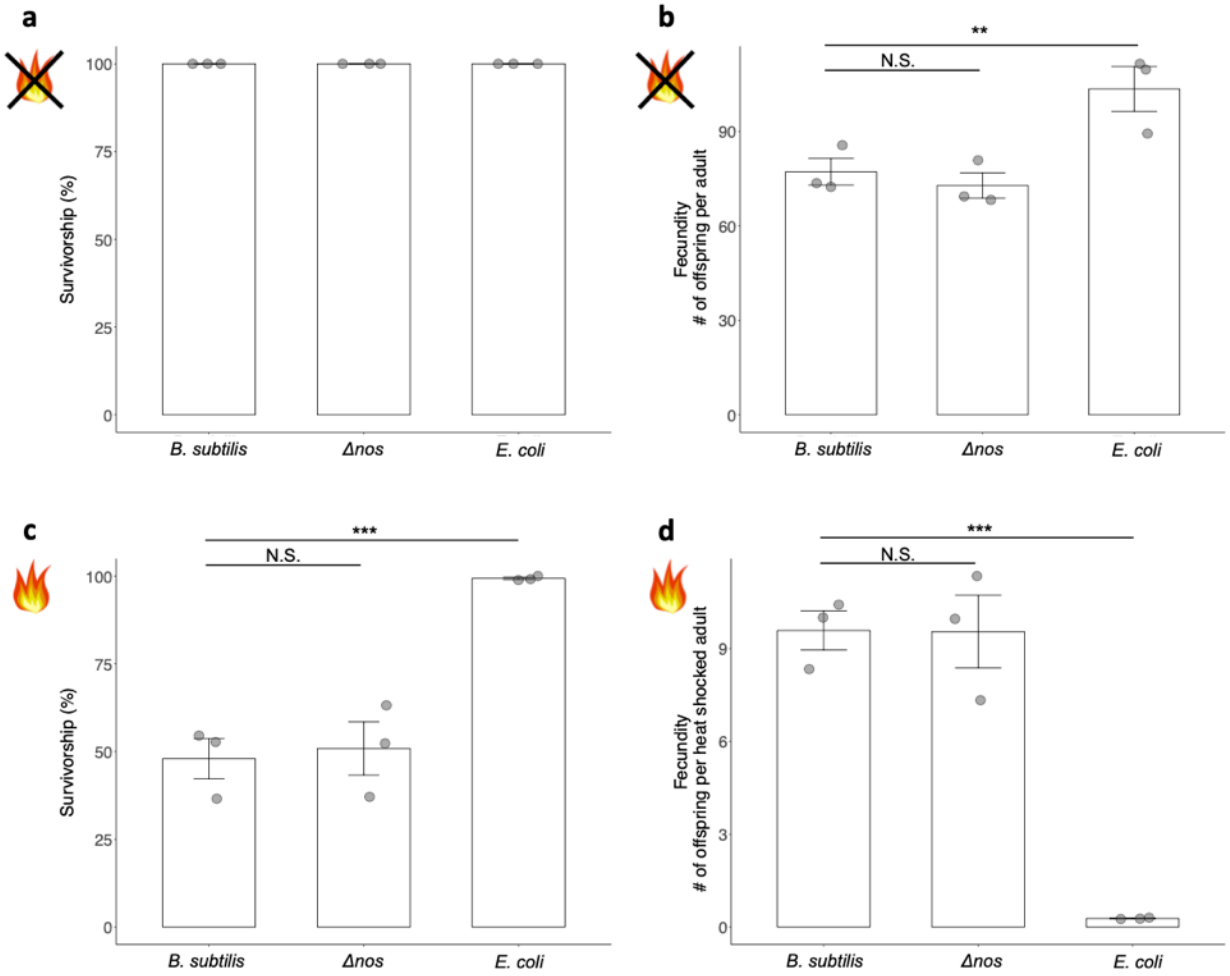

Figure 3.4. Survival and fecundity of young adult (three-day-old) nematodes under no heat shock and heat shock conditions

Nematodes were reared on the indicated bacterium until three days old, then were either left at standard conditions or heat shocked. ***a)*** *Survival after six hours and* ***b)*** *fecundity of nematodes under standard conditions.* ***c)*** *Survival after six hours and* ***d)*** *fecundity of nematodes under heat shock conditions. Error bars indicate standard errors. Each data point represents a replicate population, with each population consisting of ~200 nematodes. **p<0.01, ***p<0.001, N.S. denotes no significance.*

Development on B. subtilis is necessary for reproductive benefit

To determine whether the decrease in survival and increase in offspring production on *B. subtilis* was due to host larval development on *B. subtilis* or simply due to exposure to the bacterium during heat shock, we compared nematodes reared on *B. subtilis* that were then heat shocked on *E. coli*, and vice versa. Nematodes that developed on *B. subtilis* and were heat shocked on *E. coli*

exhibited the lowest survival (Figure 3.5a; χ_1^2= 97.10, $p < 0.001$). However, development on *B. subtilis* resulted in higher fecundity after heat shock regardless of which bacterium nematodes were exposed to during heat stress (Figure 3.5b; χ_1^2 = 15.96, $p < 0.001$). The reproductive benefit conferred by *B. subtilis* was therefore predominantly dependent upon the development of hosts on *B. subtilis*.

Early exposure to B. subtilis is more beneficial for hosts than later exposure

We then asked whether the age at which nematodes are exposed to *B. subtilis* has an effect on the hosts' survival and fecundity. We varied exposure time to *B. subtilis* by transferring nematodes to the indicated bacterium each day (Figure 3.2). We found that the time at which the host is exposed to *B. subtilis* affects both survival and fecundity upon heat stress (Figure 3.6a; χ_4^2 = 114.61, $p < 0.001$; Figure 3.6b; χ_4^2 = 35.02, $p < 0.001$). Specifically, exposure to *B. subtilis* during the first two days of host development is critical for nematodes to obtain the reproductive benefit conferred by *B. subtilis* upon heat stress (Figure 3.6b, early exposure treatment versus all other treatments). Furthermore, compared to when nematodes were exposed to *B. subtilis* throughout development and during heat shock, early exposure to *B. subtilis* was more beneficial in terms of fecundity and survival (Figure 3.6, full exposure vs. early exposure). Overall, these results demonstrate that nematodes gained the most benefits when exposed to *B. subtilis* early, whereas later exposure to *B. subtilis* conferred no greater benefit than exposure to *E. coli* alone (Figure 3.6b).

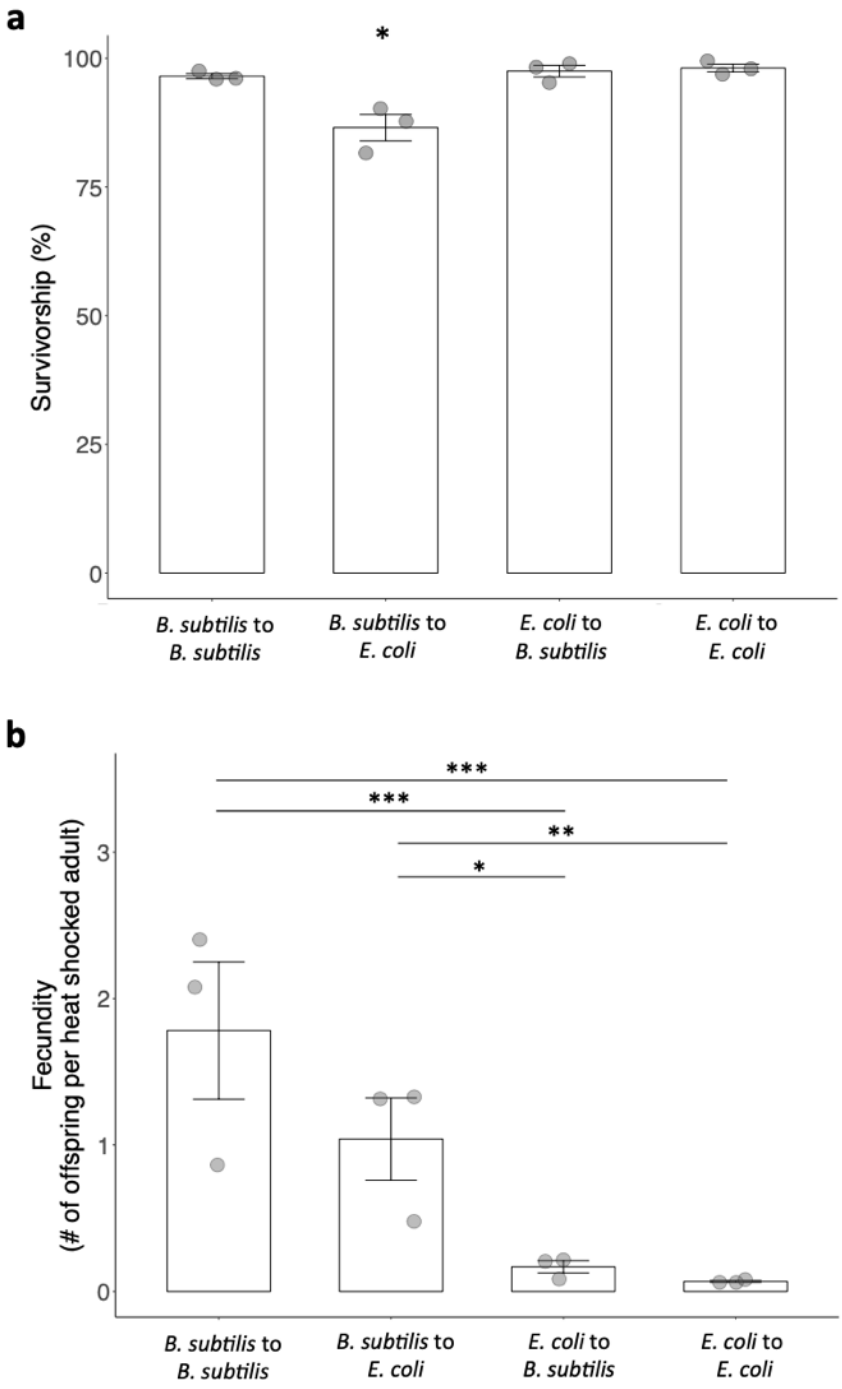

Figure 3.5. Survival and fecundity of young adult nematodes heat shocked on either *B. subtilis* or *E. coli*

Nematodes were reared on the first bacterium indicated before being transferred to the bacterium they were heat shocked on when they reached adulthood. ***a)*** *Survival after six hours and* ***b)*** *fecundity of nematodes under heat shock conditions. Error bars indicate standard errors. Each data point represents a replicate population, with each population consisting of ~100 nematodes. *p<0.05, **p<0.01, ***p<0.001.*

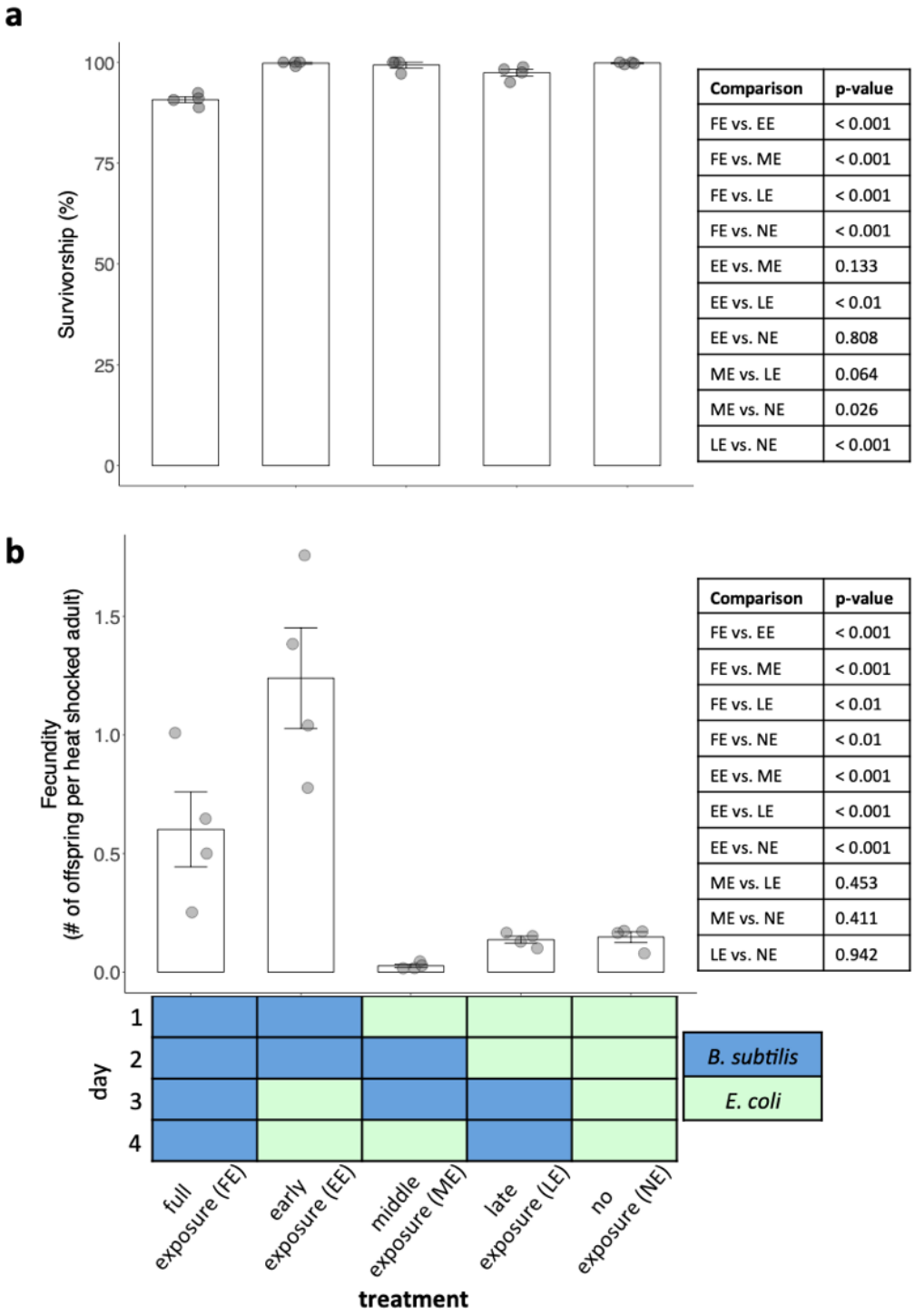

Figure 3.6. Survival and fecundity of young adult nematodes exposed to *B. subtilis* at different points throughout larval development

Nematodes were transferred to each bacterium as indicated each day before being heat shocked on day 4. ***a)*** *Survival after six hours and* ***b)*** *fecundity of nematodes under heat shock conditions. Error bars indicate standard errors. Each data point represents a replicate population, with each population consisting of ~100 to 200 nematodes.*

Bacterial colonization of host

Because nematodes benefit the most when either exposed to *B. subtilis* completely or early on in development, we asked whether these benefits were associated with live *B. subtilis* in the nematode gut. We first extracted and grew *B. subtilis* colonies from replicate groups of N2 nematodes reared on *B. subtilis* until day 2 of Figure 3.2, observing means ranging from 0.42 – 0.84 colony forming units, or CFU, per larva. The live colonies we found indicate that *B. subtilis* cells are able to enter young host larvae, pass through the grinder intact, and survive in the host gut. In addition, we recovered live *B. subtilis* in heat shocked adults (mean of 2 CFU per nematode), showing that live *B. subtilis* was present in the host after heat stress.

Discussion

Here, we evaluated the effects of specific host-microbe interactions on host survival and fecundity after environmental change, via heat shock. Overall, we found that while *C. elegans* interactions with *E. coli* resulted in greater host fitness compared to *B. subtilis* under standard conditions (Figure 3.4b), interactions with *B. subtilis* conferred significantly greater host fitness, via increased fecundity, after heat shock (Figure 3.4d). Reproduction is vital for population growth and evolution in the long term—if an individual does not reproduce, it will have no fitness regardless of whether it survives after heat stress. Here, we demonstrated that, under a scenario of heat shock, survival did not necessarily correlate with fecundity. Rather, hosts of reproductive age had lower survival overall on *B. subtilis* compared to *E. coli*, but had greater fecundity on *B. subtilis* upon heat shock. Further, in corroboration with previous research, we showed that *C. elegans* can reproduce after several hours of high heat stress (Aprison and Ruvinsky, 2014, 2015). Even though hosts undergo some sperm damage, they can produce

viable offspring if allowed sufficient time to recover and reproduce (Aprison and Ruvinsky, 2014). Thus, given this reproduction after heat shock, there is potential for *B. subtilis* protection to shape host evolution.

Our survival results for heat shocked older adult nematodes support those of previous studies, in that *B. subtilis* led to increased host survivorship compared to *E. coli* (Donato et al., 2017; Gusarov et al., 2013). While we did not find a significant difference between association with the two *B. subtilis* strains, we did not extend the heat shock period past six hours, where a larger difference in survival may be observed. By contrast, when we heat shocked younger adult nematodes, we found lower survival of hosts reared on *B. subtilis* (both wild-type and nitric oxide mutant) compared to *E. coli*. We hypothesize that the survival difference is due to the different age of hosts that were heat shocked: young adults (three-day-old nematodes) in our study instead of old adults (six to eight-day old nematodes) in the prior studies. Interestingly, the increased survival gained by nematodes under the circumstances of the prior experiments would have had little to no effect on host fitness, which was not measured, as these nematodes were past reproductive age. While we have not identified the mechanisms by which *B. subtilis* increases fecundity in young adult nematodes upon heat shock, bacterial NO does not appear to be a critical driver in the reproductive output of these nematodes. Taking the survival and reproduction data together, we see that *B. subtilis* can confer a reproductive advantage to *C. elegans* hosts while reducing their survival under heat stress.

Investment in longevity is hypothesized to trade-off with reproduction (Mukhopadhyay and Tissenbaum, 2007). While we did not measure total host lifespan, our heat shock data suggests that increased survival of *C. elegans* on *E. coli* led to a reproductive cost, the converse of which is true for hosts on *B. subtilis.* Furthermore, because nematodes have more offspring on

E. coli than *B. subtilis* in the absence of heat shock (Figure 3.4b), the interaction between *B. subtilis* and *C. elegans* is context-dependent—hosts incur a cost to harboring *B. subtilis* in the absence of heat stress, but benefit reproductively under heightened temperatures. Furthermore, while our heat shock temperature (34˚C) is much higher than the range at which *C. elegans* is generally reared (15 – 25 ˚C), hosts were able to reproduce at a rate at which the population could at least replace itself on *B. subtilis*, compensating for the reduced number of surviving adults compared to *E. coli.*

Exposure to beneficial microbes during the early stages of host development could be important for host resistance to biotic and environmental stresses during adulthood. For example, a study found that prior diet can affect *C. elegans* preference for harmful *Burkholderia* bacteria (Cooper et al., 2009). Another study examining the consequences of early exposure to pathogens in *C. elegans* found increased resistance to pathogens and heat stress during adulthood (Leroy et al., 2012). Host fecundity may also differ depending on the bacteria the host is exposed to during development and adulthood. Our study provides support for this phenomenon: exposure to *B. subtilis* during early stages of development was enough for *C. elegans* to remain reproductively viable after a period of heat shock (Figure 3.6b). By contrast, exposure to *B. subtilis* as an older larva or adult did not benefit hosts greatly when they underwent heat stress (Figures 3.5b/3.6b). This suggests that exposure to the bacterium at an early point during nematode development may be critical in priming the host to respond to heat shock as an adult. The bacterium may have entered nematodes as spores or formed spores upon entrance, and so early exposure to the *B. subtilis* may have allowed more time for spores to become vegetative and thus benefit nematodes when they were heat shocked. Furthermore, heat shock on *E. coli* after exposure to *B. subtilis* for the first two days of development offset the cost of reduced survival when heat shocked on *B.*

subtilis (Figure 3.6, early exposure vs. full exposure). The nature of this interaction is unclear, particularly given that the impact of *B. subtilis* on *C. elegans* fecundity does not appear to be mediated by NO production.

Given that *B. subtilis* can be both a gut colonizer and a food source, the results observed may be due to the effects of nutrition obtained via digestion of *B. subtilis*. However, several lines of evidence indicate that the increased fecundity of hosts on *B. subtilis* is not likely to be solely from diet alone. First, our colonization result suggests that a small number of *B. subtilis* cells can colonize young larvae, and that adults harbor a greater abundance of cells after heat shock. Therefore, *B. subtilis* can survive the larval grinder and take up residence in the nematode, as well as persist in adults after heat shock. Because early exposure to *B. subtilis* resulted in the greatest number of offspring, we hypothesize that early exposure allowed more *B. subtilis* to accumulate inside nematodes, thus leading to greater host fecundity after heat shock. Second, if the results were due to the effects of diet, we would expect that exposure to *B. subtilis* for an equal amount of time (Figure 3.6b, all treatments except for full exposure) should result in similar offspring output, whereas feeding solely on *B. subtilis* would lead to the highest benefit obtained. Further, since all host individuals were of the same genotype and were exposed to a homogenous lawn of *B. subtilis*, we would expect similar levels of nutrient acquisition among individuals both within and between treatment groups, thus resulting in approximately equivalent levels of fecundity between treatments and replicates. However, we observed substantial variance between replicates and significant differences between treatments (Figure 3.6). Therefore, the results are more likely due to *B. subtilis* colonization than nutrient acquisition via *B. subtilis* digestion. This variance is also consistent with previous work examining bacterial growth in *C. elegans*, where stochasticity has a significant effect on bacterial abundance (Vega

and Gore, 2017). Finally, a recent study has shown that *B. subtilis* extends *C. elegans* lifespan post-heat shock through the production of biofilm (Donato et al., 2017), demonstrating that live cells are present in and actively colonizing the host gut. Taken together, these results suggest that the fitness benefits conferred by *B. subtilis* post-heat shock is likely largely due to host-microbe interactions within the host.

Our study demonstrates that interacting with the appropriate microbe under stressful conditions can benefit hosts in terms of reproduction, which could have significant implications for host population growth and evolution in the long term. This could select for association with the microbe in future generations, leading to the potential for coevolution of the partners within the framework of a mutualistic symbiosis. Importantly, the mechanistic nature of the beneficial interaction between *C. elegans* and *B. subtilis* may not dictate the system's capacity for mutualistic coevolution. Studies have shown that certain bacteria may serve roles in *C. elegans* distinguishable from diet (Berg et al., 2016; Cabreiro and Gems, 2013; Dirksen et al., 2016; Gerbaba et al., 2017; Zhang et al., 2017). Because *B. subtilis* can survive within the host, the host and microbe have the potential for mutualistic coevolution when their fitness aligns. However, even if the fitness differences we observed are due to nutrients obtained via *B. subtilis* digestion and not due to the impact of having maintained association with live bacteria, then mutualistic coevolution is still possible. For example, leaf-cutter ants and the fungi they cultivate have been coevolving with each other for millions of years, even though the fungi serve primarily as the ant's food source (Schultz and Brady, 2008; Weber, 1966). Furthermore, as evident in extant models of symbiosis (Davitt et al., 2011; Heath and Tiffin, 2007; McMullen et al., 2017; Vorburger et al., 2013; Weldon et al., 2013), the *B. subtilis*-*C. elegans* interaction is likely context-dependent: fitness benefits are obtained optimally only under certain environments (e.g.,

heat stress), at a certain stage in the host's life cycle, and with the right microbe. While more work is necessary to determine the mechanism by which *B. subtilis* increases *C. elegans* fecundity after heat stress, our work provides further evidence for the critical role that bacteria can play in the evolution and ecology of their hosts.

Acknowledgements

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CHAPTER IV

ASSOCIATION WITH A NOVEL BENEFICIAL MICROBE FACILIATES HOST ADAPTATION TO A STRESSFUL ENVIRONMENT

Kim L. Hoang, Nicole M. Gerardo, and Levi T. Morran
In revision

Abstract
Beneficial symbionts can allow hosts to occupy otherwise uninhabitable niches. Despite the importance of symbionts in host evolution, we know little about how beneficial associations arise. Encountering a microbe that can improve host fitness in a stressful environment may favor persistent interactions with that microbe, potentially facilitating a long-term beneficial association. Here, we ask how evolving with a novel protective microbe affects host adaptation to stress. The bacterium *Bacillus subtilis,* compared to the standard *Caenorhabditis elegans* diet, *Escherichia coli*, protects *C. elegans* nematodes from heat shock by increasing host fecundity. In this study, we passaged nematodes on *B. subtilis* or *E. coli*, under heat stress or standard conditions for 20 host generations of selection. We found that hosts exhibited the greatest fitness increase when evolved with *B. subtilis* under stress compared to when evolved with *E. coli* or under standard conditions. Furthermore, despite not directly selecting for increased *B. subtilis* fitness, we found that hosts evolved to harbor more *B. subtilis*. Thus, our findings demonstrate that the context under which hosts evolve is important for the evolution of beneficial associations. Additionally, beneficial microbes can facilitate host adaptation to stress, which can subsequently benefit the microbe.

Introduction

Many eukaryotic organisms associate with beneficial microbes that help them occupy niches that would otherwise be inhospitable. Symbionts help their hosts by producing nutrients or protecting their hosts from enemies or harsh conditions (Douglas, 2014; Klepzig et al., 2009; Kukor and Martin, 1983; Oliver et al., 2013). Adaptation to these environments can lead to niche expansion and utilization of additional resources for the host, presumably resulting in fitness gains for individual hosts that are associated with symbionts compared to hosts without symbionts. Despite the important role symbionts have in shaping host evolution, we know little about how beneficial symbioses are initially established. Symbionts can provide hosts with additional traits or relax selection on genes that hosts may need in the absence of the symbiont, thus host-symbiont interactions can substantially alter the evolutionary trajectory of a host population. Indeed, long-term interactions with a symbiont can increase host dependency on the symbiont, such that removing the microbial partner becomes detrimental for the host (Bennett and Moran, 2015). Associating with symbionts could also impede host adaptation, such that hosts rely more on symbiont genes than their own genes to adapt (Martinez et al., 2016), which could be detrimental for hosts under certain contexts (Keeling and McCutcheon, 2017). Conversely, harboring a symbiont may provide hosts with enough time to adapt to the environment itself. Under this scenario, removing the symbiont will not affect host fitness after a period of host adaptation. Therefore, understanding the initial conditions and short-term evolutionary effects of host-symbiont association is critical for our overall understanding of mutualistic interactions.

In many established symbioses, the context of the interaction, such as abiotic conditions or presence of other organisms, is important in determining the nature of the association between the host and symbiont (Heath and Tiffin, 2007; Klepzig et al., 2009; Lowe et al., 2016; Oliver et

al., 2009). For example, the amount of benefits leguminous plants obtain from their rhizobial partners depends on the level of nitrogen in the environment (Heath and Tiffin, 2007). Therefore, it is also likely that the context is critical in the formation of a newly established host-microbe association. A beneficial symbiosis may evolve from a parasitic interaction, or from an initially beneficial interaction. In the latter case, an increase of fitness in one (commensalism) or both (mutualism) of the species involved might lead to continual association across generations, establishing a long-term beneficial symbiosis. Incipient hosts likely initially obtained their symbionts from the environment; even the ancestors of the most host-dependent microbes—intracellular symbionts—are predicted to have been free-living (Moran et al., 2008). As the microbe increases host fitness over time through continual association, it might evolve to benefit from the association, such as through increased growth within hosts or dispersal to new locations by hosts. These interactions would create more opportunities for a more intimate association to evolve, such as transmission of the microbe from the host to its own offspring. Over time, the microbe can lose genes necessary to be free-living (Fisher et al., 2017; Toft and Andersson, 2010), potentially resulting in an obligate symbiosis where both host and microbe need each other to survive and reproduce.

Our experimental system allows us to compare both the fitness of a eukaryotic host and its bacterial partner after multiple generations of interaction. Particularly, we can dictate the conditions of the initial host-microbe interaction and assess how those conditions impact the establishment of a novel beneficial association and the resulting short-term evolutionary trajectories of host populations. Furthermore, we use a model system where the host ingests, but does not completely digest, microbes as a way to establish an intimate association between the partners. We previously found that the bacterium *Bacillus subtilis,* compared to the nematode's

standard diet, *E. coli,* confers a fitness benefit to the nematode *Caenorhabditis elegans* under heat shock (Hoang et al., 2019). While the nematodes were stressed at a temperature that is normally detrimental in terms of both survival and reproduction (Aprison and Ruvinsky, 2014), *B. subtilis* is able to increase host fitness by increasing the number of offspring the host produces post-heat shock. Moreover, increased host reproduction is only observed under the stressful condition compared to the standard rearing temperature, indicating that the microbe-derived benefits are dependent on the environmental context. In addition to measuring host fitness, we can also measure bacterial fitness, as *B. subtilis* cells can survive inside the host both before and after heat shock. Leveraging these findings with the experimental tractability of both *C. elegans* and *B. subtilis*, we developed a host-microbe system with which to determine how nematode and bacterial fitness might increase through experimental evolution.

In this study we examine how the context under which the host evolves affects the fitness benefits it obtains from its microbial partner, thereby influencing the niches the host can occupy, and ultimately how these conditions shape the host's evolutionary trajectory. We experimentally evolved nematodes for 20 generations of selection under two different environmental treatments (heat stress and no heat stress), in the presence or absence of a non-evolving *B. subtilis*. To determine the effects of evolving with a novel and protective bacterium, we also evolved nematodes with a non-evolving *E. coli* under the two environmental conditions. As our nematodes had been maintained solely on *E. coli* in the laboratory, it is not a novel bacterium, nor is it beneficial to the host under heat shock conditions (Hoang et al., 2019). After experimental evolution we conducted fitness assays to measure host fecundity and *B. subtilis* colonization within hosts, allowing us to evaluate the influence of *B. subtilis* and the environment towards host adaptation.

Methods

Strains and media

We independently mutated four populations of *C. elegans* N2 using ethyl methane-sulfonate (catalog #M0880, Sigma-Aldrich, St. Louis, MO) following Morran et al. 2011, then combined and froze the four populations to establish a single ancestral host population, which we name LTM-EE1. *Bacillus subtilis* strain 168 and *E. coli* strain OP50 were used as bacterial food sources. For all experiments, we grew *B. subtilis* and *E. coli* on Nematode Growth Medium Lite (US Biological, Swampscott, MA) containing 2% glucose and 0.5mM arginine. For steps involving GFP-labeled OP50 (OP50-GFP), we grew the bacterium on NGM Lite.

Experimental evolution

Starting with the ancestral host population (composed of roughly 93.7% hermaphrodites and 6.3% males), we passaged the hosts for 40 generations under heat shock or no heat shock treatments, on either ancestral *B. subtilis* or *E. coli* (Figure 4.1). Populations in the heat shock treatment experienced heat shock every-other generation for a total of 20 generations of selection. We began the experiment by surface sterilizing the ancestral host eggs using an established alkaline hypochlorite protocol (Stiernagle, 2006) and putting roughly 700 larvae onto either *B. subtilis* (ten replicate populations) or *E. coli* (ten replicate populations). We kept them in an incubator at 20˚C. Once the nematodes reached adulthood (after three days), we heat shocked half of the *B. subtilis* plates and half of the *E. coli* plates at 34˚C for six hours, while the other half were left at 20˚C. The heat shocked plates were then left on the benchtop to cool down for 20 minutes, after which all nematodes were washed with M9 and transferred to OP50-GFP,

where they were kept at 20˚C to recover from the heat shock and produce offspring. We used OP50-GFP as the recovery bacterium because it is phenotypically different from both *E. coli* OP50 and *B. subtilis* 168, but is still relatively neutral with respect to its effects on *C. elegans.* Furthermore, movement through the OP50-GFP lawn should remove any bacteria that were on the surface of nematodes when they were transferred over. Four days after heat shock, once the offspring had reproduced themselves, we transferred roughly 700 of their larvae onto fresh *B. subtilis* or OP50 *E. coli* revived from glycerol stock. We heat shocked them three days later, thus starting the next passage. After ten generations of selection we froze each population, after which we thawed them again to resume the experiment. After 20 generations of selection we again froze each replicate population, then thawed them to conduct fecundity and colonization assays. We called populations that evolved in the presence of *B. subtilis "*B+ populations", and those with *E. coli "*B- populations". Likewise, populations evolving under heat stress are "H+ populations", and at the standard temperature "H- populations" (Figure 4.1).

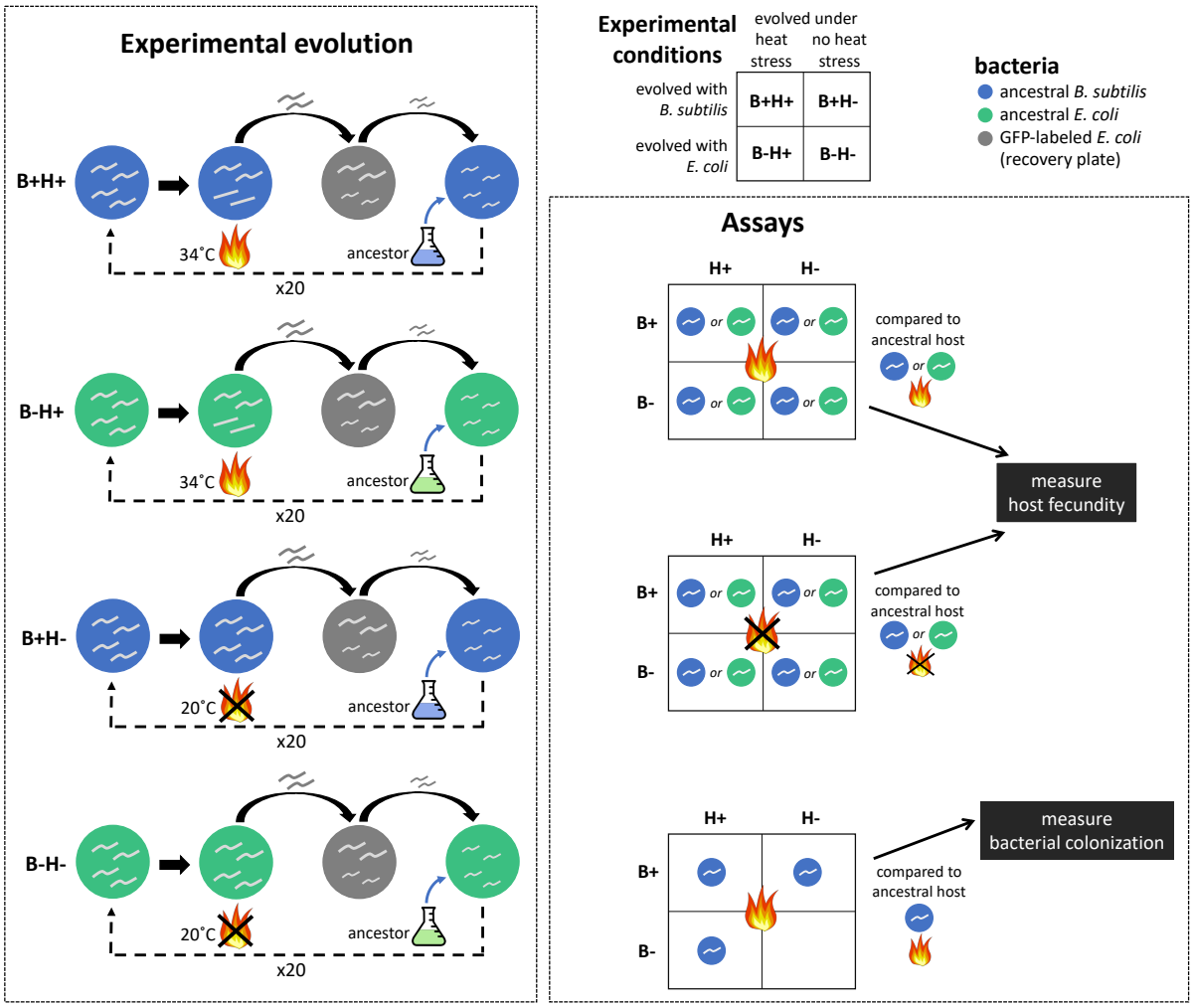

Figure 4.1. Schematic of experimental evolution and assays

Top right: Abbreviations for experimental evolution conditions

Left: nematodes were passaged on the ancestral B. subtilis (B+ hosts, blue) or on the ancestral E. coli (B- hosts, green), under heat shock (34˚C, H+) or no heat shock (20˚, H-) conditions, for 20 generations of selection (40 total generations). After each heat shock, hosts recovered on GFP-labeled E. coli (gray) to produce offspring. The offspring of these offspring were then placed on fresh plates of their respective bacteria to be heat shocked, starting the next generation. There were five replicate populations for each of the four treatments.

Right: To measure host fecundity, after 20 generations of selection, we reared hosts from each of the 20 replicate experimental populations and the ancestral population on either the ancestral B. subtilis or E. coli, then heat shocked them at 34˚C or left them at 20˚C, following the same schedule for one passage of experimental evolution. Two days after the heat shock, we measured the number of offspring per total number of initial adults.

To measure B. subtilis colonization, we heat shocked 15 replicate populations (excluding the five B-H- populations) on B. subtilis following the schedule for one passage of experiment evolution. Immediately after heat shock we washed and crushed nematodes and plated them on media to quantify CFUs in individual hosts.

Host fecundity

To determine host fitness changes that occurred, we quantified the number of offspring produced by nematodes after 20 generations of selection and by the ancestral host population (Figure 4.1). We followed the schedule for one passage of experimental evolution, as described above, for each of the evolved replicate populations and the ancestor, heat shocking about 100 – 200 nematodes on either *B. subtilis* 168 or *E. coli* OP50. After heat shock, nematodes were transferred to OP50-GFP and kept at 20˚C. Two days later we determined the number of offspring produced per heat shocked adult, which is influenced both by survival and fecundity of the surviving individuals. For each replicate population we heat shocked three replicate plates; for the ancestor we heat shocked five replicate plates, for a total of 130 plates (4 evolved treatments x 5 replicate populations x 2 bacteria x 3 replicate plates) + (1 ancestor x 2 bacteria x 5 replicate plates). We also quantified nematode fitness via fecundity assays at generation 20 when not heat shocked, following the same procedure as the heat shock assay but keeping nematodes at 20˚C throughout. To gain insight into when changes occurred in the host lineages, we similarly surveyed fecundity of the host populations after 10 generations of selection, focusing on two treatments where hosts evolved under heat shock with *B. subtilis* or *E. coli*.

Bacterial colonization

To determine bacterial abundance within nematodes, we grew the populations evolved for 20 generations of selection (excluding the five B-H- populations because they were neither exposed to *B. subtilis* nor heat shock during their evolution) and the ancestral population on *B. subtilis* for three days, then heat shocked them for six hours at 34˚C. After heat shock, we crushed individual nematodes following a previously established protocol to determine *B. subtilis* abundance (Vega

and Gore, 2017). Briefly, we washed nematodes off their heat shocked plates with M9 into 1.5ml Eppendorf tubes. We then washed them three times with cold 0.01% Triton-X 100 in M9, then incubated them at 4˚C for 15 minutes. Afterwards we soaked them in 1:1000 diluted bleach for 15 minutes at 4˚C to further remove surface bacteria. We subsequently incubated them in 0.25% sodium dodecyl sulfate (SDS) + 3% dithiothreitol (DTT) for 20 minutes, then transferred nematodes to a 96-well plate containing a small amount of sterile silicon carbide grit and 0.01% Triton X-100 in M9. We then briefly disrupted the samples using a Qiagen TissueLyser II homogenizer and plated out the samples onto LB plates, quantifying the number of colony forming units (CFUs) two days later. We heat shocked one plate for each replicate population, and three replicate plates for the ancestor. From each population we crushed ten individuals separately to quantify abundance of *B. subtilis* within a single nematode and the frequency at which each host was colonized. In total, we quantified bacterial abundance for 180 individuals (3 evolved treatments x 5 replicate populations x 10 individuals) + (1 ancestor x 3 populations x 10 individuals).

Statistical analysis

We tested impact of the bacteria on which hosts were assayed (*B. subtilis* or *E. coli*) on host fecundity using a generalized linear model (GLM) with Poisson distribution and log link function using maximum likelihood estimation. Within each bacterium hosts were assayed on, we used an analysis of variance (ANOVA) with a Bonferroni correction for multiple testing to test the main effects of evolutionary treatment (B+H+, B-H+, B+H-, B-H-), run, and treatment by run interaction. Treatment was treated as a fixed effect and run as a random effect. We confirmed that the data conformed to the assumptions of an ANOVA by implementing the

Shapiro-Wilk test to check for normality and the Levene's test to assess homogeneity of variance. We then performed Student's t-tests to compare means between treatments. Figure S4.1 shows the distribution of all individual data points for Figure 4.2. We used the total fecundity means (i.e., the mean of all replicate populations from each experimental evolution treatment) for both analyses.

To analyze the CFU abundance per host data, we utilized a negative binomial regression implemented using maximum likelihood estimation on the total CFU means. We tested the main effects of evolutionary treatment (B+H+, B-H+, B+H-), run, and treatment by run interaction. We then performed Student's t-tests to compare means between treatments. For the frequency of colonized hosts data, we used a GLM with a binomial distribution and logit link function implemented using Firth adjusted maximum likelihood. We tested the main effects of evolutionary treatment (B+H+, B-H+, B+H), run, replicate population nested within evolutionary treatment, treatment by run interaction, and population nested within treatment by run interaction. Replicate population was incorporated into this analysis due to the variation across replicate populations within treatments. We then performed contrast tests to compare means between treatments. We did not include the ancestral host directly in our analyses because it did not have replicate populations. To determine the association between host fecundity and *B. subtilis* colonization, we fit the average fecundity by average CFU with a linear fit. All analyses were conducted on JMP Pro 13.

Results

Host fecundity

We determined whether hosts evolved under different conditions exhibited differences in fecundity. By assaying hosts in the conditions under which they evolved, in addition to conditions that other host treatments experienced, we were able to distinguish host adaptation to heat stress versus the evolution of greater benefits derived from *B. subtilis* association.

When we surveyed host fitness after generation ten, there was no significant difference between B+H+ and B-H+ hosts when heat shocked on *B. subtilis* (Student's t-test $p = 0.11$; Table S4.1) or *E. coli* (Student's t-test $p = 0.97$; Table S4.2) (Figure S4.2). After generation 20, however, we found that when hosts were heat shocked on *B. subtilis*, they produced more offspring compared to *E. coli* overall ($\chi_1^2 = 14.30$, $p < 0.001$; Table S4.3). Across hosts that were assayed on *B. subtilis*, nematodes that evolved with *B. subtilis* under heat stress (B+H+) exhibited the highest fecundity (Figure 4.2a; Student's t-test $p = 0.0036$; Table S4.4). Compared to B+H- hosts, this result suggests that evolution with the beneficial bacterium is not sufficient for host adaptation; rather, the stressful environment plays a key role in the increased fecundity of B+H+ hosts. Similarly, B-H+ hosts did not adapt as well as B+H+ hosts, suggesting that solely evolving under heat stress is not enough—evolution with the protective microbe is also necessary. To determine the extent to which the increased fecundity of these hosts was driven by the nematodes themselves, we also heat shocked all hosts on *E. coli.* We did not find a significant difference between hosts (Figure 4.2b, Student's t-test $p = 0.19$; Table S4.5), indicating that in the absence of *B. subtilis* after 20 generations of selection, hosts that evolved with *B. subtilis* performed similarly to hosts that evolved with *E. coli*, regardless of the environmental condition they evolved under. Furthermore, since B+H+ hosts did not exhibit a reproductive increase on *E. coli*, association with the protective microbe during the heat shock event is also critical for host fitness. These results demonstrate that exposure to both the

beneficial bacterium (during evolution and during heat shock) and the stressful environment together were necessary to facilitate increased levels of host adaptation to the heat shock.

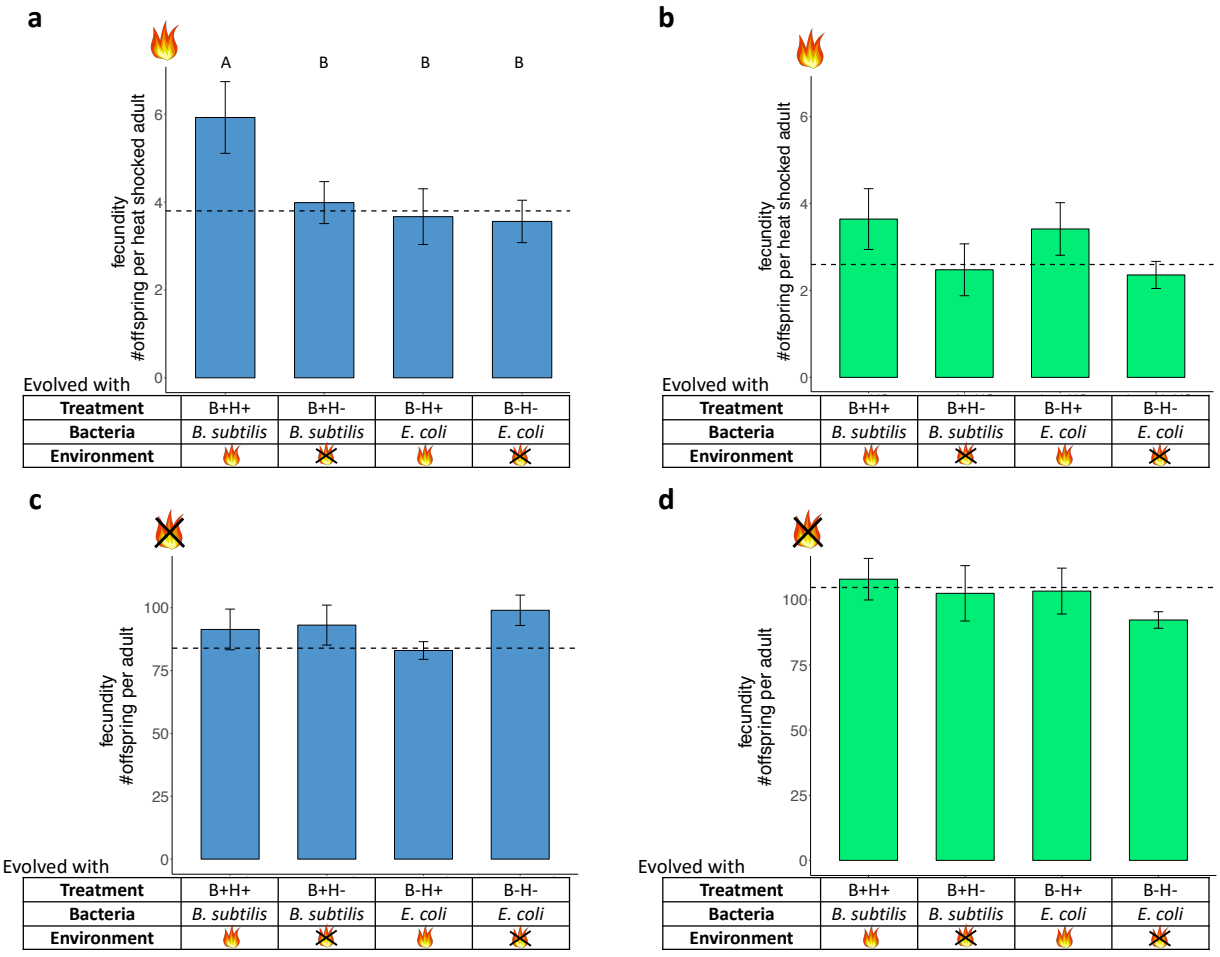

Figure 4.2. Fecundity of evolved hosts after 20 generations of selection

The x-axis indicates the condition under which nematodes evolved. (Top) Nematodes from the four experimental treatments were heat shocked at 34˚C on ***a)*** *B. subtilis or* ***b)*** *E. coli. Each plate contained roughly 200 nematodes. The data is combined across three runs. (Bottom) Nematodes from the four experimental treatments were kept at 20˚C on* ***c)*** *B. subtilis or* ***d)*** *E. coli. Each plate contained roughly 150 nematodes. The dashed line indicates the average value for the ancestral host. Error bars indicate the standard errors. Treatments that are not the same letter are significantly different.*

In parallel assays without heat shock after generation 20, we found that bacteria did not have a significant effect on reproduction (Figures 4.2c/4.2d, $F_{1,38} = 3.70$, $p = 0.062$; Table S4.6). Hosts produced similar quantities of offspring on *B. subtilis* compared to *E. coli*. Moreover, there

were no significant differences in offspring production across hosts reared on either *B. subtilis* ($F_{3,16}$ = 0.98, p = 0.43; Table S4.7) or *E. coli* ($F_{3,16}$, = 0.66, p = 0.59; Table S4.8). Despite nematodes evolving the greatest overall fecundity on *B. subtilis* under heat stress, they did not gain a similar proportional increase in fitness when the heat shock was removed at generation 20. Thus, the benefits of evolving in the presence of *B. subtilis* were limited to the heat shock environment.

Bacterial colonization

To determine whether differences in host fitness are associated with changes in *B. subtilis* colonization, we quantified *B. subtilis* abundance and the frequency of colonization in the evolved hosts when heat shocked after generation 20. We found significant treatment differences in the number of CFUs (Wald χ^2_2= 8.73, p = 0.013) and in the proportion of nematodes harboring *B. subtilis* (χ^2_{59}= 91.37, p = 0.0044) (Figure 4.3, Tables S4.9 and S4.10). Specifically, B+H+ hosts harbored more CFUs than B+H- (Student's t-test p < 0.001) and B-H+ (Student's t-test p = 0.0013) hosts, and had a greater proportion of nematodes harboring the bacterium (Figure 4.3b, χ^2_1 = 6.40, p = 0.011; χ^2_1 = 7.52, p = 0.0061, respectively). We then plotted host fecundity against *B. subtilis* CFUs (Figure 4.4). Overall, we found that increased *B. subtilis* abundance is positively correlated with increased host reproduction (R^2 = 0.34; $F_{1,14}$ = 7.23, p = 0.018; Table S4.11), where B+H+ hosts had the highest fecundity and *B. subtilis* abundance compared to the ancestor and other evolved hosts when heat shocked after generation 20.

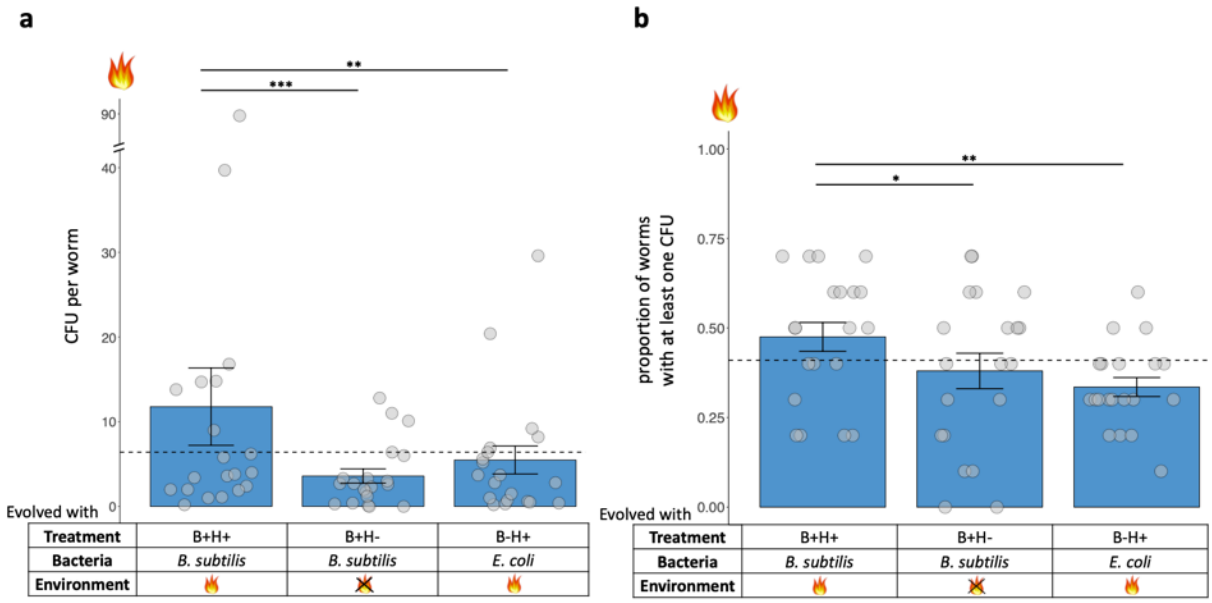

Figure 4.3. *B. subtilis* colonization in evolved hosts

Evolved nematodes were heat shocked on B. subtilis, washed, and individually crushed to quantify within-host bacterial colonization. The x-axis indicates the condition under which nematodes evolved. ***a)*** *the number of colony forming units (CFUs) in each nematode.* ***b)*** *the proportion of nematodes harboring at least one CFU. Each data point is the average of ten nematodes from each replicate population from experimental evolution. The data is combined across four runs. The dashed line indicates the average value for the ancestral host. Error bars indicate the standard errors. ***p < 0.001, **p<0.01, *p<0.05*

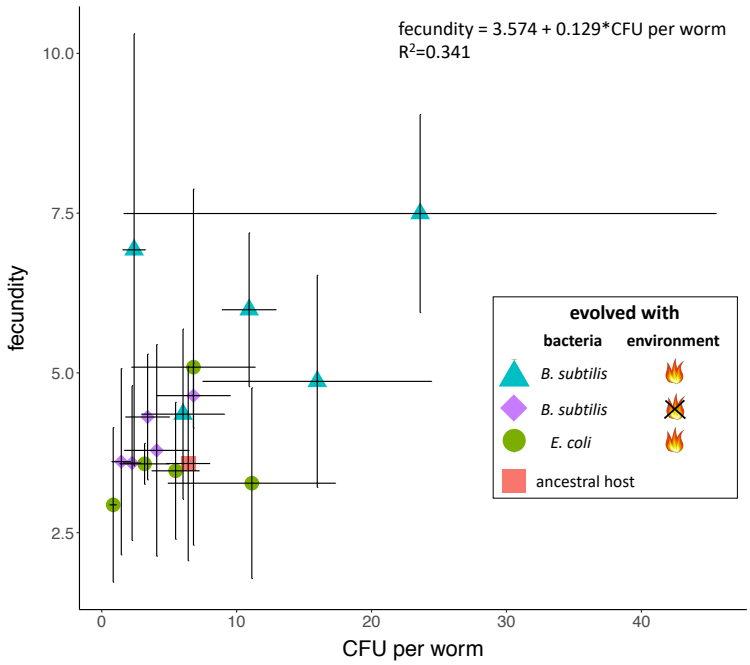

Figure 4.4. Host fecundity vs. *B. subtilis* colonization when heat shocked after generation 20

Fecundity is plotted against CFUs per host. Error bars indicate standard errors.

Discussion

Beneficial symbioses are widespread across all domains of life, but we know little about how they initially evolved (Hoang et al., 2016). Work on extant symbioses has shown that context-dependency plays a large part in the maintenance and exchange of benefits between hosts and symbionts (Heath and Tiffin, 2007; Keeling and McCutcheon, 2017; Weldon et al., 2013). Facilitation of host adaptation to a new or stressful environment by a microbe may be a way in which novel associations begin, where the symbiont provides its host with abilities it previously lacked (Douglas, 2014). In this study, we examined how evolution in the presence of a beneficial bacterium affected host adaptation to a stressful environment. In accordance with previous results, we found that *B. subtilis* conferred greater host fecundity under heat shock relative to *E. coli* (Figures 4.2a vs. 4.2b). Conversely, in the absence of environmental stress, evolved nematodes produced a similar number of offspring on *B. subtilis* compared to *E. coli* (Figures 4.2c vs. 4.2d). Importantly, we found that nematodes that evolved in the presence of a protective bacterium (B+H+ hosts) exhibited the greatest increase in post-heat shock fecundity after generation 20 when exposed to the beneficial microbe (Figure 4.2a). While nematodes did not adapt to heat shock in the absence of the beneficial bacteria, evolving in the presence of *B. subtilis* led to rapid adaptation under heat shock. Further, because hosts did not show signs of improved fitness at generation ten (Figure S4.2), increased fecundity at generation 20 provides evidence for host evolution instead of transgenerational effects, as would be suggested from past studies on *C. elegans* subjected to high temperatures (Klosin et al., 2017). B+H- hosts that evolved in the presence of *B. subtilis*, but in the absence of heat shock, did not exhibit increased fecundity under heat shock (Figure 4.2a). Thus, the presence of both the beneficial bacteria and the stressful environment were critical for facilitating host adaptation. Additionally, the

adaptation exhibited by the B+H+ hosts was context-dependent, as B+H+ host populations only exhibited relative increases in fecundity when paired with *B. subtilis* (Figures 4.2a vs. 4.2b). Therefore, adaptation was not solely driven by an overall increase in heat tolerance on the part of the host, but rather by the host's utilization of the protective bacteria. Indeed, the presence of a protective bacteria in a stressful environment altered the evolutionary trajectories of host populations, ultimately facilitating host adaptation.

Interestingly, the fitness benefits of microbial-facilitated host adaptation were not restricted to only the host populations. While we did not select for increased *B. subtilis* colonization throughout our experiment, we found that hosts evolving with *B. subtilis* under heat shock (B+H+) allowed increased within-host *B. subtilis* growth and a greater propensity to be colonized by *B. subtilis* (Figure 4.3). Therefore, both the host and microbe benefited from host adaptation, despite the fact that *B. subtilis* did not evolve during the experiment. This suggests that the microbial partner can directly benefit from host evolution, even without evolving itself. While changes to host or microbial fitness may often be influenced heavily by microbial evolution (Ford et al., 2016; King et al., 2016), our study presents a case in which changes in the host affected both its own fitness and that of its microbe. These data indicate that initial host evolution may play a critical role in the establishment of novel beneficial associations between hosts and microbes. We hypothesize there could be several mechanisms involved in the greater number of CFUs in evolved hosts, such as from nematodes eating more, allowing more live bacteria to pass through the nematode grinder, or suppressing microbial regulation in the gut. Regardless of the underlying mechanism, we found live *B. subtilis* inside hosts after heat shock. Any live bacteria to reach the host gut have the potential to colonize or survive passage through the gut and excretion. A greater propensity for hosts to harbor live microbes could have

substantial long-term benefits for bacteria. If host adaptation permits greater bacterial colonization, essentially creating a novel niche for the microbe, then selection could favor host-associated microbes and result in greater microbial fitness. However, colonization is not necessary for the bacteria to derive host-associated benefits. Indeed, hosts are typically more mobile than microbes, and association with the host could facilitate microbial dispersal when microbes exit their host and proliferate in the external environment (Brock et al., 2011; Lee and Ruby, 1994; Thutupalli et al., 2017).

Despite observing conditional adaptation of the B+H+ hosts and increased microbial fitness within B+H+ hosts, we found substantial variation in host fecundity and bacterial colonization both across and within host populations (Figures 4.2 and 4.3; Figure S4.1). This variation may be a product of the host, the beneficial microbe, or their interaction. Variance due to the host may have resulted from substantial levels of standing genetic variation in our ancestral host population that may have been maintained over the course of the experiment. However, microbial establishment likely also plays a role in the manifestation of fecundity and CFU variance. Variation in *B. subtilis* colonization is consistent with previous research in within-host bacterial growth in *C. elegans*, where stochasticity is an important factor in determining microbial community composition between individual hosts, even when those hosts are genetically identical (Vega and Gore, 2017). As a whole, we observed substantial variation in host populations that exhibited the greatest increase in reproduction and *B. subtilis* colonization (i.e., B+H+ hosts). Portions of these populations have evolved the ability to maximize their fitness under heat stress with the aid of *B. subtilis*, but these traits have not fixed in the population. Conversely, traits permitting hosts to derive greater benefits from *B. subtilis* colonization may have swept through the host population, but stochasticity in bacterial

colonization generated variance in fecundity. Nonetheless, the amount of variation present may diminish if these populations were to continue evolving as favorable host traits sweep to fixation or hosts evolve even greater propensity for *B. subtilis* colonization.

Even though the host ingests bacteria in our study system, there are several lines of evidence supporting the role of *B. subtilis* as a model for an incipient symbiont. First, symbionts need not be distinguished from food. In many extant symbioses, symbionts serve primarily as the host's food source (e.g., fungus-farming insects (Aanen et al., 2002; Menezes et al., 2015; Six, 2012; Weber, 1966)). Second, in other symbioses, symbionts that are food also serve other important roles (Forst et al., 1997; Jäckle et al., 2019; Kodama and Fujishima, 2008; Kukor and Martin, 1983; Lindquist et al., 2005). Third, while the majority of consumed *B. subtilis* are digested by the host, live *B. subtilis* colonize the host gut after consumption (Donato et al., 2017). Therefore, like known established symbionts, *B. subtilis* has the potential to serve dual roles in the association with its host. It is also possible that a long-term association can evolve despite a high turnover of bacteria within the host. For example, in the bobtail squid-*Vibrio fischeri* association, the host squid expels 95% of its bacteria each day, yet this is still considered a canonical, protective symbiosis that has shaped host evolution (McFall-Ngai, 2014; Ruby, 1996). One primary result of our study is that *B. subtilis*' status as a food source and/or an incipient protective symbiotic bacterium is malleable. What is solely food in one environment, can be food and a protective, beneficial microbe in another.

Our study sheds light into the conditions under which novel beneficial associations may arise. We examine how a host-microbe interaction evolves when the association is initially beneficial for the host under stress. We find that associating with the bacterium led to hosts gaining a fitness advantage under heat stress, allowing them to occupy a hostile environment

better than their counterparts that evolved without the protective partner. Our findings illustrate that evolution with a novel protective microbe can facilitate rapid adaptation to a stressful environment. While associating with beneficial microbes may constrain the evolutionary trajectories of some host populations (Martinez et al., 2016), our study demonstrates that early beneficial interactions between hosts and microbes do not uniformly constrain host evolution. Rather, association with the beneficial microbe facilitated the adaptation of hosts without decreasing host fitness in the absence of the microbe (Figures 4.2a and 4.2b). However, such costs may yet evolve over time. Eventually, increased fitness from associating with the microbe may lead to increased dependency, further reinforcing interactions between host and microbe. If the microbe eventually gains more benefits within the host, or through association with the host (see above), than the external environment, it would be more advantageous for the microbe to associate with the host. Increased microbial fitness within hosts can evolve over time because the host can provide its symbiont with a more optimal environment than the external environment, such as nutrient availability and fewer or no competitors (Bozonnet et al., 2017; Davidson et al., 2004; Wilson et al., 2010). Once the partners evolve to depend on one another, hosts and microbes that associate with one another gain a fitness advantage over those that do not associate with a partner. These short-term evolutionary effects of the interaction may then facilitate reciprocal adaptation and coevolution. Ultimately, these conditions may provide important stepping stones towards the evolution of obligate dependency and long-term beneficial symbioses.

Acknowledgments

Nematode strain N2 Bristol and *E. coli* strains OP50 and OP50-GFP were provided by the Caenorhabditis Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). We thank I. Gusarov for providing the *B. subtilis* 168 strain, Nic Vega and Megan Taylor for help with the bacterial colonization protocol, and Kayla Stoy for help with experimental procedures. We are grateful for thoughtful discussions with Tim Read, Daniel Weissman, and Will Ratcliff. This material is based upon work supported by the NSF Graduate Research Fellowship Program under Grant No. DGE-1444932 to KLH.

Supplemental information

Table S4.1. ANOVA table for fecundity of hosts heat shocked on *B. subtilis* after ten generations of selection

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	1.26	0.42	1.25
Error	12	4.02	0.33	**Prob > F**
C. Total	15	5.28		0.33

Table S4.2. ANOVA table for fecundity of hosts heat shocked on *E. coli* after ten generations of selection

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	0.64	0.21	3.55
Error	12	0.72	0.06	**Prob > F**
C. Total	15	1.35		0.05

Table S4.3. Summary of statistics for fecundity of hosts heat shocked on *B. subtilis* vs. *E. coli* after 20 generations of selection

Model	-LogLikelihood	L-R ChiSquare	DF	Prob>ChiSq
Difference	55.54	111.08	5	<0.001
Full	218.24			
Reduced	273.78			

Table S4.4. ANOVA table for fecundity of hosts heat shocked on *B. subtilis* after 20 generations of selection

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	242.30	22.03	7.87
Error	48	134.37	2.80	**Prob > F**
C. Total	59	376.67		<0.001

Table S4.5. ANOVA table for fecundity of hosts heat shocked on *E. coli* after 20 generations of selection

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	156.93	14.27	5.00
Error	48	136.92	2.85	**Prob > F**
C. Total	59	293.85		<0.001

Table S4.6. ANOVA table for fecundity of hosts reared at standard conditions on *B. subtilis* vs. *E. coli* after 20 generations of selection

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	**1**	987.21	987.21	3.70
Error	**38**	10152.91	267.18	**Prob > F**
C. Total	**39**	11140.12		0.06

Table S4.7. ANOVA table for fecundity of hosts reared at standard conditions on *B. subtilis* after 20 generations of selection

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	655.05	218.35	0.98
Error	16	3552.22	222.01	**Prob > F**
C. Total	19	4207.27		0.43

Table S4.8. ANOVA table for fecundity of hosts reared at standard conditions on *E. coli* after 20 generations of selection

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	655.79	218.60	0.66
Error	16	5289.85	330.62	**Prob > F**
C. Total	19	5945.64		0.59

Table S4.9. Summary of statistics for *B. subtilis* colonization (CFU)

Source	Nparm	DF	Wald Chi-Square	Prob > ChiSquare
treatment	2	2	8.73	0.01
run	3	3	7.40	0.06
treatment*run	6	6	9.33	0.16

Table S4.10. Summary of statistics for *B. subtilis* colonization (proportion)

	Chi-square	DF	Prob>ChiSq
Whole model test	91.37	59	0.004
Effect tests			
population[treatment]	12.19	12	0.43
run	8.17	3	0.04
treatment	9.45	2	0.009
run*treatment	18.33	6	0.006
population*run[treatment]	50.72	36	0.05

Table S4.11. ANOVA table for host fecundity vs. *B. subtilis* CFU

Source	DF	Sum of squares	Mean square	F Ratio
Model	1	9.01	9.01	7.23
Error	14	17.45	1.25	**Prob > F**
C. Total	15	26.46		0.02

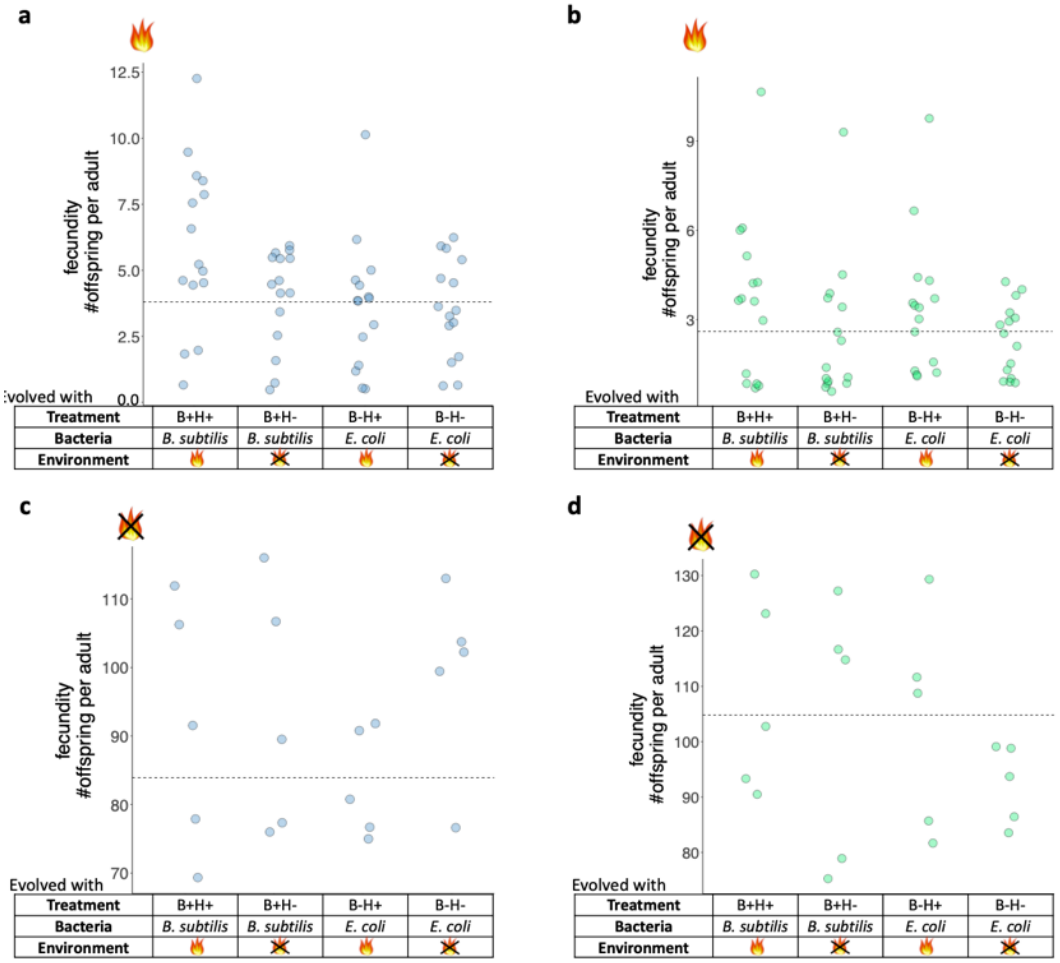

Figure S4.1. Distribution of data points for fecundity means of evolved hosts in Figure 4.2
Each data point is the average of three replicate plates for each of the five replicate populations from experimental evolution. The x-axis indicates the condition under which nematodes evolved. (Top) Nematodes from the four experimental treatments were heat shocked at 34˚C on ***a)*** *B. subtilis or* ***b)*** *E. coli. Each plate contained roughly 200 nematodes. The data is combined across three runs. (Bottom) Nematodes from the four experimental treatments were kept at 20˚C on* ***c)*** *B. subtilis or* ***d)*** *E. coli. Each plate contained roughly 150 nematodes. The dashed line indicates the average value for the ancestral host.*

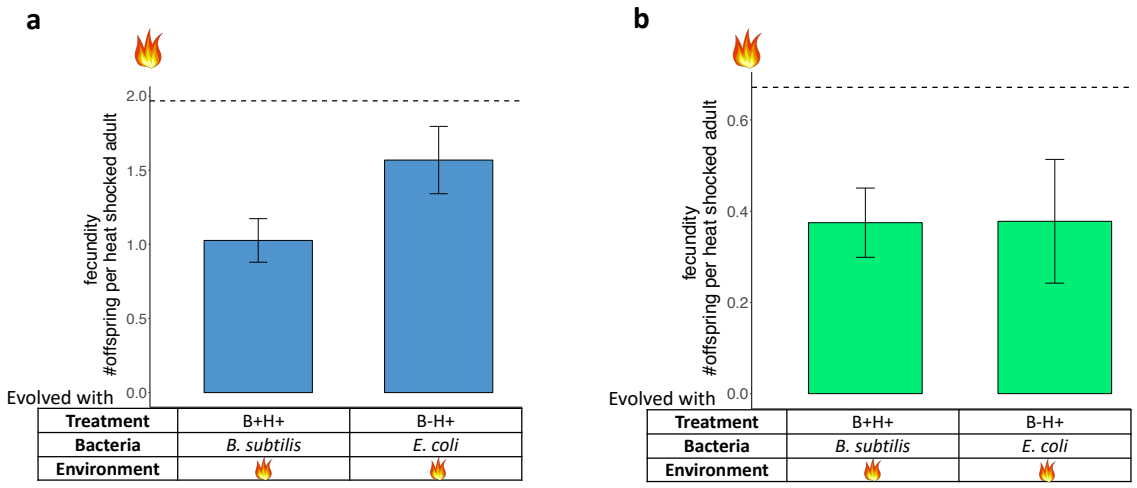

Figure S4.2. Fecundity means of evolved hosts at generation ten

The x-axis indicates the condition under which hosts evolved. To survey hosts after generation ten, nematodes were heat shocked at 34˚C on ***a)*** *B. subtilis or* ***b)*** *E. coli. Each plate contained roughly 200 nematodes. The data is combined across two runs. The dashed line indicates the average value for the ancestral host. Error bars indicate the standard errors.*

CHAPTER V

THE EFFECTS OF MICROBIAL EVOLUTION AND COEVOLUTION ON HOST FITNESS

Kim L. Hoang, Heidi Choi, Nicole M. Gerardo, and Levi T. Morran

Abstract

Different evolutionary histories may influence how microbes interact with their present hosts, which can subsequently affect host fitness. Microbial passage through non-evolving hosts has been shown to influence the evolved microbes' impact on host fitness with both positive and negative consequences. However, a host that is co-passaged with the evolving microbe can alter the evolutionary trajectory of the microbe in a manner that a non-evolving host cannot. Here, we examine how the evolutionary history of a beneficial microbe affects host fitness in terms of survival and number of progenies produced. We previously found that the bacterium *Bacillus subtilis* protects the nematode *Caenorhabditis elegans* from heat shock by increasing fecundity. In this study, we passaged *B. subtilis* through ancestral *C. elegans*, on nematode growth media in the absence of the host, or through a co-passaged host 20 times under heat shock conditions. While the bacteria that evolved in the absence of host evolution improved ancestral host fecundity, we also found a similar improvement from bacteria that were passaged on media, indicating that host adaptation was not necessarily needed for the increased benefits. In addition, we found that co-passaged bacteria tended to reduce fecundity of the ancestral host. We then asked whether the co-passaged bacteria were only beneficial when paired with their co-passaged hosts. Contrary to our prediction, we found that co-passaged hosts exhibited reduced fecundity with their co-passaged bacteria compared to when heat shocked with the ancestral bacteria. Taken together, our results indicate that co-passage resulted in evolution towards reduced benefits, suggesting that coevolution may impede the establishment of novel beneficial host-microbe associations.

Introduction

Many organisms obtain their beneficial symbionts from the environment, which can facilitate the uptake of new microbes. Acquisition of novel microbes can extend host niche and/or provide hosts with additional traits. Evolutionary history with the host may significantly impact the effects of the microbe on the host. Microbes adapted to the external environment, for example, would likely have different effects on a host compared to those adapted to a host. Of note, free-living microbes tend to harbor more genetic diversity and larger genomes than their host-associated relatives (McCutcheon and Moran, 2011; Toft and Andersson, 2010). Passage between hosts, such as from parent to offspring, can greatly reduce variation in the microbial population (Bennett and Moran, 2015). Thus, microbes that have previously adapted to the environment, to a host, or to both, can follow drastically different evolutionary trajectories. As a result, host fitness is altered from interactions with these microbes.

Host-microbe associations exist on a continuum between mutualism and parasitism. Previous work across plant and animal models has shown that microbial passage through hosts can lead to increased microbial and host fitness, shifting the interaction toward mutualism (Barroso-Batista et al., 2014; Burghardt et al., 2018; Mikonranta et al., 2015; Robinson et al., 2018). For example, passage of *Enterococcus faecalis* through the nematode *Caenorhabditis elegans* increased its protective effects against the pathogen *Staphylococcus aureus*, but only in the presence of the pathogen (King et al., 2016). In another study, pathogenic *Serratia marcescens* evolved reduced virulence when passaged through *Drosophila melanogaster* compared to when it was passaged outside the host (Mikonranta et al., 2015). In rhizobia-legume associations, traits involved in host adaptation, such as the ability to induce less plant defense (Marchetti et al., 2014), evolved after passage through the host (Guan et al., 2013; Marchetti et

al., 2010). Some hosts are found to exert stronger selection than the external environment (Burghardt et al., 2018; Morran et al., 2016). By contrast, the microbe could also evolve increased harm towards the host (Le Clec'h et al., 2017; Matthews et al., 2019; Sachs and Wilcox, 2006). For example, horizontal transmission has been shown to facilitate evolution towards parasitism in the jellyfish symbiont *Symbiodinium microadriaticum* (Sachs and Wilcox, 2006). Thus, microbial passage through hosts can have significant consequences on how host-microbe associations evolve. However, host evolution is seldom static in nature. What would happen to host fitness if hosts evolved alongside their beneficial microbes, such that the partners have the potential to coevolve?

In extant symbioses, coevolution can lead to specificity between the host and its symbiont (Murfin et al., 2015; Parker et al., 2017). Previous research has demonstrated reduced antagonism when hosts and parasites coevolve (Gibson et al., 2015), and even increased benefits for both partners (Rafaluk-Mohr et al., 2018). However, despite having a net fitness benefit, interactions between symbionts and hosts can be viewed as antagonistic, such that the partners are locked in an evolutionary arms race (Bennett and Moran, 2015; Keeling and McCutcheon, 2017; Sachs et al., 2011). Indeed, studies have shown that hosts and symbionts often compete with one another, acting on their own interests and not necessarily their partner's. For example, the protist *Paramecium bursaria* has been shown to exploit its algal symbiont by controlling the algae's population size (Lowe et al., 2016). Conversely, the bacterial symbionts, *Buchnera aphidicola* and *Hamiltonella defensa*, of pea aphids can grow to high titers within their host; such over-proliferation is correlated with decreasing host fitness (Chong and Moran, 2016; Weldon et al., 2013). Ultimately, coevolution may be a driving force behind the evolution of beneficial associations because it can create genotypes that fit well together (Guimarães et al.,

2011), but the role of coevolution versus single-partner evolution in the establishment of novel beneficial associations remains unclear.

We previously developed a model system to examine the evolution of novel beneficial host-microbe associations using the bacterium *Bacillus subtilis* and the nematode *Caenorhabditis elegans.* We evolved the nematodes in the presence or absence of non-evolving *B. subtilis* for 20 generations of selection under heat stress or no heat stress. Hosts that evolved with *B. subtilis* under heat stress had the greatest fecundity and harbored the most *B. subtilis* colonies (Hoang et al., *in review*). Since both host and bacteria benefited from changes that evolved solely in the host, in the current study we assess the impact of evolution of a protective microbe in a non-evolving host. We passage *B. subtilis* through *C. elegans* host populations under heat stress conditions, then measure host fitness at the end of the experiment. We also examine the impact of host and bacteria co-passaged, in which the host has the potential to reciprocally respond to the evolving bacteria. We compare fitness of the ancestral host when heat shocked with the coevolved bacteria versus the singly-passaged bacteria. Finally, we evaluate the fitness of co-passaged hosts against singly-passaged hosts from our previous experiment to determine whether coevolution might facilitate the evolution of increased fitness benefits.

Methods

Strains and media

Bacillus subtilis strain 168 was used as the starting bacterial strain for experimental evolution. All evolved bacterial populations came from one ancestral *B. subtilis* 168 colony. The ancestral host was the same population used in our previous study, LTM-EE1 (Hoang et al. 2019, *in review*), which composed of roughly 93.7% hermaphrodites and 6.3% males. For all

experiments, we grew *B. subtilis* on Nematode Growth Medium Lite (US Biological, Swampscott, MA) containing 2% glucose and 0.5mM arginine (NGMga). We grew GFP-labeled OP50 (OP50-GFP) on NGM Lite.

Experimental evolution

Microbial evolution treatments

We surface sterilized the ancestral host eggs using an established alkaline hypochlorite protocol (Stiernagle, 2006) and put roughly 500 – 700 larvae onto *B. subtilis*, kept at 20˚C. Once the nematodes reached adulthood (after three days), we heat shocked nematodes at 34˚C for six hours. The plates were then left on the benchtop to cool down for 20 minutes, after which all nematodes were washed with M9 and transferred to OP50-GFP, where they were kept at 20˚C until the following day. We picked a maximum of 40 live nematodes to crush (there were not 40 nematodes that survived to the next day on occasion) and plate onto LB containing 10ug/ml streptomycin to limit OP50-GFP growth. After incubation at 28˚C, we inoculated at most 20 colonies into LB broth. We then plated 100ul of the overnight culture onto NGMga, then put roughly 500 – 700 larvae of the ancestral nematode onto the overnight bacterial lawn. We heat shocked them three days later, thus starting the next passage. After ten passages we froze each population, after which we revived them again to resume the experiment. After 20 passages we froze each replicate population, then thawed them to conduct host fitness assays. We called this treatment "singly-passaged bacteria". We also had a treatment where *B. subtilis* was passaged in the absence of nematodes ("*in vitro*" treatment), following a similar protocol as the singly-passaged treatment. Because there were no nematodes in this treatment, we dabbed the inoculation pick 40 times on the bacterial lawn to simulate picking 40 nematodes. Note that even

though 34˚C is within the growth range of *B. subtilis*, we called these treatments "heat shocked" because we are referring to the stress hosts experience at this temperature. In addition, we also passaged the bacteria *in vitro* where we kept them at 20˚C instead of moving them to 34˚C for six hours ("*in vitro* 20˚C" treatment; for the rest of the manuscript, "*in vitro*" refers to the 34˚C treatment unless indicated otherwise). All three treatments had five replicate populations each.

Co-passage treatment

Concurrently as the above treatments, we co-passaged hosts and bacteria together under heat stress (five replicate populations). We followed the same protocol as the microbial evolution treatments, except after picking 40 nematodes we left the remaining hosts to produce offspring on OP50-GFP plates at 20˚C. Three days later, we washed nematodes off the OP50-GFP plates and aliquot roughly 500 – 700 larvae onto the *B. subtilis* extracted from the heat shocked hosts. We then heat shocked them three days later to start the next passage.

Host evolution treatment

Concurrently as the above treatments, we passaged hosts in the presence of the ancestral *B. subtilis* under heat stress (five replicate populations). After heat shock we let nematodes produce offspring on OP50-GFP and aliquot roughly 500 – 700 larvae onto ancestral *B. subtilis* to start the next passage. We called this treatment "singly-passaged hosts", which is the B+H+ hosts in our previous study (Hoang et al. 2019, *in review*).

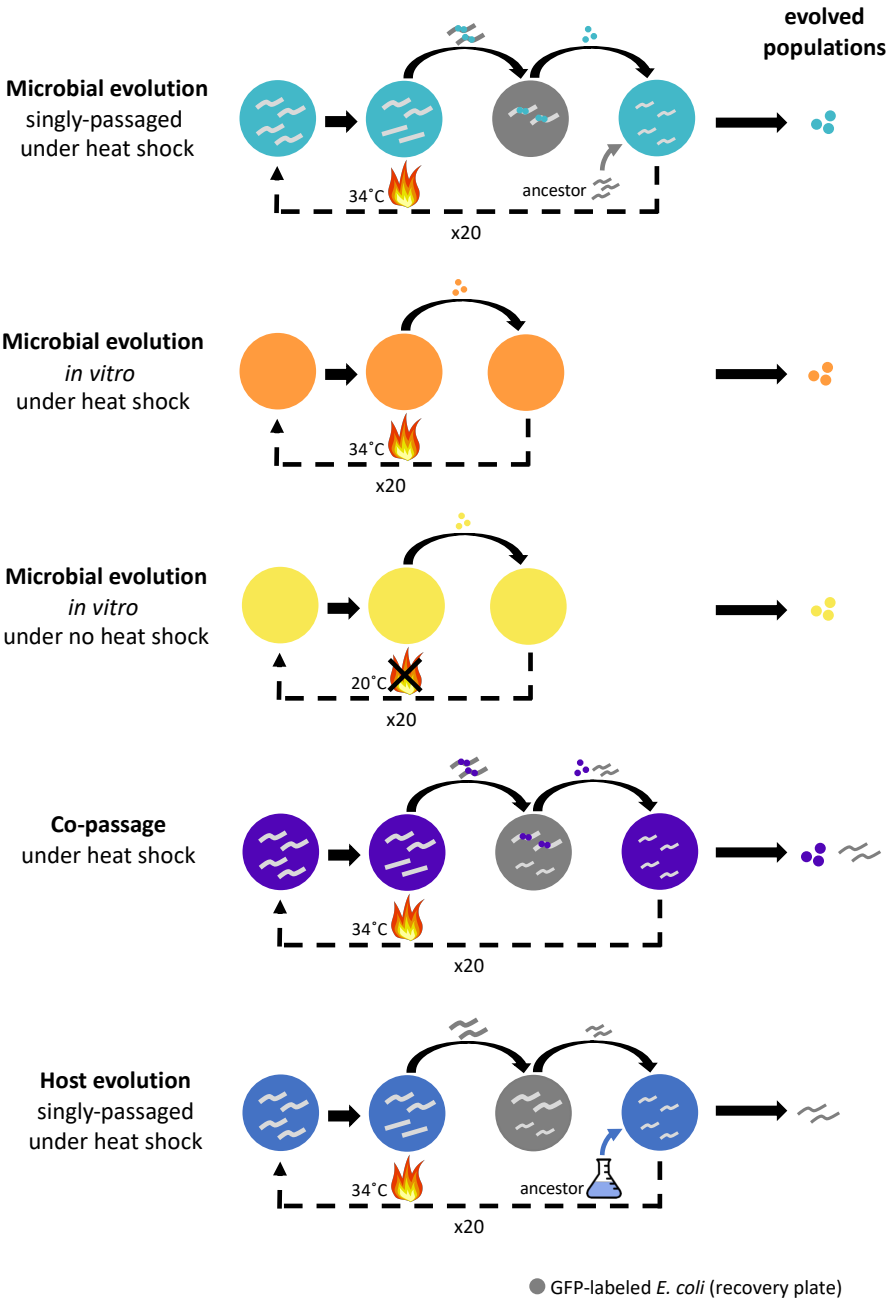

Figure 5.1. Setup of experimental evolution

Ancestral B. subtilis was passaged through ancestral C. elegans, in vitro under heat stress conditions or without heat stress conditions, or co-passaged with evolving C. elegans (each color representing an evolved treatment). The last treatment consisted of C. elegans passaged on ancestral B. subtilis. Treatments involving nematodes had a recovery period after heat stress where nematodes produced progeny on GFP-labeled E. coli. Each treatment had five replicate populations.

Host survival and fecundity assessment

Following the protocols described in Hoang et al. 2019 (*in review*), we conducted assays to evaluate host survival and fecundity as proxies for fitness. To determine host fitness changes that may have resulted from passage through a host, we quantified survival and fecundity of ancestral nematodes reared on ancestral, singly-passaged, *in vitro*, and co-passaged *B. subtilis* populations after 20 passages under heat shock conditions (Figure 5.1). We followed the schedule for one passage of experimental evolution, as described above, for each of the evolved replicate populations and the ancestral bacteria, heat shocking about 100 – 200 nematodes. After heat shock, we quantified the number of live hosts by prodding nematodes with a platinum pick to determine signs of movement, then transferred the nematodes to OP50-GFP and maintained them at 20˚C. We quantified the number of offspring produced per heat shocked adult two days later. For each replicate population we heat shocked three replicate plates (3 evolved bacteria x 5 experimental evolution replicate populations x 3 replicate plates) + (1 ancestral bacteria x 3 replicate plates). We followed similar protocols for comparisons between *in vitro* bacteria passaged under heat shock and non-heat shock conditions (2 evolved bacteria x 5 experimental evolution replicate populations x 3 replicate plates). For comparisons between co-passaged hosts and singly-passaged hosts on co-passaged and ancestral bacteria, we paired co-passaged hosts with either the bacterial population with which they were co-passaged or the ancestral bacteria, and each singly-passaged host population with one of the co-passaged bacteria or the ancestral bacteria (2 hosts x 2 bacteria x 5 experimental evolution replicate populations x 3 replicate plates).

Statistical analysis

Using survival data to compare microbial evolution treatments, we performed a General Linear Model (GLM) with an exponential distribution and reciprocal link function to test the main effects of bacteria, run, and bacteria x run interaction. To compare survival data between *in vitro* bacteria treatments, we performed a GLM with a Poisson distribution and log link function to test the main effects of run, bacteria, and bacteria x run interaction. We performed the same test to compare between co-passaged and singly-passaged hosts and evaluated the main effects of host, microbe, run, and all possible interacting terms. For all fecundity data we performed a GLM with a Poisson distribution and log link function, examining the same main effects as survival, using the total fecundity means (i.e., the mean of all replicate populations from each experimental evolution treatment). Subsequently, for both survival and fecundity analyses, we performed contrast tests to compare means between treatments.

Results

To determine fitness consequences for hosts that interacted with bacteria adapted to different environments, we heat shocked ancestral hosts on *B. subtilis* previously passaged through ancestral *C. elegans* (singly-passaged microbial evolution), on nematode growth media in the absence of hosts (*in vitro* microbial evolution) or through co-passaged hosts. For comparison, we also heat shocked ancestral hosts on ancestral *B. subtilis*.

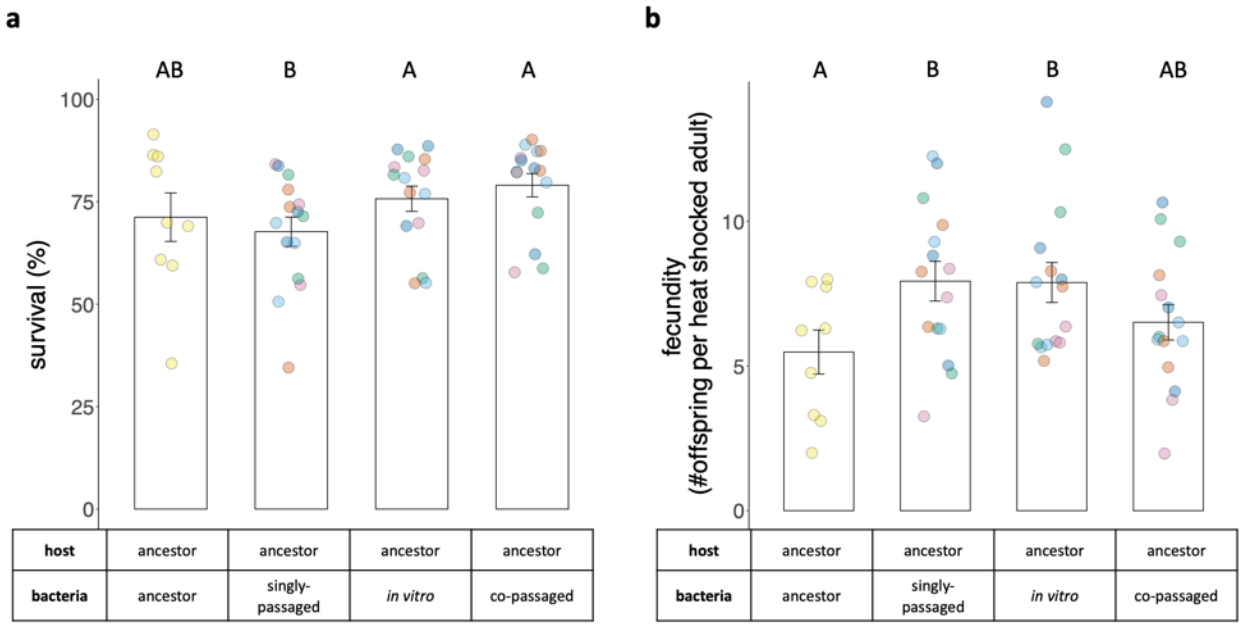

Figure 5.2. Host fitness on evolved bacteria

The x-axes indicate the host-bacteria combination that underwent heat shock after experimental evolution. Each plate contained about 100 – 200 nematodes. Each data point is the average of the three replicate plates for each of the experimental evolution populations. The same colored points within a host-bacteria combination represent the same experimental evolution population from different runs. Colors across different combinations are not necessarily from the same experimental evolution population. The data is combined across three runs. Error bars indicate the standard errors. Treatments that are not the same letter are significantly different.

Hosts differed in their survival when heat shocked on the different bacteria (Figure 5.2a; $\chi^2_{11} = 47.81$, $p < 0.0001$). Specifically, exposure to co-passaged bacteria resulted in greater survival than singly-passaged bacteria ($\chi^2_1 = 8.74$, $p = 0.003$), and *in vitro* bacteria also increased survival compared to singly-passaged bacteria ($\chi^2_1 = 4.06$, $p = 0.04$). Overall, co-passaged bacteria resulted in significantly greater survival than the other three bacteria ($\chi^2_1 = 5.56$, $p = 0.02$). Both singly-passaged bacteria and *in vitro* bacteria increased the number of offspring compared to the ancestral *B. subtilis* (Figure 5.2b; $\chi^2_1 = 6.07$, $p = 0.01$ and $\chi^2_1 = 5.59$, $p = 0.02$, respectively). Overall, exposure to experimentally passaged *B. subtilis* resulted in increased host fecundity relative to ancestral *B. subtilis* ($\chi^2_1 = 4.86$, $p = 0.03$).

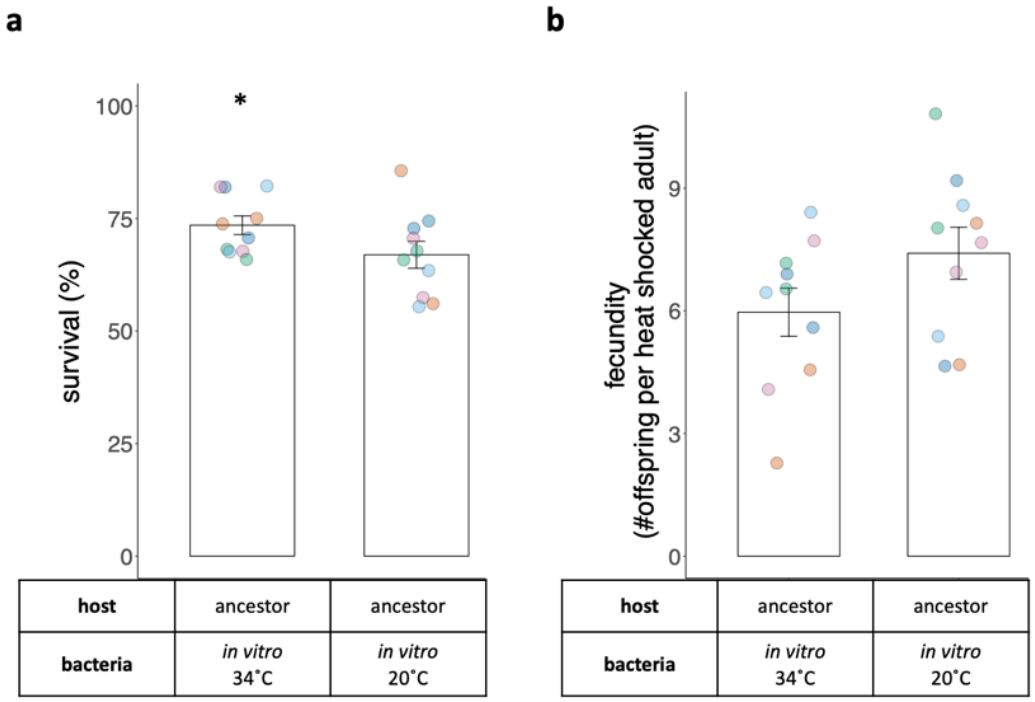

Figure 5.3. Host fitness on in vitro bacteria
*The x-axes indicate the host-bacteria combination that underwent heat shock after experimental evolution. Each plate contained about 100 – 200 nematodes. Each data point is the average of the three replicate plates for each of the experimental evolution populations. The data is combined across two runs. Error bars indicate standard errors. * $p < 0.05$.*

Because hosts exhibited similar fitness gains when heat shocked on singly-passaged bacteria and *in vitro* bacteria, we asked whether the *in vitro* bacteria effects were environment-mediated. We compared hosts that were heat shocked on bacteria passaged under heat shock or non-heat shock conditions, and found a significant difference in survival (Figure 5.3a; $\chi^2_1 = 4.14$, $p = 0.04$), but not fecundity (Figure 5.3b; $\chi^2_3 = 2.93$, $p = 0.40$).

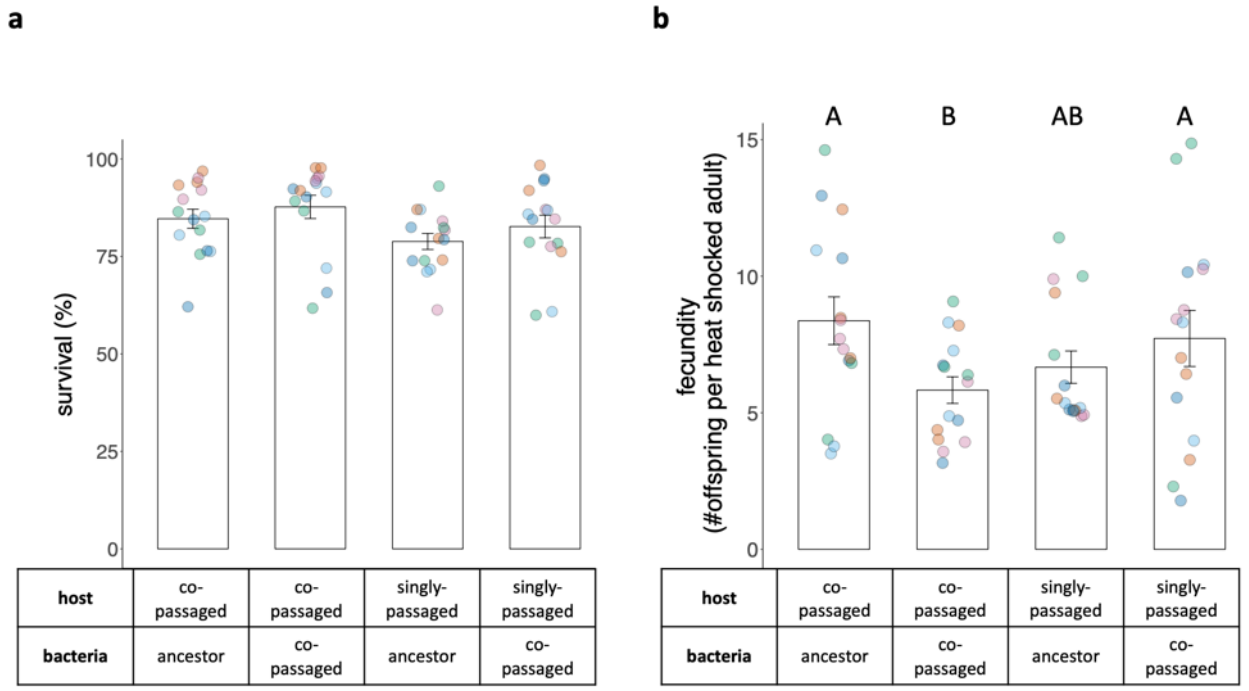

Figure 5.4. Fitness of co-passaged vs. singly-passaged hosts
The x-axes indicate the host-bacteria combination that underwent heat shock after experimental evolution. Each plate contained about 100 – 200 nematodes. Each data point is the average of the three replicate plates for each of the experimental evolution populations. The data is combined across three runs. Error bars indicate standard errors. Treatments that are not the same letter are significantly different.

As ancestral hosts had a tendency to exhibit reduced fecundity with co-passaged bacteria, we examined host fitness when co-passaged bacteria were paired with their co-passaged hosts. When we compared co-passaged and singly-passaged hosts, we found co-passaged hosts exhibited greater survival than singly-passaged hosts (Figure 5.4a; $\chi^2_1 = 5.97$, p = 0.01). There was also a significant difference in fecundity (Figure 5.4b; $\chi^2_5 = 23.88$, p = 0.0002), including a host x bacteria interaction ($\chi^2_1 = 7.07$, df = 0.008). Between treatments involving co-passaged hosts, those that were heat shocked on ancestral *B. subtilis* had greater fecundity than hosts heat shocked with their co-passaged bacteria ($\chi^2_1 = 7.08$, p = 0.008). Between treatments involving co-

passaged bacteria, those that were paired with singly-passaged hosts resulted in greater host fecundity than when paired with their co-passaged hosts ($\chi_1^2 = 4.10$, p = 0.04).

Discussion

Selection on microbes when in hosts has been shown to benefit both host and microbe (Guan et al., 2013; King et al., 2016; Marchetti et al., 2014; Mikonranta et al., 2015). Here, we examined the fitness consequences of naïve hosts interacting with bacteria passaged through either hosts or the external environment. Ancestral hosts gained a fitness increase via fecundity when heat shocked on bacteria evolved under both conditions compared to the ancestral bacteria (Figure 5.2b). We determined that this similarity was not due to the *in vitro* bacteria being passaged under heat shock versus non-heat shock conditions (Figure 5.3). This result suggests that the increase in fecundity for ancestral hosts heat shocked on *in vitro* bacteria is not mediated by the heat shock conditions during experimental evolution. Overall, the difference in fitness between association with the ancestral bacteria and passaged bacteria indicates that the bacteria evolved changes throughout experimental evolution. Moreover, increased ancestral host fecundity on singly-passaged and *in vitro* bacteria demonstrates that changes in the bacteria were sufficient to increase ancestral host fitness, and that this benefit did not require microbial adaptation to the host itself. However, passage through hosts could have increased bacterial growth within hosts; a positive correlation between microbial fitness and host fitness would support our previous study with singly-passaged hosts (Hoang et al., *in review*). Future studies will be directed towards measuring evolved *B. subtilis* growth within hosts and in media. We did find that bacteria passaged through a host that was allowed to evolve had a tendency to reduce the fecundity of ancestral hosts (Figure 5.2b), which prompted us to further explore the co-passaged pairings.

We hypothesized that specificity had evolved throughout experimental evolution between co-passaged partners, such that host and bacteria had gained traits that work well together and fitness gains could be obtained only when co-passaged bacteria were paired with their respective co-passaged hosts. Previous studies suggested that repeated associations between host and microbe, and therefore the potential for coevolution, can lead to increased benefits or at least decreased harmful effects (Gibson et al., 2015; Rafaluk-Mohr et al., 2018). Unexpectedly, we found that co-passaged hosts heat shocked with their co-passaged bacteria exhibited increased survival but decreased fecundity compared to the singly-passaged hosts, which exhibited reduced survival but greater fecundity (Figure 5.4). Moreover, co-passaged bacteria did not reduce singly-passaged host fecundity, suggesting that they conferred reduced benefits specifically towards their co-passaged hosts. Co-passaged hosts also had greater fecundity when heat shocked on the ancestral bacteria, suggesting that these hosts had the potential to adapt to heat stress, but their co-passaged bacteria were impeding them. Our selection regime during experimental evolution involved picking hosts that were alive from which to extract bacteria. Therefore, co-passaged bacteria would benefit if their hosts survived after heat shock, but, because the bacteria were not transmitted directly from mother to offspring, there was no incentive for the bacteria to promote host reproduction. Thus, our results suggest that the co-passaged bacteria were acting in their own selfish interests. By contrast, singly-passaged hosts did not necessarily need to survive for an extended period of time as much as they needed to reproduce to increase their fitness during our experiment. Because reproduction is an important factor in an organism's fitness, we argue that the co-passaged bacteria is harming their co-passaged hosts by reducing their fitness. The inverse relationship between survival and fecundity was also consistent with our previous study examining the fitness consequences of the initial

interaction between *C. elegans* and *B. subtilis* (Hoang et al., 2019), where hosts heat shocked on *B. subtilis* have more offspring but exhibit reduced survival, the opposite as seen for hosts heat shocked on *E. coli*. In general, our results indicate that co-passaging led to detrimental effects for the host, where continual interaction with the co-passaged bacteria may lead to complete exploitation of the host population, moving the interaction towards parasitism. The singly-passaged host, by contrast, may continue to adapt to its static partner, maximizing the benefits it can gain.

Adaptation to the host may involve changes in the microbe that can result in fitness consequences for the host. However, there was not a difference in fecundity between ancestral hosts that were heat shocked on singly-passaged bacteria or *in vitro* bacteria. Our findings contrasted previous work in which host adaptation resulted in altered host fitness compared to environmental adaptation (Mikonranta et al., 2015; Morran et al., 2016). Genes required for adaptation to the host may undergo similar selection to adaptation to the external environment in our experiment. The host environment may be limited in terms of nutrients and space, especially if the microbe is not already host-associated (*B. subtilis* is a soil-dwelling bacterium). Even though the heat shock temperature (34˚C) is within the growth range of *B. subtilis*, the medium itself could present a challenge since it is optimized for nematode growth and may be lacking nutrients for bacterial growth (Gusarov et al., 2013; Stiernagle, 2006). Alternatively, the singly-passaged bacteria or *in vitro* passaged bacteria can still differ, for example, in terms of how they grow *in vitro* or within hosts, without directly impacting host fitness. Our results also suggest that bacterial passage through the host is not the only condition that facilitates the evolution of a beneficial association when only the microbe evolves. This is in contrast with our previous study

on host evolution, where host fitness increased only when hosts evolved with non-evolving *B. subtilis* under heat stress (Hoang et al, in review).

The co-passage pairings resulted in the lowest host fecundity, indicating that the interaction evolved towards less benefit for the host, despite *B. subtilis* being beneficial under heat shock. We hypothesize that the host did not maintain or had exhausted the genetic variation necessary to combat its co-passaged partner and may have reached its adaptative potential. Even though the ancestral host population started with standing genetic variation, it was composed of a low percentage of males, which were more heat sensitive than hermaphrodites. Furthermore, we observed little to no males by approximately generation ten in most experimental evolution treatments. Combined with the bottleneck hosts underwent from repeated heat shock selection, these events would lead to a drastic decrease in host genetic diversity. An influx of genetic variation, such as the addition of males, may help hosts keep up with the bacteria (Stoy et al., *pers. comm./in prep.*). While theory suggests that evolutionary rates can affect the evolution of beneficial associations, such that the slower evolving partner obtains more benefits (Bergstrom and Lachmann, 2003), our study suggests that, at least for the evolution of a novel beneficial association, rapid evolution may be better for the host. Further support comes from our previous study, where we found that hosts adapted readily when the microbe was not evolving. If given the chance to evolve first and adapt to the bacteria, the host evolved to gain more benefit from its partner. Interestingly, this also led to a correlated increase in bacterial fitness.

Conclusion

Microbial adaptation to different environments can impact the fitness of hosts that interact with these microbes, subsequently altering the evolutionary trajectories of the hosts

themselves. As part of this study, we examined how host fitness is affected when only the microbe evolves, either through exposure to the internal host environment or the external environment that hosts were reared on, and found that passage in both environments resulted in increased host fecundity. We also introduced another factor where the host was allowed to evolve and determined that hosts did not reap as much benefit from these co-passaged bacteria. Furthermore, co-passaging resulted in reduced fitness for co-passaged hosts when they were paired with their co-passaged bacteria, contrary to the singly-passaged hosts. Because co-passaged bacteria only reduced co-passaged host fecundity and not singly-passaged host fecundity, this would suggest that co-passaging facilitates specificity between host and microbe. As the partners become trapped in their interactions, it may be difficult for either to gain a significant benefit. Overall, our findings shed light on the fitness consequences of tight coupling of host and microbe in a nascent interaction, suggesting that coevolution may impede the establishment of novel beneficial associations despite the benefits it brings to established long-term symbioses.

CHAPTER VI

CONCLUSION

Summary and discussion of previous chapters

The goal of this dissertation was to determine the conditions that favor the evolution of novel beneficial symbioses. Based on a vast body of work that has demonstrated the importance of environmental and evolutionary contexts in established symbioses, I examined how these conditions impacted the evolution of a nascent host-microbe association. I developed a model system using *C. elegans* and *B. subtilis* to test predictions, passaged them with or without heat stress while varying how each partner evolved, and measured how host fitness and microbial fitness were altered. Because fitness depends on passing on an individual's genes to their offspring, I will mainly focus the discussion on changes in host fecundity and bacterial abundance.

The role of environmental context

While many host-microbe associations are obligate and mutualistic, others may be beneficial only under specific environmental conditions. Many interactions are context-dependent because of costs for hosts associated with harboring microbes, and conversely, microbes can be exploited by their hosts. Persistence in a new environment may require long-term association between hosts and microbes that help each other adapt. In the absence of this environment, however, costs of the association may impede further interactions. In Chapter III, I showed that *B. subtilis* was beneficial towards *C. elegans* when heat shocked but not under standard rearing temperature. In Chapter IV, I showed that nematodes gained the greatest fitness increase and harbored the most *B. subtilis* when passaged under heat stress with non-evolving *B. subtilis*. These results indicate

that not only is the initial interaction between *C. elegans* and *B. subtilis* context-dependent, but in order for the host to derive the most protection from the bacteria, it has to be passaged under conditions that optimize the benefits of association.

The role of evolutionary context

While many host and symbiont traits have resulted from selection that each partner imposed upon the other, coevolution is not a requirement for beneficial symbioses (Moran and Sloan, 2015). The results from Chapter IV demonstrated that changes in the host alone can lead to increased fitness for both the host and the microbe. Similarly, the results from Chapter V indicated that changes in the bacteria alone can lead to increased host fitness; however, adaptation to the host was not necessary for fitness gain, because hosts also benefited when heat shocked with *in vitro* passaged bacteria.

Because I found that co-passaged bacteria had a tendency to reduce ancestral host fecundity, I examined how co-passaged bacteria affected the fitness of their co-passaged hosts. I found that they reduced their respective host's fecundity compared to the hosts that were passaged with non-evolving *B. subtilis*. These findings have implications for the evolution of novel beneficial symbioses: coevolution may act as a barrier preventing prolonged association between incipient hosts and their new microbial partners. One of the next steps would be to examine co-passaged microbial fitness within co-passaged versus singly-passaged hosts. If co-passaged bacteria exhibited decreased fitness in their co-passaged hosts, this would provide further evidence of evolution towards reduced benefits. Taken together, the results suggest that hosts may need to evolve ahead of the microbe before coevolution can occur. The tight-knit association I enforced with the co-passage treatment can be likened to vertical transmission, such

that host survival and fecundity depended on the microbe extracted from the previous generation of hosts. Singly-passaged hosts and singly-passaged bacteria, on the other hand, may be analogous to horizontal transmission, where fitness of the host is not coupled with fitness of the bacteria, and vice versa. It would be interesting to further explore these dynamics to test whether horizontal transmission is better for the establishment of novel symbioses.

Future directions

We still have much to learn about how nascent symbioses evolve. For example, genomic changes and population genetics of host and bacteria after experimental evolution remain unexplored. Microbial passage through ancestral and co-passaged hosts likely resulted in bottlenecked *B. subtilis* populations, which can lead to reduced competitive abilities. For example, in the extreme case of obligate intracellular symbioses, the symbiont has lost the ability to grow outside of the host (Toft and Andersson, 2010). If we found higher growth rates of *in vitro* bacteria in media compared to singly-passaged bacteria, this would indicate that adaptation to a host comes with a cost. Hosts also underwent bottlenecks when heat shocked, which has implications for how much genetic variation they had to respond to the non-evolving or co-passaged bacteria.

Since I determined that singly-passaging hosts can lead to increased host and bacterial fitness after 20 generations of selection, it would be interesting to examine how the association evolves over a longer period of time—has the host plateaued evolutionarily, and what happens to microbial fitness when it does? We can also explore the potential to disrupt the association, which is hinted by the co-passaged treatment. When both partners are able to evolve in response to one another, host fitness decreased. There is also an entire set of experimental evolution treatments containing hosts and bacteria that were passaged for 20 generations without heat

shock. Of particular interest are the co-passaged populations that did not experience heat shock: four out of five treatments were not able to move past generation 15; the fifth was not able to move past generation five. The main reason they did not move forward was because I was unable to extract bacteria from the hosts. Perhaps under no heat stress, hosts had no need for live bacteria, so they were digested immediately. From the perspective of the bacteria, *B. subtilis* may grow faster under heat shock conditions and persist longer in hosts; this would suggest that the environment is a determinant of bacterial fitness.

Finally, to explore mechanisms involved in *B.* subtilis-conferred heat shock protection, I attempted to use the nitric oxide mutant from the Gusarov et al. 2013 study to determine if NO was involved in altering nematode fitness under my experimental setup. I did not find NO to be a driver of increased fecundity. Another study found that *B. subtilis* biofilm was an important factor in extending host survivorship (Donato et al., 2017). It is possible biofilm is involved because it can increase the stability of bacterial populations within hosts, supporting the Chapter IV finding where increased singly-passaged host fecundity is correlated with increased bacterial abundance. It would be interesting to explore biofilm formation in the evolved bacterial lineages. Additionally, it would be interesting to examine heat shock proteins (or more general stress proteins) in *C. elegans* and determine if they influenced reproduction, then ascertain whether *B. subtilis* affects the expression of these proteins. Since we tend to find an inverse correlation between survival and fecundity when hosts are heat shocked, examining genes involved in longevity would also be reasonable.

Experimental evolution has only been used to explore beneficial interactions relatively recently. In combination with classical approaches in symbiosis research, we can form a more encompassing understanding of symbioses and address outstanding questions in host-microbe

associations. Although there is no clear cut-off amount of evolutionary time a host and its microbe should interact before they are found to be in “symbiosis”, all extant symbioses started out with nascent interactions between the partners. While *C. elegans* and *B. subtilis* are far from traditional symbiosis models, they provide the groundwork necessary to investigate an elusive question in symbiosis research and provide opportunities for further exploration.

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