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Raythe Owens April 7, 2020

Evolution of Parasite Avoidance Impacts Investment in Alternative Host Defenses

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Abstract

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Host-parasite interactions can lead to a variety of adaptations by both the host and parasite. Host fitness can be elevated through the evolution of beneficial behaviors or physiological responses that inhibit a parasite's virulence. However, the development of one defense may reduce the selective potential for alternative defenses. In a previous study, experimentally evolved *Caenorhabditis elegans* populations developed varying degrees of avoidance of the bacterial parasite, *Serratia marcescens*. Here, I attempted to isolate the effects of innate immune resistance in these same *C. elegans* populations to determine if the evolution of parasite avoidance traded off the evolution of the immune response. These host populations were exposed to *S. marcescens* in liquid media, limiting the effects of the evolved avoidance. Host populations that were experimentally evolved with exposure to a coevolving parasite developed increased resistance relative to host populations experimentally evolved without exposure to parasite. Increased resistance could not be detected for host populations experimentally evolved with a fixed parasite, but the host-mortality was much lower in these liquid media assays than what is observed on solid media. Further analysis is required to adequately assess these experimental host populations' innate immune response to their ancestral bacterial parasite, but with this analysis there is evidence for the evolution of distinct host defenses for at least a subset of the experimentally evolved populations.

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Introduction

Host-parasite dynamics occur when a parasite gains fitness throughout an interaction with a host, who loses fitness (de Roode and Lefevre 2012). From the host's perspective, this interaction takes place across three steps. First, the host and parasite must overlap temporally and spatially to allow the possibility of infection (Orr 1992, Amano, Hayashi et al. 2008). Second, the parasite must accumulate in or on the host at a high enough infection load to sufficiently infect the host (Gaugler, Wang et al. 1994). This leads into the third step, where the parasite utilizes the host's resources to proliferate, typically causing host fitness loss (de Roode and Lefevre 2012). These negative fitness effects range from immediate death and castration to minor decreases in fecundity, applying selective pressure for the host if the host-parasite dynamic evolves over time.

Selection often favors hosts that evolve defenses against infection (Morran, Schmidt et al. 2011). Host defenses can be characterized by how or when a specific defense mitigates host-parasite interactions (de Roode and Lefevre 2012). From the standpoint of infection, the first type of host defenses limits the parasite's infectivity toward the host. These are constitutive defenses, active regardless of infection. Although physical barriers to infection such as the skin are clearly constitutive, many these defenses are behavioral. These behavioral host defenses include the host recognizing and avoiding the parasite's location, ingesting medicinal compounds to work against the parasite, practicing hygiene, and more (Lozano 1991, Orr 1992, Amano, Hayashi et al. 2008, Hart 2011). The second type are inducible physiological host defenses, where once infected the host undergoes a change to become less habitable for the parasite (de Roode and Lefevre 2012). This can be through the activation of an immune system, varying innate temperature, or otherwise using hygiene to diminish the parasite's environment (Kluger

1979, Singer, Mace et al. 2009, de Roode and Lefevre 2012). The third type of host defense is through increased tolerance for the parasite (de Roode and Lefevre 2012). Tolerance mechanisms reduce the parasite's virulence without attacking its fitness (Vitale and Best 2019). Instead, the host uses alternative resource investments to compensate for the infection, such as by accumulating additional resources to accommodate the parasite or by diverting resources to maximize fecundity prior to death (Minchella and Loverde 1981, Karban and English-Loeb 1997).

Although the variance in host defense mechanisms has been widely explored (Schmid-Hempel 2005, de Roode and Lefevre 2012, Curtis 2014), there is a dearth of evidence regarding how the evolution of one defense might influence subsequent investment in alternative forms of defense within a single host population. There are studies that suggest a trade-off between different types of defenses. While collecting resin to use as a barrier to the environment within their nests, the wood ant's immune system is downregulated (Castella, Chapuisat et al. 2008). Insects which demonstrate social immunity, such as the honeybee, exhibit a reduced repertoire of immune defenses when compared to nonsocial insects (Evans, Aronstein et al. 2006). Certain plant toxins are shown to regulate the transcriptional immune response in monarch butterflies (Tan, Acevedo et al. 2019). Given the variety in host defenses, it begs the question of whether there is an evolutionary opportunity cost associated with how these defenses accumulate in populations. Part of the evolution within a host-parasite dynamic is maintained by the selective pressures applied by the parasite on the host, namely the parasite's infectivity and virulence (Morran, Parmenter et al. 2009). Presumably, these selective pressures would change depending on what type of host defense arose in the host population. As a host enacts avoidance behaviors, selection for physiological immune responses may be reduced due to the lack of exposure to

infection (Curtis 2014). Similarly, if a physiological host defense is maintained, parasite avoidance effectively decreases selective efficacy and nutritional sources that are used to maintain the host defense, costing the host. The proposed evolutionary trajectory of host defenses under these conditions implies that the evolution of one type of host defense will impact the evolution of subsequent host defenses.

This possibility of an evolutionary opportunity cost for host defenses may be explored experimentally. Host-parasite driven evolution can generate multiple host defenses across separate populations, despite similar circumstances (Penley and Morran 2018). Experimental evolution in tractable systems can permit comparisons across replicate host populations that have evolved elevated levels of defense to determine if specific forms of defense preclude investment in additional defenses.

Caenorhabditis elegans is a microscopic species of soil nematode that has claimed a robust position as a laboratory model organism (Zhang, Lu et al. 2005, Morran, Parmenter et al. 2009, Glater, Rockman et al. 2014). Along with the essential qualities of fast generation time, tractable genome, compact size and simple growth requirements, it has the added benefit for population evolution studies by having a variety of life stages, varied reproductive mechanisms, and multiple mechanisms of host defenses(Morran, Schmidt et al. 2011, Slowinski, Morran et al. 2016, Gibson, Baffoe-Bonnie et al. 2020). The virulent parasite, *Serratia marcescens*, is often used to study *C. elegans* host defense and host-parasite interactions in general (Morran, Schmidt et al. 2011, Penley, Ha et al. 2017). During their interaction, *S. marcescens* infects a *C. elegans* nematode by entering through the nematode's oral cavity and into the host gut upon consumption(Morran, Schmidt et al. 2011). Once in the host's gut, *S. marcescens* proliferates using the gut as a nutrition source, eventually penetrating the gut wall and causing a systemic

infection (Mallo, Kurz et al. 2002). Systemic infections often result in host death within several hours. On solid media, infection first occurs when the nematode eats a *S. marcescens* colony (Penley and Morran 2018). However, *C. elegans* can employ both behavioral and physiological defenses against *S. marcescens*. First, *C. elegans* can recognize and avoid *S. marcescens*, although the nematode naturally prefers to consume *S. marcescens* relative to most food sources (Zhang, Lu et al. 2005, Glater, Rockman et al. 2014, Penley and Morran 2018). Second, *C. elegans* possess innate immune defenses that can mitigate *S. marcescens* infection upon consumption. The strength of each type of host defense must be assessed to determine whether the development of one affects the development of the other.

In a previous experiment, populations of *C. elegans* were experimentally evolved with *S. marcescens* for thirty passages on solid media (Morran, Schmidt et al. 2011). Hosts were either evolved in the presence of a single parasite genotype (Evolution treatment) or coevolved with parasites (Coevolution treatment). Each experimentally evolved host population demonstrated increased fitness towards the ancestral parasite relative to control groups of *C. elegans* that were passaged thirty times without parasite exposure. Each population's ability to detect and choose a non-parasitic, standard lab food source *Escherichia coli* over the parasitic food source *S. marcescens* was evaluated (Penley and Morran 2018). Across populations of these treatments, ancestral and control hosts preferentially chose the parasitic food source, while the evolved and coevolved hosts generally lost preference for *S. marcescens*. Nonetheless, the assayed populations demonstrated a range in this avoidance phenotype. While the *C. elegans* populations evolved varying degrees of avoidance behavior against the parasite, the variance in parasite avoidance did not completely account for the variance in the change in host fitness during experimental evolution. As such, it is expected that an additional physiological host defense was

selected for and evolved during the experimental evolution. In order to isolate the effects of innate resistance, the nematodes must be unable to escape steps one and two of the host-parasite interaction, and thus be unable to avoid the parasite.

Liquid media assays are an ideal way to limit the impacts of nematode choice behaviors (Schulenburg and Muller 2004). *C. elegans* are filter feeders, meaning that in a liquid environment they take up their liquid surroundings, filter particles that are too large, and retain the small to moderately-sized bacterial colonies (Fang-Yen, Avery et al. 2009, Suzuki, Kikuchi et al. 2019). It has been shown that particulates up to 3 μm show up in the nematode's gut. *S. marcescens* colonies range from 0.5-0.8 μm in diameter, indicating that parasite's ability to get into the nematode's gut should be uninhibited by the filter feeding mechanism (Lai, Burge et al. 2004). *C. elegans* have previously been grown in the liquid buffer S-medium, an effective salt buffer without nutrients essential for *S. marcescens* growth (Stiernagle 2006). Experimentally evolved *C. elegans* populations demonstrating a range in host defenses were exposed to the parasite *S. marcescens* in liquid media. The populations' mortality to the parasite was assayed to determine if their innate resistance varied between evolved populations. These differences were then compared to differences in the avoidance phenotype previously analyzed in these populations to determine the extent of an evolutionary trend in host defense evolution.

Methods

Establishment and Maintenance of *C. elegans* host populations with range in host defenses

The 30th passage of PX382 *C. elegans*, mutated with EMS and evolved against Sm2170 as described in Morran et al (2011) were thawed and maintained for this experiment (Morran, Schmidt et al. 2011). These populations will be referred to by their evolution treatment. Control

populations were passaged without exposure to the parasite. Evolved populations were passaged with exposure to a fixed ancestral parasite. Coevolved populations were passaged with exposure to a coevolving parasite. Five replicate populations exist for each treatment. The ancestral strain of these *C. elegans* (CB4856) was also thawed and maintained. In short, the nematodes were thawed onto 10 cm NGM-Lite plates seeded with *Escherichia coli*, the standard lab nutrition source for the nematodes (Brenner 1974, Morran, Parrish et al. 2014, White, Penley et al. 2019). The populations were grown for two weeks before experimentation in order to reduce any impacts due to freezing. Once experimentation began, the populations were held in a 15℃ incubator and chunked onto fresh seeded plates every two weeks.

S. marcescens **parasite strain**

The gram-negative bacterial parasite *S. marcescens* was chosen for experimental evolution because of its capability of killing *C. elegans* after establishing a systemic infection via consumption, despite its lack of natural evolutionary history with the organism (Morran, Schmidt et al. 2011, Penley and Morran 2018). The ancestral Sm2170 strain was utilized in this study because the strength of parasite avoidance was already quantified for the evolved and coevolved *C. elegans* populations.

Assessment of Parasite Avoidance: Bacterial Choice Index

The Bacterial Choice Index (BCI) is a measure of avoidance. The BCI data for the experimentally evolved populations of *C. elegans* was gathered in a previous study, but the relevant methods are summarized here. A 10 cm NGM-Lite petri dish was spotted with 25 µL of *S. marcescens* and *E. coli* at opposite ends and incubated at room temperature for 5 hours (Penley and Morran 2018). ~200 synchronized L4 nematodes from each experimentally evolved

population was plated onto the center of the petri dish. After 4 hours, the quantity of nematodes in each bacterial patch was scored, and the populations' BCI was calculated using the equation;

$$BCI = \frac{(\#\ nematodes\ in\ parasite) - (\#\ nematodes\ in\ E.coli)}{Total\ \#\ nematodes\ plated}$$

Preparation of *C. elegans* for Liquid Media Mortality Assays

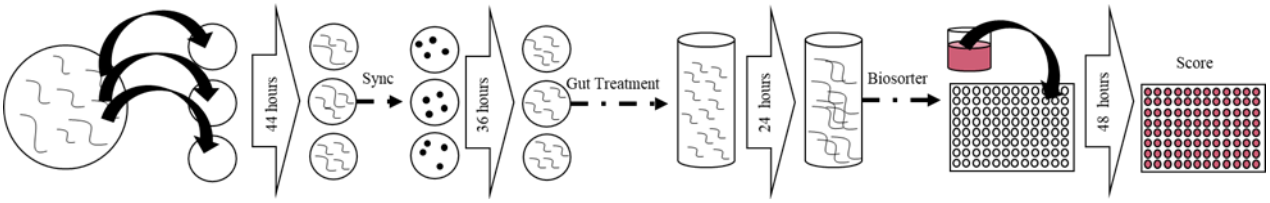

Figure 1: Liquid Media Mortality Assay Experimental Design. Pictorial representation of steps necessary to perform the mortality assays done in this study. Refer to written methods "Preparation of *C. elegans* for Liquid Media Mortality Assays" for more detailed information.

Synchronization

Each population was chunked onto three separate OP50 seeded plates in order to grow adequate population sizes (Fig. 1) (Penley and Morran 2018). The plates were incubated at 20℃ for two days. Each population was then washed with an M9 buffer into centrifuged tubes. After centrifuging at 1000 rpm for one minute, the supernatant was replaced with 3 mL M9 buffer. The populations were synchronized by the addition of 1.2 mL 2:1 Bleach:5 M NaOH solution. After visual inspection under the microscope that the nematodes were dead and releasing their eggs, each nematode solution was centrifuged and washed with M9 buffer three times. The pellet of unharmed eggs was plated onto three fresh OP50 seeded plates per population and allowed to grow at 20℃ for 36 hours, until arrival at the L3 life stage.

Gut Treatment

Once in the L3 life stage, the nematode populations were transferred to liquid media (Fig. 1). In order to prepare them for infection, the live OP50 in the nematode's gut was killed (Vega

and Gore 2017). Each population of synchronized nematodes was washed with M9 buffer three times as previously described to decrease the number of bacteria in the environment. The populations were resuspended in 5 mL S-medium after the third wash (Stiernagle 2006). 200 µL of 50x Heat-Killed OP50 was added to each population as a nutrition source to allow for continued growth of the nematodes (Vega and Gore 2017). 40 µL of 25 mg/mL gentamycin stock was added to each population, killing live OP50 in the gut after incubation for 24 hours. Each population was vortexed for 24 hours at 160 rpm at 20°C until maturity to the L4 life stage.

Biosorter Preparation

Liquid media mortality assays call for multiple replicates of single nematode systems. As such, the Biosorter is a technology that can be used to precisely deposit single nematodes into wells (Vega and Gore 2017). However, it requires clean samples. In order to grant this, each population of nematodes was washed ten times with M9 buffer. After washing, the populations were resuspended in 25 mL of S-medium + 0.01% Triton-X detergent at a concentration of 100 nematodes/mL. Each population was sorted into their own 96-well plate, previously filled with 50 µL S-medium per well (Fig. 1). Washing procedures were used to prevent between population contamination. Samples were verified under a microscope after the Biosorter to ensure the nematode in each well was alive. Only replicates alive at this stage were considered viable and used in final assays.

Preparation of *S. marcescens* for Liquid Media Mortality Assays

Inoculation

One day before using the Biosorter, two 100 mL LB broth beakers were inoculated with ancestral Sm2170. These beakers were vortexed at 160 rpm at 28℃ for 24 hours, resulting in concentrations of approximately $7.8*10^8$ CFUs (White, Penley et al. 2019).

Transfer to S-medium

The culture was separated into 40 mL aliquots and set inside a 4℃ incubator for twenty minutes to settle (Vega and Gore 2017). The aliquots were then centrifuged at 3900 rpm for twelve minutes to pellet the colonies. The supernatant was poured off, and the colonies were resuspended in S-medium. The necessary volume for resuspension was determined by the experimental treatment

Dosage

The concentration of *S. marcescens* in each well was the experimental treatment of the liquid media mortality assay. This treatment was adjusted by varying the concentration of the parasite after resuspension in S-medium. The initial concentration of *S. marcescens* in LB was $7.8*10^8$ CFUs (White, Penley et al. 2019). Resuspending the parasite solution in the same volume of S-medium as the LB volume poured out would result in the same concentration. By decreasing the resuspension volume, the effective treatment was increased in a way that is calculable by dilution theory ($M_1V_1=M_2V_2$).

Preparation of *E. coli* for Liquid Media Mortality Assays

Inoculation

One day before using the Biosorter, 10 mL LB broth was inoculated with the standard nematode lab food OP50. This volume was vortexed at 160 rpm at 28℃ for 24 hours.

Transfer to S-medium

The culture was separated into microcentrifuge tubes and set in a 4℃ incubator for twenty minutes to settle. The aliquots were then centrifuged at 9000 rpm for two minutes. The supernatant was poured off, and the pellets were resuspended in the same volume of S-medium.

Dosage-Response Assay

Four 96-well plates filled with 50 µL S-medium per well were filled with a single nematode per well from a single population (Schulenburg and Muller 2004). After replicate verification, each half-plate was subjected to one of eight dosage treatments. 50 µL *S. marcescens* solution of varying dosages [7.8, 15.6, 23.4, 31.2, 39.0, 46.8 (*10^8 CFUs)], S-medium alone or an OP50 solution was pipetted into each well. The effective concentrations were halved by this procedure. Each 96-well plate was covered with a Breath-Easy Membrane and placed into a 20℃ incubator vortexing at 160 rpm for 48 hours. Viable replicates were scored under the microscope for mortality after the infection period.

Liquid Media Mortality Assay

After replicates were verified post-Biosorter, 50 µL of 15.6*10^8 CFUs *S. marcescens* solution was pipetted into each well for all 16 nematode populations, for a final concentration of 7.8*10^8 CFUs (Fig. 1). Two controls were utilized in this experiment. The first subjected an additional well-plate of a single nematode population to an OP50 treatment, to account for any mortality due to the liquid media environment. The second subjected four additional well-plates of a single nematode population to the same 7.8*10^8 CFUs *S. marcescens* solution treatment but spaced out their inoculation and subsequent scoring to account for mortality due to time spent verifying replicates (~5 hours). Mortality was negligible in the OP50 control, and mortality

between replicates was negligibly different across scoring times. Each 96-well plate was covered with a Breath-Easy Membrane and placed into a 20℃ incubator vortexing at 160 rpm for 48 hours. These were analogous conditions of a mortality assay performed on solid media (Penley and Morran 2018). Viable replicates were scored under the microscope for mortality after the infection period.

Statistical Analysis

All statistical analysis was performed through JMP Pro15 (SAS Institute, Cary, North Carolina).

Mortality in liquid media was fitted against varied parasite dosages using a Binomially distributed Generalized Linear Model while testing for overdispersion. Significant levels of overdispersion were not detected. Contrast tests were used to determine significant increases in mortality relative to baseline controls.

Mortality in liquid media was fitted against the evolutionary treatment groups of the *C. elegans* populations using a Normally distributed Generalized Linear Model while testing for overdispersion. Significant levels of overdispersion were not detected. Contrast tests were used to determine statistical significance between treatment groups. This data included Ancestral, Control, Evolved and Coevolved *C. elegans*, as the results were used to assess expectations between the groups.

Results

Dosage-Response Assay

Limits of the liquid media mortality assay were explored by performing a dosage-response assay. Increased mortality relative to the controls was not detected until a liquid media treatment of $7.8*10^8$ CFUs *S. marcescens* [χ^2_1=4.235; p=0.0396] (Fig. 2A). However, the *S. marcescens*' dense red coloring prevents higher doses from being precisely scored due to inhibited visibility under the microscope (Fig. 2B). Although mortality is considerably lower than what is observed in solid media assays (Penley and Morran 2018), the remaining assays were done using the highest dosage of *S. marcescens* that still allowed accurate mortality scoring.

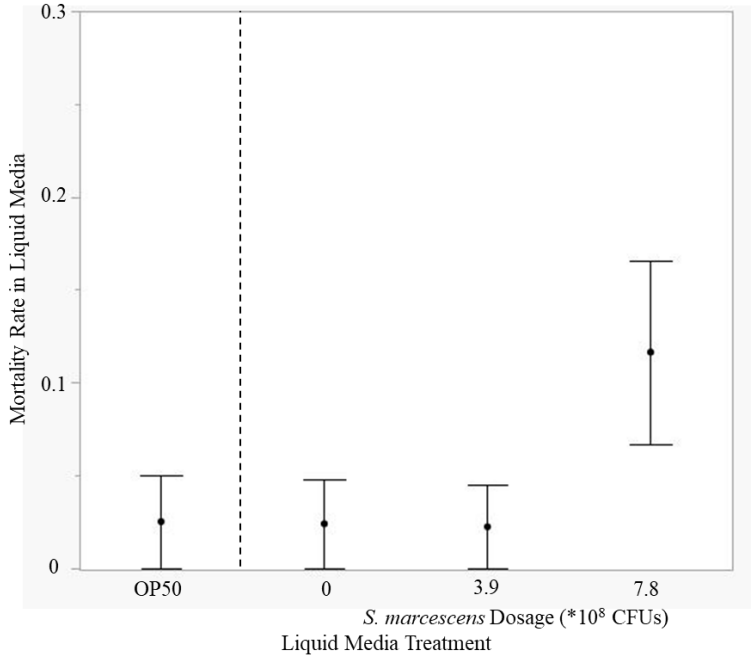

Figure 2A: *C. elegans* mortality in response to liquid media treatment. Scoring above $7.8*10^8$ CFUs is omitted due to difficulties with visibility.

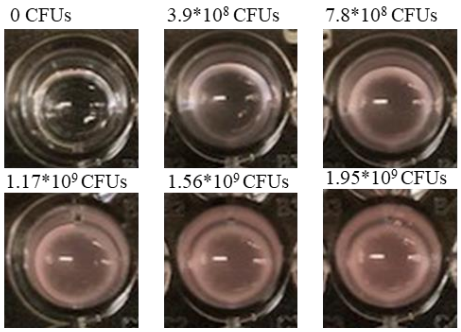

Figure 2B: Visibility of liquid media wells with different dosages of *S. marcescens*.

Liquid Media Mortality Assay

Effects of Evolutionary Treatment on Mortality in Liquid Media

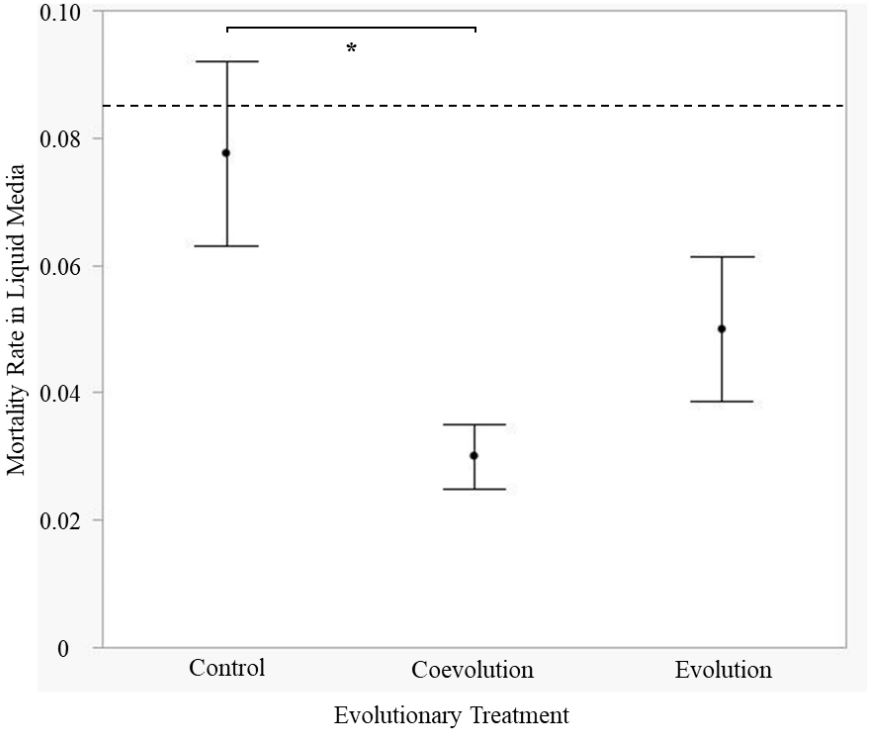

Figure 3: Effect of evolutionary treatment on host resistance. Sixteen *C. elegans* populations were assayed in liquid, though one was omitted as an outlier [5 Control, 5 Evolved, 5 Coevolved, 1 Ancestor; 1 Evolved replicate is omitted]. The mean mortality in liquid media of each treatment group is displayed with their standard error. The ancestor's mortality due to infection is noted by the dashed line.

The liquid media mortality assays ensure that the nematodes cannot escape infection, and thus comparisons in mortality rates should directly relate to the strength of individual population's evolved innate immune response (Schulenburg and Muller 2004). Coevolved host populations demonstrated increased resistance relative to control host populations [χ^2_1=9.353; p=0.0022] (Fig. 3). Evolved host populations did not exhibit increased resistance relative to control host populations [χ^2_1=3.466; p=0.0626]. Control host populations displayed no change in resistance relative to ancestral host population [χ^2_1=0.107; p=0.74]. In part, these results qualitatively support previous assays on solid media (Morran, Schmidt et al. 2011), indicating that evolved responses are not strictly media specific.

Discussion

Having multiple host defenses active against parasitism can be redundant and costly (Curtis 2014). However, this work shows that an innate resistance evolved in *C. elegans* populations due to parasite exposure (Fig. 3), in addition to the evolved parasite avoidance investigated in a previous assay (Penley and Morran 2018). The evolution of multiple host defenses contingent on parasite exposure signifies that specific evolutionary treatments can select for multiple different outcomes. This work was unable to address the theoretical predictions that there is an opportunity cost associated with hosts evolving defenses against parasitism, due to a lack in replicate data for each population's innate immune response to the parasite.

This work exemplifies a powerful experimental approach of utilizing novel environments to investigate the same system. However, it is worth mentioning that the common measure of host mortality varied widely from solid media (~80% mortality) (Penley and Morran 2018) to liquid media (~10% mortality) analyses. As population mortality rate is an effective measure of the host response to parasite activity (Morran, Schmidt et al. 2011), these vast differences indicate that the host-parasite dynamic is altered across medias. This could be due to differences in infection load, which is partially supported by the increased host mortality in response to increasing parasite dosage (Fig. 2A). During solid media parasite exposure, infection load is determined by the amount of parasite that enters the host's intestines. In liquid media, there is potentially a subtractive effect due to the *C. elegans*' filter feeding mechanics. Liquid constantly flows through the nematode, exerting pressure on the intestines to pass the colonizing parasite (Fang-Yen, Avery et al. 2009). This could cause a lower effective infection load, reducing host mortality. Alternatively, the differences in host response could be due to additional host defenses

that have effects dependent on the environment. One example of this is the effect of host tolerance. *C. elegans* are highly susceptible to dehydration (Brenner 1974, Erkut, Penkov et al. 2011, White, Penley et al. 2019), and as such when tolerance is used to mitigate effects of parasitism a portion of resources are used maintaining proper salt concentrations. In a liquid media environment, the environment is by design an ideal salt buffer for the nematode (Stiernagle 2006), resulting in less resources used for this important maintenance. The relatively increased amount of resources may allow the nematodes to live longer into the parasite infection than observed on solid media. Both effects might be mitigated by increasing the parasite dose in liquid media assays (Regoes, Ebert et al. 2002), thereby making the host response to parasite activity more comparable between medias. Further assays with this system should focus on reducing the optical density during scoring while maintaining greater concentration of the parasite. If these necessary alterations are met, then coevolved and evolved host populations could be successfully assayed to investigate variations in the strength of their innate immune response, thus allowing exploration of the evolutionary opportunity cost that might exist when investing in different defenses.

The experimentally evolved host populations developed defenses in response to their evolutionary treatment. Control populations demonstrated unaltered parasite avoidance and innate resistance relative to the ancestral host population, as expected due to the lack of evolutionary history with the bacterial parasite *S. marcescens*. Contrast this with the coevolved host populations, who developed a quantifiable range in both host defenses. The selective pressure on the host due to the parasite is largely the effect of host mortality. These results show that selectively beneficial attributes for these host populations are numerous and cumulative, working together to decrease the pressure of host mortality. However, they also demonstrate the

unpredictability of evolution; multiple evolutionary trajectories exist, despite common genetics and environments.

In natural host populations, the multiplicity of evolutionary trajectories would be magnified indefinitely. Not only do environments and genotypes vary widely (Penley and Morran 2018), but also the process of evolving individual host defenses will shape the evolutionary outcome of these populations under host-parasite dynamics. Taken to the extreme, these results might suggest that host populations are likely to specialize in single host defenses, as actively using one type of host defense reduces selective pressure for investment in subsequent defenses. However, it is hasty to make these conclusions given the abundance of evidence for multiple coinciding host defenses (de Roode and Lefevre 2012). A reason for this might be the coevolutionary nature of host-parasite dynamics; the host and parasite must respond to each other's evolutionary changes in order best perform their functions (Morran, Schmidt et al. 2011). Overcoming a single, well-adapted host defense might be a rare evolutionary occurrence, but this event would result in an evolutionary dead end for the host population. Continuous shifts in the selective benefits of different host defenses in response to the evolution of the parasite allows for the accumulation of host defenses, despite any redundancy or cost.

The evolution of host defense is dependent on evolutionary exposure to parasite but is influenced by the selective pressures generated by utilizing different host defenses. These varying pressures represent an evolutionary opportunity cost, resulting in accumulating host defenses of varied strengths against a common parasite. Though host defenses might be redundant, their variety is selectively beneficial in increasing reproductivity of individuals and is driven by selection changes throughout coevolution with the parasite.

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