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Trade-offs and heterogeneities in host-parasite interactions

By

Elisa M. Visher

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Doctor of Philosophy

in

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University of California, Berkeley

Committee in charge:

Professor Michael Boots, Chair
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Abstract

Trade-offs and heterogeneities in host-parasite interactions

By

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Doctor of Philosophy in Integrative Biology

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One question that has endlessly fascinated ecologists and evolutionary biologists is why there is so much biotic diversity on earth. At the advent of the field, Darwin grappled with these questions to propose that trade-offs between fitness traits constrained the evolution of hypothetical all-fit organisms termed 'Darwinian demons'. Trade-offs in the co-evolution of antagonistic biotic partners are thought to be particularly important for diversification processes. Despite the centrality of trade-off theory to evolutionary biology, the genetic underpinnings of and selection dynamics on trade-offs remain poorly understood.

My dissertation therefore focuses on trade-offs: what forms them, what influences them, and how they interact. To do this, I use experimental evolution methods in the *Plodia interpunctella*, or Indian meal moth, and granulosis virus model system. I focus on two trade-offs   one for each partner in a host-parasite interaction.

On the host side, I focus on the trade-off to resistance to viral in infection. Understanding this trade-off is important for predicting when we expect to see resistance evolve and how it would alter ecological dynamics or persist in the absence of the pathogen. In chapter 1 of the dissertation, I ask whether the trade-off to resistance is symmetric such that selection for the longer development time phenotype constituting a cost to resistance produces symmetric gains in resistance. In chapter 2 of the dissertation, I ask why resistance inconsistently evolves in the system and how time scales and resource levels affect such resistance evolution and its costs. With these two chapters, I find that the shape of the trade-off between resistance to infection and development time can change depending on the population's specific selection conditions when trade-offs are influenced by many genes. This is important because such differences in trade-off shape would alter the outcome of evolution.

On the virus side, I examine how trade-offs between adaptation to different host genotypes influences host range evolution in our system. This trade-off is important because specificity in biotic interactions promotes diversity in co-evolutionary systems. In chapter 3, I examine the genetic and phenotypic dynamics of host genotype specialization in the granulosis virus. In chapter 4, I experimentally evolve granulosis virus in mesocosms of its *Plodia interpunctella* host with varying degrees of spatial structure and host genetic diversity. With these two chapters, I show that trade-offs between host genotypes might not follow simple functions and may depend on their interactions with other selection pressures. This is important because it suggests that costless generalism can exist in some evolutionary and ecological scenarios, thus interrupting evolutionary dynamics that depend on specialist interactions.

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Introduction

"Doing science is not such a barrier to feeling or such a dehumanizing influence as if often made out. It does not take the beauty from nature. The only rules of scientific method are honest observations and accurate logic. To be a great science it much also be guided by a judgement, almost an instinct, for what is worth studying. No one should feel that honestly and accuracy guided by imagination have any power to take away nature's beauty" (MacArthur, 1984)

<u>Why do we study evolutionary biology?</u>

What does it mean to make predictions in evolutionary biology? While some fields seek to discover and describe unassailable laws and others seek to discover and describe unassailable things, evolutionary biology is often concerned simply by describing patterns that exist and the processes that might occasionally drive them. Thus, predictions in evolutionary biology are not absolute laws and are instead merely probabilities that emerge through the stochastic background of biology. Evolutionary biology is such a messy process because any biological organism experiences countless processes and pressures acting upon it. From the many dimensions of the abiotic environment to the spatial and temporal structuring of populations themselves to the everchanging biotic environment, populations are constantly exposed to multiple interacting and often contradictory selection pressures that must act through sampling processes of available variation and overcome a background of random, stochastic factors. When we predict how a population 'ought' to evolve, we are often attempting to pick apart just one process amongst this set.

Any prediction in biology is therefore messy and not absolute, but that doesn't mean that it is useless. If we consider how impossible it is to just describe everything about every organism and every environment, then we must consider that ecology and evolutionary biology must attempt to find patterns amongst what we do know to make guesses about what we cannot know. And both these patterns and the deviations from them are what makes biology beautiful.

So how then can we find patterns in evolution? To study evolutionary biology, we must combine methods. Naturalist observations from the field tell us what does happen, mathematical theory can tell us what processes could drive those patterns, and evolution experiments in the lab and field tell us what biologically can happen and whether the assumptions of theory are fair. Only through this combination of observation and logic can we create robust hypotheses for the ecological and evolutionary processes governing nature. At the same time, we must be cautious about how human factors bias our assumptions and logic. What factors bias us towards delighting in the prevalence of simple patterns in nature or reveling in the complexity of biology? How can we hold this contradictory but true standpoints in our minds

simultaneously? Navigating these biases and our responses to them is central to the fundamental question of whether, and when, and what we call predictions in ecology and evolutionary biology. We must be honest about what factors exist that complicate our ability to discern patterns or, more dangerously, create false patterns where none exist. And, perhaps more so than in other fields, we must continuously gather evidence as any process in evolutionary biology is a matter of probability rather than a law.

Why is there so much diversity in nature?

One question that has endlessly fascinated ecologists and evolutionary biologists is why there is so much biotic diversity on earth. At the advent of the field, Darwin grappled with these questions to propose that trade-offs between fitness traits constrained the evolution of hypothetical all-fit organisms termed 'Darwinian demons'. With trade-offs, higher performance in one environment is costly in the alternate, thus constraining niche breadth and promoting diversification through niche partitioning (Ackermann & Doebeli, 2004; A. A. Agrawal et al., 2010; Levins, 1968). This trade-off framework has since been central to eco-evolutionary theory exploring how varying population and environmental conditions reshape a system's evolutionarily stable strategies and potential for diversification (Boots et al., 2014; Poisot et al., 2012). Particularly implicated in these diversification processes are co-evolutionary antagonistic biotic interactions, including those between predators and prey, hosts and parasites, and competitors (Yoder & Nuismer, 2010).

Why and how do we study evolution in host-parasite interactions?

I study evolution in host-parasite interactions because of their implications for broad ecological and evolutionary processes and because they produce fascinating and dynamic patterns (R. M. Anderson & May, 1992, 1982; Yoder & Nuismer, 2010). Simultaneously, they have applied consequences across many systems (Elton, 1958; Strauss et al., 2012; Woolhouse et al., 2005). These applied consequences sometimes mean that host-pathogen interactions are treated as a totally unique process, but, in many instances, they are governed by the same processes driving other patterns in nature. Many of the effects of infectious diseases in systems are like those of predators or pests and parasites more generally (Dieckmann et al., 1995; Kasada et al., 2014). We should therefore not consider this field to be one where theory must be constantly invented, but rather as one where theory can often be adapted from the deep theoretical and empirical literatures on these other biotic interactions.

We also need to be cognizant of how infectious disease evolution is unlike these other areas. Problems of the relative population sizes and generation times between hosts and parasites, of selection happening at multigenerational timescales as parasites replicate within hosts and then must transmit to start new infections, and of steep bottlenecks

during such processes can all limit the applicability of theory from other regions of ecology and evolution to infectious disease evolution (Visher & Boots, 2020).
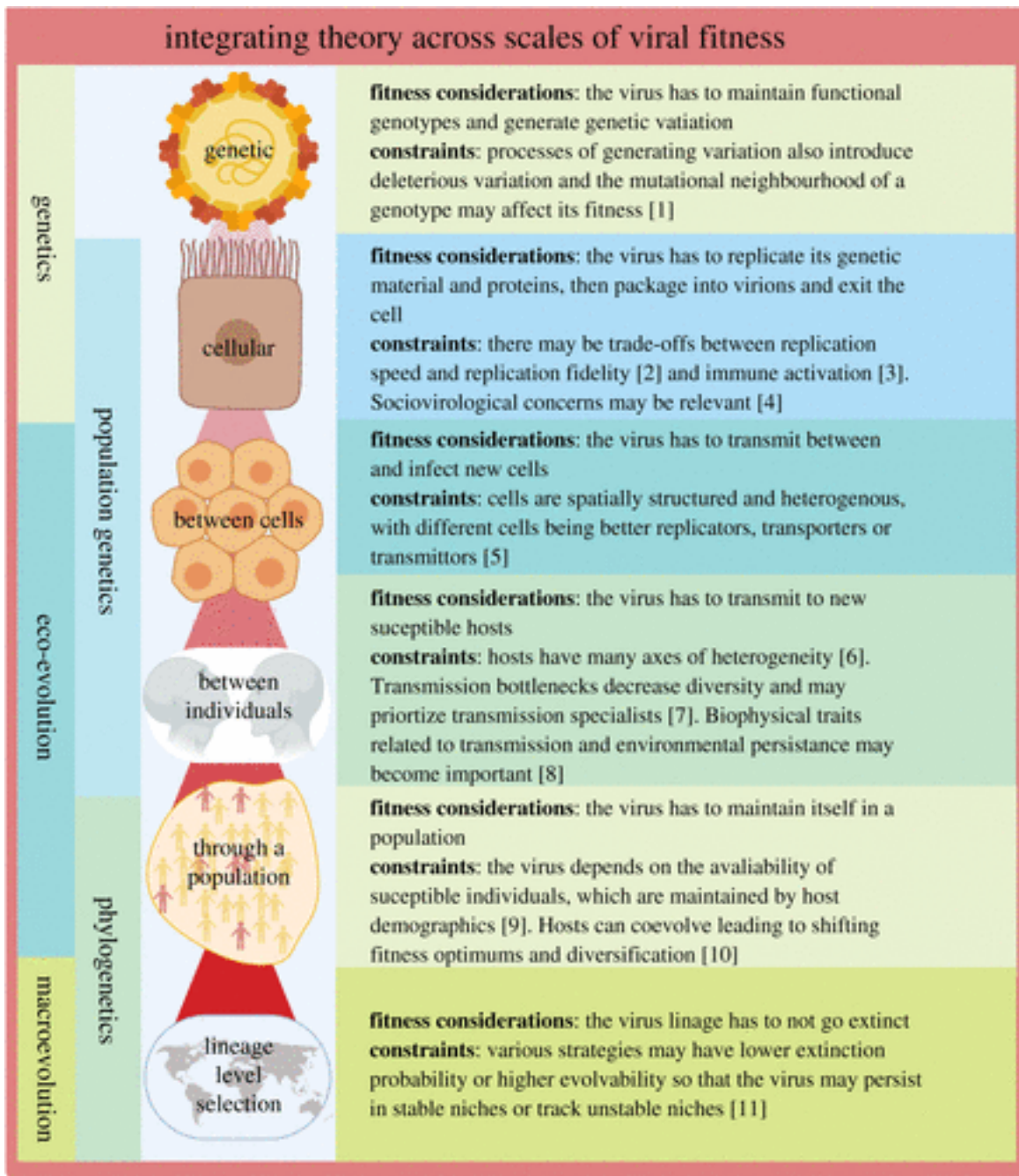
Figure 2: Integrating theory across scales of viral fitness. 1, (Lauring & Andino, 2010); 2, (Fitzsimmons et al., 2018); 3, (Alizon, 2008); 4, (Díaz-Muñoz et al., 2017); 5, (Cuevas et al., 2003); 6, (Regoes et al., 2000); 7, (McCrone & Lauring, 2018); 8, (Handel et al., 2014); 9, (Lion & Metz, 2018); 10, (Boots et al., 2014); 11, (Bono et al., 2019). Created with Biorender.com. Reprinted from (Visher & Boots, 2020).

How can we better understand pathogen evolution in nature?

While data from more empirical systems will help us understand how nuances of biology caveat theory and contribute to the myriad of exceptions from patterns, there also remain major gaps in our theoretical and conceptual understandings of how infectious diseases should evolve in the simplest of systems. One such gap is how evolutionary processes at different scales link to determine how individual mutations randomly occurring in a single organism scale up into eco-evolutionary dynamics at the population scale. Specifically, when we think through evolutionary genetics frameworks, they tell us that phenotypes are determined by combinations of genetic variants that have distributions of fitness effects and, sometimes more importantly, distributions of pleiotropic fitness effects (Ardell & Kryazhimskiy, 2021; Crow & Kimura, 2009). Additionally, epistasis between these variants can lead to the same allele having different effects on different genetic backgrounds (Flynn et al., 2013). Selection acts upon what variation does exist to increase the frequency of variants beneficial in a specific environment but can be confounded by dynamics like clonal interference and genetic drift (Lang et al., 2013). Which mutations arise is therefore not determinate, even with static selection pressures. Furthermore, variants under selection are only influenced by the specific environment exerting selective pressures (Kinsler et al., 2020). Their effects in alternate environments are determined by their pleiotropic fitness effects. Understanding these pleiotropic fitness effects, or trade-offs, is therefore necessary to understand the effects of evolution on heterogeneous or changing environments.

Selection pressures, however, are certainly not static. They vary at every scale and the evolutionary dynamics of the population itself can reshape the environment and shift selection pressures. Eco-evolutionary theory tries to understand how optimal fitness strategies are shaped by these shifting dynamics in different environments and under different assumptions (Geritz et al., 1998). These models depend on assumptions about trade-offs to explain how ecological feedbacks can lead to frequency dependent selection (A. A. Agrawal et al., 2010). Trade-offs therefore link genetic processes to selection dynamics through to eco-evolutionary feedbacks and are the crux of understanding evolution.

Despite this centrality of trade-off theory to evolutionary biology, the genetic underpinnings of and selection dynamics on trade-offs remain poorly understood (Bono et al., 2019; Draghi, 2021; Fry, 1996; Remold, 2012). Thus, we still have little idea about how pleiotropic variants are selected through shifting selection pressures and how well the trade-off assumptions of eco-evolutionary models match the realities of genetic processes. The key predictions of eco-evolutionary trade-off theory are often qualitatively reproduced in nature and experiments, but attempts to directly empirically measure trade-offs frequently fail (A. A. Agrawal et al., 2010; Remold, 2012). These failures may be in part due to measurement limitations, but there also seem to be trends in which evolutionary conditions are more likely to produce genotypes with measurable trade-offs (Bono et al., 2017). This could suggest that trade-offs function inconsistently

across evolutionary contexts, but that underlying genetic mechanisms create trends amongst this inconsistency. Our limited understanding of the genetics of trade-offs presents a critical gap in our ability to understand patterns of biotic diversity as eco-evolutionary models of niche partitioning are crucially altered by trade-off shape. The assumed shape of the trade-off often determines whether a system branches to form a diverse assemblage of specialists or stabilizes on a single generalist (Boots et al., 2014).

What do I do in this dissertation?

My dissertation therefore focuses on trade-offs: what forms them, what influences them, and how they interact. To do this, I use experimental evolution methods in the *Plodia interpunctella*, or Indian meal moth, and granulosis virus model system (Gage, 1995; Mohandass et al., 2007; Vail & Tebbets, 1990a). Experimental evolution can be a powerful method to determine how specific factors influence the evolution of a trait as it allows theoretical predictions and assumptions to be tested in a controlled manner in the lab (Ebert, 1998; Kawecki et al., 2012). It therefore brings theory into actual biology while allowing specific questions to be examined.

I focus on two trade-offs. On the host side, I concentrate on the trade-off to resistance to viral in infection. Understanding trade-offs to resistance and when we expect costly or costless resistance strategies to evolve is necessary to predict when we expect to see resistance evolve and how it would alter ecological dynamics or persist in the absence of the pathogen (Boots & Bowers, 1999; Boots & Haraguchi, 1999). Understanding this trade-off has therefore been a major focus of previous work in the *P. interpunctella* and granulosis virus system (Bartlett et al., 2018; Boots, 2011; Boots & Begon, 1993, 1995; Boots & Roberts, 2012; Mealor & Boots, 2006). In chapter 1 of the dissertation, we extend this work to ask whether the trade-off to resistance is symmetric such that selection for the longer development time phenotype constituting a cost to resistance produces symmetric gains in resistance (Bartlett et al., 2020). However, we show that selecting for longer development time in this system selects for reduced resistance. This is important because it shows how phenotypes typically characterized by a trade-off can deviate from that trade-off relationship when there are many genetic variants that can shape both traits.

In chapter 2 of the dissertation, we ask why resistance inconsistently evolves in the system and how time scales and resource levels affect such resistance evolution and its costs. We find that populations selected for resistance with the dual stressor of low resource quality are slowed, but not prevented, from evolving resistance and that variation in starting populations or early sampled adaptations can lead to contingency towards context-dependent evolved resistance. Additionally, we find that some costs to resistance observed at early time points were compensated over longer evolutionary time scales. This is important because it informs perspectives for the predictability of adaptation and how variation in specific evolutionary conditions can alter the

evolutionary trajectories of a population towards costly or costless resistance strategies. In sum, this work means that the shape of a trade-off between two phenotypes can change depending on the specific selection conditions when trade-offs are influenced by many genes. This is important because such differences in trade-off shape would alter the outcome of evolution.

The second trade-off we focus on is on the virus side. Specifically, we examine how trade-offs between adaptation to different host genotypes influences host range evolution in our system. This trade-off is important because specificity in biotic interactions promotes diversity in co-evolutionary systems (Sexton et al., 2017). In chapter 3 we examine the genetic and phenotypic dynamics of host genotype specialization in the granulosis virus (Visher, Uricchio, et al., 2021). We find that the *Plodia interpunctella* granulosis virus consistently evolves increases in overall specialization, but that our two fitness components evolve independently such that lines can specialize in productivity or infectivity. We also find that specialization in our experiment is a highly polygenic trait best explained by a combination of evolutionary mechanisms. This is important for understanding the evolution of specialization in host-parasite interactions and its broader implications for co-existence, diversification, and infectious disease management.

In chapter 4, we experimentally evolve granulosis virus in mesocosms of its *Plodia interpunctella* host with varying degrees of spatial structure and host genetic diversity. We find that virus evolves specific interactions with its locally familiar host genotype in both homogeneous and spatially heterogeneous host populations, but that the impact of local adaptation depends on the spatial structuring of contacts. We also find that virus in heterogeneous mesocosms is more local adapted when there are higher migration rates between the host types. This is important because it demonstrates that trade-offs optimizing exploitation rates at intermediate values, like those governing pathogen infectivity in spatial structure, may interact with trade-offs determining niche breadth in ways that can reverse the impact of local adaptation on pathogen phenotypes. In sum, these results show that trade-offs between host genotypes might not follow simple functions and may depend on their interactions with other selection pressures. This is important because it suggests that costless generalism can exist in some evolutionary and ecological scenarios, thus interrupting evolutionary dynamics that depend on specialist interactions.

Acknowledgements

Pretty early in the PhD, I took that standpoint that my dissertation would not be the 'point' of my time here. Instead, it would simply be a way to showcase the work that I would do and all that I would learn along the way. And I cannot showcase that work and growth without celebrating all the people (and a cat) who have been so truly essential to this process. I wrote this whole essay once on how science is done by human beings in conversation with the communities around them. I stand by that. This dissertation is a testament to the many people who have pushed, supported, and inspired my growth in science, career, and life. Each of you has shaped me into the scientist who produced this dissertation and have made me a stronger, better, and more thoughtful human being. In everything that I do and have done, I can pick apart the threads of your influences and so I cannot picture who I would be as a scientist or person without all of you. So this one goes out to all of you.

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Chapter 1

The target of selection matters: An established resistance development-time negative genetic trade-off is not found when selecting on development time

Authors: Lewis J. Bartlett[*], Elisa Visher[*], Yazmin Haro, Katherine E. Roberts, Mike Boots

ABSTRACT

Trade-offs are fundamental to evolutionary outcomes and play a central role in eco-evolutionary theory. They are often examined by experimentally selecting on one life-history trait and looking for negative correlations in other traits. For example, populations of the moth *Plodia interpunctella* selected to resist viral infection show a life-history cost with longer development times. However, we rarely examine whether the detection of such negative genetic correlations depends on the trait on which we select. Here, we examine a well-characterized negative genotypic trade-off between development time and resistance to viral infection in the moth *Plodia interpunctella* and test whether selection on a phenotype known to be a cost of resistance (longer development time) leads to the predicted correlated increase in resistance. If there is tight pleiotropic relationship between genes that determine development time and resistance underpinning this trade-off, we might expect increased resistance when we select on longer development time. However, we show that selecting for longer development time in this system selects for reduced resistance when compared to selection for shorter development time. This shows how phenotypes typically characterized by a trade-off can deviate from that trade-off relationship, and suggests little genetic linkage between the genes governing viral resistance and those that determine response to selection on the key life-history trait. Our results are important for both selection strategies in applied biological systems and for evolutionary modelling of host parasite interactions.

INTRODUCTION

Trade-offs remain fundamental to modern ecological and evolutionary thinking for trade-offs interact (Acerenza, 2016; Shoval et al., 2012). More specifically, they are central

to our understanding of the evolution and ecology of infectious diseases (Alizon et al., 2009; Alizon & Michalakis, 2015; May & Anderson, 1983; Shoval et al., 2012)(and to resistance evolution to both chemical and biological pressures (Best et al., 2011; Best & Hoyle, 2013; Boots & Bowers, 1999; Boots & Haraguchi, 1999; Foster et al., 2011; Gandon et al., 2002, 2008; Gandon & Vale, 2014; Gemmill & Read, 1998; Gillespie, 1975; Gwynn et al., 2005; M. R. Miller et al., 2007; Sheldon & Verhulst, 1996; Shirley & Sibly, 1999). This large body of theoretical and empirical work has made substantial progress in our fundamental understanding of evolutionary trade-offs, but there remains the need to better integrate theory and empirical findings (Cressler et al., 2015; McKean et al., 2008; Schmid-Hempel, 2003). A clear understanding of fundamental evolutionary processes is crucial to the evolutionary management of resistance in pest and pathogen control (Brosi et al., 2017; Brown, 2002; Seifi et al., 2013; Yan et al., 1997), and therefore, investigating the fundamental nature of trade-offs with both empirical and theoretical studies remains of considerable importance.

Theory generally implicitly assumes that trade-offs are symmetrical such that selection on either trait results in a response in the other (Abrams, 2001; Geritz et al., 1998; Kisdi & Geritz, 1999). However, when traits within a trade-off are highly polygenic in their underpinnings, they may be able to evolve outside a simple genotypic trade-off, and therefore, selection on each trait may not result in the same reciprocal response to selection in the other (Stearns & Partridge, 2001). Fundamentally, traits involved in negative genotypic correlations may be tightly linked, but, equally, differences in the genetic architecture of the traits may mean that the effect of selection in the two traits is far from symmetrical (Stearns & Partridge, 2001). Testing whether the impact of selection is reciprocal on many trade-offs is difficult, partly because detecting trade-offs is already challenging (Cressler et al., 2015; McKean et al., 2008); however, it is central to the complete understanding of trade-off relationships (Stearns & Partridge, 2001).

One particularly well-evidenced trade-off is that of the resistance of *Plodia interpunctella* (Hübner) to the baculovirus, 'Plodia interpunctella Granulosis Virus' (PiGV). *Plodia interpunctella*, or the Indian meal moth, is a grain-feeding agricultural pest that is naturally infected by the baculovirus PiGV, an obligate killer that transmits by being orally ingested during larval cannibalism. The larvae of *P. interpunctella* are a widespread grain-feeding pest (Mohandass et al., 2007), which have been used as experimental study species for its ease of population maintenance and agricultural importance (Mohandass et al., 2007; Silhacek & Miller, 1972). Eggs are laid into cereal media by adults in a semelparous event, larvae then develop in the food media until pupation, and, following pupation, adult moths emerge, mate and lay a new generation of eggs (Gage, 1995). Adults do not have functional feeding physiology; their reproductive success is broadly determined by how quickly they can develop and their pupal mass (Boots & Begon, 1993; Silhacek & Miller, 1972). *Plodia* larvae can be infected by the baculovirus Plodia interpunctella Granulosis Virus (PiGV) (Vail & Tebbets, 1990b). PiGV infections are obligately lethal following infection via consumption of viral

occlusion bodies. Resistance is thought to occur mostly at the gut wall through mechanical barriers such as the peritrophic membrane and apoptosis of infected gut wall cells (Begon et al., 1993; Tidbury, 2012).

Two notable selection experiments have shown that *Plodia* experiencing selection through exposure to the pathogen evolve resistance to it at the cost of increased development time (Boots, 2011; Boots & Begon, 1993). This trade-off was further confirmed to be both genetic and constitutive (demonstrable even in the absence of exposure to infection), in a recent third experiment through the comparison of inbred lines (Bartlett et al., 2018). As such, this genotypic trade-off is particularly well defined since it has been shown in replicated selection experiments, under different resource conditions, and using inbred lines. This same system has also been used to infer trade-off shape from population level patterns of resistance (Mealor & Boots, 2006), and similar developmental trade-offs in *Plodia* have been demonstrated in the context of bacterial and parasitoid resistance (Niogret et al., 2009; Oppert et al., 2000). However, in all cases, selection has been applied only to resistance and the correlated changes in development time observed (Boots & Begon, 1993; Mealor & Boots, 2006). We do not therefore know how tight the correlation between the traits is and whether resistance is a constraint on the evolution of development time. Here, we test whether selection in the opposite direction along this trade-off that is, selection on the development rate, both faster and slower leads to correlated changes in resistance.

METHODS

Population maintenance and artificial selection

We maintained *Plodia* populations following well-established protocols. Our selection lines all originated from the same outbred laboratory stock population, which we have shown in previous studies to maintain appreciable amounts of genetic variation in life-history characteristics and resistance to PiGV (Bartlett et al., 2018). We originated eight lines, each as a starting cohort of 60 randomly selected, recently emerged *Plodia* adults (of unknown sex) placed on 200 g of fresh food media inside 1000-ml straight-side wide-mouth Nalgene jars (Thermo Fisher Scientific). We prepared food media in batches consisting of 250 g 'Ready Brek' (Weetabix Ltd.), 150 g wheat bran (Bob's Red Mill), 100 g rice flour (Bob's Red Mill), 100 g brewer's yeast (MP Biomedicals), 125 ml glycerol (VWR), 125 ml clear organic honey (Dutch Gold Honey Inc.), 2.2 g methyl paraben (VWR) and 2.2 g sorbic acid (Spectrum Chemicals). We homogenized the media with industrial mixers before it was sealed and frozen for a minimum of 24 hr prior to thawing at ambient- temperature for use.

We allowed adult moths to reproduce and then selected 60 of their adult moth offspring to found the next generation in a new jar of food media. How we select these sixty moths is how we differentiate our two selection regimes, dubbed 'early-' or 'late-' selected; of our 8 lines, 4 were assigned to the 'early' treatment, and 4 to the 'late'.

For the early-selected lines, we collected the first sixty next-generation adults that were counted during daily checks to found the next generation. Under this regime, only the very fastest developing larvae (relative to the rest of their population) were allowed to reproduce. For the late-selected lines, populations were checked daily. So long as abundant 5th-instar larvae were present, any adult moths were removed from the population and frozen. Once there were no 5th instar larvae, sixty adults were then randomly selected from the remaining population and transferred to a new jar of food media. In this way, we allowed only slower developing larvae to found the next generation for that line, although we could not guarantee these were the absolutely most slowly developing of their generation. Previous studies in this system have shown no difference in development time (our trait under selection) between male and female moths; for example, (Boots & Begon, 1994, 1995) show no effect of sex on development time across two large multifactor experiments. (Boots & Begon, 1993) did find a small significant difference according to sex in their analysis of two populations; if we analyse the data presented (Welch's t-test), we find that only one population shows a significant difference on development time based on sex (resistant population: $t_{107.0} = 1.72, p = .087$; control population: $t_{97.4} = 2.21, p = .029$) with Cohens' d for each equal to $d = 0.32$ and $d = 0.41$ respectively; we point readers to (Boots & Begon, 1993, 1994, 1995) for development time means and standard errors for male and female moths. We therefore have good evidence that there is broadly no, or only a small, difference in development time between sexes, leading us to believe our selection regime should not have skewed operational sex ratios and altered sexual selection in a meaningful way.

We maintained these selection regimes for approximately four years; however, the number of generations this time period represents is different for each line due to their differences in development time (Table 1). We maintained all selection lines in a single incubator throughout the experiment, where they experienced a constant climate of $27 \pm 2°C$ and $35 \pm 5\%$ humidity, with 16:8 hr light:dark cycles. Following this period of maintenance and selection, we removed the selection pressure for two generations of *Plodia* where next-generation founders were randomly selected within each line. This is typical in such *Plodia* studies as ours to try and mitigate plasticity or parental effects (Boots, 2011; Roberts et al., 2020). We then assayed the lines' life history and level of resistance.

Table 1. Number of generations of selection experienced by each line at the point of assaying, and which assay block each line was assigned to.

Line	Selection regime	Generations of selection	Assay block
E1	Early	52	A
E2	Early	55	A
E3	Early	48	B
E4	Early	51	B
L1	Late	37	A
L2	Late	38	B
L3	Late	40	B
L4	Late	37	A

Resistance and life-history assays

We undertook assaying of resistance and development in two blocks: four lines were assayed per block, with two early-selected and two late-selected lines in each block. The two blocks were separated by approximately one calendar month. This protocol was used due to the limitation of asynchronous generation timings between lines.

We characterized the life-history traits of each line using two measures: time to pupation (development time) and pupal mass. For each line, we took sixty adult *Plodia*, known to have eclosed in the last 24 hr, placed them in jars of new food media and incubated them under the conditions described above. After 11 days, we selected fifty larvae on the 1st day of their 3rd instar from each line and placed them in individual compartments on 25-cell compartmentalized square petri dishes (Thermo Fisher Scientific) (two petri dishes per isoline) with ample food media. We can identify 1st-day 3rd-instar larvae based on the size of their head (which changes only during moulting and identifies different instars) and the size of their body (which if smaller in diameter than the head signifies their 1st day at that instar). Petri dishes were then incubated as above and checked daily to monitor larval development. We recorded the date of each larva's pupation, and two days later, the pupa was extracted from its silk cocoon and weighed using a 1-µg precision microbalance. Growth rate of each individual was calculated as its mass at pupation divided by its days to pupation. Not all larvae were recovered, as some inevitably die due to handling or other causes of stochastic mortality, or are damaged during pupal extraction from cocoons ($\overline{\overline{n}}$ = 22.375/50 pupae recovered per line).

We measured the resistance of each line to PiGV by comparing infection rates of larvae to different PiGV doses. We took 150 1st-day 3rd-instar larvae from each line, following the same protocol as described above. We placed larval cohorts of fifty larvae into circular petri dishes (three cohorts per line) and starved the larvae for one hour. We then pipetted droplets of virus solution into these petri dishes, with each cohort given one of three solutions. Virus solutions represented three doses, each diluted by an order of magnitude (such that the strongest dose is 100 times stronger than the weakest). We diluted solutions with distilled water, and all solutions contained 2% sucrose (Thermo Fisher Scientific) and 0.1% Coomassie Brilliant Blue R-250 dye (Thermo Fisher Scientific). We left larvae to voluntarily feed on the solution droplets, encouraged by their brief starvation and the solution sucrose content. We considered an individual larva dosed when 50% of their alimentary track was stained blue (visible due to the blue dye and translucent larval body) at which point we removed them from the petri dish and placed them individually into cells of 25-cell compartmentalized square petri dishes, before incubation for twenty days as above. After twenty days, we froze the petri dishes to kill all remaining live larvae, before opening them for counting. Infected larvae are apparent due to their bright white cadavers, a consequence of the accumulation of viral occlusion bodies in the haemolymph. Uninfected larvae were distinguishable as healthy larval cadavers or as developing pupae or adults. Not all larvae were recovered to be categorized as either infected or uninfected, as some inevitably die due to handling or other causes of stochastic mortality ($\overline{\overline{n}}$ = 16.75/25 larvae recovered per line per dose).

Additional assays

The eight experimental populations were additionally assayed for both life history and resistance at the beginning of the experiment after the first generation under the selection regime (generation = 1). These assays were all undertaken on different days due to the asynchrony of the experimental populations throughout the selection process and were performed in a different laboratory using a different viral stock solution, and therefore, 'doses' used between the initial generation = 1 assays and the final main assays are not the same. Therefore, the comparison across the different experiments must be interpreted with caution but we include some analyses of these initial assays to better inform the framing of our findings.

Further, following the first analysis of our main findings, we sought to better frame the results of our main experiment in the context of established trade-offs in this system by re-assaying life history and resistance of three inbred populations from (Bartlett et al., 2018). To improve comparability of these new assays, we used the same viral stock solutions (same doses) as the main experiment, and the assays we undertaken in the same laboratory in the same year as the main experiment. These assays could not be undertaken concurrently with the assaying of the early- and late-selected lines and so direction comparison is partially confounded by potential day effects.

Statistical analysis

All analyses were undertaken in R (v.3.4.4 'Someone to Lean On') (R Core Team, 2021).

With the exception of the re-assayed inbred lines, we analysed all assay data using a generalized linear mixed-effects modelling approach to account for our hierarchical experimental design. We tested for significance of fixed effects using the 'afex' package (Singmann, Bolker, Westfall, Aust, & Ben-Shachar, 2019) which integrates with the generalized linear mixed-modelling 'lme4' package (Bates, Mächler, et al., 2015; Bolker et al., 2009), and coupled this with the 'emmeans' (Lenth et al., 2022) package to estimate effect sizes where appropriate. For the main experiment, we tested for a significant effect of treatment on growth rate, development time, pupal mass, and susceptibility to the pathogen. Random effects were the blocking factor 'Block' (see Table [1]), and 'Line' nested under 'Treatment' to account for our hierarchical experimental structure. 'Treatment' was the only fixed effect for analysis of growth rate, development time, and pupal mass; 'line', 'dose', and an interaction between the two were the fixed effects for the analysis of infection data by line, and 'treatment' and 'dose' were the fixed effects for analysis of infection data by selection regime. Models for growth rate and pupal mass used a Gaussian error structure, for development time used a Poisson error structure, and models for infection assays used a binomial error structure. Because of this, all model comparisons used a likelihood-ratio test ('LRT') method (see documentation for 'afex' package, (Singmann, Bolker, Westfall, Aust, Ben-Shachar, et al., 2019)).

Initial assays of the experimental lines at generation = 1 used the same approach as above, with the exception of not including a blocking factor as each population was assayed on a different day. The three re-assayed inbred lines from Bartlett et al. (2018)were all assayed concurrently and had no hierarchical experimental structure; we calculated simple arithmetic means for their life-history assays, and for infection assays used generalized linear models with a binomial error structure including dose and line as fixed, possibly interacting effects. We examined the rank-order change in line susceptibility from the start to the end of the experiment, according to selection regime, through way of an ANOVA on regular ranks (using the 'npIntFactRep' package (Feys, 2016)).

We further investigated a potential correlation between life-history traits and susceptibility to infection, similar to that shown in (Bartlett et al., 2018). We used the GLMMs from our analysis of development time and infection to predict an expected development time and expected proportion of individuals infected at the highest virus dose for all eight lines if they were assayed concurrently. We plotted these values and tested for a correlation using a Pearson's correlation.

We provide an annotated R script and curated data for reproducibility of all analyses in association with this manuscript.

RESULTS

<u>Main experiment</u>

Experimental selection regime had a significant effect on growth rate ($p = .03$) where the late-selected line growth rate was 0.31 mg/day lower than the early-selected line (Figure **2**). This was driven by a significant effect of treatment on development time ($p = .02$) where late-selected lines pupated 7.0 days later than early-selected lines (Figure **2**); consistent with previous work in this system (Bartlett et al., 2018; Boots, 2011; Boots & Begon, 1994), there was no effect of selection on pupal mass ($p = .55$).
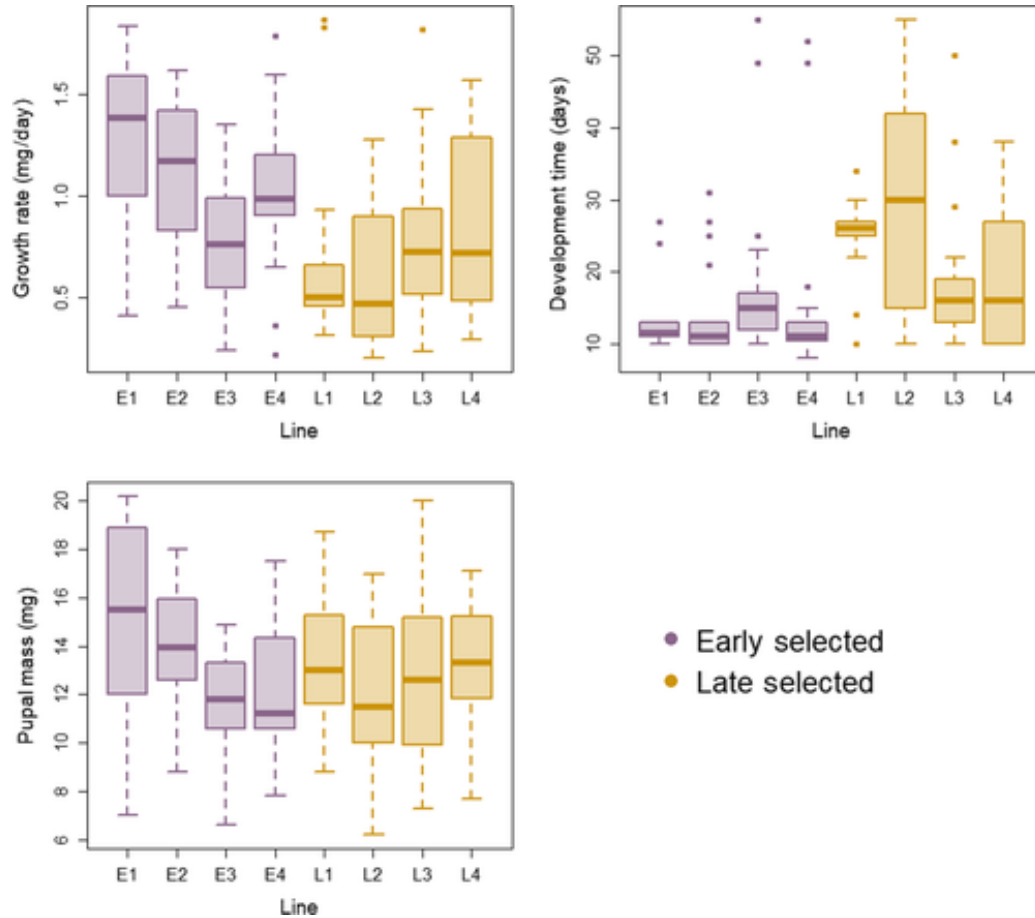
Figure 2: Paneled box plots illustrating life-history traits of each line (growth rate, development time, pupal mass). Early-selected lines are shown in purple and left-aligned on each subplot; late-selected lines are shown in orange and right-aligned on each subplot. Significant differences amongst lines and between treatments were found for growth rate and development time, but not pupal mass. Early-selected lines had shorter development times and therefore higher growth rates.

Experimentally selected lines also showed significant variation in their resistance to PiGV (Figure **3**). We found no evidence ($p = .33$) of an interaction effect between 'line' and 'dose', indicating no heterogeneity in terms of dose response (again in agreement with previous work in this system (Bartlett et al., 2018; Boots, 2011; Boots & Begon, 1994)).

However, lines did differ in their overall resistance to the pathogen ($p = .005$), illustrated in Figure 3. We found inconclusive evidence of an effect of selection regime on resistance to the pathogen at the end of the experiment, regardless of if we modelled based simply on 'treatment' (presented here) or based on an estimated cumulative selection differential (identical results, not presented in this study). Late-selected lines were possibly more susceptible to the virus than early-selected lines (Figure 3); estimated effect sizes (likelihood of infection) for early- and late-selection regimes (on a hypothetical average dose) were that early-selected individuals had a 0.09 probability of infection (95% CI: 0.04 0.19) whereas late-selected individuals had a 0.23 probability of infection (95% CI: 0.12 0.39), and the effect of selection regime was not significant ($p = .07$), although see additional analyses below.
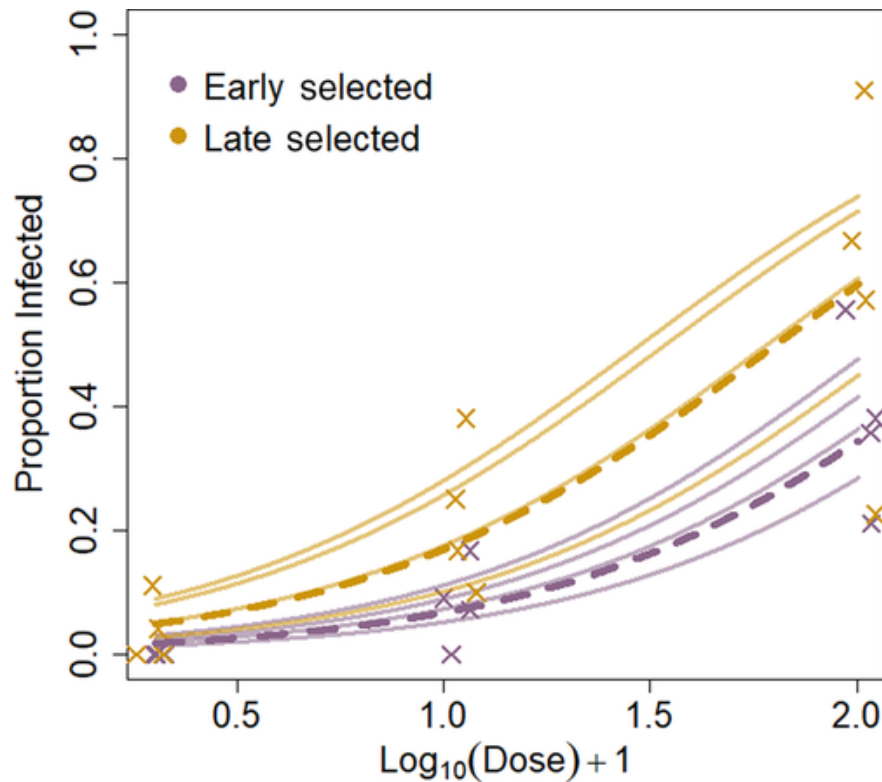
Figure 3: Plot showing resistance of each line to PiGV. Plotted crosses are proportion of larvae infected, where dose strength is plotted in logit space (note that a jitter has been applied to the plotted points along the x-axis for easier visualization). Plotted solid curves represent the expected proportion of infected larvae with increasing dose for each line, and the heavier dashed curves represent the predicted proportions infected based off treatment; all curves are predicted values from the corresponding GLMMs. The early-selected lines are plotted in purple; the late-selected lines are plotted in orange. Lines do not show heterogeneity of dose response, but do significantly differ in their resistance to PiGV

We further investigated the link between line development time and susceptibility to the pathogen by examining if there was a correlation between these two phenotypes across all eight lines (Figure 5). We found no evidence for a correlation between growth rate and susceptibility (Pearson's Correlation, $t_6 = 1.06, p = .33$). We do not have the necessary level of replication to test with any power for a correlation between line development time and susceptibility within each treatment, and caution that even for a 'collated' correlation, eight replicate populations remain a low level of replication for even simple correlative analysis.

Additional assays

We analysed two additional data sets following our main analysis, to better frame the results of our main experiment. We undertook a similar analysis as above on assays of life history and resistance of the 8 experimental lines at generation = 1 (after one single generation of selection). These analyses are confounded by each line being assayed on a separate day, as during the selection experiment the populations are asynchronous in their reproductive bouts, and so results should be interpreted cautiously. We found no significant difference in growth rate at the treatment level between the lines set-up to be late-selected or early-selected ($p = .93$), illustrated in Figure **S1** (Chapter 1 Supplementary Material). We did find evidence of a bias in resistance at set-up between the two treatment groups ($p = .005$), however this was in the direction of the late-selection populations being more resistant than the early-selection population opposite to the direction of the difference in susceptibility according to treatment at the end of the experiment. We cannot directly, quantitatively compare start and end of experiment population susceptibilities as they were assayed using different viral stock; however, we can examine the rank-order change of line resistances across selection regimes between the start and end of the selection experiment (see Figure **4**). A regular-ranks ANOVA shows a significant effect of selection regime on the change in line resistance rank order from the start to the end of the experiment ($F_{1,6} = 18.3, p = .005$), where early-selected lines were significantly more likely to move up the resistance rank order and late-selected more likely to move down. This suggests that during the experiment, late-selected lines became more susceptible, early-selected lines became less susceptible, or both.
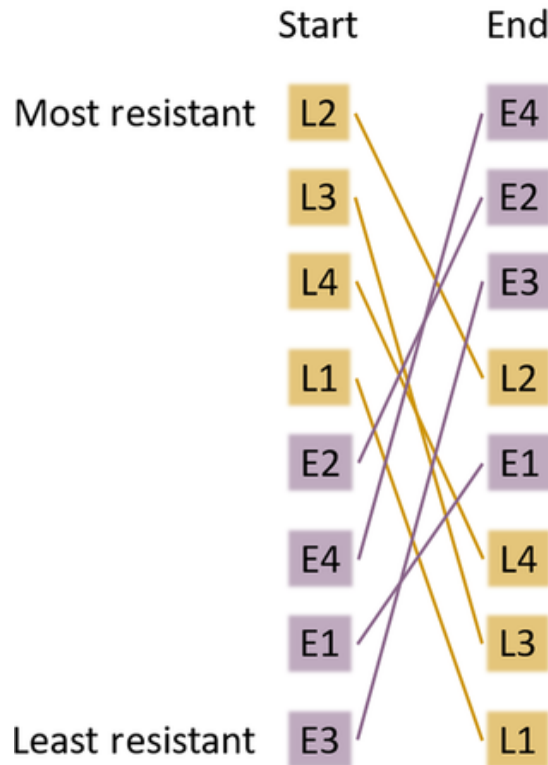
Figure 4: Rank-order change in resistance to PiGV of replicate Plodia lines at the start (after one generation) and end (after ≥37 generations, see Table 1) of the selection experiment. We find a significant effect of selection regime on the rank-order change between the start and end of the experiment (ANOVA on regular ranks: F1,6 = 18.3, p = .005). We present evidence (confounded by different assay dates) that there was a stochastic bias at the start of experiment, where late-selection lines were significantly more resistant (p = .005).

For comparison to our experiment end-point results, we analysed the susceptibility to infection and growth rates of our three re-assayed inbred lines surviving from Bartlett et al., (2018), calculating a mean development time and estimated likelihood of infection at the highest dose; notably for these populations, assays were undertaken shortly after the assay of our main selection experiment using the same conditions and same virus stock, however were not assayed on the same days as our early and late lines. We present a replotting of Figure **5** in the Supplementary Material (Chapter 1 Figure **S2**) showing how these inbred lines compare to the selection lines in susceptibility/ development time phenotype space.
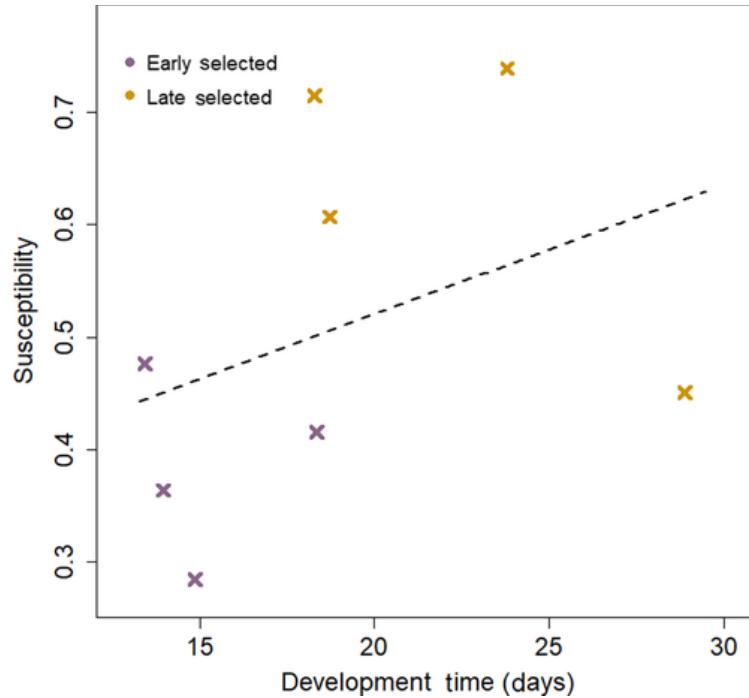
Figure 5: Graph showing expected likelihood of infection at max dose (susceptibility) and expected development time of each line, based on model predictions. Although the general finding that early-selected lines (purple) were significantly faster growing and possibly less susceptible than late-selected lines (orange) is apparent, there was no strong compelling evidence for a correlation between growth rate and susceptibility on a line-by-line basis.

DISCUSSION

Our results emphasize the importance of examining the fundamental underpinnings of both sides of genotypic trade-offs, as results from selecting on one trait may not be reflected when selection acts on the other. We successfully selected on development time (Figure 2), and consequently overall growth rate, with no change to pupal mass. Our finding of no significant effect on pupal mass is in agreement with previous work selecting on resistance (Bartlett et al., 2018; Boots, 2011; Boots & Begon, 1993; Oppert et al., 2000), but is notable in that here we were directly selecting on a life-history trait. Our key result is that early-selected lines were equally or less susceptible to infection (Figures 3 and 4) compared to late-selected lines, counter to the well documented trade-off between pathogen resistance and development time in this species found when we select on resistance (Bartlett et al., 2018; Boots, 2011; Boots & Begon, 1993; Oppert et al., 2000). That is resistance and development time are negatively correlated when we select on resistance or when populations evolve by genetic drift, but are unlinked or possibly positively correlated when we select on development time. Our results should not be interpreted as evidence against the existence of an otherwise robustly supported genotypic trade-off between resistance and development time in our system. Rather, our findings offer some insight into the likely polygenic underpinnings

of these traits, the mechanisms of immunity in this system, as well the potential caveats of the interpretation of selection experiments.

Our key result is that when we selected on development time, we found evidence of a correlated response on resistance that is inconsistent with, or even opposite to, the established trade-off in this system. The previously documented trade-off is constitutive and genetic (Bartlett et al., 2018), but we have no explicit data on how many genes may be involved in resistance and linked to development time. Ongoing work in this system putatively points to multiple, context-dependent evolutionary routes to resistance (Roberts et al., 2020), and that resistance traits are polygenic. It is clear that variation in insect life-history phenotypes are also often highly polygenic (Comeault et al., 2014; Jha et al., 2015), with many interacting genes responsible for determining fecundity, growth rates, or size at maturation. Our results are likely to be a consequence of such polygenic underpinnings where the set of genes that responded to selection on development time here were not those that affect resistance in the predicted direction. This shows that the full set of genes governing life-history phenotypes have mixed correlations with those involved in viral defence. An informative ideal test of these observations in this system would be a single experiment where populations are subjected to a 2×2 selection regime, combining early versus late selection on development time (as here) and exposure or no-exposure to PiGV (as in previous selection experiments). Such a selection experiment would illustrate the possible multiple routes to immunity which may involve in different contexts, as has been recently done in this system comparing nutritional selection regimes in tandem with virus exposure regimes (Roberts et al., 2020).

Similar experiments selecting along both traits in a trade-off have been conducted in the life-history experimental evolution literature (summarized in **Stearns & Partridge, 2001**). For example, Hillesheim & Stearns (1991) and Zwaan et al. (1995) both examined the life-history trade-off between weight at eclosion and development time. Hillesheim & Stearns (1991) selected on body size, whereas Zwaan et al. (1995) selected on development time. In this case, the trait under selection did not affect the correlation of these traits in both experiments larger flies had longer development time. In a second analysis, Hillesheim & Stearns (1992) examined the lifespan of fly lines selected for divergence in body size and found that larger flies had shorter lives than smaller ones. However, when Partridge et al. (1999) selected for longevity, they found no difference in weight between long and short lived flies. Additionally, when Stearns et al. (2000)) selected for increased intrinsic mortality by increasing extrinsic mortality rates, they found that flies were both shorter lived and smaller. In these cases, the sign of the genetic correlation of these traits in this system varied depending on the trait under selection as it did in our experiment here in comparison to previous experiments elsewhere.

As molecular genetic and sequencing methods become increasingly widespread and affordable, efforts to link specific genes to selection experiments or evolutionary trade-

offs have become more common (Korte & Farlow, 2013). However, identifying the genetic bases of trade-offs remains challenging, especially when traits are determined by more than a few pleiotropic or linked genes. There are systems where there has been success in identifying pathogen-resistant quantitative trait loci (Zhong et al., 2005); however, our findings suggest that it will be challenging for current genomics approaches to determine the full set of genes that co-regulate the resistance-development time trade-off in *Plodia* (Gassmann et al., 2009). Our selection experiment emphasizes that the set of genes identified in quantitative trait locus studies will depend on the population's selective history and therefore that these results from experimental evolution should be interpreted cautiously. However, reciprocal selection experiments such as ours could be harnessed to identify more complete sets of pleiotropic genes since selection experiments along multiple axes may select on a broader set of pleiotropic genes that underlie linked traits.

The differences in resistance that we observed have multiple plausible explanations with potential insight for related future experiments. One possible hypothesis is that haemocoelic immunity, which has been shown to vary between populations in this system (Saejeng et al., 2011), is unaltered between our selection lines, whereas midgut immunity is inherently greater in the fast-developing lines due to greater likelihood of shedding viral occlusions bodies before infection occurs through accelerated ecdysis. Engelhard & Volkman (1995) showed in a similar lepidoptera-baculovirus system that an age difference between larval instars of just a few hours significantly affects infection likelihood and that larvae were able to fully clear early infections from the midgut epithelium during ecdysis. Interestingly, similar work in mosquitoes has shown that faster development correlates with increased resistance to an ingested pathogen (Koella & Agnew, 1999; Yan et al., 1997), yet some of those same authors show that late-selected mosquito lines exhibit higher haemocoelic immune activity than early-selected lines (Koella & Boëte, 2002), counter to their previous findings. Furthermore, there is fundamental theoretical work exploring how accelerated development can be an adaptive response to age-structured infection (Hochberg et al., 1992). It is therefore plausible that this study has indirectly increased or maintained resistance in the faster-developing lines by allowing larvae to escape the establishment of successful infection by developing quickly, potentially through more rapid ecdysis after inoculation. Although there are numerous insect-resistance studies that similarly seem to identify such 'costless resistance' (Faria et al., 2015; Milks et al., 2002; Undorf-Spahn et al., 2012), these findings are difficult to reconcile with theory and widespread observation in variation of resistance (Koskella, 2018; Schmid-Hempel, 2003; Susi & Laine, 2015), and it is acknowledged that trade-offs are difficult to tractably characterize experimentally (Cressler et al., 2015). If our early-selected lines have evolved to develop faster without a loss of, or even with a corresponding gain in resistance, we speculate it is at the expense of some other competitive axis which doesn't manifest in these experiments.

It must also be emphasized that the early-selected lines may not be benefiting from higher resistance, but that the nature of our experimental design may have led to late-selected lines being particularly vulnerable to infection. It is well established that many mutations are purely detrimental, constituting 'genetic load' (Crnokrak & Barrett, 2002; Whitlock & Bourguet, 2000; Wielgoss et al., 2013); captive managed (laboratory) populations may harbour much larger genetic loads than their wild counterparts (Bryant & Reed, 1999) on the basis of relaxed selection. Reduced growth rate is a disadvantageous trait (Boots & Begon, 1993; Bowers et al., 1994a; Silhacek & Miller, 1972), and therefore by selecting for a broadly less-fit phenotype, we may have simply inadvertently selected for individuals of broadly low quality that harbour large numbers of deleterious alleles and significantly elevated genetic loads across the population. As such, our 'late-selected' treatment may have manifested as selection for poor performing low-fitness phenotypes, and therefore it may be no surprise that these populations show elevated susceptibility to infection. Without characterizing the ancestral phenotypes at the same time as our two selection lines, we cannot determine if both our early-selected and late-selected lines show changed susceptibility and development time compared to their ancestral state. We have data on the ancestral case demonstrating changes in relative development times and susceptibility, but without assaying a non-selected 'ancestral' treatment alongside the early- and late-selection treatments, we cannot make informative direct comparisons.

Future investigations of these selection lines may provide insight if assayed in direct comparison to a larger number of inbred lines similar to those described in Bartlett et al. (2018) under one single experiment. We partially attempted this using the three surviving inbred lines from the Bartlett et al. (2018)study; however, note that those lines were assayed on a separate date to blocks A and B of this main experiment (Table **1**) and so direct comparisons are difficult. However, we still present a tentative appraisal of how our selected lines compare to these inbred lines in Figure **S2** (Chapter 1 Supplementary Material); we note that the inbred lines from Bartlett et al. (2018) were originated from the same outbred starting population at approximately the same as the selection lines presented in this study. Although our interpretation is speculative, the apparent result is that the early-selected lines mostly sit along the same trade-off as the three assayed inbred lines, whereas the late-selected lines have moved away into phenotype space which would under a wild-type scenario be seen as noncompetitive. This suggests that the late-selected lines have not evolved along the trade-off, but may rather have evolved away from it by increasing development time with either no change, or a decrease, in resistance to the virus. This could be through accumulation of broadly deleterious mutations, or a demonstration of the highly polygenic nature of life-history traits, as discussed above.

In conclusion, our results suggest a polygenic underpinning of the established trade-off in *Plodia* and its immunity to PiGV. These results highlight potential mechanisms of immunity which may be worth further investigation, notably accelerated growth to

escape infection, and in this case rapid ecdysis clearing midgut epithelial infection. Finally, this study illustrates some of the challenges of selection experiments investigating trade-offs, such as the potential of inadvertently selecting for evolution away from, rather than along, a phenotypic trade-off, especially by accumulation of broadly deleterious mutations.

Chapter 2

The evolution of host resistance to a virus is determined by resources, historical contingency, and time scale

Authors: Elisa Visher, Hannah Mahjoub, Khadija Soufi, Nilbert Pascual, Vivian Hoang, Lewis J. Bartlett, Katherine Roberts, Sean Meaden, Mike Boots

ABSTRACT

To predict the evolution of any trait, we must understand the evolutionary constraints and trade-offs acting on that trait. In the context of hosts evolving resistance to parasites, these trade-offs will determine optimal strategies, cycling, diversification, and maintenance of resistance. However, trade-offs are often inconsistently measured across experiments and can depend on environmental conditions. Here, we extend a selection experiment evolving resistance to viral infection under variable resource quality in the *Plodia interpunctella* model system to explore the evolutionary conditions leading to an incongruent earlier measurement of costless resistance. We find that environmental resource quality, historical contingency, and the time scale of selection all affect trade-offs in our long-term selection experiment. Specifically, populations selected for resistance with the dual stressor of low resource quality are slowed, but not prevented, from evolving resistance. Second, variation in starting populations or early sampled adaptations led to contingency towards context-dependent evolved resistance. Finally, some costs to resistance observed at early time points were compensated over longer evolutionary time scales. Our work therefore informs perspectives for the predictability of adaptation and how variation in specific evolutionary conditions can alter the evolutionary trajectories of a population towards costly or costless resistance strategies. (196/200)

INTRODUCTION

Trade-offs between life-history characteristics are critical to evolutionary outcomes and are central to many of our theories for adaptation and diversification (Ackermann & Doebeli, 2004; A. Agrawal & Lively, 2002; Darwin, 1859; Garland et al., 2022; Levins, 1968). In the case of a host evolving resistance to a pathogen (or other stressor), trade-offs between resistance and other fitness-relevant traits can lead to the evolution of: (1) intermediate (optimal) resistance strategies, (2) diversification through negative frequency dependent selection from ecological feedbacks, (3) resistance cycling, and/or (4) the loss of resistance in the absence of the stressor (Andersson & Hughes, 2010; Best

et al., 2010; Boots, Best, et al., 2009; Boots et al., 2014; Boots & Bowers, 1999; Boots & Haraguchi, 1999; Bowers et al., 1994b; Cotter et al., 2004; Fuxa & Richter, 1989; Gillespie, 1975; Graham et al., 2005; Kirk et al., 2021; Koskella, 2018; Moret & Schmid-Hempel, 2000; Roy & Kirchner, 2000; Schmid-Hempel, 2003). Because of this importance, costs to resistance have been measured in many empirical systems (Auld et al., 2013; Brown, 2002; Cory, 2017; Duncan et al., 2011; Herren & Baym, 2022; Kawecki, 2020; McGonigle et al., 2017; Susi & Laine, 2015; Vale et al., 2015; Zhong et al., 2005), but see (Faria et al., 2015; Milks et al., 2002; Penley et al., 2018; Tavalire et al., 2018; Undorf-Spahn et al., 2012). Understanding the costs to resistance is essential to being able to predict when resistance has a high potential to evolve and persist in scenarios ranging from antibiotic resistance of pathogens (Herren & Baym, 2022), resistance management in the control of invasive species and pests (P. J. Kerr et al., 2017), and coevolutionary arms races (Brodie et al., 2002). There are therefore both fundamental and applied reasons to measure both the strength and shape of trade-off relationships (de Mazancourt & Dieckmann, 2004; Duncan et al., 2011; Ehrlich et al., 2020; Farahpour et al., 2018; Hoyle et al., 2008; Jessup & Bohannan, 2008; Kamo et al., 2007; Kasada et al., 2014; Maharjan et al., 2013; Mealor & Boots, 2006).

Despite the central importance of trade-offs, evolutionary biology has consistently been plagued by the issue that trade-offs are difficult to measure and inconsistently observed (Bono et al., 2017; Cressler et al., 2015; Fry, 1996; Visher & Boots, 2020). This has led to a rich body of empirical work that attempts to discern factors that influence when trade-offs are observed (ex. Bono et al., 2017; Fry, 2003; Stearns, 1989). When trade-offs are not observed, this can sometimes be due to measurement error where costs to some adaptive phenotype exist in fitness dimensions that are not measured (Kawecki, 2020; Kinsler et al., 2020). In other cases, high environmental quality may obscure trade-offs as the organism can allocate resources to buffer costs (Gómez et al., 2015; Jessup and Bohannan, 2008; Kraaijeveld and Godfray, 1997; Luong and Polak, 2007; McKay et al., 2016; McKean et al., 2008; but see Zeller and Koella, 2017), though the relationship between resources and resistance evolution may be less clear if we consider that parasites can mediate trophic changes that alter the availability of resource (Pascua et al., 2014; Walsman et al., 2021) or if parasite resistance mechanisms correlate with other traits (Dargent et al., 2013; Stephenson, 2014). In yet other cases, the likelihood of a population evolving costly (or costless) strategies may be influenced by its specific evolutionary conditions including the environment that it is adapting to, the time scale of selection, the heterogeneity (spatial or temporal) of the environment, and the starting genotypes in the population (Bono et al., 2017; Card et al., 2019; Remold et al., 2008). A better understanding of how these processes define both the nature and our potential to measure trade-offs requires the detailed analysis of well described trade-off relationships.

One of the better characterized trade-offs in evolutionary ecology has been the trade-off between resistance to viral infection and development time in the *Plodia*

interpunctella (Indian Meal Moth) (Hübner) and *Plodia interpunctella* granulosis virus (PiGV) model system (Boots & Begon, 1993). The trade-off whereby increased resistance comes at a cost of longer development time has been established repeatedly by laboratory experimental evolution selecting for resistance (Boots, 2011; Boots & Begon, 1993; Niogret et al., 2009; Oppert et al., 2000), by assaying natural populations with phenotypic variation (Boots & Begon, 1995), and by assaying inbred genotypes with phenotypic variation in the absence of infection (Bartlett et al., 2018). It has also been established that costs to resistance can be mediated by the quality of resources provided to the population during evolution so that populations evolving with low-quality resources evolve lower, more costly resistance (Boots, 2011). However, this trade-off has recently proven breakable because laboratory experimental evolution selecting on development time (rather than resistance) results in fast development selected populations actually having higher resistance than their slow development selected counterparts (Bartlett et al., 2020). Additionally, a second laboratory evolution experiment evolved resistance under high and low resource quality a second time, as in (Boots, 2011), to explore the genetic basis of resistance (Roberts et al., 2020). In this experiment, the authors saw inconsistent, context-dependent resistance evolution and did not find trade-offs between resistance and growth rate in virus-selected populations evolved in high-quality resources. Furthermore, virus-selected populations evolved in low-quality resources did not evolve significant resistance, and what resistance they did evolve only showed trade-offs in the low-quality, but not common garden, environment. These results provide an exciting opportunity to explore how variation in specific ecological and evolutionary conditions can alter the evolutionary trajectories of a population towards costly or costless resistance strategies.

Compared to (Boots, 2011), (Roberts et al., 2020) use the same selection conditions of constant PiGV-exposure and different resource qualities, but, in contrast, they selected from a genetically distinct starting population. The variation in evolution outcomes may be explained by this difference in starting population genetics. However, the fact that the populations in (Roberts et al., 2020) inconsistently evolve resistance also suggests that the lack of trade-offs could be because these populations are evolutionarily 'behind' (despite the (Roberts et al., 2020) lines being assayed after 14 generations of selection, compared to the 10 in (Boots, 2011)). If the populations are simply 'behind' in evolution, they may still be able to fix only costless resistance strategies and are not yet running into evolutionary costs (Bono et al., 2017; Y. Li et al., 2019). In this paper, we examine the repeatability of trade-off observation by testing the hypothesis that differences in evolved resistance and its costs are simply due to slower evolution in the second experiment (Roberts et al., 2020). To do this, we extend the time scale of selection on a subset of the (Roberts et al., 2020) populations and re-assay resistance and development time in common garden and home quality environments. Extending the time scale of selection also allows us to test additional questions about the temporal dynamics of costs and whether they can be compensated over longer time scales (Andersson & Hughes, 2010) and about the longer-term dynamics of evolution under dual stressors (in

this case virus infection and low resource quality) (Hiltunen et al., 2018). We therefore explore whether: 1) resistance will evolve if given more time under selection, 2) if the lack of trade-offs in these populations can be explained by them being 'behind' in evolution and therefore not at Pareto fronts where resistance phenotypes are constrained (Y. Li et al., 2019; Shoval et al., 2012), and 3) whether longer evolutionary time scales will allow populations evolving under low resource quality to 'catch-up' to those evolving in high resources. By exploring these questions, we gain insight into the historical contingency of resistance evolution and its trade-offs.

METHODS

Study system

Plodia interpunctella (Hübner), the Indian meal moth, is a stored grain pest with cyclical population dynamics in the lab. During their five larval instar stages, *P. interpunctella* live at high population densities within the food that they were laid into and consume. After the fifth instar, *P. interpunctella* pupate and eclose. *P. Interpunctella* adult moths do not eat and primarily disperse, mate, and reproduce (Gage, 1995; Mohandass et al., 2007; Silhacek & Miller, 1972). Plodia interpunctella granulosis virus (PiGV) is an obligately lethal, dsDNA baculovirus (R. L. Harrison et al., 2016a) that infects *P. interpunctella*. Larvae orally ingest PiGV occlusion bodies that have been released into environment or during the process of larval cadaver cannibalism. For successful infection to occur, the virus must shed its protein coat in the gut and infect gut epithelial cells in the budded virus form, cross the gut membrane to establish systemic infection though the larval fat body, and then package into the infectious, protein-coated occlusion body form, at which point the infection kills the larvae. Infection can be cleared before the host is fully infected through various resistance mechanisms including gut ecdysis during molting stages, freeing the host to carry out its natural life cycle and pupate into an adult moth (Boots & Begon, 1993; Engelhard & Volkman, 1995).

Host line selection and maintenance

Populations of *P. interpunctella* were initially established from an outcrossed *P. interpunctella* population by (Roberts et al., 2020) and selected under 4 treatment conditions: high-quality food with virus (VHF), low-quality food with virus (VLF), high-quality food without virus (CHF), and low-quality food without virus (CLF). Food quality was manipulated by replacing a portion of the cereal mix (50% Ready Brek ©, 30% wheat bran, and 20% ground rice by weight) with either 10% (high-quality food) or 55% (low-quality food) methyl cellulose, a non-digestible fibrous bulking agent, by weight. This alters the amount of nutrition available to the larvae without altering feeding rates (Boots, 2011; Boots & Begon, 1994; Boots & Roberts, 2012). These dry cereal mixtures were then mixed with brewer's yeast (100g per 500g dry mix), honey (125mL per 500g dry mix), and glycerol (125mL per 500g dry mix) to form the control (CHF and CLF) food types. For virus food types (VHF and VLF), virus from a stock solution of

PiGV was also mixed into the food at a dose corresponding to LD20 for the ancestral *P. interpunctella* population (see (Roberts et al., 2020)).

For our experiment, we maintained 5 replicate selection lines per treatment (20 lines total) from the populations established by (Roberts et al., 2020) based upon their history of population bottlenecking and health, but not due to their resistance or development time. We continued selection of these lines for >36 generations past their initial 14, so that each line had a cumulative selection time of >50 generations (corresponding to ~4 years). Note that differences in generation time compounded over the years so the generation numbers of selection lines at the second (final) set of assays varied up to 10 generations.

To continue selection, populations were reared under the same conditions as in (Roberts et al., 2020): 1000mL straight-side wide-mouth Nalgene pots (ThermoFisher Scientific, U.K.) with 200g of their appropriate food medium in separate virus and control incubators set at 27±2 °C and 35±5% humidity, with 16:8hr light: dark cycles. Each generation (~1 month), we recorded day of first adult emergence for each selection line, cleared the pot of all adult moths after 2 days to prevent selection for early emergence, and then moved 50 newly emerged adult moths onto a new pot of the appropriate food medium 2 days after that to establish the next generation.

Resistance and development time assays

For each selection line, we measured resistance (proportion infected) and development time (days until pupation and mass at pupation) on both a common garden (standard food: 0% methyl cellulose) and virus-free home (CLF or CHF) environment at both the early and final time point (Boots, 2011). To prepare for the assay, selection was relaxed, and populations were split by approximate next emergence date into 5 batches with 1 selection line from each of the 4 treatments. Once each line in the batch had enough adult moths, 50 adults from each line in the batch were moved onto new pots containing virus-free food of the appropriate quality for each set of assays. For common garden assays, all treatments were moved onto standard food. For home environment assays, VLF and CLF lines were moved onto virus-free low-quality (CLF) food and VHF and CHF lines were moved onto virus-free high-quality (CHF) food. This 'relaxation' step prevent maternal effects from confounding our assays (Boots & Roberts, 2012). When adult moths emerged in parental pot, 80 adult moths were moved onto a new pot of the same food type to set up an assay pot. 11 days after setting up this pot, third instar larvae were collected for resistance and development time assays.

For resistance assays, 200 third instar larvae from each selection line were exposed to four 10-fold dilutions of virus (50 larvae dosed per dilution) corresponding to LD0-LD60. The 50 third instar larvae for each selection line x dose x assay combination were collected in separate petri dishes and then starved under a damp paper towel for 1 hour. Tiny droplets of virus solutions containing the appropriate dilution, 2% sucrose, and 0.2% Coomassie Brilliant Blue R-250 dye (ThermoFisher Scientific, U.S.A.) were placed

into each dish using a syringe. The sucrose entices the larvae to consume the virus and the dye allows for confirmation that larvae have ingested half their body length of solution. These larvae were considered successfully exposed and used to fill 25-cell compartmentalized square petri dishes (ThermoFisher Scientific, U.S.A.) filled with the appropriate food type (standard for common garden assays, high or low-quality for home environment assays) with 1 grid plate per selection line x dose x assay (home or common garden) combination and 1 exposed larva per cell. Assay grids were then placed into a single incubator at the same conditions that populations were reared and allowed to develop. After 21 days, grids were frozen and destructively sampled to measure the proportion of larvae infected and uninfected. Infected larvae were distinguishable as a successful PiGV infection turns the larvae opaque, chalky white, while non-infected larvae will continue their life history as normal to pupate.

For development time assays, 100 3rd instar larvae from each selection line were placed individually into four 25-cell compartmentalized square petri dishes containing either common garden or home food with 1 larva per cell and 2 grids for each of the assay environments. Grids were then moved to a single incubator at the same conditions that populations were reared in and checked every other day. Day of pupation was recorded for each larva and then each pupa was weighed 2 days later. Mass at pupation was then divided by days to pupation to calculate growth rate.

Statistical analysis

Analyses were conducted using a generalized linear mixed modeling approach in R ("R version 4.1.2 (2021-11-01)") (R Core Team 2021) using packages 'lme4'(Bates et al. 2015) and 'glmmTMB' (Brooks et al., 2017) to build models; 'DHARMa' (Hartig & Lohse, 2021) to check residuals; 'afex' (Singmann, Bolker, Westfall, Aust, & Ben-Shachar, 2019) and 'car' (Fox & Weisberg, 2019) to determine significant model terms; 'emmeans' (R. Lenth, 2019) to extract effects; 'tidyverse' (Wickham et al., 2019) to manipulate data; and 'ggplot2', 'ggforce', and 'patchwork' to plot results (Pedersen, 2020; Pedersen & RStudio, 2021; Wickham, 2009). For all response variables, we determine error structures for models iteratively by testing fitted model residuals with 'DHARMa' and then adjusting error structures to best match model assumptions. We corrected residual distributions by sequentially testing models with observation level random effects (Harrison, 2014), negative binomial distributions, then zero-inflated negative binomial or quasi-Poisson distributions as needed. Once model fits were satisfactory, we tested for significance of predictor terms. Models of resistance used a binomial error structure to account for the binary outcome of 'Infected/uninfected'. Growth rate models used gaussian, Poisson, negative binomial, or generalized Poisson error structures as required (See Supplementary model tables). Annotated R code and model output tables are attached in the supplement (note that models are named (M1-22) and that the model name that estimates and p-values are drawn from are included alongside these numbers).

For the first part of our analysis, we re-analyze the early time point (generation 14) data from (Roberts et al., 2020) for our subset of populations to see if the lineages we selected varied in their susceptibilities and growth rates in these initial assays and newly analyze the final time point (generation 50+) data to ask whether treatment affects susceptibility and growth rate after further generations of selection. For each of these time points, we separately analyze common garden assay and home food assay data sets to see if differences in a population's conditions (virus/control and high-quality/low-quality food) led to evolved differences in resistance or growth rate. For each response variable for each data set, we ran two models to test the effects of treatment on our response variables. The first model included treatment as an interaction between 'evolution resource environment' and 'virus exposed/control' and the second model included 'treatment' directly. This allowed us to explore the interacting effects of our two experimental manipulations as well see the differences between individual treatments. All models include a resistance ('proportion infected') or life history ('growth rate') metric as the response variable with 'replicate line' nested under 'treatment' as random effects to account for our experimental structure. For final time point data, we include an additional random effect of 'batch' to account for the batched assay structure. For resistance models, 'dose' was included in the model as a fixed effect.

For the second part of our analysis, we ask whether the different evolution treatments lead to differences in the change in resistance or growth rate between the early and final time points. For each selection line, we calculate the change in both growth rate and resistance effect size estimates between the early and final time points. We then use the same linear modelling framework as above to determine whether 'treatment' informatively predicts the change in resistance or growth rate for each line in the common garden and home assay conditions between generation 14 (early) and the end of the experiment (final).

For the final part of our analysis, we test whether a given line's growth rate is significantly predicted by its measured degree of resistance to infection, and whether this changes over time. We again analyze common garden and home assay data separately using the same linear modelling framework as above with 'growth rate' as the response variable and 'susceptibility estimate', 'treatment', and 'time point' as fixed effects with potential interactions.

RESULTS

Selection Line Resistance

For the subset of lines that we further select, lines from virus selected conditions have significantly lower susceptibility to infection when assayed on home food at the early time point (estimate = -0.75, p = 0.02, Supplementary Model Tables M4) . This effect is primarily driven by the decreased susceptibility of populations from the virus high-quality (VHF) treatment (estimate = -0.75, p = 0.03, M3), as the virus low-quality

(VLF) populations do not have significantly lower (estimate = -0.19, p = 0.57) susceptibility than control lines. However, this effect does not hold when lines are assayed in the common garden environment, as neither 'treatment' (p = 0.15, M1) nor 'virus/control' (p = 0.95, M2) significantly affects proportion infected in these models. Across all models at the early time point, assay virus 'dose' significantly affects proportion infected (p < 0.001, M1-4) and neither 'evolution food type' (common garden: p = 0.09, M2; home: p = 0.081, M4) nor the interaction effect between 'evolution food type' and 'control/virus' (common garden: p = 0.95, M2; home: p = 0.59, M4) have significant effects. Therefore, selection from virus led to lower proportions infected at this early time point when populations were assayed in the home food environment, but not when assayed in the common garden environment (Fig 1 A-B, E-F). We did not, however, see a significant effect of resource quality on evolved resistance at the early time point (Fig 1 A-B, E-F).

After additional generations of selection, lines from virus selected conditions continue to have significantly lower susceptibility to infection in the home food assays (estimate = -0.83, p = 0.03, M8). In this case, neither 'evolution food type' nor the interaction between 'evolution food type' and 'virus/control' is significant (evolution food type: p = 0.84, M8 ; interaction: p = 0.227, M8) and the effect is driven by both virus high-quality (estimate = -0.83, p = 0.01) and virus low-quality (estimate = -0.59, p = 0.06) selected treatments, though only the virus high-quality treatment is statistically significant on its own. However, in the common garden assays, there is a strong interaction effect between 'evolution food type' and 'virus/control' (p = .009, M6) so that only control low-quality selected populations significantly differ in their susceptibility to virus infection (estimate = 0.66, p = 0.003, M5). In these common garden assays, virus low-quality and virus high-quality lines are no more resistant than the control high-quality lines. Across all models at the final time point, the virus dilution assayed at for the dose curve significantly affects proportion infected, as expected (p < 0.001, M5-8). Therefore, further selection from virus continues to result in lower susceptibility to infection when assayed on home food conditions, but not common garden conditions (Fig 1 C-D, G-H). After additional evolution, however, this result is not as independently driven by the lower susceptibility of virus high-quality lines, as virus low-quality lines have gotten closer to the virus high-quality lines in resistance, though still not 'caught up'. This suggests that the context dependent resistance seen at the earlier time point is not a transient evolutionary strategy and that the dual stressor effect of low-quality resources preventing the evolution of resistance diminishes over time.

Finally, 'treatment' does not predict a selection line's change in susceptibility between the early and final time points in either the common garden (p = 0.5) or home (p = 0.5) assays (Figure S1, Chapter 2 Supplementary Material).
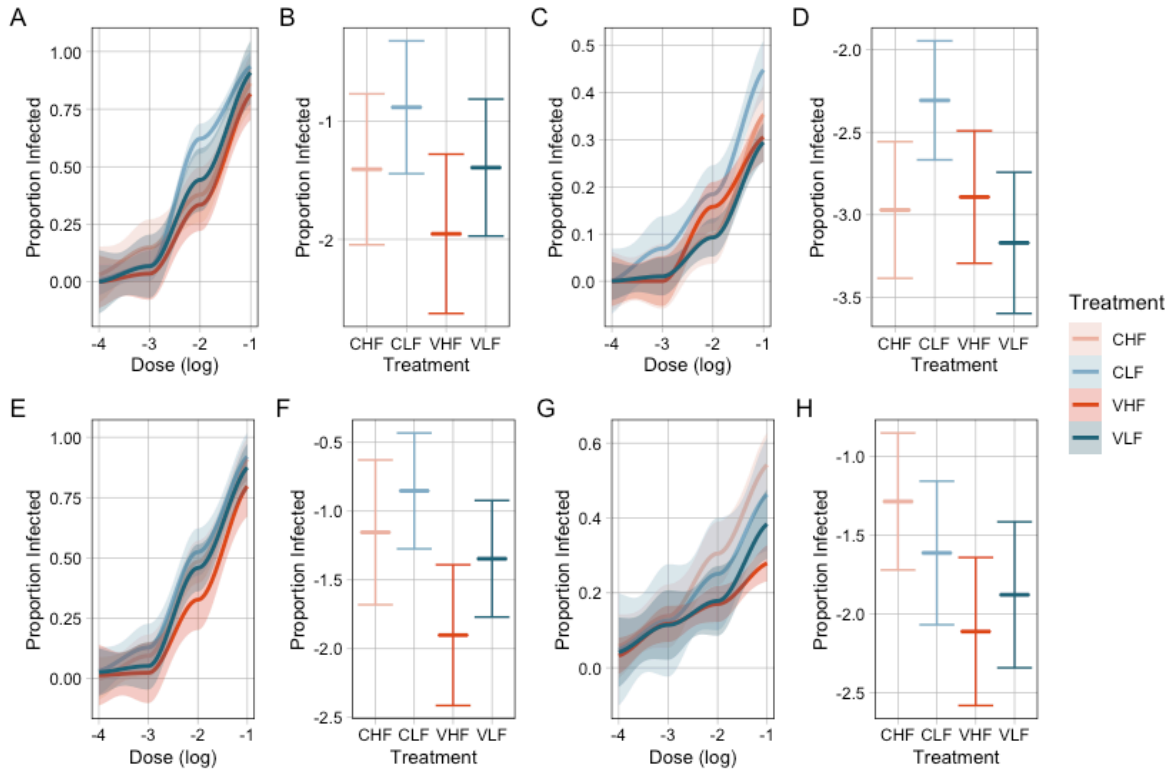
Figure 6: Resistance of selection lines after 14 (A-B, E-F) and 50+ (C-D, G-H) generations of section when assayed in common garden (A-D) and home food (E-H) environments. Panels A, C, E, F show smooths of the raw proportions infected at each dose, with dose log transformed on the x-axis. Panels B, D, F, G present model estimates for each treatment's effect on proportion infected across doses from models M1, M3, M5, M7 (See Supplementary Model Tables).

Selection Line Life Histories

 For the subset of lines that we further evolve, lines from virus selected conditions do not have differences in growth rate when assayed in common garden (p = 0.4, M11) or home quality food (p = 0.4, M13) at the early time point. There are no differences between treatments in the common garden assays (p = 0.8, M12), but treatments do significantly vary in the home assays (p = 0.007, M14). This is driven by lines evolved and therefore assayed on low-quality food developing more slowly (estimate = -0.75, p < 0.001, M13). Therefore, the only factor affecting growth rate at the early time point is whether the line is being assayed on low-quality or high-quality food, and the evolved resistance of virus high-quality lines in the home assays (Fig 1E-F) does not seem to come at a treatment-level cost of slower growth rate (Fig 2A).

After additional generations of selection, lines from virus selected conditions continue to show no differences in growth rate when assayed in common garden (p = 0.89, M15) or home quality food (p = 0.77, M17) at the final time point. There also continue to be no differences between treatments in the common garden assays (p = 0.45, M16), and

treatment is no longer significant in the home assays at the final time point (p = 0.179, M18). However, lines evolved and therefore assayed on low-quality food still develop more significantly more slowly when considered together (estimate = -0.27, p = 0.03, M17). Therefore, growth rate is still affected by whether the line is being assayed on low-quality or high-quality food at the final time point, and the evolved resistance of VHF lines, and now VLF lines, in the home assays (Fig 1G-H) continues to not be associated with a treatment-level cost of slower growth rate (Fig 2B).

Finally, treatment does not affect the change in growth rate between early and final time points in common garden assays (p = 0.8, M20, Fig S3C-D). In home assays, treatment does not have an overall significant effect on growth rate (p = 0.13, M19), but CLF lines have a borderline significant increase in growth rate (estimate = 0.52, p = 0.059, M19, Fig S3A-B).
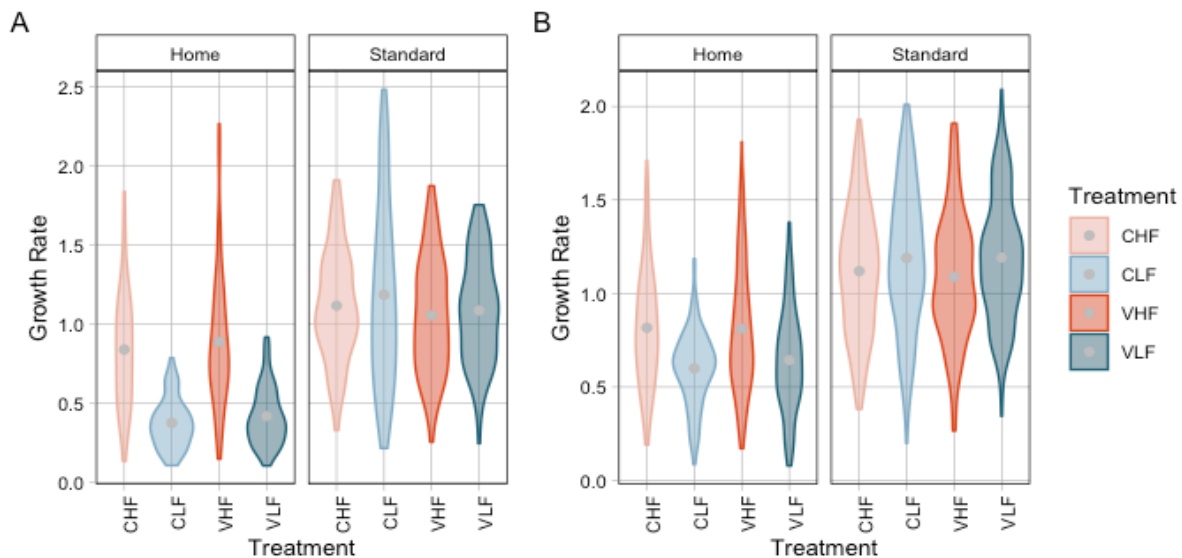
Figure 7: Growth rates for lines at early (A) and final (B) time points in standard and home food assays. Growth rate is the mass at pupation divided by the days until pupation. Violins represent raw growth rate data separated by treatment, assay condition, and assay time point. Gray dots represent means.

Correlations between Resistance and Growth Rate

In common garden assays, neither susceptibility (p = 0.15), treatment (p = 0.34), time point (p = 0.98), nor any interaction term therewithin have significant relationships with growth rate (M22, Fig3A). The sole significant single term is the interaction between susceptibility and the VLF treatment where the relationship between growth rate and susceptibility is significantly positive (estimate = 0.0005, p = 0.03) so that the fastest growers are the most susceptible.

In home assays, however, susceptibility (p = 0.005), treatment (p = 0.007), time point (p = 0.037), susceptibility: treatment (p < 0.001), susceptibility: time point (p = 0.02), and susceptibility: treatment: time point (p = 0.038) all have significant effects on growth

rate (M21, Fig3B). The only non-significant model term is the interaction between treatment and time point (p = 0.11). Notably, the relationship between susceptibility and growth rate becomes significantly more negative at the final time point (estimate = -0.005, p = 0.02). From Figure 8B, we can see that this is largely because low-quality food lines shift from a positive relationship between growth rate and susceptibility in the early assays where the fastest growers are the most susceptible to a negative relationship in the final assays where the fastest growers are the most resistant. At the same time, VHF lines shift from a negative relationship between susceptibility and growth rate (fastest growers are most resistant) to a positive one (fastest growers are least resistant). We can also see that, for the same growth rate, virus selected lines from both low-quality and high-quality backgrounds are less susceptible than their control counterparts at the early time point. At the final time point this effect holds for VHF and CHF lines, but an unusually fast-growing, high-resistance CLF 5.1 means that the trend reverses for the VLF and CLF lines. Therefore, there are within-treatment level trade-offs between resistance and development time in the VLF (but not VHF) lines in the early assays, but these not only disappear, but reverse, after additional evolution.
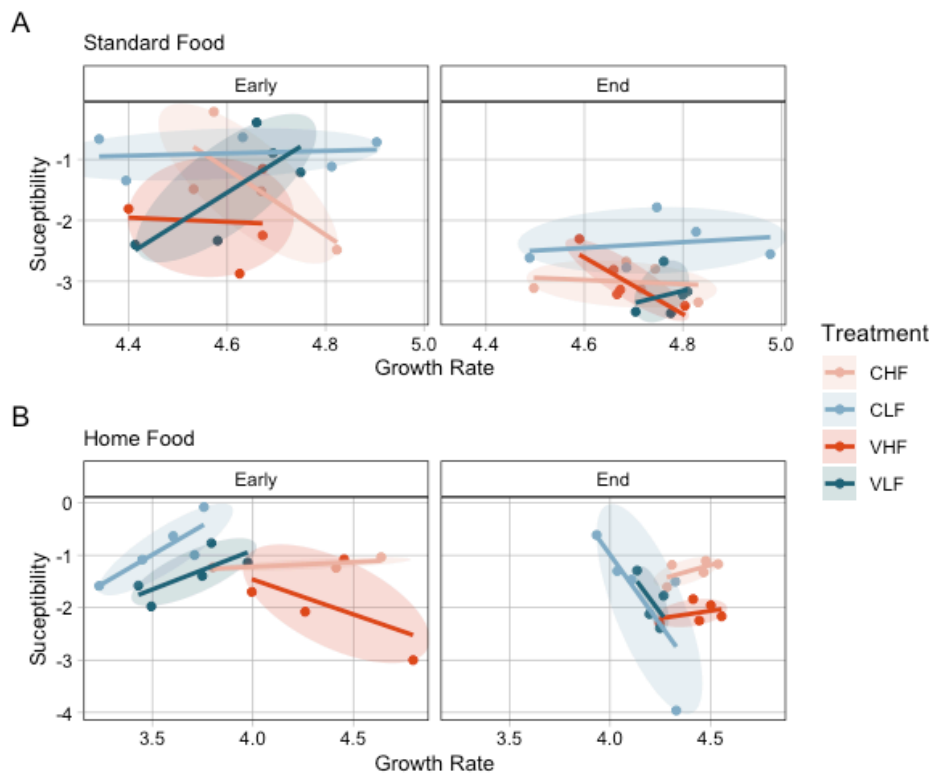
Figure 8: Correlations between growth rate and susceptibility in (A) common garden and (B) home environment assays at early and final time point.

DISCUSSION

A key result is that we found that virus-selected lines show evidence of evolved resistance when assayed on their home food quality environment (the one in which

selection occurred), but not in the common garden standard food environment. This is found at both the earlier and final time points, pointing towards a consistent effect of context-dependent resistance where selection lines' resistance mechanisms depend on their assay environment. While the resistance of virus-selected lines in the home environment at the earlier time point is primarily driven by lines that were selected in the high-quality environment, after 36+ additional generations of selection, lines selected for resistance in the low-quality environment evolve more resistance and partially catch up to lines selected in the high-quality environment. This points to an effect where the dual stress of evolving in a low resource quality environment with exposure to virus can slow, rather than completely restrict, the evolution of resistance to infection.

Despite this evolution of resistance in the home environment, however, we do not see treatment-level costs to resistance in growth rate. This is in contrast to previous results from the *Plodia interpunctella* and PiGV system where resistance has shown to be associated with slower growth rates (Bartlett et al., 2018; Boots, 2011; Boots & Begon, 1993, 1995). Interestingly, this pattern of costless resistance observed in the early time point (Roberts et al., 2020) does not disappear after further generations of selection and increased resistance in the home environment. This suggests that the lack of detected costs was not caused by a transient effect where initial costless resistance alleles can be fixed early on, but only costly alleles remain as the population approaches the Pareto front (Y. Li et al., 2019; Shoval et al., 2012; Visher, Evensen, et al., 2021). It also doesn't follow general trends where trade-offs are more likely to be observed in longer selection experiments (Bono et al., 2017), though these trends are from microbial experiments where the number of generations is much higher.

While costless resistance at the treatment level even after 36+ generations of further selection suggests that this is not a transient effect, it is also possible that our populations do still sit below the Pareto front between resistance and growth rate, but that their small, bottlenecked effective population sizes means that drift is preventing them from reaching the Pareto front and accessing costly resistance strategies (Chavhan et al., 2020; White et al., 2021, but see Bisschop et al., 2022). It is also possible that, since we did not sequentially increase the concentration of virus that populations were exposed to, virus-selected lines evolved 'enough' resistance with costless strategies and did not explore more costly resistance strategies.

In the *Plodia interpunctella* and PiGV system, resistance that comes at the cost of slower development time may be selected against since faster growth rates themselves increase resistance to infection by shortening the time window for infection (Bartlett et al., 2020; Boots & Begon, 1993; Hochberg et al., 1992). This is because resistance in our system can be developmentally mediated as infection must establish before the gut epithelia is shed during larval molting, so shorter intervals between molts can confer higher resistance (Bartlett et al., 2020; Engelhard & Volkman, 1995). This means that resistance may come at a cost of slower development time, but slower development time may come

at a cost of lower resistance. This effect is potentially supported by the result where low-quality selected lines have negative relationships between growth rate and susceptibility in the home environment at the end of the experiment, suggesting that the fastest growers are the least susceptible to infection for both these treatments. Thus, the slower development times experienced by lines evolving and being assayed on low-quality food might put them against the portion of the non-linear relationship between resistance and development time where their slower development time is itself a challenge to their ability to effectively resist infection. The context dependency of evolved resistance prevents us from making comparisons across common garden and home assays, but it is notable that the correlation between susceptibility and growth rate is significantly positive for VLF in the common garden at the final time point, as growing on standard quality food moves them higher on the growth rate axis and may move them out of the region of resistance-conferring effect of growth rate. This relationship does not hold for the high-quality selected lines, which have faster growth rates in the home environment as they are growing on more nutritious food. It is interesting to note that this positive relationship between resistance and development time for the lines evolved and being assayed in low-quality food was not found earlier in the lineages' evolution. This suggests that earlier costs to resistance were either compensated for or that the low-quality selected lines started exploring different resistance mechanisms with different cost structures.

As a whole, our results emphasize the impact of time scales, resources, and context dependence on trade-offs between life-history traits. We show that the nature and, in particular, the costs of resistance in this long-term experiment (Roberts et al., 2020) differ from previous results in the system that found consistent costs to resistance with development time (Bartlett et al., 2018; Boots, 2011; Boots & Begon, 1993, 1995). This can be explained because there may well have been differences in the starting populations such that different resistance conferring variants existed in the standing genetic variation of the ancestral population for each experiment. Alternatively, even if the starting variation was identical, different sampling of early adaptive alleles could have led towards different historically contingent evolutionary trajectories. It does seem that selected lines selected for resistance in low resource quality were partially able to catch up to their high-resource selected counterparts when given longer to evolve and, finally, that lines were able to compensate what costs to resistance did exist when given a longer evolutionary time scales. Therefore, it is also clear that trade-offs do not consistently evolve even over extended timescales and that more work is therefore needed to better understand their conditionality to better predict the evolution of resistance. Understanding when costly or costless resistance strategies evolve and how that depends on resource availability and time scale of selection would help to predict when we expect to see resistance evolve and how it would alter ecological dynamics or persist in the absence of the pathogen.

Chapter 3

The evolution of host specialization in an insect pathogen

Authors: Elisa Visher, Lawrence Uricchio, Lewis Bartlett, Nicole DeNamur, Aren Yarcan, Dina Alhassani, and Mike Boots'

ABSTRACT

Niche breadth coevolution between biotic partners underpins theories of diversity and co-existence and influences patterns of disease emergence and transmission in host-parasite systems. Despite these broad implications, we still do not fully understand how the breadth of parasites' infectivity evolves, the nature of any associated costs, or the genetic basis of specialization. Here, we serially passage a granulosis virus on multiple inbred populations of its *Plodia interpunctella* host to explore the dynamics and outcomes of specialization. In particular, we collect time series of phenotypic and genetic data to explore the dynamics of host genotype specialization throughout the course of experimental evolution and examine two fitness components. We find that the *Plodia interpunctella* granulosis virus consistently evolves increases in overall specialization, but that our two fitness components evolve independently such that lines can specialize in productivity or infectivity. Furthermore, we find that specialization in our experiment is a highly polygenic trait best explained by a combination of evolutionary mechanisms. These results are important for understanding the evolution of specialization in host-parasite interactions and its broader implications for co-existence, diversification, and infectious disease management. (190/200)

INTRODUCTION

The question of why some species are specialists and others are generalists has been central to evolutionary biology since its inception (Darwin, 1859). This co-existence of strategies is commonly explained by there being some cost to generalism such that specialists are favored under certain ecological conditions (Futuyma & Moreno, 1988) because "jacks-of-all-trades are the masters of none" (MacArthur 1984). The theory of costly generalism has been extensively applied in the host-parasite eco-evolutionary literature to explain parasite niche breadth and specialization at the levels of both host species and host genotype (Gandon & Poulin, 2004; Osnas & Dobson, 2012; Regoes et

al., 2000). Niche breadth at the level of host species has important implications for pathogen emergence (Guth et al., 2019) and species co-existence (Connell, 1971; Janzen, 1970); while niche breadth and specialization at the genotype level underpins the monoculture effect (Elton, 1958), local adaptation (Kawecki & Ebert, 2004), and the Red Queen Hypothesis of Sex (Jaenike, 1978).

Despite these broad implications for niche breadth evolution in antagonistic coevolutionary systems, there is still debate about whether costs to niche breadth are, in fact, universal and what the dominant genetic mechanisms driving such costs would be (Jaenike, 1990; Remold, 2012). Several mechanisms for the evolution of specialization have been proposed. The classic trade-off hypothesis expects that increases in fitness on one host negatively trade-off with fitness on foreign hosts (Levins, 1968; Regoes et al., 2000). These strict negative trade-offs are not universal though, so several additional theories have been proposed including host specialization due to weakly positive or neutral genetic correlations leading to asymmetrical fitness gains (Fry, 1996) and host specialization due to the accumulation of deleterious mutations on alternate hosts (Kawecki, 1994; Whitlock, 1996). The number of genes involved in specialization could also vary so that it is driven by few mutations of large effect or by many mutations of small effect.

Experimental evolution approaches have been used to explore the evolution of specialism and the nature of costs to generalism in a wide range of taxa evolving to a variety of selective environments (Bono et al., 2017; Cooper & Lenski, 2000; Kassen, 2002; Visher & Boots, 2020). Host genotype specialization, specifically, has been studied with great success in host-parasite systems including mice and RNA virus (Kubinak et al., 2012a), mosquitos and microsporidia (Legros & Koella, 2010), daphnia and bacteria (Little et al., 2006), protists and bacteria (Nidelet & Kaltz, 2007), *C. elegans* and bacteria (Schulte et al., 2011), and wheat and fungus (Zhan et al., 2002). Generally, these studies find that serial passage on a single host genotype increases fitness on that host genotype while decreasing or at least resulting in smaller fitness gains on other genotypes.

However, there has been limited empirical exploration of the genetic mechanisms of such specialism. Similar work has explored the genetics of virus specialization to different host cell lines, finding that specialization was driven by a mix of antagonistic pleiotropy and mutation accumulation depending on a lineage's evolutionary history (Remold et al. 2008), but this inquiry has not yet been extended to specialization to different host genotypes. A better understanding of the genetics of specialization is important because the number of potential mutations involved in host genotype specialization and the genetic mechanism of such specialization will affect the evolutionary dynamics of specialization and may create divergent predictions for eco-evolutionary theory (Remold, 2012; Visher and Boots, 2020).

In this paper, we explore the evolutionary dynamics of host genotype specialization in the *Plodia interpunctella* (Hübner) and *Plodia interpunctella* granulosis virus (PiGV) laboratory model system. *Plodia interpunctella*, the Indian meal moth, is a stored grain

pest that has been extensively used to characterize trade-offs and test eco-evolutionary dynamics in the lab (Bartlett et al., 2018, 2020; Boots, 2011; Boots & Mealor, 2007). We experimentally evolve virus populations to determine whether PiGV evolves to specialize on familiar host genotypes, collect multiple fitness metrics at multiple time points to explore the phenotypic dynamics of specialization, and sequence virus populations at multiple time points to explore the genetic mechanisms of specialization. We find that serially passaging virus leads to consistent increases in specialization on familiar host genotypes through the course of experimental evolution, and that specialization can occur in multiple fitness components. MCMC-based inference analysis of time series data shows that this specialization is not driven by few mutations of large effect (Schraiber et al., 2016). Combining these lines of evidence suggests that a combination of genetic mechanisms is likely to explain specialization in our system.

METHODS

Study System

Our study system is *Plodia interpunctella* (Hübner), the Indian meal moth, and the Plodia interpunctella granulosis virus (PiGV). *Plodia interpunctella* is a pest that lives in grain stores (Mohandass et al., 2007). During its five larval instar stages, it develops within its food medium before pupating and emerging into an adult moth. For this experiment, we use inbred lines previously generated in Bartlett et al. (2018). These lines were made by mating individual brother-sister pairs for more than 27 generations. At this point, inbred populations should represent near-clonal populations of a single genotype that was randomly selected from the genetically diverse founder population via drift. Limited data suggests that these inbred lines had levels of resistance similar to other selection lines in our lab (Bartlett, Visher, et al., 2020), though it is possible that inbreeding could have affected resistance quality. However, we would not expect this to alter our characterizations of the dynamics of specialization since all our specialization metrics are relative across equally inbred lines.

Plodia interpunctella granulosis virus (PiGV) is a dsDNA baculovirus that is an obligate killer (Vail & Tebbets, 1990b). The natural life cycle is as follows: a larvae ingests virions in the occlusion body form, the virions shed their protein coats and infect gut epithelial cells, the virions either pass through the gut to establish a successful infection or are cleared during molting (freeing the larvae to carry out the rest of their life history), the virus begins to proliferate through the entire body of the larvae, and, once at a critical mass, packages into the protein-coated occlusion body form and kills its host (Rohrmann, 2013). It can then be transmitted to susceptible larvae when they cannibalize infected cadavers and ingest occluded virus. Critically, the virus must kill its host in order to transmit and larvae can only pupate and become adult moths if they were not successfully infected (Boots & Begon, 1993).

Host Selection and Maintenance

We selected three inbred *Plodia interpunctella* populations with similar overall levels of resistance for this experiment, as measured by a preliminary resistance assay of all twelve of the inbred populations (Table S1). The chosen inbred populations (Lines 2, 9, and 17) represent genotypes with similar medium overall levels of resistance compared to the full set of potential inbred lines (Table S1). Populations of these genotypes were maintained in the absence of the virus as in (Bartlett et al., 2020) (See Supplemental Methods for details).

Setting Up Experimental Evolution

Virus evolution was initiated with a single genetically diverse virus stock that we diluted to a passaging dose that would cause high mortality ($\sim$7.5x10^8 occlusion bodies per mL). A 0.5mg early third instar larvae eats <0.1mg of the solution (unpublished data), corresponding to an exposure dose of <75,000 occlusion bodies. This value is consistent with natural field doses of baculovirus, which tend to be very high (Eakin et al., 2015; Kennedy & Dwyer, 2018).

We counted the concentration of this passaging dose on a Petroff-Hauser counting chamber with a darkfield microscope at 400x magnification. This dilution was combined with 2% sucrose (ThermoFisher Scientific, U.S.A.) and 0.2% Coomassie Brilliant Blue R-250 dye (ThermoFisher Scientific, U.S.A.). The sucrose encourages the larvae to consume the virus solution and the dye allows us to recognize larvae that have consumed half their body length of virus solution and are therefore considered successfully inoculated.

We set up three replicate evolving lines of virus on each of the three inbred host genotypes (See Figure S1 for passaging scheme, Chapter 3 Supplementary Material). For each virus line, we collected 100 third instar larvae of the appropriate genotype in a petri dish and starved them under a damp paper towel for 2 hours. We then syringed tiny droplets of our virus-sucrose-dye solution onto the petri dish for the larvae to orally ingest. After about an hour, we moved 50 successfully inoculated larvae into two 25-cell compartmentalized square petri dishes (ThermoFisher Scientific, U.S.A.) with standard food. The grid plates were then transferred to a single incubator for 20 days.

Serial Passage

After 20 days, we harvested virus from each virus line under sterile conditions by collecting up to 10 virus killed cadavers per line and transferring these to sterile 15 mL disposable tissue grinders (ThermoFisher Scientific, U.S.A.). Infected larvae were recognizable by their opaque, chalky, white coloration. We were not able to collect 10 infected cadavers from all virus lines at all passages, so, when we could not find 10 infected cadavers, we collected every infected cadaver that we could find (Table S2). To extract virus from infected cadavers, we added 2mL of sterile DI water to the tissue grinders and homogenized the solution until all cadavers had been thoroughly crushed.

We then transferred 1mL of the supernatant to a sterile 1.5mL Eppendorf tube and centrifuged the solution for 1 minute at 3,000 rpm to remove larger particulate matter from the supernatant. We transferred 600uL of this solution to a sterile 1.5 mL Eppendorf and centrifuged this for 3 minutes at 13,000 rpm to pellet the virus. We removed the supernatant from the pellet and resuspended in 1mL sterile water.

After extracting the virus, we diluted the solution 10x and added 600uL of the dilution to a .65 micron filter spin column (Millipore Sigma, U.S.A.) that we centrifuged at 13,000rpm for 3 minutes to semi-purify the virus of possible bacterial and fungal contaminants (for method details see Table S3). Importantly for later comparisons, this purification method differed from the sucrose gradient purification method used to generate the ancestral virus stock (Harrison et al., 2016) and may have resulted in differences in infectivity per particle. We counted each of the semi-purified virus solutions as above and diluted them to the passaging dose concentration of $\sim 7.5 \times 10^{\wedge}8$ occlusion bodies per mL in 2% sucrose and .2% dye to form our final passaging solutions for each virus line. A portion of these virus dilutions were then used to infect the next set of third instar larvae of the appropriate genotype following the protocol above and the rest was stored at -20C for assays and sequencing. Virus was serially passaged for nine passages (Figure S1, Chapter 3 Supplementary Material). The number of passages was determined at the start of the experiment and was based on numbers standard for similar experiments (Kubinak et al., 2012a; Legros & Koella, 2010; Nidelet & Kaltz, 2007; Zhan et al., 2002).

Assaying

We assayed each virus line at multiple passages to track evolution over the course of the experiment (Figure S1, Chapter 3 Supplementary Material). We assayed the starting population of virus as well as virus harvested from passages 1, 4, 6, and the final passage 9. For each assay, we inoculated all 3 host genotypes with all 9 virus lines at both the passaging dose and 10% of the passaging dose. We inoculated 25 larvae for each host genotype x virus line x dose combination using the standard inoculation protocol above. Because of time constraints, inoculations for each passage were conducted across three days with one host genotype each day being inoculated with all of the virus lines. By assaying all the virus populations from each of the evolutionary histories on all of the host genotypes, we were able to measure how the evolving virus line changed in fitness on the familiar (the genotype that the virus evolved on) and foreign (genotypes that the virus was unexposed to) host genotypes.

After 20 days, we froze the grid plates and counted the number of infected and uninfected individuals in each grid. This proportion infected is our viral 'infectivity' metric. We collected all the infected larvae from each assay grid that had been inoculated with the higher dose and froze them in a pooled sample per grid plate. We extracted virus from these samples via tissue grinding and the two centrifugation steps (without filtering) and counted the virus in a Petroff-Hauser counting chamber as above. From these virus counts and the number of infected larvae, we were able to determine

how many occlusion bodies each virus line produced per infected cadaver on average when infecting each host genotype at the high dose. This average number of occlusion bodies per infected cadaver at the high dose is our viral 'productivity' metric.

Finally, we multiplied the average number of virions produced per infected cadaver by the proportion of larvae infected to get a composite measure of fitness for each virus line on each host genotype at the high dose. This is hereto after referred to as 'fitness'.

Sequencing and Variant Calling

The ancestral virus population and virus populations for each line at the four assayed time points (37 samples) were next prepared for sequencing. First, extracted occlusion bodies were rinsed in 0.1% SDS and purified in a Percoll gradient as in (Gilbert et al., 2014). Occlusion bodies were then dissolved in 0.5M Na_2CO_3 and DNA was extracted with a QIAamp DNA kit. Library preparation and sequencing was conducted at the UC Berkeley QB3 center on non-amplified DNA. 150bp paired end libraries were generated with Kapa Biosystems library preparation kits and multiplexed to run on one lane of an Illumina MiSeq platform. Reads were then de-multiplexed and aligned to the PiGV reference genome [GenBank: KX151395] using bowtie2 (R. L. Harrison et al., 2016a; Langmead & Salzberg, 2012). The resulting alignments for each sample had 99.99-100% genome coverage, 51-100 mean coverage depth, and 40.2-41 mean MapQ scores. The ancestral population .bam file was then re-aligned to the reference, indel and alignment quality scores were added (dindel method), and variants were called (SNV and indel, minimum coverage=20, default parameters) using LoFreq (Version 2.1.5) (Wilm et al., 2012) in usegalaxy.org (Afgan et al., 2018). LoFreq filter was used to select variants above 0.5 frequency to create a new consensus fafsa file using bcftools consensus (Version 1.10). FastQ files from all samples were then realigned to this consensus using bowtie2 (Version 2.4.2) and Samtools (Version 1.13) (H. Li et al., 2009) and variants were called using LoFreq as above. Variants were then filtered using LoFreq filter to select those above 0.01 frequency. The Galaxy history can be viewed here: https://usegalaxy.org/u/evisher/h/reviews2022final.

Phenotypic Assay Data Analyses

We analyzed all phenotypic assay data using a linear mixed modelling approach in R (v.4.0.3) using packages 'lme4'(Bates et al. 2015) and 'glmmTMB' (Brooks et al., 2017) to build models, 'DHARMa' (Hartig & Lohse, 2021) to check residuals, 'afex' (Singmann, Bolker, Westfall, Aust, & Ben-Shachar, 2019) and 'car' (Fox & Weisberg, 2019) to check model effects, 'emmeans' (R. Lenth, 2019) to extract effects, and 'tidyverse' (Wickham et al., 2019) to manipulate data. Our response variables were either fitness, infectivity, or productivity of the virus line. Error structures for models were determined by testing model residuals with 'DHARMa' and then adjusting error structures to best normalize the residuals. We corrected residual distributions by sequentially testing models with observation level random effects (X. A. Harrison, 2014), negative binomial distributions,

then zero-inflated negative binomial or quasi-Poisson distributions as needed (See annotated R code).

The first part of our analysis looked at data from the end of the evolution experiment (passage 9). We tested for an effect of specialization by using a 'self' factor that was either true (virus was assayed on same host genotype it was evolved on) or false (virus was assayed on a host genotype it was not evolved on). We included this as a fixed effect alongside 'assay genotype' and 'evolution genotype' (the host genotype used for the assay and that the virus was evolved on, respectively). In the case of the 'infectivity' data analysis, 'dose' was also included as a fixed effect. Our random effects were 'evolution genotype' and 'virus line', with 'virus line' nested under 'evolution genotype' to account for our experimental structure. Our infectivity model used a binomial error structure and our productivity and fitness models used Poisson error structures with observation level random effects. To see if there were differences in which fitness metrics the 'evolution genotypes' specialized on, we built 'fitness', 'infectivity', and 'productivity' models specified the same as above, but with 'self' only included as in interaction term with 'evolution genotype'. To see if there were differences in the ability of each virus selection line to evolve any specialism, we further analyzed the effect of 'self' on fitness by including it as an interaction effect with 'virus line' in a model specified the same as above, but with 'virus line' replacing 'evolution genotype' as a fixed effect and a negative binomial error structure. All model tables are provided in the Supplementary Model Tables file and organized by test. For full model structure, see Supplementary Model Tables M1.1-M1.7.

We also analyzed our infectivity, productivity, and fitness data across the whole experiment, including passages 1, 4, 6, and 9 to interrogate how specialization evolved with time. We did not include passage 0 data in this analysis because of clear differences in passage 0 to 1 fitness (likely due to different virus storage and extraction conditions) and differences in the underlying data structure of passage 0 data compared to evolved passage data (due to ancestral virus not yet being 'split' into virus lines). We used the same general approach as detailed above, where fixed effects were 'assay genotype', 'evolution genotype', 'self', 'passage number', and an interaction between 'passage number' and 'self'. Our error structure included 'evolution genotype', 'virus line' and 'passage number', with 'virus line' nested under 'evolution genotype' as above, and 'passage number' nested under 'virus line' to account for multiple generations acting as repeated measures. Our infectivity model used a binomial error structure with observation level random effects and our productivity and fitness models used zero-inflated negative binomial error structures. For full model structure, see Supplementary Model Tables M2.1-2.3.

We further used a similar modelling approach to test for correlations between virus fitness on familiar and foreign hosts across the whole experiment by building a model for 'fitness' including the interacting fixed effects of 'assay genotype' and 'passage number' and the same 'passage number', 'virus line', 'evolution genotype' nested error

structure as above and then extracting the residuals for each measurement of fitness of each virus line on each assay genotype. These residuals were used to build a 'fitness on familiar genotypes' and 'average fitness on foreign genotypes' dataset that we used to test whether 'fitness on familiar hosts' was predicted by 'average fitness on foreign hosts' and whether this effect interacted with the 'evolution genotype'. We further used the same modelling approach to test for a correlation between a virus line's virion production and its infectivity by including proportion infected as an additional fixed predictor in a separate model of viral productivity at the highest dose. For full model structure, see Supplementary Model Tables M3.1-M3.5.

We used the 'ggplot2' (Wickham, 2009) and 'patchwork' (Pedersen, 2020) packages to plot graphs of our results. See supplement for annotated code.

<u>Variant Analysis</u>

Variant frequencies were analyzed to: 1) identify genetic regions of variation in our population, 2) determine whether variant community composition was predicted by treatment, and 3) identify signatures of positive selection across the time series.

To determine regions of variation, we plotted variant frequencies against genome position, identified genome regions with high genetic variation, and compared these genetic regions to the annotated PiGV reference genome by hand to identify potentially interesting nearby genes (R. L. Harrison et al., 2016a). To determine whether passage 9 variant community composition was predicted by treatment, we made multidimensional scaling plots in 'vegan' using the 'metaMDS' function with the 'Canberra' method, which deemphasizes zero values (Middlebrook et al., 2021; Oksanen et al., 2020). We then used constrained ordination analysis on Hellinger and Chi-square pre-transformed SNP frequencies in 'vegan' and performed a Monte Carlo permutation test to determine whether treatment significantly predicted SNP frequency variance amongst the virus populations (Oksanen et al., 2020). See supplement for annotated code.

Finally, to identify signatures of positive selection, we used an MCMC-based inference procedure to infer the strength of selection acting at variable positions in our genomic time series data (Schraiber et al., 2016). This software estimates selection coefficients given an observed frequency trajectory, accounting for uncertainty in true allele frequencies due to binomial sampling. While we knew the average virus population size within a single individual at the end of infection, we did not know the exact number of virus particles that founded each infection. However, we can apply functions derived for another baculovirus and lepidoptera system in (Kennedy & Dwyer, 2018) to estimate that infections are founded by about 37-42 virions as third instar larvae ingest <0.1mg of virus solution (unpublished data), corresponding to a likely exposure dose of ~7500-750,000 occlusion bodies. Thus, we chose several possible demographic models based upon a range of reasonable inoculums (from 35 to 200 viral particles) and a range of growth rates (including "slow" and "fast" exponential processes with ~1.2-5

fold growth per generation) and calculated the harmonic mean of population size and the number of generations needed to reach 10^{10} particles for each scenario (Harpak & Sella, 2014). We then repeated our estimates of selection strength using each of these effective population sizes, which ranged from small to moderate (N_e = 92 to N_e = 2869). We call 'significant' alleles using the most conservative demographic model (N_e=92) by a loose threshold, where the 90% HPD interval did not overlap 0 (Figure S5, Chapter 3 Supplementary Material). For details, see supplementary methods and annotated code.

RESULTS

Specialization of Viruses at the Final Passage

After nine passages of experimental evolution, we find good evidence that viruses evolved to specialize on their familiar host genotype, indicated by a significantly positive effect of 'self' on viral infectivity (estimate = 0.33, p = 0.014, Figure 9A/D, M1.1), productivity (estimate = 0.76, p = 0.016, Figure 9B/E, M1.2), and fitness (estimate = 0.91, p = 0.01, Figure 9C/F, M1.3) (See Figure 9, Supplementary Model Tables M1.1-M1.3). Therefore, the evolved virus lines infected relatively higher proportions of individuals, produced more virions per infection, and therefore had higher fitness when infecting the host genotype that they had evolved on than when infecting foreign host genotypes.

We found a significant effect of 'dose' (p < 0.001, M1.1) for infectivity, as expected, and a significant effect of 'assay genotype' (host) for infectivity (p < 0.001, M1.1) but not productivity (M1.2) or fitness (M1.3). We did not find significant effects of 'evolution genotype' on any of our three metrics (M1.1-M1.3), meaning that specific host genotypes did not lead to the evolution of generally more infectious or higher fitness virus populations when averaged across all three assay genotypes.

Next, we asked whether different evolution treatments led to differences in specialization on different fitness metrics. In our fitness model, we do see a significant interaction between 'evolution genotype' and 'self' (p=0.03, M1.5) driven by higher specialization of lines evolved on host genotype 17. We do not find a significant interaction in our infectivity models (p=0.104, M1.6). We do see a significant (p=0.017, M1.7) interaction in our productivity model, however, driven by higher productivity specialization of lines evolved on host genotype 17 (fitness: estimate =1.91, p=0.002). Finally, we test whether virus lines differ in their fitness and find that they have significant fitness differences (p=0.012) and interaction effects with 'self' (p=0.0028). See Supplemental Model Tables M1.1-1.7 for full models and results.
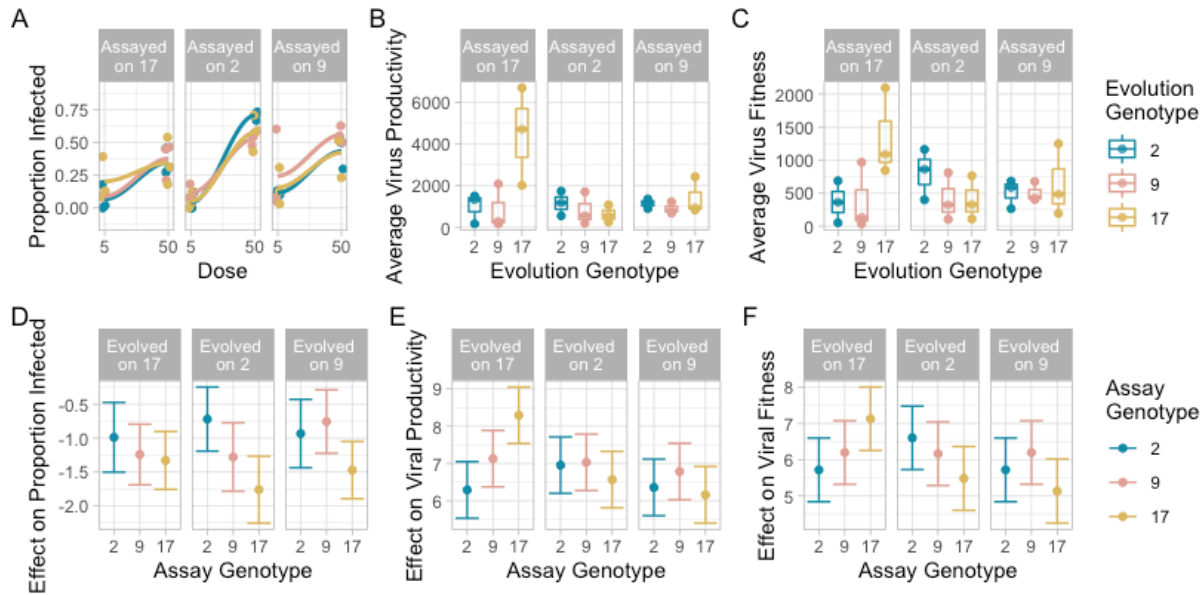
Figure 9: Specialization of virus at the end of the experiment. Paneled plots show the effect of the virus's evolutionary history on its (a,d) infectivity, (b,e) productivity, and (c,f) composite fitness when infecting each of the assay lines. Panels on plots A-C are organized by the assay genotype as assays were conducted on different days. Panels on plots D-F are organized by the evolution genotype, as this better matches our question of how virus lines evolved to specialize on their familiar host. Productivity metrics were only collected at the high dose. Fitness is the proportion infected at the high dose x the average number of virions produced per infected cadaver. Panels a-c present raw data while panels d-f present effect size estimates and errors from the GLMM models.

Evolution of Specialization over Time

Our analysis of fitness data across all evolved passages (1, 4, 6, and 9) showed significant effects of passage number (p = 0.001), evolution genotype (p=0.0006), and assay genotype (0.035) on virus fitness (Figure 10, Figure S2, M2.1, Chapter 3 Supplementary Material). Virus lines had significantly (p=0.025) lower fitness when assayed on host genotype 17, while virus lines evolved on host genotype 17 was significantly more fit (p=0.034) (M2.1). There is a significant effect of passage number on virus fitness (p=0.001), with virus lines generally increasing in their total fitness from passage 1 to passage 4 and no further meaningful change from passage 6 to 9 (Pass4-1: estimate=0.69, p=0.04; Pass6-4: estimate=0.2, p=1.0; Pass9-6: estimate=-0.16, p=1.0) (Figure 10B, Figure S2B, M2.1). There is not a significant interaction between the effect of infecting a familiar host and passage number (p=0.16), and viruses only become significantly specialized at passage 9 (FALSE-TRUE: estimate=-0.68, p=0.03) (Figure 10C, M2.1). This is because fitness on foreign hosts inconsistently changes (Pass4-1: estimate=1.07, p=0.005 ; Pass 6-4: estimate=0.11, p=1.0 ; Pass 9-6: estimate=-0.5, p=0.15), even though fitness on familiar hosts has non-significant, consistent increases (Pass4-1: estimate=0.31, p=1.0 ; Pass 6-4: estimate=0.29, p=1.0 ; Pass 9-6: estimate=0.19, p=1.0) (Figure 10C).
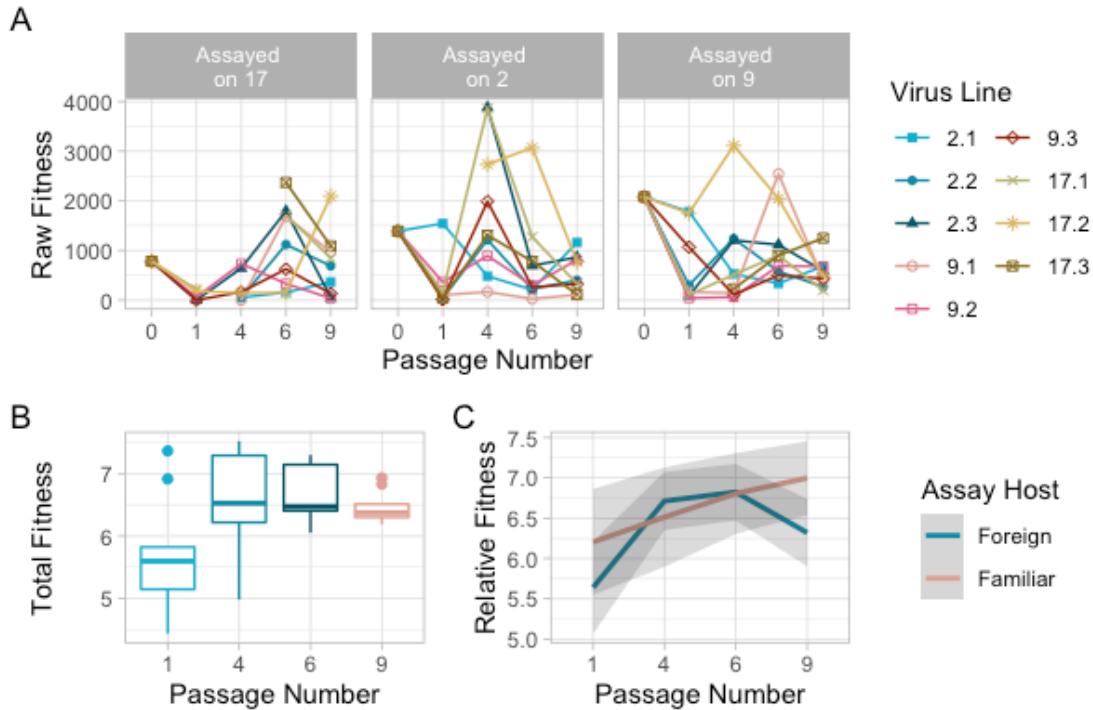
Figure 10: Evolution of specialization over time. Paneled plot showing (A) raw data of each virus line's fitness on each assay line across the experiment, (B) the statistical effect of passage on total fitness across hosts and (C) the statistical effect of whether the virus was assayed on its familiar host genotype (red) or on a foreign one (blue) on viral fitness over time. Y-axis effect sizes and errors for (B) and (C) are taken from the GLMM models using the 'emmeans' package.

Correlation between Fitness on Familiar and Foreign Hosts

We next determined the correlation between a virus line's fitness on their familiar host genotype and on the foreign host genotypes. A negative correlation would mean that the virus lines with the highest fitness on their familiar genotype had the lowest fitness on foreign genotypes and indicate a strict trade-off. Across the passage 1-9 dataset, we find that there is not a generally significant correlation between fitness on familiar and foreign hosts (p = 0.13, M3.1), nor does this relationship significantly change over time (p = 0.738) (Figure S3, M3.3, Chapter 3 Supplementary Material). However, there is a significant interaction effect between the genotype that the lineage evolved on and the relationship between fitness on familiar and foreign hosts (p = 0.001, M3.1). Specifically, the relationship between fitness on familiar and foreign hosts is negative for lines evolved on genotype 17, positive for lines evolved on genotype 2 (p = 0.002), and not significant for lines evolved on genotype 9 (p = 0.16) (Figure 11, M3.1).
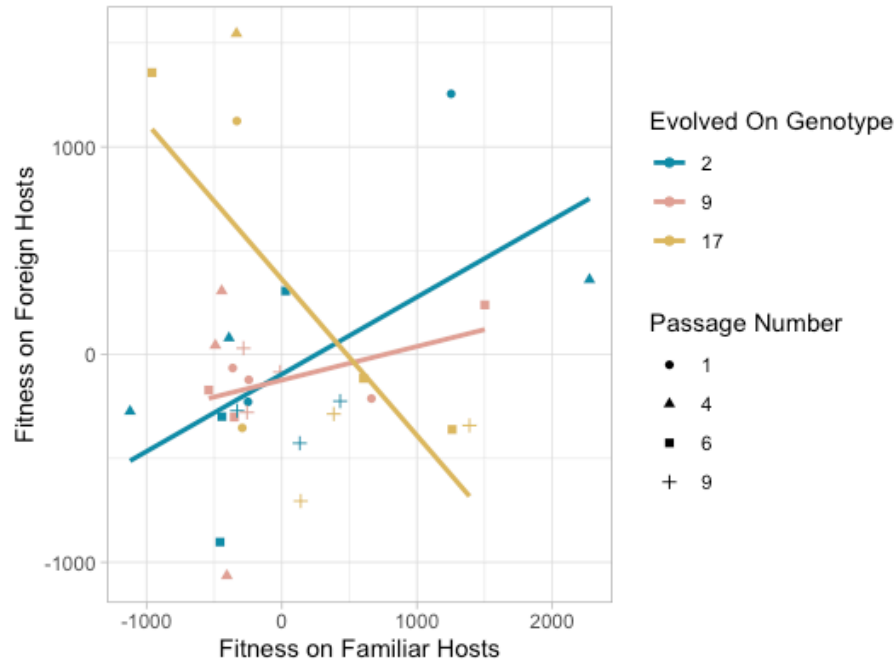
Figure 11: Correlation Between Fitness on Familiar and Foreign Hosts. Plot showing the correlation between each virus line's fitness on familiar and foreign hosts at passages 1, 4, 6, and 9. Effect sizes are taken from the GLMM models.

Relationship between Virus Productivity and Infectivity

When we examine the passage 9 dataset, we find that the relationship between virus productivity and infectivity significantly (p = 0.007, M3.5) interacts with whether the virus is infecting familiar or foreign hosts so that the relationship is negative when lines are assayed on their familiar genotype and positive when they are assayed on foreign. However, when we analyzed the full dataset with all passages, we do not find a significant three-way interaction between the effects of virus infectivity, virus productivity, and being assayed on the familiar genotype. Therefore, we fit and tested a model with an interaction effect between 'self', 'productivity', and 'passage number' (M3.4). We do not find a generally significant interaction between these three metrics (p=0.067, M3.4), but do find that the interaction between infecting a familiar host and proportion infected becomes significantly negative at passage number 9 (p = .01, M3.4) after being generally positive across the rest of the passages. This effect is mostly driven by the evolution genotype 17 lines, which have significantly higher specialization in productivity. Therefore, the direction of the relationship between viral productivity and infectivity changes from positive to negative depending on the passage number and whether the virus is infecting a familiar or foreign host (see Figure 12). This indicates that productivity and infectivity are not strictly positively correlated traits and that specialism can evolve independently in either trait.
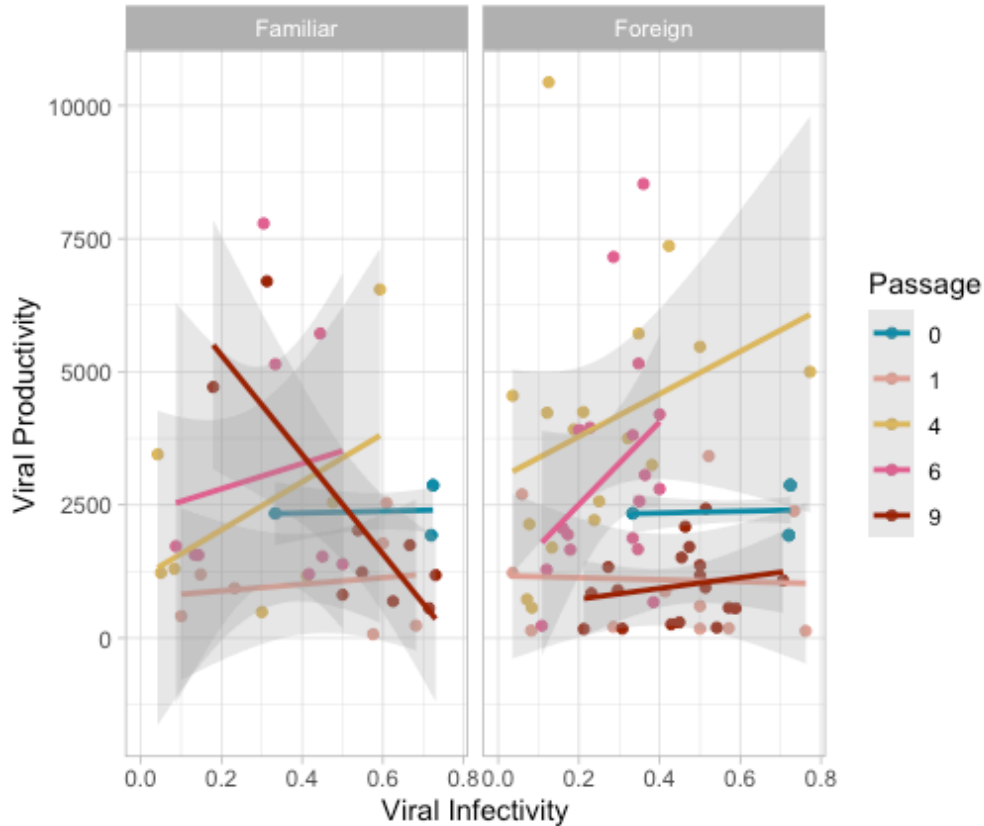
Figure 12: Relationship between virus productivity and infectivity. Paneled plot showing the relationship between viral productivity and infectivity on both familiar (left panel) and foreign hosts (right panel) at each passage.

Genetic Variation

Most variants are at low (<10%) frequencies, but there are several genomic regions that consistently have high genetic variation (Figure S10, Chapter 3 Supplementary Material). These regions correspond with several ORFs homologous with genes in AcMNPV that have known functions including occluded virus production, oral infection, time to kill, and host range (Table S4) (R. L. Harrison et al., 2016a; Rohrmann, 2019). We do not find that treatment significantly predicts variance in variant community composition at passage 9 in constrained ordination analyses with permutation tests (23% variance explained, p = 0.69), indicating that evolution genotype is not significantly predicting the frequencies of genetic variants (Figures 13, S4).

Among the 18 alleles that were called as significant in the analysis of the N_e = 92 model (Figure 13), we found 3 that were called as significant in 2 or more biological replicates from the same treatment (Table S5). Some putatively selected variants were shared across virus populations from two or more of the inbred lines, suggesting they may represent generalist adaptation to experimental conditions rather than adaptation to specific host genotypes (Figure 13A, S6-8). In general, we note that the inferred

selection coefficients are mostly indicative of weak positive selection. If we suppose an effective population size of 92 (as in the demographic model we used for the selection inference), then the inferred values of 2Ns indicate per-allele effects ranging from 0.014 to 0.23.
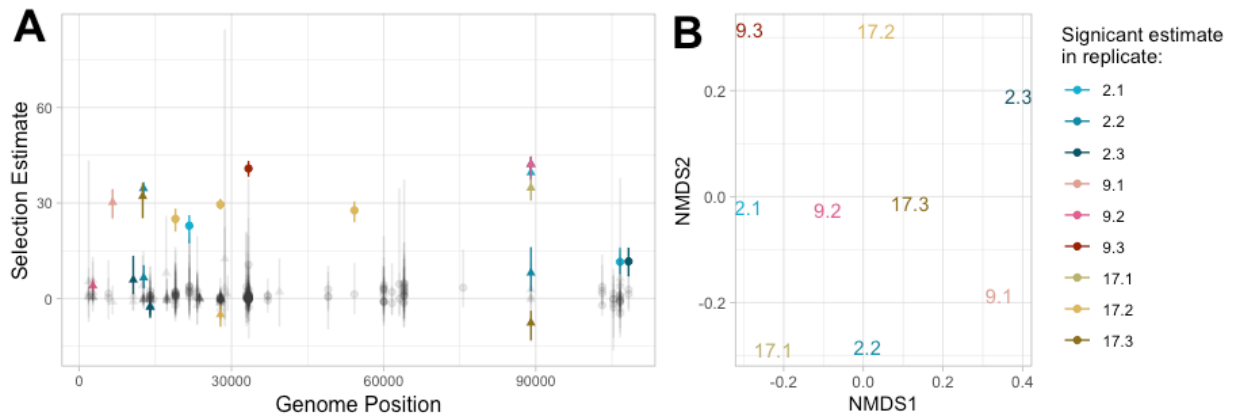
Figure 13: (A) ***Genome-wide selection inferences for SNP and indel variants in each replicate.*** *Significant variants (positive and negative) are colored by the replicate that they are detected in, and non-significant variants are light gray. Circle points represent SNPs and triangles represent Indels. See figures S6-8 for expanded versions of this figure. (B)* ***Multidimensional scaling plots for passage 9 variant communities.*** *Variant communities are transformed using the 'canberra' method and plotted using 'vegan'. See figure S4 for 'rare' and 'common' variant NMDS plots.*

DISCUSSION

Specialization is critical to many of our theories of coevolution and the maintenance of diversity (Futuyma & Moreno, 1988). In particular, specialization between parasites and their hosts is crucial for understanding patterns of disease emergence and spread (Woolhouse & Gowtage-Sequeria, 2005). Here, we use experimental evolution techniques to test whether a granulosis virus can evolve to specialize on specific genotypes of its moth host. We find that the virus evolved to specialize in infectivity, productivity, and fitness on familiar host genotypes (Figure 9).

A unique feature of our experiment is that we collect time series phenotypic and genetic data that allow us to explore the dynamics of specialization in novel ways. First, a key finding of our experiment is that the virus can evolve both higher viral infectivity and productivity on familiar host genotypes, thus specializing (Figure 9). Several previous similar studies have also measured multiple fitness components related to specialization to find that pathogens could variably specialize on parasite virulence and/or transmission (Kubinak et al., 2012a; Legros & Koella, 2010; Nidelet & Kaltz, 2007; Zhan et al., 2002). Kubinak et al. (2012) found that Friend complex virus evolved both higher viral productivity and virulence on familiar host genotypes and Zhan et al. (2002)

found that fungal strains could specialize in both virulence and frequency, though this effect was inconsistent depending on the pathogen strain considered. However, Legros and Koella (2010) found that microsporidia specialized in infectivity, but not productivity, while Nidelet and Kaltz (2007) found that parasites specialized in growth assays, but not horizontal transmission. However, none of these previous studies have examined the correlations between their fitness components across time.

With our phenotypic time series data, we can see that the relationship between our two fitness components (infectivity and productivity) is positive at the start of the experiment but, by passage 9, evolves to be negative when infecting familiar hosts (Figure 12). This correlation is likely to be an emergent property of selection where different virus lines are primarily selected to increase specialization by improving either viral productivity or viral infectivity, rather than an actual genetic trade-off between these traits. The likelihood of specializing on different fitness metrics may be related to evolution background as virus lines evolved on host genotype 17 seem to be more specialized in their productivity at passage 9, while virus lines evolved on host genotype 2 seem to be more specialized in their infectivity at passage 9 (Figure 9). This finding highlights the importance of measuring multiple fitness components when pathogen populations can use many strategies to increase their fitness.

Next, we can ask questions about the number of potential genes involved in specialism evolution. If specialization were to be driven by few mutations of large effect, we would expect to see some degree of genetic parallelism in replicates and strong signatures of selection (if specialization is not driven by mutation accumulation). If there were many genetic options for specialization, we would not necessarily expect to see the phenotypic parallelism of the experiment reflected at the genetic level and selection on any one variant would be weaker.

In our experiment, the evidence indicates that specialization was driven by many variants of small effect (Figure 13). We did not observe any clear signals of selective sweeps where low frequency alleles swept to high frequency. Given the relatively high depth of coverage of our samples and the quality of the sequencing data, it is unlikely that we failed to detect many (if any) sweeps. Furthermore, our selection analysis does not identify any variants with strong parallel signatures of selection across replicates (Figure 13). These results are likely influenced by the facts that our starting population is genetically diverse, so our experiment is more likely to select on standing variation (Long et al., 2015), and that we serially passage through hosts, so transmission bottlenecks likely genetically bottleneck our lineages (Kennedy & Dwyer, 2018). It is also possible that selection for specialization may have been obscured by the initial selection for generally improved fitness in experimental conditions, though we did not see strong, parallel signatures of selection for either general or specialist fitness.

Our results finding many candidate genes with lower selection coefficients are generally in line with previous evolve and re-sequence experiments that start with standing genetic variation and less-specific environmental stressors, though clonal

interference may have been less prominent in our experiment due to the relatively smaller bottleneck sizes in vivo infection processes (Lang et al., 2013; Long et al., 2015; C. R. Miller et al., 2011; Schlötterer et al., 2015; Tenaillon et al., 2012). In the context of virus adaptation to host genotype, Middlebrook et al. (2021) also do not see parallel genetic evolution when FVC virus specializes on mice with different MHC genotypes from a clonal starting population, though they did see evidence that virus populations adapted to each MHC genotype are more similar to each other than to those adapted to foreign MHC types.

Second, we can ask questions about whether specialization is driven by antagonistic pleiotropy, conditionally positive adaptation resulting in fitness asymmetries, or mutation accumulation in alternate environments. If specialization were to be driven by antagonistic pleiotropy, we would expect to see that the most fit replicates on the familiar host are the least fit on the foreign host and that positive selection acts on variants. We would not have clear predictions for how total fitness across all the genotypes would change over time as this would depend on the symmetry of the trade-off shape. In the case of conditionally positive alleles resulting in fitness asymmetries between familiar and foreign hosts, we would expect to see slightly positive or neutral fitness correlations between familiar and foreign hosts, positive selection on variants, and overall increases in total fitness across all the genotypes. In the case of mutation accumulation, we would expect negative fitness correlations between familiar and foreign hosts (the most specialized are those that are worst on foreign hosts), no evidence of positive selection since MA is driven by drift, and overall decreases in total fitness across all the genotypes.

Of course, these mechanisms are not exclusionary, especially in our case where many variants can affect specialization. These predictions may therefore be muddied if multiple mechanisms are driving specialization. Additionally, any directional fitness changes to overall experimental conditions might hamper our ability to fully assess whether fitness correlations between genotypes are positive or negative (as some replicates may just be the 'most adapted' to the general environment) and our ability to assess changes in total fitness across genotypes in the system.

We find that correlations between fitness on familiar and foreign hosts significantly vary depending on the evolutionary history of the virus (Figure 11). There is a negative correlation between fitness on host genotype 17 and foreign genotypes, suggesting that specialization on this host could be consistent with any mechanism. However, correlations between fitness on familiar and foreign host genotypes are positive for virus specializing on host genotypes 2 and not significant for virus specializing on host genotype 9. This suggests specialization driven by asymmetric conditional positivity. Therefore, it is likely that multiple mechanisms contribute to specialization in our system.

From our sequence analysis, we do not see evidence of strong, parallel positive selection on any variants. We observe many instances of subtle frequency

differentiation during the course of the experiment, which seems a likely candidate to explain the genetic mechanism for adaptation (Figures 13, S6-8). Thus, the sequencing data cannot help to exclude potential specialization mechanisms as it is unclear whether these weakly positively selected alleles collectively have strong enough effects to explain phenotypic specialization (as would be predicted by positive selection on antagonistically pleiotropic or conditionally positive alleles) or whether additional drift-based mutation accumulation processes are also needed to explain the specialization in our system.

Finally, the total fitness of virus lineages across all host genotypes does not increase continuously though the experiment (Figure 10). Total fitness does increases from passage 1 to passage 4 but plateaus from passage 6 to passage 9, which is also when we see our largest changes in specialization. This would suggest that antagonistic pleiotropy or a balance of conditional positivity and mutation accumulation is driving specialization. It also suggests that PiGV quickly reached a point of being fairly well adapted to experimental conditions so that directional selection to overall experimental conditions is less likely to obscure patterns resulting from specialization. However, a caveat to these trends in total fitness is that our assay scheme was designed to best test the changes in relative fitness on different genotypes over time and so assayed viruses from different passages on different days. Therefore, these trends in total fitness (but not relative fitness) might be confounded by random day effects.

In this experiment, we have shown that *Plodia interpunctella* granulosis virus can evolve to specialize on specific genotypes of its host and that specialization is not driven by strong selection on few alleles. However, we cannot precisely determine the evolutionary mechanism of this specialization. Putting our evidence together, it seems most likely that the evolution of specialization in our experiment is driven by many genetic variants and by multiple mechanisms. For lines evolved on host genotype 17, which also showed the most specialization via viral productivity, specialization may be most parsimoniously explained by antagonistic pleiotropy as this would explain their negative fitness correlations with overall stable fitness. For lines evolved on genotypes 2 and 9, specialization may be most parsimoniously explained by a combination of weakly positive fitness asymmetries and mutation accumulation in alternate environments as these mechanisms could have collectively driven specialization while their opposing effects on total fitness would result in no total fitness changes. The weak signatures of selection and lack of genetic parallelism in our sequence analysis would fit with these hypotheses if antagonistic pleiotropy and conditionally positive fitness asymmetries are driven by many variants of small effect.

Of course, the findings of our experiment may be limited in their universality as the *Plodia intepunctella* and PiGV system is but one model system with unique biological features like obligate killing and, while our serial passaging protocol closely mimics the natural transmission pathway of oral ingestion of virus killed cadavers, it is not exactly natural transmission in that we homogenize cadavers and transmission is constrained

to happen on a certain day after exposure (day 20), to a specific larval instar (3rd), and at a specific dose. Thus, further studies on this topic in different model systems will only help to strengthen our understandings of the dynamics of specialization.

In conclusion, we used an experimental evolution approach to determine whether a baculovirus could evolve to specialize on specific genotypes of its moth host. We find that virus does evolve higher infectivity, productivity, and fitness on familiar host genotypes (Figure 9). This specialization may be variably driven by combinations of antagonistic pleiotropy, conditionally positive alleles leading to fitness asymmetries, and mutational accumulation on foreign host genotypes in our different evolutionary conditions. Time series data shows that specialization in fitness evolves over the time course of the experiment and that the different fitness components of virus lineages may be independently selected on (Figures 10, 12, S2). Our results demonstrate that gene-by-gene interactions are evolvable in the *Plodia interpunctella* and PiGV model system and suggests that the system has promise for experiments on the ecological conditions that shape selection on specialization and niche breadth.

Chapter 4

Spatial structure reverses the impact of local adaptation on pathogen exploitation rates

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ABSTRACT

Host genetic diversity and the spatial structuring of transmission both influence pathogen evolution, but how these processes interact has not been well considered. Here, we experimentally evolve granulosis virus in mesocosms of its *Plodia interpunctella* host with varying degrees of spatial structure and host genetic diversity. We find that virus evolves specific interactions with its locally familiar host genotype in both homogeneous and spatially heterogeneous host populations, but that the impact of local adaptation depends on the spatial structuring of contacts. In one treatment, exploitation rates are higher on the familiar host, while, in the other, they are lower. While we find that food viscosity had unexpected impacts on the spatial structuring of contacts, spatially structured transmission may have led to the virus evolving more prudent exploitation rates on only the familiar host, leading to locally maladapted high exploitation rates on foreign hosts. Additionally, we also find that virus in heterogeneous mesocosms is more locally adapted when there are higher migration rates between the host types. Our experiment therefore demonstrates that trade-offs optimizing exploitation rates at intermediate values, like those governing pathogen infectivity in spatial structure, may interact with trade-offs determining niche breadth in ways that can reverse the impact of local adaptation on pathogen phenotypes.

INTRODUCTION

When trade-offs between different phenotypes act upon an organism, they can lead to a variety of evolutionary outcomes including the evolution of intermediate optimal strategies, evolutionary branching and niche partitioning, and cyclical dynamics (Ackermann & Doebeli, 2004; Dieckmann et al., 1995; Farahpour et al., 2018; Geritz et al., 1998). Thus, trade-off theory underpins a wide array of ecological and evolutionary theory on adaptation and diversification (A. A. Agrawal et al., 2010; Chesson, 2000). One area where trade-offs are of particular importance is in explaining how biotic diversity is generated and maintained (Godsoe et al., 2021; Levins, 1962; Macarthur & Levins,

1967). The idea here is that 'jacks-of-all-trades are the master of none', meaning that trade-offs between adaptation to different abiotic environments or biotic partners require an organism to choose between being a fit specialist or mediocre generalist (MacArthur, 1984). For host-parasite interactions, this fundamental problem of niche breadth evolution becomes a question of host range evolution (Antonovics et al., 2013; Best et al., 2010; Boots et al., 2014) and has implications for theories ranging from the Red queen hypothesis of sex (Jaenike, 1978; Lively & Dybdahl, 2000), Janzen-Connell effects (Connell, 1971; Janzen, 1970), Geographic mosaic theory (Thompson, 2005), and zoonotic emergence (Guth et al., 2019; Woolhouse & Gowtage-Sequeria, 2005).

However, it is an obvious fact that any organism is subject to multiple environmental pressures and thus a single phenotype may be subject to multiple, potentially interacting trade-offs simultaneously (Sexton et al., 2017). For example, while an organism's growth rates on different environments might trade-off with each other, that organism's growth rate might also be subject to life history trade-offs that select for intermediate optimal strategies (Stearns, 1989). Less appreciated is how the specific geometries and interactions between trade-offs can lead to wildly different evolutionary outcomes for a system (Cressler et al., 2010; Stearns, 1989; Steiner & Pfeiffer, 2007). Depending on the order in which we consider our trade-offs, for above example, we could consider that: 1) growth rates could be lower than maximal across the system, but still highest for the specialist on its familiar environment or 2) growth rates could be at the intermediate optimal level for both the specialist on its familiar environment and the 'mediocre' generalist on both. While the first type of interaction more easily allows for predictions from single trade-off theory to be extended to multiple, there is no reason to expect that it should be more common. It is therefore necessary to better understand how trade-offs interact to determine how predictions of single trade-off theory scale into more ecologically realistic scenarios.

Here, we experimentally evolve granulosis virus in mesocosms of its *Plodia interpunctella,* or Indian meal moth (Hübner), host that vary in their host genetic diversity and spatial structuring. Altering host genetic diversity allows us to examine the consequences of trade-offs between host types on virus evolution (Regoes et al., 2000), while altering the spatial structure of infectious contacts selects on pathogen exploitation rate (Boots & Sasaki, 1999). Additionally, the spatial structuring of genetic diversity might further influence host range evolution due to differences in contact rates between hosts (Gandon & Poulin, 2004). Thus, our experiment examines how pathogen evolves when experiencing a combination of selection pressures that are governed by separate, but potentially interacting, trade-offs.

On its own, spatially structured transmission is predicted to select for less infective, more prudent pathogens as higher proportions of local, rather than global, transmission mean that the pathogen competes with more closely related strains to deplete its local susceptible population and 'self-shade' (Berngruber et al., 2015; Boots & Sasaki, 1999, 2000; Lion & Boots, 2010). Previous evolution experiments in the Plodia interpunctella

and PiGV system support this theory (Boots & Mealor, 2007), as do experiments in microbial systems (B. Kerr et al., 2006). On its own, host heterogeneity is also predicted to select for less infective and virulent pathogens, but, in this case, these lower exploitation rates are due to evolutionary constraints where adapting to two host types mean that the pathogen is less able to specialize on either one (Elton, 1958; Levins, 1962; Regoes et al., 2000; Visher & Boots, 2020). Some previous experiments in empirical systems confirm these hypotheses (Cornwall et al., 2018; Gibson, 2022; González et al., 2019; Hughes & Boomsma, 2004; Kubinak et al., 2012b; White et al., 2020), but these results are not always consistent and the exact nature of such constraints and whether they are due to inevitable antagonistic pleiotropy or limitations to selection processes is still unclear (Bono et al., 2017, 2019; Burmeister & Turner, 2020; Remold, 2012; Visher & Boots, 2020; Whitlock, 1996). Our experiment therefore examines how an optimizing life history trade-off mediated by susceptible density and acting at the population level interacts with a constraint-based trade-off acting at the individual level.

Finally, the spatial structuring of host genetic diversity has important consequences for how pathogens are expected to evolve in metapopulations and locally adapt to different patches (J. T. Anderson et al., 2013; Kawecki & Ebert, 2004; Nuismer, 2006). Theory suggests that contact rates between hosts, or migration between patches, should alter how pathogens are select to specialize on their familiar host or become generalists. Higher contact rates between hosts should select for generalists (Gandon & Poulin, 2004; Osnas & Dobson, 2012; Williams, 2012), but can also lead to greater local adaptation when migration increases the amount of genetic variation that selection can act upon and increases local competition for patches (Gandon, 2002; Gandon et al., 1996; Gandon & Michalakis, 2002).

Our experiment therefore explores how trade-offs mediated by spatial structure and host heterogeneities interact and how the spatial structuring of host heterogeneity affects its impact on pathogen evolution. We find that manipulating the spatial structure of a population can reverse the impact of local adaptation on pathogen phenotypes so that they are less exploitative of their familiar host than of foreign hosts. We also find that virus in heterogeneous mesocosms is more local adapted when there are higher migration rates between the host types. These results imply that interactions between trade-offs mean that specialization or local adaptation might not always correlate with the highest exploitation rates, or per-infection fitness, on the familiar environment when optimizing trade-offs are simultaneously selecting for lowered exploitation rates.

METHODS

Study system

Plodia interpunctella, or the Indian meal moth, is a stored grain pest that naturally lives at high population density within its cereal food medium for 5 larval instar stages before it pupates into an adult moth that disperses, mates, and lays eggs, but does not eat (Gage, 1995; Mohandass et al., 2007). It is naturally infected by the *Plodia interpunctella* granulosis virus (PiGV), a dsDNA baculovirus that transmits when larvae directly cannibalize infection killed cadavers or consume virus that was released into the environment from such cadavers (Consigli et al., 1983; R. L. Harrison et al., 2016b). Thus, the virus is an obligate killer that will only transmit if it kills its host. Notably, the virus can only infect *P. interpunctella* during the larval stages and, if exposed larvae clear the infection, they can pupate and carry out the rest of their life history as normal (Tidbury, 2012). Infection killed cadavers are recognizable because successful infection turns larvae an opaque, chalky white color due to the high density of viral occlusion bodies (Begon et al., 1993). The system has proved a powerful tool to examine the effects of spatial structure on pathogen exploitation rates and, more recently, host genotype specialization (Boots & Mealor, 2007; Visher, Uricchio, et al., 2021).

Host line selection and maintenance

For this experiment, we select 2 inbred populations of *P. interpunctella* that were previously generated in the lab by brother-sister mating individuals for >27 generations (Bartlett et al., 2018). Each population should therefore be essentially a clonal population of two distinct host genotypes (Il-9 and Il-17). We have previously shown that virus evolved in homogeneous populations of these specific inbred lines can specialize on its familiar host genotype by increasing their infectivity and/or productivity (Visher, Uricchio, et al., 2021).

We maintain these lines in wide mouthed Nalgene pots (ThermoFisher Scientific, U.K.) in the absence of infection with 200g 'Standard' food medium (500g cereal mix (50% Earth's Best oatmeal, 30% wheat bran, 20% rice flour), 100g brewer's yeast, 2.2g sorbic acid, 2.2g methyl paraben, 125ml honey, and 125ml glycerol) in incubators set at 27 ± 2 °C and $35\pm5\%$ humidity, with 16:8hr light: dark cycles (Bartlett et al., 2020). To maintain populations, ~50 adult moths are moved into a new pot with new food when they emerge monthly. Thus, host lines are maintained in the absence of infection and do not evolve throughout the experiment.

Manipulating spatial structure

We manipulate spatial structure in the experiment by changing the viscosity of the food medium, as in (Boots, Childs, et al., 2009; Boots et al., 2021; Boots & Mealor, 2007). Because larvae live within their food medium, this alters individuals' dispersal patterns and thus the spatial structure of their infectious contacts. We make food of two viscosities, 'loose' and 'sticky', which differ solely in the amount of glycerol added. 'Loose' food contains 500g Earth's Best oatmeal, 100g brewer's yeast, 2.2g sorbic acid, 2.2g methyl paraben, and 175ml glycerol. 'Sticky' food contains 500g Earth's Best

oatmeal, 100g brewer's yeast, 2.2g sorbic acid, 2.2g methyl paraben, and 450ml glycerol. 'Loose' and 'sticky; food is then added to wide-mouthed Nalgene pots and frozen overnight to kill any insect eggs potentially contaminating the food. The mass of food added to these pots (300g 'loose' and 550g 'sticky' food) was determined by the volume needed to entirely fill half of one of our plastic mesocosm containers.
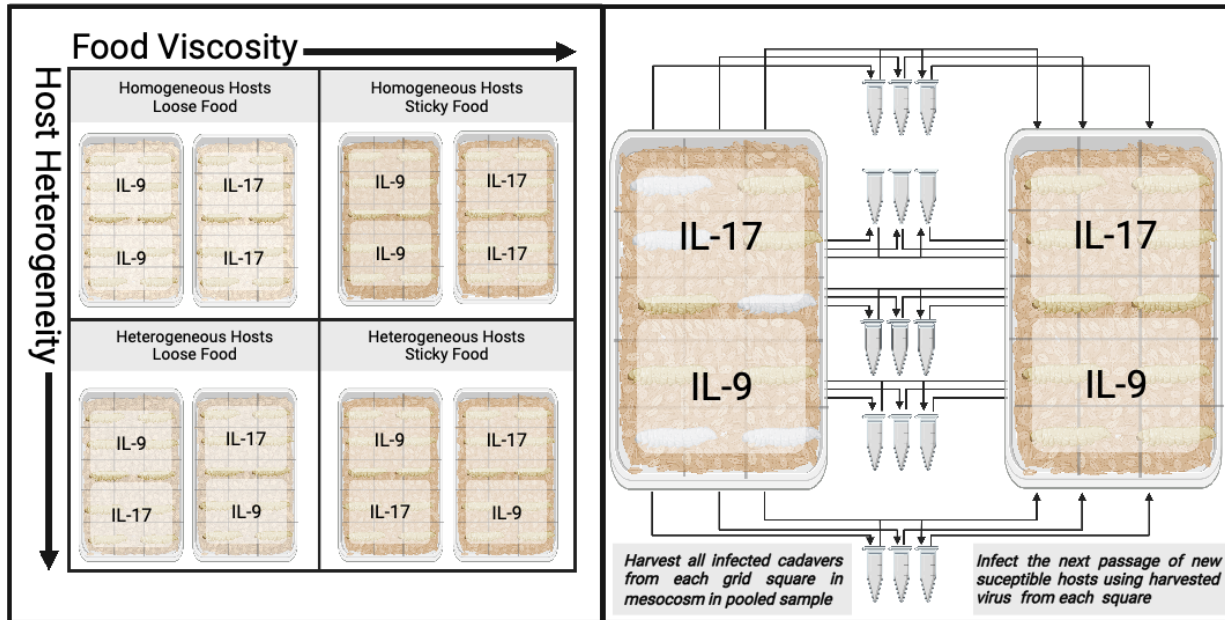
Figure 14: Experimental design and passaging scheme. (Left) Each mesocosm is set up by adding 2 pots of food containing 11-day old larvae to opposite vertical ends of a plastic mesocosm with a 3x5 grid. The 3rd row of the grid is therefore a contact zone between the sides. There are 3 replicates for each of the 8 mesocosms. There are 4 different treatment types of homogeneous or heterogeneous hosts by 'loose' or 'sticky' food. Each homogeneous treatment is set up with both host types and each heterogeneous host treatment is set up alternating which host is on 'top' or 'bottom' of the grid. (Right) After 20 days incubating with virus, mesocosms are frozen and dissected into grid squares. All infected larvae from each grid square are harvested and added to a 1.5mL Eppendorf tube (1 square per tube) and homogenized to release occlusion bodies. 500mL of the virus solution is then pipetted in droplets over the same grid square of a new mesocosm of the same treatment set-up with fresh 11-day old hosts. Therefore, the spatial force of infection is preserved across passages. We experimentally evolve virus for 10 passages.

Setting up host populations for mesocosms

After thawing the pots of 'sticky' and 'loose' food, we add ~50 similarly aged adult moths to each pot from either our Il-9 or Il-17 host maintenance populations and incubate them for 10 days as above. These single-genotype adults then mate and lay eggs into the 'sticky' or 'loose' food. 10 days after adding adults, these pots containing Il-17 larvae in 'loose food, Il-17 larvae in 'sticky' food, Il-9 larvae in 'loose' food, or Il-9 larvae in 'sticky' food are used to set up mesocosms.

Mesocosms are set up in 9.25" x 12.08" x 0.9" inch plastic A4 document cases (Daiso Japan, U.S.A.) that have been marked with an even 3 x 5 grid of 3.08" x 2.42" squares.

We set up mesocosms with 6 treatments: 1) homogeneous Il-9 hosts in 'loose' food; 2) homogeneous Il-17 hosts in 'loose' food; 3) homogeneous Il-9 hosts in 'sticky' food; 4) homogeneous Il-17 hosts in 'sticky' food; 5) heterogeneous hosts (Il-9 + Il-17) in 'loose' food; and 6) heterogeneous hosts (Il-9 + Il-17) in 'sticky' food (Figure 14). Each mesocosm therefore has a single food type (either 'sticky' or 'loose'), according to its treatment. To arrange hosts in the mesocosm, we add 2 pots of the food containing 10-day old larvae to opposite vertical ends of the grid, so that food fully fills the plastic containers. For homogeneous host treatments, we add 2 pots containing larvae of the same genotype (either both Il-9 or both Il-17) and, for heterogeneous treatments, these 2 pots contain larvae of different genotypes. Thus, homogeneous genotype mesocosms have a single genotype of host throughout the grid, while heterogeneous mesocosms have two genotypes separated vertically across the grid with a contact zone at the 3rd row. To balance our treatments and account for potential effects due to position within the grid, we set up 2 mesocosms of each of the 2 heterogeneous treatments and alternate which genotype is on 'top'. There are therefore 8 unique mesocosm types.

These mesocosms are then incubated overnight, as above, so that virus can be added to them on day 11. We repeat this process each passage to set up new mesocosms so that evolving virus can be added to a fresh mesocosm. This allows us to maintain 2 non-interbreeding and non-evolving host genotypes in our mesocosms and preserve the spatial structuring of such genotypes across passages.

Experimental evolution

We set up 3 replicates of each of our 8 unique mesocosm types (24 serially passaged mesocosms total). Experimental evolution is initiated by pipetting 5ml of 10^{-2} stock virus solution in small droplets evenly over the top of each mesocosm. Each mesocosm is then incubated for 20 days and then frozen overnight to kill larvae for harvesting. Passage lengths (10 days before set-up, 1 day overnight, 20 days with virus) therefore roughly correspond to the host's natural ~monthly cyclical demography (Boots, Childs, et al., 2009). We stagger the replicates' passaging schedules weekly due to the time intensive nature of passaging.

To harvest virus, we dissect each microcosm by grid square and separately collect infection killed cadavers from each square in sterile 1.5ml Eppendorf tubes (Figure 14). We add 1ml sterile, MilliQ water to each square's tube and use a sterile pellet pestle (Fisher Scientific, U.S.A.) to manually burst larvae and release viral occlusion bodies. We droplet 500ul of this solution from each square onto the same square of that treatment's freshly set-up microcosm to infect the next passage and freeze the rest of the solution. Therefore, we preserved the force of and spatial structure of infection within the grid across passages. We record the number of infected cadavers in each square at each passage to track the spatial ecology of infection in the experiment.

The mesocosms newly infected with passaged virus were then placed back into incubators for 20 days to allow the virus to infect larvae for the next passage. We

experimentally evolve virus for 10 passages, a number standard for microbial evolution experiments. There are several occasions where we had to use the frozen virus solutions reserved during passaging to re-set up passages due to low numbers of infected cadavers or contamination with another pathogen. Contamination was recognizable because the most common contaminant, *Bacillus thuringiensis* (Bt), turns infected larvae black rather than white (Oppert et al., 2000). We could generally expect any co-infected larvae to display the black phenotype since Bt kills larvae more quickly (Yitbarak, unpublished data), so re-setting up passages when Bt was visually detected would clear the virus population. Congruously, we did not see any signs of persistent contamination within our passages nor in any of our assays.

<u>Assay</u>

After passage 10, we collect virus infected cadavers from each square as above. We next pool the virus populations from the 3 squares in each row of the 3x5 grid in equal proportion so that we have 5 virus samples per mesocosm. Each pooled sample is therefore the virus population at a certain vertical position within the mesocosm, along the gradient of heterogeneity for the heterogeneous host mesocosms. We purify virus by centrifuging for 1 min at 3000 rpm to remove larger particulate matter and then 3 min at 13000 rpm to pellet virus. We run these samples through a .65 micron filter to semi-purify our virus of larger bacterial and fungal contaminants, as in (Visher, Uricchio, et al., 2021). Next, we quantify the concentrations of each sample by counting occlusion bodies on a Petroff-Hauser counting chamber with 400x darkfield microscopy and dilute each to an assay dose concentration of ~$7.5x10^8$ occlusion bodies per mL in 2% sucrose and .2% dye (Visher, Uricchio, et al., 2021). The sugar entices the larvae to ingest the virus and the dye allows the experimenter to determine which have ingested half their body lengths of solution and are considered exposed.

We assay each pooled virus sample on both of our 2 host genotypes, Il-9 and Il-17, to determine the proportion of hosts each virus sample infects on each genotype and the average number of occlusion bodies each virus sample produces per infection on each genotype. With 5 virus samples for each of the 8 mesocosms that have 3 replicates, this results in 240 virus sample x assay line combinations. We batch these assays to infect 1 genotype with all 5 virus samples for 4 of the mesocosms of 1 replicate each day. Since we can only assay half of a replicate's mesocosms each day, we balance the batches so that the 4 mesocosms assayed each day have equal homogeneity/heterogeneity, Il-9/Il-17 proportions, and loose/sticky food. Because only 1 assay genotype is assayed per day, we include assay genotype in all statistical models to account both for general differences in resistance and assay day effects.

To set up infectivity assays, we first move ~70-80 adult moths of the appropriate inbred line for the batch into new pots with 200g 'standard' food. 11 days after setting up assay pots, we collect 100 third instar larvae for each assay combination in a petri dish and stave them under a damp paper towel for 2 hours. After starvation, we syringe tiny droplets of the appropriate virus-sucrose-dye solution onto the petri dish for the larvae

to consume. We add 50 larvae that have orally ingested half their body lengths of virus solution to 2 25-cell compartmentalized square petri dishes (ThermoFisher Scientific, U.S.A.) with 'standard' food and incubate them for 20 days (Visher, Uricchio, et al., 2021). Assay grids are labelled with random identifiers to blind assay combinations and prevent bias. After 20 days, assay grids are frozen and destructively sampled to count the number of infected and uninfected individuals in each grid. Infected cadavers from each assay grid are then saved in a pooled sample for virus quantification.

To determine the average number of occlusion bodies produced per infected individual, we extract virus from the pooled samples from each assay grid using a sterile pellet pestle (Fisher Scientific, U.S.A.) to manually burst larvae and release viral occlusion bodies. We then centrifuge these samples as above, but do not filter them, and count occlusion bodies on the Petroff-Hauser counting chamber. The concentration of occlusion bodies is then divided by the number of infected cadavers that were in the pooled sample to get the average number of occlusion bodies produced per infection for each virus sample on each host genotype.

Statistical analyses

All models were run in R version 4.2.0 (2022-04-22) -- "Vigorous Calisthenics" (R Core Team, 2021). We used packages 'glmmTMB' and 'lme4' to build models, 'DHARMa' to check model residuals, 'afex' and 'car' to determine significant model terms, 'emmeans' to extract effects, 'tidyverse' to manipulate data, and 'patchwork' and 'ggplot2' to plot results (Bates, Mächler, et al., 2015, p. 4; Brooks et al., 2017; Fox & Weisberg, 2019; Hartig & Lohse, 2021; R. V. Lenth et al., 2022; Pedersen, 2020; Singmann, Bolker, Westfall, Aust, Ben-Shachar, et al., 2019; Wickham, 2009; Wickham et al., 2019). We determine error structures for models by testing fitted models with 'DHARMa' and then adjusting to best fit residuals. On occasion, observation level random effects are used to correct overdispersion in our models (X. A. Harrison, 2014). Where necessary, contrasts are used with 'emmeans' to determine effect estimates for treatments.

Infection time series analysis

We examine the spatial structuring of transmission using the data collected on the number of infected individuals in each square at each passage for each mesocosm. To determine whether food viscosity affected the spatial structuring of infection, we built a generalized linear mixed model (GLMM) with a negative binomial distribution ('nbinom1') that asked whether the number of infected individuals in a square is significantly predicted by the number of infected individuals in that same square at the previous passage and whether this term interacted with food viscosity. The model also included a fixed effect of the position of the square itself (to account for larval preferences in where they moved) and random effects of passage number, replicate, the pot of larvae used to set up that half of the mesocosm (to roughly account for susceptible density), and observation ID.

We additionally run a Levene's Test for Homogeneity of Variance on the infected count data to determine if our 'sticky' and 'loose' food treatments differ in their variance, a measure of how 'clumpy' infection is within a mesocosm.

Assay analysis

Infectivity and productivity assay data was analyzed in a generalized linear mixed modeling framework using the same packages and process as above (Bolker et al., 2009). We build models treating proportion infected, average number of occlusion bodies per infected individual, and the composite exploitation rate (proportion infected multiplied by the average occlusion body count) as our response variables. We transform average virus counts and composite exploitation rates by multiplying and rounding them to produce integer counts for Poisson and negative binomial distribution assumptions. In each model, we include random effects for replicate, assay grid, and the nested effect of virus sample under replicate under treatment.

First, we ask whether virus populations significantly differ in their specialization at the whole mesocosm level by building models that include an interaction effect between the assay being on the host that the population was evolved on ('familiar') or the foreign host and the type of food the population evolved in. Virus from heterogeneous populations is coded as 'heterogenous' as they do not have a familiar or foreign host at the whole mesocosm level. We also include assay line as a fixed effect to account for overall differences in resistance between our assay host genotypes. The infectivity model uses a binomial error structure with observation level random effects, the productivity model uses a Poisson error structure with observation level random effects, and the exploitation rate model uses a zero-inflated negative binomial error structure with observation level random effects. To better see the effects of specialization in the homogeneous populations, we also run these same models for the homogeneous host treatments only with the infectivity model using a binomial error structure, the productivity model using a Poisson error structure, and the exploitation rate model using a negative binomial error structure with observation level random effects.

Next, we ask whether spatial structure or host heterogeneity have significant effects on viral phenotypes across genotypes (without accounting an effect of local adaptation). We build models for infectivity and productivity with the same random effects as above, and without fixed effects for being assayed on the familiar or foreign host. For host heterogeneity models, we include the assay line, food type, and whether the treatment had homogeneous or heterogeneous hosts as fixed effects, allowing for an interaction between food type and treatment heterogeneity. The infectivity model uses a binomial error structure with observation level random effects and the productivity model uses a Poisson error structure with observation level random effects. For spatial structure models, we include the food type and assay line as fixed effects with the same random effects as above. The infectivity model uses a binomial error structure with observation level random effects and the productivity model uses a Poisson error structure with observation level random effects.

Finally, we ask whether virus phenotypes are spatially structured within the heterogeneous host treatments. First, the local proportion of host genotype 17 is determined for each virus population by its distance from the host contact zone so that the edge of the mesocosm where host genotype 17 was added is coded as 1, the next row is of the grid is coded .75, the middle contact zone is coded as 0.5, the following row is coded as .25, and the edge of the mesocosm where host genotype 9 was added is coded as 0. We build models for infectivity, productivity, and exploitation rate that include the local proportion of host genotype 17, the host genotype of the assay, the food type, and interaction effects between all three fixed terms. We include the same random effects as in the previous models for replicate, assay grid, and nestedness, but also include random terms for the assay set-up date and the specific position and order of lines to account for the fact that we have two heterogeneity treatments that differ in which line is on 'top' or 'bottom' of the mesocosm. The infectivity model uses a binomial error structure with observation level random effects, the productivity model uses a Poisson error structure with observation level random effects, and the exploitation rate model uses a zero-inflated negative binomial error structure with observation level random effects.

RESULTS

Food viscosity spatially structures of infection

Across the whole experiment, the density of infected hosts in a position positively correlates with the density of infected hosts in that position in the previous passage (Figure 15, estimate = 0.009, p = 0.011). There are also significant effects of the position itself (p < 0.001), where infection is more likely to be found in corner and edge squares of the mesocosm and on the side closer to light, and the type of food, where infection is higher in 'sticky' food (estimate = 0.26, p < 0.001). Importantly, there is a significant interaction term between the effect of infected density at a position in the previous passage and the type of food (p=.04). However, the predictive power of the previous passage's density of infected hosts in a position is significantly weaker for 'sticky' food that for 'loose' food (Figure 15, estimate = -0.009, p = 0.04). This means that, contrary to our expectations, 'loose' food spatially structures infection at the grid square level more than 'sticky' food in our experiment. From experimenter observation, it seems likely that this is because 'loose' food packed mesocosms more precisely than 'sticky' food, leaving fewer gaps for larvae to exit the food into and move unimpeded along. At the same time, however, the food types also differ in the variance of their infected count data (p < 0.001), with 'sticky' food having more clustered infected individuals.
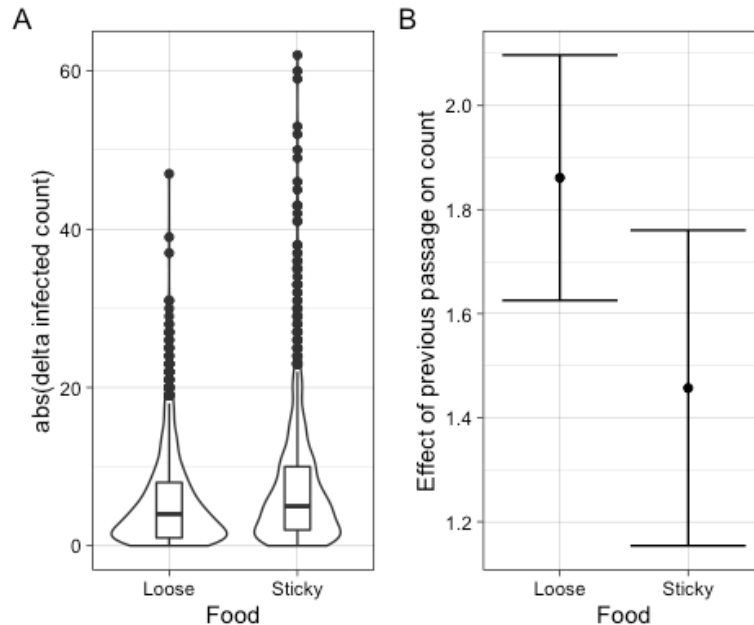
Figure 15: (A) Change in the number of infected cadavers in a square between passages (y-axis) for 'loose' and 'sticky' food (x-axis). (B) Effect estimates drawn from a GLMM for the interaction between type of food evolved on and the effect of the previous passage's infection count at a square on the current passage's infected count on a square.

The impact of local adaptation on virus phenotypes depends on spatial structure

Averaged across host genotypes, the spatial structure manipulation does not affect virus populations evolved per particle infectivity (p = 0.90) or per infection virus productivity (p = 0.81) (Supplemental Figure 1, Chapter 4 Supplemental Material). This is counter to previous results that only assay virus on genetically diverse local hosts (Boots & Mealor, 2007). However, any impact of spatial structure may also depend on local adaptation as evolved virus populations are assayed on both familiar and foreign hosts. When test whether spatial structure interacts with host genotype specialization, we find that there are significant interaction effects between which host genotype the population was evolved on, the host genotype assayed on, and the type of food evolved on (Figure 16). Specifically, for virus populations evolved in homogeneous host populations, viruses from 'loose' food populations are less infective on their familiar host (estimate = -0.311, p = 0.013) while those from 'sticky' food populations do not differ in infectivity between familiar and foreign hosts (estimate = 0.011, p = 0.37) (Figure 16B). Simultaneously, viruses from 'loose' food populations are less productive on their familiar host (estimate = -0.437, p < 0.001) while those from 'sticky' food are significantly more productive on familiar hosts (estimate = 0.602, p < 0.001) (Figure 16D). Collectively, this means that the viruses are more prudent on their familiar hosts in 'loose' food and more exploitative on familiar hosts in 'sticky' food (Figure 16F).
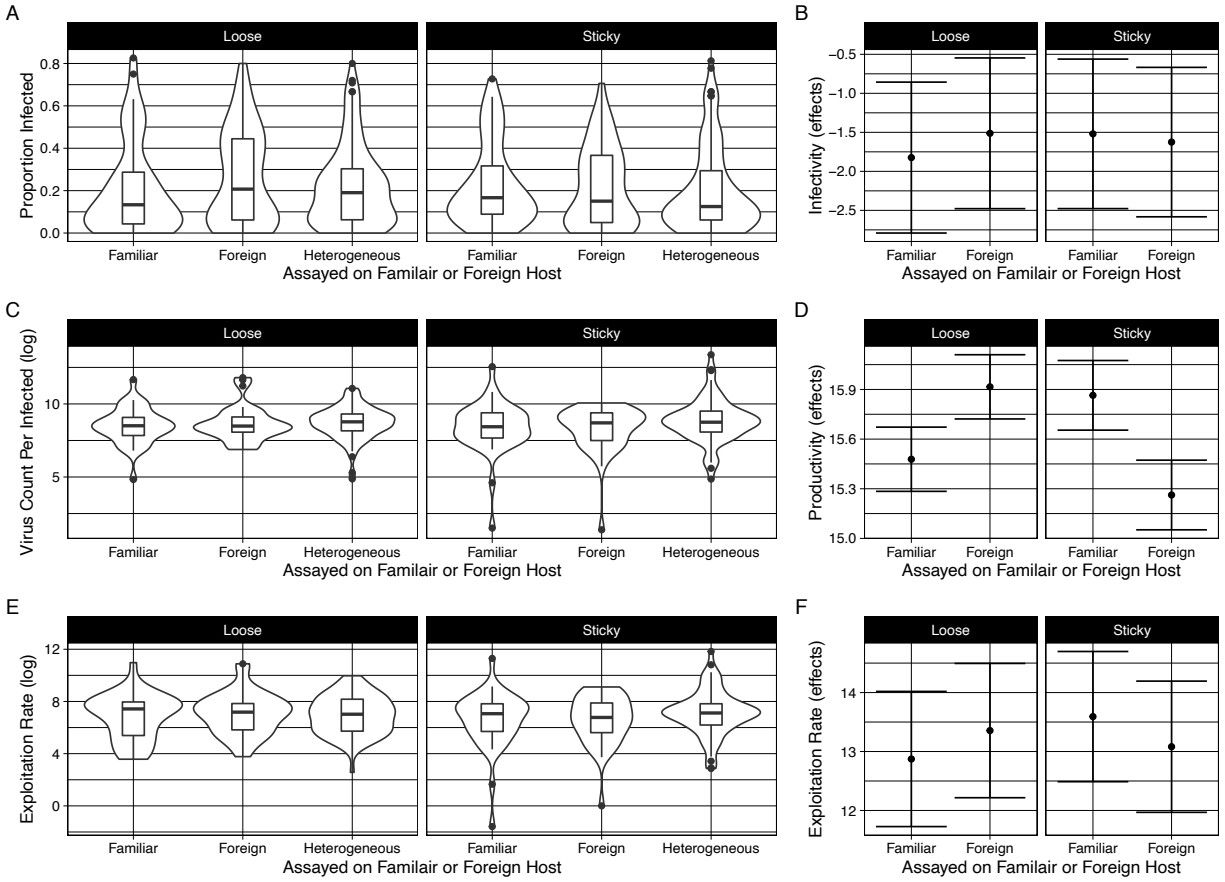
*Figure 16: Interactions between local adaptation and the spatial structuring of infection. Panels A, C, E plot raw data and panels B, D, F show model effect estimates drawn from GLMMs for the homogeneous host dataset. Y-axes represent: (A) proportion infected, (B) effect estimates for the infectivity model for homogeneous host treatments only, (C) average number of occlusion bodies produced per infectious cadaver on a log scale, (D) effect estimates for the viral productivity model for homogeneous host treatments only, (E) composite exploitation rate (proportion infected * average number of occlusion bodies) on a log scale, and (F) effect estimates for the exploitation rate model for homogeneous host treatments only. X-axes represent whether the virus from homogeneous host treatments was being assayed on the host genotype that it evolved on or on the foreign host genotype (A-F) or whether the virus came from a heterogeneous host treatment (A, C, E). Panels show whether the virus came from a treatment with 'loose' food or 'sticky' food.*

Spatially structured host genetic diversity leads to local adaptation

On average, virus populations evolved in heterogeneous host populations do not significantly differ in infectivity (p = 0.79) or productivity (p = 0.087) from those evolved in homogeneous host populations. However, heterogeneous host populations have gradients of host mixing within them as the different positions are different distances from the other host genotype. Additionally, we expect spatial structure to impede host movement and thus further alter the degree of host mixing for different positions. Therefore, we explore whether there is an effect host specialization within the heterogeneous host mesocosms and whether this effect is altered by the spatial

structuring of the population. We find that there is an interaction between the local proportion of a host genotype (i.e. the position's distance from the contact zone) and the bias in exploitation rate (a composite metric of virus infectivity and productivity) that is mediated by the type of food evolved in (p = 0.059, Figure 17). As in homogeneous populations, virus populations are biased towards higher exploitation rates on familiar hosts in 'sticky' food populations (estimate = 1.57, CI = [0.06, 3.08]) and somewhat lower exploitation rates on familiar hosts in 'loose' food populations (estimate = -0.524, CI = [-2.14, 1.09]).
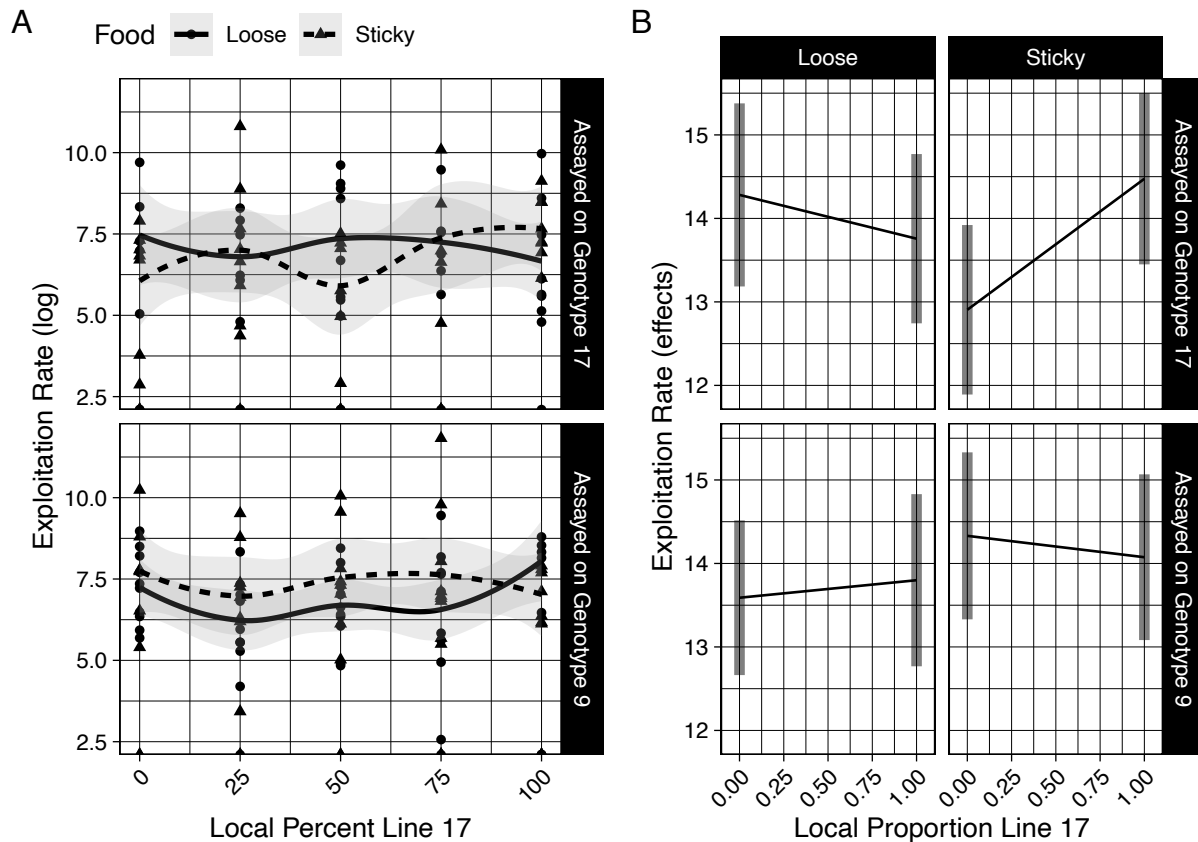
*Figure 17: Interactions between local adaptation and the spatial structuring of infection within heterogeneous host populations. (A) Raw data for the composite exploitation rate (proportion infected * average number of occlusion bodies, log-scale, y-axis) for virus sampled from different spatial positions within the mesocosm with different degrees of mixing between the 2 host types (x-axis). Virus from 'loose' food treatments is represented with circle points and solid line and virus from 'sticky' food treatments is represented with triangle points and dashed line. Panels represent which host genotype the virus was assayed on. (B) Model effect estimates drawn from a GLMM for the effects of food type and assay genotype on exploitation rate (y-axis) for virus sampled from different spatial positions within the mesocosm with different degrees of mixing between the 2 host types (x-axis). Panels are separated horizontally by food type and vertically by which host genotype the virus was assayed on.*

DISCUSSION

In our experiment, we find that pathogens can evolve to be more exploitative of their familiar host on one food type and less exploitative of their familiar host on the other food type. This effect holds at both the treatment level for virus lines evolved in homogeneous host environments and as a gradient affect in heterogeneous host environments where the degree of local adaptation depends on the distance from the host contact zone (Figure 16, Figure 17). Therefore, our experiment suggests that section pressures related to host diversity and spatial structure interact to reverse the outcome of local adaption on pathogen exploitation rate. This result has important implications for eco-evolutionary theory.

Most theory depending on specialist interactions assumes that the pathogen should always have the highest exploitation rate on its familiar host (Abrams, 2006; Chesson, 2000; Lively & Dybdahl, 2000). This is partially because most of these models focus only on the infectivity component of pathogen specialization and do not consider post-infection processes like replication and virulence where high levels might be expected to lower fitness (Dybdahl & Storfer, 2003). Even then, our results suggest that infectivity might also not always be maximized on the familiar host when more prudent strategies are fitter (Lion & Metz, 2018). The implications of this phenomena for theory on how antagonistic biotic interactions maintain diversity have not been well considered.

Classically, theory on how antagonistic biotic interactions maintain diversity depends on the idea that pathogens have the highest exploitation rates on their familiar or locally common host so that rare hosts have a fitness advantage (Gibson et al., 2020). When transmission between host types is low, maladaptively high virulence on a host would lead to stuttering transmission chains and local extirpation of the pathogen in the rare host and preserve rare genotype advantage (Guth et al., 2019; Plowright et al., 2017). However, when transmission between host types freely occurs, selection for lower exploitation rates on the familiar host resulting in maladaptively high exploitation on foreign or rare hosts means that rare genotype advantage is not ubiquitous (Dybdahl & Storfer, 2003). Therefore, environments selecting for lower exploitation rates may also be less likely to conform to the predictions of eco-evolutionary theory that depends on this rare genotype advantage like the Red Queen Hypothesis of Sex (Jaenike, 1978) and Janzen-Connell effects (Connell, 1971; Janzen, 1970). Additionally, we should not always expect pathogens to have the highest exploitation rates on their familiar host, even in the absence of co-evolutionary dynamics where the host might be further 'ahead' in the arms race (Greischar & Koskella, 2007). Our data shows that patterns approximating the host being 'ahead' in a coevolutionary arms race can be found even in the absence of host evolution.

Our result that virus evolves lower exploitation rates on the familiar host in one of our food types could be understood if food viscosity was spatially structuring infectious contacts so that pathogens were selected to lower their exploitation rates, but that they were only selected to do so on the familiar host. While the pathogen would be selected

to have the highest 'fitness' on its familiar host in each treatment, the optimal exploitation rate associated with that peak fitness would depend on the spatial structuring of infectious contacts because self-shading in spatial structured environments selects for more prudent pathogen strategies (Lion & Boots, 2010). This means that the pathogens selected in spatially structured environments could actually have higher exploitation rates on foreign host genotypes than familiar.

However, our evolution experiment resulted in lower exploitation rates on the familiar host in 'loose' food, despite our expectations that 'sticky' food would spatially structure the population more. Previous experiments with similar food types used 'sticky' food to spatially populations as they, and our preliminary experiments, showed that 'sticky' food impedes larval movement (see Supplementary material) (Boots et al., 2021; Boots & Mealor, 2007). Therefore, we were expecting to see lowered exploitation rates in 'sticky' food, rather than 'loose' food.

However, we do have evidence that our food viscosity manipulation behaved in unexpected ways. Our infection ecology data seems to suggest that 'loose' food spatially structured infection more than 'sticky' food at the level of force of infection within a 3.08" x 2.42" grid square between passages. One reason for the different outcome in our experiment may be that slight differences in 'sticky' food viscosity can lead to higher rates of larvae exiting the food to move unimpeded along the surface. While we added enough food to entirely fill our plastic mesocosms, the 'sticky' food was clumpier and left more air gaps on the surface of the food than the 'loose' food. Therefore, larvae in 'sticky' food may have been more likely to travel between grid squares along the surface of the food so that infection was more spatially structured in 'loose' food treatments. Alternatively, it is possible that the virus had different rates of environmental decay on the different food types leading to differences in where larvae were infected. Another potential explanation is that eggs and early instar larvae may have been more 'clumped' in 'sticky' food so that the density of susceptible individuals was more heterogeneous and influenced the local infection density more than the dose of virus added to the square. The fact that 'sticky' food mesocosms are more heterogeneous in the clustering of infected individuals potentially supports this interpretation. Therefore, the infection ecology results suggest that 'loose' food spatially structured infection more than 'sticky' food at the grid square level between passages, but we have no way of determining how food structured infection at the sub-square level, when larvae were earlier instar with lower movement rates. It is possible that 'sticky' food less structured infection between passages at the square spatial scale, but structured contacts at smaller spatial scales early in each passage.

However, greater spatial structuring of infectious contacts in the 'loose' food would provide a plausible mechanism for why lower exploitation rates evolved on the familiar host in these lines. It is difficult to think of another plausible hypothesis. As force of infection was higher during passaging in the 'sticky' food lines, it is possible that 'sticky' lines had stronger selection to specialize on the familiar host, but this could explain why

effect sizes of specialization could vary, but not why they would switch directions (Bisschop et al., 2022). Alternatively, it is plausible that infection in 'sticky' food infected earlier instar larvae as the high numbers of infected individuals in certain squares during selection passages were observationally correlated with clusters of early instar larvae and 'sticky' food moderately slows development (Supplemental results). However, we would expect this to lead to lower exploitation rates on the familiar host in 'sticky' food as earlier instar larvae are both less resistant and, potentially, more spatially structured (Boots & Begon, 1993). Therefore, the most likely hypothesis to explain these results seems to be that the higher spatial structuring of spatial contacts at the grid-square level between passages in the 'loose' food imposed a spatial structuring effect that selected for lower exploitation rates on the familiar host.

Another key, but slightly counterintuitive, result of our experiment is that the effects of local adaptation in heterogeneous host environments are stronger in better mixed populations than in more spatially structured ones (Figure 17). As host genotype mixing only depends on spatial structure effects above the square scale, we can better assume that 'loose' food more strongly structured contacts between genotypes than 'sticky' food. While more contact between host genotypes should, in theory, select more strongly for generalism (Gandon & Poulin, 2004; Osnas & Dobson, 2012; Williams, 2012), theory suggests that higher migration rates between patches can lead to stronger local adaptation due to migration conferring more genetic diversity to select from and increased competition to strengthen such selection (Gandon & Michalakis, 2002). Our experiment seems to support the latter theory, implying that certain rates of migration between populations may support, rather than homogenize, phenotypic diversity.

Our ability to make conclusions is hampered by the fact that our experimental manipulation for spatial structure seemed to have worked in the opposite direction as intended. However, our results are suggestive that evolution in response to multiple selection pressures can be governed by interacting trade-offs and result in different locally adapted optimal strategies. Specifically, when optimal exploitation strategies on the familiar host are more prudent, pathogens may have maladaptively high exploitation rates on foreign hosts. These results have important implications for eco-evolutionary theory on diversity that depends on rare genotype advantage. Furthermore, our experiment shows that higher pathogen migration rates between host types can lead to stronger effects of local adaptation, suggesting that some population mixing might support metapopulation diversity.

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Appendix

Supplementary information, data, and code for chapter 1 can be found at:
Bartlett, L. J., Visher, E., Haro, Y., Roberts, K. E., & Boots, M. (2020). The target of selection matters: An established resistance development-time negative genetic trade-off is not found when selecting on development time. *Journal of Evolutionary Biology*, *33*(8), 1109 1119. https://doi.org/10.1111/jeb.13639

Supplementary information, data, and code for chapter 2 can be found at:
Visher, E., Mahjoub, H., Soufi, K., Pascual, N., Hoang, V., Bartlett, L.J., Roberts, K., Meaden, S., Boots, M. (2022). The evolution of host resistance to a virus is determined by resources, historical contingency, and time scale. bioRxiv.

Supplementary information, data, and code for chapter 3 can be found at:
Visher, E., Uricchio, L., Bartlett, L., DeNamur, N., Yarcan, A., Alhassani, D., & Boots, M. (2021). The Evolution of Host Specialization in an Insect Pathogen. bioRxiv. https://doi.org/10.1101/2021.10.11.463986

Data and code for chapter 4 is available upon request. Supplementary information is available below:
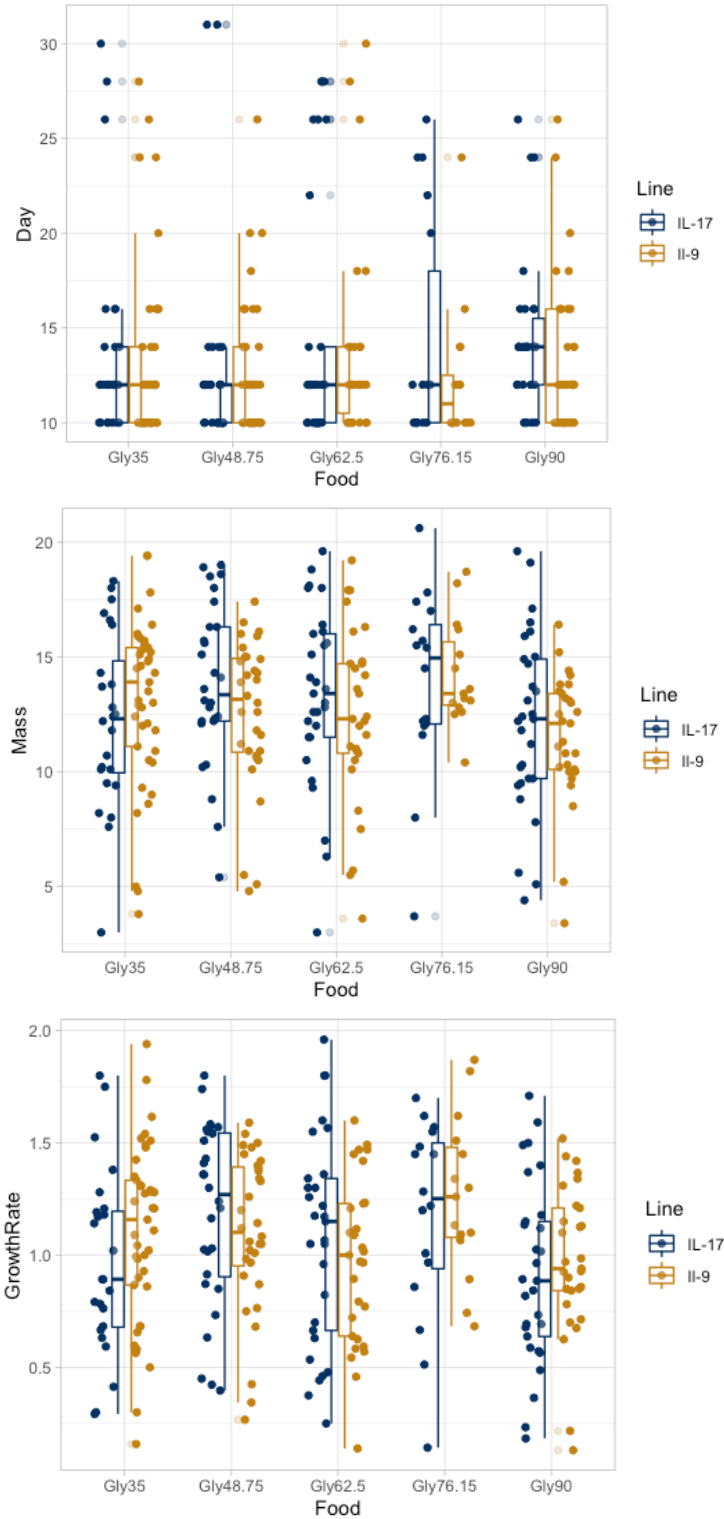
Figure S1: Box plots illustrating life-history traits (growth rate, development time, pupal mass) of Inbred Line 9 & 17 for different glycerol levels: 35mL Gly (Food 1), 48.75mL Gly (Food 2), 62.5mL Gly (Food 3), 76.25mL Gly (Food 4), 90mL Gly (Food 5). All food was made with Earth's Best breakfast cereal (100g), brewer's yeast (20g), sorbic acid (0.2g), and methyl paraben (0.2g). From GLMMs, we find that Line and Food type do not significantly affect key life history metrics.

	35mL Glycerol	48.75mL Glycerol
Sample Size	9	10
Average (cm)	2.82	1.77
Standard Deviation (cm)	3.47	2.00
Standard Error (cm)	1.16	0.56

Table S1: Summary statistics (average, standard deviation, standard error) of IL-9 larvae movement data in spatial structure assay. Spatial assays are set up with 18cm PVC tubes with a diameter of 4cm. Both ends of the tube are double layered with mesh cloth and rubber bands to ensure that food and larvae don't escape. Food is lightly packed into the tube by knocking it against a surface and second instar larvae are placed into one end of the tube clearly marked as the starting point. After incubating for 10-14 days, the pipes are frozen to ensure that larvae no longer move after the allotted amount of time. Frozen food is then pushed out using a flat surface to not disturb the larvae location. Starting from the larvae starting point, the food is sifted through 0.5cm at a time until the larvae is uncovered, and distance traveled by the larvae is recorded.