Leveraging natural variation to study fitness costs and trade-offs in host immunity



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"El escéptico no significa el que duda, sino el que explora o investiga, a diferencia del que afirma y piensa que ha encontrado."

"The skeptic does not mean him who doubts, but him who investigates or researches, as opposed to him who asserts and thinks that he has found."

-Miguel de Unamuno

"It would be instructive to know not only by what physiological mechanism a just apportionment is made between the nutriment devoted to the gonads and that devoted to the rest of the parental organism, but also what circumstances in the life-history and environment would render profitable the diversion of a greater or lesser share of the available resources towards reproduction."

-R.A. Fisher

"And everything that I did or did not do
In the miniature of my life, has a lineage back to you."

-Josh Pyke

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Thesis abstract

During health and infection, hosts allocate energy to growth, maintenance, defense, and reproduction. Current theory in ecological immunology suggests that mounting an energetically-costly defense will lead to trade-offs against host fitness. In this thesis, we used lab-adapted and wild *Caenorhabditis elegans* as model hosts to directly test for fitness costs of immune-upregulation and pathogen damage, as well as trade-offs with reproduction, after transient exposure to an opportunistic pathogen, *Staphylococcus aureus*. Utilizing a spectrum of wild host genotypes allowed us to explore the breadth of genetic and functional variation for naturally-relevant alleles. We found that despite short-term delays in reproduction after *S. aureus* exposure, all host isolates recovered and had similar total brood sizes as nematodes fed only control bacterial food (Chapter 2). We subsequently examined the impact of pathogen exposure across several generations in population size assays. We found a genetic correlation across host isolates such that those with a relatively smaller population size after transient *S. aureus* exposure had relatively larger population sizes after exposure to a second opportunistic pathogen, *Pseudomonas aeruginosa* (Chapter 3). This finding suggests that *C. elegans* has 'balanced immunity' against these two distinct pathogen species, evidenced at the population level. Finally, we found no evidence of a beneficial pathogen-induced maternal effect but did identify that lab-adapted nematodes have the highest magnitude of cost from maternal pathogen exposure (Chapter 4) and this corresponds to the magnitude of the delay reported in Chapter 2. Surprisingly, our findings in genetically-diverse isolates identify trade-offs in pathogen-tailored defense strategies as more important drivers of population size than energy-based trade-offs from mounting an immune response or suffering pathogen damage. We discuss the potential of studying nematode-pathogen systems with more naturalistic conditions to address ecological immunology questions using naturally-derived isolates with ecologically-relevant parameters.

Chapter 1

Thesis introduction

Outline

We investigated costs and trade-offs in host-pathogen interactions across a diverse panel of wild host isolates. In this introduction, we start by giving an overview of defense-related costs and trade-offs, and largely focus on invertebrate examples in the literature. This section encompasses the over-arching theme of the project, examined in all chapters. We then more specifically discuss host immune balance between distinct pathogens (Chapter 3) and pathogen-induced maternal effects (Chapter 4). The conceptual overview is followed by an introduction to *Caenorhabditis elegans* as a model host for two opportunistic pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Finally, we discuss our choice of fitness metrics and present chapter summaries.

Costs and trade-offs of defense

Pathogens are a major selective force on hosts (Schmid-Hempel, 2013). Such strong selection has favored the maintenance of natural genetic variation in host defense, which leads to a critical question in ecological immunology (Rolff & Siva-Jothy, 2003): what generates variation in defense and what are the consequences? This variation has been shown to exist across several invertebrate taxa. These include water fleas (e.g. Ebert *et al*., 1998), aphids (e.g. Henter & Via, 1995), mosquitoes (e.g. Riehle *et al*., 2006), nematodes (e.g. Schulenburg & Muller, 2004), fruit flies (e.g. Lazzaro *et al*., 2006), flour beetles (e.g. Wegner *et al*., 2009), and bumble bees (e.g. Wilfert *et al*., 2007). More specifically, natural genetic variation has been found in the fitness costs due to pathogen challenge: for example, upon exposing multiple genotypes of wild nematodes to pathogenic bacteria and measuring their population growth (Petersen *et al*., 2015). This finding indicates that within a species, different strategies to respond to pathogens have evolved over time, given different selective pressures and

ecological contexts. It is important to understand genetic variation in defense and associated fitness costs because it can deepen our understanding of the numerous strategies that exist in nature for trading off immunity and reproduction as well as balancing immune responses against different types of pathogens. These costs and trade-offs have implications for population dynamics in the wild, where hosts can face diverse biotic and abiotic challenges (Pedersen & Babayan, 2011).

Upon infection, hosts may suffer from pathogen damage as well as the metabolic demands of mounting an immune response (Zuk & Stoehr, 2002). Therefore, hosts must re-allocate limited energy and resources between defense and traits such as maintenance and reproduction (Sheldon & Verhulst, 1996). Costs have been detected in experimental studies involving the evolution of a defense component as well as the deployment of defense. For example, selection of honeybees for increased resistance to bacterial pathogen slowed larval growth (Sutter *et al*., 1968) and antigenic challenge of wild pied flycatchers led to decreased foraging and reduced number of offspring (Ilmonen *et al*., 2000) (see Schmid-Hempel, 2003 for further review).

Trade-offs in nature have been documented historically (e.g. Darwin, 1859) and their mechanisms, as well as ecological and evolutionary implications have been hypothesized in the last century (e.g. Fisher, 1930). Since then, trade-offs between life-history traits have been studied across taxa, in particular relating to defense. For example, between resistance to parasitoids and larval competitive ability in fruit flies (Kraaijeveld & Godfray, 1997), strength of antibody response and ornamentation in domestic chickens (Verhulst *et al*., 1999), and resistance to bacteriophage parasites and population growth in bacterial hosts (Koskella *et al*., 2012). In nature, host organisms must weigh the risks and costs of pathogen challenge

with the repercussions of reallocating energy and resources away from other biological functions to find an optimal allocation strategy (Stearns, 1992).

The evolution and maintenance of host defense mechanisms and the upregulation of immune responses can both have negative consequences for reproduction. The costs of evolving defenses have been studied with regimes selecting for increased pathogen resistance, the costs of maintenance have been explored in the absence of pathogen, and the costs of using defenses have been investigated by challenging hosts with treatments that upregulate immune responses (reviewed in Schmid-Hempel, 2003). Fitness costs to invertebrate hosts have been shown using pathogenic (e.g. live bacteria) and non-pathogenic (e.g. heat-killed bacteria or immune elicitors) treatments (reviewed in Schwenke *et al*., 2016). While some of these studies focused on metrics such as egg quantity and quality, more comprehensive fitness measures encompass lifetime reproduction and measurements of offspring that survive to reproduce themselves (Lack, 1954). Fitness costs of pathogens have been demonstrated across generations: in terms of decreased lifetime reproduction and decreased population sizes (e.g. Feistel *et al*., 2019). In fact, host defenses can be defined as mechanisms that minimize fitness loss upon infection (Schmid-Hempel, 2013). As for fitness costs in nature, the host genotype with the highest levels of defense may not be the most successful, rather, the host genotype making optimal compromises between defense, maintenance, and reproduction will be selected for (discussed in McKean *et al*., 2008 and Viney, 2005).

Although current evidence supports the idea that immunity confers a fitness cost, the relative contributions of maintaining and deploying immunity to this overall cost, and to variation in immunity are not well understood. Maintenance costs (also known as constitutive costs) are derived from investment towards defense in the absence of infection, while deployment costs

(also known as inducible costs) come from mounting an immune response (discussed in Schmid-Hempel, 2003). These physiological-level costs are usually examined in terms of energy-based costs resulting from maintaining defenses or upregulating an immune response. Further, the costs of infection can be split into the costs of pathogen damage and immune upregulation (Sheldon & Verhulst, 1996). At the population level, one can study the evolutionary costs of immunity (Schmid-Hempel, 2003). Evolutionary costs are revealed in the form of a negative correlation of various host genotypes between fitness in the absence of a pathogen versus immunocompetence with a pathogen present (discussed in McKean *et al*., 2008). While the physiological and evolutionary costs of immune maintenance and deployment have been investigated before in invertebrates (e.g. McKean *et al*., 2008), it remains unclear whether the costs of immune upregulation versus pathogen damage might vary across host genotypes (Sheldon & Verhulst, 1996; Schwenke *et al*., 2016). This latter question will expand our understanding on the extent to which there is host variation in deploying defenses and suffering pathogen harm.

Immunological trade-offs between pathogen species

Pathogen-driven selection has shaped host defenses to include constitutive and inducible immunity, as well as general and specific responses across pathogens (Schmid-Hempel & Ebert, 2003). A broad response can benefit hosts facing variable and diverse pathogens but may not work as efficiently as a tailored defense strategy (reviewed in Schulenburg *et al*., 2004). A few decades ago, it was believed that invertebrates only mounted general, indiscriminate immune responses (e.g. Lackie, 1980; Klein, 1989) though it is now known they exhibit specificity in their defenses (e.g. Messier-Solek *et al*., 2010; Ghosh *et al*., 2011). Invertebrates can mount distinct responses within classes of microbes (e.g. Wong *et al*., 2007) and display specificity down to the strain level (reviewed in Pees *et al*., 2016).

The presence of specific defenses makes sense given that hosts have evolved immunity in environments with multiple classes of pathogens present. Therefore, although some host responses to infection can work effectively across multiple pathogen strains (e.g. Fellowes *et al*., 1999), in other cases, a defense response against one type of pathogen trades off with susceptibility to other types (e.g. Marsh *et al*., 2011). This concept of balanced immunity has been studied across diverse species. Examples include biotrophic and necrotrophic pathogens in plants (Spoel & Dong, 2008), tolerance to a bacterial pathogen trading off with sensitivity to a fungal pathogen in nematodes (Marsh *et al*., 2011), trade-offs between resistance to bacterial species in fruit flies (Ayres & Schneider, 2008), and the balance between helper T cell classes influencing the murine immune response (Koguchi & Kawakami, 2002). The extent to which these immunological trade-offs between pathogen classes vary across host genotypes remains to be elucidated. That is, in a given species some host genotypes might be better at responding to one class of pathogen and this may trade off with increased susceptibility to another class. This trend can be revealed across host genotypes in the form of a negative genetic correlation.

Pathogen-induced maternal effects

Offspring phenotypes can be influenced by a mother's genetic contribution and also be a product of her environment (Bernardo, 1996). Passing immunity down to future generations has been prominently investigated in terms of vertebrate antibodies (e.g. Grindstaff *et al*., 2003). In invertebrates, maternal effects have been reported in studies involving pathogen and immune-eliciting challenges in the parental generation leading to increased immunity in the next generation (e.g. Little *et al*., 2003; Moret, 2006; Sadd *et al*., 2005). Pathogen-

induced maternal effects, sometimes termed immune priming, can last one-to-two (intergenerational) or three-plus (transgenerational) generations (reviewed in Perez & Lehner, 2019). More specifically, intergenerational effects could be attributed to direct exposure of embryos to the immunogenic maternal environment, while transgenerational effects exclude direct exposures (discussed in Burton *et al*., 2020).

Adaptive traits have a positive impact on life history. There is debate as to how to define adaptability of maternal effects, whether it is in terms of increasing the fitness of the parent possibly at the expense of offspring quality (Marshall & Uller, 2007), or in terms of increasing offspring fitness (Dey *et al*., 2016). While one recent meta-analysis reported strong, persistent benefits for up to three generations in plants and animals (Yin *et al*., 2019), there is discussion about the generality of benefits, given concerns with experimental design in distinguishing anticipatory parental effects from condition-transfer parental effects and biases in reviewing the literature (see Sánchez-Tójar *et al*., 2020 and Zhang *et al*., 2020).

While maternal effects may be beneficial in certain scenarios, they can carry costs in other contexts. Namely, benefits are evident when parental and offspring environments are matched and costs are evident when there is a mismatch (reviewed in Roth *et al*., 2018). In relation to defense, costs can be apparent in the absence of pathogen in the F1 generation (Contreras-Garduño *et al*., 2014) or when F1 are challenged with a different kind of pathogen to what the parental generation has seen (Sadd & Schmid-Hempel, 2009). Further, the magnitude of maternal effects related to immune priming can be limited by the extent of the parental generation's defenses. An evaluation of trade-offs in mealworm beetles showed that the immune response of pathogen-challenged mothers negatively correlated with the egg's antibacterial activity (Moreau *et al*., 2012). Namely, trade-offs between progeny quantity and

quality are relevant, as well as between parental and progeny fitness, though the costs and benefits of preconditioning offspring depend on whether they will be fitter for the environment they will develop and grow in (Marshall *et al*., 2010).

Maternal effects can be important in impacting evolution on an ecological timescale, since priming can rapidly generate change on the phenotypic level (Mousseau & Fox, 1998; Räsänen & Kruuk, 2007; Uller, 2008). More specifically, immune priming will be selected for in cases where offspring are prepared and better able to respond to the pathogens that were also prevalent in the parental environment (Roth *et al*., 2018). Factors to consider in predicting the conditions under which immune priming might evolve include: the specificity or generality (i.e. cross-reactivity) of the mechanisms for different pathogen challenges, the prevalence of pathogen, in terms of space and time, and the ability for the hosts to be motile and disperse broadly (Roth *et al*., 2018). When pathogen and/or host dispersal is constrained, it would be likely for multiple host generations to be encountering the same kind of pathogen (Little & Kraaijeveld, 2004). Therefore, environments with consistent host-pathogen interactions would be expected to select for immune priming (reviewed in Roth *et al*., 2018), and we would expect to see immune priming persist in philopatric individuals of a species which also includes dispersing individuals (Pigeault *et al*., 2016). One exception where immune priming could be found in dispersing individuals is when transmission of a pathogen occurs vertically, directly from parent to offspring (e.g. Yue *et al*., 2013).

It remains to be determined how immune upregulation and/or pathogen damage in mothers might differentially affect offspring, and whether fitness benefits and costs from maternal effects, if any, vary by host genotype.

Model system

In order to rapidly assess fitness costs and trade-offs across multiple host generations, we used a model system with a *Caenorhabditis elegans* nematode host, and *Staphylococcus aureus* and *Pseudomonas aeruginosa* opportunistic bacterial pathogens. To disentangle the costs of immune upregulation from pathogen damage, we used heat-killed and live pathogen treatments. To explore evolutionary costs, we used a genetically-diverse panel of wild nematode isolates with a wide geographic range and the canonical wildtype, lab-adapted N2 isolate. The wild isolates were selected randomly from those studied by Andersen *et al.* (2012) and it was verified that this selection of isolates spanned the spectrum of the phylogeny (Figure 1) and various isolation locations (Supplementary Table 1).

Nematode host

C. elegans are small, free-living, hermaphroditic nematodes that reproduce through self-fertilization. The selfing mode of reproduction facilitates the maintenance of isogenic populations without inbreeding depression (reviewed in Frézal & Félix, 2015). Also, *C. elegans* in the lab have a short generation time of about three days and a high proliferation rate, with one hermaphrodite producing about 300 offspring in one brood (Hodgkin & Barnes, 1991). The maintenance protocols are well-defined; they involve rearing nematodes on Nematode Growth Medium (NGM) seeded with a bacterial food source (Brenner, 1974; Stiernagle, 1999). The stress-resistance phenotypes allow for already-hatched nematodes to be cryopreserved and for embryos to survive a bleach treatment that synchronizes populations and removes microbes (reviewed in Frézal & Félix, 2015). Cryopreservation is especially useful in limiting the accumulation of mutations from lab adaptation in wild isolates (Sterken *et al*., 2015).

For *C. elegans* to grow, they undergo four molting cycles to shed the cuticle. The four larval stages between hatching and adulthood are known as L1 – L4 (Supplementary Figure 1). The lifetime reproduction of *C. elegans* is limited by the number of sperm that can be produced during L4 stage (Ward & Carrel, 1979). The hermaphrodite generates about 300 sperm before switching irreversibly to egg production for the rest of adulthood (Kimble & Ward, 1988). In particular, hermaphroditism is a useful trait for investigating fitness costs because the investment in mating and variability present in an outcrossing mode of reproduction does not have to be taken into account in self-fertilizing *C. elegans*. Moreover, populations are generated relatively quickly compared to other invertebrate model organisms and offspring are isogenic (Hedges, 2002; Corsi *et al*., 2015).

The lab environment of *C. elegans* involves two-dimensional agar with monoaxenic bacterial food and controlled temperatures. This environment is in stark contrast to nature: three-dimensional compost and rotting fruit with diverse microbes and fluctuations in abiotic and biotic factors (Félix & Braendle, 2010; Schulenburg & Félix, 2017). The N2 reference strain has become adapted to the lab since its isolation in Bristol, England in 1951; N2 nematodes spent 18 years growing in the lab before being frozen (Sterken *et al*., 2015). In fact, several mutations, some affecting behavior, reproduction and pathogen susceptibility, have appeared as a result of this domestication, compared to other isolates (e.g. Weber *et al*., 2010; McGrath *et al*., 2011; Andersen *et al*., 2014; Sterken *et al*., 2015).

C. elegans is a well-established model for host-pathogen interactions (Gravato-Nobre & Hodgkin, 2005). They rely on innate immunity for protection, with defenses including behavioral avoidance, physical barriers, and non-cellular physiological immune responses.

The secretion of defense components such as lectins, lysozymes, and antimicrobial factors is regulated by several immune signaling pathways (reviewed in Marsh & May, 2012). Healthy (i.e. uninfected) nematodes can constitutively express lysozymes, for example (Alper *et al.*, 2007), and there are inducible components of the immune response as well (Mallo *et al.*, 2002; Shapira *et al.*, 2006; Troemel *et al.*, 2006). Given that the nematode immune response is genetically encoded (Gravato-Nobre & Hodgkin, 2005), and that genetically-diverse isolates of nematodes have been sampled from across the world (Figure 1; Supplementary Table 1) *C. elegans* is a powerful model to investigate natural variation in host defense.

C. elegans wild isolates exhibit natural genetic variation in response to pathogens. This variation has been shown for bacteria that can co-exist with nematodes in nature, such as *Bacillus thuringiensis* (Schulenburg & Muller, 2004; Petersen *et al*., 2015), several bacterial species co-isolated with nematodes in France (Volkers *et al*., 2013), and also for a natural viral pathogen (Ashe *et al*., 2013). Moreover, in studies with non-co-isolated pathogens such as *Serratia marcescens* (Schulenburg & Ewbank, 2004), *Photorhabdus luminescens* (Sicard *et al*., 2007) and *Pseudomonas aeruginosa* (Styer *et* al., 2008; Reddy *et al.,* 2009; Martin *et al*., 2017), nematodes also exhibit variation in responses ranging from mortality, to morbidity, to avoidance behavior. While these pathogens were not isolated from infected nematodes, *S. marcescens* and *P. aeruginosa* could potentially be encountered by wild nematodes (Schulenburg & Félix, 2017). The impact of *B. thuringiensis* on population growth of German isolates has been reported (Petersen *et al*., 2015), but the fitness costs from opportunistic pathogen exposure across a geographically widespread and genetically-disparate panel of hosts have yet to be investigated. The fact that nematode isolates exhibit natural genetic variation in their pathogen responses, in addition to variation in life-history

traits such as reproduction (Harvey & Viney 2007; Diaz & Viney 2014) and development (Volkers *et al*., 2013) make them a useful animal to explore the fitness costs of infection.

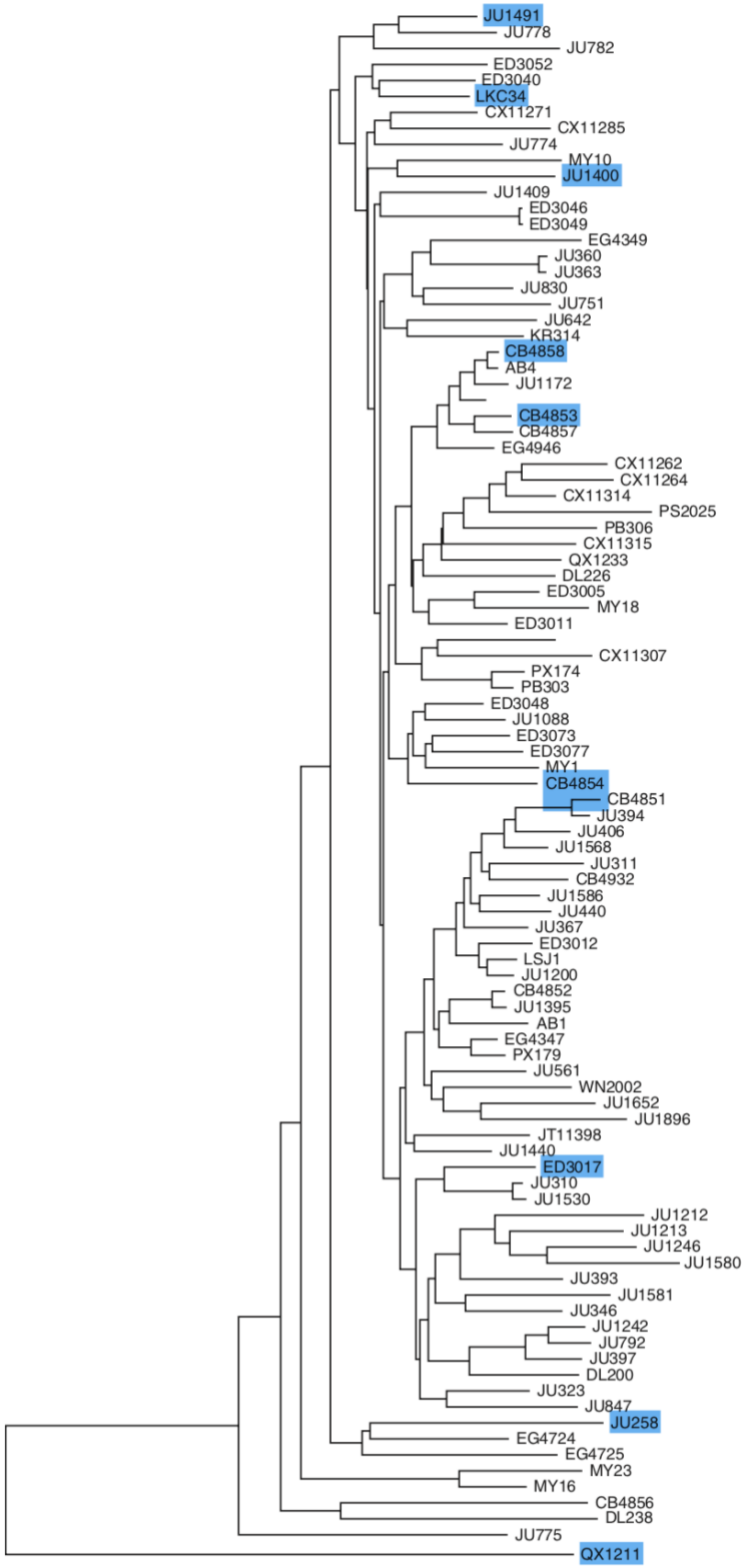


Figure 1: Neighbor-joining tree of 97 *C. elegans* isolates (termed isotypes by Andersen *et al.*) Blue highlights indicate nine isolates randomly selected to capture genetic diversity for this study. The lab-adapted N2 is not included in the phylogeny. Base image sourced from Andersen *et al.* (2012).

Opportunistic pathogens

Opportunistic pathogens can be defined in a clinical context as commensal bacteria that become pathogenic when they infect immunocompromised hosts (Pirofski & Casadevall, 2012). Here, we take a more ecological approach and define opportunistic pathogens as bacterial strains that *C. elegans* have not encountered in nature before (due to the strains' clinical isolation: e.g. Holden *et al*., 2004; Tata *et al*., 2016) and that may cause host morbidity due to high dose of exposure and/or compromised host immunity. Opportunistic pathogens may co-occur in the host habitat but are not co-evolving with hosts, and although not modeled herein, can be beneficial in studies accompanying ecological stressors (e.g. fire, flooding, drought) which can increase host exposure to novel pathogens (reviewed in Boughton *et al*., 2011). In nature, *C. elegans* can disperse to new locations on isopod and snail vectors (Schulenburg & Félix, 2017), so it is likely that nematodes encounter microbes with which their lineage has no specific evolutionary history. This study utilizes the clinical isolates *Staphylococcus aureus* MSSA476 and *Pseudomonas aeruginosa* PA14. Bacteria from the *Staphylococcus* and *Pseudomonas* genera have been identified in the mushroom environment of nematodes (Grewal & Richardson, 1991) with *Pseudomonas* spp. being the most frequently-isolated species (eleven species) and four species of *Staphylococcus* being identified on button mushrooms (Rossouw & Korsten, 2017). While the interactions between our hosts and the clinical isolates are novel, and therefore modeling opportunistic pathogen challenge, *C. elegans* have likely encountered bacteria from the same genera in nature. We used *S. aureus* throughout the thesis and *P. aeruginosa* was chosen to study immune balance phenotypes in the third chapter.

Given our aim to separate the costs of pathogen damage and immune upregulation, we made use of two bacterial treatments. Another dimension of our host-pathogen system is that both

live and heat-killed *S. aureus* have been reported to elicit an immune response in nematodes after 8 hours of exposure, but only live *S. aureus* causes damage (Irazoqui *et al*., 2010). In contrast, hosts upregulate ten key genes on live *P. aeruginosa* but exhibit minimal or no induction on heat-killed *P. aeruginosa*, allowing us to use the latter treatment as a negative control. Overall, *C. elegans* hosts suffer distinct pathology and generate partially non-overlapping immune responses to *S. aureus* and *P. aeruginosa* (as discussed below). These contrasting patterns allowed us to investigate divergent host defense strategies across host isolates. It is known that *C. elegans*, (specifically the lab-adapted N2) recover in terms of survival after brief pathogen exposures to *S. aureus* (Sifri *et al*., 2003) or *P. aeruginosa* (Tan *et al*., 1999), but the impact of transient infection and/or immune upregulation on fitness remain to be studied, both in N2 and wild nematode isolates.

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium responsible for minor and major infections in animal hosts (Lindsay, 2010). In *C. elegans,* live bacteria rapidly colonize and accumulate in the gut within 4 to 8 hours of exposure (Supplementary Figure 2). Several hours later, hosts suffer from lysed cells, disrupted gut epithelia, destroyed internal organs (Irazoqui *et al*., 2010), and will die within a few days (Garsin *et al*., 2001; Sifri *et al*., 2003). By 12 hours of exposure, *S. aureus*-infected nematodes show signs of morbidity: they are slower-moving, smaller, and appear to lay fewer eggs than control nematodes (Irazoqui *et al*., 2010). Rescue from mortality is possible through time-sensitive transfer to a non-pathogenic food. In fact, nematodes transferred at or before 8 hours of *S. aureus* exposure have normal lifespans and can clear the infection from the gut (Sifri *et al*., 2003). Host defense pathways critical to responding to *S. aureus* are primarily the β-catenin (*bar-1*) pathway (Irazoqui *et al*., 2008) and additionally the p38 MAPK cascade (Sifri *et al*., 2003). Microarrays of N2 hosts

show 382 genes with changed expression after 8 hours of exposure; many of the upregulated genes include potential antimicrobial and detoxification molecules. And as mentioned before, heat-killed and live *S. aureus* upregulate ten key immune genes to the same extent after 8 hours of host exposure (Irazoqui *et al*., 2010). In sum, these prior results with incipient morbidity (but not mortality), as well as immune upregulation in the early infection period led us to choose 8-hour transient exposures. The specific strain MSSA476 was chosen given well-established host-pathogen mortality outcomes in 24-hour long-term exposures (e.g. King *et al*., 2016, Ford *et al*., 2016 Rafaluk-Mohr *et al*., 2018) but unknown impacts of short-term exposures on host morbidity.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative bacterium common in many environments and pathogenic to both animals and plants (reviewed in Marsh & May, 2012). Although it has not been co-isolated from natural nematodes, *P. aeruginosa* has been reported to coexist with *C. elegans* in a mushroom farm, and bacteria isolated from this farm decreased nematode population growth (Grewal, 1991). In the lab setting, *C. elegans* mortality is dependent on media: in low osmolarity agar like NGM, *P. aeruginosa* PA14 infects the nematode intestine and accumulates slowly, killing the worm over the course of several days. In high osmolarity agar, the mortality is mediated by toxins and occurs in a few hours (Tan *et al*., 1999). In the present study, we focused on slow killing in order to induce host morbidity but not mortality. Colonization dynamics of this pathogen differ from that of *S. aureus*: gut accumulation does not begin until 6 to 8 hours of exposure (Supplementary Figure 2). At 8 hours, host intestines are distended, but have partial (not full, as in *S. aureus*) accumulation of live bacteria, and intestinal cells appear abnormal (Irazoqui *et al*., 2010). Nematodes can be rescued from mortality after 12 hours or fewer of PA14 exposure and transfer onto non-pathogenic food.

However, longer exposures (i.e. 18-30 hours) result in an increasing proportion of dead nematodes (i.e. 25-95%, respectively), thus highlighting that hosts can fully survive a transient exposure (Tan *et al*., 1999). The p38 MAPK cascade is critical for host defense, as nematode mutants in components of this pathway had decreased lifespan on *P. aeruginosa* compared to control food (Kim *et al*., 2002). Also, the insulin-like signaling pathway might be functioning in parallel to the p38 MAPK cascade to promote host defense (Troemel *et al*., 2006). We chose the PA14 strain given it is a well-established bacterium for studying nematode-pathogen interactions.

Choice of bacterial food

Given that *C. elegans* are bacterivores and that in the lab they derive their nutrition from monoaxenic bacterial lawns, we favored continuity between the maintenance bacteria and pathogenic exposure. Control bacterial food was chosen so that *S. aureus*-exposed worms were reared on Gram-positive *Bacillus subtilis* PY79 and *P. aeruginosa*-exposed worms were reared on Gram-negative *Escherichia coli* OP50. The former pair are both in the phylum Firmicutes and the latter pair are both Proteobacteria. OP50 was historically chosen to maintain *C. elegans* in the lab because it was readily available and since it forms thin films on NGM agar, which facilitate nematode visualization (Brenner, 1974; Stiernagle, 1999). Since then, OP50 has become standard in protocols although it is not a food source *C. elegans* would encounter in nature and is suboptimal in terms of development (Pang & Curran, 2014), metabolism (Brooks *et al*., 2009), and lifespan (MacNeil *et al*., 2013). Maintenance of *C. elegans* on PY79 is not as widespread as that on OP50. Nevertheless, PY79 has been used as a control for *S. aureus* in previous studies (e.g. Garsin *et* al., 2001; Sifri *et al*., 2003; Begun *et al*., 2007) and PY79 does not upregulate the specific immune genes upregulated by live and heat-killed *S. aureus* (Irazoqui *et al*., 2010). While N2 lifespan

is longer on *B. subtilis* than *E. coli* food (Lezzerini *et al*., 2015; Sánchez-Blanco & Kim, 2011), nematode reproduction is similar in terms of kinetics and brood size (Garsin *et* al., 2003; Sánchez-Blanco *et al*., 2016). Given we are only interested in the reproductive period of adult *C. elegans* (i.e. not the post-reproductive timeframe), the lifespan difference is not a limitation in our study.

Brood size and population growth as fitness metrics

To measure the costs of pathogen exposure, we focused on fitness in the form of lifetime reproduction (brood size) and population growth. Survival assays have been increasingly used to investigate the pathogenicity of microbes in *C. elegans* (Marsh & May, 2012), and sterile mutants are often used to facilitate tracking of nematode survival, neglecting effects of pathogens on reproduction (discussed in Feistel *et* al., 2019; Abbate *et al*., 2015). To date, few studies have evaluated the effects of pathogens on *C. elegans* reproduction (e.g. Grewal, 1991; Coolon *et al*., 2009; MacNeil *et al*., 2013; Watson *et al*., 2013; Diaz *et al*., 2015; Petersen *et al*., 2015; Feistel *et al*., 2019). Even so, these studies focus on the effects of longer-term exposures, though it is unclear that these would recapitulate the briefer microbial interactions likely found in nature. Additionally, since reproduction lasts about four days into *C. elegans* adulthood, a decrease in survival after this timeframe is not an appropriate proxy for fitness (discussed in Diaz *et al*., 2015). Therefore, we measured fitness impacts through reproduction in multiple host generations to better investigate the ecology and evolution of these host-pathogen interactions.

Scope of thesis

This thesis examines fitness costs and trade-offs between reproduction and defense, including evolutionary costs across multiple host genotypes. Within a host isolate type, we hypothesized that nematodes exposed to immunogenic (all treatments except heat-killed *P.*

aeruginosa) and/or pathogenic (live *S. aureus* and live *P. aeruginosa*) bacterial treatments would exhibit reduced lifetime fecundity (evaluated for *S. aureus*) and population growth (evaluated for *S. aureus* and *P. aeruginosa*) compared with food controls. Among host isolates, we predicted negative genetic correlations between fitness on food and minimized fitness loss after transient pathogen exposure, as well as an immunocompetence trade-off across two pathogen species. Finally, we speculated that successive maternal and offspring pathogenic exposures would reveal the benefits and/or costs of any intergenerational effects.

In **Chapter 2**, we investigated fitness costs and trade-offs between reproduction and immunocompetence (i.e. minimized fitness loss) in terms of cumulative progeny and lifetime fecundity using brood assays. We found that hosts recover lifetime reproduction to control levels after a delay induced by transient *S. aureus* exposure. Given the absence of a distinction in costs of immune upregulation and pathogen damage in this timeframe, we assessed fitness costs in subsequent generations in the next chapter.

In order to look at the performance of the lineage of pathogen-challenged host parents, we looked at a longer-term fitness cost in **Chapter 3** by measuring population sizes after two generations. These assays were done with both *S. aureus* and *P. aeruginosa* exposures to investigate immune balance phenotypes. Hosts experienced a fitness cost from pathogen damage associated with live *S. aureus* exposure and a fitness boost from live *P. aeruginosa* exposure (both compared to relative population sizes of their respective heat-killed treatments). We also demonstrate that hosts experience an immunocompetence trade-off across the two opportunistic pathogens.

While the previous chapters focused on consequences of pathogen exposure on the challenged host's fitness, **Chapter 4** added the additional dimension of investigating whether maternal pathogen exposure can confer benefits and costs to their offspring when these offspring are infected (benefits) or not (costs). In measuring offspring performance in terms of population size, we found no evidence of intergenerational priming effects of *S. aureus* pathogen exposure, but we note a connection between the magnitude of the reproductive delay in Chapter 2 and the magnitude of the F1 fitness cost from maternal exposure.

In each of the chapters, we also comment on natural variation across the host isolates.

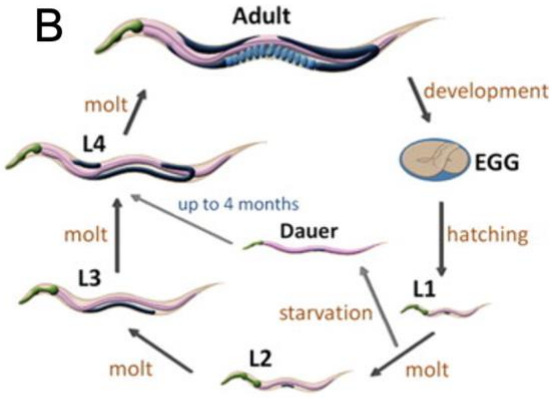
Chapter 5 summarizes our findings, discusses the benefits and limitations of our model system, and proposes means of studying nematode-pathogen interactions in more naturalistic conditions to better understand the host biology in an ecological and evolutionary context.

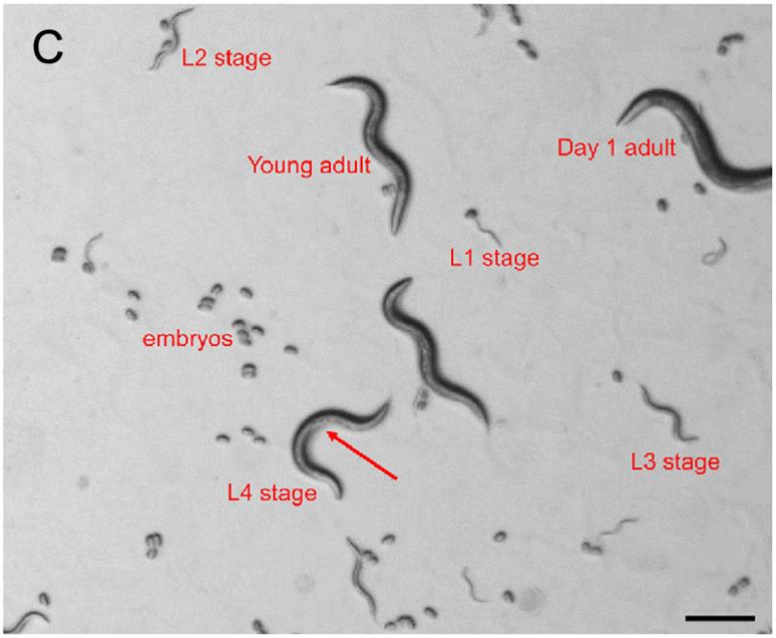
Supplementary Table 1: *C. elegans* wild isolates used in this project and source information. Isolate names represent "isotype sets" as described by Andersen *et al.* (2012). Locations, GPS coordinates, and dates are presented with the amount of detail provided by the original researcher. Comments as compiled by Andersen *et al*., where available. Adapted from Supplementary Table 1 in Andersen *et al*.

Isolate	Location	GPS Latitude	GPS Longitude	Isolation Date	Sampled by	Comments
CB4853	Altadena, USA	34.189	-118.131	1974/05	C.D. Johnson	Isolated in May 1974 from vegetable garden on Garfias St, Altadena, California USA, Noted as identical to CB3198 by Hodgkin and Doniach
CB4854	Altadena, USA	34.189	-118.131	1974/05	C.D. Johnson	Isolated in May 1974 from vegetable garden on Garfias St, Altadena, California USA, Noted as identical to CB3198 by Hodgkin and Doniach
CB4858	Pasadena, USA	NA	NA	1973	E.M. Hedgecock	
ED3017	Edinburgh, United Kingdom	55.92	-3.19	2004/12/03	A. Cutter	
JU258	Ribeiro Frio, Madeira	32.73	-16.89	2001/10	M-A. Félix	From Madeira Island, Portugal, Oct 01. Ribeiro Frio 1.1. Along a path through an orchard.
JU1400	Sevilla, Spain	37.3845	-5.988	2008/03	M-A. Félix	Isolated from rotting orange fruits, Garden Catalina de Ribera, Sevilla, Spain sampled on 29 Mar 08 by MAF. Not bleached.
JU1491	Le Blanc, France	46.63	1.06	2008/08/17	M-A. Félix	Isolated from rotting Arum maculata stems sampled on 17 Aug 2008 by MAF in woods near Le Blanc (Indre, France). Plated on 18 Aug, picked as a L4 larva on 18 Aug.
LKC34	Unknown city, Madagascar	-18	46	2005/06/17	V. Stowell	
QX1211	San Francisco, USA	37.7502	-122.4331	2007/11/26	M. Rockman	

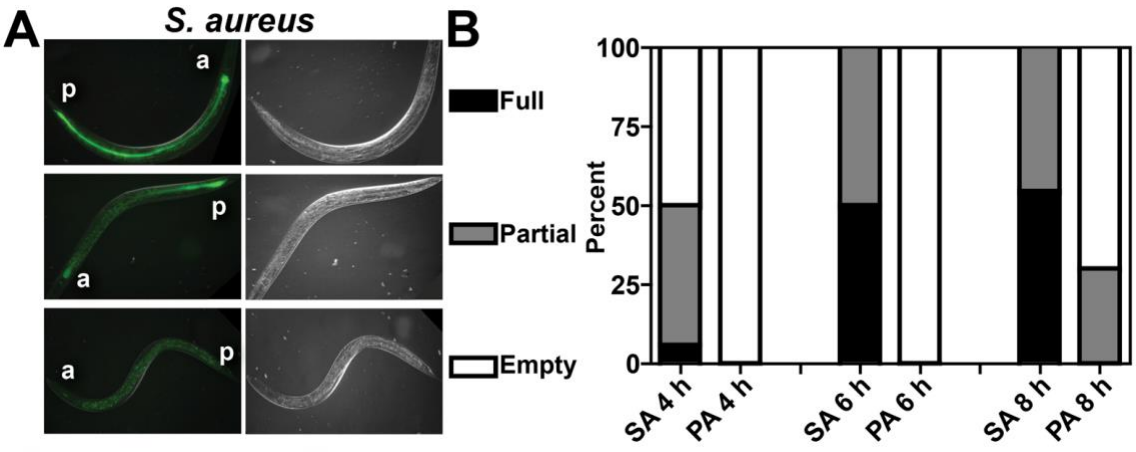
A

T	Time (h after hatch)				
	L1/L2 molt	L2/L3 molt	L3/L4 molt	L4/Adult molt	Egg-laying
16°C	24	38	55	74	94
20°C	15	24	34	48	58
25°C	11	18	26	35	45





Supplementary Figure 1: Timing of *C. elegans* life cycle in the laboratory at different temperatures (A) with life stages represented graphically (B) and as seen under a dissecting stereomicroscope (C). Eggs are laid after about 150 minutes of *in utero* development and can spend 9 hours developing *ex utero*. After hatching, L1 nematodes can arrest if they encounter unfavorable conditions (this is part of the overnight synchronization protocol), and nematodes can also divert development to an alternative dauer stage upon encountering a stressful environment. T = Temperature. Note that our experiments took place at 20°C from L1-L4 stage and 25°C afterwards. The arrow in panel C indicates a half-moon shape of L4 stage nematodes, which is the developmental stage at which exposure took place. The scale bar represents 250 microns. Image in panels A and B sourced from WormAtlas and adapted by Gonzalez-Moragas *et al*. (2015). Image in panel C from Rieckher and Tavernarakis (2017).



Supplementary Figure 2: Timecourse of *S. aureus* accumulation in the nematode intestine (A, B) compared to slower *P. aeruginosa* accumulation (B). Panel A depicts representative epifluorescence (left) and Nomarski (right) micrographs of animals infected with GFP-expressing *S. aureus*, illustrating three categories of intestinal accumulation observed. a, p, indicate anterior and posterior ends respectively. Panel B presents quantification of intestinal accumulation of *S. aureus* (SA) and *P. aeruginosa* (PA) at different times. N≥18 nematodes for each condition. Panels A and B and corresponding caption descriptions used from Supplementary Figure 4 from Irazoqui *et al*. (2010).

Chapter 2

Transient pathogen exposure and lifetime fecundity across a diversity of nematode isolates

Abstract

To maximize fitness upon pathogenic infection, host organisms might reallocate energy and resources among life-history traits, such as reproduction and defense. The fitness costs of infection can result from both immune upregulation and direct pathogen damage. However, the relative impacts of each to host fitness are unclear, particularly in environments where hosts can encounter opportunistic pathogens. The extent to which these costs vary by host genotype is also unknown. We attempted to disentangle the costs of host immune defense and pathogen damage by transiently exposing nine wild isolates and one lab-adapted strain of *Caenorhabditis elegans* nematodes to *Staphylococcus aureus* as a model opportunistic pathogen. While exposure to heat-killed *S. aureus* does induce an immune response but does not damage hosts, infection with live pathogen does both. After exposure, we tracked offspring production throughout the host's lifetime. We were unable to observe any differences in the fitness costs imparted by immunity and pathogen damage but found that nematodes exhibit a short-term delay in offspring production when exposed to live or heat-killed *S. aureus*. Nematode hosts had reduced cumulative progeny after *S. aureus* exposure relative to the food control on the first day of reproduction, but then their fecundity recovered to control levels. We did find variation by host genotype for both cumulative progeny production and magnitude of fitness costs. Moreover, we did not find trade-offs between fecundity on control food and minimized fitness loss after pathogen exposure, across host isolates. Lifetime fitness costs and evolutionary costs were not induced in our experimental conditions for the measured traits. In nature, nematodes are constantly interacting with microbes, and thus the ability to recover levels of fecundity after a brief insult would be selectively advantageous.

Introduction

Upon encountering a pathogen, hosts can pay costs from both pathogen damage and immune upregulation. During infection, hosts are hypothesized to invest more energy and resources to defense thereby reducing allocation to maintenance, growth, and reproduction, among other traits (Sheldon & Verhulst, 1996). Costs of immunity have been described theoretically and studied empirically in a diversity of host species (Stearns, 1992). Studies have measured the negative effect of pathogens and/or immune stimulation on host metabolism (e.g. Bashir-Tanoli & Tinsley, 2014), growth rate (e.g. Bartlett *et al*., 2018) and survival (e.g. Moret & Schmidt-Hempel, 2000). In terms of fecundity, infection has been found to drive delays (Feistel *et al*., 2019), reductions (Rigby & Jokela, 2000), or even accelerate reproductive output to offset higher host mortality (Jokela *et al*., 1999).

The aforementioned studies consider the costs of immune stimulation through non-pathogenic antigens or the entire cost of defense against pathogenic infection. The relative impacts of immune upregulation versus pathogen damage on host reproductive output, in total and over time, remain unclear. While previous work distinguishes between the costs of induced versus constitutive immunity, that is, the cost of upregulating the immune system versus maintaining it (Armitage *et al*., 2003), it is difficult to disentangle immune upregulation from pathogen damage (e.g. Sheldon & Verhulst, 1996; Schwenke *et al*., 2016). To determine the cost incurred solely by the pathogen independent of host immune stimulation, one could manipulate the immune response without causing damage and pair this treatment with a complementary exposure that induces both costs. It is expected that exposing a host to two stresses (pathogen damage and immune upregulation) is more costly than exposing a host to one stress (immune upregulation only).

The costs of defense might additionally vary across wild populations. The host genotypes in a species can differ in their ability to defend themselves against infection (e.g. McKean *et al.*, 2008; Bartlett *et al*., 2018) and might pay different costs of immune stimulation and/or pathogen damage. Moreover, costs can be impacted by environmental factors such as nutrient quantity and quality (reviewed in Sandland & Minchella, 2003). Nutrient limitation has revealed infection costs in invertebrate models (e.g. Moret & Schmid-Hempel, 2000; Hoang, 2001; McKean *et al*., 2008). Hosts in wild populations may also be able to escape an infectious environment. That is, pathogen exposure might be transient and not lifelong, as is simulated in many host-pathogen studies with constant adult exposure (e.g. Petersen *et al.*, 2015; Feistel *et al*., 2019). Studies that have looked into varying exposure time found critical thresholds for when an infection overwhelms a host and they can no longer recover, though before these timeframes, hosts transferred from pathogen to control conditions can maintain lifespans found in control animals (e.g. Tan *et al*., 1999; Sifri *et al*., 2003). It remains to be determined whether costs, namely fitness costs, of immunity and infection are of different magnitude across host genotypes in the context of short-term pathogen exposure.

To attempt to disentangle the fitness consequences of the immune response and pathogen damage, we transiently exposed nine genetically-disparate wild isolates and the lab-adapted N2 isolate of *Caenorhabditis elegans* to live and heat-killed *Staphylococcus aureus* (Irazoqui *et al*., 2010) as well as control *B. subtilis* PY79 food, and subsequently tracked offspring production. By measuring lifetime fecundity over multiple days, we were able to examine the reproductive schedule and total viable progeny. *C. elegans* constantly interacts with microbes in its natural habitat (Félix & Braendle, 2010). It is an established model for microbial pathogenesis

(Gravato-Nobre & Hodgkin, 2005) and immune response to pathogens (Pukkila-Worley & Ausubel, 2012). *C. elegans* launches an immune response to both live and heat-killed *S. aureus* after a short-term, 8-hour exposure, but pathology is only observed in the live treatment (Irazoqui *et al*., 2010). Assessing fitness costs across a panel of wild isolates allows us to investigate the evolutionary costs of pathogen exposure. Wild *C. elegans* isolates are known for demonstrating genetic variation in many phenotypes, including their response to pathogens (reviewed by Barrière & Félix, 2005), in terms of infection levels, pumping rate (a metric of pharyngeal behavior which indicates feeding), and evasion behavior (Schulenburg and Müller, 2004). Previous work on interactions between *C. elegans* and the pathogen *Bacillus thuringiensis* suggests there is an evolutionary cost for maintaining immunity. Schulenburg and Müller (2004) found that the wild isolate CB4851 had the highest survival on *B. thuringiensis* after a 24-hour exposure, and Hodgkin and Doniach (1997) found this isolate produced the fewest offspring among other isolates under normal food conditions. It is therefore possible for higher defense against pathogens in *C. elegans* to be costly when pathogens are absent.

In this study, we specifically investigated: (i) differences in offspring production over time among food control, live pathogen, and heat-killed pathogen treatments, to explore potential delays in reproductive timing and/or decreases in total brood size after transient pathogen exposure; (ii) whether there was variation in cumulative progeny among host isolates; (iii) differences in the magnitude of fitness costs between live and heat-killed pathogen exposures; and (iv) trade-offs in the form of a negative correlation of host genotypes for minimized fitness loss after pathogen exposure (i.e. immunocompetence against both heat-killed and live pathogen) versus fecundity in the absence of exposure. While we did not detect any difference between costs of immunity and pathogen damage, we found that nematodes recover offspring

production after a delay induced by transient pathogen exposure (live and heat-killed, compared to food). We also confirm the existence of natural variation for lifetime fecundity across nematode isolates (as in Hodgkin & Barnes, 1991). We did not find evidence for fitness costs or trade-offs in this host-pathogen system, but this outcome may be due to the short period of exposure.

Materials and Methods

Nematode and bacterial strains

We chose nine isolates of *C. elegans* collected across several continents that span the phylogenetic tree of Andersen *et al*. (2012) and the canonical wildtype strain N2, which was isolated from Bristol (Nicholas *et al*., 1959) and then became lab-adapted before it was frozen (Brenner, 1974, as detailed in Sterken *et al*., 2015). Specifically, we used *Caenorhabditis elegans* wild isolates CB4853, CB4854, CB4858, ED3017, JU1400, JU1490, JU258, LKC34, and QX1211 provided by the Woollard laboratory at the Department of Biochemistry. These isolates were randomly chosen from the phylogenetic tree of Andersen *et al*. (2012) to represent a wide panel of nematodes from various geographical locations and genetic backgrounds (see Chapter 1).

Nematode populations were maintained at 20°C on Nematode Growth Medium (NGM; Brenner, 1974) with *Bacillus subtilis* PY79 food before being exposed to the pathogen *Staphylococcus aureus* MSSA476. MSSA476 was grown in 3-5 mL of Todd-Hewitt Broth (THB) and PY79 was grown in 13-15 mL aliquots of LB (to make both maintenance and exposure plates). NGM plates were seeded with 800 μL of bacterial food. For exposures, 55 mm Tryptic Soy Agar (TSA) plates were seeded with 60 μL of the MSSA476 or PY79 control at OD_{630}=0.15. This

concentration of liquid culture balanced the necessity to see nematodes under the microscope with providing sufficiently thick lawns for food. All maintenance and exposure plates were incubated at 30°C overnight.

For exposures involving heat-killed bacteria, overnight cultures were first diluted to OD_{630}=0.15, then 1 mL transferred to microcentrifuge tubes and incubated in a water bath set to 88°C for 1 hour. Incubation conditions were determined upon surveying the literature and trialing a range of temperatures (data not shown) to ensure no future bacterial growth upon plating. Heat-killed samples were allowed to cool and then plated as described above for 55 mm TSA exposure plates.

Egg preparation and synchronization

The bleaching protocol was used to prepare batches of age-synchronized eggs. Prior to the start of each experiment, starved nematodes from maintenance plates were transferred to new 90 mm NGM plates with bacterial food by taking a small agar chunk, one for each nematode isolate. Three days later, each plate was washed with M9 + Triton-X (0.01%) and all gravid nematodes and eggs transferred to a 15 mL conical tube, filled up to 4 mL, and then 1 mL of freshly-prepared bleach solution (1:1 of 10% sodium hypochlorite and 5M NaOH) was added and tubes were left to shake at 88 rpm in an orbital shaker for 10-15 minutes. Once nematode bodies released eggs, tubes were centrifuged at 3200 rpm for 2 minutes, and pellets were re-suspended up to 4 mL with M9 + Triton-X, mixed by inversion, and then centrifuged again for three washing steps. After the final washing step, pellets were resuspended in 2 mL M9 + Triton-X and left to shake at 88 rpm overnight at 20°C. The following day, the resulting synchronized larval stage 1 (L1) nematodes were plated on 90mm NGM maintenance plates to a density of

about 1800 nematodes per plate for each isolate. All nematodes were reared at 20°C for 44 hours, until they reached L3/L4 stage.

Transient exposure of nematodes to bacterial pathogen

Nematodes were exposed to either food or bacterial pathogen (heat-killed or live) at L3/L4 stage, 44 hours post-L1 synchronization (Figure 1). About one hour before exposure, nematodes were washed off NGM maintenance plates with M9 + Triton-X into microcentrifuge tubes, and then washed three times by allowing nematodes to pellet by gravity and be re-suspended. Approximately 100 nematodes were transferred to one of six replicate plates with a lawn of either MSSA476 or food. This time was noted as zero hours since L4, and used for subsequent timepoints. Exposure plates were incubated at 25°C for 8 hours, the point at which nematodes have expressed immune genes specific to *S. aureus* but have not yet started to lay eggs (Irazoqui *et al*., 2010; Aprison & Ruvinsky, 2014). This exposure time enhanced immune-upregulation effects prior to the host reproductive period.

Lifetime fecundity assays

Host fecundity for single nematodes was assessed after transient exposure to live and heat-killed pathogens. Four nematodes were picked from each exposure plate (there were in total six plates for each nematode strain and exposure combination) into four corresponding wells of a randomized set of 12-well plates. Well plates were prepared in advance by filling with 3 mL NGM agar and seeded the day before with a 30 μL spot of bacterial food. Nematodes were maintained at 25°C for the remainder of the experiment and were transferred to new wells with food each day, at 30 hour- and 54 hour-post L4. Two days after each picking time point, live progeny from each single nematode were counted for 8-30 hour, 30-54 hour, and 54-126 hour time intervals. Treatments consisted of two biological replicates per assay and each experiment

was repeated three times to have six replicate plates for a given bacterial exposure. Overall, we tracked 720 founder worms over three counting points. Worms that crawled off the plate or died from picking action at any time point were censored from the analyses (Supplementary Table 1).

Statistical analyses

Data were analyzed in R version 3.6.1 and RStudio version 1.2.1335 (R Core Team, 2019). All count data were checked for a normal distribution at each time point. The cumulative progeny from each founder worm at each time point, for a given host isolate, and a specific bacterial exposure, were averaged for a replicate plate (Supplementary Figure 1). These means were further averaged to combine the six replicate plates into one mean.

Comparisons of total brood size were analyzed using two-way analysis of variance (ANOVA) with factors of host isolate and bacterial exposure as main effects, and also their interaction. The number of offspring produced at 30 hours (i.e. the first day of reproduction after pathogen exposure) was analyzed using a two-way ANOVA to determine the presence of a reproductive delay among control and exposure treatments, across host isolates. The Tukey multiple comparison of means test was used to determine significant differences within host strains and bacterial exposures. Given normally-distributed data, Pearson's product-moment correlations were used to examine the relationship of mean brood sizes between nematodes exposed to control food and exposure treatments (Supplementary Figure 2). These plots indicate trends towards hosts with consistently high reproduction (independent of the exposure) and the need for ratios to compare fitness costs across host isolates.

That is, employing ratios of progeny from pathogen exposure over progeny from control food to compare across strains allows us to investigate significant differences in the magnitudes of costs,

not just brood reductions in raw count data. Additionally, the ratios represent a proxy for immunocompetence since host defenses are mechanisms employed to minimize fitness loss upon infection (Schmid-Hempel, 2013). For subsequent analyses, count data were transformed into fitness cost ratios of progeny counts on pathogen (either live or heat-killed) over progeny on control food (Supplementary Figure 3). Since exposures to both live and heat-killed *S. aureus* cause an upregulation in the host immune response, the difference in fecundity between hosts exposed to live and heat-killed pathogen can be interpreted as the fitness cost from pathogen damage. The ratio data were analyzed at each time point with a binomial generalized linear model (GLM) with worm isolate as an additional independent factor, followed by two-way ANOVA and Tukey Contrasts for multiple comparisons of means with the car (Fox & Weisberg, 2019) and multcomp (Hothorn *et al*., 2015) R packages.

Lastly, we looked into tradeoffs between reproduction in control conditions versus immunocompetence. Spearman's rank correlation tests were used on non-normally distributed data comparing brood sizes on food (count data) versus minimized fitness loss (ratio data).

Figures were made using the ggplot2 (Wickham, 2016) and RColorBrewer (Neuwirth, 2014) packages in R.

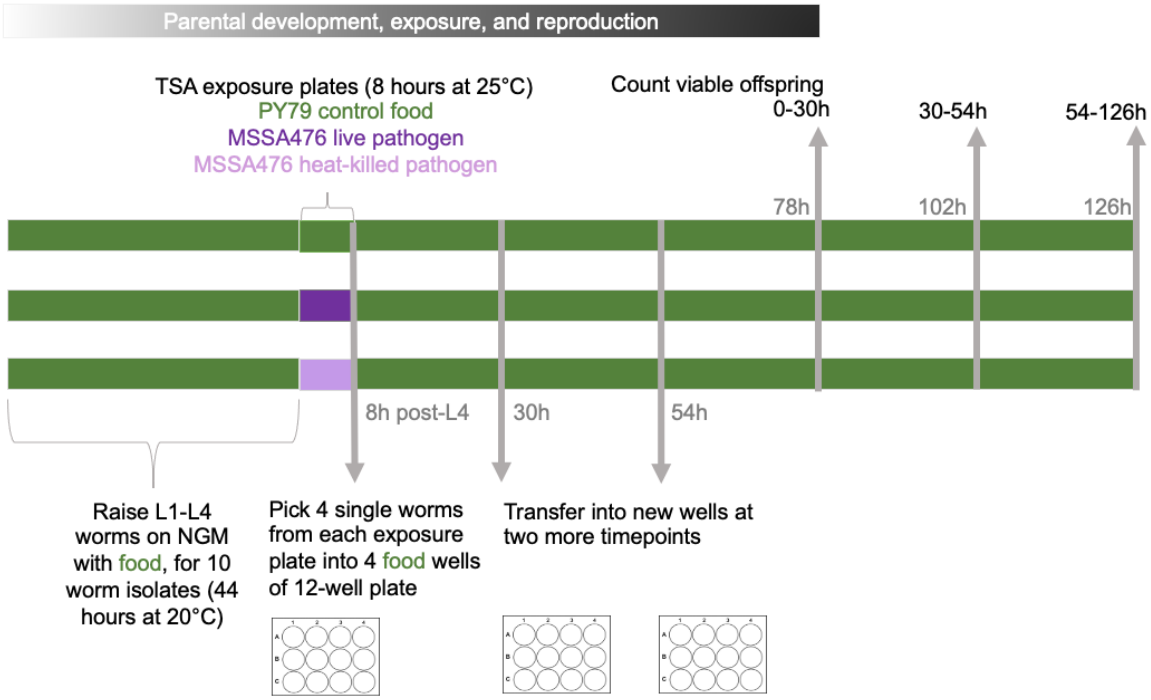


Figure 1: Experimental method for brood assays to determine cumulative progeny over time and lifetime fecundity in Chapter 2. The figure is representative for one nematode isolate; experiments were performed across ten isolates. Parental nematodes developed on *B. subtilis* PY79 control food (green) for two days, were exposed to control food, live *S. aureus* MSSA476 (dark purple), or heat-killed *S. aureus* MSSA476 (light purple) for 8 hours, and then all transferred back to control food for the duration of the experiment. Transfers of parental worms occurred at 8 hours, 30 hours, and 54 hours post-L4 stage. Offspring from the first timeframe (eggs laid between 0-30 hours) were counted two days later (approximately 78 hours post-L4), offspring from the second timeframe (eggs laid between 30-54 hours) were counted two days later (approximately 102 hours post-L4), and offspring from the third timeframe (54-126 hours) were counted on the final day, at which point most parental nematodes had few additional offspring as they had ceased reproduction around 78 hours post-L4. Color changes (or in the control case, green with light outlines) indicate transfer of nematodes between plates. Grey gradient indicates progression in parental development. Timepoints during experimental course noted in grey font. Timeframes at which eggs were laid are noted in black font.

Results

Lifetime fecundity and reproductive timing

We sought to determine whether transient exposure to the live and heat-killed opportunistic pathogen *S. aureus* MSSA476 reduced host lifetime fecundity. We predicted lower brood sizes after pathogen exposures that would be attributed to delays in reproduction and general decreases of offspring at each time point. After 30 hours post-L4 stage, there were significant differences in cumulative offspring by bacterial treatment (Figure 2: ANOVA, F = 58.44, df = 2, $P < 0.0001$) and worm isolate (ANOVA, F = 6.16, df = 9, $P < 0.0001$). Offspring counts at 30 hours from nematodes transiently exposed to live (Tukey multiple comparisons of means, $P < 0.001$) and heat-killed ($P < 0.001$) MSSA476 were lower than those exposed to food, pointing to a delay in reproduction. However, we found no significant reduction in total brood sizes across the nematode isolates when comparing the three bacterial treatments (Figure 2: ANOVA, F = 0.96, df = 2, $P = 0.39$) but did find significant differences between nematode isolates (Figure 2: ANOVA, F = 3.92, df = 9, $P = 0.0002$), meaning that some isolates had higher brood sizes than others. These results highlight that nematodes were able to have control exposure levels of lifetime fecundity after an initial delay from a transient pathogen exposure.

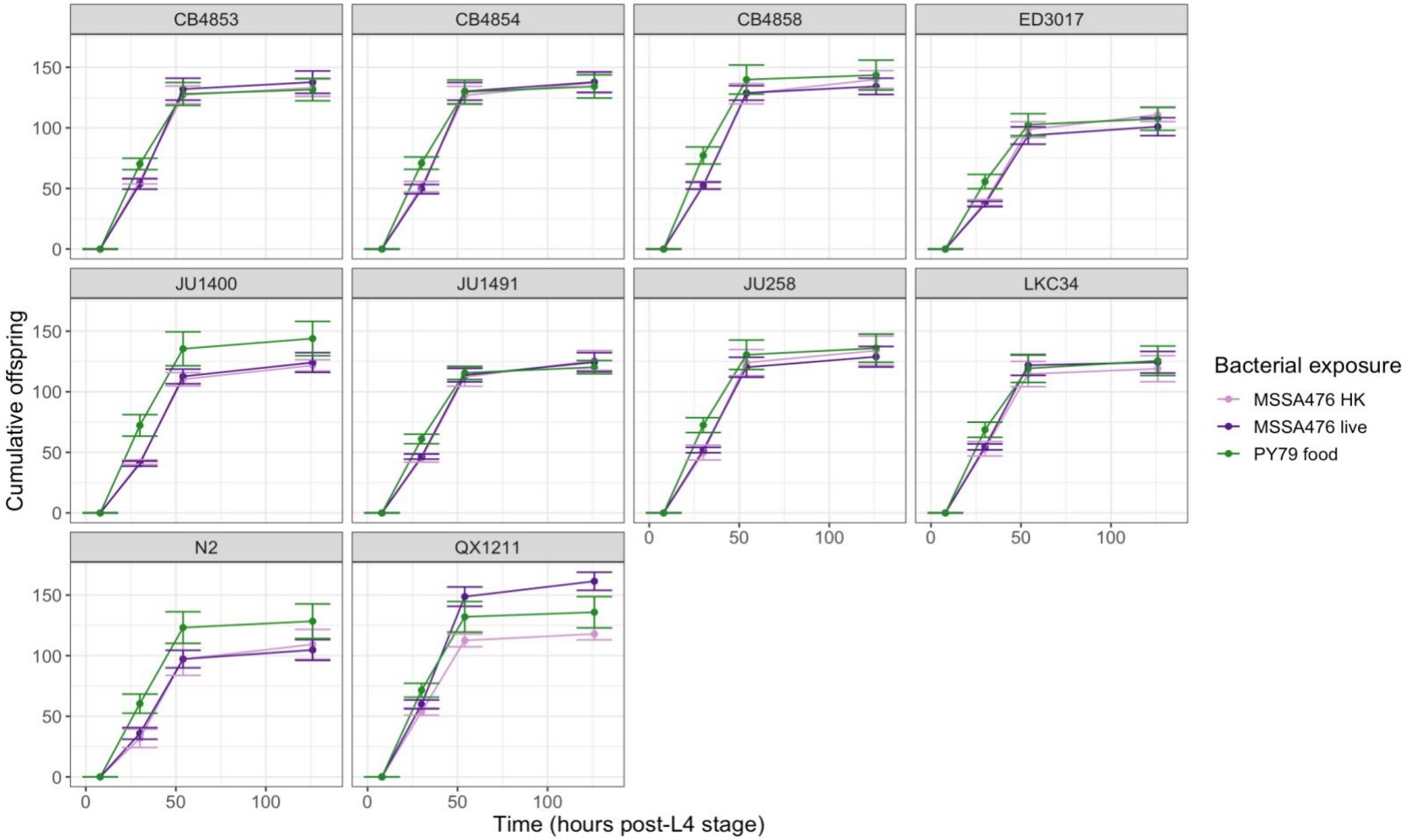


Figure 2: Cumulative viable offspring over time (mean ± 1 SE) for wild nematode isolates and lab-adapted N2 isolate. Nematodes were reared on food, then transiently exposed to *S. aureus* MSSA476 live pathogen (dark purple), heat-killed pathogen (HK, light purple), or *B. subtilis* PY79 food control (green) in the first 8 h post-L4 stage, and then all moved onto food for the rest of their reproductive period. Host isolate names are indicated above the grid.

Costs of immune upregulation and pathogen damage

We aimed to disentangle the cost of immune upregulation from the cost of pathogen damage by comparing the fitness cost of exposure to the live pathogen (immune upregulation and damage) to the cost of exposure to the heat-killed pathogen (immune upregulation only). Differences in relative fecundity among isolates were observed at 30 hours (Figure 3: Binomial GLM, χ^2 = 44.79, df = 9, $P < 0.0001$), 54 hours (Binomial GLM, $\chi^2 = 46.06$, df = 9, $P < 0.0001$), and 126 hours (Binomial GLM, $\chi^2 = 40.77$, df = 9, $P < 0.0001$), irrespective of bacterial exposure. Notably, the lab-adapted N2 strain exhibited statistically significantly lower ratios (i.e. higher fitness cost) compared to CB4853 (Tukey Contrasts, $P = 0.0109$), LKC34 ($P = 0.0293$), and QX1211 ($P = 0.0495$) at 30 hours. There were no statistically significant comparisons between pairs of hosts at subsequent timepoints. These comparisons show that the lab-adapted strain had the biggest fitness cost during the delay phase and was unable to minimize its fitness loss to the extent of various wild isolates. Across all timepoints, differences associated with live versus heat-killed bacterial exposure were not significantly different (Figure 3; Supplementary Table 2). We did not detect any difference between the costs of immune upregulation and pathogen damage under our experimental conditions.

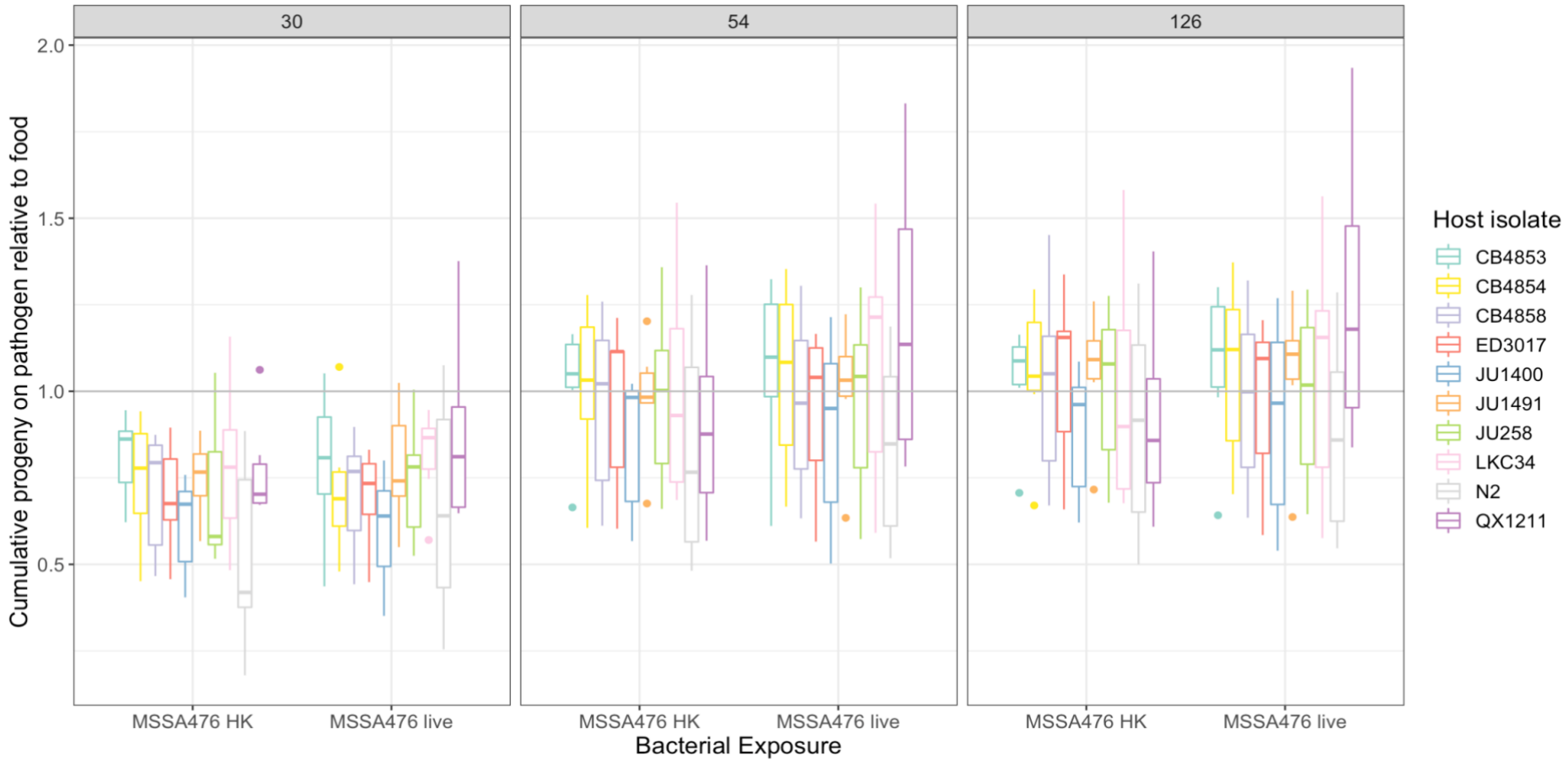


Figure 3: Fitness cost expressed as host cumulative progeny on *S. aureus* MSSA476 heat-killed (HK) or live pathogen relative to food for nematode isolates across time. Relative progeny shown at 30 h, 54 h, and 126 h post-L4. Grey line at y = 1 indicates when host reproduction after pathogen exposure equals host reproduction on food. Boxes show first quartile, median, and third quartile of the data, whiskers extend 1.5 times the interquartile range from the first and third quartiles, and circles indicate outliers.

No evidence of evolutionary costs

We tested whether there was a negative correlation across isolates in the total brood size produced on food versus immunocompetence after exposure to live or heat-killed *S. aureus*. However, we did not find a significant relationship between control brood size and immunocompetence after transient exposures to live (Figure 4A: Spearman's rank correlation: $P = 0.838$, rho = -0.08) or heat-killed *S. aureus* (Figure 4B: $P = 0.154$, rho = -0.49). The brood size versus immunocompetence for the lab-adapted N2 across the treatments were on the intermediate-to-low end, compared to the range of wild isolate brood sizes.

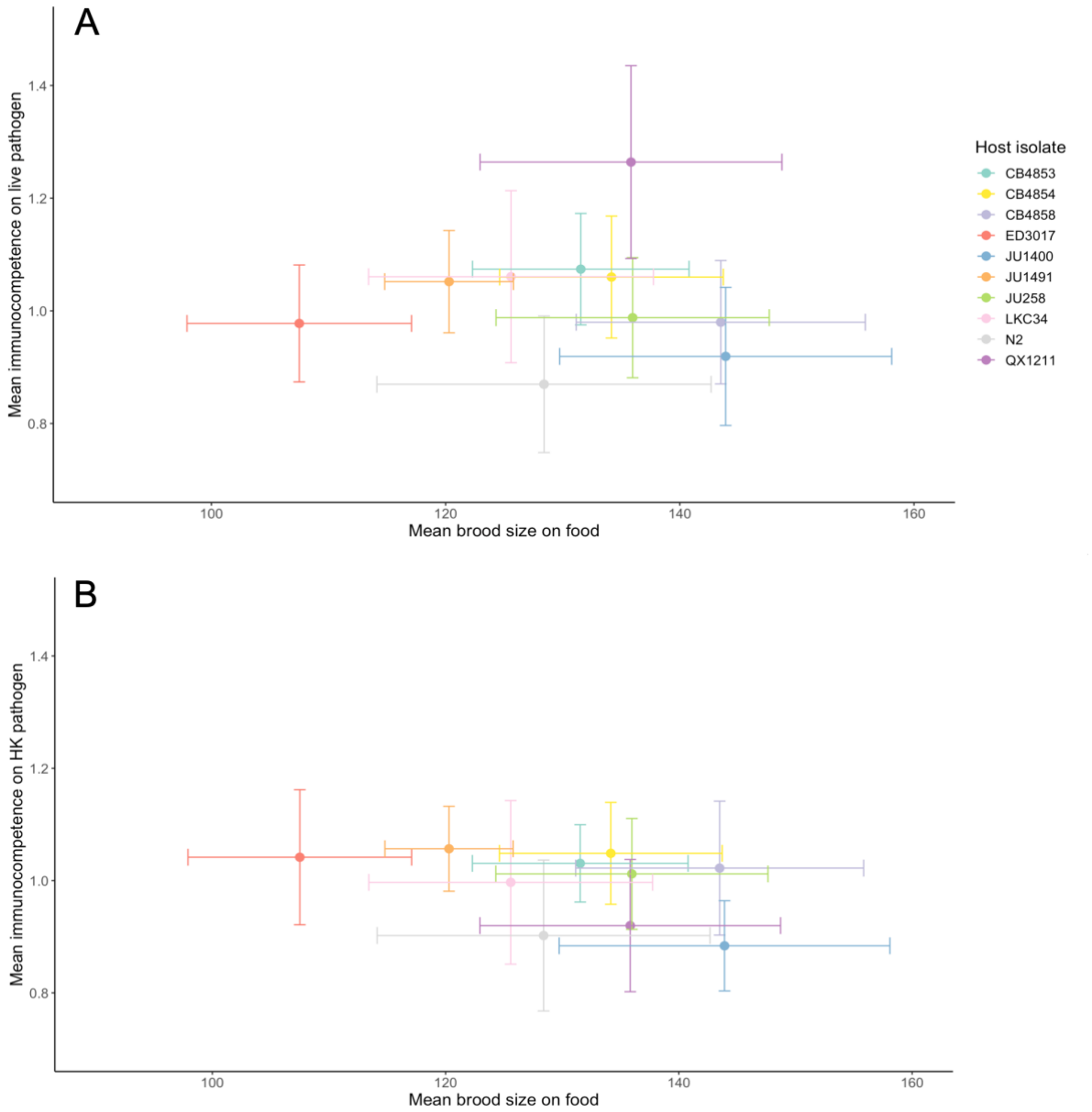


Figure 4: Total number of offspring per nematode isolate on food (mean ± 1 SE) versus mean immunocompetence (ratio of total offspring after pathogen exposure relative to total offspring on food ± 1 SE) after exposure to either live (A) or heat-killed (HK, B) *S. aureus* MSSA476, across ten host isolates.

Discussion

Host defense against pathogens is regarded as a costly life-history trait that trades off with host reproduction (Stearns, 1992). In our study, we detected no significant reductions in lifetime reproduction after transient pathogen exposure but did find delays (compared to food controls) after the first day of reproduction. To date, the relative lifetime fitness costs of immune upregulation versus pathogen damage more generally are unknown, and this is true for our system under our experimental conditions. We did not find significant differences in the fitness costs between live and heat-killed pathogen exposures, nor did we find trade-offs between total brood size on food and immunocompetence with either live or heat-killed pathogen exposures. However, we did find natural variation in lifetime fecundity among the nematode isolates, which confirms previous findings (Hodgkin & Barnes, 1991; Hodgkin & Doniach, 1997).

Delays in reproduction attributed to host-pathogen interactions have been documented. Feistel *et al*. (2019) found significant delays in N2 nematodes exposed to fungal pathogen, with pathogen-exposed hosts producing few offspring initially and nearly half of their brood in the late-reproductive window, from day four onwards. Further, they found reduced brood sizes in pathogen-exposed hosts, however, this was with continuous exposure to the pathogen. In the present study, it is possible that we did not detect reduced lifetime fecundity given the brief, transient exposure, and that increased exposure time may have negatively impacted host reproduction. However, Baeriswyl *et al*. (2010) showed that timing of nematode reproduction across lifelong bacterial exposures can change without affecting total brood. While our experiment was done in controlled lab settings, it is possible that high extrinsic mortality in the wild would expose fitness costs if infected parental worms are preyed on before finishing reproduction. Also, *C. elegans* reproduction is sperm-limited. That is, the hermaphrodites

undergo spermatogenesis first, then initiate oogenesis (Kimble & Ward, 1988). Perhaps in exposing hosts during oogenesis at 44-52 hours, we did not maximally impact reproduction in the way that an earlier pathogen exposure, during spermatogenesis, could.

The delay we observed may be attributed to the intestinal context of infection by *S. aureus*. Connors and Nickol (1991) refer to the relationship between intestinal pathogens and metabolism in the context of adult starling infection. It could be that *S. aureus*, in colonizing the intestine (Sifri *et al.*, 2003), reduces host energy intake and absorption in the first day post-exposure. This mechanism of pathogenesis could negatively impact reproduction, and then once hosts can clear the infection, they are able to recover normal levels of reproduction. Though in light of the reproductive delay also being observed for hosts of non-infecting, heat-killed treatments, it could be likely that we are seeing acute consequences of host immune upregulation.

There are several steps in the reproductive cycle where the short-term delay is possible. For example, mechanisms involving sperm production, the timing of switching from spermatogenesis to oogenesis, oocyte production, mitosis, and meiosis (Hubbard & Greenstein, 2005) might be impacted by pathogen exposure. Moreover, the SARM-ASK1-MKK3 module, the neurotransmitter serotonin, and the neuropeptide encoded by *ins-11* have roles in both egg laying and pathogen response (Shivers *et al*., 2009; Ringstad & Horvitz, 2008; Lee & Mylonakis, 2017). A technique like DAPI staining (e.g. Zanetti *et al*., 2012) or use of serotonin-mutant hosts could point to which step or steps in reproduction are delayed upon pathogen exposure. Future work could investigate how results might be different for a stronger transient pathogen challenge: perhaps a more ‘damaging’ pathogen might extend the delay past the first

day of reproduction and even impact lifetime fecundity counts. Upon being infected with a highly virulent pathogen, it is possible hosts would not survive past the exposure period, in which case there would be little or no progeny.

Fitness costs of defense are not universally detected in host-pathogen interactions (e.g. Williams *et al*., 1999; Penley *et al*., 2018). The rarity of 'no cost' studies could be due to publication bias against negative results (Malagoli & Ottaviani, 2014). Further, some studies find costs only in certain scenarios, such as depending on the density of competitors, nutrient availability, nutrient quality, and predation frequency (reviewed in Sandland & Minchella, 2003). McKean *et al.* (2008) found evolutionary costs between resistance and fecundity only in food-limited, but not food-unlimited environments. It is possible that nematode hosts in the present study were well-fed throughout the experimental period; they were placed on food immediately after pathogen exposure for the rest of their reproductive life. We may only be able to detect fitness costs and trade-offs in a more stressful, food-limited environment. Even so, it could be stressful for hosts to experience a change in nutrition, independent of the food quantity or immunological effects. Future experimentation could include an additional control in which hosts are reared on food, transferred to a different bacterial food (known to be non-pathogenic) and then moved back on to the maintenance food strain (e.g. paper that was published after completion of experiments and thesis submission: Gabaldón *et al.*, 2020). Also, it is possible that while offspring quantity did not vary between treatments, offspring quality may have differed. Aside from measuring fecundity, Perez *et al*. (2017) investigated 'quality' metrics such as size at hatching, rate of development, starvation resistance, and vitellogenin provisioning in the context of nematode maternal age and found that younger mothers gave rise to lower-quality offspring. The

connection between parental transient pathogen challenge and *C. elegans* offspring quality remains to be determined.

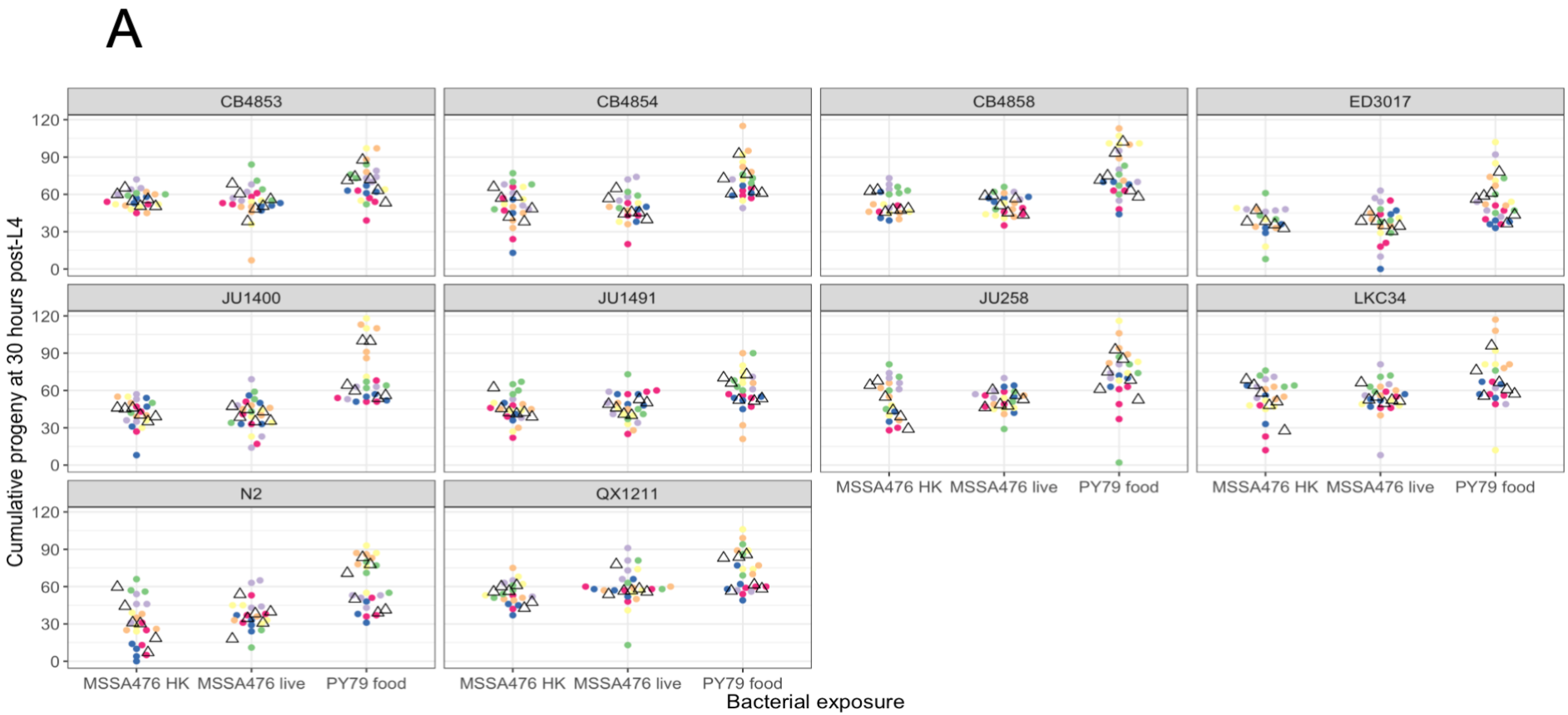
While we did not find the hypothesized lifetime fitness costs and trade-offs in terms of immunocompetence and total brood size, we revealed that upon encountering an opportunistic pathogen, wild *C. elegans* suffer short-term consequences of exposure but recover in a well-fed environment. These results may suit the lifestyle of these animals; *Caenorhabditis* constantly encounter microbes, ranging from mutualists to pathogens, in their compost and orchard environments (Félix & Braendle, 2010). Perhaps the ability to robustly and quickly recover from transient insult by opportunistic pathogens is favored in these microbe-rich habitats.

Supplementary Table 1: Number of observations for each host isolate and exposure combination at the final timepoint. Observations indicate technical replicates: non-censored nematodes with a maximum of 24 in treatments where no hosts were censored. All treatment combinations had a sample size of 6 biological replicate plates except ED3017 hosts exposed to heat-killed MSSA476 had 5 biological replicate plates. HK = heat-killed.

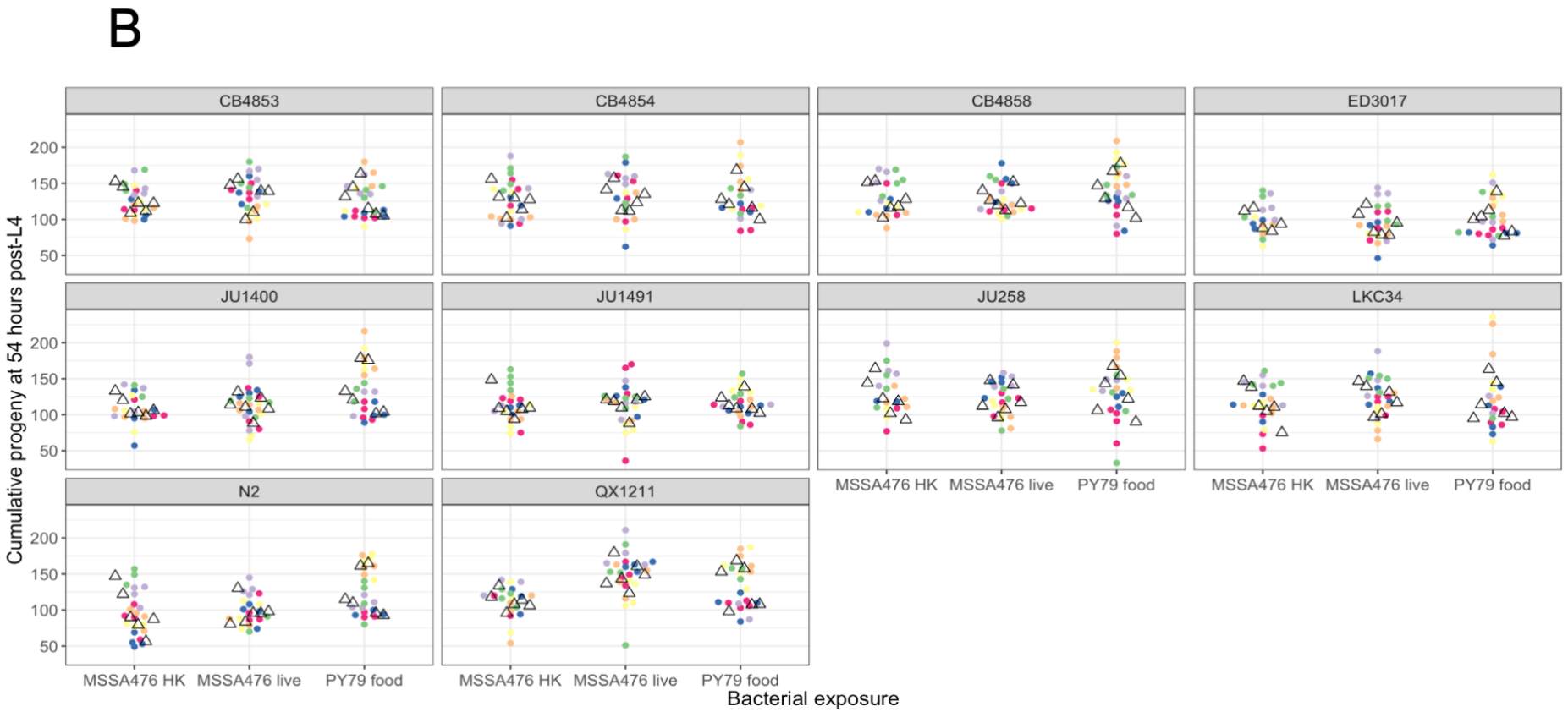
Host isolate	Exposure	Observations
CB4853	MSSA476 HK	21
	MSSA476 live	23
	PY79	22
CB4854	MSSA476 HK	22
	MSSA476 live	19
	PY79	20
CB4858	MSSA476 HK	20
	MSSA476 live	21
	PY79	23
ED3017	MSSA476 HK	17
	MSSA476 live	21
	PY79	24
JU1400	MSSA476 HK	20
	MSSA476 live	24
	PY79	21
JU1491	MSSA476 HK	21
	MSSA476 live	23
	PY79	22
JU258	MSSA476 HK	18
	MSSA476 live	22
	PY79	22
LKC34	MSSA476 HK	22
	MSSA476 live	23
	PY79	19
N2	MSSA476 HK	22
	MSSA476 live	20
	PY79	22
QX1211	MSSA476 HK	19
	MSSA476 live	24
	PY79	21

Supplementary Table 2: Results from statistical tests by figure and time point (in hours). Metric indicates the type of data analyzed, with fitness costs representing progeny from *S. aureus* treatments relative to food. Exposure refers to the three bacterial treatments and Host indicates nematode isolate type. Exposure*Host denotes interaction effect.

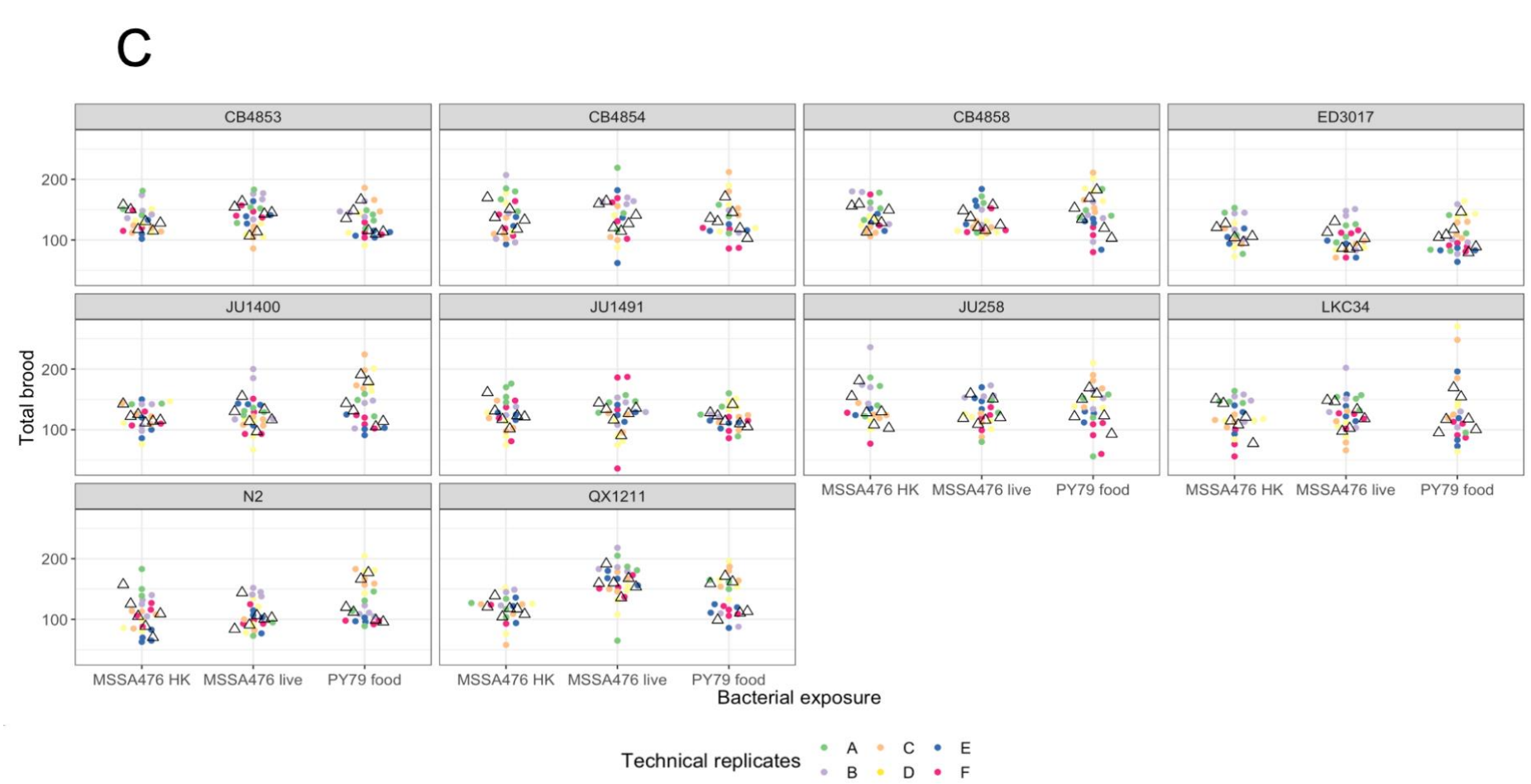
Figure	Time	Metric	Results
2	30	Cumulative progeny	Exposure: ANOVA, F = 58.44, df = 2, $P < 0.001$ Host: ANOVA, F = 6.16, df = 9, $P < 0.001$ Exposure*Host: ANOVA, F = 0.54, df = 18, $P = 0.93$
	126	Total brood	Exposure: ANOVA, F = 0.96, df = 2, $P = 0.39$ Host: ANOVA, F = 3.92, df = 9, $P = 0.0002$ Exposure*Host: ANOVA, F = 0.79, df = 18, $P = 0.487$
3	30	Fitness cost	Exposure: Binomial GLM, $\chi^2 = 0.30$, df = 1, $P = 0.584$ Host: Binomial GLM, $\chi^2 = 44.79$, df = 9, $P < 0.0001$ Exposure*Host: Binomial GLM, $\chi^2 = 2.37$, df = 9, $P = 0.984$
	54	Fitness cost	Exposure: Binomial GLM, $\chi^2 = 3.35$, df = 1, $P = 0.067$ Host: Binomial GLM, $\chi^2 = 46.06$, df = 9, $P < 0.0001$ Exposure*Host: Binomial GLM, $\chi^2 = 13.17$, df = 9, $P = 0.155$
	126	Fitness cost	Exposure: Binomial GLM, $\chi^2 = 1.28$, df = 1, $P = 0.257$ Host: Binomial GLM, $\chi^2 = 40.77$, df = 9, $P < 0.0001$ Exposure*Host: Binomial GLM, $\chi^2 = 21.56$, df = 9, $P = 0.01$
4A	126	Total brood vs. immunocompetence	Spearman's rank correlation: S = 178, $P = 0.838$, rho = -0.08
4B	126	Total brood vs. immunocompetence	Spearman's rank correlation: S = 246, $P = 0.154$, rho = -0.49



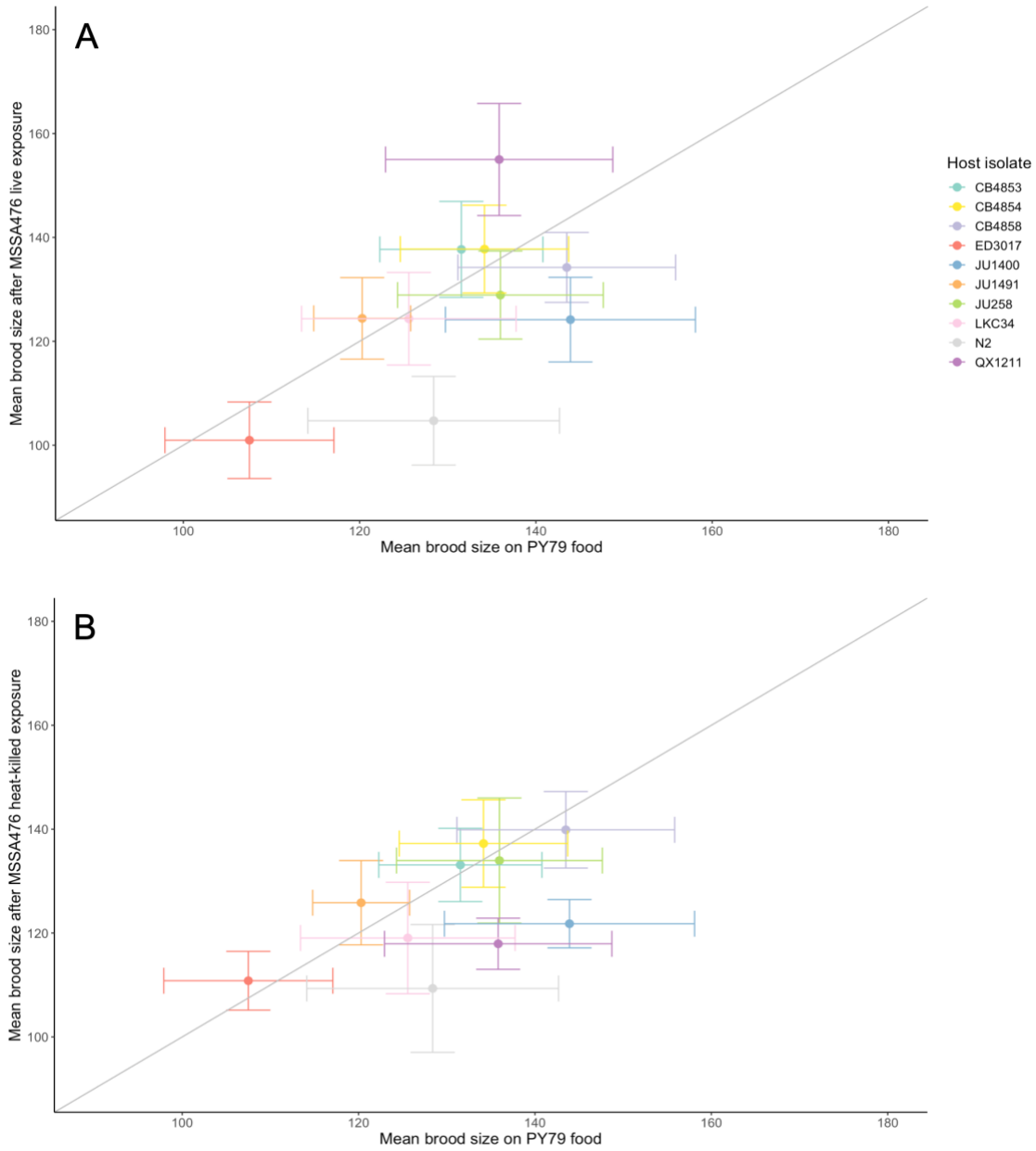
Supplementary Figure 1: Raw data showing variation in cumulative progeny of technical and biological replicates across 30-hour (A), 54-hour (B), and 126-hour (C) timepoints. Up to four technical replicates (founder nematodes tracked for reproduction over time) are depicted as points colored by their corresponding technical replicate code, A-F (A = green, B = purple, C = orange, D = yellow, E = blue, F = pink). The means of up to six biological replicates (exposure plates) are depicted as black triangles. Note different y axes to accommodate the distribution of technical replicates at each timepoint. Host isolate names indicated above each grid.



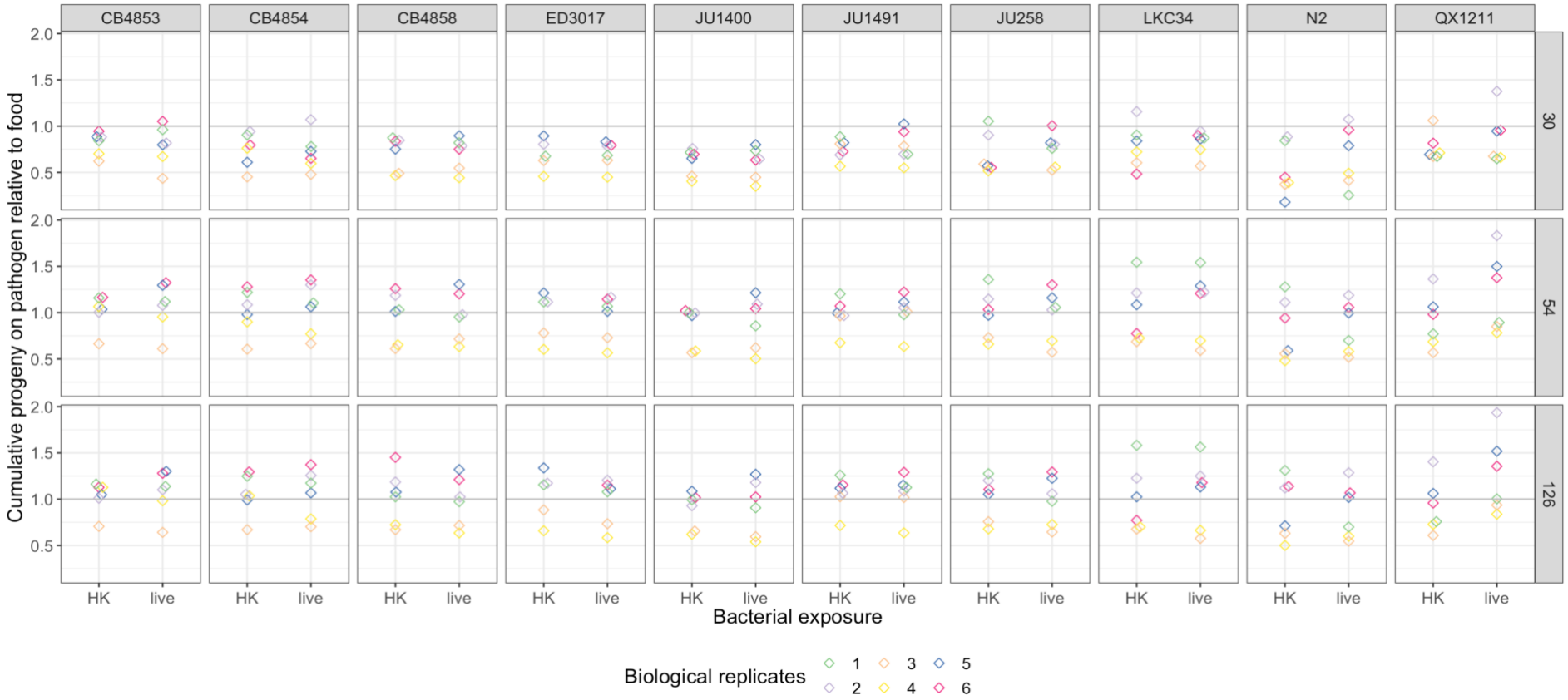
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Supplementary Figure 2: Total number of offspring per nematode across ten isolates (mean ± 1 SE) on food and after exposure to either live (A) or heat-killed (B) *S. aureus* MSSA476. Grey line indicates y = x. Colors indicate ten host genotypes.



Supplementary Figure 3: Raw data showing the six ratios to represent fitness costs for a given bacterial exposure, host isolate, and timepoint. Ratios were constructed at the level of the biological replicate, by dividing cumulative progeny from hosts after MSSA476 heat-killed (HK on x axis) or MSSA467 live (live on x axis) treatments by the corresponding cumulative progeny on food with the same batch identity, 1-6. Host isolates are indicated along the top and timepoints (in hours) are indicated on the right. Grey line at y = 1 indicates when host reproduction after pathogen exposure equals host reproduction on food.

Chapter 3

Investigating the costs and trade-offs of transient exposure to two pathogen species at the host population level

Abstract

Host organisms live in a multi-microbial environment that includes diverse pathogen species. Increased host defenses towards one type of pathogen may increase susceptibility to other pathogen types. We used genetically-diverse *Caenorhabditis elegans* host isolates to test for natural genetic variation in fitness costs of immune-upregulation and pathogen damage, as well as trade-offs with reproduction compared to uninfected hosts and tradeoffs in immunocompetence to two pathogens, after transient exposure to *Staphylococcus aureus* and *Pseudomonas aeruginosa* live and heat-killed bacterial pathogens. We examined the fitness impacts of pathogen exposure by measuring host population sizes, which allowed us to simultaneously capture changes in reproductive output, developmental time, and survival. We found significant decreases in relative population sizes for hosts exposed to live versus heat-killed *S. aureus* and found increased reproductive output after live *P. aeruginosa* exposure, compared to heat-killed challenge. We also tested for genetic correlation across host isolates for responses to the two pathogen species. Nematode isolates producing relatively smaller population sizes after live *S. aureus* exposure were better able to respond to live *P. aeruginosa* challenge, as measured by their relatively larger population sizes on this bacterium. This finding reveals that *C. elegans* genotypes display different immune balance phenotypes, that are evident in the generations beyond the infected host, against two distinct pathogen species.

Introduction

There is a wide array of microbes in natural ecosystems that can be beneficial or harmful to their animal hosts. Because of the negative impacts of harmful microbes, or pathogens, on host fitness, hosts have evolved a variety of defense strategies. Hosts can (i) evade pathogens: avoidance, (ii) limit their within-host growth: resistance, and/or (iii) limit their damage:

tolerance (reviewed in Rivas *at al*., 2014). Within these broad defense categories, host responses can vary from general to specific, through a variety of recognition mechanisms, behaviors, signaling pathways, and effector molecules (reviewed in Medzhitov & Janeway, 1997). Trade-offs in defense can exist when improved immunity to one class of pathogens leads to increased susceptibility to another class (see Marsh *et al*., 2011 for further references). This concept of balanced immunity has been evidenced in model hosts such as *Drosophila melanogaster* (Ayres & Schneider, 2008) and *Caenorhabditis elegans* (Marsh *et al*., 2011). The potential for host adaptation to one pathogen impacting infection outcomes of other pathogens is critical to understand how hosts evolve in natural ecosystems with a pathogen community (e.g. Betts *et al*., 2018). Nevertheless, the impact of balanced immunity in nature remains unclear (Martins *et al*., 2014).

Given that most pathogen encounters in nature do not cause host mortality, it is important to evaluate non-lethal impacts of pathogens on host fitness. However, many studies quantify pathogenesis in terms of reduced survival (e.g. Tan *et al*., 1999; Mylonakis *et al*., 2002; Sifri *et al*., 2003; Apidianakis & Rahme, 2009; Marsh *et al*., 2011), and disregard the non-lethal, morbidity effects pathogens can have on traits like host development and reproduction (Abbate *et al*., 2015). In some cases, measuring survival alone might not encompass the fitness effects that pathogens can have particularly if hosts finish reproduction within a shorter period (Diaz *et al*., 2015). Many studies measuring survival have done so with sterile mutants to simplify the assessment of host mortality (Park *et al*., 2017). This approach, however, takes reproduction, an important life-history trait, out of the balance of energy and resource allocation, and does not reflect the fitness impacts of pathogen exposure. In contrast, employing a metric such as host population size (Feistel *et al*., 2019) over a fixed time concurrently accounts for the lethal and non-lethal impacts of infection on a variety of host

life-history traits, including reproductive rate, development time, and survival.

The nematode *Caenorhabditis elegans* has been used to study host-pathogen interactions across various microbes of clinical and natural relevance (reviewed in Sifri *et al*., 2005). While decades ago it was believed that invertebrates had rudimentary and general immune responses, it is now known that nematodes are capable of tailoring defenses to bacteria, fungi, and viruses (Ermolaeva & Schumacher, 2014; Jian & Wang, 2018). They also have distinct responses within classes of microbes (Wong *et al*., 2007) and specificity down to the pathogen strain level (reviewed in Pees *et al*., 2016). Most *C. elegans* research is conducted with the canonical wildtype strain N2, which is lab adapted (Sterken *et al*., 2015). However, significant natural variation in the response (i.e. behavior, morbidity, and mortality) of *C. elegans* wild isolates exists against bacterial pathogens, such as *Bacillus thuringiensis* (Schulenburg & Müller, 2004) and *Serratia marcescens* (Schulenburg & Ewbank, 2004). Population growth also varies upon exposure to naturally-associated bacteria, including *B. thuringiensis*, *Serratia* sp., and *Serpens* sp. (Petersen *et al*., 2015). The responses of natural host genotypes should be investigated to make broader conclusions about the connection between immunity and host fitness from ecological and evolutionary perspectives (Gasch *et al*., 2016). Further justification includes studying the range of disease outcomes that hosts of the same species experience after infection and seeing the strategies with which hosts successfully balance reproduction and defense.

In this study, we investigated the longer-term consequences of pathogen damage and immune responses to host fitness, beyond the challenged host generation. We exposed a panel of ten *C. elegans* isolates (N2 alongside nine genetically-diverse wild isolate hosts) to both *S. aureus* and *P. aeruginosa* (both live and heat-killed). We conducted experiments separately

since investigating co-infections was not in the remit of this project. Hosts were exposed to the pathogens transiently for 8 hours to mimic the ephemeral habitat found in natural settings and to ensure immune upregulation before reproduction. We then measured the resulting host population size, two generations later, derived from the exposed parental founder worm. The two pathogen species used herein, one Gram positive and the other Gram negative, are both 'novel' to nematodes since they were isolated from a clinical setting. In *C. elegans*, the pathogens generate different host responses: Irazoqui *et al*. (2010) found that *S. aureus* and *P. aeruginosa* differ in their infection mechanisms and elicit distinct *C. elegans* host responses, from cellular pathology to upregulation of immune genes that indicate pathogen specificity. In that study, while the same *S. aureus*-specific genes were upregulated by hosts against live and heat-killed *S. aureus*, *P. aeruginosa*-specific genes were only upregulated during live pathogen exposure. That nematodes do not produce an immune response of the same specificity or magnitude against heat-killed *P. aeruginosa* allowed us to use this treatment as a negative control. The fitness costs of hosts to *S. aureus*-induced immune upregulation and pathogen damage could be disentangled by comparing the outcomes of heat-killed and live treatments. However, by comparing the fitness costs of exposure to live and heat-killed *P. aeruginosa*, we can evaluate the impact of the immune response and pathogen damage together versus a negative control. We thus predicted the biggest differences in host population sizes between hosts in *P. aeruginosa* treatments, rather than in the *S. aureus* treatments, because the former comparison of hosts should have the more disparate responses.

Across our diverse panel of nematode isolates and pathogens, we examined whether there were: (i) trade-offs in the minimized fitness loss of hosts experiencing pathogen damage and/or immune upregulation with optimal host reproductive success, as measured by

correlations of host relative population sizes after live or heat-killed pathogen exposure versus host population sizes on food, (ii) differences in fitness costs caused by pathogen damage and/or immune upregulation determined by comparing relative host population sizes between live and heat-killed pathogen exposures (both relative to food) within and among host isolates, and (iii) trade-offs in fitness costs associated with balanced immunity between two pathogen species as evidenced by a negative genetic correlation across all host isolates. More specifically, we predicted a negative correlation between the minimized fitness loss after pathogen exposure (relative population size on both heat-killed and live pathogens) and in the absence of exposure (population size on food alone). We found no evidence of tradeoffs between immunocompetence and control-treatment population sizes across host genotypes with transient pathogen exposures. We distinguished between the higher dual costs of immunity and pathogen damage against *S. aureus* in nematode hosts versus hosts who only experienced immune upregulation. Moreover, live *P. aeruginosa*-exposed hosts produced larger populations than hosts exposed to heat-killed *P. aeruginosa*. Notably, we found a trade-off in relative population sizes across our host isolates after live *S. aureus* and *P. aeruginosa* exposures. These results indicate that in our system, the trade-off in defense strategies between pathogen species is more important in driving host population growth than trade-offs in energy and resources upon host infection.

Materials and Methods

Nematode and bacterial strains

We used *Caenorhabditis elegans* N2 nematodes and the wild isolates CB4853, CB4854, CB4858, ED3017, JU1400, JU1490, JU258, LKC34, and QX1211. These isolates were randomly chosen from across a phylogenetic tree (Andersen *et al*., 2012) to represent a wide

panel of nematodes from various geographical locations and genetic backgrounds (see Chapter 1).

Nematode populations were maintained at 20°C on Nematode Growth Medium (NGM) with *Bacillus subtilis* PY79 or *Escherichia coli* OP50 before being exposed to the pathogens *Staphylococcus aureus* MSSA476 or *Pseudomonas aeruginosa* PA14, respectively. We ensured that nematodes were consistently exposed to Gram-positive (MSSA476 treatments paired with PY79 food) or Gram-negative (PA14 treatments paired with OP50 food) bacteria throughout the assays.

MSSA476 was grown in 3-5 mL of Todd-Hewitt Broth (THB), PA14 was grown in 3-5 mL of Luria broth (LB), and food sources (PY79 and OP50) were grown in 13-15 mL aliquots of LB. NGM plates were seeded with 800 μL of bacterial food diluted to OD_{630}=0.15. For the Gram-positive assays, 55 mm Tryptic Soy Agar (TSA) plates were seeded with 60 μL of the exposure strain diluted to OD_{630}=0.15. We used 55 mm NGM plates (as well as 60 μL) for Gram-negative assays, to have slow-killing nematode exposures as opposed to fast-killing (Tan *et al*., 1999). All maintenance and exposure plates were incubated at 30°C overnight.

For exposures involving heat-killed bacteria, overnight cultures were first diluted to OD_{630}=0.15, then 1 mL transferred to microcentrifuge tubes and incubated in a water bath set to 88°C for 1 hour. Incubation conditions were determined upon surveying the literature and trialing a range of temperatures (data not shown) to ensure no future bacterial growth upon plating. Heat-killed samples were allowed to cool and then plated as described above for 55 mm TSA and NGM exposure plates.

Egg preparation and synchronization

Before each experiment, starved nematodes from maintenance plates were transferred to new 90 mm NGM plates with bacterial food by taking a small agar chunk, one for each host isolate. Three days later, each plate was washed with M9 and Triton-X (0.01%) and all gravid nematodes and eggs transferred to a 15 mL conical tube. The volume of the nematode suspension was adjusted to 4 mL, and then 1 mL of freshly-prepared bleach solution (1:1 of 10% sodium hypochlorite and 5M NaOH) was added and tubes were left to shake at 88 rpm for 10-15 minutes in an orbital shaker. Once nematode bodies bent to release eggs, tubes were centrifuged at 3200 rpm for 2 minutes, and pellets were re-suspended up to 4 mL with M9 + Triton-X, mixed by inversion, and then centrifuged again for three washing steps. After the final washing step, pellets were resuspended in 2 mL M9 + Triton-X and left to shake at 88 rpm overnight at 20°C. The following day, the resulting synchronized larval stage 1 (L1) nematodes were plated on 90 mm NGM maintenance plates to a density of about 1800 nematodes per plate for each isolate. All nematodes were reared at 20°C for 44 h, until they reached L3/L4 stage.

Transient exposure of nematode hosts to bacterial pathogens

Nematodes were exposed to either food or bacterial pathogen (heat-killed or live) at L3/L4 stage, around 44 h post-L1 synchronization. To expose nematodes to bacteria, nematodes were washed off NGM maintenance plates with M9 + Triton-X into microcentrifuge tubes, and then washed three times by allowing nematodes to pellet by gravity and be re-suspended. Approximately 100 nematodes were transferred to one of six replicate plates with a lawn of either MSSA476, PA14, or food (Figure 1, Figure 2). This time was noted as 0 h post-L4. Exposure plates were incubated at 25°C for 8 h, the point at which nematodes have expressed immune genes specific to each pathogen but have not yet started to lay eggs (Irazoqui *et al*.,

2010; Aprison & Ruvinsky, 2014). The exposure timeframe was chosen to enhance the immune-upregulation effects leading up to the host reproductive period.

Population growth assays

To measure the impact of transient pathogen exposure on population growth, the number of individuals in a single host lineage across three generations (P, F1, and F2) was counted. The lineage expansion assay method was followed as described in Feistel *et al.* (2019), with a few modifications. This metric allows us to look at pathogen effects beyond the lifetime of a single host. Changes to host population sizes indicate exposure-mediated effects on a host isolate that encompass host reproductive rate, developmental time, and survival concurrently. After 8 h exposure to either MSSA476, PA14, or control food, four founder nematodes were picked off each exposure plate onto four separate 90 mm NGM plates seeded with bacterial food. Plates were left at 25°C for 5 days while nematodes produced F1 and F2 generations. On the final day, each plate was washed with M9 + Triton-X until most nematodes were displaced. The suspension was diluted to a total volume of 4 mL in a conical tube, and then six 10 μL droplets were counted to determine the population size for each plate. Treatments consisted of three biological replicates per assay and each experiment was repeated twice to have six replicate plates for a given bacterial exposure. Overall, we set up six replicate plates × four founder worm replicates × ten host isolates × three bacterial treatments × two pathogen types for a total of 1,440 tracked founder worms. We censored a small number of worms that left the plate or were injured/killed while picking (Supplementary Table 1, Supplementary Table 2). Given that the lineage expansion method was developed relatively recently, we present the variation in the number of worms per droplet in Supplementary Table 3.

Statistical analyses

Data were analyzed in R version 3.6.1 and RStudio version 1.2.1335 (R Core Team, 2019). To calculate the population size from a founder worm, the average was taken from six 10 µL droplets and back-calculated to give the number of worms per plate. Population sizes from four replicate worms from an exposure plate were combined to give a mean population size for that plate (Supplementary Figure 1, Supplementary Figure 5). A mean of those six plates was calculated to give a mean population size for a given host isolate for a specific bacterial exposure.

To determine the correlation between pathogen-exposed host population sizes and control host population sizes, we used Pearson's product moment correlation and adjusted for multiple testing with false discovery rate correction. This correlation test was done for each *S. aureus* MSSA476 (Supplementary Figure 2) and *P. aeruginosa* PA14 treatment (Supplementary Figure 6), compared to *B. subtilis* PY79 or *E. coli* OP50 food, respectively. These plots indicate that some host genotypes have consistently high or consistently low population sizes (independent of the exposure) therefore, we employed ratios to compare fitness costs across host isolates. For subsequent analyses, count data were transformed into fitness cost ratios of mean population size on pathogen (either live or heat-killed) over mean population size on control food, matched by batch identity (Supplementary Figure 3, Supplementary Figure 7). The ratios represent a proxy for immunocompetence since host defenses are mechanisms employed to minimize fitness loss upon infection (Schmid-Hempel, 2013). To determine a) the correlation between relative population size after transient pathogen exposure and food control population size and b) the correlation of relative population sizes between hosts exposed to *S. aureus* and hosts exposed to *P. aeruginosa*, we used Spearman's rank correlation tests.

Differences between live and heat-killed bacterial treatments, for both *S. aureus* and *P. aeruginosa,* were examined using generalized linear models (GLM) with quasibinomial error distribution as the data were over-dispersed. We tested for differences between live and heat-killed exposures for a given bacterial species using analysis of variance (ANOVA) to test for main effects by host isolate, bacterial exposure, and the interaction of the two, for both *S. aureus* and *P. aeruginosa* data sets using the car (Fox & Weisberg, 2019) and multcomp (Hothorn *et al*., 2015) R packages. To quantify fitness reductions and fitness boosts between live and heat-killed exposures, we used the percentage difference formula from Cole and Altman (2017) to compare mean relative population sizes after each pathogenic treatment.

Figures were made using the ggplot2 (Wickham, 2016) and RColorBrewer (Neuwirth, 2014) packages in R.

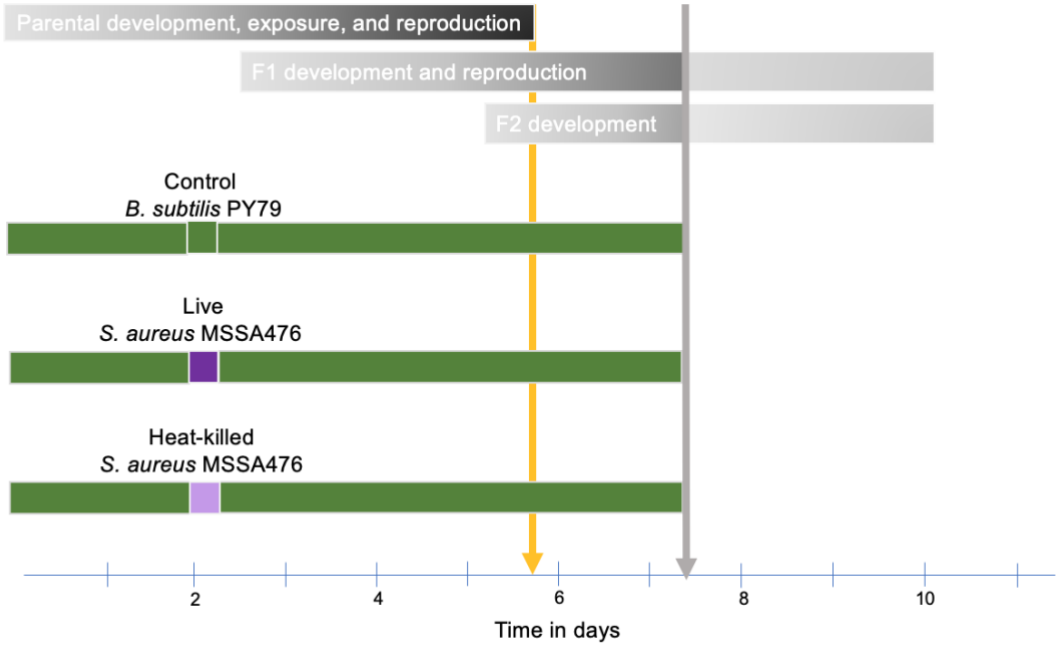


Figure 1: Experimental method for lineage expansion assays to determine population sizes in Chapter 3. The figure is representative for one nematode isolate; experiments were performed across ten isolates. Parental nematodes developed on *B. subtilis* PY79 control food (green) for two days, were exposed to control food, live *S. aureus* MSSA476 (dark purple), or heat-killed *S. aureus* MSSA476 (light purple) for 8 hours, and then all transferred back to control food for the duration of the experiment, during which F1 and F2 nematodes expanded in population for five days. Color changes (or in the control case, green with light outlines) indicate transfer of nematodes between plates. Grey gradients indicate progression in development. Yellow arrow indicates approximate end period for parental reproduction during total brood assays (Chapter 2). Grey arrow indicates final timepoint at which nematodes were washed off plates and counted to determine population size. The time scale is extended for ease of comparison with the method in Chapter 4.

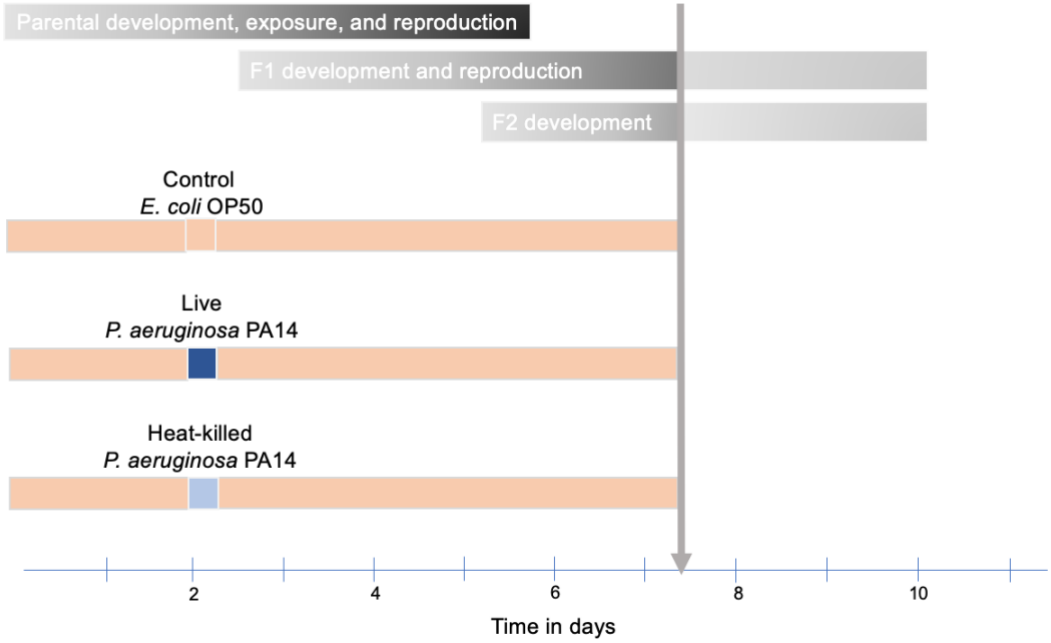


Figure 2: Experimental method for lineage expansion assays to determine population sizes in Chapter 3. The figure is representative for one nematode isolate; experiments were performed across ten isolates. Parental nematodes developed on *E. coli* OP50 control food (orange) for two days, were exposed to control food, live *P. aeruginosa* PA14 (dark blue), or heat-killed *P. aeruginosa* PA14 (light blue) for 8 hours, and then all transferred back to control food for the duration of the experiment, during which F1 and F2 nematodes expanded in population for five days. Color changes (or in the control case, orange with light outlines) indicate transfer of nematodes between plates. Grey gradients indicate progression in development. Grey arrow indicates final timepoint at which nematodes were washed off plates and counted to determine population size.

Results

Effect of transient *S. aureus* exposure on host population growth

We investigated the effect of transient *S. aureus* exposure on the population sizes of one lab-adapted and nine wild nematode isolates. The numbers for population sizes after pathogenic exposure were generally lower than numbers for population sizes from the control food treatment, which is evident in seeing points below the y=x line in Supplementary Figure 2. We found that reproduction after pathogen exposure to live (Supplementary Figure 2A: Pearson's product-moment correlation: t = 4.92, df = 8, $P = 0.001$, $R^2 = 0.87$) or heat-killed (Supplementary Figure 2B: Pearson's product-moment correlation: t = 7.48, df = 8, $P = 0.0001$, $R^2 = 0.94$) *S. aureus* correlated with reproduction on food. Isolates that performed well on food also performed well after pathogen exposure, relative to other isolates (Supplementary Table 4). Host isolates JU258 and QX1211 consistently had the highest population sizes across all *S. aureus* treatments and the food control (JU258: food mean pop. size = 17483 ± 2227; live pathogen = 12843 ± 1322; heat-killed pathogen = 15122 ± 1299. QX1211: food mean pop. size = 14792 ± 1812; live pathogen = 12005 ± 757; heat-killed pathogen = 13236 ± 654). In contrast, JU1491 and ED3017 yielded population sizes that were consistently lower (JU1491: food mean pop. size = 10690 ± 1721; live pathogen = 8440 ± 683; heat-killed pathogen = 8272 ± 1135. ED3017: food mean pop. size = 11000 ± 607; live pathogen = 8068 ± 146; heat-killed pathogen = 9936 ± 857). The lab-adapted N2 host population sizes were intermediate across bacterial exposures (food mean pop. size = 14147 ± 1653; live pathogen = 9539 ± 653; heat-killed pathogen = 12644 ± 760). Because of the trend of genotypes to be ‘high performing’ or ‘low performing,’ we analyzed subsequent data as relative population sizes to better compare fitness cost magnitudes across genotypes.

We then investigated the distinct costs of immune upregulation versus pathogen damage to host population growth. While there was no significant variation amongst host relative population sizes (Figure 3: Quasibinomial GLM, $\chi^2 = 1.71$, df = 9, $P = 0.995$), we did find a significant difference by live versus heat-killed *S. aureus* exposure (Figure 3: Quasibinomial GLM, $\chi^2 = 4.66$, df = 1, $P = 0.031$). That is, hosts exposed to live *S. aureus* (versus heat-killed *S. aureus*) were less able to minimize their fitness loss after pathogen exposure compared to their control-treatment population sizes (i.e. experienced greater fitness costs). One aspect to note is the larger variation in the relative population sizes for the heat-killed treatments. This was a resulting artefact of analysis: the pairing by batch identity to generate ratios led to higher variation in the heat-killed treatment than live treatment (Supplementary Figure 4). Across host genotypes, there was an 18% reduction in relative population sizes on live MSSA476 (average rel. pop. size = 0.82) compared to heat-killed MSSA476 (average rel. pop. size = 0.98). This decrease is associated solely with live pathogen damage, and not the immune upregulation that hosts in both treatments experience (Supplementary Table 5). Notably, the lab-adapted N2 strain exhibited the biggest loss in relative population size (33.26%) from heat-killed to live; that is, N2 populations suffer the biggest fitness cost due to pathogen damage compared to other strains.

We looked into whether there was an evolutionary cost of immunocompetence by comparing the relative population sizes (ratio metric indicating minimized fitness loss after pathogen exposure) to population sizes on control food. We found no evidence of a tradeoff between the immune and reproductive traits after either live (Figure 4A: Spearman's rank correlation, $P = 0.4$, rho = -0.3) or heat-killed (Figure 4B: Spearman's rank correlation, $P = 0.4$, rho = 0.4) *S. aureus* exposure. The population size versus immunocompetence for the lab-adapted N2 across the treatments were on the intermediate-to-low end, compared to the range of wild isolate data.

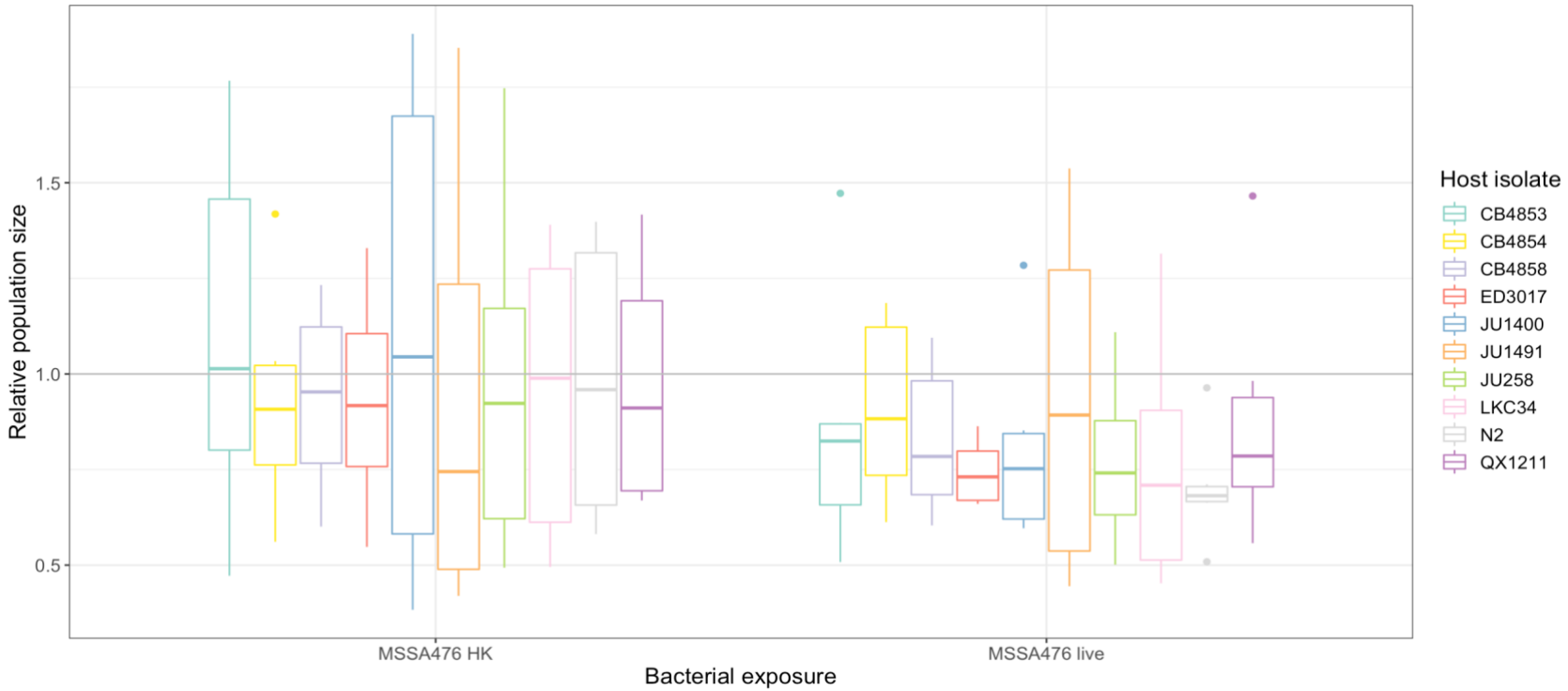


Figure 3: Relative population sizes for ten nematode isolates after transient exposure to live and heat-killed (HK) *S. aureus*. Values are relative to population sizes on PY79 food. Boxes show first quartile, median, and third quartile of the data, whiskers extend 1.5 times the interquartile range from the first and third quartiles, and circles indicate outliers. Grey line at y = 1 represents no difference between host population size on *S. aureus* treatment and control food. Colors indicate ten host genotypes.

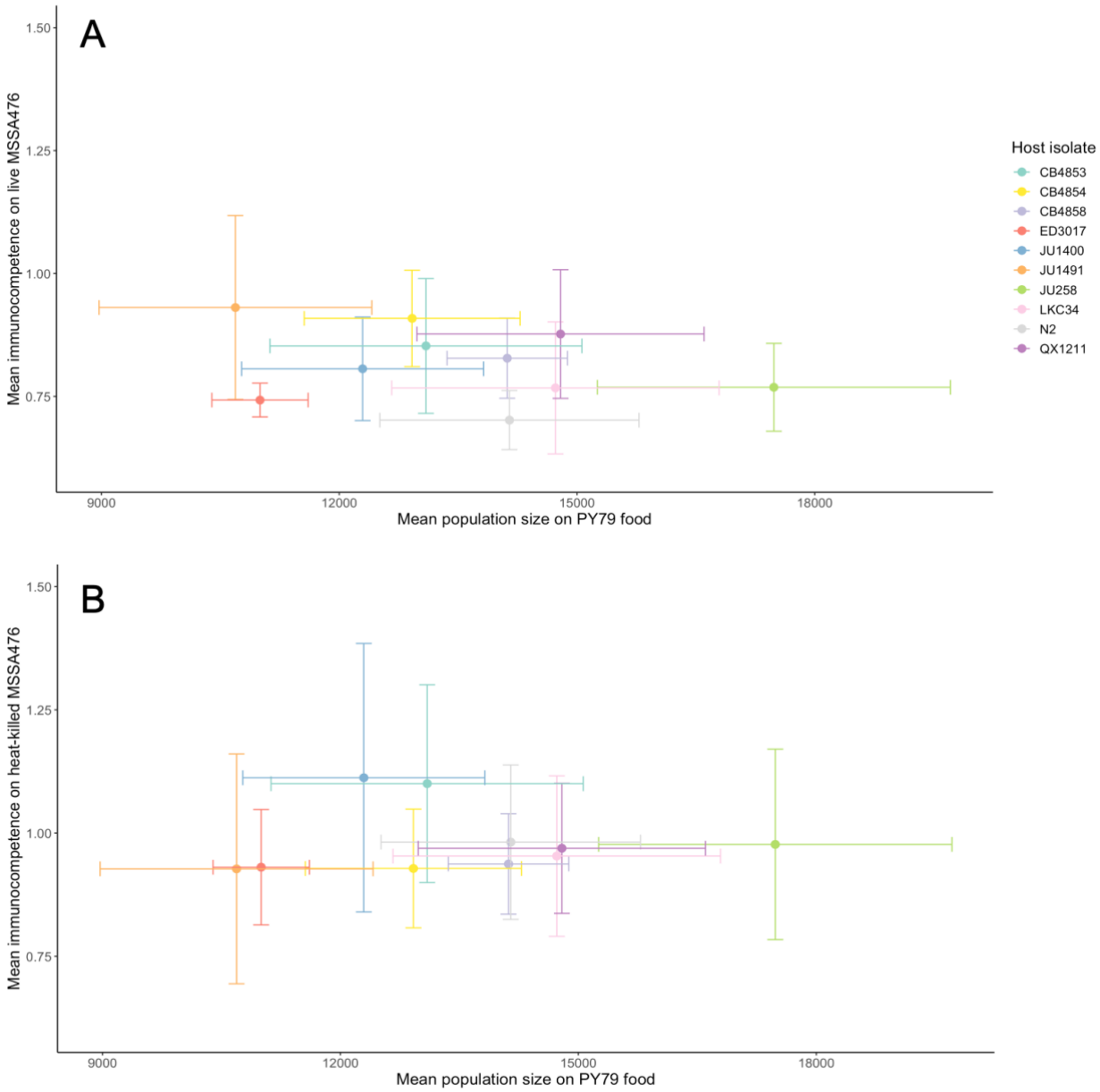


Figure 4: Population size of each nematode isolate on food (mean ± 1 SE) versus mean immunocompetence (ratio of population after pathogen exposure relative to population on food ± 1 SE) after exposure to either live (A) or heat-killed (B) *S. aureus* MSSA476, across ten host isolates.

Effect of transient *P. aeruginosa* exposure on host population growth

We next investigated the effect of transient *P. aeruginosa* exposure on nematode host population size. In both live and heat-killed pathogen exposure treatments, we found that nematode population sizes were correlated positively with population sizes on food (Supplementary Figure 6A: PA14 live vs. food: Pearson's product-moment correlation: t = 2.85, df = 8, $P = 0.02$, $R^2 = 0.71$; Supplementary Figure 6B: PA14 heat-killed vs. food: t = 4.18, df = 8, $P = 0.006$, $R^2 = 0.83$). The trends with host isolate performance on Gram-negative bacteria (Supplementary Table 6) were not as clear as with the aforementioned trends for hosts on Gram-positive bacteria (Supplementary Table 4). While the low-performing isolates continued to be JU1491 and ED3017 (JU1491: food: mean pop. size = 12789 ± 1262; live pathogen = 12950 ± 1129; heat-killed pathogen = 11924 ± 913. ED3017: food: mean pop. size = 9944 ± 579; live pathogen = 16703 ± 1156; heat-killed pathogen = 12016 ± 557), the higher-performing isolates varied. On *E. coli* OP50 food, CB4854 and CB4858 had the highest mean populations (CB4854: mean pop. size = 19056 ± 1401; CB4858: mean pop. size = 17328 ± 797), after live *P. aeruginosa* exposure JU258 and CB4854 had the highest populations (JU258: mean pop. size = 24475 ± 2181; CB4854: mean pop. size = 23514 ± 1798), and after heat-killed *P. aeruginosa* exposure CB4858 and JU1400 had the highest populations (CB4858: mean pop. size = 19679 ± 1447; JU1400: mean pop. size = 18267 ± 1305). The lab-adapted N2 host population sizes were on the lower end for food (mean pop. size = 14633 ± 1140) and heat-killed *P. aeruginosa* (mean pop. size = 14431 ± 777), and intermediate for live pathogen (mean pop. size = 21731 ± 2165).

Nematodes had significantly higher population sizes after live *P. aeruginosa* exposure compared with heat-killed exposure (Figure 5: Quasibinomial GLM, $\chi^2 = 24.323$, df = 1,

P<0.0001), both relative to food (*E. coli* OP50). Also, we found significant differences among the host isolates (Figure 5: Quasibinomial GLM, $\chi^2 = 20.51$, df = 9, $P = 0.015$) indicating that some nematode isolates increased their reproduction more than others. There was no significant interaction between bacterial exposure and host isolate (Figure 5: Quasibinomial GLM, $\chi^2 = 9.76$, df = 9, $P = 0.37$). Across host genotypes, there was a 20% boost in relative population sizes on live PA14 (average rel. pop. size = 1.33) compared to heat-killed PA14 (average rel. pop. size = 1.08) (Supplementary Table 7). In line with the percentage difference result after Gram positive exposures, the lab-adapted N2 strain exhibited the greatest increase in relative population size (36.47%) from heat-killed to live.

We examined whether there was an evolutionary cost of immunocompetence by comparing the relative population sizes of each host genotype to their population sizes on control food. We found no evidence of the expected negative correlation between defense and reproduction after either live (Figure 6A: Spearman's rank correlation, $P = 0.2$, rho = -0.4) or heat-killed (Figure 6B: Spearman's rank correlation, $P = 0.5$, rho = -0.3) *P. aeruginosa* exposure. The population size versus immunocompetence for the lab-adapted N2 across the treatments was on the intermediate-to-low end for heat-killed PA14 and higher for live PA14, compared to the range of wild isolate data.

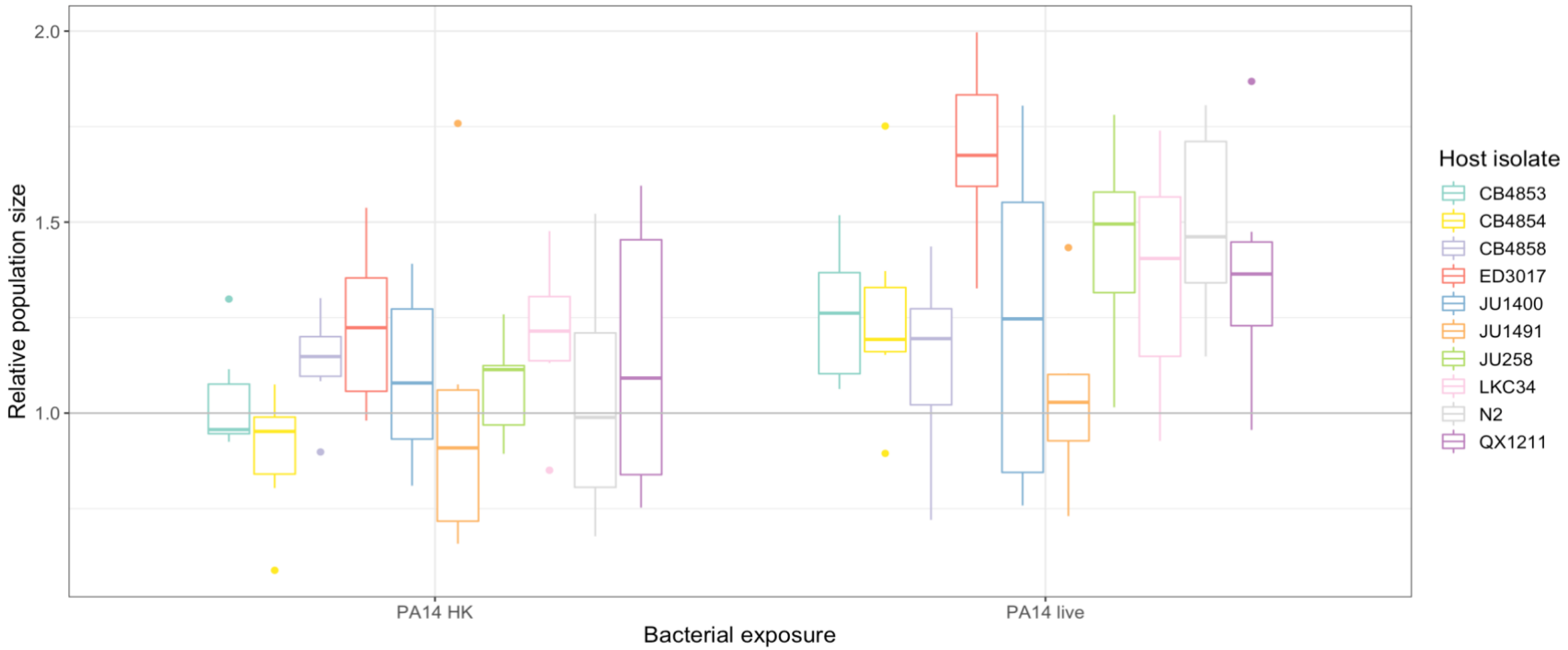


Figure 5: Relative population sizes for ten nematode isolates after transient exposure to live and heat-killed (HK) *P. aeruginosa*. Values are relative to population sizes on OP50 food. Boxes show first quartile, median, and third quartile of the data, whiskers extend 1.5 times the interquartile range from the first and third quartiles, and circles indicate outliers. Grey line at y = 1 represents no difference between host population size on *P. aeruginosa* treatment and control food. Colors indicate ten host genotypes.

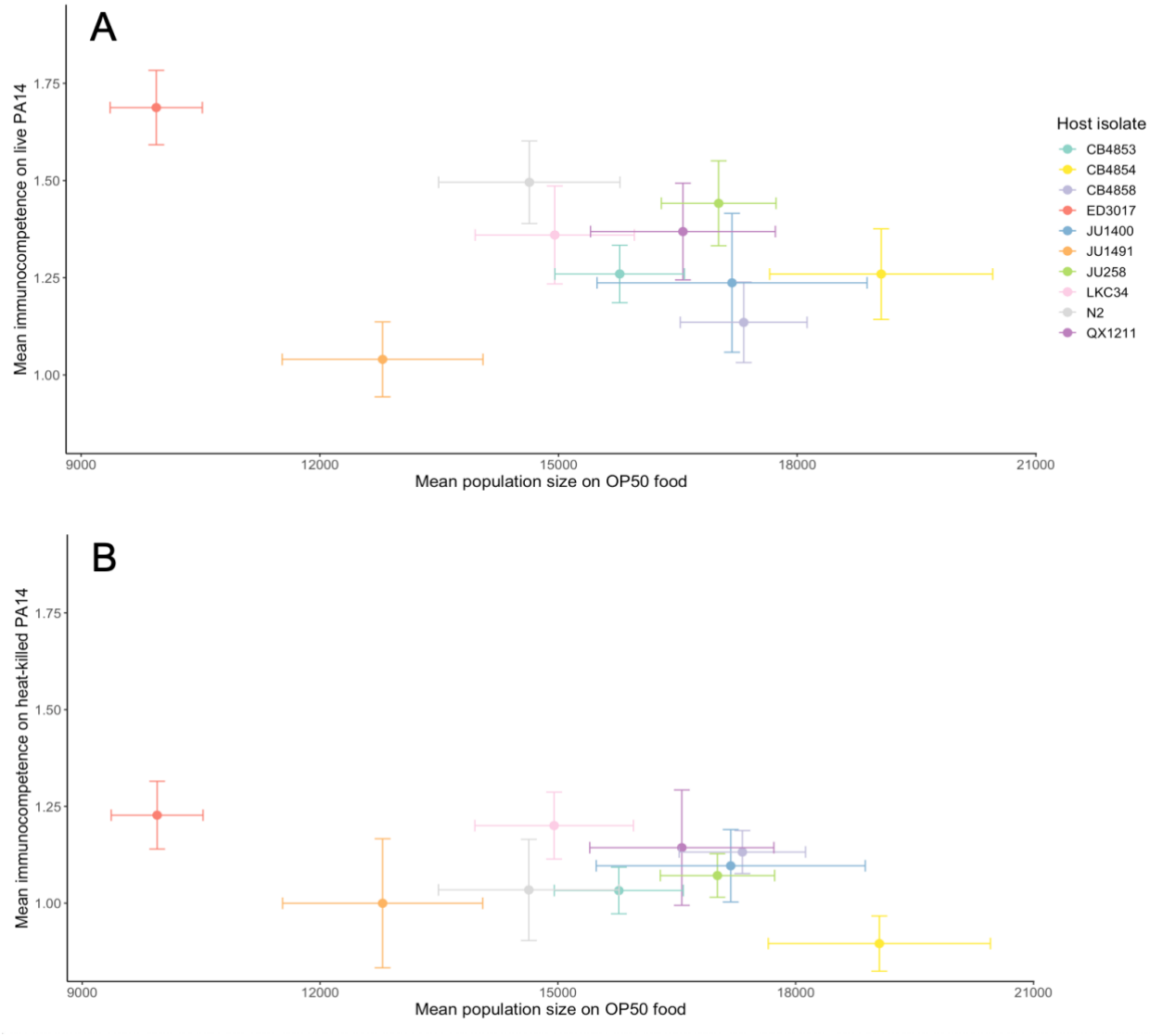


Figure 6: Population size of each nematode isolate on food (mean ± 1 SE) versus mean immunocompetence (ratio of population after pathogen exposure relative to population on food ± 1 SE) after exposure to either live (A) or heat-killed (B) *P. aeruginosa* PA14, across ten host isolates. Colors indicate ten host genotypes.

Comparisons between two opportunistic pathogens

As mentioned before, hosts on Gram-positive bacteria displayed population size trends with consistent high and low-performing host isolates (Supplementary Table 4 and Supplementary Figure 2), whereas hosts on Gram-negative bacteria were not as consistently high-performing across the bacterial treatments (Supplementary Table 6 and Supplementary Figure 6). We aimed to see whether there was a relationship between the degree to which nematode population sizes were affected by exposure to the two opportunistic pathogen species. We found a trade-off in immunocompetence across host isolates after live opportunistic pathogen exposures (Figure 7A: Spearman's rank correlation rho = -0.72, $P = 0.02$). Conversely, we did not find a significant relationship among relative population sizes resulting from exposure to heat-killed treatments (Figure 7B: rho = 0.079, $P = 0.84$). Since the control food is in the denominator of each ratio, this accounts for food-driven population differences. The negative correlation is driven by the live pathogens in Figure 7A and no correlation is present with heat-killed pathogen treatments over food in Figure 7B. The lab-adapted N2 isolates performed better, in terms of relative population size, after live *P. aeruginosa* exposure than after *S. aureus* exposure, compared with the wild isolates.

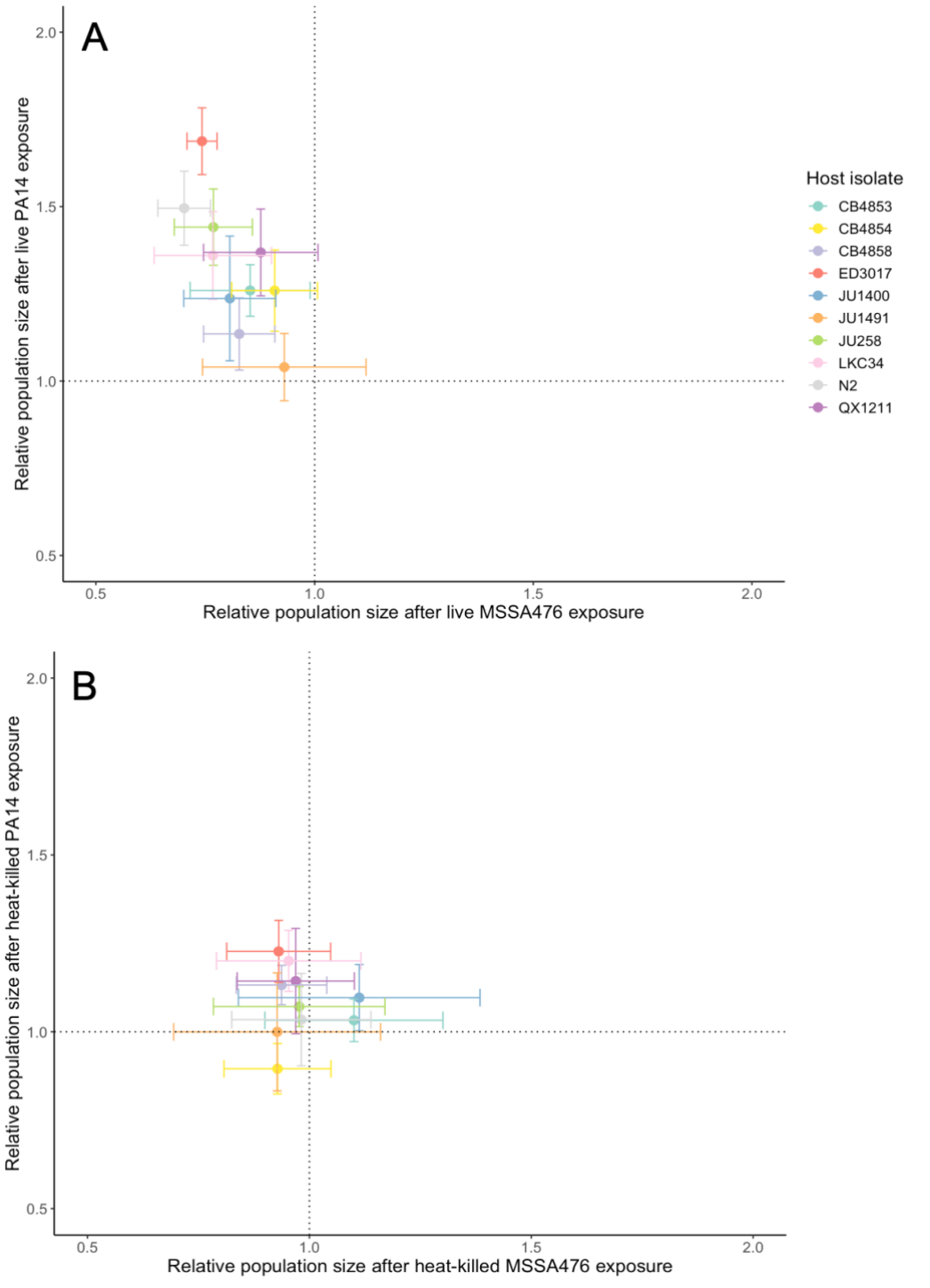


Figure 7: Relationship between immunocompetence across ten nematode isolates after exposure to *S. aureus* and *P. aeruginosa*. Trade-off between population sizes after exposure to the two live pathogens (A) and no trade-off between population sizes after exposure to the two heat-killed pathogens (B). Values are relative to population sizes on food (*B. subtilis* PY79 for *S. aureus* MSSA476 exposures, *E. coli* OP50 for the *P. aeruginosa* PA14 exposures). Mean relative population sizes are shown with 1 standard error. Dotted lines at x = 1 and y = 1 represent no difference between pathogen treatment and food. Colors indicate ten host genotypes.

Discussion

The long-term fitness consequences of transient exposures to multiple pathogen species are relatively unexplored across systems. That is despite the fact that most host organisms live in multi-microbial environments with a diversity of pathogen species. Here, we have revealed substantial natural genetic variation in population size across host isolates in the absence and presence of pathogen species. Overall, we found more evidence of genetic variation in relative population sizes in response to *P. aeruginosa* exposure than to *S. aureus*. This contrast may emerge from the nematodes having a more extensive evolutionary history with members of the *Pseudomonas* genus both as food sources and pathogens. These are prominent microbes in the natural compost environment of the nematodes (Samuel *et al*., 2016), and Proteobacteria (including the Pseudomonadaceae family) are abundant in the native microbiome (Dirksen *et al*., 2016). The concept of bacteria acting as both food sources and pathogens is further discussed in Chapter 5.

We found a trade-off in fitness costs among host isolates between exposures to two distinct, live pathogen species. This result suggests further evidence of an immunological balance in *C. elegans* (e.g. Marsh *et al*., 2011; Nakad *et al*., 2016), and the work herein demonstrates that these different responses to infection are a strong driver of population-level effects. While not explored in the context of fitness across two pathogen species, previous work does show that resistance and tolerance (two defense strategies) are negatively genetically correlated in five mouse genotypes responding to malaria parasites (Råberg *et al*., 2007). Nematodes suffer distinct host pathologies and raise different transcriptional responses to *S. aureus* and *P. aeruginosa* (Irazoqui *et al.*, 2010). These authors found certain C-type lectins, which have an antimicrobial function, were induced specifically after *S. aureus* infection, but not *P. aeruginosa.* When these genes were over-expressed in mutant nematodes, the hosts

were better able to survive on *S. aureus* (compared to control N2 worms) but showed mildly enhanced susceptibility to *P. aeruginosa*. These genes could be under diversifying selection in nature, whereby extreme values (e.g. high or low expression of a gene like *clec-60*) are favored rather than intermediate expression. Future work in our system could measure changes in the expression of immune effector genes, particularly *clec-60*, to determine if this gene is relevant in the wild for immunological balance between Gram-positive and Gram-negative bacteria. Nakad *et al*. (2016) found that a single neuropeptide gene mediates nematode defense responses to Gram-positive *B. thuringiensis* and Gram-negative *P. aeruginosa,* which resulted in decreased susceptibility to *P. aeruginosa*, but increased susceptibility to *B. thuringiensis.* After performing transcriptional profiling, Nakad *et al*. suggested that changes in p38 MAPK signaling, insulin-like signaling, and C-type lectins are responsible for these two opposing phenotypes. The exact mechanisms underpinning balanced immunity and natural variation in *C. elegans* to both pathogen species here remain to be investigated. The p38 MAPK signaling pathway is an interesting candidate for investigating differences between genotypes given its well-elucidated function in pathogen defense, implications for egg-laying (Kim *et al*., 2002), and role in controlling *ins-11* neuropeptide expression in the intestine (Lee & Mylonakis, 2017). This neuropeptide goes from the nematode intestine to sensory neurons and triggers avoidance behavior: worms with higher *ins-11* expression have extended survival but less fat storage and fewer progeny. Further, the insulin-like signaling pathway acts as a bridge between defense and metabolism, governing how resources are allocated (Lee *et al*., 2018) and is of interest for future research given the tradeoffs investigated in this project.

On the evolutionary level, further study is needed regarding genetic trade-offs between two distinct pathogens (which we found) and energetic trade-offs between defense and

reproduction in the absence and presence of pathogens (which were not evident in our study). This has implications for longer-term evolutionary responses versus short-term plasticity and can inform us about host-pathogen interactions in natural settings: do the same host and pathogen genotypes persist in an area for several generations or do pathogen threats come and go? The former scenario would select for the evolution of long-term responses whereas the latter would encourage for plastic strategies.

Across host isolates, we found that transient exposure to live *P. aeruginosa* significantly increased population sizes relative to heat-killed challenge. Essentially, there was a fitness benefit from short-term exposure to this pathogen. This result could indicate that the outcome of host-microbe interactions here are time-dependent, with live *P. aeruginosa* acting as a nutritious food source compared to live *E. coli*, normally used to rear nematodes in the lab. Although existing metabolomics work has shown that *P. aeruginosa* PA14 (the strain used here) infection decreases nematode amino-acid metabolism compared to *E. coli* OP50 (Witting *et al*., 2014), this work was done on sterile mutants feeding on PA14 for 24 hours. In one of the initial studies characterizing *C. elegans-P. aeruginosa* interactions, heat-killed PA14 on NGM agar was found to support normal N2 growth and development after multi-day exposures, and these worms went on to have broods that entirely consumed the heat-killed bacterial lawn (Tan *et al*., 1999). This is in contrast with the live treatment of the same study in which N2 nematodes exposed to live PA14 had very few offspring, which all died. More work is needed to characterize how the time period of host-microbe interactions can cause shifts between nutrition and pathogenicity. The extent to which host immunity against *P. aeruginosa* or potential nutritional benefits result in the increased population growth should be disentangled. One could assess nematode population sizes after exposure to avirulent mutants of PA14 (e.g. *gacA* and *toxA* used by Tan *et al*., 1999 or other mutants

detailed by Feinbaum *et al*., 2012). If a nutritional effect is responsible, then hosts would maintain high reproduction across the avirulent strains as well. If an immune and/or stress effect was at play, then the virulence components responsible would be lacking, and lower population sizes would result after the avirulent exposure.

We also observed significant variation among the isolates in their population sizes across treatments. Andersen *et al*. (2012) found variation across wild *C. elegans* isolates (including those used in this study) in avoidance behavior to PA14, which they mapped to chromosome IV. Prior work comparing the CB4856 Hawaiian wild isolate with N2 revealed lack of wild host avoidance to be responsible for increased PA14 susceptibility (Reddy *et al*., 2009). Similar results have been elucidated with the RC301 German isolate compared to N2 (Martin *et al*., 2017). Avoidance can be an advantageous defense strategy because it is less energetically expensive compared to resistance and tolerance, minimizes infection, and prevents the self-harm derived from a poorly-controlled immune response (reviewed in Rivas *et al*., 2014; also see Schmid-Hempel, 2003 and Singh & Aballay, 2009). In our experiments, we selected founder worms from the bacterial lawn and not those that had moved to the plate edges after the 8 hours. Evaluating our results in light of avoidance behavior is outside of the current scope of this work. Nevertheless, in nature, nematodes must distinguish between food microbes and pathogens, so avoidance of *P. aeruginosa* is a relevant trait. Future investigation could make use of chemosensation mutants that have impaired ability to discriminate between bacteria (e.g. *che-3*) to disentangle the effects of high avoidance in terms of nutrition (i.e. missed feeding opportunities, perhaps decreasing reproductive output) and defense (i.e. preventing a high-burden infection, perhaps increasing reproductive output). Whether *P. aeruginosa* growing in natural conditions would be nutritious and/or harmful for

nematode hosts across time as part of a mixed diet is important for better understanding host-microbe interactions in the wild.

We disentangled the fitness costs of immune upregulation from pathogen damage induced by *S. aureus*. That is, for a transient *S. aureus* exposure, there were significant decreases in the subsequent population sizes from live compared to heat-killed bacterial treatments (both relative to food). This is in contrast with the equivalent result in Chapter 2 for relative brood sizes. This points to how modest decreases in brood size and small differences between heat-killed and live *S. aureus* treatments are magnified at the population level (e.g. Feistel *et al*., 2019), given two more generations for the pathogen's delay in the first day of reproduction (Chapter 2) to become evident. The pathogen damage from the 8-hour exposure in a parent is detrimental enough to cause reduced population sizes, even when the nematode hosts are moved to food plates for reproduction. One aspect to confirm in a future study is the extent (time and amount) to which live *S. aureus* persists in the *C. elegans* gut after the 8-hour period. Interestingly, N2 hosts had the highest fitness cost from the live treatment, and therefore suffered the most from pathogen damage compared to wild isolates. Lab-adapted nematodes might not be used to contending with pathogen damage after so many generations of relatively benign food (Sterken *et al*., 2015), but they may be accustomed to persistent baseline levels of immune upregulation from the lab-based bacterial diet (Alper *et al*., 2007).

However, we did not find the expected tradeoff between immunocompetence to pathogen and reproduction in the absence of pathogen. The exposure time was potentially not long enough to make this negative correlation evident. Consequences of nematode exposure to *S. aureus* have been examined under a range of timeframes, mostly longer than that used in this study. Garsin *et al*. (2001) and Sifri *et al*. (2003) tracked decreasing host survival over 4 to 6 days.

Irazoqui *et al*. (2010) found increasing pathogen burden and host cytopathology over 4 hours, 8 hours, 12 hours, 24 hours, and 36 hours. King *et al*. (2016) measured mortality after 24 hours of infection. Regardless, we used an 8 hour time period to mimic a transient, pre-reproductive exposure consistent with a previous study of host gene expression on live and heat-killed *S. aureus* (Irazoqui *et al*., 2010), whereas others with longer exposures sought to perform lifespan assays (e.g. Garsin *et al*., 2001; Sifri *et al*., 2003). Perhaps a longer exposure, or an earlier-age exposure during spermatogenesis, would have had a significant impact for a host where reproduction of hermaphrodites is sperm-limited (Kimble & Ward, 1988). It remains to be determined how hosts are infected in natural settings: at what stage nematodes are most susceptible to infection and the extent to which pathogens persist or are transient, given the nematodes' avoidance behavior. Another possibility is that our hosts were not constrained by resources, since food depletion can reveal costs of immunity that are masked by unlimited food (McKean *et al*., 2008).

While this project focused on single bacterial infections, a future direction could be to conduct co-infections with multiple pathogens. Perhaps hosts co-infected with diverse bacterial species would be further stressed: costs and trade-offs could be more apparent in these conditions. A further extension could be to see how co-infections with bacteria, fungi, and/or viruses (e.g. Pukkila-Worley *et al*., 2011) would impact the host's capacity to deploy defenses, repair damage, and continue growth and reproduction, and how the pathogens interact with each other within the host (e.g. Peleg *et al*., 2008).

In conclusion, our findings highlight that host-pathogen interactions are often complex and context-dependent. We found that the genetic trade-offs in host defense between two distinct pathogens may be a more important driver of population size than energy-based trade-offs

(i.e. transient presence or absence of pathogen). This result suggests that host immune responses are calibrated by evolution in a multi-microbial environment since fitness in the presence of one pathogen may be traded-off with fitness to another class of pathogen. We determined that the live *S. aureus* infection carries additional fitness costs stemming from the dual burden of immune upregulation and pathogen damage. Compared with Chapter 2, this result highlights the necessity to look beyond the infected host generation in studying fitness consequences. We also found increased reproductive output after transient, live *P. aeruginosa* exposure, pointing to the usefulness of future research into how bacterivores balance pathogen avoidance with nutrition.

Supplementary Table 1: Number of observations for each host isolate and Gram positive exposure combination. Observations indicate technical replicates: non-censored nematodes with a maximum of 24 in treatments where no hosts were censored. All treatment combinations had a sample size of 6 biological replicate plates. HK = heat-killed.

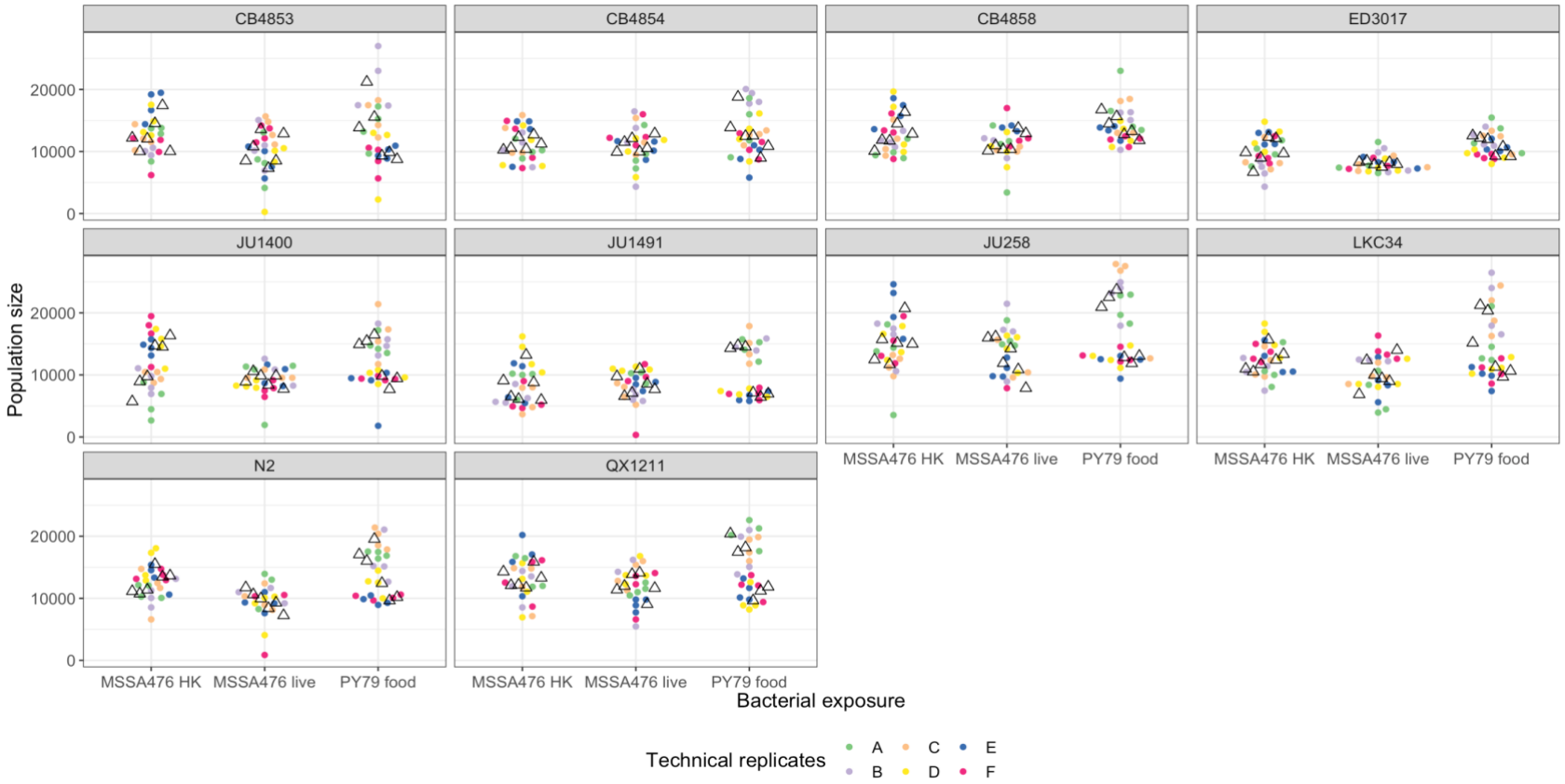
Host isolate	Exposure	Observations
CB4853	MSSA476 HK	23
	MSSA476 live	24
	PY79	23
CB4854	MSSA476 HK	24
	MSSA476 live	23
	PY79	24
CB4858	MSSA476 HK	24
	MSSA476 live	21
	PY79	24
ED3017	MSSA476 HK	24
	MSSA476 live	23
	PY79	24
JU1400	MSSA476 HK	24
	MSSA476 live	23
	PY79	24
JU1491	MSSA476 HK	24
	MSSA476 live	23
	PY79	23
JU258	MSSA476 HK	24
	MSSA476 live	20
	PY79	24
LKC34	MSSA476 HK	24
	MSSA476 live	23
	PY79	24
N2	MSSA476 HK	24
	MSSA476 live	21
	PY79	24
QX1211	MSSA476 HK	24
	MSSA476 live	23
	PY79	24

Supplementary Table 2: Number of observations for each host isolate and Gram negative exposure combination. Observations indicate technical replicates: non-censored nematodes with a maximum of 24 in treatments where no hosts were censored. All treatment combinations had a sample size of 6 biological replicate plates. HK = heat-killed.

Host isolate	Exposure	Observations
CB4853	PA14 HK	23
	PA14 live	24
	OP50	24
CB4854	PA14 HK	24
	PA14 live	24
	OP50	24
CB4858	PA14 HK	22
	PA14 live	24
	OP50	24
ED3017	PA14 HK	22
	PA14 live	24
	OP50	23
JU1400	PA14 HK	22
	PA14 live	24
	OP50	24
JU1491	PA14 HK	21
	PA14 live	24
	OP50	24
JU258	PA14 HK	24
	PA14 live	24
	OP50	23
LKC34	PA14 HK	22
	PA14 live	23
	OP50	24
N2	PA14 HK	24
	PA14 live	24
	OP50	24
QX1211	PA14 HK	23
	PA14 live	24
	OP50	24

Supplementary Table 3: Average variation in droplet counts within a sample as coefficient of variation (CV). The CV was calculated for every technical replicate and then summarized by host isolate genotype and bacterial exposure. Summarizing by biological replicates (exposure plate groupings), as in other analyses, was excluded to portray the variation at the level of technical replicates.

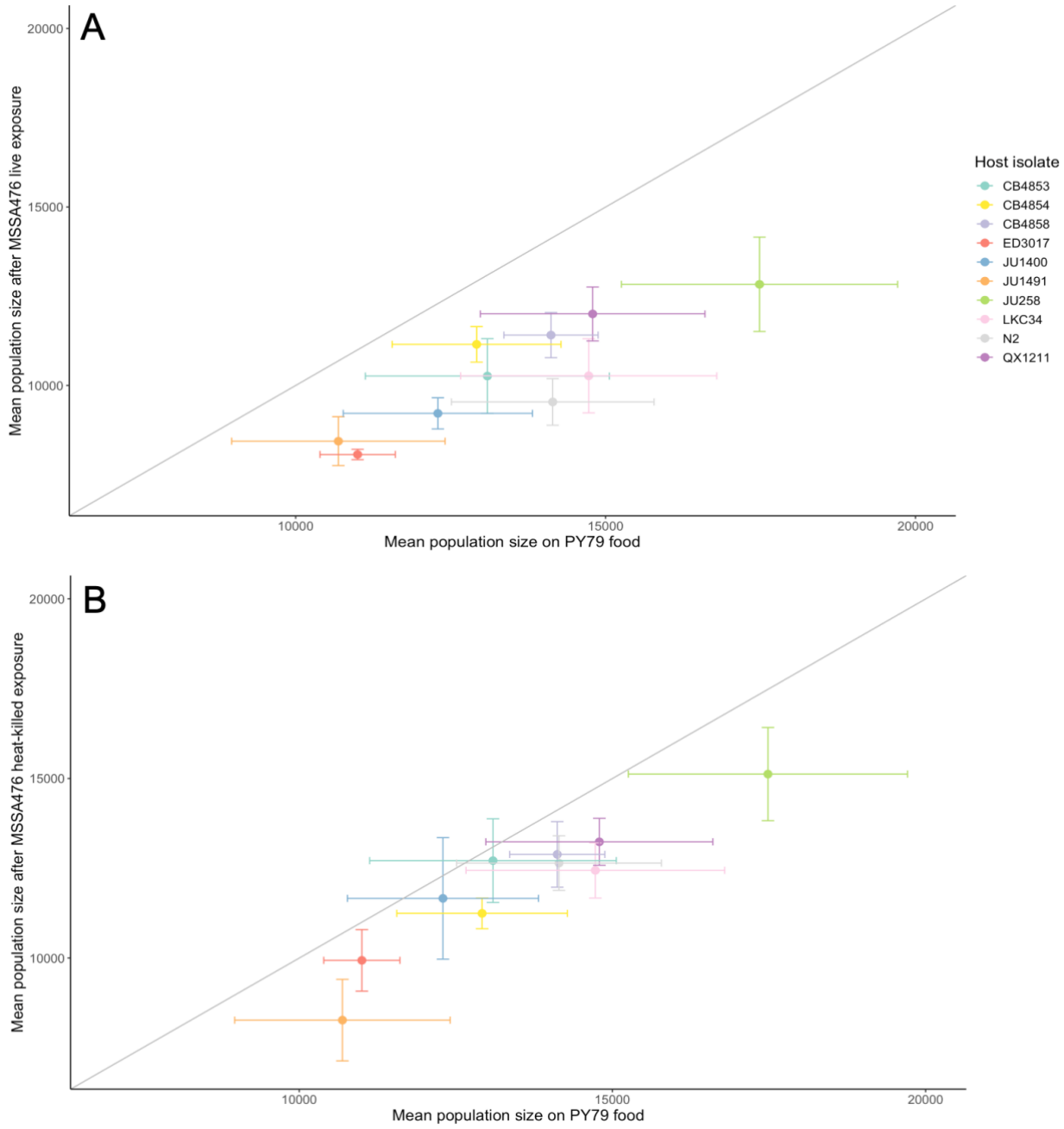
Host isolate	Exposure	CV
CB4853	MSSA476 HK	14.8
	MSSA476 live	20.3
	PY79	15.5
CB4854	MSSA476 HK	14.5
	MSSA476 live	16.2
	PY79	15.9
CB4858	MSSA476 HK	15.0
	MSSA476 live	13.3
	PY79	16.0
ED3017	MSSA476 HK	15.2
	MSSA476 live	17.3
	PY79	16.4
JU1400	MSSA476 HK	16.8
	MSSA476 live	15.3
	PY79	15.8
JU1491	MSSA476 HK	21.3
	MSSA476 live	23.2
	PY79	16.7
JU258	MSSA476 HK	14.8
	MSSA476 live	14.6
	PY79	12.8
LKC34	MSSA476 HK	13.5
	MSSA476 live	14.6
	PY79	14.5
N2	MSSA476 HK	13.5
	MSSA476 live	16.7
	PY79	16.8
QX1211	MSSA476 HK	15.2
	MSSA476 live	17.1
	PY79	14.4



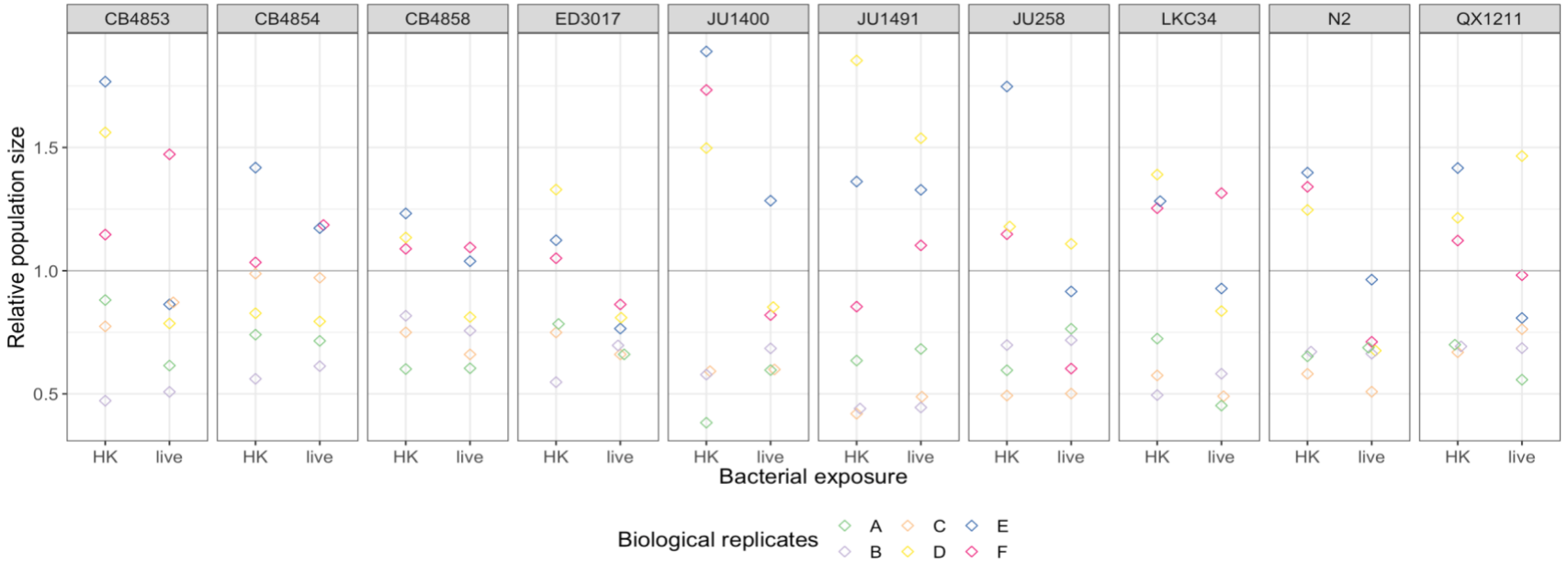
Supplementary Figure 1: Raw data showing variation in population size of technical and biological replicates for Gram positive exposures. Up to four technical replicates (founder nematodes tracked for population size) are depicted as points colored by their corresponding biological replicate code, A-F (A = green, B = purple, C = orange, D = yellow, E = blue, F = pink). The means of six biological replicates (exposure plates) are depicted as black triangles.

Supplementary Table 4 Mean population sizes for ten nematode host isolates after exposure to three Gram-positive bacterial treatments. Data are displayed in groups of uninfected worms on food (*B. subtilis* PY79) and after pathogen exposure (*S. aureus* MSSA476), and sorted within each bacterial treatment by low to high mean population size. ±1 standard error is shown. HK = heat-killed.

Exposure	Host isolate	Population mean	Standard error
PY79	JU1491	10690	1721
	ED3017	11000	607
	JU1400	12294	1526
	CB4854	12919	1362
	CB4853	13094	1969
	CB4858	14119	760
	N2	14147	1635
	LKC34	14728	2066
	QX1211	14792	1812
	JU258	17483	2227
MSSA476 live	ED3017	8068	146
	JU1491	8440	683
	JU1400	9219	437
	N2	9539	653
	CB4853	10264	1046
	LKC34	10270	1037
	CB4854	11152	496
	CB4858	11411	633
	QX1211	12005	757
	JU258	12834	1322
MSSA476 HK	JU1491	8272	1135
	ED3017	9936	857
	CB4854	11244	428
	JU1400	11661	1691
	LKC34	12442	769
	N2	12644	760
	CB4853	12712	1165
	CB4858	12886	912
	QX1211	13236	654
	JU258	15122	1299



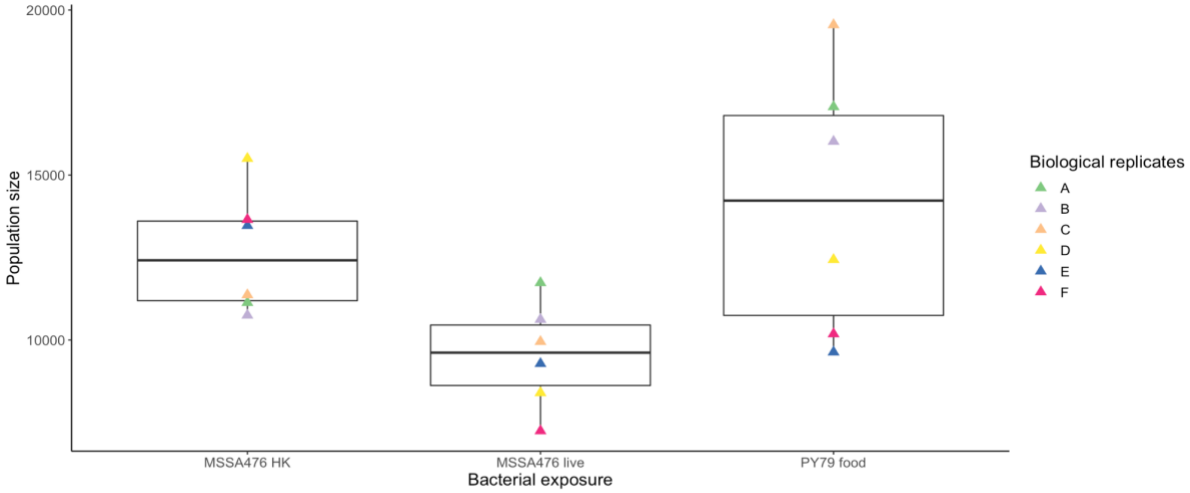
Supplementary Figure 2: Population sizes of ten nematode isolates after exposure to *S. aureus* MSSA476 live (A) and heat-killed (B) versus population sizes on PY79 food. Nematodes were reared on food, then transiently exposed to pathogen or food for 8 hours post-L4 stage. After exposure, single founder worms (P generation) were moved to food and allowed to reproduce undisturbed for five days (F1 and F2 generations). Mean population sizes are shown with 1 standard error. Grey line represents y = x. Colors indicate ten host genotypes.



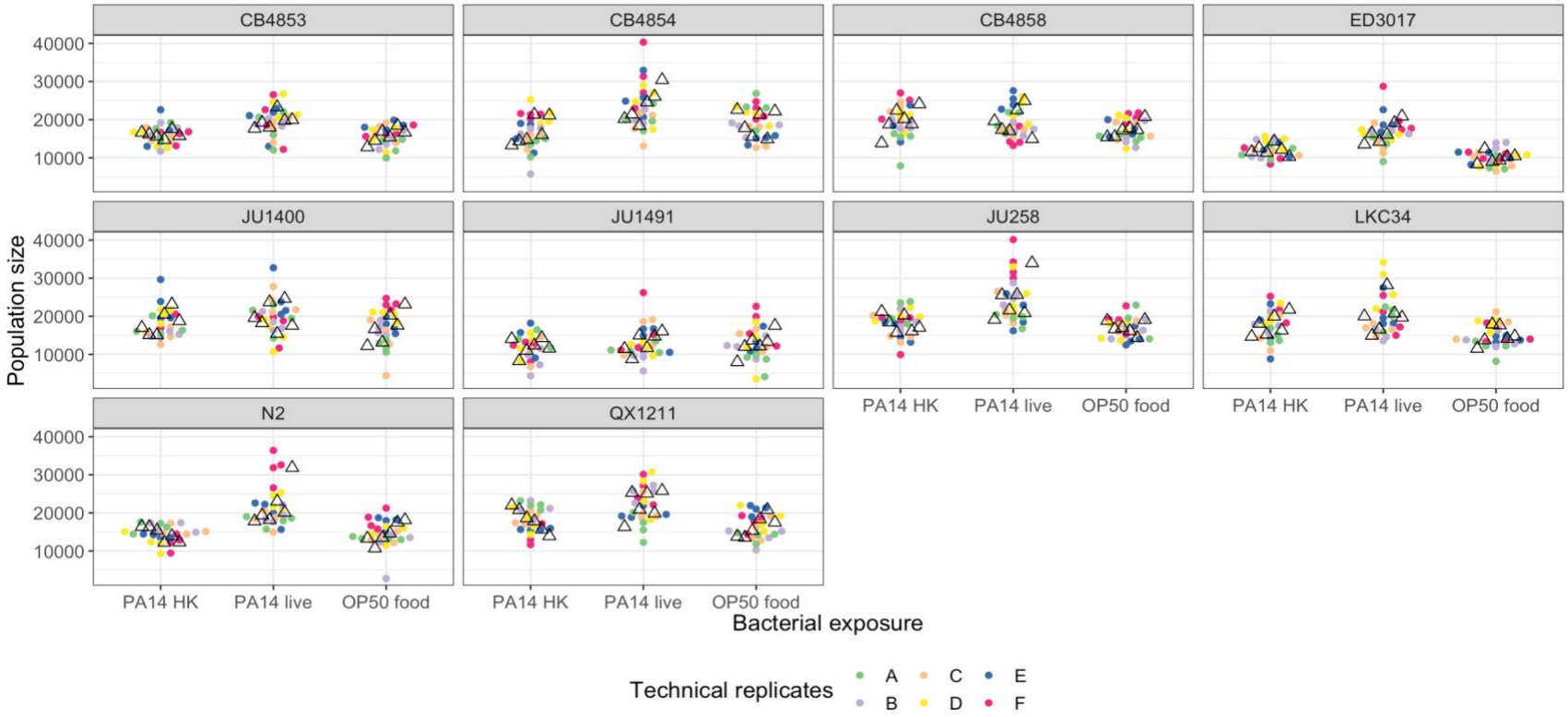
Supplementary Figure 3: Raw data showing the six ratios to represent fitness costs for a given Gram positive bacterial exposure and host isolate. Ratios were constructed at the level of the biological replicate, by dividing population size from hosts after MSSA476 heat-killed (HK on x axis) or MSSA467 live (live on x axis) treatments by the corresponding population size on food with the same batch identity, A-F. Host isolates are indicated along the top. Grey line at y = 1 indicates when host populations after pathogen exposure equals host populations on food.

Supplementary Table 5: Percentage difference between mean relative population sizes after MSSA476 heat-killed and live exposures. Population sizes are relative to PY79 control food, with values closer to 1 representing reproduction near control levels for that host genotype. Percentage difference was calculated as in Cole & Altman (2017): the difference between the two groups divided by the mean of the two groups, multiplied by 100. Percentages represent the average relative population loss from hosts between heat-killed and live treatments, indicating the average fitness cost of experiencing pathogen damage from the live treatment, independent of the immune upregulation of both heat-killed and live challenges. Percentage differences are shown sorted from high to low. The average percentage difference (across all host genotypes) indicates an 18% fitness cost from live MSSA476 exposure compared to heat-killed MSSA476. HK = heat-killed.

Host isolate	Mean relative population size after MSSA476 HK exposure	Mean relative population size after MSSA476 live exposure	Percentage difference (%)
N2	0.982	0.702	33.26
JU1400	1.112	0.806	31.92
CB4853	1.100	0.853	25.36
JU258	0.977	0.769	23.89
ED3017	0.931	0.742	22.50
LKC34	0.953	0.767	21.63
CB4858	0.937	0.828	12.42
QX1211	0.969	0.877	10.00
CB4854	0.928	0.909	2.12
JU1491	0.927	0.931	-0.36



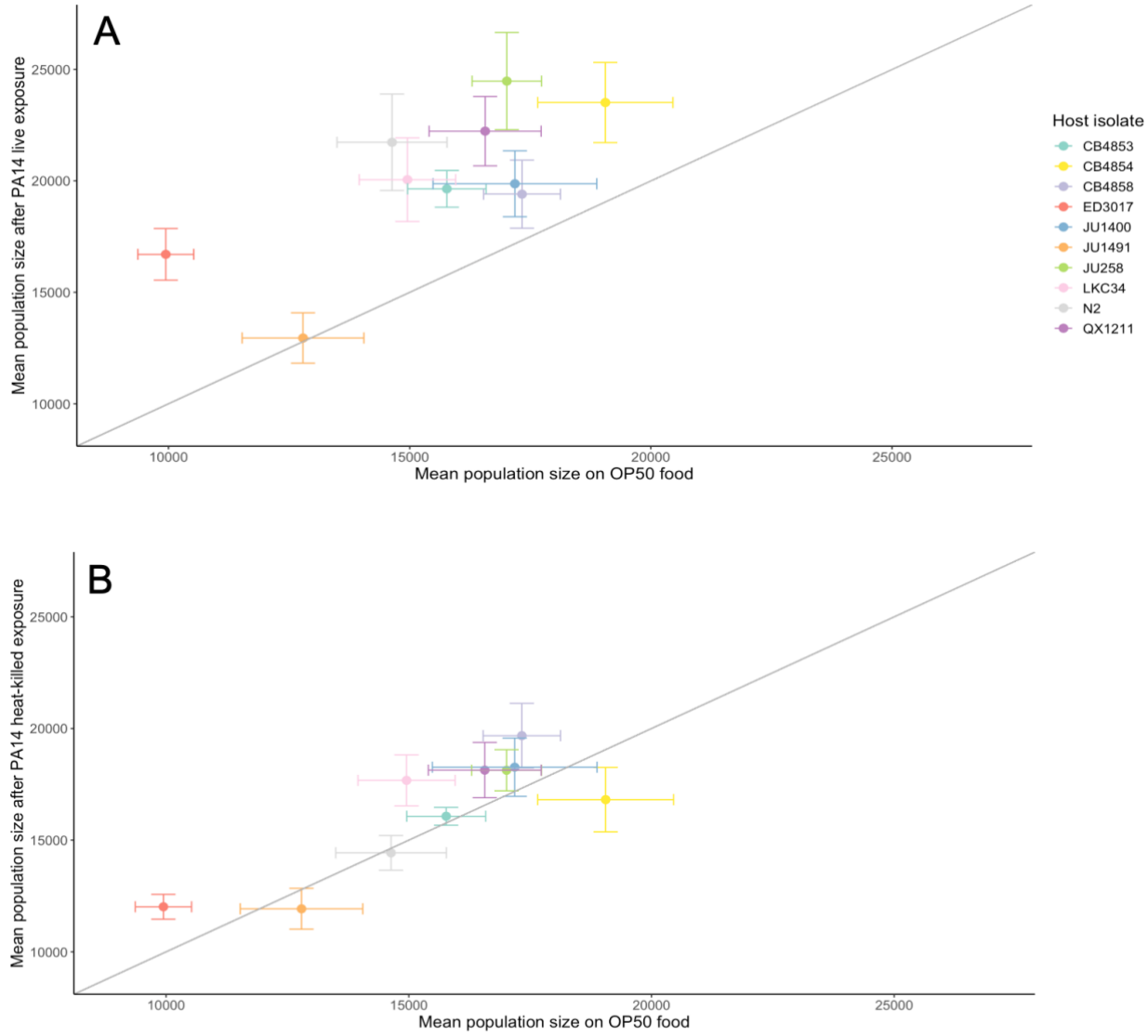
Supplementary Figure 4: Population sizes of N2 hosts across three exposures at the level of biological replicates. Showing data summarized at this level demonstrates that the means of biological replicates A, B, and C are above the median and D, E, F are below the median for nematodes exposed to PY79 food. Nematodes exposed to MSSA476 live follow this pattern as well, while nematodes challenged with MSSA476 heat-killed (HK) have mean biological replicate A, B, and C below the median and D, E, and F above the median for this treatment. The pairing by A-F identity to generate ratios (fitness cost metric) led to higher variation in the HK treatment than live treatment, represented here with N2 nematodes and evident in other host isolates (see Figure 3) .



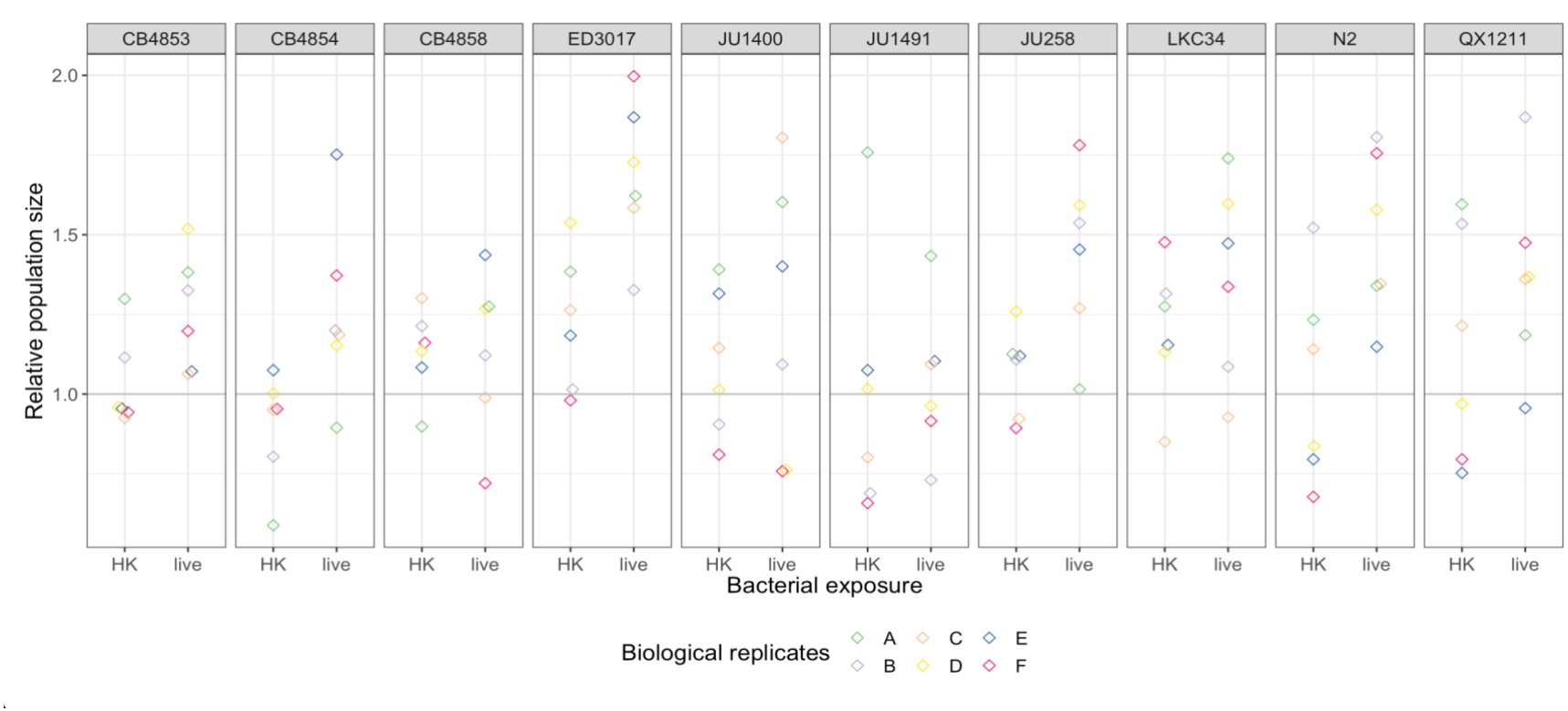
Supplementary Figure 5: Raw data showing variation in population size of technical and biological replicates for Gram negative exposures. Up to four technical replicates (founder nematodes tracked for population size) are depicted as points colored by their corresponding biological replicate code, A-F (A = green, B = purple, C = orange, D = yellow, E = blue, F = pink). The means of six biological replicates (exposure plates) are depicted as black triangles.

Supplementary Table 6: Mean population sizes for ten nematode host isolates after exposure to three Gram-negative bacterial treatments. Data are displayed in groups of uninfected worms on food (*E. coli* OP50) and after pathogen exposure (*P. aeruginosa* PA14), and sorted within each bacterial treatment by low to high mean population size. ±1 standard error is shown. HK = heat-killed.

Exposure	Host isolate	Population mean	Standard error
OP50	ED3017	9944	579
	JU1491	12789	1262
	N2	14633	1140
	LKC34	14953	1000
	CB4853	15769	813
	QX1211	16564	1162
	JU258	17013	721
	JU1400	17181	1697
	CB4858	17328	797
	CB4854	19056	1401
PA14 live	JU1491	12950	1129
	ED3017	16703	1156
	CB4858	19403	1527
	CB4853	19642	826
	JU1400	19869	1478
	LKC34	20053	1875
	N2	21731	2165
	QX1211	22231	1557
	CB4854	23514	1798
	JU258	24475	2181
PA14 HK	JU1491	11924	913
	ED3017	12016	557
	N2	14431	777
	CB4853	16069	400
	CB4854	16814	1447
	LKC34	17678	1142
	JU258	18131	922
	QX1211	18136	1241
	JU1400	18267	1305
	CB4858	19679	1447



Supplementary Figure 6: Population sizes of ten nematode isolates after exposure to *P. aeruginosa* PA14 live (A) and heat-killed (B) versus population sizes on OP50 food. Nematodes were reared on food, then transiently exposed to pathogen or food for 8 hours post-L4 stage. After exposure, single founder worms (P generation) were moved to food and allowed to reproduce undisturbed for five days (F1 and F2 generations). Mean population sizes are shown with 1 standard error. Grey line represents y = x. Colors indicate ten host genotypes.



Supplementary Figure 7: Raw data showing the six ratios to represent fitness costs for a given Gram negative bacterial exposure and host isolate. Ratios were constructed at the level of the biological replicate, by dividing population size from hosts after PA14 heat-killed (HK on x axis) or PA14 live (live on x axis) treatments by the corresponding population size on food with the same batch identity, A-F. Host isolates are indicated along the top. Grey line at y = 1 indicates when host populations after pathogen exposure equals host populations on food.

Supplementary Table 7: Percentage difference between mean relative population sizes after PA14 heat-killed and live exposures. Population sizes are relative to OP50 control food, with values greater than 1 representing reproduction above control levels for that host genotype. Percentage difference was calculated as in Cole & Altman (2017): the difference between the two groups divided by the mean of the two groups, multiplied by 100. Percentages represent the average relative population boost from hosts exposed to live PA14 versus heat-killed PA14. Percentage differences are shown sorted from high to low. The average percentage difference (across all host genotypes) indicates a 20% fitness benefit from live PA14 exposure compared to heat-killed PA14. HK = heat-killed.

Host isolate	Mean relative population size after PA14 HK exposure	Mean relative population size after PA14 live exposure	Percentage difference (%)
N2	1.034	1.495	36.47
CB4854	0.895	1.259	33.77
ED3017	1.227	1.688	31.58
JU258	1.071	1.441	29.45
CB4853	1.033	1.260	19.82
QX1211	1.143	1.369	17.94
LKC34	1.200	1.360	12.46
JU1400	1.097	1.237	12.02
JU1491	1.000	1.040	3.95
CB4858	1.132	1.135	0.26

Chapter 4

Examining costs and benefits of maternal effects after transient pathogen exposure

Abstract

A parent's environment can have intergenerational consequences that impact offspring fitness. Pathogens impose selective pressure on hosts to evolve mechanisms to reduce the costs of infection both within and across generations. Using five *Caenorhabditis elegans* host genotypes and two *Staphylococcus aureus* pathogen treatments, we investigated whether there is genetic variation for benefits (evident when the next generation encounters the pathogen) and costs (evident in the absence of infection) of maternal exposure to pathogen. We evaluated offspring performance in terms of F1 population sizes and assessed maternal effects by comparing offspring with the same F1 exposure whose mothers had been challenged/infected with heat-killed/live *S. aureus* versus offspring whose mothers who were maintained in the absence of pathogen. We found no evidence of intergenerational benefits resulting from maternal pathogen exposure though we do note a trend towards a cost of maternal heat-killed pathogen challenge in the lab-adapted N2 nematode strain. Moreover, there was no correlation across the host genotypes for magnitudes of benefits versus magnitudes of costs of maternal pathogen exposure, but we do report a link to Chapter 2 with a positive correlation between the magnitude of the pathogen-induced delay in the mother's first day of reproduction and the magnitude of the cost to offspring from maternal heat-killed pathogen exposure, with the highest magnitudes exhibited by the lab-adapted N2. Given the lack of statistically significant maternal effect costs in our study, these findings highlight the capacity for nematode lineages to recover from transient pathogen exposure, particularly for wild host isolates.

Introduction

Parental exposure to abiotic and biotic stressors can have lasting impacts on offspring. That is, parents can affect offspring phenotype independent of offspring genotype (Mousseau & Fox, 1998; Räsänen & Kruuk, 2007; Uller, 2008). Parents, and mothers more substantially, can provision their offspring with differing quantity or quality of nutrients, and/or they can have epigenetic effects through modifications of chromatin and RNA (reviewed in Perez & Lehner, 2019). This transmitted information can be altered based on factors parents have encountered before offspring conception or during embryo development (reviewed in Heard and Martienssen, 2014). Intergenerational (lasting one or two generations) and transgenerational (lasting multiple generations) effects can precondition the offspring so that they are prepared for similar environments or challenges in the future (reviewed in Perez & Lehner, 2019). Meta-analyses of transgenerational effects (also termed anticipatory parental effects) in plants and animals have found evidence that they are generally beneficial in both stressful and benign environments (Yin *et al*., 2019) though they are not always adaptive (Uller *et al*., 2013). That is, parental effects can be beneficial, neutral, or disadvantageous (reviewed in Yin *et al*., 2019) and the extent to which parental effects impact the growth and success of offspring can depend on the environmental context.

Various environmental cues have been reported to induce parental effects across taxa. Parental exposure to mild osmotic stress preconditions offspring for harsher osmotic stress in both *Arabidopsis thaliana* (Wilbowo *et al*., 2016) and *Caenorhabditis elegans* (Frazier & Roth, 2009). Increased temperature during egg maturation results in larger *Salma salar* egg mass (Jonsson & Jonsson, 2016) and transient, mild heat stress in *C. elegans* influences mRNA levels in three subsequent generations (Schott *et al*., 2014). Nutritional stress in the form of dietary restriction leads to larger offspring in *Daphnia magna* (Garbutt & Little,

2017), *Drosophila melanogaster* (Valtonen *et al*., 2012), and *C. elegans* (Harvey & Orbidans, 2011). In the case of *D. magna*, the larger offspring also have improved resistance to a bacterial pathogen. Garbutt and Little (2017) propose that larger offspring can obtain more resources and support higher defense responses and that this maternal effect could lead to offspring performing better in various sub-optimal conditions. While this is not a specific adaptation for combating a pathogen, there are many examples of intergenerational and transgenerational immune priming, as discussed below.

Immune priming, where previous infection experience confers a more effective response upon a second exposure, can occur within an organism and between generations (e.g. Tidbury *et al*. 2010; Dhinaut *et al*., 2017). The latter is not as well studied as the former (reviewed in Roth *et al.*, 2018). Some of the earliest work suggesting intergenerational and/or transgenerational immune priming (henceforth, intergenerational immune priming) was done in the last century with moths (Ishimori & Metalnikov*,* 1924) and since then, this phenomenon has been revealed in multiple invertebrate species (reviewed in Pigeault *et al*., 2016). Apart from live pathogens, immune priming has been shown in studies with immune-eliciting components (Moret, 2006) and heat-killed pathogen challenge (Sadd & Schmid-Hempel, 2007). Across several animals (reviewed in Roth *et al.,* 2018), the parental and/or grandparental experience of a live or dead pathogen challenge or immune elicitor can aid in the fitness and survival of progeny. Although it can be beneficial, intergenerational immune priming can also carry costs for the recipient offspring. The costs can become evident when the pathogenic environment of parents and offspring are mismatched (reviewed in Roth *et al*., 2018), such as when parents are exposed and then no pathogen is present in the next generation (Contreras-Garduño *et al*., 2014), or a different type of pathogen challenges the offspring (Sadd & Schmid-Hempel, 2009). While most studies of immune priming

investigate benefits on host organisms and/or their offspring, more attention is needed to costs of priming that might impede the persistence of these traits (discussed in Contreras-Garduño *et al*., 2014). The existence and persistence of intergenerational immune priming depends on a variety of ecological factors.

For intergenerational immune priming to be maintained in populations, the overall benefits for the parents and offspring must outweigh the costs (Sadd & Schmid-Hempel, 2009). Another dimension to consider is whether these costs and benefits vary by host genotype, because for intergenerational immune priming to evolve, it must be inherited from parent to offspring and vary between host populations (Vu *et al*., 2015). Previous work in plants has shown that genotypes differ in the type of mechanism utilized to pass information from parent to offspring (e.g. Galloway & Etterson, 2007; Castro *et al*., 2013; Galloway, 2001). For example, there was variation among four genotypes of *Medicago truncatula* in transgenerational plasticity for germination timing after parents were grown under salinity stress (Vu *et al*., 2015). The impact of maternal infection effects on the ecology and evolution of wild invertebrate populations remains to be fully determined in the context of multiple host genotypes.

We established in Chapter 2 that several host genotypes (one lab-adapted and nine wild isolates) of parental generation *C. elegans* nematodes who are exposed to pathogen (both live or heat-killed *S. aureus* MSSA476) experience a delay in reproduction during the first day, but recover number of progeny to food-control levels by the end of their reproductive lifetime. While Chapter 2 investigated the effect of the pathogenic exposure on the challenged host's lifetime fecundity, this chapter focuses on the benefits and costs of maternal effects (induced by pathogen exposure) on offspring for a subset of five host genotypes (one lab-adapted and four wild isolates). *C. elegans* is a well-established model to

research adaptivity of multigenerational effects (Perez & Lehner, 2019; Baugh & Day, 2020) and the trade-offs of benefits and costs from defense-related multigenerational effects are not yet established (Willis *et al*., 2020). Benefits of maternal effects are evaluated in the context of challenging offspring with live pathogen and costs of maternal effects are revealed when offspring are uninfected (i.e. continuous exposure to food). The distinction between the preceding chapters and this one is that pathogens are 'by definition' costly (i.e. harmful; Pirofski & Casadevall, 2012) and maternal effects can be beneficial and costly at the same time, or have no effect (e.g. Contreras-Garduño *et al*., 2014, Robinson *et al*., 2020). As in the previous chapters, we employed two *S. aureus* exposures to have the live treatment which induces both immune upregulation and pathogen damage in hosts, and the heat-killed treatment which induces immune upregulation but does not generate damage.

More specifically, we had two targeted questions about benefits and costs for each host genotype. We asked whether the offspring from the first day of reproduction of pathogen-challenged mothers i) respond well to live pathogen compared to offspring whose mothers have continuously been exposed to food and ii) carry a fitness cost compared to offspring whose mothers have continuously been exposed to food. Looking at benefits and costs concurrently meant having four tests per host genotype to look into both live and heat-killed exposures. We assessed offspring performance in the form of population size (F1 - F3 generations) where a higher population size was interpreted as beneficial and lower population size was interpreted as costly. Three scenarios exist linking the reduced number of first-day progeny from Chapter 2 to maternal effects in this chapter. If pathogen-exposed mothers prepare offspring for future pathogen encounters (benefit), then offspring from mothers who encountered the pathogen will have higher populations than offspring whose mothers only saw control food when all offspring are challenged. A potential cost can be

revealed when offspring are exposed to food: offspring from mothers who experienced pathogen might have lower populations (versus offspring whose mothers only had food) due to expressing a 'primed' defense phenotype in a noninfectious environment. An alternate scenario is that pathogen-exposed mothers suffer from pathogen damage and/or immune upregulation and produce low-quality offspring (see Perez *et al*., 2017 for a discussion of low-quality and sub-optimal offspring). Exposing the low-quality offspring to pathogen would mean lower population sizes from the lineages which are pathogen-challenged twice (compared to offspring whose mothers saw food). When low-quality offspring are exposed to food only then they would still be expected to have lower population sizes than offspring whose mothers saw food. Finally, if pathogen-exposed mothers (despite having delayed fecundity and reduced number of progeny on the first day of reproduction) produce offspring that are of comparable quality to offspring from uninfected lineages (i.e. only food) then we would expect no differences in population sizes between offspring whether their mothers were exposed to pathogen or food (upon either food or pathogen challenge in the F1 generation).

Our final two questions investigated relationships across genotypes. We looked into iii) tradeoffs between benefits and costs of maternal effects and iv) correlations between the magnitude of the delay in the first day of reproduction reported in Chapter 2 with the magnitude of the cost of maternal pathogen exposure. When correlating the magnitude of benefits and magnitude of costs, we would expect to see a positive correlation between the two metrics: genotypes with a high benefit might experience a high cost and genotypes with a low benefit might experience a low cost. That is, one host genotype's offspring might perform well upon pathogen challenge if their mothers have provisioned them for defense but then their siblings might perform poorly on food (compared to offspring of mothers who

were uninfected): the maternal provisioning is beneficial in the pathogenic context but costly in the absence of pathogen. Another genotype's offspring might not perform well after pathogen challenge, but their siblings could thrive in the uninfected context. In the second correlation, we expect to see higher delays in the mother's reproduction corresponding with higher costs of maternal exposure experienced in the subsequent uninfected generations.

Overall, we found a statistically significant correlation between the magnitudes of the brood delay and the cost of maternal heat-killed pathogen exposure in the un-challenged offspring. However, we found no significant differences in offspring population sizes to indicate intergenerational effects and no evidence of a tradeoff between benefits and costs of maternal exposure, though we do note natural variation amongst the host genotypes and highlight the consequences of working with lab-adapted versus natural isolates.

Materials and Methods

Nematode and bacterial strains

We used *Caenorhabditis elegans* N2 nematodes and the wild isolates CB4853, JU258, LKC34, and QX1211. These four isolates are a subset of the nine isolates from the previous two chapters, which were randomly chosen from across a phylogenetic tree (Andersen *et al*., 2012) to represent a wide panel of nematodes from various geographical locations and genetic backgrounds. A subset of genotypes was chosen to obtain a manageable number of plates and reasonable processing time for each batch of samples. We chose the isolates for Chapter 4 based on the distribution of 'delay' in fecundity from Chapter 2: nematodes ranged in exhibiting low delay (CB4853, QX1211), intermediate delay (JU258, LKC34) and high delay (N2). This was in line with our hypothesis of determining whether genotypes that are

suffering delays in terms of quantity (number of progeny) might have low quality offspring (if resources are lacking in infected mothers) or high quality offspring (if mothers were investing in priming, for example) from the initial delay point. Nematode populations were maintained at 20°C on Nematode Growth Medium (NGM) with *Bacillus subtilis* PY79 before being exposed to the pathogen *Staphylococcus aureus* MSSA476.

MSSA476 was grown in 3-5 mL of Todd-Hewitt Broth (THB), and food source (PY79) was grown in 13-15 mL aliquots of Luria Broth (LB). NGM plates were seeded with 800 μL of bacterial food. For the assays, 55 mm Tryptic Soy Agar (TSA) plates were seeded with 60 μL of the exposure strain diluted to OD_{630}=0.15. All maintenance and exposure plates were incubated at 30°C overnight.

For exposures involving heat-killed bacteria, overnight bacterial cultures were first diluted to OD_{630}=0.15, then 1 mL transferred to microcentrifuge tubes and incubated in a water bath set to 88°C for 1 hour. Incubation conditions were determined upon surveying the literature and trialing a range of temperatures (data not shown) to ensure no future bacterial growth upon plating. Heat-killed samples were allowed to cool and then plated as described above for 55 mm TSA exposure plates.

Egg preparation and synchronization

Before each experiment, starved nematodes from maintenance plates were transferred to new 90 mm NGM plates with bacterial food. Three days later, each plate was washed with M9 and Triton-X (0.01%). All gravid nematodes and eggs were transferred to a 15 mL conical tube, the total volume adjusted to 4 mL, and then 1 mL of freshly-prepared bleach solution (1:1 of 10% sodium hypochlorite and 5M NaOH) was added and tubes were left to shake at

88 rpm in an orbital shaker for 10-15 minutes. Once nematode bodies bent to release eggs, tubes were centrifuged at 3200 rpm for 2 minutes, and pellets were re-suspended up to 4 mL with M9 + Triton-X, mixed by inversion, and then centrifuged again. After the final of three washes, pellets were resuspended in 2 mL M9 + Triton-X and left to shake at 88 rpm overnight at 20°C. The following day, the resulting synchronized larval stage 1 (L1) nematodes of the parental generation (P) were plated on 90 mm NGM maintenance plates to a density of about 2,000 nematodes per plate for each isotype. All nematodes were reared at 20°C for two days, until they reached L3/L4 stage.

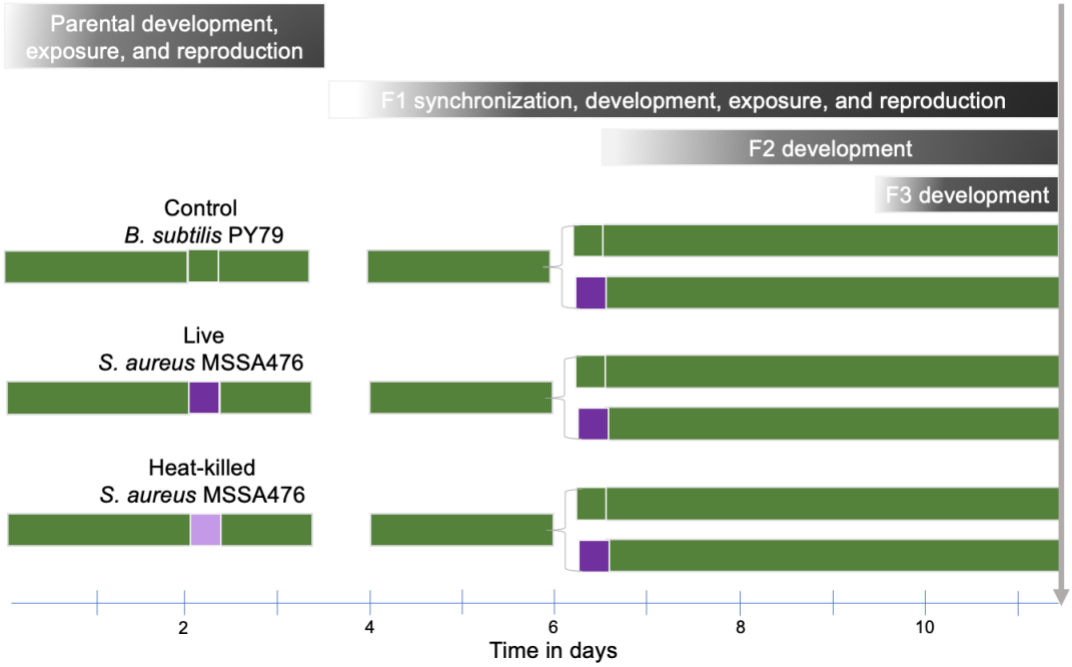


Figure 1: Experimental method for intergenerational exposures in Chapter 4. The figure is representative for one nematode isolate; experiments were performed across five isolates. Parental nematodes developed on *B. subtilis* PY79 control food (green) for two days, were exposed to control food, live *S. aureus* MSSA476 (dark purple), or heat-killed *S. aureus* MSSA476 (light purple) for 8 hours, and then allowed to reproduce for one subsequent day (the offspring synchronization through bleaching kills parents without harming eggs). The F1 generation was harvested by bleaching and synchronized in M9 salt solution overnight, then allowed to develop on food for two days. The 8-hour exposures to food and live MSSA476 pathogen occurred for matched F1 nematodes derived from the same parental exposure plate. After exposure, single founder F1 worms expanded in population (F2 and F3) for five days. Color changes (or in the control case, green with light outlines) and left braces indicate transfer of nematodes between plates. Grey gradients indicate progression in development. Downward grey arrow indicates final timepoint at which nematodes were washed off plates and counted to determine population size.

Intergenerational exposures

The impact of repeated pathogen exposures in the parental (P) and offspring (F1) generations was evaluated by performing lineage expansion assays with CB4853, JU258, LKC34, and QX1211 wild worm isolates, as well as with N2 lab isolate (Figure 1). An initial round of bleaching was performed to synchronize the P generation and approximately 6,000 L1 worms of each isolate were distributed across three NGM maintenance plates. After 48 h, L3/L4 stage nematodes were washed and approximately 1,000 parental nematodes were exposed to food or pathogen (live or heat-killed MSSA476) for 8 h at 25°C and then, via a brief M9 washing step, moved to food for about 24 h to allow for egg production. The exposure timeframe (8 h) was chosen to maximize the immune-upregulation effects leading up to the host reproductive period, as this is the point at which nematodes have been reported to express *S. aureus*-specific immune genes (Irazoqui *et al.*, 2010), but have not yet started to lay eggs (Aprison & Ruvinsky, 2014).

One day after pathogen exposure, the mothers were bleached to yield the F1 generation, which were reared at a density of about 1800 worms per maintenance plate. The bleaching time point corresponds with the first time point in Chapter 2 by capturing the offspring from the 'delayed reproduction' timeframe. This time point means that offspring had no direct contact with the bacterial exposure plate. After 44 h of development, matched L3/L4 stage F1 worms (derived from a common parental exposure plate) were exposed to either PY79 food (100 worms) or MSSA476 live pathogen (100 worms) for 8 h. After exposure, three founder nematodes were picked off each exposure plate onto three separate 90 mm NGM plates seeded with bacterial food (Supplementary Table 1).

Population growth assays

To measure the impact of transient pathogen exposure in P and/or F1 generations on subsequent population growth, the number of individuals in a single host lineage across three generations (F1, F2, and F3) was counted. The lineage expansion assay method was followed similar to that described in Feistel *et al.* (2019). After 8 h exposure to either live MSSA476 or PY79 food, three founder nematodes were picked off each exposure plate onto three separate 90 mm NGM plates seeded with bacterial food. Plates were left at 25°C for 5 days while F1 nematodes produced F2 and F3 generations. On the final day, each plate was washed with 4 mL M9 + Triton-X, until most nematodes were displaced, and transferred to a conical tube. Four 5 μL droplets were counted to determine the population size for each plate. Compared with lineage expansion assays in Chapter 3, a smaller volume and number of droplets was chosen to reduce final-day sample processing time to less than 12 hours per batch. Treatments consisted of three technical replicates per assay and each experiment was repeated four times to have four replicate plates for a given bacterial exposure. Population sizes were collected for a total of 356 founder worms (originally 360, with four worms censored for either leaving the Petri dish or injury/death during transfer) (Supplementary Table 1; Supplementary Figure 1). Given that the lineage expansion method was developed relatively recently, we present the variation in the number of worms per droplet in Supplementary Table 2.

Statistical analyses

Data were analyzed in R version 3.6.1 and RStudio version 1.2.1335 (R Core Team, 2019). To calculate the population size from an F1 founder worm, the average was taken from four 5 μL droplets and back-calculated to give the number of worms per plate. Population sizes from three replicate worms from an exposure plate were combined to give a mean population

size (Supplementary Figure 2). To test for differences in F1 population sizes, we performed four Welch's two-sample t-tests per host isolate (Supplementary Table 3) after verifying normal distribution of the population size data. Given the absence of population differences within each genotype, we analyzed data across genotypes with a linear mixed-effects model with host genotype, parental exposure, and F1 exposure (and their interactions) as fixed effects and batch as a random effect using the lme4 (Bates *et al*., 2015) and car (Fox & Weisberg, 2019) packages in R. We conducted Tukey Contrast multiple comparisons of means tests with the multcomp package (Hothorn *et al*., 2015) to determine which host genotypes were significantly different from each other in terms of population size.

In order to look into the magnitude of benefits and costs of maternal effects, we created ratios from the population sizes (Supplementary Figure 3). The benefit ratios represent the extent to which offspring whose mothers have been exposed to heat-killed or live pathogen reproduce more after live pathogen exposure (>1) than offspring whose mothers have continuously been on control food. The cost ratios represent the extent to which offspring whose mothers have been exposed to heat-killed or live pathogen reproduce less after food exposure and experience a greater cost (>1) than offspring whose mothers have continuously been on control food. Higher benefit and higher cost are both indicated as values >1. The mean of each condition (orange squares in Supplementary Figure 3) corresponds to the values in the correlation plots. To determine whether there was a genetic correlation of benefits versus costs of maternal effects, we plotted the mean benefit and mean cost of each condition for both live and heat-killed exposures and used Spearman's rank correlation tests.

To connect the current chapter with Chapter 2, we correlated the magnitude of the brood delay from the first day of reproduction after transient pathogen exposure with the cost of

maternal transient pathogen exposure. To calculate the magnitude of the brood delay, we took the inverse of the relative cumulative progeny from the 30 h timepoint in Chapter 2 (see Supplementary Figure 3 in Chapter 2) as the cumulative progeny on the food control divided by the cumulative progeny after pathogen exposure. It was conceptually easier to express the magnitude of the delay as >1 in Chapter 4 compared to the minimized fitness loss <1 in Chapter 2. This was compared to the aforementioned magnitude of the maternal effect cost using Spearman's rank correlation tests for both live and heat-killed conditions.

Figures were made using the ggplot2 (Wickham, 2016) and RColorBrewer (Neuwirth, 2014) packages in R.

Results

We aimed to determine whether the pattern of delayed fecundity in Chapter 2 could be having longer-lasting impacts when the maternal stress matches the offspring stress and when the maternal stress is absent in the offspring's generation. That is, we investigated the benefits (mother and offspring both exposed to stress) and costs (mother exposed and offspring not) of maternal effects induced by pathogen exposure to live and heat-killed *S. aureus*. We also investigated the tradeoffs in the magnitude of benefits and magnitude of costs across one lab-adapted and four wild isolate *C. elegans* genotypes and the correspondence of the reported delay in Chapter 2 with the cost of maternal exposure in this chapter.

In making pairwise comparisons within a host genotype to determine differences in offspring performance, we found no statistically significant differences (Figure 2; Supplementary Table 3). In measuring offspring quality using a population size metric, pathogen-exposed (either

live or heat-killed) mothers produce offspring that are of comparable quality to offspring produced by non-infected mothers. This is despite the delayed fecundity from the first day of reproduction as reported in Chapter 2. More specifically, within a host genotype there are no statistically significant differences in population size for pathogen-exposed F1 whether their mothers saw pathogen or food, and no statistically significant differences in population size for uninfected F1 (food control) whether their mothers saw pathogen or food. The lab-adapted N2 strain tended toward a cost from maternal heat-killed *S. aureus* exposure (i.e. a difference in population sizes between F1 all on food, whose mothers each saw heat-killed *S. aureus* or control food) but this difference cannot be generalized at the alpha = 0.05 level (Welch two-sample t-test, t = 2.63, df = 3.40, $P = 0.0689$).

To test for cross-genotype effects, we employed a linear mixed-effects model. There was a significant difference in population sizes across host genotypes (Figure 2: Linear mixed-effects model, $\chi^2 = 34.13$, df = 4, $P < 0.0001$). The LKC34 wild isolate had significantly lower population sizes compared to JU258 (Tukey Contrasts, $P = 0.0151$) and N2 (Tukey Contrasts, $P = 0.0349$), independent of exposures in any generation. Similar to what was established in the prior chapters, there was a significant effect of exposure (Linear mixed-effects model, $\chi^2 = 32.06$, df = 1, $P < 0.0001$) with offspring producing lower population sizes on the live pathogen treatment compared to food (Tukey Contrasts, $P = 0.0005$). We also found an interaction between host genotype and F1 exposure (Linear mixed-effects model, $\chi^2 = 15.26$, df = 4, $P = 0.004$). However, we found no interaction between the exposures of the P and F1 generations (Linear mixed-effects model, $\chi^2 = 1.54$, df = 2, $P = 0.46$). While we did not discover evidence of intergenerational effects under these experimental conditions, we did find natural variation for population growth across host

genotypes and confirmed the long-term fitness costs of pathogen exposure in infected offspring.

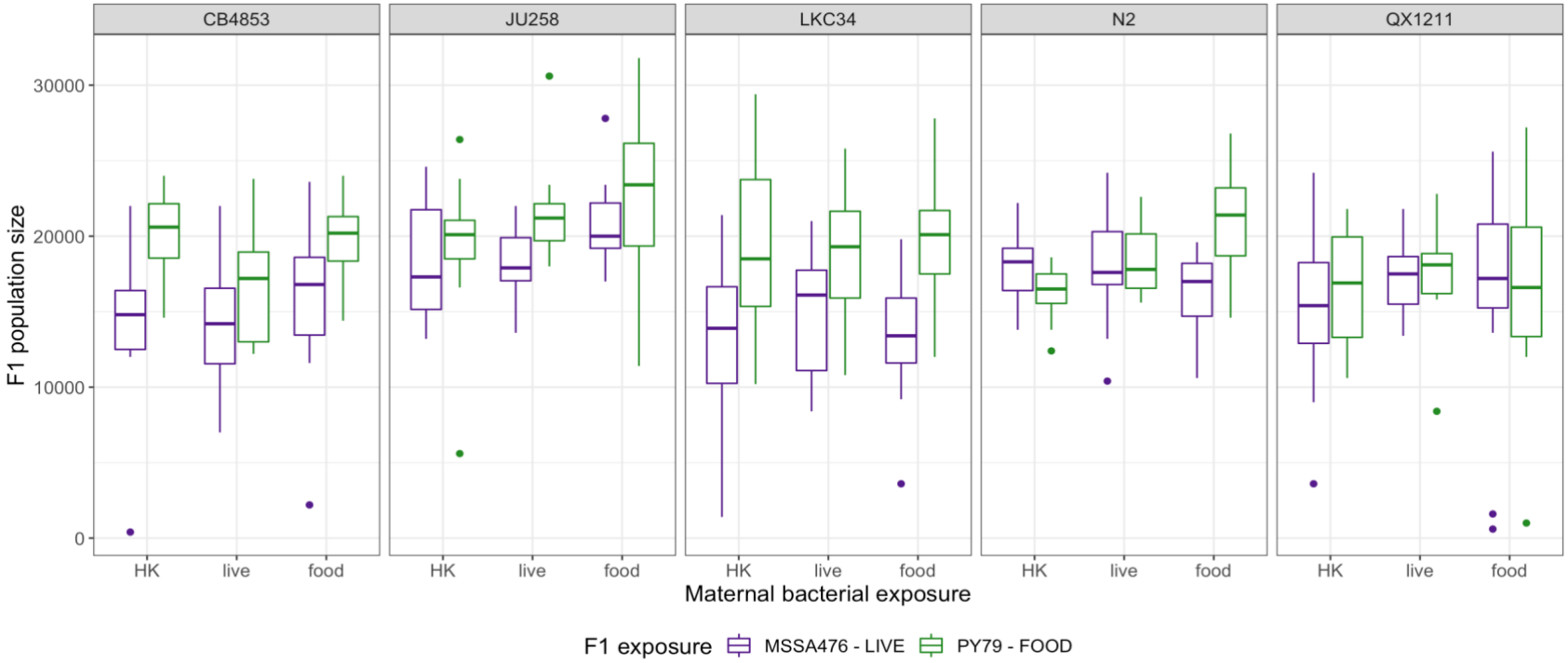


Figure 2: Distribution of F1 population sizes across five nematode isolates in three maternal treatments and two F1 treatments. Boxplots display summarized data at the level of technical replicate (Supplementary Figure 1), with each boxplot representing data aggregated from 12 lineage expansion plates (i.e. four batches times three founder worms). Nematode isolate names are shown on the top and maternal exposure is indicated on the x-axis as MSSA476 heat-killed (HK), MSSA476 live (live), and PY79 control (food). F1 exposure is represented by purple boxplots (MSSA476 live treatment) or green boxplots (PY79 control food). Boxes show first quartile, median, and third quartile of the data, whiskers extend 1.5 times the interquartile range from the first and third quartiles, and circles indicate outliers.

We also tested for the correlation between the magnitude of benefits of maternal pathogen exposure and the magnitude of such costs. We found no evidence of a correlation between the two metrics after either live (Figure 3A: Spearman's rank correlation, $P = 0.78$, rho = -0.2) or heat-killed (Figure 3B: Spearman's rank correlation, $P = 0.35$, rho = 0.6) maternal *S. aureus* exposure. One distinction to note is that in the maternal heat-killed pathogen exposure condition, the N2 lab-adapted strain exhibits higher benefit and higher cost than wild isolates. Additionally, upon maternal live pathogen exposure, the QX1211 isolate mean point indicates a benefit (>1) in line with the magnitude of N2 but of lower cost. While we did not find a genetic correlation of high benefits paired with high costs, and low benefits paired with low costs, we do report natural variation in how the host isolates are spread across the spectrum of magnitudes of costs and benefits of maternal exposures, both live and heat-killed.

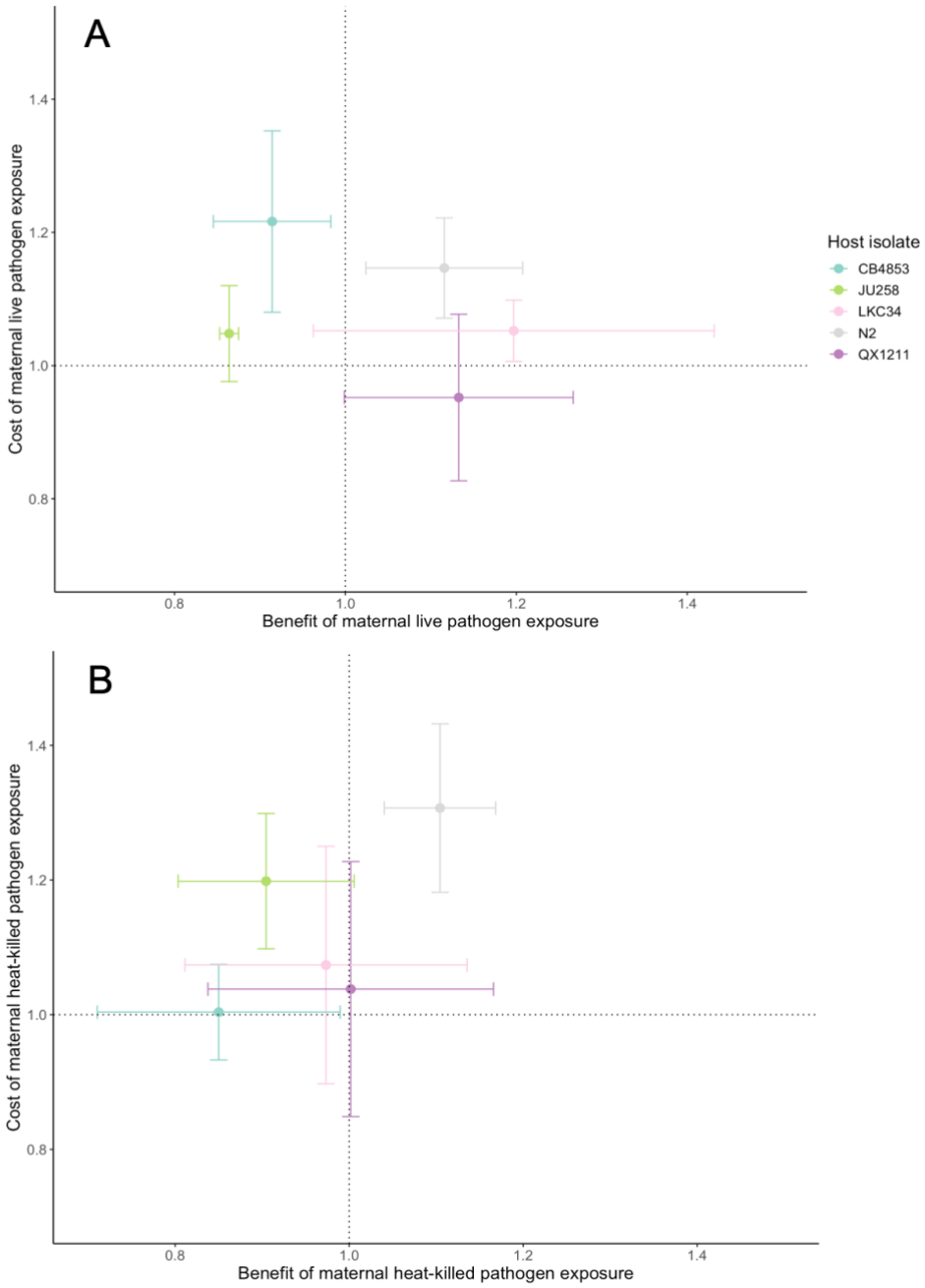


Figure 3: Benefits versus costs of maternal live (A) and heat-killed (B) MSSA476 pathogen exposure on F1 lineage expansion. The relative benefits (x>1) are evidenced by the F1 live pathogen treatment and the relative costs (y>1) are evidenced by the F1 control food exposure (in both cases, comparing F1 from pathogen-challenged mothers to F1 from control food mothers). Dotted line at x = 1 represents threshold where F1 encountering pathogen expand their population equally well whether their mothers have been exposed to food or pathogen (i.e. no benefit of maternal pathogen exposure). If x>1, it is advantageous for mothers to have been exposed to pathogen, compared to mothers on control food. If x<1, it is disadvantageous. Dotted line at y = 1 represents threshold where F1 encountering food expand their population equally well whether their mothers have been exposed to food or pathogen (i.e. no cost of maternal pathogen exposure). If y>1 it is disadvantageous for mothers to have been exposed to pathogen, compared to mothers on control food. If y<1 it is advantageous. Note that higher benefit and higher cost are both indicated as values >1. Points and error bars represent mean ± 1 SE. Host isolates are indicated by colors in the legend.

Finally, we connected the current chapter with the brood delay reported in Chapter 2. We found a statistically significant correlation across host isolates for mothers exposed to heat-killed *S. aureus* (Figure 4B: Spearman's rank correlation, $P = 0.017$, rho = 1) but no evidence of a correlation for mothers exposed to live *S. aureus* (Figure 4A: Spearman's rank correlation, $P = 0.45$, rho = 0.5). Note that a rho value of 1 indicates the delay and cost metrics are monotonically increasing and is not comparable with a 'perfect' Pearson correlation value for evaluating linear relationships. The correlation in the heat-killed condition indicates that the extent of the short-term fitness delays evident on the first day of reproduction of the challenged parent are evident to a similar degree in the future, un-challenged offspring (which are continuously maintained and exposed to control food). Moreover, the heat-killed-exposed N2 hosts exhibit higher delay and cost magnitudes than the wild isolate genotypes.

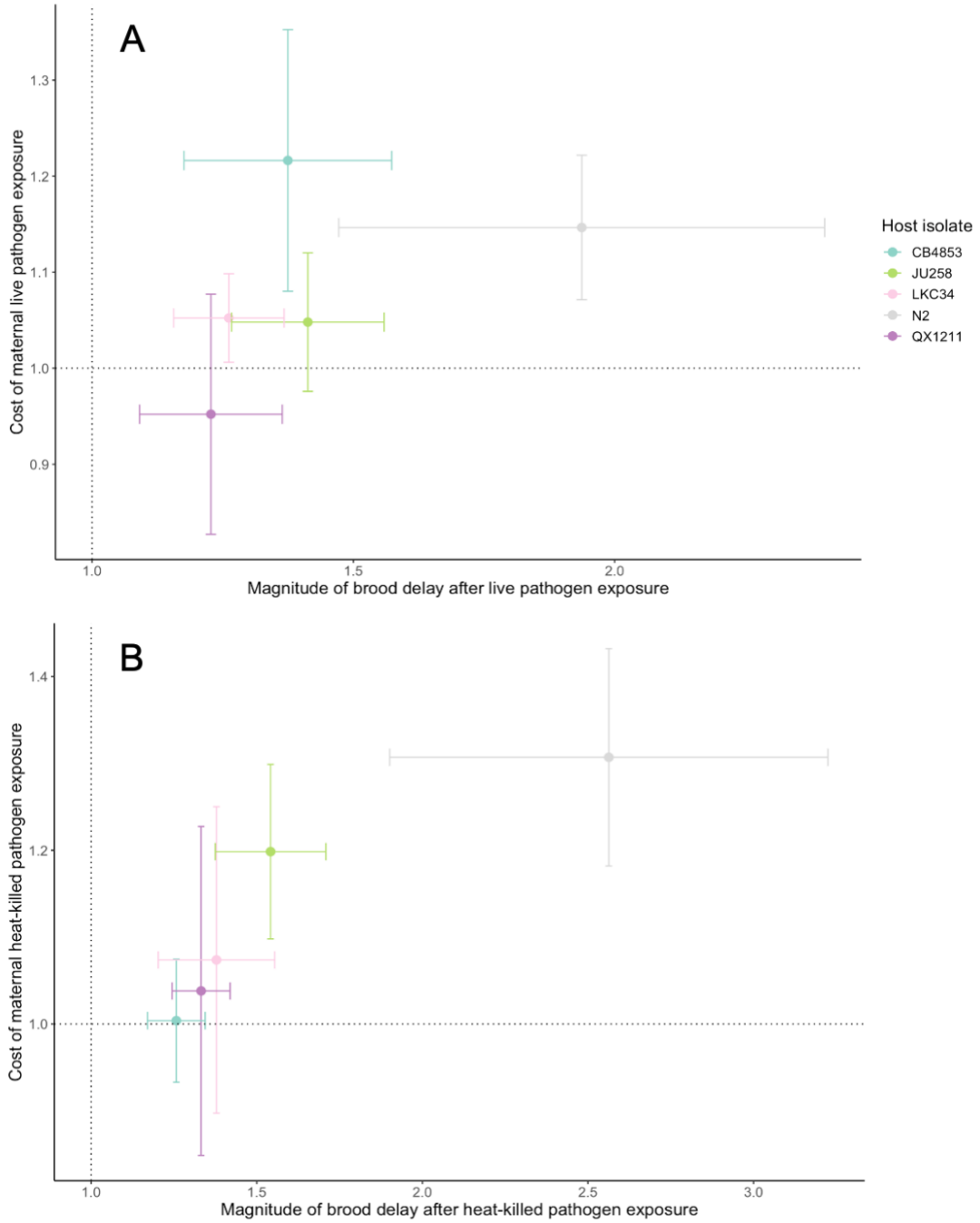


Figure 4: Magnitude of brood delay after pathogen exposure versus the cost on F1 of maternal pathogen exposure for live (A) and heat-killed (B) treatments. Dotted line at x = 1 represents threshold where cumulative progeny from mothers exposed to pathogen equals reproduction on food for the first day of adulthood (30 hours post-L4). Where x>1, pathogen exposure is disadvantageous and higher x values indicate poorer performance after pathogen exposure relative to food for a given host isolate. Dotted line at y = 1 represents threshold where F1 encountering food expand their population equally well whether their mothers have been exposed to food or pathogen (i.e. no cost of maternal pathogen exposure). If y>1 it is disadvantageous for mothers to have been exposed to pathogen, compared to mothers on control food. If y<1 it is advantageous. Note that more extreme delays in brood production and higher F1 population cost of maternal effects are both indicated as values >1. Points and error bars represent mean ± 1 SE. Host isolates are indicated by colors in the legend.

Discussion

A parent's environment can have implications for offspring fitness. Specific to host-pathogen interactions, immune priming between generations can prepare offspring for a future pathogen encounter (Roth *et al*., 2018). However, maternal effects can also be costly for populations if pathogen exposure reduces parental and/or offspring fitness (e.g. Contreras-Garduño *et al*., 2014; Sadd & Schmid-Hempel, 2009). In other cases, the parental generation's environment may have no effect on certain fitness metrics in their offspring (e.g. Leung *et al*., 2013; Pansch *et al*., 2014). While we did not find statistically significant benefits or costs of pathogen-induced maternal effects, we do report, in our sample, lower populations for food-exposed offspring whose mothers had been transiently exposed to heat-killed pathogen (compared to food-exposed offspring whose mothers had continuously seen food) for the N2 lab-adapted nematode strain. Further, we did find a positive correlation between the magnitude of delay in Chapter 2 and magnitude of the cost of maternal pathogen challenge experienced in uninfected offspring. We also describe natural variation in population sizes by host genotypes and natural variation in the magnitude of costs and benefits, though there is no discernable genetic correlation in our study.

While there is evidence of interactions between the environment experienced in the parental generation and parental genotype (e.g. Stjernman & Little, 2011; Groot *et al*., 2017; Alvarez *et al*., 2020) we did not find evidence for such interactions nor benefits of maternal effects under these experimental conditions. This is in line with other studies that report no effects (e.g. Leung *et al*., 2013; Pansch *et al*., 2014), subtle costs without any benefit (Littlefair *et al*., 2016), and weak effects (e.g. Uller *et al*., 2013) of parental treatment though the extent of 'neutral' effects in the literature is difficult to determine due to publication bias for positive results. In our study conditions, this points to the resilience of nematodes in overcoming the

effects of the previous generation's transient pathogen exposure, especially in wild isolates. Herein, the lab-adapted N2 tend toward lower-quality offspring after pathogen challenge. Perhaps a stronger cost would be discernible under more naturalistic conditions: varying the duration of pathogen exposure and/or host developmental stage during infection initiation (e.g. Pereira *et al*., 2020; Balla *et al*., 2015), multiple infections with limited food (e.g. Littlefair *et al*., 2016), or exposure to a co-isolated pathogen of wild *C. elegans* that persists in the hosts' compost habitat and may have a co-evolutionary history with nematodes (Felix & Braendle, 2010; Willis *et al*., 2020). However, the prevalence and time scale of various stressors in natural conditions is unknown so it is difficult to translate laboratory-based findings to population growth in the wild (Baugh & Day, 2020). Theoretical studies put forward that evolution of adaptive parental effects necessitates environmental conditions experienced by ancestors that can serve as dependable cues for descendants (e.g. Botero *et al*., 2015).

Further, we did not find a correlation between offspring performance after pathogen exposure (benefit of maternal treatment) and performance of uninfected offspring (cost of maternal treatment). That is, there is no indication of infected offspring lineage expansion (performance after pathogenic infection) trading off with uninfected lineage expansion (performance in absence of pathogen) when comparing lineages whose mothers were exposed to *S. aureus* with those continuously fed control *B. subtilis*. Future work could look into a host-pathogen system with an established intergenerational and/or transgenerational defense mechanism (e.g. Sterken *et al*., 2014; Moore *et al*., 2019; Burton *et al*., 2020) to see if there is a correlation of higher defense benefits being paired with higher fitness costs, and low benefits with low costs. To date, published studies of multigenerational immune priming in *C. elegans* have not utilized wild isolates though there is evidence of natural genetic

variation in a transgenerational phenotype involving heat stress (Frézal *et al*., 2018). One bioRxiv manuscript (Kaletsky *et al*., 2020a) that presented findings on transgenerational avoidance of *P. aeruginosa* with N2 and the wild isolate JU1580 did not include the results of the latter genotype in the final publication (Kaletsky *et al*., 2020b). Peer-reviewed evidence of intergenerational and/or transgenerational immunity in natural *C. elegans* has yet to be presented.

We found a positive correlation between the magnitude of the brood delay in Chapter 2 and the magnitude of the cost of maternal pathogen exposure, though only for heat-killed challenge and not for live *S. aureus*. We also found a trend towards a cost for N2 offspring whose mothers had been exposed to heat-killed pathogen (compared to control food-exposed mothers), but not live pathogen. In this system, hosts exposed to live *S. aureus* experience both pathogen damage and immune upregulation and hosts exposed to heat-killed *S. aureus* only experience immune upregulation. In relation to the gene expression, we only know that live and heat-killed *S. aureus* induce the same levels of immune genes at 8 hours, and we know the time course of some of these genes in live-exposed hosts (Irazoqui *et al*., 2010), but we do not know the kinetics of gene expression for hosts on heat-killed *S. aureus*. It is possible that in exposing mothers to 'processed' bacterial fragments in the heat-killed treatment, these hosts had higher immune upregulation earlier than 8 hours (e.g. peak at 4 hours) and then gene expression levels decreased (but were still high) at 8 hours. In the live *S. aureus* exposure, Irazoqui *et al*. reported several genes that were still increasing in expression from 8 to 12 hours. Therefore, the heat-killed treatment may have stressed mothers sooner during the exposure period than the live treatment, where mothers ingest live bacteria rather than fragments.

We already evidenced lower quantity of first-day progeny in Chapter 2 and herein we could be seeing lower-quality N2 offspring (when offspring performance is measured in terms of population size) as a result of earlier maternal investment in immune upregulation. There is ample evidence of reproduction-immunity trade-offs in parents who are immune challenged having lower quantity and/or quality offspring (reviewed in Schwenke *et al*., 2016) and for transgenerational immune priming incurring fitness costs in the parent (Zanchi *et al*., 2012), though we did not find evidence of immune priming against *S. aureus*. Future work could investigate whether vitellogenin provisioning impacts offspring quality in the context of host-pathogen interactions; this protein has consequences for offspring quality metrics in *C. elegans* experiencing transient starvation (Perez *et al*., 2017). Our finding of reduced numbers of potentially lower-quality offspring is in contrast to an existing finding in *C. elegans* by Harvey and Orbidans (2011) where mothers reared in a low-food environment have lower numbers of larger eggs, and these offspring are well-prepared for resource-poor conditions, though this study did not look into a pathogenic context.

The positive correlation between the costs in Chapter 2 and Chapter 4 should not be labelled as a genetic correlation *per se* because we are comparing distinct attributes in different generations rather than the same organism. One axis represents the extent of the delay experienced by mothers challenged with heat-killed *S. aureus* and infected with live *S. aureus* compared to uninfected mothers (i.e. lack of ability to minimize fitness loss upon pathogen exposure, or lack of immunocompetence) and the other axis represents offspring quality as the extent of the population decrease of uninfected offspring, comparing progeny with pathogen-challenged or pathogen-infected mothers to progeny with uninfected mothers (i.e. lack of ability to expand in population as a result of maternal environment). We see evidence

of lower-quantity offspring (Chapter 2) correlating with lower-performing offspring (Chapter 4) though the latter is likely due to environmental factors rather than inherited factors.

Further, we did not find any statistically significant differences nor trends for the four wild isolate genotypes in terms of benefits and costs of maternal effects. In correlating the magnitudes of benefits and costs across the five genotypes, the N2 lab-adapted strain exhibited higher magnitudes of benefits and costs in the heat-killed condition (i.e. >1 but not statistically significant in terms of pairwise population size comparisons) than the four wild isolate genotypes. N2 nematodes have higher fitness in the lab compared to wild isolates (Duveau & Felix, 2012; Andersen *et al*., 2014), which was evident in the lineages that were continuously exposed to food (though note that our control exposure and post-exposure conditions of feeding *B. subtilis* at 25°C does not correspond to the traditional maintenance protocol established in the last several decades and we do not necessarily present 'optimal' lab fitness conditions; Gómez-Orte *et al*., 2018). Further, the studies of N2 versus wild isolate fitness measured lifetime fecundity and not population size, which can be impacted by the tradeoff of brood size and age at maturity (Hodgkin & Barnes, 1991). However, N2 nematodes' adaptations to a benign lab environment may carry consequences. When exposed to pathogen (which the 'maintained' N2 lineage has not encountered in decades; Weber *et al*., 2010; Sterken *et al*., 2015) the N2 hosts are not as prepared to defend themselves and reproduce compared to wild isolates, whose recent ancestors faced a multi-microbial environment (Felix & Braendle, 2010). Another consideration is the outcome assessed. Wild beetle isolates were found to differ from lab beetles in terms of transgenerational immune priming, but only in some metrics (Tate & Graham, 2017). Evidently, the chosen metric and the experimental context (laboratory versus naturalistic conditions) can influence the interpretation of comparisons between host strains.

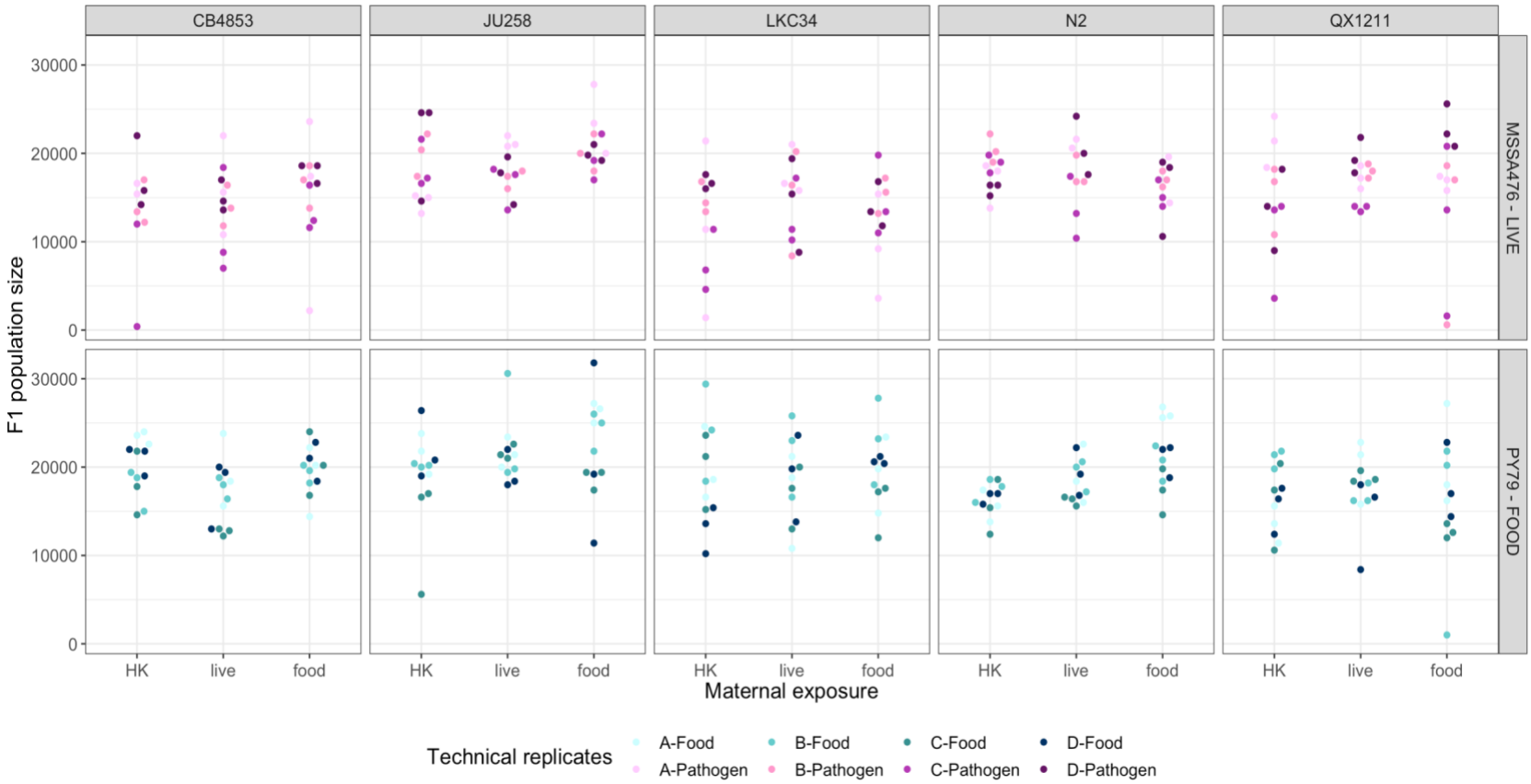
In relating Chapter 2 with this chapter, we report that for most host isolates and exposure conditions, pathogen-exposed mothers (despite having delayed fecundity and reduced number of progeny on the first day of reproduction) produce F1 that are of comparable quality to food-exposed mothers. This is relevant to the natural setting of nematodes: naturally-derived hosts are not adversely impacted (in terms of lineage reproductive performance) from a maternal transient pathogen challenge. This serves the nematodes well in nature, where offspring from challenged hosts can quickly recover from a pathogen encounter and continue reproducing. Overall, nematodes are resilient in the face of a transient challenge, and wild nematodes from multi-microbial environments may be better equipped to overcome challenges versus lab-adapted nematodes that are maintained in a benign environment.

Supplementary Table 1: Number of observations for each host isolate, parental exposure, and F1 exposure combination. Observations indicate technical replicates: non-censored nematodes with a maximum of 12 in treatments where no hosts were censored. All treatment combinations had a sample size of 4 biological replicate plates. HK = heat-killed.

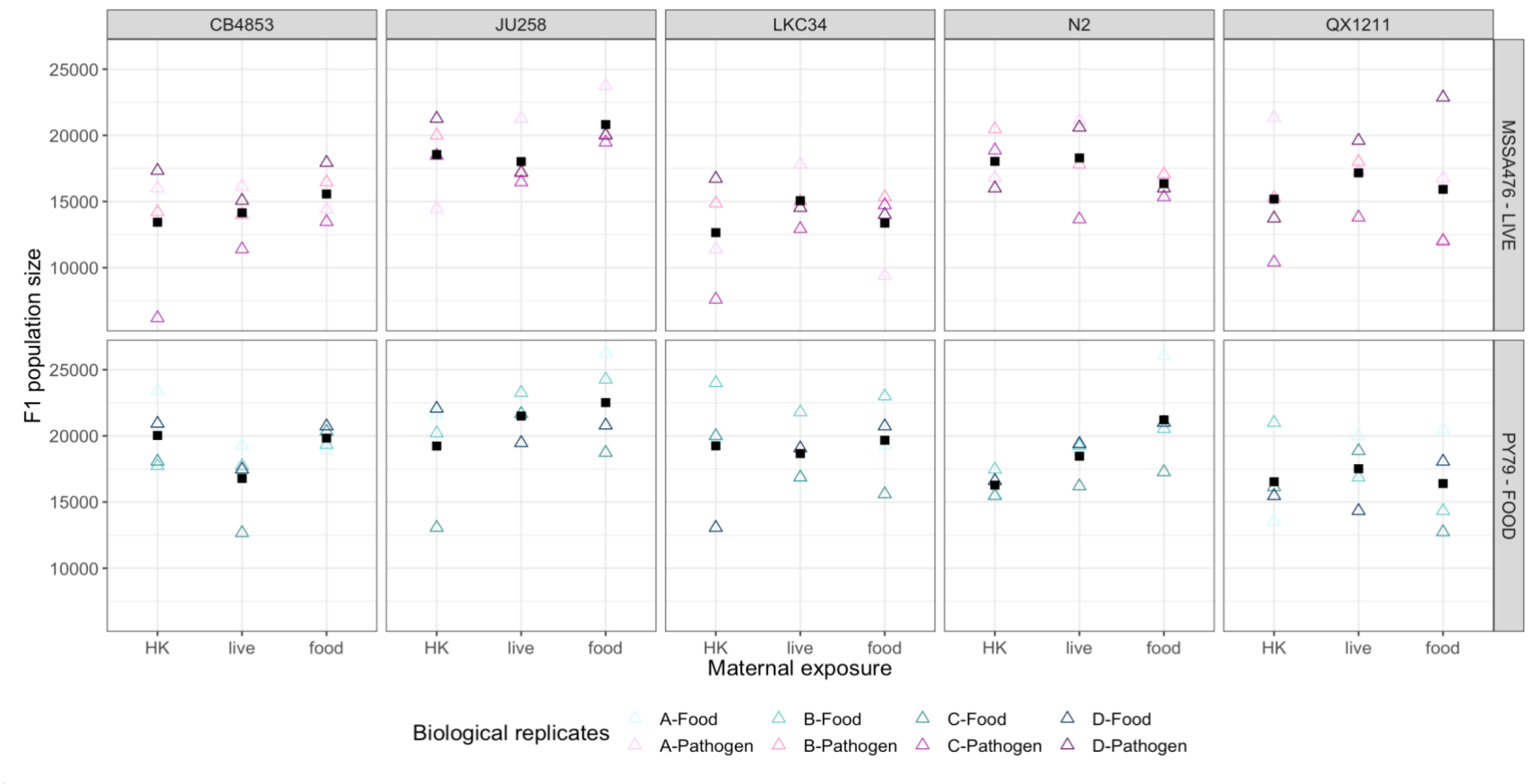
Host isolate	Maternal exposure	F1 exposure	Observations
CB4853	MSSA476 HK	MSSA476 live	10
JU258			12
LKC34			12
N2			12
QX1211			12
CB4853	MSSA476 live		12
JU258			12
LKC34			12
N2			11
QX1211			12
CB4853	PY79		12
JU258			12
LKC34			12
N2			11
QX1211			12
CB4853	MSSA476 HK	PY79	12
JU258			12
LKC34			12
N2			12
QX1211			12
CB4853	MSSA476 live		12
JU258			12
LKC34			12
N2			12
QX1211			12
CB4853	PY79		12
JU258			12
LKC34			12
N2			12
QX1211			12

Supplementary Table 2: Average variation in droplet counts within a sample as coefficient of variation (CV). The CV was calculated for every technical replicate and then summarized by host isolate genotype and bacterial exposure of the parental generation and F1. Summarizing by biological replicates (exposure plate groupings), as in other analyses, was excluded to portray the variation at the level of technical replicates.

Host isolate	Maternal exposure	F1 exposure	CV
CB4853	MSSA476 HK	MSSA476 live	34.4
		PY79	16.0
	MSSA476 live	MSSA476 live	21.6
		PY79	12.5
	PY79	MSSA476 live	19.4
		PY79	13.5
JU258	MSSA476 HK	MSSA476 live	15.1
		PY79	16.8
	MSSA476 live	MSSA476 live	14.3
		PY79	16.7
	PY79	MSSA476 live	17.2
		PY79	16.0
LKC34	MSSA476 HK	MSSA476 live	32.8
		PY79	15.7
	MSSA476 live	MSSA476 live	17.5
		PY79	20.7
	PY79	MSSA476 live	25.4
		PY79	11.7
N2	MSSA476 HK	MSSA476 live	18.9
		PY79	16.1
	MSSA476 live	MSSA476 live	13.7
		PY79	14.8
	PY79	MSSA476 live	15.5
		PY79	13.5
QX1211	MSSA476 HK	MSSA476 live	18.1
		PY79	19.0
	MSSA476 live	MSSA476 live	15.1
		PY79	12.9
	PY79	MSSA476 live	25.3
		PY79	19.8



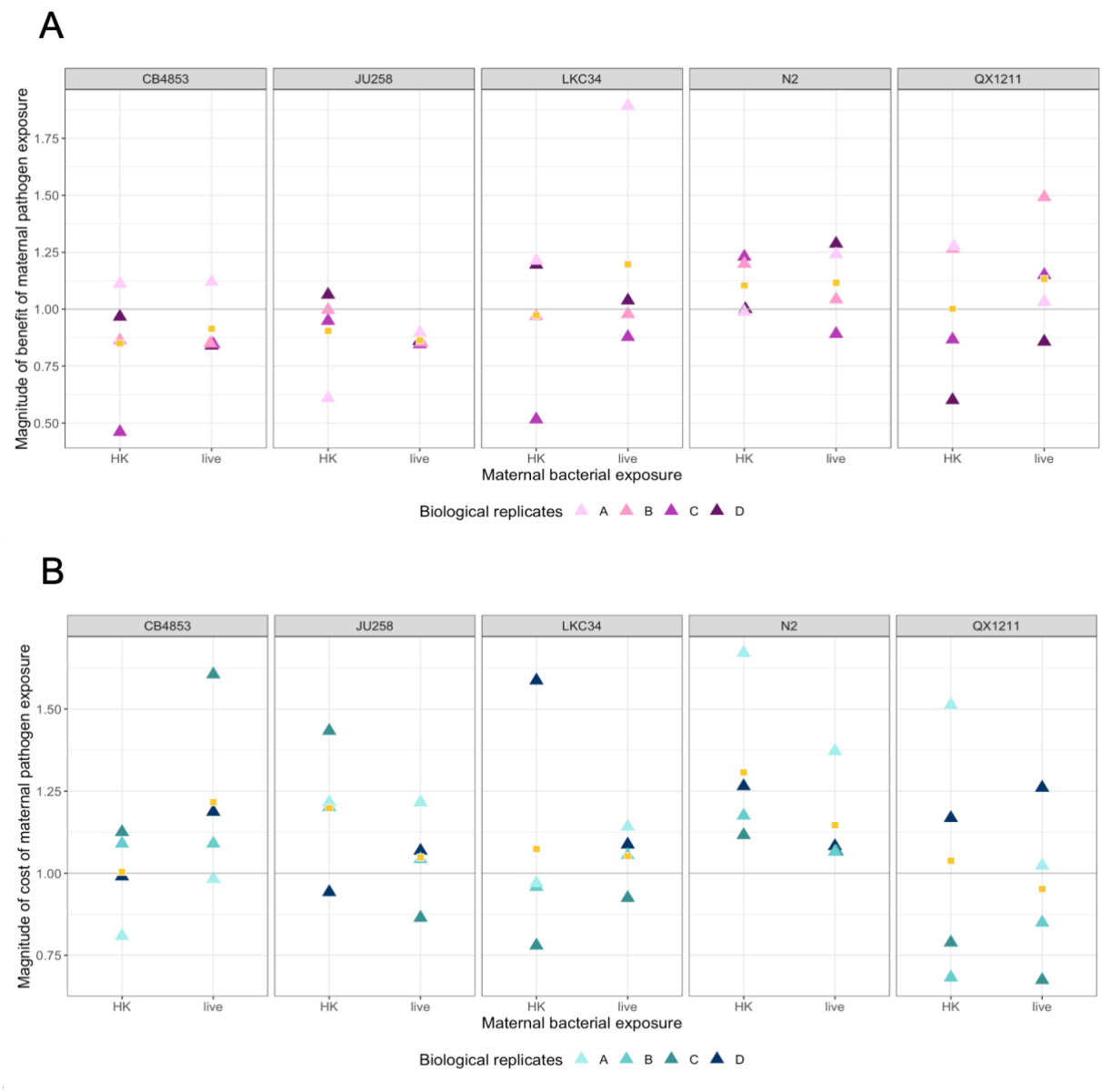
Supplementary Figure 1: Raw data showing variation in F1 population size of technical and biological replicates. Up to four technical replicates (founder nematodes tracked for population size) are depicted as points colored by their corresponding biological replicate code (A, B, C, or D). F1 pathogen exposure replicates are marked in the pink-purple gradient and control food replicates in the blue-green gradient. Nematode isolate names are shown on the top and F1 treatment is shown on the right. Maternal exposure is indicated on the x-axis as MSSA476 heat-killed (HK), MSSA476 live (live), and PY79 control (food).



Supplementary Figure 2: Processed data showing the biological replicate averages of grouped technical replicates. The mean F1 population size of each biological replicate is shown as a triangle and the overall treatment mean is shown as a black square. Four biological replicates are shown (each colored for A, B, C, D) for each combination of worm isolate, maternal exposure treatment, and F1 exposure treatment. F1 pathogen exposure replicates are marked in the pink-purple gradient and control food replicates in the blue-green gradient. Nematode isolate names are shown on the top and F1 treatment is shown on the right. Maternal exposure is indicated on the x-axis as MSSA476 heat-killed (HK), MSSA476 live (live), and PY79 control (food).

Supplementary Table 3: Summary of Figure 3 comparisons and results. Values from the noted parental exposure treatment and F1 exposure combination were always compared to the control condition: maternal food exposure with F1 exposed to MSSA476 to assess benefits and PY79 to assess costs. The Welch two-sample test statistic is indicated by t with the corresponding degrees of freedom (df) and p-value adjacent. No comparisons were statistically significant at the alpha = 0.05 level.

Host isolate	Maternal exposure	F1 exposure	t	df	p-value
CB4853	MSSA476 live	MSSA476 live	-0.99	6.00	0.3601
	MSSA476 HK		-0.79	3.95	0.4728
JU258	MSSA476 live		-1.90	5.93	0.1063
	MSSA476 HK		-1.28	5.22	0.2550
LKC34	MSSA476 live		1.01	5.56	0.3556
	MSSA476 HK		-0.30	5.24	0.7789
N2	MSSA476 live		1.11	3.36	0.3413
	MSSA476 HK		1.54	3.99	0.1988
QX1211	MSSA476 live		0.44	4.30	0.6815
	MSSA476 HK		-0.21	5.92	0.8383
CB4853	MSSA476 live	PY79	-2.05	3.52	0.1195
	MSSA476 HK		0.14	3.59	0.8938
JU258	MSSA476 live		-0.55	4.22	0.6130
	MSSA476 HK		-1.22	5.75	0.2703
LKC34	MSSA476 live		-0.52	5.56	0.6259
	MSSA476 HK		-0.15	5.30	0.8852
N2	MSSA476 live		-1.40	4.02	0.2349
	MSSA476 HK		2.63	3.40	0.0689
QX1211	MSSA476 live		0.52	5.40	0.6244
	MSSA476 HK		0.06	5.94	0.9569



Supplementary Figure 3: Relative benefit (A) and cost (B) of maternal MSSA476 heat-killed and live exposures across nematode isolates. Triangles represent the magnitude of cost or benefit from maternal treatment and are colored by their biological replicate batch code. The benefit ratios (A) represent the extent to which offspring whose mothers have been exposed to heat-killed or live pathogen reproduce more after live pathogen exposure (>1) than offspring whose mothers have continuously been on control food. The cost ratios (B) represent the extent to which offspring whose mothers have been exposed to heat-killed or live pathogen reproduce less after food exposure and experience a greater cost (>1) than offspring whose mothers have continuously been on control food. The mean of each treatment is indicated with an orange square. They grey line at y = 1 represents the value where offspring encountering pathogen expand in population equally well whether their mothers have seen food or pathogen before (i.e. no benefit, A), and the value where offspring on control food expand in population equally well whether their mothers have seen food or pathogen before (i.e. no cost, B). Note that higher benefit and higher cost are both indicated as values >1. F1 pathogen exposure replicates (A) are marked in the pink-purple gradient and control food replicates (B) in the blue-green gradient. Nematode isolate names are shown on the top and maternal exposure is indicated on the x-axis as MSSA476 heat-killed (HK), MSSA476 live (live).

Chapter 5

Thesis general discussion

Connecting the chapters

Pathogens can impact host reproduction. Host reproductive schedules, individual fecundity, and the population growth of a lineage can all vary during infection. Therefore, we decided to measure fitness costs as lifetime fecundity in terms of total brood size and cumulative progeny to investigate the reproductive impacts of infection and immunity (Chapter 2). Upon reading a pre-print by Feistel *et al*. (now published; 2019) and learning how modest decreases in brood size could translate into large reductions in population size, we employed their lineage expansion assay to investigate fitness costs in terms of population growth (Chapter 3). Finally, we sought to determine longer-term impacts: how the maternal environment can be beneficial or detrimental for their progeny's response whether the same pathogen is present or absent in the subsequent generation (Chapter 4). Throughout, we utilized a diversity of wild host isolates which provided us with variation in naturally-relevant alleles to examine the range of effects of immunogenic and pathogenic exposures on host proliferation. Overall, we investigated the degree to which transient pathogen exposure can impact hosts over several generations, with variation observed among multiple host genetic backgrounds, as well as between pathogen species and heat-killed versus live bacterial treatments.

Chapter summaries

In Chapter 2, we examined the costs of immunity and infection and tradeoffs between immunocompetence and reproduction for the host reproductive lifetime. We expected to see significant reductions in brood size for pathogenic treatments, though instead we found nematode hosts recovered their brood size to control levels after a delay induced by transient pathogen exposure. The mechanism causing the short-term delay remains unknown but future work could look into the various steps of the reproductive cycle that

might be inhibited and/or molecules that bridge immunity and reproduction, such as serotonin and insulin. In an attempt to distinguish fitness costs of immune upregulation from pathogen damage across time points, we found that lab-adapted nematodes and wild isolates showed no difference in brood size relative to food, for either live or heat-killed pathogen exposures. While we predicted a trade-off among host genotypes between brood sizes on food and immunocompetence after pathogen exposure, we found no correlation between the control and these treatments. We suggest that a brief exposure time and unlimited nutrition may be masking fitness costs and trade-offs. Overall, we showed that nematodes can recover in terms of reproduction from a transient pathogen exposure.

In Chapter 3, we moved beyond individual host broods to looking at population growth encompassing two further generations. An additional dimension included using two distinct bacterial pathogens to study immunological trade-offs in defense strategies across host genotypes. Although we predicted trade-offs for each pathogen species at the population level, we actually found that immunocompetence (i.e. minimized fitness loss) after *S. aureus* exposure and *P. aeruginosa* exposure did not negatively correlate with reproduction on the respective food. In disentangling costs of immune upregulation and pathogen damage, we found that live *S. aureus* exposures (which confer both stresses) significantly reduce relative population sizes. This result contrasts with Chapter 2 in which there was no distinction between relative brood sizes for the two bacterial treatments and highlights the advantages of inspecting multiple generations. Surprisingly, we observed that host isolates had larger population sizes after live *P. aeruginosa* exposure compared to heat-killed exposure. In combining relative population sizes from hosts on either live pathogen species, we revealed an immunocompetence trade-off across host genotypes, suggesting an immune balance in the host response to two distinct pathogens. We also

propose that *P. aeruginosa*, in the context of a short exposure, could be a nutritious nematode food source compared to control food.

In Chapter 4, we transiently exposed two generations of nematodes to *S. aureus* to investigate the presence of maternal effects and whether these effects are beneficial or costly in terms of offspring performance. Upon pathogen exposure, mothers may prime offspring, preparing them for future defense (benefit) or the mothers may suffer from pathogen exposure and produce low-quality offspring (no benefit). Moreover, priming mechanisms, if any, could be costly for offspring if they upregulate defenses but are not infected. We found no evidence of strong intergenerational effects, but we do report a distinction between wild isolates and the lab-adapted nematode strain: the latter strain exhibited the highest magnitude of a cost for maternal pathogen exposure and the highest magnitude of delay from Chapter 2. This points to the resilience of natural isolates in particular, which can overcome repeated transient challenges without a large impact to their population growth.

Fitness as a measure of immunocompetence

We used reproduction as our main indicator of fitness costs. Since pathogens often have non-lethal consequences in hosts, using survival to measure the costs of infection (as is common in many of the host-pathogen studies already cited) limits our understanding of morbidity effects in favor of mortality. In some cases, measuring survival or lifespan alone might not encompass the fitness consequences of pathogens particularly if hosts finish reproduction within a shorter period (Diaz *et al.*, 2015). Moreover, we did not measure mortality because nematodes can recover survival to control levels after an exposure of 8 hours or less to *S. aureus* (Sifri *et al.*, 2003) and 12 hours or less to *P. aeruginosa* (Tan *et*

al., 1999), upon transfer to non-pathogenic food; mortality was not as relevant as morbidity under our transient exposure conditions.

Many metrics are used to evaluate immunocompetence (reviewed in Sheldon & Verhulst, 1996). Immunocompetence can be determined by measuring expression levels of genes encoding antimicrobial effectors, immune cell counts, pathogen load, and survival, among other traits (reviewed in Schwenke *et al*., 2016). However, higher levels of defense-related genes, molecules, or cells do not necessarily mean the measured trait is more effective (Sheldon & Verhulst, 1996). In fact, a maximal immune response is not optimal across contexts given the physiological costs of immunity (Viney *et al.*, 2005). Additionally, an immune metric may adequately capture an effective response to one pathogen but be inappropriate for another type of pathogen, for example, if different immune pathways are upregulated (Hawley & Altizer, 2011). Also, an upregulation in one pathway may be accompanied by downregulation in other immune pathways, which highlights the need to uncover trade-offs within immune responses (reviewed in Boughton *et al*., 2011). Given an immune pathway, it may be difficult to know what component to measure because focusing on only one molecule of the cascade might not capture the overall effectiveness of that response. Therefore, it is important to measure immunocompetence in terms of functional activity, for example as pathogen clearance, wound healing, or fitness (e.g. in terms of reproductive output, growth, development, or host condition; reviewed in Boughton *et al*., 2011). In order to capture the ecological and evolutionary process underlying defense strategies (Viney *et al*., 2005), we measured brood size and population size. Further work in our system is required to connect the molecular biology of host defense to ecological contexts and evolutionary trajectories.

Not all studies use the term ‘fitness’ or ‘fecundity’ in the same way. Some authors define fitness to include host survival as well (e.g. Feistel *et al*., 2019), but we focused on lifetime reproduction in Chapter 2, and the number of offspring that reproduce themselves, according to Lack’s definition of fitness (1954) for Chapter 3 and Chapter 4. Fecundity has been measured as offspring per brood, offspring per breeding season, and offspring per lifetime, though these can vary widely across taxa (reviewed in Pincheira-Donoso & Hunt, 2015). These measures are not applicable in the *C. elegans* model system given that nematodes produce one brood in their lifetime, and this is fully captured during our experimental period.

Leveraging natural variation from wild populations

In nature, organisms are exposed to a diversity of pathogens. However, most of our knowledge on host responses to infection comes from lab-adapted hosts in controlled environments with limited or no prior pathogenic exposures (discussed in Pedersen & Babayan, 2011). While model organisms have been fundamental in elucidating molecular mechanisms, these studies underemphasize natural genetic variation and the functional analysis of allelic variation (discussed in Gasch *et al*., 2016). Gasch *et al*. recommend that studies in model systems should integrate organisms from natural populations in order to determine the extent to which lab-adapted, canonical strains represent the range of members in their species and to unravel mechanisms and their variability. Studying natural variation has been specifically recommended in *C. elegans* research, where most studies have been done with the lab-adapted N2 isolate (Félix & Braendle, 2010; Volkers *et al*., 2013; Petersen *et al*., 2015). Aside from examining natural genetic variation to pathogen responses, by leveraging natural variation we were also able to investigate evolutionary costs of defense in terms of genetic correlations. In this thesis, our panel of wild isolates

exhibited variations in brood sizes, population sizes, magnitudes of costs, and differing immunocompetence to two distinct pathogens: conducting this project with only N2, or with a limited number of genetically-similar strains, would not have provided this wealth of information. A further conclusion can be made about the variation: despite having lower or higher reproduction and population growth under certain conditions, nematode genotypes derived from nature have evolved from successful lineages that have optimized immune balance phenotypes and resource allocation between defense and reproduction (among many other traits). We have provided further evidence that various successful strategies exist though our results should be evaluated in more natural conditions involving co-isolated bacteria, multiple infections, and resource-limited, fluctuating environments (e.g. Félix & Braendle, 2010; Littlefair *et al*., 2016; Dey *et al*., 2016).

Experiments conducted in lab settings, while controlled, may not be generalizable to the field given stark environmental changes. Namely, *ad libitum* food and the stress of a novel setting can influence the immune response (Calisi & Bentley, 2009). Additionally, *C. elegans* reproducing in a semi-natural soil media had lower reproductive output than control nematodes under standard lab conditions (Freyth *et al*., 2010). In a review of 'outdoor immunology,' Boughton *et al*. (2011) suggest comparing results of lab-based studies to wild organisms in their natural environment, and even moving laboratory-bred animals into field studies. Recent examples of this are found in the murine model. 'Wilding' mice have been produced by transferring embryos of lab strains into wild mice and these offspring have a more natural microbiota (Rosshart *et al*., 2019). Also, adult mice released into an outdoor enclosure had more diverse microbiota (including increased fungi) and altered host immunity (Yeung *et al*., 2020). Incorporating the complexity of

nature into experimental design will help reveal how adaptation to lab versus natural conditions influences reproduction, defense, and other life-history traits.

A bacterivore's perspective: food, pathogen, or symbiont?

Bacteria are a natural food source of *C. elegans* (Schulenburg & Félix, 2017). The bacterial diet supplies macronutrients and micronutrients to support growth, development, and reproduction (reviewed in Zhang *et al*., 2017). In the lab, various bacterial diets have been found to influence life-history traits of *C. elegans* (reviewed in Pang & Curran, 2014). Coolon *et al*. (2009) tested four native soil bacteria and found that *C. elegans* food preference correlates with its fitness. Further, when nematodes were fed monocultures of naturally-isolated bacteria, 80% of strains supported growth and 20% decreased growth and induced a stress reporter. Follow-up experiments showed that the latter strains were not just nutritionally deficient, but that they might be producing damaging toxins (Samuel *et al*., 2016).

A pathogen has traditionally been defined as an organism that damages a host (Pirofski & Casadevall, 2012) though there are studies that categorize microbes as pathogens if there is evidence of immune gene upregulation (e.g. Alper *et al*., 2007; Samuel *et al*., 2016). Our heat-killed *S. aureus* treatment does cause an immune response but does not damage nematode hosts as evaluated by cytopathology (Irazoqui *et al*., 2010): does this make heat-killed *S. aureus* pathogenic or non-pathogenic in our model system? And what happens when brief exposures do not reduce host lifespan or host reproduction, is that microbe still considered pathogenic? We found that transient exposures to live *P. aeruginosa* increased population sizes relative to control food, though in another study, longer exposures resulted in decreased populations (Grewal, 1991). How do temporal dynamics of pathogen

exposure influence host reproduction and immunity? This question is particularly interesting for bacterivores, who must assess whether a microbe is a meal or danger, and may pass up on much-needed resources if they are overly cautious, or become infected if they misinterpret cues (Schulenburg & Félix, 2017).

As discussed in Chapter 3, one way to dissect the nutritional versus immune role of live *P. aeruginosa* would be to feed nematodes avirulent mutant strains to see the extent to which the increased reproductive output is mediated by an immune or stress response. This finding, in comparison with previous studies demonstrating reduced reproduction with longer-term *P. aeruginosa* exposures (Grewal, 1991; Tan *et al*., 1999), leads us to question: at what exposure duration and under what conditions does *P. aeruginosa* turn from potentially-nutritious food to damaging pathogen? Recent work found an intergenerational effect whereby a 4-hour parental exposure to PA14 generated an attraction response in F1 and an 8-hour parental exposure generated an avoidance response in F1 (Pereira *et al*., 2020). Although we did not specifically look at intergenerational effects with PA14, further work manipulating exposure timing (duration e.g. Pereira *et al*., 2020 and host age e.g. Balla *et al*., 2015) will allow us to see if time and life stages are factors in whether *P. aeruginosa* helps or harms nematode hosts, and whether there is a spectrum of effects (i.e. attraction to aversion) for progeny. Another key experiment to probe the effects of immunity on reproduction in *C. elegans* would be to manipulate the immune response in a sterile way. That is, to under- and over-express specific immune pathways rather than attempting to regulate immunity solely through different bacterial exposures, which carry nutritional consequences. Such an experiment would also delve into questions of 'immunosufficiency,' in other words: what is an optimal immune

response? Does over-expression of a key pathway benefit the host during pathogen exposure but then carry fitness costs in the absence of pathogens?

Aside from acting as food, some microbes can also protect *C. elegans* from potential pathogens. Early studies into protective microbes demonstrated nematodes reared on *Bacillus megaterium* and *Pseudomonas mendocina* had increased resistance to *P. aeruginosa* compared to *E. coli*-fed controls (Montalvo-Katz *et al*., 2013). Later studies have looked into the protective effects of the natural microbiome, showing bacterial members with anti-fungal properties (Dirksen *et al*., 2016) and *Pseudomonas fluorescens* isolates with protective effects against *Bacillus thuringiensis* (Kissoyan *et al*., 2019). In relation to the present study, *C. elegans* fed *Bacillus subtilis* GS67 resisted *B. thuringiensis*; the protection came in the form of *B. subtilis* reducing pathogen colonization in the intestine (Iatsenko *et al*., 2014). This is not likely to be occurring with our *B. subtilis* PY79 food, since PY79 is effectively ground up by the pharynx and no intact PY79 cells are evident in the nematode intestine (Garsin *et al*., 2001). While it does not seem likely that PY79 would be directly suppressing *S. aureus* growth in the gut, future study into the spectrum of dietary and protective microbes is key in identifying how naturally-occurring bacteria in the nematode habitat can be a meal and a symbiont, supporting growth as well as protecting from potential infection (Hoang *et al*., 2019).

Considerations of exposure

A further element of our bacterial exposures in the context of infection is that we employed a natural infection route of nematodes for *S. aureus* and *P. aeruginosa* bacterial pathogens. Cuticular and anal infections of *C. elegans* are documented, though most pathogens enter through the mouth and infect the intestine (reviewed in Gravato-Nobre & Hodgkin, 2005). While other studies in organisms such as insects directly inject pathogens

into host, this bypasses external defenses that hosts would use in nature to protect themselves (reviewed in Boughton *et al*., 2011). In our system, *C. elegans* orally intake the bacteria and can utilize the grinding action of the pharynx to prevent some or most microbial cells from surviving the passage into the gut (Seymour *et al*., 1983; Gravato-Nobre & Hodgkin, 2005). Studies in the model organism *Drosophila melanogaster* demonstrate that infection mode is an important factor. Experimental evolution with infection by two distinct routes led to different host evolutionary trajectories (Martins *et al*., 2013). Also, natural infection of *D. melanogaster* through ingestion of a bacterial pathogen uncovered previously-unreported immune genes (Vodovar *et al*., 2005). Mimicking the natural infection process should elicit the relevant immune responses that are as close to normal as possible in order to produce realistic host responses (reviewed in Boughton *et al*., 2011). While we used a natural route of infection, one limitation is not knowing the exact quantity the nematodes are ingesting, since even isogenic nematodes exposed for the same duration will become differentially colonized (Twumasi-Boateng & Shapira, 2012).

Another limitation is the difficulties of generating a dead and/or non-pathogenic bacterial treatment. *C. elegans* exhibit decreased pharyngeal pumping rates when fed heat-killed *E. coli* (Wood *et al*., 2004) and the overall effect of a dead microbial diet is not clear (Muschiol *et al*., 2009). We attempted to UV-treat *S. aureus* in order to at least retain the physical properties of a living bacterial lawn, but it proved resistant to killing and this was later confirmed by a separate lab group (Javier Irazoqui, personal communication, 13 August 2018). We also attempted work with an avirulent strain of *S. aureus* (RN6390 wildtype and its *agr* mutant, found in Pollitt *et al*., 2014), but the dense, mucilaginous lawns proved difficult to work with; Politt *et al*. used liquid cultures to infect wax moth

larva. One factor that remains to be determined is whether heat-killed and live *S. aureus* elicit comparably costly immune upregulation. Irazoqui *et al*. (2010) found comparable upregulation of ten *S. aureus*-specific immune genes, but the genome-wide host impact of these two treatments is not yet known. Upon infecting *C. elegans* with live and heat-killed *Candida albicans*, Pukkila-Worley *et al*. (2011) found that 56% of genes upregulated in the live treatment were also induced in worms exposed to heat-killed *C. albicans*. Further work is needed to establish how heat-killed bacterial treatments, which are regularly used as a control in *C. elegans* research, induce certain (but not all) aspects of an immune response and might not be nutritionally complete or attractive food sources. Moreover, the aforementioned future work with avirulent mutants would help us dissect nutritional versus immunological effects of pathogen exposures.

'Rewilding' nematode research

We used clinical isolates of *S. aureus* and *P. aeruginosa.* They are not natural pathogens of *C. elegans* and were therefore useful in modeling novel host-pathogen interactions. Nevertheless, Firmicutes are found, albeit at low abundance, in the native *C. elegans* microbiome and Proteobacteria (including the Pseudomonadaceae family) are found in high abundance (Dirksen *et al*., 2016). Thus, *C. elegans* in the wild may have encountered a wider array of Pseudomonadaceae compared to Staphylococcaceae, and these interactions may have influenced taxonomic abundance of the host microbiome and have consequently fine-tuned the host responses over time. This may underpin the higher range of host variation we observed in responses to *P. aeruginosa* compared to *S. aureus* in Chapter 3.

Similarly, *D. melanogaster* hosts exhibit greater variation in their response to viruses with which they have co-evolved (Duxbury *et al*., 2019). Natural genetic variation of *C. elegans* isolates to *P. aeruginosa* has been revealed in relation to avoidance behavior (Andersen *et al*., 2012; Martin *et al*., 2017), but such relationships have not been explored for *S. aureus* or other Firmicutes. Another possibility in explaining this result in Chapter 3 involves the common nematode isolation protocol (Barrière & Félix, 2006). Sampling may be biased by the use of *E. coli*, a Gram-negative microbe, as an attractant, which may select for nematode genotypes that readily respond to Gram-negative bacteria (discussed in Petersen *et al*., 2015). Petersen *et al*. advocate for using unbiased sampling protocols (e.g. sieves) to obtain a more realistic picture of *C. elegans* ecology in the wild. A further element to consider of the common isolation protocol is that in attempting to identify natural pathogens of *C. elegans*, only mild pathogens may be discovered because one cannot easily collect a worm that is paralyzed or has been completely dissolved (Schulenburg & Félix, 2017). Studying natural host-pathogen interactions across isolates will likely lead to further discovery of allelic and phenotypic variation in the host response (reviewed in Frézal & Félix, 2015) and microbial co-colonization dynamics (Ford *et al*., 2016) in a natural setting.

Inter-isolate competition, while important in nature, was beyond the scope of the current work. That is, how would fitness in our host genotypes be impacted by pathogen exposure with the addition of competition against other genotypes for resources? Further, how might competition against other Rhabditida and bacterivores in natural habitats influence host-pathogen outcomes? Although members of *Caenorhabditis* are similar morphologically, they are highly divergent genetically and it remains to be determined how this might lead to distinct niches at the community level (Félix & Braendle, 2010). Non-‘Elegans’ group

nematode species exhibit variation in the time to reach maximum population density in the lab, ranging from 8 to 15 days (Sohlenius, 1969), though this is with separate cultivation and not in a competition scenario. An evolution experiment selecting for increased pathogen resistance in fruit flies showed that resistant lines displayed elevated defense compared to susceptible lines but had reduced ability to acquire nutrients when competing against conspecifics (Kraajiveld & Godfray, 1997; Kraajiveld *et al*., 2001). Moreover, *C. elegans* population sizes can vary seasonally, with denser populations in autumn and bottlenecks occurring during the winter, which could lead to extinction and re-colonization by other nematode genotypes (Petersen *et al*., 2014), but further study is needed to examine the population dynamics over time at the level of individual fruits or stems with various *C. elegans* genotypes and other nematodes competing for resources (Barrière & Félix, 2007). We predict that competition would limit resources and exacerbate (or reveal) trade-offs between reproduction and immunity.

Intergenerational effects have yet to be evidenced in *C. elegans* wild isolates. While intergenerational (and transgenerational) effects are found in studies with N2 (e.g. Moore *et al*., 2019; Burton *et al*., 2020), it would be useful to replicate the published studies with non-lab-adapted hosts to see if these specific maternal effects are more widespread in *C. elegans*. As mentioned in Chapter 4, a bioRxiv manuscript (Kaletsky *et al*., 2020a) detailing transgenerational avoidance of *P. aeruginosa* with N2 and the wild isolate JU1580 excluded the latter genotype in the peer-reviewed publication (Kaletsky *et al*., 2020b). Further, one might attempt to create more naturalistic settings for wild isolates (e.g. perhaps conducting experiments in compost or with rotting fruit) and also see if N2 would retain previously-reported multigenerational mechanisms in this more natural, and potentially stressful, environment. Another avenue for future work could be assessing the

degree of specificity of the maternal effects. Offspring of infected parents may not suffer costs in terms of future pathogen exposure but perhaps challenging offspring with an abiotic stress would reveal reduced offspring quality. The converse has been identified, with adaptation to osmotic stress in N2 nematode mothers preventing adaptation to pathogen infection in progeny (Nicholas Burton, personal communication, 20 March 2019). It remains to be determined whether this trade-off would exist in natural, unpredictable, fluctuating environments.

Relationship between brood size and population size

We did not find a genetic correlation between brood sizes and population sizes across our wild isolates (data not shown). This result makes sense in light of the biology of *C. elegans*: the shift from spermatogenesis to oogenesis means that reproduction is sperm-limited and that sperm number (i.e. brood size) trades off with the timepoint at which the first egg is fertilized (i.e. generation time) (discussed in Cutter, 2004). Consequently, nematodes producing fewer sperm will have smaller broods, but then lay eggs sooner and increase in population faster, compared with nematodes producing more sperm (Hodgkin & Barnes, 1991). Therefore, brood size can trade off with population size, though this outcome might depend on the time interval assessed and we might not have captured the point at which this negative correlation is evident and statistically significant.

Moreover, population sizes predicted from single founder nematodes overestimate observed population sizes (Harvey *et al*., 2008). This discrepancy indicates density effects of nematodes growing with other individuals can affect reproductive output, and this could potentially have implications for pathogen exposure as well. Diaz *et al*. (2015) found that nematodes in cohorts of 25 had lower reproductive output than individuals or cohorts of 10 (for nematodes on *E. coli* and *Salmonella enterica*, but not *P. aeruginosa* PAO1), and

there were no differences for survival. While Diaz *et al.* rule out starvation effects, they do consider density-dependent pheromone effects (e.g. Artyukhin *et al*., 2013). The method with which we measured population size (i.e. uninterrupted lineage expansion on a single plate) versus brood size (i.e. daily transfer of the founder worm to new wells) could have been subject to social effects of founder worms and their offspring being exposed to density-dependent signals. Namely, brood assays were conducted with relatively low density effects and lineage expansion assays were conducted with relatively high density effects. The reasons for lack of correlation between data in Chapter 2 and Chapter 3 could be attributed to nematode biology and/or experimental setup. Further, as shown by Hodgkin and Barnes, brood size should not be considered a proxy for population size or population growth rate, and researchers aiming to investigate population dynamics should consider the trade-off between brood size and generation time that is found in nematodes but does not extend to all model organisms.

Many factors influencing costs and trade-offs

Immunity and reproduction are both important traits, but it is rarely possible to optimize both characteristics. During infection, immune responses are upregulated and host reproductive output may decrease, but costs are not always present when taking into account factors from the environment and host context. Costs of immunity can be hidden and revealed depending on factors such as nutrient quantity and quality, predation frequency, density of competitors, and multiple immune challenges (reviewed in Sandland & Minchella, 2003). The availability of resources (i.e. nutrient quantity) is especially well-studied. There is evidence of low nutrient quantity revealing costs of infection for bumblebees (Moret & Schmid-Hempel, 2000) and fruit flies (Hoang, 2001; McKean *et al*., 2008) that were hidden with a replete environment. However, this is not always the case,

as *ad libitum* food did not prevent the fitness costs of chronic immune activation in crickets (Stahlschmidt *et al*., 2013). Moreover, Stahlschmidt *et al*. reported a trade-off between offspring quantity (i.e. estimated lifetime fecundity) and quality (i.e. size).

In Chapter 2, we found total brood size after pathogen exposures recovered to control food levels by the end of the reproductive period, though this result emerged after an initial delay. One hypothesis, which was tested in Chapter 4, was that mothers maintained the same reproductive output, but sacrificed offspring quality after pathogen exposure. Trade-offs between offspring quality and quantity are documented, and they usually are studied in the context of resource-depleted and/or stressed mothers producing a smaller quantity of better-provisioned eggs (discussed in Harvey & Orbidans, 2011). We therefore took measurements of population sizes in Chapter 3 and performed intergenerational exposures in Chapter 4. We have no conclusive evidence of a trade-off between offspring quantity and quality under our experimental conditions. Perhaps providing nematodes with limited resources or a longer pathogen exposure time would have revealed both lifetime fitness costs of immunity and/or a trade-off between offspring quantity and quality.

Future research could consider the impact of host status on pathogen-induced costs and trade-offs. For example, Feistel *et al.* (2019) showed immunocompromised mutant nematodes had lower reproduction compared to healthy nematodes across unexposed, live, and heat-killed fungal pathogen treatments. At present, more work is needed to characterize immunocompetence versus immunocompromised statuses of lab-maintained versus natural populations of nematodes hosts (but see Balla *et al*., 2015). It is expected that that multiple infections would affect host immunity; nematodes are often isolated with various microbes in their gut and on their exterior (Félix & Braendle, 2010; Félix &

Duveau, 2012). In fruit flies, hosts infected in cooler conditions unexpectedly had greater immune gene upregulation than warm-condition flies (Linde *et al*., 2008). One explanation is that at the higher temperature, immune upregulation occurred quickly, cleared the infection, and returned to basal levels before the experimental timepoint. Such a possibility speaks to the importance of considering immune dynamics and infection progression in future studies, beyond just single timepoints.

Temperature is known to influence overall physiology and fitness (Gilchrist & Huey, 2001), and would be relevant in natural nematode seasonal cycles and varying sampling latitudes. Perhaps some host genotypes would be better adapted to eliciting an immune response and/or sustaining reproduction at the 25°C exposure temperature maintained in the lab (Harvey & Viney, 2007). This temperature was reported to lead to decreased egg-laying in N2 compared to 16°C (Byerly *et al*., 1976). In relation to competition, *C. elegans* and *Caenorhabditis briggsae* have been found sharing natural habitats, with *C. elegans* populations expanding more quickly at lower temperatures and *C. briggsae* population growth increasing at higher temperatures (Félix & Duveau, 2012). Future work could investigate these population-level temperature effects in light of pathogen infection and inter-species competition.

Is defense in nature costly for nematodes?

The risk of low or no investment in defense is death before reproduction. Alternatively, relatively high investment in defense could mean low investment in reproduction (discussed in Viney *et al*., 2005). What is the optimal response? Theoretical work has examined when organisms should maintain constitutive defenses and deploy inducible defenses, when to mount general versus specific immune responses, and how immune pathways trade-off with each other (reviewed in Rolff & Siva-Jothy, 2003). Optimal

immune responses are context-dependent. Consequently, measuring fitness, rather than immune output, is the best way of evaluating optimal defense (discussed in Viney *et al*., 2005).

While costs of immunity have been detected across numerous host-pathogen systems, they are not always evident (reviewed in Rigby *et al*., 2002). Specific to a *C. elegans* evolution experiment, after generating host lines with elevated defense levels to a bacterial pathogen, Penley *et al*. (2018) did not detect maintenance costs; elevated defense was maintained whether the pathogen was present or absent. Similarly, we did not identify fitness costs across all types of pathogen exposures in our study. We found that following a transient, pre-reproductive pathogen exposure nematodes recovered in terms of brood size (Chapter 2) and did not pass on significant costs to subsequent generations (Chapter 4). In terms of life-history tradeoffs, we did not find evidence for a negative correlation between immunocompetence and reproduction at the brood (Chapter 2) and population (Chapter 3) levels. Though we did discover a trade-off in host reproduction across isolates responding to two distinct bacterial pathogens (Chapter 3). Taken together, we suggest that population size in nature is more strongly determined by the pathogen type (i.e. trade-offs in defense strategies) that exist in the host environment and its evolutionary history, rather than the costs of defense. This trade-off model would predict whether certain host genotypes will expand in population more, in relation to other host genotypes in the community, after brief encounters with certain types of pathogens. However, studies done to date cannot yet answer whether defense itself in nature would be costly for nematode reproduction. Many outstanding questions remain: investigating costs with variables such as co-infections, resource limitation and acquisition, food choice behavior, competition and predation, and differences by life stage.

Conclusion

The outcomes of host-pathogen interactions are complex and context-specific. These interactions are shaped by past exposures, the current abiotic and biotic environment, and the future motivation to produce well-provisioned progeny, as well as many other factors. Defense responses across different host genotypes are variable and are calibrated by evolution, which points to the importance of using the breadth of variation exhibited by wild isolates, whose genes and traits have been shaped in nature. It is with experimental approaches that consider relevant natural circumstances that we will advance our understanding of fitness costs and trade-offs of host defense.

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