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Zachary R. Lynch Date

Effects of parasites on host adaptation: immune system trade-offs, alternative behavioral defenses, and outcrossing rates

By

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B.S., University of Rochester, 2011

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Abstract

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By Zachary R. Lynch

Coevolution between hosts and parasites drives adaptation in both antagonists; hosts are selected to resist or tolerate infection and parasites are selected to optimize their infectivity and transmission. Host immune systems comprise behavioral, cellular, humoral, social, and symbiont-mediated defenses, which can alleviate the fitness consequences of infection but may carry maintenance and deployment costs. Therefore, hosts are expected to specialize in only a subset of possible defenses. I tested this hypothesis by measuring behavioral and cellular defenses used by fruit flies against parasitoid wasps. However, I found no evidence for trade-offs in the relative strengths of these defenses across eight fly species and two wasp species. Although one wasp species was more virulent, each fly species behaved similarly towards both wasps. *Drosophila melanogaster* exhibited the weakest cellular immunity and the strongest behavioral avoidance, suggesting that it may specialize in alternative defenses against wasps, such as medication with ethanol. I found that fly larvae experienced a two-fold reduction in parasitization intensity when they consumed ethanol during exposure to the generalist wasp *Leptopilina heterotoma*, leading to a 24-fold increase in survival to adulthood. However, larvae did not self-medicate with ethanol after being parasitized. Instead, my results suggest that female flies have an innate preference for laying eggs in ethanol food, a behavior that protects their offspring from wasps but occurs independent of wasp exposure. My final chapter addresses a central mystery in evolutionary biology: why is outcrossing ubiquitous in plants and animals despite its reduced population growth potential relative to self-fertilization? The best-supported explanation is that host–parasite coevolution generates shifting adaptive landscapes that favor outcrossed offspring. I tested whether parasite turnover could have a similar effect in the absence of coevolution. Using experimental evolution with the nematode *Caenorhabditis elegans* and the pathogenic bacterium *Serratia marcescens*, I found that exposure to novel parasite strains led to elevated host outcrossing rates, which facilitated host adaptation. My results suggest that recurring episodes of parasite turnover could favor the long-term maintenance of outcrossing. Future studies should investigate behavioral defenses using more ecologically realistic experimental setups and host–parasite combinations with more recent coevolutionary histories.

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Chapter 1

Introduction

Coevolution between hosts and parasites drives adaptation in both antagonists; hosts are selected to resist or tolerate infection and parasites are selected to optimize their infectivity and transmission. In my dissertation, I focus on how parasites affect host adaptation. This is a more complicated issue than we might intuitively predict; it seems that host immune traits should increase in strength over time because parasite infection can have serious fitness consequences, but several factors may prevent this from happening. Parasites evolve counter-defenses that can render host immune responses ineffectual, host immune systems comprise various types of defenses, and the expression of defenses, like any other trait, involves resource costs. Considering these factors leads to different predictions: (1) trade-offs will occur between host defenses that act against particular types of parasites, and (2) hosts will evolve novel defenses as their existing defenses become too costly to maintain or are defeated by parasites. I explore prediction (1) in Chapter 2 and prediction (2) in Chapter 3. In this chapter, I provide background on the different types of immune responses, their costs, and the current evidence for immune trade-offs.

It is also important to consider host characteristics that may generally affect rates of adaptation and how parasites could affect the evolution of those characteristics. The maintenance of outcrossing in animal species that are also capable of asexual reproduction or self-fertilization is puzzling because outcrossing lineages have lower reproductive potential over time. However, outcrossing may result in greater offspring genetic diversity and allow beneficial

mutations to be combined into single genotypes more rapidly. Thus, outcrossing is expected to be favored when recurring environmental change causes adaptive landscapes to shift over time. The best-supported mechanism for generating these shifts is host–parasite coevolution, but frequent host migration or local turnover in parasite populations could have similar effects. I explore this prediction in Chapter 4. In this introductory chapter, I explain the current evidence that host–parasite coevolution favors the maintenance of host outcrossing and discuss the possible role of host migration based on recent studies.

History of trade-off research

The study of trade-offs has been considered crucial to our understanding of ecology and evolution since Darwin (1859) and continues to be an active area of research (Sheldon & Verhulst, 1996; Zera & Harshman, 2001; Fry, 2003; Rolff & Siva-Jothy, 2003; Roff & Fairbairn, 2007; Moore & Hopkins, 2009; Agrawal *et al.*, 2010; Ardia *et al.*, 2011; Asplen *et al.*, 2012). Trade-offs occur when an increase in one trait implies a decrease in another; they are expected to result from the fitness costs and benefits of expressing different traits. Classic examples of life history trade-offs, including age vs. size at reproductive maturity, reproductive investment vs. survival, and quality vs. quantity of offspring, have been characterized in many systems (Stearns, 1989; 1992). Physiological trade-offs occur because individuals have limited resources that must be allocated to a broad range of traits. At the population level, microevolutionary trade-offs can result from genetic linkage between traits such that an adaptive change in one

trait is tied to a maladaptive change in another trait (Stearns, 1992). These negative genetic correlations can constrain rates of adaptation (Ardia *et al.*, 2011). Macroevolutionary trade-offs are observed when species have different fixed strategies for resource allocation across a set of traits, resulting in negative correlations between those traits across species (Stearns, 1992).

However, trade-offs are not always observed when they are expected. As a theoretical example, we can consider each individual's resource pool (A_i) as being divided between reproduction (R_i) and survival (S_i), such that $A_i = R_i + S_i$, with each individual investing a certain proportion of resources (B_i) into reproduction, such that $R_i = B_i * A_i$. If the variation in A is sufficiently high relative to the variation in B among individuals in a population, then the correlation between R and S will be positive within the population, even though a physiological trade-off between reproduction and survival would be expected (van Noordwijk & de Jong, 1986). Therefore, we are most likely to observe physiological trade-offs when individual variation in resource acquisition is low. However, artificial selection studies testing for microevolutionary trade-offs have different considerations regarding resource availability. Previous studies in which fruit fly populations were selected for resistance against parasitoid wasps only found trade-offs between cellular immunity and larval competitive ability in food-limited environments (Kraaijeveld & Godfray, 1997; Fellowes *et al.*, 1998), suggesting that costs of immunity might not be evident in the absence of resource limitations. To enable strong responses to artificial selection, the starting populations used for these studies must also have sufficient additive genetic variation for the traits of interest; thus, large, outbred, wild-caught host

populations are commonly used. This approach revealed trade-offs in the previously mentioned fly–wasp studies (Kraaijeveld & Godfray, 1997; Fellowes *et al.*, 1998), but trade-offs were not observed when the same fruit fly population was later selected for resistance to a fungal parasite (Kraaijeveld & Godfray, 2008). Perhaps this difference occurred because fungal infections may be mild and easily tolerated, whereas parasitoid infections necessarily result in either host or parasitoid death. Therefore, factors such as resource availability, standing genetic variation, and expected strength of selection must be considered when designing artificial selection experiments to test for trade-offs.

Trade-offs between immune functions and other aspects of life history have been studied extensively (Zuk & Stoehr, 2002). They are evident across diverse taxa, including birds (Ardia, 2005; Tieleman *et al.*, 2005; Lee *et al.*, 2008; Hasselquist & Nilsson, 2012), mammals (Graham *et al.*, 2010), and insects (Fellowes *et al.*, 1999a; McKean & Nunney, 2001; McKean *et al.*, 2008). For example, across 70 tropical bird species, higher levels of constitutive antibodies were associated with longer offspring development times (Lee *et al.*, 2008). Although stronger immune responses are often thought to be better, Graham *et al.* (2010) showed that high levels of self-reactive antibodies, which are markers for autoimmune disease, were associated with reduced fitness in a natural population of Soay sheep. However, in comparison to the rich history of literature regarding trade-offs between immunity and other life history traits, trade-offs among immune mechanisms have been relatively ignored (Moore & Hopkins, 2009; Parker *et al.*, 2011; Asplen *et al.*, 2012).

Insects are relevant models for studying immune trade-offs because different species possess different combinations of costly humoral, cellular, and behavioral defenses (Fellowes *et al.*, 1999a; Evans *et al.*, 2006; Lemaitre & Hoffmann, 2007; McKean *et al.*, 2008; Gerardo *et al.*, 2010; de Roode & Lefèvre, 2012). Many insect species are also convenient models due to their small sizes and short generation times, which make them highly amenable to the investigation of microevolutionary and macroevolutionary trade-offs. Therefore, I will focus my discussion on insects, particularly *Drosophila melanogaster*, a model organism for immunity and genetics that possesses a complex set of defenses (Lemaitre & Hoffmann, 2007; Carton *et al.*, 2008; Lefèvre *et al.*, 2012b; Milan *et al.*, 2012; Kacsoh *et al.*, 2013). In order to demonstrate how the study of macroevolutionary immune trade-offs will advance our understanding of host–parasite coevolution, I will discuss: (i) costs of humoral, cellular, and behavioral immunity, (ii) immune trade-offs within species, and (iii) immune trade-offs across species.

Costs of humoral and cellular immune responses

Insect humoral immune responses involve the activity of non-cellular components of the hemolymph, including pathways such as the phenoloxidase melanization cascade and chemicals such as reactive oxygen species and antimicrobial peptides (Nyholm & Graf, 2012). McKean *et al.* (2008) found a negative correlation between fecundity of uninfected females and humoral resistance to a natural bacterial pathogen across 40 genetically variable lines of *D. melanogaster*. This correlation was only found in food-limited environments,

supporting the idea that costs of immunity might only be evident when resources are scarce (van Noordwijk & de Jong, 1986). Similarly, bumblebees (*Bombus terrestris*) that were challenged with lipopolysaccharide showed increased hemolymph antibacterial activity, but only starved individuals suffered increased mortality (Moret & Schmid-Hempel, 2000). McKean and Nunney (2001) identified a physiological trade-off between humoral immunity and mating activity in fruit flies. Male *D. melanogaster* housed with increasing numbers of females were less able to clear bacterial infections and were significantly more likely to engage in courtship and mating.

Insect cellular defenses include clot formation, encapsulation, nodulation, and phagocytosis (Lavine & Strand, 2002). Trade-offs and fitness costs associated with melanotic encapsulation in *D. melanogaster* are particularly well-understood. This defense involves surrounding a parasitoid wasp egg with a multilayered capsule of hemocytes, then employing phenoloxidase and reactive oxygen species to melanize the hemocyte capsule and asphyxiate the wasp egg (Strand & Pech, 1995; Carton & Nappi, 1997). Kraaijeveld and Godfray (1997) selectively bred *D. melanogaster* that survived parasitism by the wasp *Asobara tabida* and observed a rapid increase in melanotic encapsulation rate, from 5% to ~55% within five generations. Larvae from the selected lines showed reduced competitive ability in food-limited environments. Similar results were found when *D. melanogaster* lines were artificially selected for resistance to a more virulent wasp species, *Leptopilina boulardi* (Fellowes *et al.*, 1998). Fitness costs of mounting this immune response have been found by comparing traits of adult flies with and without melanized capsules in the *D. melanogaster–A. tabida*

system. Melanized capsules indicate that flies survived parasitism, whereas the lack of a capsule indicates that flies were not parasitized. Fellowes *et al.* (1999a) found that females with capsules were smaller and had significantly lower early-life fecundity regardless of the capsule status of their mates. Adult flies with capsules were also found to have significantly lower resistance to desiccation and starvation stresses (Hoang, 2001). These effects could be direct consequences of parasitoid-induced damage, but they might also reflect physiological trade-offs. The latter hypothesis is plausible because host fitness is reduced to zero if a successful anti-parasitoid defense is not mounted, so parasitized larvae must invest scarce resources into a cellular immune response in order to survive, presumably at the cost of reducing investment in other fitness-related traits such as body size, fecundity, and stress resistance.

Costs of behavioral immune responses

Behavioral immune responses, which include any altered behaviors that hosts use to increase their direct fitness when threatened or infected by parasites, have recently been recognized as crucial defense mechanisms that are common across animals, even those that are not normally recognized for having complex cognition (Parker *et al.*, 2011; de Roode & Lefèvre, 2012; de Roode *et al.*, 2013). In particular, recent studies have provided evidence that insects can medicate themselves and their offspring against parasites by using natural plant chemicals. Medication behaviors may be fixed or phenotypically plastic. To predict how these behaviors will evolve, the costs of maintaining plasticity, costs of searching for medicinal plants, and side effects of medicinal chemicals must be balanced

with the benefits of curing parasite infection. Plasticity is expected to be maintained unless parasites are extremely virulent (Choisy & de Roode, 2014). Female monarch butterflies (*Danaus plexippus*) that are infected with protozoan parasites are very likely to pass the infection to their offspring through their larval food plant. Lefèvre *et al.* (2010) showed that infected female monarchs respond by preferentially ovipositing on milkweed plants that contain high levels of toxic cardenolides, and infected offspring that fed on those plants had reduced parasite loads and increased longevity. However, consuming some milkweed plants with especially high cardenolide concentrations can lead to reduced survival to adulthood and reduced adult life span in both infected and uninfected monarchs (Tao *et al.*, 2016), suggesting that selection would not favor constitutive expression of this behavior.

Recent studies have reported multiple behavioral defenses used by *D. melanogaster* against parasitoid wasps, including reduced oviposition in the presence of wasps and medication with ethanol. Lefèvre *et al.* (2012b) found that female flies avoid laying eggs at wasp-infested sites and significantly reduce their oviposition rates when forced to live with wasps. The fitness consequences of those behaviors have not been fully characterized, but I investigate whether the latter behavior represents an offspring quality vs. quantity trade-off in Chapter 2. Milan *et al.* (2012) found that fly larvae fed on ethanol-laden food were less likely to be attacked by wasps. Additionally, fly larvae preferentially consumed ethanol after being attacked, which killed developing wasps and increased survival to adulthood. Ethanol is a likely candidate for anti-wasp medication because it is available in naturally fermenting fruits where fruit fly larvae feed on yeast

(Gibson *et al.*, 1981) and *D. melanogaster* has evolved particularly high ethanol tolerance compared to other fruit fly species (David & Van Herrewege, 1983). However, consuming high concentrations of ethanol may lead to slower development and increased mortality (McKenzie & Parsons, 1972; McKechnie & Geer, 1984; Milan *et al.*, 2012), suggesting that this behavior would be detrimental to uninfected larvae. In follow-up studies, female flies that were exposed to wasps preferentially laid eggs in ethanol-containing food (Kacsoh *et al.*, 2013; Kacsoh *et al.*, 2015), suggesting that mothers respond to the threat of their offspring being parasitized by preemptively medicating them. However, other studies have found that uninfected *D. melanogaster* larvae preferentially consume ethanol (Parsons, 1977; Parsons & King, 1977) and that female flies prefer to lay eggs in ethanol food even when they have not been exposed to wasps (Richmond & Gerking, 1979; Siegal & Hartl, 1999; Azanchi *et al.*, 2013; Zhu & Fry, 2015). In Chapter 3, I attempt to resolve these inconsistencies by testing whether ethanol is an effective treatment before, during, or after exposure to wasps, whether parasitized larvae have increased ethanol preference, and whether wasp-exposed mothers lay more eggs in ethanol food, using a generalist wasp and a specialist wasp.

Immune trade-offs within species

Given the costs of humoral, cellular, and behavioral immune mechanisms, it is reasonable to expect that trade-offs will occur and that certain defenses might be lost over evolutionary time (Parker *et al.*, 2011). Pea aphids (*Acyrthosiphon pisum*) have lost certain immune system components that are

common across insects, such as c (chicken)-type lysozymes, antimicrobial peptides, peptidoglycan receptor proteins, and the immunodeficiency signaling pathway (Altincicek *et al.*, 2008; Gerardo *et al.*, 2010). However, other components, such as the Toll and Janus activated kinase/Signal transducers and activators of transcription (JAK/STAT) signaling pathways, heat shock proteins, and phenoloxidase, are conserved in pea aphids (Gerardo *et al.*, 2010). Each of these conserved immune system components also have roles in non-immune functions, which include embryonic development (Leulier & Lemaitre, 2008), hematopoiesis (Kisseleva *et al.*, 2002), general stress responses (Pockley, 2003), and wing melanization (Hooper *et al.*, 1999) respectively. This striking loss of humoral immune capabilities suggests that pea aphids have alternative defenses.

Although parasitoid wasps are capable of significantly limiting the growth of natural populations of pea aphids (Snyder & Ives, 2003), cellular encapsulation of parasitoid eggs has never been reported in pea aphids (Bensadia *et al.*, 2006). Significant variation in resistance to parasitoid wasps and fungal pathogens was previously found across pea aphid clones, but there was no response to selection by wasps and no evidence for microevolutionary trade-offs; instead, there were positive correlations in resistance to different parasites (Henter & Via, 1995; Ferrari *et al.*, 2001). Later studies found a reasonable explanation for those surprising patterns: symbiont-mediated defenses are crucial for protecting pea aphids against parasitoids and fungal pathogens. The bacterial secondary symbionts *Hamiltonella defensa* and *Regiella insecticola* confer resistance to the parasitoid wasp *Aphidius ervi* (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Moran *et al.*, 2005; Oliver *et al.*, 2005; Hansen *et al.*, 2012). *R.*

insecticola also confers resistance to the entomopathogenic fungus *Pandora neoaphidis* (Scarborough *et al.*, 2005; Lukasik *et al.*, 2013). However, the evolutionary history of these interactions remains unclear: decreased humoral immunity may have allowed for increased prevalence of microbial symbionts in pea aphids, or the defensive efficacy of symbionts may have rendered host defenses expendable over evolutionary time (Gerardo *et al.*, 2010).

Recent studies of honey bees and wood ants have suggested that the expression and diversification of canonical immune pathways may trade off with investment in social defenses. Although honey bees share the basic components of multiple immune pathways with *D. melanogaster*, these immune gene families have expanded significantly more on the fruit fly lineage, which suggests that honey bees might be less able to mount defenses against a diverse range of parasites and pathogens (Evans *et al.*, 2006). One possible explanation for this finding is that honey bee defenses are highly specialized to a limited set of coevolved parasites. Alternatively, honey bees might allocate fewer resources towards individual immunity and more towards social defenses, including hygienic behavior, use of propolis in nest construction, and offspring quarantining (Evans *et al.*, 2006). Simone et al. (2009) housed honey bees in experimental colonies supplemented with propolis (a mixture of antimicrobial plant resins and wax used to line interior nest walls) and found that they had reduced bacterial loads and reduced expression of certain immune genes. Similarly, wood ants (*Formica paralugubris*) incorporate antimicrobial conifer resin into their nests, which protects ants from infection and leads to reduced immune expression in workers (Castella *et al.*, 2008a; Castella *et al.*, 2008b).

Expanding these studies to include broad cross-species comparisons and other types of defense, such as cellular immunity, could further improve our understanding of immune trade-offs.

Immune trade-offs across species

Immune mechanisms provide beneficial resistance or tolerance to parasite infections, but are costly to deploy and may be functionally redundant if used against similar parasites, suggesting that trade-offs are likely to occur within immune systems (Sheldon & Verhulst, 1996; Rolff & Siva-Jothy, 2003; Sadd & Schmid-Hempel, 2009; Ardia *et al.*, 2011; Parker *et al.*, 2011). Future studies should explore immune trade-offs in a broad macroevolutionary context, an approach that has made significant contributions to our understanding of life history trade-offs (Stearns, 1992), but has not been sufficiently applied to immunity. Comparative approaches may be especially informative because traits in general, including immune specializations, are expected to be more variable between species than within species (Harvey & Pagel, 1991; Stearns, 1992). For example, bee species with different levels of sociality might differentially specialize in social versus individual-level defenses against bacterial pathogens, or butterfly species that have not evolved to handle high levels of toxins could use humoral or cellular defenses against protozoans.

Groups of closely related host species that can use multiple defenses against shared, coevolved parasites are ideal for investigating macroevolutionary immune trade-offs. For example, fruit flies in the *D. melanogaster* species subgroup (Lachaise *et al.*, 1988) share a long coevolutionary history with

parasitoid wasps in the genus *Leptopilina* (Cynipoidea, Figitidae) (Allemand *et al.*, 2002), against which they can deploy cellular (Carton & Nappi, 1997; Carton *et al.*, 2008) and behavioral (Lefèvre *et al.*, 2012b; Milan *et al.*, 2012; Kacsoh *et al.*, 2013) immune mechanisms. Several studies have reported variation across fly species in the strength of cellular immunity and shown that *D. simulans* is much more resistant than its sister species *D. melanogaster* against the parasitoid wasp *Leptopilina boulardi* (Carton & Kitano, 1981; Schlenke *et al.*, 2007; Lefèvre *et al.*, 2012b). Although *D. melanogaster* typically outcompetes *D. simulans* in the laboratory and in the field when *L. boulardi* prevalence is low, presence of *L. boulardi* can mediate coexistence of the two fly species, suggesting that alternative behavioral defenses might be under strong selection in *D. melanogaster* (Fleury *et al.*, 2004). Lefèvre *et al.* (2012b) reported a possible behavioral defense: females of both species avoid ovipositing at wasp-infested sites, but in the forced presence of wasps, *D. melanogaster* significantly reduces its oviposition rate whereas *D. simulans* does not (Lefèvre *et al.*, 2012b). This appears to be a permanent behavioral switch, because female *D. melanogaster* still have significantly reduced lifetime egg output when wasps are removed from the experiment after one day (Lefèvre *et al.*, 2012b). Although the adaptive value of oviposition reduction remains unclear, it might be associated with avoidance of wasp-infested sites in nature or reflect an offspring quality vs. quantity trade-off. The current evidence suggests that *D. simulans* has stronger cellular immunity whereas *D. melanogaster* has a stronger behavioral response. In Chapter 2, I assay these traits across all combinations of eight fruit fly and two parasitoid wasp species to evaluate this potential trade-off across a broader range of species.

The role of outcrossing in host adaptation

Outcrossing is the most prevalent method of reproduction in plants and animals, but its ubiquity is surprising given its costs relative to self-fertilization. These costs include a numerical disadvantage at the group level (the two-fold cost of males) and reduced genetic contribution to offspring at the individual level (the cost of meiosis). Half of the individuals in a typical outcrossing lineage are males that cannot bear offspring, resulting in a two-fold reduction in per-capita birth rate compared to selfing lineages, in which all individuals can produce offspring (Maynard Smith, 1971; 1978). Cross-fertilization between unrelated individuals leads to a 50% reduction in parent-offspring relatedness compared to self-fertilization (Williams, 1975; Charlesworth, 1980). However, these costs do not apply simultaneously and the relevant cost depends on the genetic factors that control production of uniparental offspring (Lively & Lloyd, 1990).

Outcrossing is maintained in the vast majority of plants and animals despite these costs, suggesting that outcrossing must also carry significant benefits. The Red Queen hypothesis predicts that changing environmental conditions produce shifting adaptive landscapes that can favor the production of genetically diverse offspring and thus maintain outcrossing in the long term (Jaenike, 1978a; Hamilton, 1980; Bell, 1982). Host–parasite coevolution is the best-supported mechanism for generating this persistent change (Lively & Morran, 2014). Host populations undergo negative frequency-dependent selection as parasites are selected to infect the most common host genotypes. Self-fertilizing hosts will continue producing offspring with similar genotypes and

common selfed genotypes will suffer disproportionately high levels of infection. Conversely, outcrossing hosts can exchange genetic material across lineages and recombine beneficial mutations into novel genotypes, resulting in genetically diverse offspring that may suffer lower levels of parasite infection on average. Outcrossing will be favored whenever outcrossed offspring gain a fitness advantage from reduced parasitism that outweighs the costs of sex, but the balance between fitness benefits and costs is expected to fluctuate over time.

Studies of natural interactions between a freshwater snail and its trematode parasite have provided strong support for the Red Queen hypothesis. Populations of the freshwater snail *Potamopyrgus antipodarum* consist of asexual females or a mixture of sexual and asexual individuals. The sterilizing trematode parasite *Microphallus* sp. commonly infects these snails. Trematodes adapt to infect locally common snail clones, leading to oscillations in the frequencies of snail clones and the maintenance of clonal diversity (Dybdahl & Lively, 1998; Lively & Dybdahl, 2000). A long-term field study showed that initially common and resistant snail clones became rare and increasingly infected over time, allowing initially rare clones to increase in frequency, while sexual individuals persisted at a relatively consistent frequency (Jokela *et al.*, 2009). A more recent field study found that the ratio of uninfected sexual females to uninfected asexual females was periodically greater than 2:1 and that geometric mean fitness did not differ between the two reproductive modes over five years (Vergara *et al.*, 2014). Therefore, evidence from natural populations suggests that the advantage of reduced parasitism may periodically outweigh the costs of sex and allow for the long-term coexistence of asexual and sexual individuals.

Experimental evolution studies have shown that outcrossing can facilitate adaptation to changing environmental conditions. The nematode *Caenorhabditis elegans* has an androdioecious mating system that is especially amenable to tracking changes in outcrossing rates over time. *C. elegans* populations consist of males and hermaphrodites. Hermaphrodites cannot mate with each other but can self-fertilize or outcross with males (Brenner, 1974). During a 30-generation evolution experiment, Morran *et al.* (2011) passaged nematode populations with either non-evolving bacterial pathogens or pathogens that were taken from host carcasses every generation. Thus, the former treatment imposed directional selection for host resistance, whereas the latter treatment allowed for host–parasite coevolution. Both treatments resulted in elevated outcrossing rates at the beginning of the experiment, but only coevolving parasites favored the maintenance of high outcrossing rates throughout the experiment. The rotifer *Brachionus calyciflorus*, which normally reproduces asexually but excretes a sex-inducing protein at high population densities, is also a useful model for studying the evolution of outcrossing. Becks and Agrawal (2010) showed that rotifer subpopulations that periodically migrated between variable environments were more responsive to the sex-inducing stimulus and produced more sexual offspring as compared to rotifers that experienced constant environmental conditions. Becks and Agrawal (2012) found that sexual offspring were more common and had higher fitness during the initial stages of adaptation to new environments. However, as populations became well-adapted to the novel conditions, asexual offspring had higher fitness and fewer sexual offspring were produced. The combined evidence suggests that single episodes of change can

lead to short-term advantages for outcrossing, but long-term maintenance of outcrossing requires frequently changing selective pressures.

Overview of dissertation research

Fruit fly species in the *Drosophila melanogaster* subgroup show substantial variation in the strengths of their cellular immune responses against parasitoid wasps. Coupled with recent evidence that *D. melanogaster* exhibits stronger avoidance of wasps during oviposition than *D. simulans*, it seems likely that immune specializations differ across fly species and trade-offs might be observed between different defenses. In Chapter 2, I assay behavioral and cellular immunity across all combinations of eight fruit fly and two parasitoid wasp species to test for immune trade-offs. However, I find no evidence for trade-offs between these defenses across fly species; instead, behavioral avoidance seems to be generally employed regardless of wasp virulence. I also find no evidence that wasp-induced oviposition reduction behavior is adaptive, because wasp-exposed mothers produced fewer offspring that showed equal or weaker resistance against wasps. By testing mutants deficient in either sight or smell, I find that both senses are involved in wasp avoidance. This chapter, entitled "Evolution of behavioural and cellular defences against parasitoid wasps in the *Drosophila melanogaster* subgroup" was published in the Journal of Evolutionary Biology in February 2016.

My results from Chapter 2 supported previous studies that found weak cellular immune responses in *D. melanogaster*, suggesting that alternative defenses likely exist. Medication with ethanol is a strong candidate based on the

natural history of this species and recent empirical evidence. However, the behaviors underlying ethanol acquisition in *D. melanogaster* are unclear: earlier studies reported innate attraction to ethanol in larvae and adults, but recent studies showed that exposure to wasps triggers increased ethanol preference. In Chapter 3, I test the effectiveness of ethanol as an anti-wasp treatment before, during, and after exposure to the specialist wasp *Leptopilina boulardi* and the generalist wasp *Leptopilina heterotoma*. I also test whether parasitization leads to increased ethanol food preference in larvae and whether wasp exposure leads to increased ethanol oviposition preference in females. I find that ethanol confers the strongest protection during exposure to the generalist wasp but is relatively ineffective against the specialist wasp, matching theoretical predictions that specialist parasites are more likely to evolve counter-defenses. My results also suggest that *D. melanogaster* has an innate oviposition preference for ethanol, which likely protects offspring against parasitoids that have low ethanol tolerance. This chapter, entitled "Ethanol confers differential protection against generalist and specialist parasitoids of *Drosophila melanogaster*," is in preparation for submission to PLoS ONE.

Current evidence suggests that host–parasite coevolution can favor the long-term maintenance of outcrossing, whereas episodes of environmental change may only favor outcrossing in the short term, until populations adapt to the new conditions. In chapter 4, I use the nematode *Caenorhabditis elegans* and the pathogenic bacterium *Serratia marcescens* to test whether exposure to novel parasite strains leads to increased outcrossing rates during experimental evolution. I find that the most virulent novel parasite induces the greatest

increase in host outcrossing rates over 18 generations. I also find that outcrossing generally facilitates adaptation to novel parasites without causing host populations to lose resistance to parasite strains encountered earlier in their evolutionary history. My results suggest that future studies imposing frequent parasite turnover events may observe long-term maintenance of elevated outcrossing rates in the absence of host–parasite coevolution. This chapter, entitled “Turnover in local parasite populations favors host outcrossing over self-fertilization during experimental evolution,” is in preparation for submission to the Journal of Evolutionary Biology.

Chapter 2

Evolution of behavioural and cellular defences against parasitoid wasps in the *Drosophila melanogaster* subgroup

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Abstract

It may be intuitive to predict that host immune systems will evolve to counter a broad range of potential challenges through simultaneous investment in multiple defenses. However, this would require diversion of resources from other traits, such as growth, survival, and fecundity. Therefore, ecological immunology theory predicts that hosts will specialize in only a subset of possible defenses. We tested this hypothesis through a comparative study of a cellular immune response and a putative behavioral defense used by eight fruit fly species against two parasitoid wasp species (one generalist and one specialist). Fly larvae can survive infection by melanotically encapsulating wasp eggs and female flies can potentially reduce infection rates in their offspring by laying fewer eggs when wasps are present. The strengths of both defenses varied significantly but were not negatively correlated across our chosen host species; thus, we found no

evidence for a trade-off between behavioral and cellular immunity. Instead, cellular defenses were significantly weaker against the generalist wasp, whereas behavioral defenses were similar in strength against both wasps and positively correlated between wasps. We investigated the adaptive significance of wasp-induced oviposition reduction behavior by testing whether wasp-exposed parents produce offspring with stronger cellular defenses, but we found no support for this hypothesis. We further investigated the sensory basis of this behavior by testing mutants deficient in either vision or olfaction, both of which failed to reduce their oviposition rates in the presence of wasps, suggesting that both senses are necessary for detecting and responding to wasps.

Introduction

Trade-offs between life history traits such as age vs. size at maturity, quality vs. quantity of offspring, and early vs. late fecundity are commonly observed and well-described (Stearns, 1992). Following the successful tradition of life history theory, studies in the recently established field of ecological immunology have argued that anti-parasite defenses often impose significant costs, leading to reduced investment in other traits such as somatic growth, survival, and fecundity (Boots & Begon, 1993; Sheldon & Verhulst, 1996; Moret & Schmid-Hempel, 2000; Rolff & Siva-Jothy, 2003; Otti *et al.*, 2012). It is often difficult to explain the underlying mechanisms of these physiological costs (Zera & Harshman, 2001; Rolff & Siva-Jothy, 2003), but autoimmune responses and oxidative stress may be important drivers of reduced lifespan and reproductive potential (Hasselquist & Nilsson, 2012). Trade-offs between immune responses

and life history traits can be revealed through artificial selection experiments. For example, fruit fly lineages selected for increased resistance to parasitoid wasps showed reduced competitive ability and reduced feeding rates (Kraaijeveld & Godfray, 1997; Fellowes *et al.*, 1998; 1999b). Given that many defenses are costly, it is expected that hosts have evolved incomplete immune arsenals characterized by investment in some but not all types of immunity (Parker *et al.*, 2011).

Most studies of host-parasite interactions have focused on the molecular basis for cellular and humoral immune mechanisms (Gillespie *et al.*, 1997; Lemaitre & Hoffmann, 2007; Dodds & Rathjen, 2010; Laughton *et al.*, 2011; Parham & Janeway, 2014). However, hosts can also defend themselves against their parasites through behavioral mechanisms. Behavioral immunity was first described in mammals (Janzen, 1978), including great apes, which are believed to use herbal medicines when sick (Wrangham & Nishida, 1983; Huffman & Seifu, 1989; Huffman, 2003). Although it has been argued that big brains are required for such medication behaviors (Sapolsky, 1994), an increasing number of studies indicate that small-brained insects have also evolved a wide variety of anti-parasite behaviors (de Roode & Lefèvre, 2012). For example, gypsy moth larvae prevent infection by avoiding virus-killed moth cadavers (Capinera *et al.*, 1976; Parker *et al.*, 2010), migratory locusts seek out hot temperatures to overcome fungal parasite infection (Inglis *et al.*, 1996), and woolly bear caterpillars increase the intake of anti-parasitoid chemicals in their diet (Singer *et al.*, 2009). Behavioral defenses may not necessarily benefit the individual performing the behavior, but may instead be directed towards genetic kin. For example, parasite-infected monarch butterflies preferentially lay their eggs on milkweeds with high

levels of cardenolides, and infected offspring that feed on those plants have reduced spore loads and longer lifespans (Lefèvre *et al.*, 2010; Lefèvre *et al.*, 2012a).

The use of alternative defense mechanisms may render traditional cellular and humoral immune responses superfluous. For example, wood ants that incorporate anti-microbial conifer resin into their nests have reduced investment in humoral antimicrobial activity (Castella *et al.*, 2008a; Castella *et al.*, 2008b). Honey bees have a wide range of behavioral defense mechanisms, whereas the honey bee genome lacks many genes that have immune functions in other insects (Evans *et al.*, 2006). Pea aphids harbor mutualistic bacteria that protect them from endoparasitoid wasps and fungal pathogens (Oliver *et al.*, 2003; Parker *et al.*, 2013), but appear to have lost several canonical insect immune genes (Gerardo *et al.*, 2010). Although these examples are compelling, they do not provide direct evidence for trade-offs between alternative defenses across species. Here, we address this outstanding question by comparing the relative strengths of cellular and behavioral immune responses mounted by eight fruit fly species against two parasitoid wasp species.

Fruit flies in the *Drosophila melanogaster* subgroup (Lachaise *et al.*, 1988; David *et al.*, 2007) likely coevolved with endoparasitoid wasps in the genus *Leptopilina* (Hymenoptera: Cynipoidea, Figitidae) across their ancestral African ranges (Allemand *et al.*, 2002). These wasps lay eggs in fly larvae and can impose strong selective pressure on fly populations, given that natural rates of parasitism can exceed 90% (Fleury *et al.*, 2004). Female wasps probe fly larvae with their ovipositors and inject venom along with an egg once they find a suitable host. Fly

larvae that have been attacked can activate a cellular immune response known as melanotic encapsulation, in which the wasp egg is recognized as foreign, activated plasmatocytes bind to it, and lamellocyte production is induced in the lymph gland. After plasmatocytes and lamellocytes have formed a multilayered capsule around the wasp egg, cells inside the capsule release free radicals and the melanin-generating enzyme phenoloxidase, and the developing wasp presumably dies due to toxicity, asphyxiation, or physical entrapment. Wasps have evolved a wide range of counter-defenses; for example, eggs can avoid complete encapsulation by attaching to host tissues and venom can cause host lamellocytes to lose adhesiveness or lyse. Interactions between immune activation and suppression largely determine whether the host or the parasitoid survives to adulthood. Adult flies that have survived parasitism carry melanized capsules that can be observed through the abdominal cuticle or by dissecting the fly (Rizki & Rizki, 1984; Rizki *et al.*, 1990; Carton & Nappi, 1997; Lemaitre & Hoffmann, 2007; Carton *et al.*, 2008; Keebaugh & Schlenke, 2014).

Female flies practice oviposition behaviors that may help protect their offspring from wasps, potentially serving as alternatives or complements to larval melanotic encapsulation responses. Lefèvre *et al.* (2012b) found that both *D. melanogaster* and *D. simulans* preferred to lay eggs at clean sites versus wasp-infested sites, which likely reduces the risk that their offspring will be parasitized. This behavior appears to be driven by olfactory cues, as Ebrahim *et al.* (2015) found that *D. melanogaster* avoided oviposition sites perfumed with wasp odors. In addition to the preference for wasp-free oviposition sites, Lefèvre *et al.* (2012b) showed that female *D. melanogaster* laid significantly fewer eggs when

they were forced to live in vials with wasps. We measured melanotic encapsulation (cellular immunity) and oviposition reduction (behavioral avoidance) responses mounted by seven of the nine species of the *D. melanogaster* subgroup, plus the outgroup species *D. suzukii*, against two wasp species, the specialist *Leptopilina boulardi* and the generalist *L. heterotoma* (Carton *et al.*, 1986; Schlenke *et al.*, 2007). We tested for immune system trade-offs by assessing correlations between cellular immunity and behavioral avoidance across fly species in response to both wasps. Using the same dataset, we analyzed both responses separately to determine whether immunity against the specialist wasp was a significant predictor of immunity against the generalist wasp.

Reduced oviposition in the forced presence of wasps may be related to preference for wasp-free sites in choice tests (Lefèvre *et al.*, 2012b; Ebrahim *et al.*, 2015), or may be adaptive through an alternative mechanism. Here, we tested one such adaptive explanation, which is that flies are subject to a trade-off between offspring quality and quantity (Stearns, 1992). Specifically, female flies that respond to wasp exposure by producing fewer offspring may produce higher-quality offspring that have enhanced immunity against parasitoid wasps (Lefèvre *et al.*, 2012b). We tested this offspring quality vs. quantity trade-off hypothesis by comparing the melanotic encapsulation responses of *D. yakuba* offspring derived from control and wasp-exposed parents.

We further investigated visual and olfactory cues as possible triggers of oviposition reduction behavior, using vision- and olfaction-deficient *D. melanogaster* strains. It was recently reported that female *D. melanogaster* use

the olfactory receptors Or49a and Or85f to detect *L. boulardi* odors, including the sex pheromone iridomyrmecin, and strongly avoid laying eggs at sites with those odors (Ebrahim *et al.*, 2015). Visual detection of wasps can trigger reduced neuropeptide F signaling in the fan-shaped body of fly brains (Kacsoh *et al.*, 2013), which might also lead to changes in oviposition behavior.

Materials and Methods

Insect strains and maintenance

The *D. melanogaster* subgroup consists of nine species that originated in Africa: *D. erecta*, *D. orena*, *D. mauritiana*, *D. melanogaster*, *D. santomea*, *D. sechellia*, *D. simulans*, *D. teissieri*, and *D. yakuba* (Lachaise *et al.*, 1988; David *et al.*, 2007). We did not use *D. mauritiana* or *D. sechellia* in this study, but added *D. suzukii* as an outgroup because it differs from the other fly species in ecological niche, evolutionary history, and immune traits. *D. suzukii* is an invasive fresh fruit pest that originated in east Asia and has recently spread to Europe and North America (Adrion *et al.*, 2014; Atallah *et al.*, 2014). *D. suzukii* larvae have high constitutive hemocyte loads and mount successful melanotic encapsulation responses against a broad range of wasps that successfully parasitize *D. melanogaster* (Kacsoh & Schlenke, 2012; Poyet *et al.*, 2013).

D. erecta (strain 14021-0224.01), *D. orena* (strain 14021-0245.01), *D. santomea* (strain 14021-0271.00), *D. teissieri* (strain 14021-0257.01), and *D. yakuba* (strain 14021-0261.01) were acquired from the Drosophila Species Stock Center at UC San Diego. Multiple lines of *D. melanogaster*, *D. simulans*, and *D. suzukii* were established from single females collected in Atlanta, GA in 2013

using rotting fruit traps. Ten *D. melanogaster*, ten *D. simulans*, and three *D. suzukii* isofemale lines were interbred to create genetically variable populations of each species. Four *D. melanogaster* strains were acquired from the Bloomington Drosophila Stock Center: the wild-type strains *Canton S* (strain 1) and *Oregon R* (strain 5), the white-eyed mutant w^{1118} (strain 5905), and the olfaction-deficient mutant $Orco^{2}$ (strain 23130). *Orco* is a broadly expressed odorant receptor that interacts with specialized receptors to mediate responses to a diverse range of olfactory stimuli. Insertion of the wild-type eye color marker *mini-white* into the *Orco* coding region complements the *white*-null background and leads to defective larval and adult olfactory responses in the null mutant $Orco^{2}$ (Larsson *et al.*, 2004; Vosshall & Hansson, 2011). The sight-deficient *D. melanogaster* strain GMR-*hid* was kindly provided by K.H. Moberg. Ectopic expression of the *head involution defective* (*hid*) gene under the control of GMR, an eye-specific promoter, causes cell death in the developing retina and results in ablated eyes (Grether *et al.*, 1995).

The *Drosophila* medium used for all experiments and maintenance of fly and wasp stocks was prepared by adding 20 L cold water, 1480 g yellow cornmeal (Fisher), 640 g inactive dry yeast (Genesee Scientific), 200 g *Drosophila* agar (Genesee Scientific), and 1750 mL molasses (Good Food, Inc.) to a steam kettle on the "high" stir setting. The food was simmered and stirred for 30 min, then cold water was added to a total volume of 32 L, followed by 460 g Tegosept mold inhibitor (Genesee Scientific) and 100 mL propionic acid (Fisher). After an additional 5–10 min of cooking, the food was dispensed into vials or bottles. This

recipe makes 20 trays of 100 wide vials or 25 square-bottom bottles (Genesee Scientific).

We used two larval endoparasitoid wasp species in the genus *Leptopilina* (Hymenoptera: Cynipoidea, Figitidae) for all experiments: *L. boulardi* (strain Lb17) and *L. heterotoma* (strain Lh14) are inbred strains generated from single females collected in Winters, CA in 2002 (Schlenke *et al.*, 2007). *L. boulardi* and *L. heterotoma* have different host ranges and virulence strategies, leading us to expect substantial variation in the strengths of behavioral and cellular defenses across fly species. *L. boulardi* is a more specialized wasp that often parasitizes flies in the *D. melanogaster* / *D. simulans* clade in nature, whereas *L. heterotoma* is a generalist wasp that successfully parasitizes diverse species across the genus *Drosophila* (Carton *et al.*, 1986; Fleury *et al.*, 2004). Differences in the immune-suppressive effects of *L. boulardi* and *L. heterotoma* venom in *D. melanogaster* larvae may partially explain this difference in host range (Schlenke *et al.*, 2007). *L. heterotoma* venom directly attacks circulating host lamellocytes, causing morphological changes, loss of adhesiveness, and eventually lysis (Rizki & Rizki, 1984). *L. boulardi* venom can alter host lamellocyte morphology to some extent, but does not lyse lamellocytes, and *L. boulardi* eggs can evade complete encapsulation by attaching to host tissues (Rizki *et al.*, 1990). Wasps were maintained by allowing *D. melanogaster* strain *Canton S* flies to lay eggs in food vials for 3 days, then removing the flies and adding ~10 mated female wasps. Adult wasps were kept at 18 °C in food vials with one-half rolled Kimwipe pushed into the center of the food (to control the humidity) and the cotton plug supplemented with 50% honey water (to provide a food source). Wasps were

aged 3–7 days before using them in experiments. All experiments were conducted in a 25 °C incubator with a 12 h light: 12 h dark cycle.

Cellular immunity assays

Bottles with 50 mL *Drosophila* medium and 10 mL water were microwaved to liquefy the food. 35 mm and 60 mm Petri dishes were filled approximately halfway with liquefied food and cooled at 4 °C. Large groups of flies (> 200 adults) were placed in cylindrical mesh-topped embryo collection chambers (Genesee Scientific #59-100) with 60 mm food dishes and allowed to lay eggs. The food dishes were replaced every two days until sufficient numbers of fly larvae had been collected. Before each assay, groups of 40–60 female wasps were placed on *Oregon R* egglay dishes with first and second-instar larvae and allowed to attack for 2 h. We expected these "experienced" wasps to attack more efficiently in the future. Sets of 50 second-instar fly larvae were transferred from egglay dishes to 35 mm food dishes and exposed to six experienced female wasps (Lb17 or Lh14) for 3 h. Two days later, 10 larvae from each dish were dissected. Replicates in which fewer than seven of those 10 larvae contained wasp eggs, wasp larvae, or melanized capsules (i.e. wasp attack rate was <70%) were discarded from the statistical analyses. The remaining larvae were counted and transferred to food vials with one-half rolled Kimwipe in the center of the food (to provide a pupation surface). These vials were checked daily for eclosed flies and wasps for approximately five weeks. Eclosed flies were visually examined or dissected to check for melanized capsules, which indicate that the fly survived parasitism. Flies without capsules were considered unattacked and ignored in the

analyses. All transferred fly larvae that did not eclose were assumed to have died from wasp attacks. Based on the possible eclosion outcomes for attacked larvae: (i) fly survival, (ii) wasp survival, and (iii) death, cellular immunity indices [i / (i + ii + iii)] and proportional eclosion outcomes were calculated for each dish replicate, then averaged across replicates. The number of dish replicates, total eclosion outcomes, and mean cellular immunity index for each fly-wasp combination are shown in Table S1 (Chapter 2 Appendix).

Forced co-habitation assays

Flies were allowed to eclose for three days, then collected in food vials, aged for 24 h, sorted into groups of 25 female and five male flies, and given 24 h to recover from CO_2 anesthesia. The resulting groups of 2–5 day old flies were transferred to new vials in three treatments: (i) no wasps (control), (ii) eight female Lb17, or (iii) eight female Lh14. Insects were transferred to new vials every 24 h for five days, using very light anesthesia to avoid losing insects and minimize any possible effects of CO_2 on behavior and mating success (Barron, 2000). Dead female flies and eggs laid in each vial were counted daily, then cumulative per-female egg counts over five days (PFEC) were calculated. Ideally, five replicates per treatment (control, Lb17, and Lh14) were performed simultaneously. However, for some fly species it was impossible to obtain 375 3–5 day old females (15 vials x 25 females per vial) in a single generation, so multiple experiments were conducted with 2–4 replicates per treatment. Within each experiment, oviposition maintenance indices for each Lb17 and Lh14 replicate were calculated as $[PFEC_{Lb17} / mean(PFEC_{control})]$ or $[PFEC_{Lh14} / mean(PFEC_{control})]$ and

considered independent replicates. The number of vial replicates, mean PFEC for each treatment, and mean oviposition maintenance index for each fly-wasp combination are shown in Table S2 (Chapter 2 Appendix).

Adaptive significance of behavioral avoidance

Groups of 25 female and five male *D. yakuba* aged 1–3 days were placed in vials to generate 3 parental treatments: (i) no wasps, (ii) 10 female Lb17, or (iii) 10 female Lh14. After a 3-day acclimation period, the insects were transferred to new vials daily for six days. Eggs were counted every 24 h and cumulative per-female egg counts were calculated. On days 3 and 4, fine forceps were used to transfer unhatched eggs from the vials into 35 mm food dishes. Due to variation in oviposition and hatching rates, the number of eggs per dish ranged from 20–44. Collecting unhatched eggs was necessary because some fly larvae that hatched in the egglay vials had already been attacked by wasps, making them unsuitable for controlled wasp exposures. 60 h later, when most larvae had grown to second instar, six female Lb17 were added to each dish for 3 h exposures. We focused on *D. yakuba* and Lb17 because this host-parasitoid combination resulted in substantial fly survival, wasp survival, and death in our cellular immunity assays (Chapter 2 Appendix, Table S1), suggesting that wasp-induced changes in parental investment might lead to enhanced offspring immune responses and higher fly survival. Fly larvae were not exposed to Lh14 because baseline resistance to Lh14 is extremely low (Chapter 2 Appendix, Table S1) and seems unlikely to change with different parental treatments. Immediately following the wasp exposures, the contents of each dish (food and larvae) were scooped into

food vials with one-half rolled Kimwipe. Approximately two weeks later, surviving fly offspring with and without melanized capsules were counted. We assumed that fly larvae could survive exposure to Lb17 by mounting a successful cellular immune response (adults with capsules) or avoiding parasitism (adults without capsules), and both outcomes were compared across parental treatments. A similar experiment was performed with *D. melanogaster* and *D. simulans* (Chapter 2 Appendix).

Sensory basis of behavioral avoidance

The behavioral responses of wild-type (*Oregon R*), white-eyed mutant (w^{1118}), sight-deficient mutant (GMR-*hid*), and olfaction-deficient mutant ($Orco^{2}$) *D. melanogaster* strains to Lb17 and Lh14 were measured using the previously described forced co-habitation assays. We hypothesized that the wild-type flies would reduce their oviposition rates in the presence of wasps and that one or more mutants would fail to respond, suggesting that detection of wasps requires vision, olfaction, or both.

Phylogenetic analysis

We were unable to find a published phylogeny with all eight of our chosen fly species to use for calculating phylogenetically independent contrasts. Therefore, we constructed a phylogeny using the amylase-related protein gene *Amyrel* (Da Lage *et al.*, 1998), which has been used to estimate multiple *Drosophila* phylogenies (Cariou *et al.*, 2001; Kopp, 2006; Da Lage *et al.*, 2007; Yang *et al.*, 2012). Although we were unable to find any gene in GenBank with

complete coding sequences for all eight fly species, we found a partial *Amyrel* coding sequence for *D. suzukii* and complete coding sequences for the other seven species (Chapter 2 Appendix, Table S3). These sequences were aligned and trimmed in Mesquite version 2.75 (Maddison & Maddison, 2011) using MUSCLE version 3.8.31 (Edgar, 2004), resulting in an aligned region of 1411 base pairs. The fly phylogeny was estimated using MrBayes version 3.2 (Ronquist *et al.*, 2012), which implements Bayesian inference (Larget & Simon, 1999) within a Markov Chain Monte Carlo simulation framework (Metropolis *et al.*, 1953; Hastings, 1970). Nucleotide substitution rate parameters were estimated using a generalized time-reversible model (Rodriguez *et al.*, 1990) with gamma-distributed rate variation across sites (Yang, 1993). Two separate runs with four interacting chains were executed for 1,000,000 generations and sampled every 500 generations. The first 25% of the posterior distributions were regarded as burn-in and ignored. The resulting 50% majority rule consensus tree (Fig. 1) was prepared using FigTree version 1.4.2 (Rambaut, 2014). The topology of the tree is consistent with previous studies (Da Lage *et al.*, 2007; Yang *et al.*, 2012).

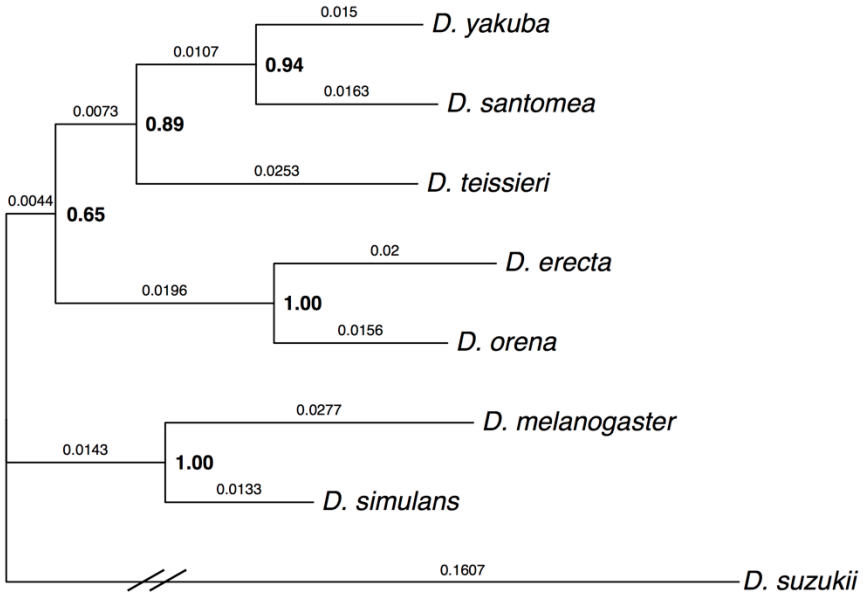

Figure 1. Phylogeny of the eight fly species. This phylogeny was constructed from *Amyrel* coding sequences using Bayesian inference; estimated branch lengths are shown and posterior probabilities are given for each node. A break was added to the *D. suzukii* branch because it is nearly 8 times longer than any other branch when drawn to scale.

Statistical analysis

Unless otherwise stated, all analyses were performed in R version 3.0.3 (R Development Core Team, 2014). For cellular immunity data, the effects of fly species, wasp species, and their interaction on proportional fly survival were analyzed using generalized linear models (GLMs) with quasi-binomial error distributions and logit link functions. Pairwise comparisons between fly species' responses to *L. boulardi* were assessed using Tukey's honestly significant difference (HSD) tests. *D. melanogaster*, which never survived parasitism by either wasp (Fig. 2), was removed from the overall analysis and manually assigned to the lowest significance group because the logistic regression algorithm does not converge when proportional fly survival equals zero. Pairwise comparisons were not carried out for responses to *L. heterotoma* because only *D.*

suzukii had an appreciable survival rate. All pairwise comparisons were assessed using the R package multcomp (Hothorn *et al.*, 2008).

For behavioral avoidance data, the effects of fly species, wasp species, and their interaction on log-transformed oviposition reduction indices were analyzed using an ANOVA. Shapiro-Wilk normality tests ($W > 0.843$, $P > 0.062$ for all combinations of fly and wasp species), visual inspection of the normal Q-Q plot for the entire dataset, and a Fligner-Killeen test comparing variances across fly-wasp combinations ($\chi^2_{15} = 21.61$, $P = 0.119$) suggested that the log-transformed oviposition reduction indices did not violate the normality or homoscedasticity assumptions of ANOVA. The effects of wasp treatment (control, Lb17, or Lh14) on cumulative per-female egg counts over five days were analyzed individually for each fly species using GLMs with quasi-Poisson error distributions and log link functions, then pairwise comparisons were assessed using Tukey's HSD tests.

Traits measured across species cannot be considered statistically independent because evolutionary changes are shared along internal branches of phylogenetic trees. We addressed this problem by calculating phylogenetically independent contrasts (Felsenstein, 1985) from the mean cellular immunity and oviposition maintenance indices for each fly-wasp combination using the *Amyrel* phylogeny (Fig. 1) and the Contrast program in PHYLIP version 3.69 (Felsenstein, 2005). Correlations between oviposition maintenance vs. cellular immunity index contrasts measured across fly species in response to (i) *L. boulardi* and (ii) *L. heterotoma* were assessed to test for immune system trade-offs. Significant positive correlations would support the trade-off hypothesis because high oviposition maintenance indices indicate weak behavioral

avoidance. Correlations between (i) cellular immunity index contrasts and (ii) oviposition maintenance index contrasts measured across fly species in response to *L. heterotoma* vs. *L. boulardi* were assessed to determine whether defenses against the specialist wasp were significant predictors of defenses against the generalist wasp. Felsenstein's (1985) method produces pairs of contrasts that can be regarded as being drawn from a bivariate normal distribution with mean = 0 and variance = 1. Therefore, a Pearson product-moment correlation coefficient test was performed in each case, with the null hypothesis that the correlation between paired contrasts was equal to zero. Transforming the branch lengths of the phylogeny based on: (i) equal branch lengths, (ii) Grafen's (1989) method, and (iii) Pagel's (1992) method, implemented in version 1.15 of the PDAP:PDTREE package for Mesquite (Midford *et al.*, 2009), did not qualitatively change our conclusions. Only the results from the PHYLIP analysis based on our *Amyrel* phylogeny are reported.

For the *D. yakuba* adaptive significance experiment, the effects of parental treatment (control, Lb17-exposed, or Lh14-exposed) on cumulative per-female egg counts over six days were analyzed using GLMs with quasi-Poisson error distributions and log link functions, followed by pairwise comparisons using Tukey's HSD tests. The effects of parental treatment on proportional offspring survival following exposure to Lb17 were analyzed using GLMs with quasi-binomial error distributions and logit link functions, followed by pairwise comparisons using Tukey's HSD tests. Separate analyses were performed for the two survival outcomes (with or without a melanized capsule) and their sum. To evaluate the sensory basis of behavioral avoidance, the effects of wasp treatment

(control, Lb17, or Lh14) on cumulative per-female egg counts over five days were analyzed using GLMs with quasi-Poisson error distributions and log link functions, followed by pairwise comparisons using Tukey's HSD tests. Separate analyses were performed for each fly strain.

Results

Fly larvae can survive wasp infection by melanotically encapsulating wasp eggs and female flies can potentially reduce infection rates in their offspring by laying fewer eggs when wasps are present. To test for trade-offs between these defenses and assess their generality across host species, we measured cellular immune responses and oviposition reduction behaviors using eight fly species and two wasp species. We defined two summary statistics: (i) cellular immunity index, the mean proportional fly survival following wasp attack; and (ii) oviposition maintenance index, the cumulative per-female egg count during 5-day assays (PFEC) for females that were forced to live with wasps divided by PFEC for females kept in wasp-free environments.

Cellular immunity indices (Fig. 2, equivalent to proportional fly survival) varied significantly across fly species ($F_{6,138} = 89.5$, $P < 0.0001$); resistance to *L. boulardi* was stronger overall ($F_{1,137} = 468$, $P < 0.0001$), and there was a significant interaction between fly species and wasp species ($F_{6,131} = 34.6$, $P < 0.0001$). *D. santomea* and *D. suzukii* had the strongest resistance to *L. boulardi*, followed by *D. teissieri*, *D. yakuba*, *D. orena*, and *D. erecta*, whereas *D. simulans* had very weak resistance and *D. melanogaster* never survived parasitism (Fig. 2a; $z > 3.78$, $P < 0.003$ for all significant pairwise comparisons). Most fly species

had no resistance to *L. heterotoma* (Fig. 2b). However, *D. suzukii* was an unsuitable host for both wasps, surviving parasitism approximately 60% of the time and never allowing either wasp to develop successfully.

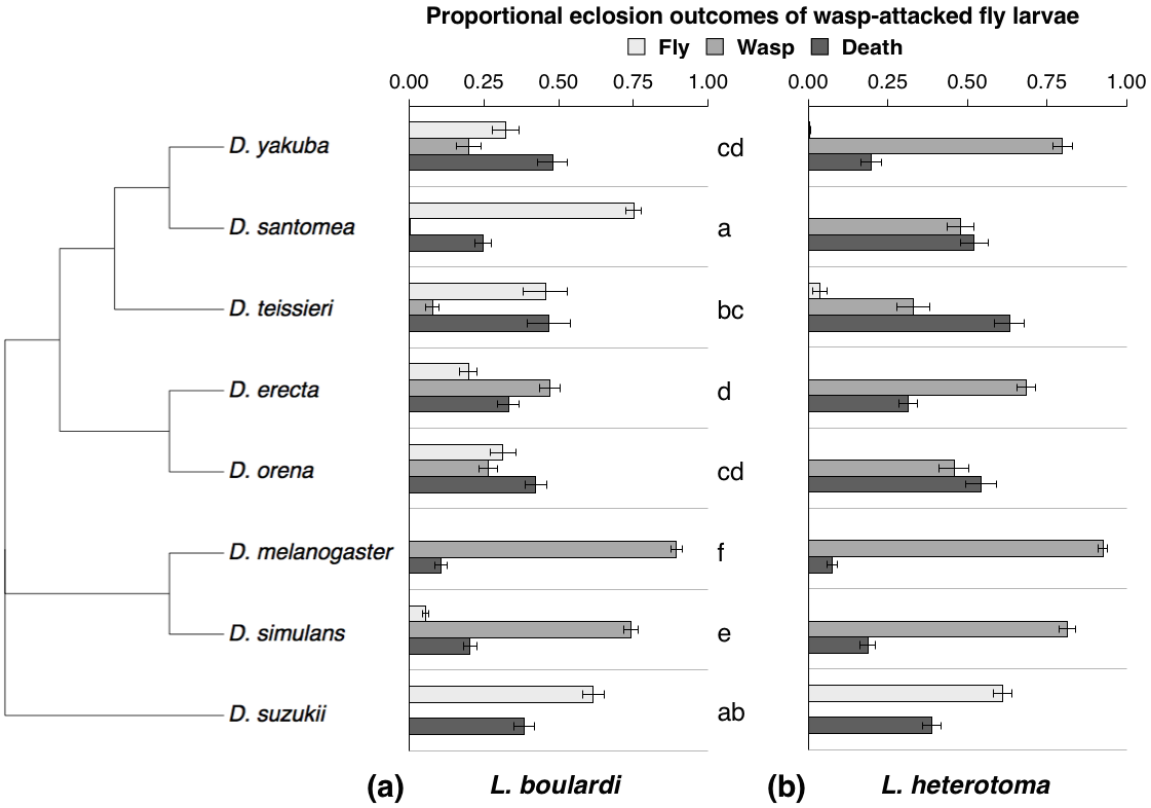

Figure 2. Cellular immunity indices. Mean proportions of fly larvae attacked by (a) *L. boulardi* and (b) *L. heterotoma* that: (i) eclosed as flies with melanized capsules, (ii) eclosed as wasps, or (iii) died (±1 SEM). Sample sizes are shown in Table S1 (Chapter 2 Appendix). Different letters in (a) indicate groups of species that had significantly different fly survival after exposure to *L. boulardi* ($z > 3.78$, $P < 0.003$). Pairwise comparisons were not carried out in (b) because most fly species had zero survival against *L. heterotoma*.

Oviposition maintenance indices (Fig. 3) varied significantly across fly species ($F_{7,92} = 14.7$, $P < 0.0001$), but there was no effect of wasp species ($F_{1,92} = 0.046$, $P = 0.831$) and no significant interaction between fly species and wasp species ($F_{7,92} = 1.31$, $P = 0.256$). Most fly species responded to both wasps by significantly reducing their oviposition rates. Specifically, *D. erecta* and *D. orena*

maintained similar oviposition rates regardless of wasp treatment ($z < 1.04$, $P > 0.554$ for control vs. *L. boulardi* and control vs. *L. heterotoma* comparisons), whereas all other fly species significantly reduced their oviposition rates in response to both wasps ($z > 2.32$, $P < 0.05$ for control vs. *L. boulardi* and control vs. *L. heterotoma* comparisons).

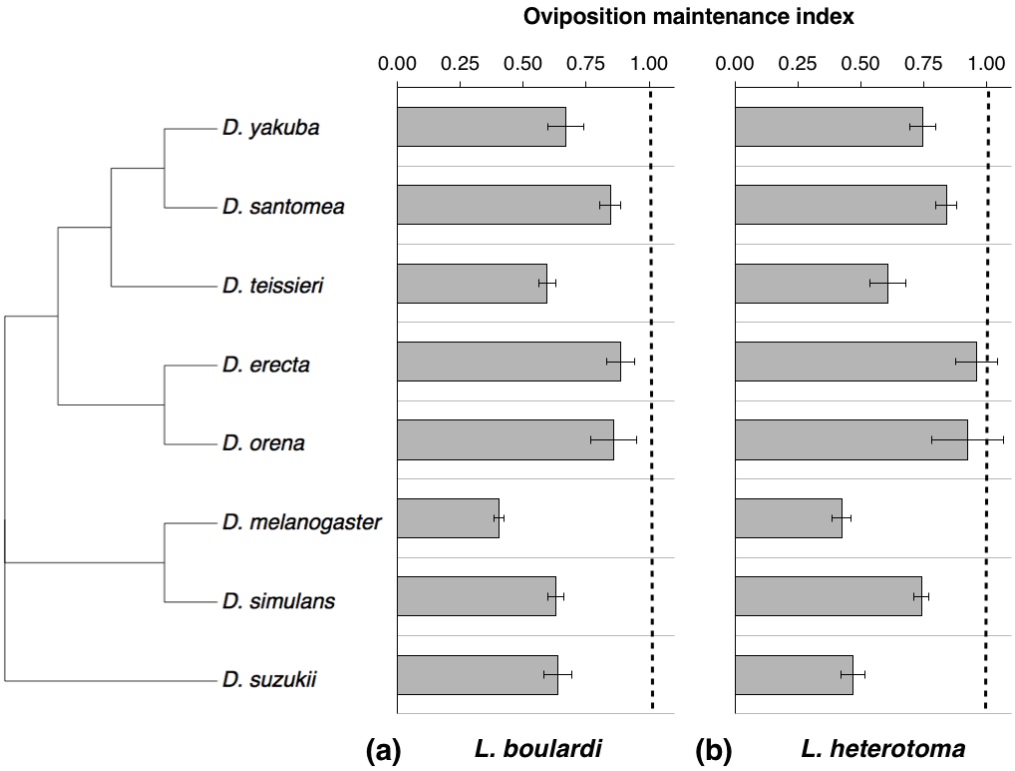

Figure 3. Oviposition maintenance indices. Mean oviposition maintenance indices for female flies housed with (a) *L. boulardi* and (b) *L. heterotoma* (±1 SEM). Sample sizes are shown in Table S2 (Chapter 2 Appendix). Oviposition maintenance indices equal to 1.00 (dashed lines) would indicate no difference in oviposition rates between control and wasp-exposed flies.

Cellular immunity and behavioral avoidance may carry significant costs (Kraaijeveld *et al.*, 2002; Lefèvre *et al.*, 2012b) and both traits varied significantly across fly species, suggesting that trade-offs might occur between these defenses. We tested for trade-offs using phylogenetically independent contrasts to control

for shared evolutionary history and non-independence of traits across fly species (Felsenstein, 1985). We found no significant correlations between cellular immunity and behavioral avoidance responses measured across fly species in response to *L. boulardi* (Fig. 4a; $r = 0.510$, $t_5 = 1.33$, $P = 0.242$) or *L. heterotoma* (Fig. 4b; $r = -0.329$, $t_5 = -0.778$, $P = 0.472$), indicating that these defenses do not trade off across the fly phylogeny.

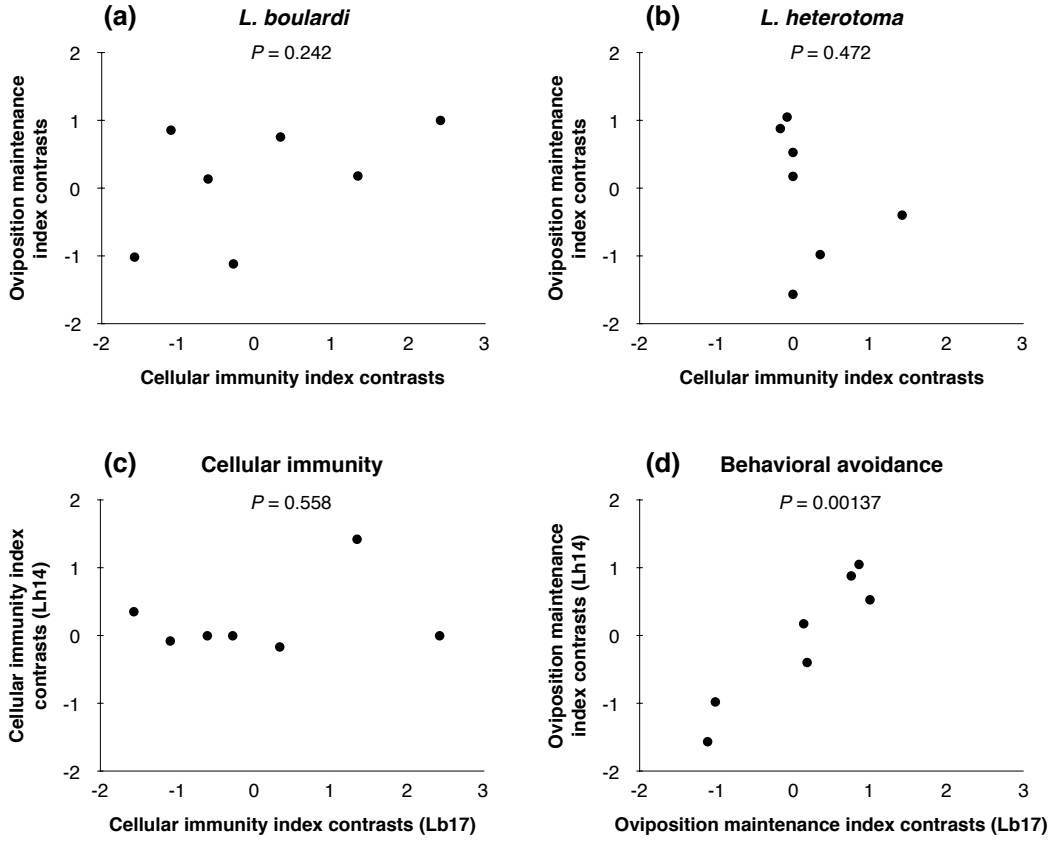

Figure 4. Cellular immunity and oviposition maintenance correlations. Correlations between phylogenetically independent contrasts for: (a) fly oviposition maintenance indices vs. cellular immunity indices using *L. boulardi*, (b) fly oviposition maintenance indices vs. cellular immunity indices using *L. heterotoma*, (c) fly cellular immunity indices comparing *L. heterotoma* vs. *L. boulardi* infection, and (d) fly oviposition maintenance indices comparing *L. heterotoma* vs. *L. boulardi* exposure. *P* values from Pearson correlation tests are shown in each panel.

L. heterotoma can develop successfully on multiple hosts across the genus *Drosophila*, whereas *L. boulardi* has a more restricted host range that includes the *D. melanogaster* group (Carton *et al.*, 1986; Schlenke *et al.*, 2007). This difference in host range might be explained by differences in immune suppression mechanisms (Rizki & Rizki, 1984; Rizki *et al.*, 1990; Schlenke *et al.*, 2007), leading us to predict that fly cellular immune responses would not necessarily be effective against both wasp species. The strengths of cellular immune responses against *L. heterotoma* vs. *L. boulardi* were not positively correlated across fly species (Fig. 4c; $r = 0.270$, $t_5 = 0.628$, $P = 0.558$), suggesting that resistance to the specialist wasp is a poor predictor of resistance to the generalist wasp. However, the strengths of behavioral avoidance responses to *L. heterotoma* vs. *L. boulardi* were positively correlated across fly species (Fig. 4d; $r = 0.944$, $t_5 = 6.41$, $P = 0.00137$), suggesting that female flies do not distinguish between these wasp species when reducing oviposition.

Laying fewer eggs in the presence of wasps could be an adaptive behavior if the resulting offspring have enhanced immunity against wasps (Lefèvre *et al.*, 2012b). We tested this hypothetical offspring quality vs. quantity trade-off (Stearns, 1992) by measuring the oviposition rates of *D. yakuba* parents in three treatments: (i) control (no wasps), (ii) exposed to *L. boulardi*, and (iii) exposed to *L. heterotoma*, then measuring offspring survival after exposure to *L. boulardi*. Wasp-exposed mothers laid significantly fewer eggs over six days than control mothers (Fig. 5a; $z > 3.10$, $P < 0.006$ for control vs. Lb17 and control vs. Lh14 comparisons). There was a significant effect of parental treatment on offspring resistance to *L. boulardi* (Fig. 5b; $F_{2,16} = 4.24$, $P = 0.0332$). Offspring of control

and Lb17-exposed parents had similar melanotic encapsulation success against Lb17 (z = 0.202, P = 0.978), whereas offspring of Lh14-exposed parents had significantly lower encapsulation success (z = 2.65, P = 0.0214). The majority of surviving offspring of Lh14-exposed parents lacked melanized capsules, indicating that they avoided parasitism. However, there was no significant effect of parental treatment on the proportion of unparasitized offspring (Fig. 5b; $F_{2,16}$ = 3.31, P = 0.0628) or total offspring survival (Fig. 5b; $F_{2,16}$ = 0.434, P = 0.655). Wasp-exposed parents laid significantly fewer eggs and their offspring did not have enhanced survival when exposed to wasps, suggesting that this behavior is costly to fly fitness. Thus, we found no evidence for a trade-off between offspring quality and quantity.

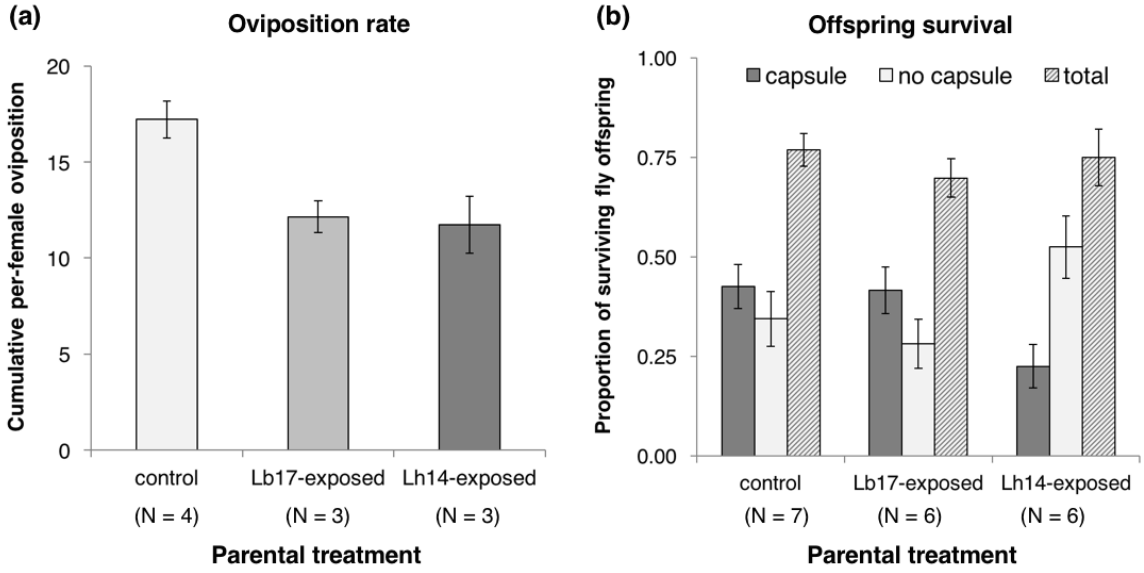

Figure 5. Testing for an offspring quality vs. quantity trade-off in *D. yakuba*. (a) Mean cumulative per-female oviposition over six days for *D. yakuba* in food vials without wasps (control), with *L. boulardi* (Lb17-exposed), and with *L. heterotoma* (Lh14-exposed) (±1 SEM). (b) Mean proportions of *D. yakuba* larvae produced in each parental treatment that eclosed as flies: (i) with melanized capsules or (ii) without melanized capsules, along with total fly survival (i)+(ii), after being exposed to *L. boulardi* (±1 SEM). Sample sizes indicate number of vial replicates in (a) and number of dish replicates in (b).

To investigate the sensory basis of oviposition reduction, we compared the behaviors of wild-type and white-eyed *D. melanogaster* to those of vision- and olfaction-deficient mutants. Both wild-type (*Oregon R*) and white-eyed (w^{1118}) flies had significantly reduced cumulative per-female oviposition over five days when they were forced to live with female wasps (Fig. 6a,b; $z > 3.44$, $P < 0.002$ for control vs. Lb17 and control vs. Lh14 comparisons). In contrast, the sight-deficient mutant GMR-*hid* and the olfaction-deficient mutant $Orco^2$ showed no significant changes in oviposition when housed with either wasp (Fig. 6c,d; $z < 1.76$, $P > 0.18$ for control vs. Lb17 and control vs. Lh14 comparisons). These results suggest that female *D. melanogaster* require both visual and olfactory cues to detect parasitoid wasps and reduce their oviposition rates.

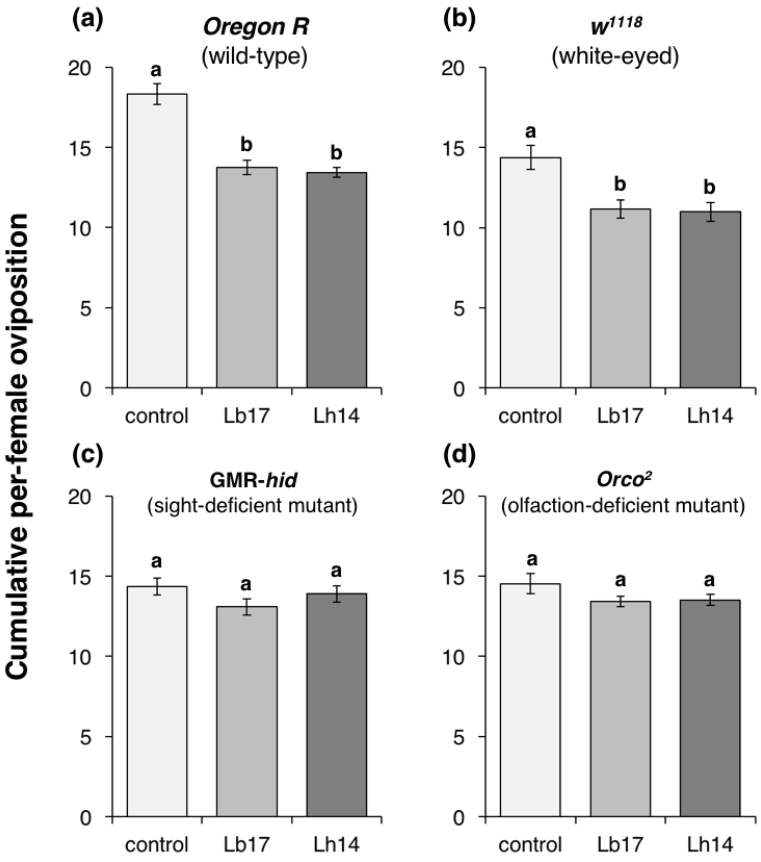

Figure 6. Behavioral avoidance in sensory mutant strains. Mean cumulative per-female oviposition over five days in food vials without wasps (control), with *L. boulardi* (Lb17), and with *L. heterotoma* (Lh14) for different *D. melanogaster* strains: (a) *Oregon R* (wild-type), (b) w^{1118} (white-eyed), (c) GMR-*hid* (sight-deficient mutant), and (d) $Orco^2$ (olfaction-deficient mutant) (±1 SEM). The y-axis label is the same for all panels. N = 5 vial replicates per treatment per strain. Different letters indicate significant differences between treatments ($z > 3.44$, $P < 0.002$).

Discussion

We measured melanotic encapsulation and oviposition reduction responses across all combinations of eight fruit fly species and two larval endoparasitoid wasp species (Figs. 2 and 3). We found no significant correlations between the strengths of these defenses and thus no evidence that they trade off across fly species (Fig. 4a,b). Resistance to *L. boulardi* was a poor predictor of

resistance to *L. heterotoma* (Fig. 4c). This is not surprising because the two wasps have different virulence mechanisms, *L. heterotoma* has a broader natural host range, and we expected *L. heterotoma* to be more virulent across our chosen host species (Rizki & Rizki, 1984; Carton *et al.*, 1986; Rizki *et al.*, 1990; Schlenke *et al.*, 2007). Oviposition reduction responses to both wasps were positively correlated across fly species (Fig. 4d), suggesting that this behavior is generalized, not attuned to different wasp species based on their virulence levels.

Previous studies using fruit flies and parasitoid wasps have revealed trade-offs between cellular immune responses and life history traits. *D. melanogaster* lines artificially selected for increased resistance to *Asobara tabida* and *L. boulardi* evolved approximately 11-fold and 100-fold increases in melanotic encapsulation ability (respectively) within just five generations, but showed reduced competitive success in parasite-free environments with severely limited food (Kraaijeveld & Godfray, 1997; Fellowes *et al.*, 1998). Subsequent studies identified physiological changes underlying this trade-off: larvae from the selected lines had increased circulating hemocyte densities (Kraaijeveld *et al.*, 2001) and reduced feeding rates (Fellowes *et al.*, 1999b). Lefèvre et al. (2012b) tested for a possible trade-off within fly immune systems by assaying behavioral and cellular immune responses mounted by the sister species *D. melanogaster* and *D. simulans* against *L. boulardi*. Females of both species avoided laying eggs in wasp-infested oviposition sites when given a choice, but only *D. melanogaster* laid fewer eggs when forced to live with wasps. Conversely, a substantial proportion of *D. simulans* larvae successfully encapsulated wasp eggs, whereas no *D. melanogaster* larvae survived parasitism, suggesting that *D. simulans*

invests more resources in cellular immunity than behavioral defense. We expanded the Lefèvre et al. (2012b) study by testing a broader range of host-parasitoid interactions but failed to confirm any consistent trade-off between melanotic encapsulation and oviposition reduction (Fig. 4a,b).

The lack of a trade-off was surprising given that the strengths of both defenses varied significantly across fly species (Figs. 2 and 3). We would expect this variation to manifest as a negative correlation between the two defenses across host species, assuming that both traits involve fitness costs and benefits. However, there are several reasons why this might not have occurred. Perhaps simultaneous investment in both strategies is favored because they are only partially successful when used alone. Another possibility is that one or both defenses do not entail a significant cost. Encapsulation success is positively correlated with level of constitutive hemocyte production (Eslin & Prevost, 1998; Kraaijeveld *et al.*, 2001; Sorrentino *et al.*, 2004; Moreau *et al.*, 2005), which is likely to require substantial resource investment. If larvae delay pupation and continue eating until they have reached a threshold weight (Robertson, 1963), then the physiological costs to larvae of mounting an encapsulation response may be disconnected from adult fitness. However, fly larvae that successfully defend themselves against wasps may suffer significant fitness costs as adults, including smaller body size and reduced fecundity (Fellowes *et al.*, 1999a), and weaker resistance to desiccation and starvation stresses (Hoang, 2001).

The immediate fitness cost of laying fewer eggs in the presence of wasps is obvious, but there are at least three ways in which this behavior could be adaptive. First, flies may withhold eggs where wasps are present, then lay them in

non-infested oviposition sites in the future. In previous work, flies continued to lay fewer eggs even after they were moved to new vials without wasps (Lefèvre *et al.*, 2012b). However, delayed reproduction may occur in natural situations where potential associations between environment and wasp presence are not as strong as in lab vials. Second, female flies might modulate their allocation of reproductive resources to produce fewer offspring with enhanced immunity. However, we found that *D. yakuba* housed in wasp-infested environments produced fewer offspring and those offspring were not better able to survive exposure to *L. boulardi* (Fig. 5). We obtained similar results when we tested *D. melanogaster* and *D. simulans* against both wasp species (Chapter 2 Appendix, Fig. S1). Third, wasp-exposed parents could produce offspring that are better able to avoid wasp attacks. Examples of larval avoidance include rolling towards the attacking wasp to disrupt ovipositor penetration (Hwang *et al.*, 2007) and crawling away from wasp semiochemicals (Ebrahim *et al.*, 2015). Unparasitized offspring will not suffer the costs of mounting anti-wasp immune responses, which can include smaller body size, reduced fecundity, and weaker stress resistance (Fellowes *et al.*, 1999a; Hoang, 2001). Future studies should compare offspring derived from control and wasp-exposed parents by measuring avoidance mechanisms in larvae and life history traits in adults that survive exposure to wasps.

We must also consider the possibility that the wasp-mediated oviposition reduction behavior is a byproduct of some other adaptive behavior and is not adaptive itself. Insects that do not exhibit parental care must lay their eggs in food sources that can reliably support offspring development, and we expect

females to avoid unsuitable oviposition sites if nearby alternatives are available (Jaenike, 1978b). For example, Lefèvre et al. (2012b) found that female *D. melanogaster* and *D. simulans* avoided wasp-infested sites during oviposition preference experiments. Although oviposition reduction behavior might appear to be a maladaptive stress response in the context of artificial forced co-habitation experiments, perhaps this behavior manifests as adaptive avoidance of wasp-infested sites when multiple choices are available. Future studies should investigate this possibility by conducting oviposition choice experiments (rather than the forced co-habitation assays used here). Measuring wasp avoidance behavior in this way might lead to different conclusions about trade-offs with cellular immunity across fly species. Before testing for trade-offs with other defenses, it would be ideal to characterize the costs and benefits of parasite-induced changes in oviposition behavior. For example, the seed beetle *Mimosestes amicus* can lay inviable eggs as "shields" to protect the eggs below from the parasitoid wasp *Uscana semifumipennis* (Deas & Hunter, 2012). However, *M. amicus* detects parasitized eggs on potential host plants and may choose to delay oviposition or seek a clean site rather than suffer the costs of laying defensive eggs (Deas & Hunter, 2013).

Olfactory cues trigger a diverse range of behavioral responses in fruit flies and their natural enemies. *D. melanogaster* avoids oviposition sites containing toxic microbes that produce the volatile compound geosmin, which is detected by the odorant receptor *Or56a* (Stensmyr *et al.*, 2012). Conversely, *D. melanogaster* selectively oviposits on *Citrus* spp. fruits, which produce volatile terpenes that activate the odorant receptor *Or19a*. Valencene, the primary ligand of *Or19a*,

strongly repels *L. boulardi*, and fly larvae feeding on valencene-containing substrates experience reduced rates of parasitism (Dweck *et al.*, 2013). *L. boulardi* and *L. heterotoma* have evolved foraging behaviors that exploit host odorants, such as water-soluble kairomones produced by larvae and aggregation pheromones deposited by female flies during oviposition (Vet *et al.*, 1993; Wiskerke *et al.*, 1993; Hedlund *et al.*, 1996). However, the possible roles of visual cues in fly-parasite interactions are not as well understood. Our present study suggests that some behavioral responses to parasitoid wasps require interpretation of both olfactory and visual cues. Wild-type *D. melanogaster* strains significantly reduced their oviposition rates in the presence of wasps, whereas mutants deficient in either olfaction or vision failed to show this response (Fig. 6).

There is one potential caveat with our result: the olfaction-deficient mutant $Orco^{2}$ was created by inserting a *mini-white* gene into the *Orco* coding region in a *white*-null background (Larsson *et al.*, 2004). Various behavioral deficiencies have been reported in *white* mutants, including impaired optomotor responses (Kalmus, 1943), reduced anesthetic sensitivity (Campbell & Nash, 2001), and enhanced male-male courtship (Anaka *et al.*, 2008; Krstic *et al.*, 2013). Furthermore, insertion of *mini-white* into *white*-null backgrounds does not always restore wild-type eye coloration (Hazelrigg *et al.*, 1984; Silicheva *et al.*, 2010) or behavior (Krstic *et al.*, 2013). We observed a range of eye coloration in $Orco^{2}$ flies, including pale red, orange, and slightly lighter than wild-type red. However, to address these concerns, we tested the *white*-null strain w^{1118}. Any deficiencies associated with *white*-null mutants should be fully expressed in w^{1118}

and may be partially rescued in *Orco²*. We found that *w¹¹¹⁸* showed the same wasp-induced oviposition reduction behavior as the wild-type *Oregon R*, suggesting that the absence of this behavior in *Orco²* is most readily explained by olfaction deficiency, not genetic background effects (Fig. 6).

Given the benefits of anti-parasite defenses, an intuitive expectation is that hosts should simultaneously maximize their investment in a wide range of defenses. However, the emerging view from the recently established field of ecological immunology is that defenses are costly and we should expect trade-offs between alternative immune responses (Sheldon & Verhulst, 1996; Norris & Evans, 2000; Rolff & Siva-Jothy, 2003; Ardia *et al.*, 2011; Parker *et al.*, 2011). Despite substantial evidence that trade-offs occur within the immune systems of individual species, comparative studies of immune arsenals used by multiple host species against their shared parasites are lacking. The *Drosophila-Leptopilina* system appears to be an excellent model for studying trade-offs between different immune mechanisms. However, we found no evidence for such a trade-off between melanotic encapsulation and oviposition reduction. Future studies incorporating multiple immune mechanisms from each host life stage and/or different measures of behavioral avoidance might be required to validate this key prediction of ecological immunology theory.

Acknowledgements

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Chapter 2 Appendix

Table S1. Cellular immunity dish replicates (reps), eclosion outcomes, and cellular immunity indices. The sum of eclosion outcomes for each fly-wasp combination equals the number of larvae that were transferred to eclosion vials. Each dish started with 50 larvae, 10 of which were dissected to estimate wasp attack rates, but the sums are less than 40 x number of replicates because some larvae died before being transferred.

Fly	Wasp	Reps	Flies without capsules	Flies with capsules	Wasps	Deaths	Cellular immunity index
D. erecta	Lb17	11	22	74	174	122	0.1987
D. melanogaster	Lb17	14	43	0	452	50	0.0000
D. orena	Lb17	12	15	90	76	116	0.3127
D. santomea	Lb17	12	28	302	1	98	0.7509
D. simulans	Lb17	12	27	22	295	81	0.0555
D. suzukii	Lb17	10	95	154	0	99	0.6160
D. teissieri	Lb17	10	26	154	28	148	0.4560
D. yakuba	Lb17	9	130	72	42	104	0.3218
D. erecta	Lh14	10	48	0	218	99	0.0000
D. melanogaster	Lh14	12	15	0	408	33	0.0000
D. orena	Lh14	9	16	0	93	99	0.0000
D. santomea	Lh14	10	28	0	146	166	0.0000
D. simulans	Lh14	13	21	0	368	83	0.0000
D. suzukii	Lh14	10	82	148	0	100	0.6109
D. teissieri	Lh14	9	21	10	95	177	0.0364
D. yakuba	Lh14	8	28	1	205	52	0.0037

Table S2. Forced co-habitation vial replicates (reps), cumulative per-female egg counts (PFEC), and oviposition maintenance indices (OMI). Control, *L. boulardi* (Lb17), and *L. heterotoma* (Lh14) treatments were performed simultaneously for each fly species such that egg counts from Lb17 and Lh14 vials could be compared to single groups of control vials.

Fly	Control reps	Control PFEC	Lb17 reps	Lb17 PFEC	Lb17 OMI	Lh14 reps	Lh14 PFEC	Lh14 OMI
D. erecta	5	4.848	5	4.344	0.8864	5	4.696	0.9586
D. melanogaster	5	10.040	5	3.953	0.4045	5	4.274	0.4242
D. orena	8	3.028	7	2.847	0.8602	6	3.364	0.9251
D. santomea	9	9.065	9	7.693	0.8463	9	7.637	0.8404
D. simulans	5	12.256	5	7.688	0.6299	5	8.526	0.7405
D. suzukii	8	2.938	8	1.972	0.6354	7	1.474	0.4688
D. teissieri	7	4.262	7	2.491	0.5950	7	2.490	0.6066
D. yakuba	9	5.885	9	3.912	0.6689	9	4.382	0.7465

Table S3. Sources for *Amyrel* coding sequences. Accession number and length of each amylase-related protein gene (*Amyrel*) coding sequence downloaded from GenBank.

Fly species	GenBank accession number	Base pairs	Reference
D. erecta	AF039562.2	1482	Da Lage et al. 2007
D. melanogaster	AF022713.2	1482	Da Lage et al. 2007
D. orena	U96158.2	1482	Da Lage et al. 2007
D. santomea	AY736503.1	1482	Da Lage et al. 2007
D. simulans	U96159.4	1482	Da Lage et al. 2007
D. suzukii	HQ631524.1	1411	Yang et al. 2012
D. teissieri	AF039557.2	1482	Da Lage et al. 2007
D. yakuba	AF039561.2	1482	Da Lage et al. 2007

Adaptive significance of oviposition reduction behavior

Wasp-induced oviposition reduction could be an adaptive behavior if the offspring of wasp-exposed flies have enhanced immunity against wasps (Lefèvre *et al.*, 2012b). We tested this hypothetical offspring quality vs. quantity trade-off (Stearns, 1992) using *D. melanogaster* and *D. simulans*. For both fly species, three groups of 125 female and 25 male flies were transferred to food vials and held for 24 h. They were then transferred into cylindrical mesh-topped embryo collection chambers (Genesee Scientific #59-100) assigned to the following three treatments: (i) wasp-free control, (ii) 40 female *L. boulardi*, and (iii) 40 female *L. heterotoma*. 60 mm food dishes were provided for oviposition and replaced every 24 h. Eggs laid on the first day of the experiment were discarded to allow the insects to acclimate to the experimental conditions. Following the daily food transfers, unhatched eggs from each dish were transferred to 35 mm food dishes using forceps and allowed to grow to second instar. Collecting unhatched eggs was necessary because some fly larvae that hatched in the embryo collection

chambers had already been attacked by wasps, making them unsuitable for controlled wasp exposures.

After the collected eggs hatched and grew into second-instar larvae, sets of 50 second-instar larvae were transferred to 35 mm food dishes and exposed to six experienced female wasps for 3 h. Two days later, the surviving larvae were counted, transferred to food vials with one-half rolled Kimwipe in the center of the food (to provide a pupation surface), and allowed to develop. Eclosed flies were visually examined for melanized capsules, which indicate that the fly survived parasitism. Flies without capsules were considered unattacked and ignored in the analyses. Eclosed wasps were also counted. Fly larvae that did not eclose were assumed to have died from wasp attacks. The effects of parental treatment (control or wasp-exposed) on the proportion of offspring that survived parasitism were analyzed using GLMs with quasi-binomial error distributions and logit link functions, considering each fly-wasp combination separately.

In our forced co-habitation assays, we found that both fly species had significantly reduced cumulative per-female oviposition rates when housed with wasps (main text, Fig. 3; $z > 5.59$, $P < 0.001$ for control vs. *L. boulardi* and control vs. *L. heterotoma* comparisons). However, when *D. melanogaster* parents were exposed to wasps, their offspring continued to have zero encapsulation success (Fig. S1a). Similarly, offspring of wasp-exposed *D. simulans* parents did not have increased survival following wasp attack (Fig. S1b; $F_{1,9} = 0.082$, $P = 0.782$ for *L. boulardi*, zero fly survival against *L. heterotoma* in both parental treatments).

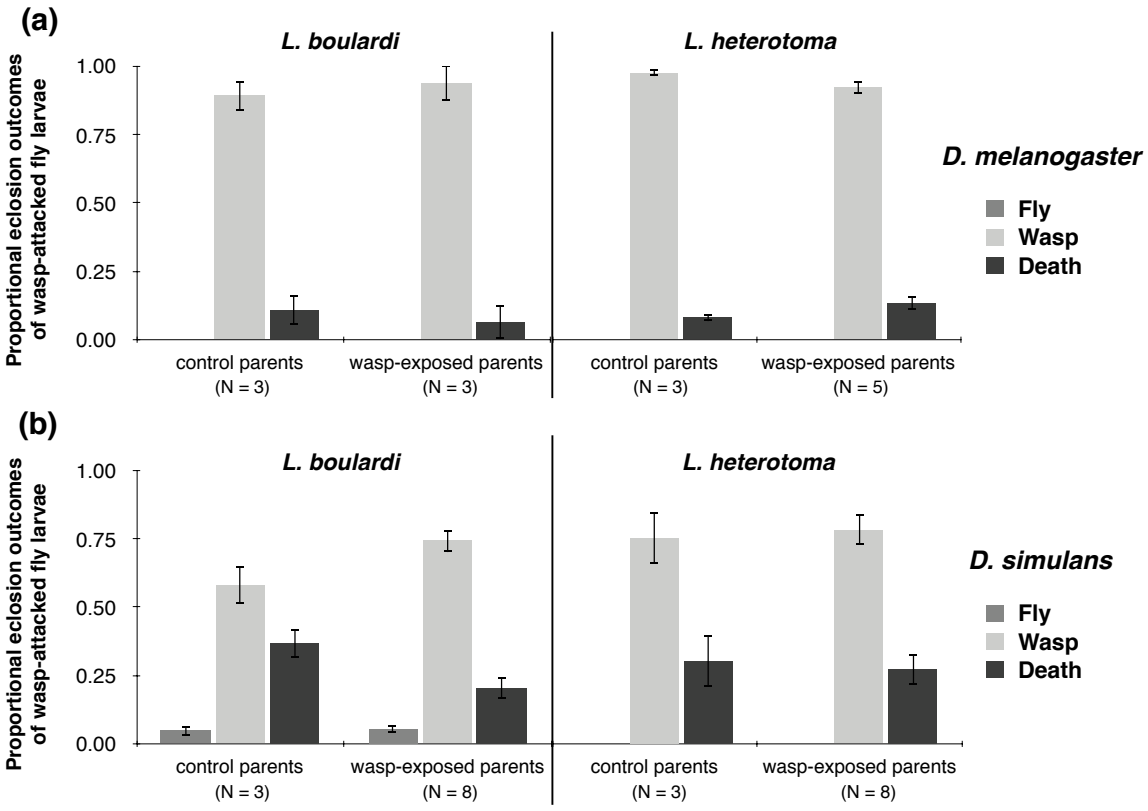

Figure S1. Testing for an offspring quality vs. quantity trade-off in *D. melanogaster* and *D. simulans*. Mean proportions of (a) *D. melanogaster* and (b) *D. simulans* offspring produced by control and wasp-exposed parents that: (i) eclosed as flies with melanized capsules, (ii) eclosed as wasps, or (iii) died, after being attacked by *L. boulardi* or *L. heterotoma* (±1 SEM). Numbers of cellular immunity dish replicates are shown.

Neither *D. melanogaster* nor *D. simulans* produced offspring with stronger cellular immune responses when housed in wasp-infested environments (Fig. S1). However, we identified two potential problems with this experiment. First, flies were housed with wasps in bigger cages than those in which we measured oviposition reduction (main text, Fig. 3), and we did not confirm that these bigger enclosures also resulted in reduced egg lay. Second, *D. melanogaster* and *D. simulans* have low encapsulation success against *L. boulardi* and *L. heterotoma* under normal conditions (main text, Fig. 2), and it is possible that

parental wasp exposure might lead to enhanced offspring resistance in fly species with higher baseline encapsulation success. We addressed both of those concerns by conducting a new experiment with *D. yakuba* (reported in the main text), which resulted in the same conclusion: we have no evidence that reduced oviposition in the presence of wasps represents a trade-off between offspring quality and quantity.

Chapter 3

Ethanol confers differential protection against generalist and specialist parasitoids of *Drosophila melanogaster*

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Abstract

As parasites coevolve with their hosts, they can evolve counter-defenses that render host immune responses ineffective. These counter-defenses are more likely to evolve in specialist parasites than generalist parasites; the latter face variable selection pressures between the different hosts they infect. Natural populations of the fruit fly *Drosophila melanogaster* are commonly threatened by endoparasitoid wasps in the genus *Leptopilina*, including the specialist *L. boulardi* and the generalist *L. heterotoma*, and both wasp species are able to incapacitate the cellular immune response of *D. melanogaster* larvae. Given that ethanol tolerance is high in *D. melanogaster* and stronger in the specialist wasp than the generalist, we tested whether fly larvae could use ethanol as an anti-parasite defense and whether its effectiveness would differ against the two wasp species. We found that fly larvae benefited from eating ethanol-containing food during exposure to *L. heterotoma*, as evidenced by reduced parasitism rates and increased survival. Although host ethanol consumption did not affect *L. boulardi* parasitism rates, it led to a modest increase in fly survival. Thus, ethanol conferred stronger protection against the generalist wasp than the specialist. We

tested whether fly larvae can self-medicate by seeking ethanol-containing food after being attacked by wasps, but found no support for this hypothesis. We also allowed female flies to choose between control and ethanol-containing oviposition sites in the presence vs. absence of wasps and generally found significant preferences for ethanol regardless of wasp presence. Overall, our results suggest that *D. melanogaster* larvae obtain protection from certain parasitoid wasp species through their mothers' innate oviposition preferences for ethanol-containing food sources.

Introduction

Populations involved in antagonistic interactions often experience episodes of rapid, coupled evolutionary change. Host or prey populations undergo natural selection for new traits that confer enhanced resistance or tolerance against their enemies, and these new traits are repeatedly countered by adaptations in enemy populations. For example, plant defenses can increase in strength due to selection pressures from herbivores (Mauricio & Rausher, 1997; Agrawal *et al.*, 2012) and parasites can evolve to avoid or impair host immune responses (Eslin & Prevost, 2000; Labrosse *et al.*, 2003). However, we expect coevolutionary trajectories to differ between parasites that infect one or a few hosts (specialists) and parasites that infect a broader range of hosts (generalists). Populations of specialist parasites are distributed across narrower host ranges every generation, causing selection pressures to be more consistent across generations. Therefore, antagonistic coevolution is more likely between specialist

parasites and their hosts, and specialist parasites will be more likely to counter host defenses than generalist parasites (Kawecki, 1998; Thompson, 1999).

Insect immune systems comprise physical, cellular, and humoral defenses (Gillespie *et al.*, 1997). Epithelial cells in the cuticle, gut, and tracheae serve as primary barriers to infection. They also produce antimicrobial peptides and reactive oxygen species that play important roles in local immune responses (Tzou *et al.*, 2000; Ha *et al.*, 2005). Hemocytes are involved in several types of defenses, including clot formation, phagocytosis, nodulation, and encapsulation (Lavine & Strand, 2002). The fat body responds to systemic infections by producing antimicrobial peptides and secreting them into the hemolymph (Lemaitre *et al.*, 1997; Lemaitre & Hoffmann, 2007). However, recent studies have increasingly focused on alternative behavioral and symbiont-mediated defenses (Parker *et al.*, 2011; de Roode & Lefèvre, 2012). For example, woolly bear caterpillars (*Grammia incorrupta*) that are parasitized by tachinid flies can improve their survival by increasing their ingestion of pyrrolizidine alkaloids (Singer *et al.*, 2009). In addition, protozoan-infected female monarch butterflies improve the fitness of their infected offspring by preferentially laying eggs on milkweed plants with high levels of cardenolides, which reduces infection in their offspring (Lefèvre *et al.*, 2010; Lefèvre *et al.*, 2012a). Lastly, the mycophagous fly *Drosophila neotestacea* harbors a maternally transmitted mutualistic bacterium, *Spiroplasma*, which protects the fly against a sterilizing nematode parasite (Jaenike *et al.*, 2010).

Larvae of the cosmopolitan fruit fly *Drosophila melanogaster* are commonly parasitized by endoparasitoid wasps in the genus *Leptopilina*

(Hymenoptera: Cynipoidea, Figitidae), including *L. heterotoma*, a generalist that successfully parasitizes many *Drosophila* species, and *L. boulardi*, a specialist that is restricted to *D. melanogaster* and *D. simulans* across most of its geographical range (Carton *et al.*, 1986; Allemand *et al.*, 2002; Schlenke *et al.*, 2007; Fleury *et al.*, 2009). Resistance against parasitoid wasps is critical to the long-term persistence of fruit fly populations, as natural rates of parasitism can exceed 90% (Fleury *et al.*, 2004). Fly larvae can use a cellular immune response known as melanotic encapsulation to kill wasp eggs that have been laid inside them. However, venom released by wasps during oviposition can disrupt this response, allowing wasp larvae to consume and kill their host before emerging from the fly pupal case (Rizki & Rizki, 1984; Rizki *et al.*, 1990; Schlenke *et al.*, 2007). *D. melanogaster* cellular immune responses have often been found to be weak or completely ineffective against *Leptopilina*, as well as *Asobara* parasitoids (Carton & Kitano, 1981; Eslin & Prevost, 1998; Lefèvre *et al.*, 2012b; Poyet *et al.*, 2013; Lynch *et al.*, 2016). Therefore, recent studies have investigated alternative behavioral defenses, such as avoidance of wasp-infested oviposition sites (Lefèvre *et al.*, 2012b; Lynch *et al.*, 2016) and use of ethanol for self-medication or kin medication (Milan *et al.*, 2012; Kacsoh *et al.*, 2013).

D. melanogaster larvae feed on yeasts that grow on fermenting fruits, in which ethanol concentrations can exceed 4%. Some *D. melanogaster* populations thrive in and around wine cellars, using piles of discarded grape residues and barrel seepages as larval habitats, in which ethanol concentrations are often 7% or higher (McKenzie & McKechnie, 1979; Gibson *et al.*, 1981). *D. melanogaster* has the highest adult ethanol tolerance among *Drosophila* species that use

fermenting fruits as larval habitats, and adult ethanol tolerance is even higher in populations that breed in beer factories and wine cellars (David & Van Herrewege, 1983; Mercot *et al.*, 1994). This trait appears to have evolved in parallel between *D. melanogaster* and its parasitoids. Bouletreau and David (1981) reported strong ethanol tolerance in six out of seven parasitoids of *Drosophila* larvae, with females having significantly higher tolerance than males, and hypothesized that this is an adaptation to avoid toxicity from the host larvae's food sources during oviposition. Milan *et al.* (2012) found that the specialist *L. boulardi* had significantly stronger tolerance to 6% and 8% ethanol than the generalist *L. heterotoma*. If *D. melanogaster* uses ethanol for defense against parasitoids, this difference in ethanol tolerance between parasitoid species may reflect the general prediction that specialist parasites are more likely to counter novel host defenses than generalist parasites. Here we follow this general prediction to test the following specific predictions: (i) *D. melanogaster* larvae consuming ethanol-containing food are better protected against generalist than specialist wasps; and (ii) *D. melanogaster* will actively use ethanol as a behavioral defense against generalist but not specialist wasps.

We tested the first prediction by growing *D. melanogaster* larvae in 0% or 6% ethanol food before, during, and/or after exposure to wasps, then measuring attack rates, parasitization intensities, and eclosion outcomes (fly survival, wasp survival, and death). With respect to our second prediction, there are two ways in which *D. melanogaster* larvae could obtain ethanol: they might seek ethanol-containing food when foraging (self-medication) or female flies might preferentially oviposit in ethanol-containing food (kin medication) (Milan *et al.*,

2012; Kacsoh *et al.*, 2013). We tested for both types of medication behavior by comparing preferences for 0% vs. 6% ethanol food between parasitized and unparasitized larvae, then comparing oviposition preferences for 0% vs. 6% ethanol food between wasp-exposed and unexposed female flies. We performed all of these experiments with the specialist *L. boulardi* and the generalist *L. heterotoma* to investigate the role of parasite-host specificity in the evolution of host defenses.

Materials and Methods

Insect strains and maintenance

The *D. melanogaster* wild-type strains *Canton S* (strain 1) and *Oregon R* (strain 5) were obtained from the Bloomington Drosophila Stock Center. They were maintained in wide fly vials (Genesee Scientific) on standard cornmeal-molasses-yeast medium (Lynch *et al.*, 2016). For all experiments involving fly larvae, large groups of *Oregon R* flies (~200 adults) were placed in mesh-topped embryo collection chambers (Genesee Scientific) with standard food in 60 mm diameter Petri dishes. Egglay dishes were collected and replaced every 24 h, then held for the appropriate amount of time (48–72 h from the midpoint of the egglay period) for larvae to reach second or third instar as required for each experiment. The endoparasitoid wasps *Leptopilina boulardi* (strain Lb17) and *L. heterotoma* (strain Lh14) are inbred strains generated from single females collected in Winters, CA in 2002 (Schlenke *et al.*, 2007). To maintain wasp stocks, *Canton S* flies were allowed to lay eggs in food vials for three days, then the flies were removed and ~10 mated female wasps were added. Newly emerged

male and female wasps were kept in food vials with one-half rolled Kimwipe pushed into the center of the food and fed with a 50-50 honey-water solution. They were held for 3–7 days before each experiment to ensure the female wasps had mated. Insect stocks were kept on the lab bench under ambient temperature, humidity, and light conditions. Environmental conditions measured during our experiments generally ranged from 23–25°C and 30–34% relative humidity, although an HVAC failure led to different conditions during one day of an oviposition preference trial, as explained later. Overhead lights were usually on during work hours and off at night. Most experiments also took place under these ambient light conditions, except for the larval food preference and adult oviposition preference experiments, which used a 15 h light: 9 h dark cycle.

Recipes for colored ethanol solutions

All experiments involved preparing fly food at 0% and 6% ethanol, which are within the range of ethanol concentrations experienced by *D. melanogaster* larvae in their natural food sources (McKenzie & McKechnie, 1979; Gibson *et al.*, 1981). Instant Drosophila medium (Formula 4-24 plain, Carolina Biological Supply) was mixed with the appropriate volume of a colored 0% or 6% ethanol solution. The stock 0% ethanol solution was made in 500 mL batches with 495 mL reverse osmosis water and 5 mL red food coloring (McCormick). This made it easier to see fly eggs and larvae in the instant food. Fresh batches of 6% ethanol solution were made immediately before each experiment by mixing appropriate amounts of the stock solution and 100% ethanol (Decon Laboratories). For example, to make 15 Petri dishes with 1 mL of liquid each, 14.1 mL stock was

mixed with 0.9 mL 100% ethanol, then pipetted onto the instant food in 1 mL aliquots.

Effects of ethanol consumption on unparasitized larvae

Fly food was prepared in 35 mm diameter Petri dishes by mixing 0.25 g instant Drosophila medium and ~10 granules of live baker's yeast (Fleischmann's) with 1 mL red 0% or 6% ethanol solution. Sets of 40 second-instar *Oregon R* larvae were placed in 0% or 6% ethanol food dishes for 24 h, transferred to new dishes with 0% or 6% ethanol food for another 24 h, then placed in cornmeal-molasses-yeast food vials to complete development. This generated four treatments: 0%–>0%, 0%–>6%, 6%–>0%, and 6%–>6%. Surviving adult flies were counted ~10 days later. The effect of food treatment on proportional fly survival was assessed using a generalized linear model (GLM) with quasi-binomial error distribution and logit link function. All statistical analyses were performed in R version 3.2.3 (R Development Core Team, 2014).

Effects of ethanol consumption before and after exposure to wasps

Fly food was prepared in 35 mm diameter Petri dishes by mixing 0.25 g instant Drosophila medium and ~10 granules of live baker's yeast with 1 mL red 0% or 6% ethanol solution. Sets of 40 second-instar *Oregon R* larvae were placed in 0% or 6% ethanol food dishes, where they fed for 24 h before being exposed to 10 female wasps (Lb17 or Lh14) for 2 h. They were then moved from their original dishes to new dishes with 0% or 6% ethanol food, where they fed for 24 h. This generated four treatments: 0%–>0%, 0%–>6%, 6%–>0%, and 6%–>6%. Five

larvae from each dish were dissected to count the number of parasitoid eggs laid inside them. The remaining larvae were transferred to cornmeal-molasses-yeast food vials to develop and eclose. Final eclosion success was calculated from the number of larvae transferred, and most vials had fewer than 35 larvae because some died or could not be found. Surviving adult flies and wasps were counted approximately one week and four weeks later, respectively.

The effects of food given before exposure to wasps on the proportion of dissected fly larvae that had been parasitized were assessed using GLMs with quasi-binomial error distributions and logit link functions. The effects of food given before exposure to wasps on the number of wasp eggs laid in each dissected fly larva were assessed using GLMs with quasi-Poisson error distributions and log link functions. The effects of food given (i) before exposure to wasps and (ii) after exposure to wasps on proportional eclosion outcomes (fly survival, wasp survival, and death of both fly and wasp) were assessed using GLMs with quasi-binomial error distributions and logit link functions.

Effects of ethanol consumption during exposure to wasps

Fly food was prepared in 35 mm diameter Petri dishes by mixing 0.25 g instant Drosophila medium and ~10 granules of live baker's yeast with 1 mL red 0% or 6% ethanol solution. Sets of 30 early third-instar *Oregon R* larvae were placed in 0% or 6% ethanol food dishes and immediately exposed to 10 female wasps (Lb17 or Lh14) for 2 h. The larvae fed in their dishes for 12 h after the wasps were removed, then 10 larvae from each dish were dissected to count the number of parasitoid eggs laid inside them. The remaining larvae were

transferred to cornmeal-molasses-yeast food vials to develop and eclose. Final eclosion success was calculated from the number of larvae transferred, and most vials had fewer than 20 larvae because some died or could not be found. Surviving adult flies and wasps were counted approximately one week and four weeks later, respectively.

The effects of food treatment on the proportion of dissected fly larvae that had been parasitized were assessed using GLMs with quasi-binomial error distributions and logit link functions. The effects of food treatment on the number of wasp eggs laid in each dissected fly larva were assessed using GLMs with quasi-Poisson error distributions and log link functions. The effects of food treatment on proportional eclosion outcomes (fly survival, wasp survival, and death of both fly and wasp) were assessed using GLMs with quasi-binomial error distributions and logit link functions.

Larval ethanol food preference

Nine sets of 150 late second to early third-instar *Oregon R* larvae were placed in 60 mm diameter Petri dishes with cornmeal-molasses-yeast food. The larvae were exposed to 20 female wasps (Lb17 or Lh14) for 3 h or left as unexposed controls (3 dishes per treatment). Larval choice environments were then set up in bisected 100 mm diameter Petri dishes (Fisher Scientific, FB08757150) with 1 g instant Drosophila medium and 10–20 granules of live baker's yeast on each side, plus 4 mL of red liquid: 0% ethanol on one side and 6% ethanol on the other side. Larvae from each 60 mm dish were divided equally between two 100 mm choice dishes, one with the larvae starting on 0% ethanol

and one with the larvae starting on 6% ethanol. Overall, there were three wasp exposure treatments (control, Lb17, and Lh14) and two starting side treatments (0% and 6%), with three replicates per combination and ~70 larvae per replicate. The larvae were given 24 h to move freely within their bisected food dishes. After this choice period, larvae on the 0% and 6% sides of each dish were counted as they were removed from the food. Ten larvae from each original wasp exposure dish (30 total per wasp species) were dissected to count the number of parasitoid eggs laid inside them. Ambient lab conditions ranged from 24–25°C and 30–32% relative humidity during this trial and a 15 h light: 9 h dark cycle was used. The effects of wasp treatment on the proportion of larvae that were on the 6% ethanol side following the 24 h choice period were assessed using GLMs with quasi-binomial error distributions and logit link functions. The 0% and 6% ethanol starting side treatments were analyzed separately.

Adult ethanol oviposition preference

We conducted oviposition preference experiments in which groups of female *Canton S* flies were allowed to choose between control (0% ethanol) and 6% ethanol food dishes in cages with or without female parasitoid wasps. We used two types of cages and tested two different methods of mixing the fly food. Every trial involved collecting data from a group of cages at multiple time points, so we placed the dishes on opposite sides of the cages and switched the position of the 6% ethanol dish between time points to ensure that we were measuring ethanol preference rather than side bias.

We ran one set of experiments in 60 x 60 x 60 cm population cages constructed from white PVC pipe covered with coarse brown/green mesh. We observed some parasitoid wasps escaping through the mesh, indicating that these cages are not ideal for experiments with wasps. However, these cages were used in a previous study (Kacsoh *et al.*, 2013), and our goal was to replicate their setup. Groups of 300 female flies (3–6 days old) were released into cages with 50 female wasps (Lb17 or Lh14) or without wasps (control) and given two food dishes, one with 0% ethanol and one with 6% ethanol. These dishes were replaced 24 h later and eggs laid in each dish were counted following both choice periods (0-24 h and 24-48 h). In our first experiment, we prepared the fly food by mixing 4 g instant Drosophila medium with 16 mL red 0% or 6% ethanol solution in 100 mm diameter Petri dishes (well-mixed food). In our second experiment, we instead prepared the 6% ethanol dishes by adding 15 mL red 0% ethanol solution to 4 g instant Drosophila medium, then slowly dispensing 1 mL 95% ethanol across the surface of the food using a micropipette (ethanol-on-top food), following published protocols (Kacsoh *et al.*, 2013; Kacsoh *et al.*, 2015) . Ambient lab conditions ranged from 23–24°C and 31–34% relative humidity during these trials and a 15 h light: 9 h dark cycle was used. The effects of choice period, wasp treatment, and their interaction on the proportion of eggs laid on 6% ethanol food were assessed using GLMs with quasi-binomial error distributions and logit link functions.

To address the problem of wasps escaping from the population cages and determine whether our conclusions were robust to changes in the experimental setup, we ran another set of experiments in BugDorm-43030 insect rearing cages

(30 x 30 x 30 cm). These cages have fine mesh that prevents wasps from escaping. Groups of 100 female flies (3–6 days old) were released into cages with 20 female wasps (Lb17 or Lh14) or without wasps (control) and given two food dishes, one with 0% ethanol and one with 6% ethanol. These dishes were replaced 24 and 48 h after the start of the experiment. Eggs laid in each dish were counted following these three choice periods (0–24 h, 24–48 h, and 48–72 h). In our first experiment, we prepared the fly food by mixing 2 g instant Drosophila medium with 8 mL red 0% or 6% ethanol solution in 60 mm diameter Petri dishes (well-mixed food). In our second experiment, we instead prepared the 6% ethanol dishes by adding 7.5 mL red 0% ethanol solution to 2 g instant Drosophila medium, then slowly dispensing 0.5 mL 95% ethanol across the surface of the food using a micropipette (ethanol-on-top food). Ambient lab conditions generally ranged from 23–25°C and 30–32% relative humidity during these trials and a 15 h light: 9 h dark cycle was used. Due to a temporary HVAC failure, ambient conditions were 20–22°C and 37–44% relative humidity during one day of one trial, but this did not have any obvious effect on our results. The effects of choice period, wasp treatment, and their interaction on the proportion of eggs laid on 6% ethanol food were assessed using GLMs with quasi-binomial error distributions and logit link functions.

Results

Effects of ethanol consumption on unparasitized larvae

To investigate the possible fitness costs of consuming ethanol when unparasitized, we fed *D. melanogaster* larvae 0% or 6% ethanol food in the 48 h

after reaching second instar. There was no difference in survival to adulthood across food treatments (Fig. 1; $F_{3,11}$ = 0.392, P = 0.761), suggesting that consuming 6% ethanol food during second and third instar is not costly to fly fitness.

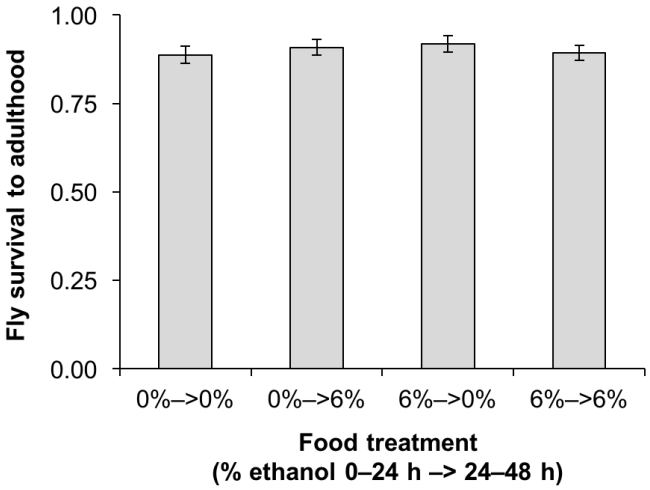

Figure 1. Effects of ethanol consumption on unparasitized larvae. Survival to adulthood of unparasitized second-instar *D. melanogaster* larvae fed 0% or 6% ethanol food from 0–24 h, moved to new 0% or 6% ethanol food from 24–48 h, then transferred to standard fly food vials to complete development (4 replicates per treatment, 40 larvae per replicate, error bars ± 1 SEM).

Effects of ethanol consumption before and after exposure to wasps

Next, we investigated the effects of consuming ethanol in the 24 h before and after exposure to wasps. Second-instar *D. melanogaster* larvae grown in 6% ethanol food for 24 h before exposure to the generalist *L. heterotoma* were not less likely to be parasitized (Fig. 2a; $F_{1,28}$ = 0.374, P = 0.546), but had significantly fewer parasitoid eggs laid inside them (Fig. 2b; $F_{1,28}$ = 12.7, P = 0.00134). This led to significantly higher fly survival (Fig. 2c; $F_{1,28}$ = 5.75, P = 0.0234) and wasp survival ($F_{1,28}$ = 11.2, P = 0.00232), along with lower death rates ($F_{1,28}$ = 24.2, P < 0.0001).

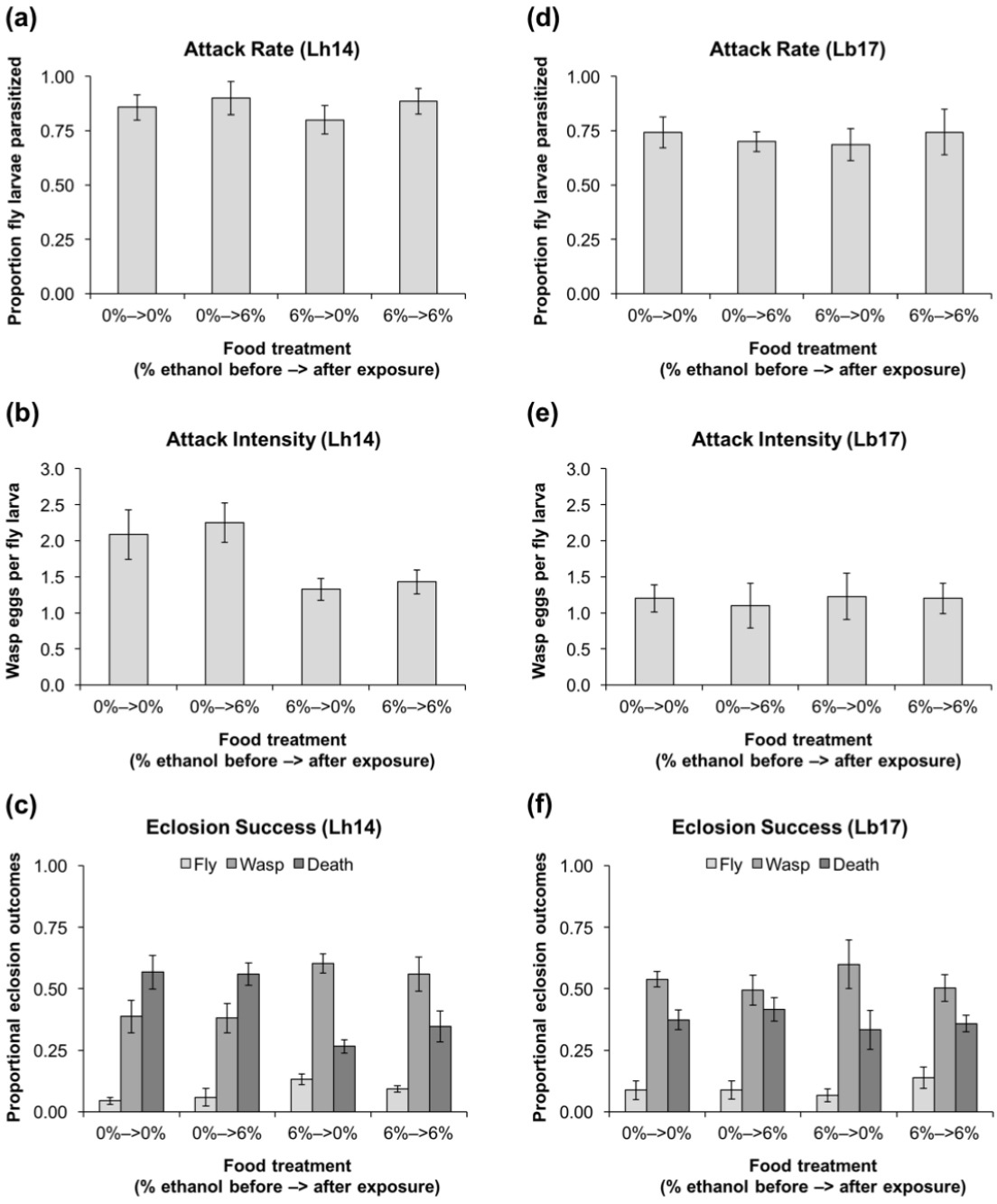

Figure 2. Effects of ethanol consumption before and after exposure to wasps. Proportion of *D. melanogaster* larvae parasitized (a,d), number of wasp eggs per fly larva (b,e), and proportion of larvae that: (i) eclosed as flies, (ii) eclosed as wasps, or (iii) died (c,f), when second-instar fly larvae were fed 0% or 6% ethanol food for 24 h, exposed to *L. heterotoma* (Lh14) (a,b,c) or *L. boulardi* (Lb17) (d,e,f) for 2 h, moved to new 0% or 6% ethanol food for 24 h, then transferred to standard fly food vials to measure eclosion success (6–8 replicates per treatment, 5 larvae for dissections and ~30 for eclosions per replicate, error bars: ± 1 SEM).

None of these effects were observed with the specialist wasp, as fly larvae grown in 6% ethanol food for 24 h before exposure to *L. boulardi* did not experience lower parasitization rates (Fig. 2d; $F_{1,25}$ = 0.0133, P = 0.909) or intensities (Fig. 2e; $F_{1,25}$ = 0.0578, P = 0.812) and there were no effects on any eclosion outcome (Fig. 2f; fly: $F_{1,25}$ = 0.161, P = 0.692; wasp: $F_{1,25}$ = 0.204, P = 0.656; death: $F_{1,25}$ = 0.729, P = 0.401).

Consuming 6% ethanol in the 24 h after exposure to wasps did not have a significant effect on any eclosion outcome when larvae were exposed to *L. heterotoma* (Fig. 2c; fly: $F_{1,28}$ = 0.490, P = 0.490; wasp: $F_{1,28}$ = 0.319, P = 0.577; death: $F_{1,28}$ = 0.656, P = 0.425) or *L. boulardi* (Fig. 2f; fly: $F_{1,25}$ = 1.19, P = 0.286; wasp: $F_{1,25}$ = 1.14, P = 0.296; death: $F_{1,25}$ = 0.313, P = 0.581). These results suggest that ethanol can protect fly larvae against the generalist *L. heterotoma* but not the specialist *L. boulardi*, and only when it is consumed before exposure to wasps.

Effects of ethanol consumption during exposure to wasps

Ethanol is continuously produced as yeasts grow on fermenting fruits, whereas ethanol concentrations decrease over time in artificial lab medium due to evaporation (Gibson *et al.*, 1981). Therefore, our previous experiments may have under-estimated the effects of 6% ethanol food on wasp oviposition behavior. We attempted to maximize these effects by placing *D. melanogaster* larvae in ethanol food immediately before wasp exposure.

Third-instar fly larvae placed in 6% ethanol food immediately before exposure to the generalist *L. heterotoma* were attacked significantly less often

(Fig. 3a; $F_{1,10}$ = 7.04, P = 0.0242) and had significantly fewer parasitoid eggs laid inside them (Fig. 3b; $F_{1,10}$ = 11.1, P = 0.00768). This led to significantly higher fly survival (Fig. 3c; $F_{1,10}$ = 54.2, P < 0.0001), whereas wasp survival and death were significantly reduced (wasp: $F_{1,10}$ = 9.98, P = 0.0102; death: $F_{1,10}$ = 12.4, P = 0.00551).

When we performed the same experiment with the specialist *L. boulardi*, fly larvae did not experience lower parasitization rates (Fig. 3d; $F_{1,11}$ = 0.2, P = 0.664) or intensities (Fig. 3e; $F_{1,11}$ = 1.31, P = 0.277). However, fly survival was significantly higher (Fig. 3f; $F_{1,11}$ = 9.91, P = 0.00927) despite no effects on wasp survival ($F_{1,11}$ = 0.537, P = 0.479) or death ($F_{1,11}$ = 0.064, P = 0.805).

These results provide additional evidence that ethanol confers protection against the generalist *L. heterotoma*. Consuming ethanol food in the 24 h before wasp exposure led to a two-fold increase in fly survival (Fig. 2c), compared to a 24-fold increase in fly survival when fly larvae were placed in ethanol food immediately before wasp exposure (Fig. 3c). Ethanol did not decrease *L. boulardi* attack rates or intensities in either experiment (Fig. 2d,e; Fig. 3d,e) but led to increased fly survival in the second experiment (Fig. 3f), suggesting that ethanol provides limited protection against the specialist wasp.

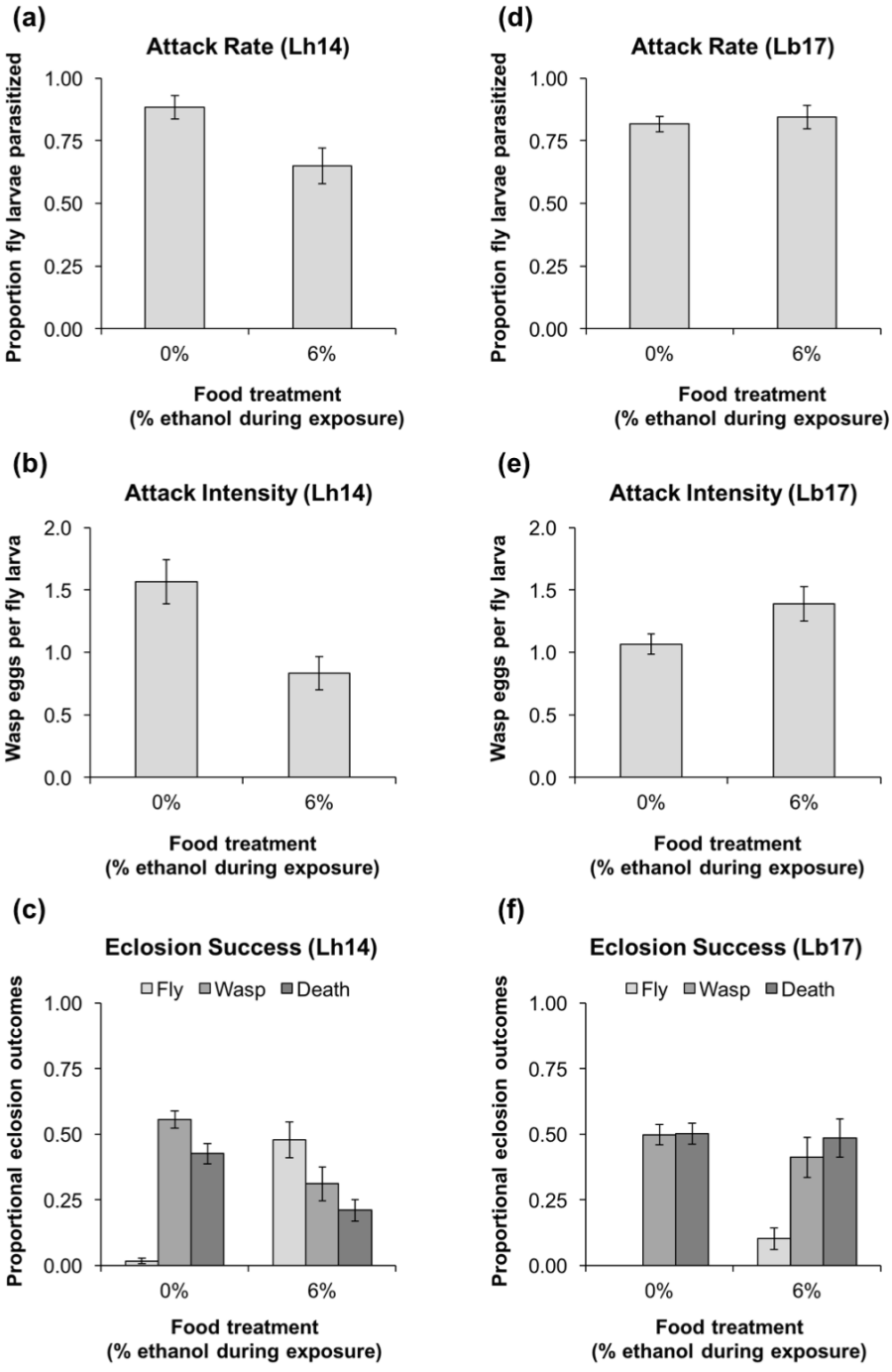

Figure 3. Effects of ethanol consumption during exposure to wasps. Proportion of *D. melanogaster* larvae parasitized (a,d), number of wasp eggs per fly larva (b,e), and proportion of larvae that: (i) eclosed as flies, (ii) eclosed as wasps, or (iii) died (c,f), when third-instar fly larvae were placed in 0% or 6% ethanol food, immediately exposed to *L. heterotoma* (Lh14) (a,b,c) or *L. boulardi* (Lb17) (d,e,f) for 2 h, allowed to feed for 12 h, then transferred to standard fly food vials to measure eclosion success (6–7 replicates per treatment, 10 larvae for dissections and ~20 for eclosions per replicate, error bars: ± 1 SEM).

Larval ethanol food preference

To determine whether fly larvae would preferentially seek ethanol food and whether this behavior would change following wasp attack, we gave wasp-exposed and unexposed *D. melanogaster* larvae 24 h to freely migrate in bisected Petri dishes with 0% and 6% ethanol food. Compared to unexposed control larvae, wasp-exposed larvae did not show increased migration from the 0% ethanol side to the 6% ethanol side (Fig. 4a; $F_{2,6} = 0.824$, $P = 0.483$). Exposure to wasps also had no effect on the propensity of fly larvae to stay in 6% ethanol food when they started there (Fig. 4b; $F_{2,6} = 0.0912$, $P = 0.914$). The overall tendency, regardless of wasp exposure treatment and starting side, was to stay on the starting side ($t_{17} = 8.12$, $P < 0.0001$). Lack of wasp exposure effects cannot be explained by low attack rates, as 83.3 ± 0.03% and 96.7 ± 0.03% of larvae exposed to *L. boulardi* and *L. heterotoma* (respectively) contained wasp eggs. Neither unparasitized nor parasitized *D. melanogaster* larvae preferentially migrated towards 6% ethanol food. Thus, we found no evidence for self-medication behavior.

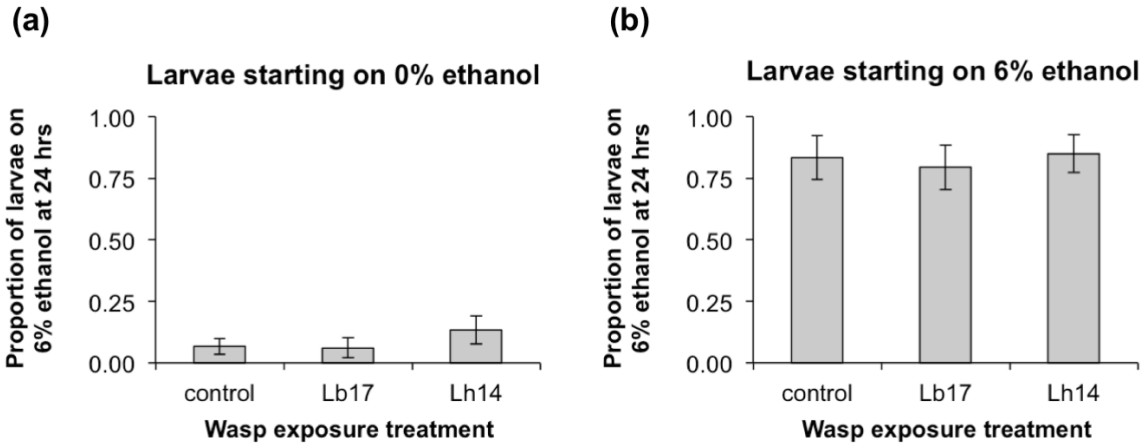

Figure 4. Larval ethanol food preference. Proportion of late second to early third-instar *D. melanogaster* larvae in three wasp exposure treatments (unexposed controls, exposed to *L. boulardi* (Lb17), and exposed to *L. heterotoma* (Lh14)) that were on the 6% ethanol side at the end of a 24 h choice experiment after starting on the 0% ethanol side (a) or the 6% ethanol side (b) of bisected Petri dishes (3 replicates per wasp exposure and starting side combination, ~70 larvae per replicate, error bars: ± 1 SEM).

The results from a similar experiment carried out previously with less control of temperature, humidity and light cycle were qualitatively equivalent to the results described here (Chapter 3 Appendix, Fig. S1).

Adult ethanol oviposition preference

To determine whether parasitoid wasps affect female flies' oviposition preference for ethanol, we placed female *D. melanogaster* in cages with or without female wasps and allowed the flies to choose between 0% and 6% ethanol oviposition sites. We investigated the generality of this behavior by using two types of cages and two different methods of mixing the fly food.

Our first set of experiments was conducted in 60 x 60 x 60 cm population cages (Fig. 5a). When the food was prepared by aliquoting 0% or 6% ethanol solution onto the instant Drosophila medium (well-mixed food), flies showed a

significant preference for 6% ethanol oviposition sites (Fig. 5c; $t_{17} = 3.02$, $P = 0.00766$) and there was no effect of wasp treatment ($F_{2,15} = 0.228$, $P = 0.800$). Similarly, when 1 mL 95% ethanol was pipetted onto the surface of the food after adding 15 mL red 0% ethanol solution (ethanol-on-top food), flies showed a significant preference for 6% ethanol oviposition sites (Fig. 5d; $t_{17} = 5.89$, $P < 0.0001$) and there was no effect of wasp treatment ($F_{2,15} = 0.244$, $P = 0.788$). For both trials, data were pooled across the two choice periods (0-24 and 24-48 h) because there was no effect of choice period on oviposition preference ($F_{1,14} < 1.70$, $P > 0.217$) and no significant interaction between choice period and wasp treatment ($F_{2,12} < 0.680$, $P > 0.525$).

The results from a similar experiment carried out previously with less control of temperature, humidity and light cycle were qualitatively equivalent to the results described here, except that the trials with well-mixed food did not reveal a significant preference for 6% ethanol oviposition sites (Chapter 3 Appendix, Fig. S2).

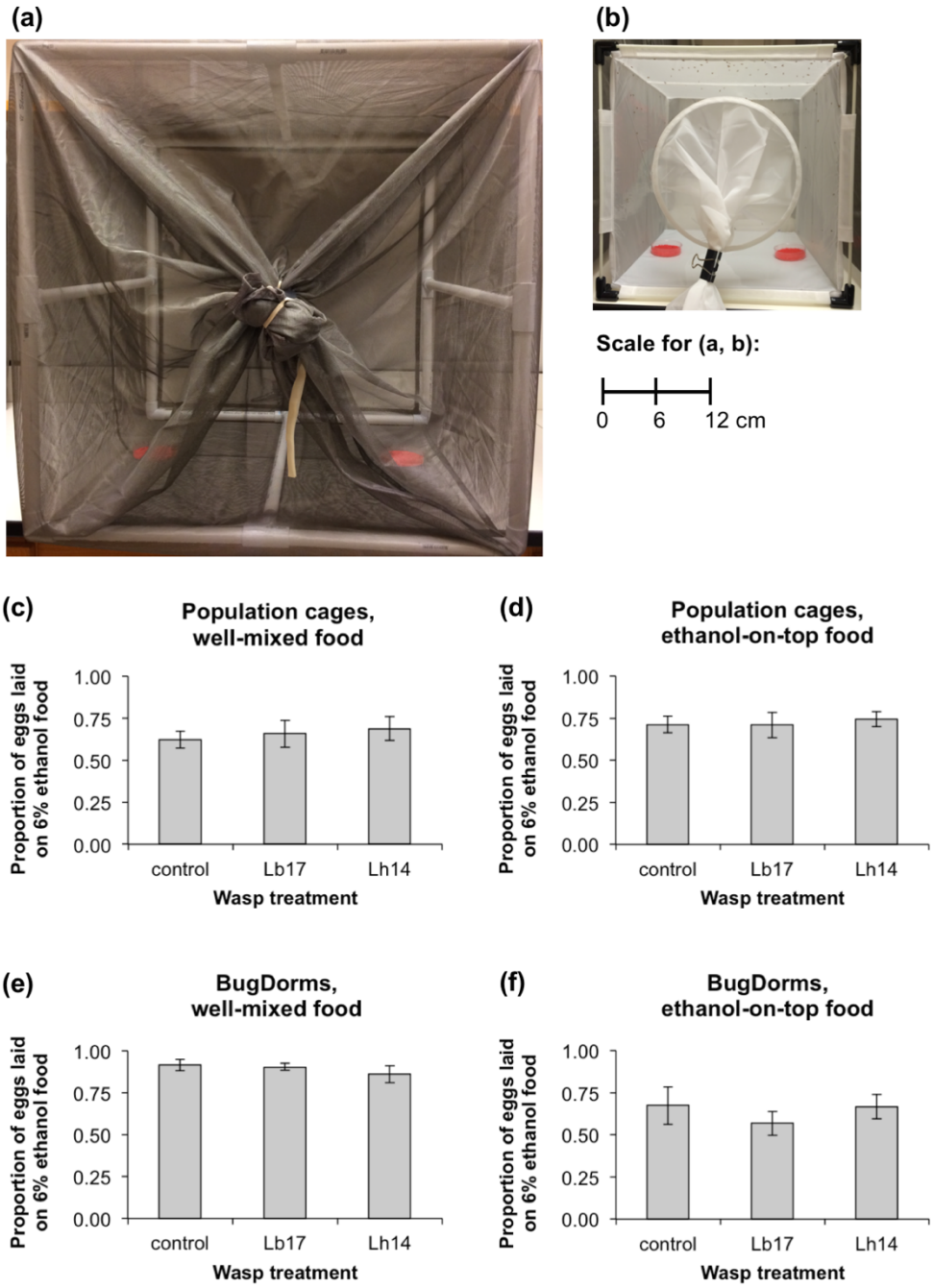

Figure 5. Adult ethanol oviposition preference. Proportion of eggs laid on 6% ethanol food when female flies were allowed to choose between 0% and 6% ethanol food in the absence of wasps (control) or in the presence of female *L. boulardi* (Lb17) or *L. heterotoma* (Lh14). Two types of cages were used: population cages (a,c,d) and BugDorms (b,e,f). Two methods of preparing the 6% ethanol dishes were compared: thoroughly mixing ethanol into the food (c,e) and pipetting 95% ethanol onto the surface of the food after adding the red 0% ethanol solution (d,f). N = 6 per treatment in (c,d), N = 6 (control) or 9 (Lb17 and Lh14) per treatment in (e,f), error bars: ± 1 SEM.

Our second set of experiments was conducted in 30 x 30 x 30 cm BugDorm-43030 cages (Fig. 5b). In the trials with well-mixed food, flies showed a significant preference for 6% ethanol oviposition sites (Fig. 5e; t_{23} = 9.60, P < 0.0001) and there was no effect of wasp treatment ($F_{2,21}$ = 0.194, P = 0.825). However, in the trials with ethanol-on-top food, flies did not show a significant preference for either oviposition site (Fig. 5f; t_{23} = 1.45, P = 0.160) and there was no effect of wasp treatment ($F_{2,21}$ = 1.37, P = 0.285). Both BugDorm trials showed significant effects of choice period on oviposition preference ($F_{2,19}$ > 6.60, P < 0.0088). Ethanol preference was weakest from 48–72 h with well-mixed food and weakest from 0–24 h with ethanol-on-top food. However, data were pooled across the three choice periods for both trials because there was no significant interaction between choice period and wasp treatment ($F_{4,15}$ < 1.86, P > 0.170). Female *D. melanogaster* preferentially laid eggs on 6% ethanol food in the majority of our experiments and the presence or absence of female wasps never had a significant effect on this behavior.

Discussion

We investigated whether *D. melanogaster* larvae can use ethanol for protection against parasitoid wasps. We also tested whether ethanol use is driven by choices at the larval or adult stages and whether its effectiveness differs against generalist vs. specialist wasps. We found that unparasitized second-instar fly larvae were equally likely to survive to adulthood when they consumed 0% or 6% ethanol food (Fig. 1), suggesting that ethanol consumption may not carry

fitness costs in the absence of parasitoids. A previous study that also used the *D. melanogaster* wild-type strain *Oregon R* similarly found that 4% to 8% ethanol food did not affect fly survival (Milan *et al.*, 2012), although studies using different wild-type strains have found reduced fly survival at ethanol concentrations above 3% (McKenzie & Parsons, 1972; McKechnie & Geer, 1984). Second-instar larvae that consumed 6% ethanol food in the 24 h before exposure to wasps were not less likely to be parasitized by the generalist *L. heterotoma* or the specialist *L. boulardi*. Consuming ethanol before exposure to *L. heterotoma* led to lower parasitization intensities and higher fly survival, but food consumed in the 24 h after exposure had no effect on fly survival against either wasp (Fig. 2). When we placed third-instar larvae in 6% ethanol food immediately before exposing them to wasps, parasitization rates and intensities were only reduced for *L. heterotoma*, although fly survival was higher against both wasp species (Fig. 3). However, significantly increased fly survival was only coupled with significantly reduced wasp survival in one scenario, when fly larvae were placed in 6% ethanol food immediately before exposure to the generalist *L. heterotoma* (Fig. 3c). When ethanol was administered 24 h before exposure, fly and wasp survival were both significantly increased (Fig. 2c). This suggests that constant access to ethanol is highly beneficial to the long-term persistence of *D. melanogaster* populations threatened by the generalist wasp. Thus, overall, we found that ethanol provides effective protection against the generalist *L. heterotoma* but limited protection against the specialist *L. boulardi*, similar to the findings of Milan *et al.* (2012).

Previous laboratory choice experiments showed that unparasitized *D. melanogaster* larvae prefer 6% ethanol food to 0% ethanol food (Parsons, 1977; Parsons & King, 1977) and that larvae parasitized by *L. boulardi* and *L. heterotoma* have even stronger preferences for 6% ethanol food (Milan *et al.*, 2012). However, the results of our fitness experiments (Figs 2 and 3) suggest that preferential consumption of ethanol would not be equally effective against the generalist and the specialist wasp. In our food choice experiments, *D. melanogaster* larvae did not migrate towards 6% ethanol food even after they had been parasitized (Fig. 4). Therefore, we found no evidence that fly larvae self-medicate with ethanol to cure wasp infections. This suggests that the ability of larvae to exploit ethanol for anti-wasp defense might be primarily dictated by adult oviposition preferences rather than larval food preferences, which seems likely because some fruits that serve as natural habitats for *D. melanogaster* have little or no ethanol (Gibson *et al.*, 1981) and flying adults are better able to move between fruits than crawling larvae.

Female flies could preferentially lay their eggs in ethanol-containing food to protect their offspring from future wasp parasitization, an example of kin medication behavior (de Roode *et al.*, 2013). *D. melanogaster* has evolved high ethanol tolerance (David & Van Herrewege, 1983) and exploits ethanol-rich food sources avoided by its sister species, *D. simulans* (McKenzie & Parsons, 1972; McKenzie & McKechnie, 1979). This suggests that female *D. melanogaster* will prefer ethanol-laden oviposition sites regardless of whether they encounter wasps, and multiple studies have reported innate ethanol preference (Richmond & Gerking, 1979; Siegal & Hartl, 1999; Azanchi *et al.*, 2013; Zhu & Fry, 2015).

However, other studies have found that ethanol oviposition preference is significantly increased following exposure to wasps (Kacsoh *et al.*, 2013; Kacsoh *et al.*, 2015). Our results support the former studies because we found significant preferences for 6% ethanol oviposition sites in three of our four experiments and none of those experiments showed an effect of wasp exposure (Fig. 5). We also found that fly larvae consuming ethanol food are significantly more likely to survive exposure to wasps (Figs 2 and 3). Taken together, these results strongly suggest that innate oviposition preference for ethanol protects fly offspring against *L. boulardi*, *L. heterotoma*, and probably other wasp species that have lower ethanol tolerance than *D. melanogaster* larvae.

Ethanol oviposition preference is a labile behavior in laboratory assays (Richmond & Gerking, 1979) and studies have often reported conflicting results (Siegal & Hartl, 1999). We conducted choice experiments in two types of cages with different insect densities and food recipes to test the generality of our conclusions. Our result that female *D. melanogaster* generally prefer to oviposit in high-ethanol food is consistent with some studies (Richmond & Gerking, 1979; Siegal & Hartl, 1999; Azanchi *et al.*, 2013; Zhu & Fry, 2015) but inconsistent with others (Kacsoh *et al.*, 2013; Kacsoh *et al.*, 2015), which found that wasp-exposed, but not unexposed, flies preferred to oviposit in food with ethanol. One possible explanation for these inconsistencies is that artificial food sources start with a fixed amount of ethanol that continuously evaporates during choice experiments. This happens slowly in plugged containers and very quickly in open Petri dishes (Gibson *et al.*, 1981). Therefore, ethanol concentrations should remain more constant when choice periods are shorter and ventilation is reduced in the

experimental containers. Studies using choice periods of 3 h or shorter have reported significant ethanol preferences (Richmond & Gerking, 1979; Azanchi *et al.*, 2013), whereas studies using 16 h choice periods have produced mixed results (McKenzie & Parsons, 1972; Cavener, 1979; Siegal & Hartl, 1999; Zhu & Fry, 2015). Two studies that ran choice assays in small bottles instead of large cages both found significant ethanol preferences despite using different choice periods: 3 h (Azanchi *et al.*, 2013) and 16 h (Siegal & Hartl, 1999). Limiting the effects of ethanol evaporation may improve the reproducibility of choice experiments, but the relevance of such experiments to our understanding of natural fly behaviors remains unclear. Laboratory setups may not accurately reflect spatiotemporal variation in ethanol concentrations across naturally fermenting fruits, and other environmental factors can contribute to fly oviposition decisions and resistance against wasps.

Experiments using artificial food sources are not sufficient to demonstrate that medication behaviors are relevant in an organism's natural environment (de Roode *et al.*, 2013). To improve the ecological relevance of choice experiments and perhaps resolve inconsistencies across studies, future experiments should better approximate the natural oviposition environments of flies and wasps. For example, flies could be allowed to choose between fruits with or without yeast in the presence or absence of wasps, using yeast species that differ in their fermentation propensities. Female *D. melanogaster* tend to prefer *Saccharomyces* species that opt for fermentation even in aerobic conditions (the Crabtree effect) (Palanca *et al.*, 2013). These yeasts dominate fruit niches by producing ethanol and heat that other species cannot tolerate (Goddard, 2008).

However, ethanol is not the only volatile compound involved in attracting female flies; the fermentation headspace of *Saccharomyces cerevisiae* also includes acetic acid, acetoin, 2-phenyl ethanol, and 3-methyl-1-butanol (Becher *et al.*, 2012). Although *S. cerevisiae* is often used in studies involving fruit flies and yeast, it is not commonly associated with *Drosophila* in nature, so future studies may benefit from testing yeast strains that have been isolated from wild-caught flies (Hoang *et al.*, 2015). Understanding natural yeast communities seems especially important because *D. melanogaster* engages in niche construction. Adult flies vector yeasts between fruits and larvae assemble them into communities with predictable compositions and densities (Stamps *et al.*, 2012). Therefore, choice experiments that only test the effects of ethanol ignore several potentially important aspects of tri-trophic interactions between yeasts, flies, and parasitoid wasps.

Furthermore, substances besides ethanol may influence the ability of *D. melanogaster* offspring to avoid or resist parasitoids. Gibson *et al.* (1981) found that apples, citrus fruits, melons, and tomatoes that served as natural hosts for *D. melanogaster* larvae contained little or no ethanol. However, citrus fruits might offer a different type of protection against wasps: volatile terpenes in their rinds attract ovipositing *D. melanogaster* but deter foraging *L. boulardi* (Dweck *et al.*, 2013). Besides producing ethanol, yeasts provide essential nutrients that can affect the cellular immune responses of fly larvae. Anagnostou *et al.* (2010) reported that *D. melanogaster* larvae had significantly different development times and melanotic encapsulation success against the braconid wasp *Asobara tabida* when they consumed different yeast species. In two-choice assays,

infected larvae preferred yeasts that enhanced their encapsulation abilities, and *S. cerevisiae* was neither the most preferred nor the most beneficial. Therefore, the optimal choice for protection against parasitoid wasps likely depends on whether enhancing encapsulation ability, medicating with ethanol, or exploiting another repulsive substance is most effective against a particular wasp species.

During the course of antagonistic coevolution, parasites may evolve counter-defenses that render host immune responses ineffective. However, evolutionary trajectories are expected to differ between specialist and generalist interactions because populations of specialist parasites experience more constant selection pressures to infect particular hosts over evolutionary time (Kawecki, 1998). This is reflected in the different strategies used by the specialist wasp *L. boulardi* and the generalist wasp *L. heterotoma* to incapacitate the immune system of *D. melanogaster:* the specialist alters and avoids host blood cells whereas the generalist directly attacks host blood cells (Rizki & Rizki, 1984; Rizki *et al.*, 1990; Schlenke *et al.*, 2007). We predicted that the specialist wasp would be better able to withstand ethanol. Our results broadly supported this prediction, as ethanol conferred more effective protection against the generalist than the specialist. However, contrary to recent studies (Milan *et al.*, 2012; Kacsoh *et al.*, 2013), we did not find that fly larvae seek out ethanol for self-medication after being attacked by wasps, or that adult flies increase their oviposition preference for ethanol after being exposed to wasps. Instead, our results suggest that female *D. melanogaster* have an innate oviposition preference for ethanol, which provides their offspring with passive protection against parasitoid wasps as a side benefit. However, in natural interactions

between fruits, yeasts, flies, and parasitoid wasps, substances besides ethanol can attract or repel ovipositing insects and mediate resistance against wasps. Future studies that elucidate the connections between oviposition preference and offspring performance in fly populations that interact with different fruit, yeast, and wasp species will allow us to better understand the evolution of behavioral defenses against parasitoid wasps.

Acknowledgements

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Chapter 3 Appendix

Additional larval ethanol food preference experiment

The results below are from our first larval food preference experiment, in which we did not measure ambient light, temperature, or humidity conditions. We later decided that it was necessary to run a 15 h light: 9 h dark cycle and measure temperature and humidity conditions in the lab, so we repeated this experiment. Otherwise, we used the same protocol described in the Materials and Methods in the main text.

D. melanogaster larvae that were exposed to wasps did not show increased migration from the 0% ethanol side to the 6% ethanol side of bisected Petri dishes during 24 h food choice experiments (Fig. S1a; $F_{2,6} = 2.23$, $P = 0.189$). Exposure to wasps also had no effect on the propensity of fly larvae to stay in 6% ethanol food when they started there (Fig. S1b; $F_{2,6} = 0.418$, $P = 0.676$). The overall tendency, regardless of wasp exposure treatment and starting side, was to stay on the starting side ($t_{17} = 12.0$, $P < 0.0001$). Lack of wasp exposure effects cannot be explained by low attack rates, as 82.8 ± 0.08% and 93.3 ± 0.03% of larvae exposed to *L. boulardi* and *L. heterotoma* (respectively) contained wasp eggs.

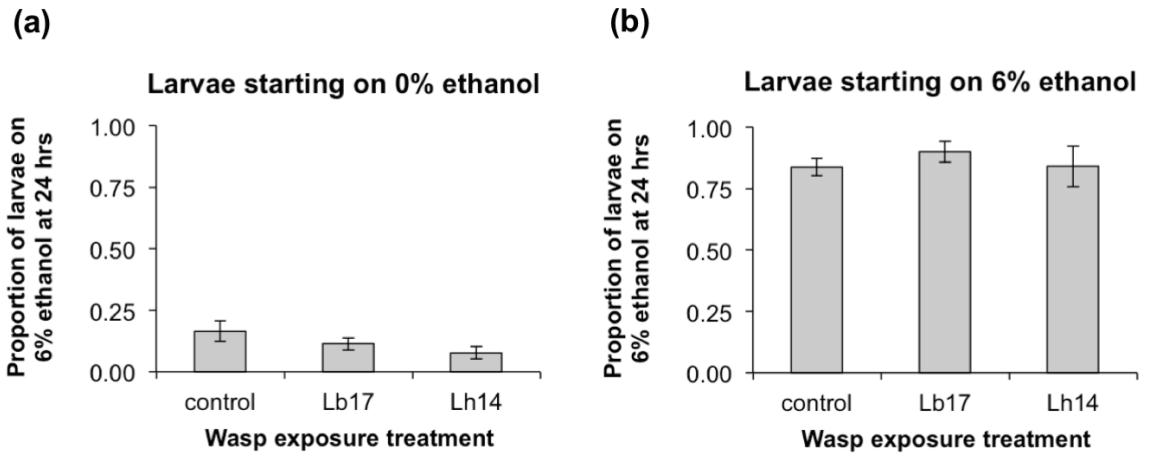

Figure S1. Additional larval ethanol food preference experiment. Proportion of late second to early third-instar *D. melanogaster* larvae in three wasp exposure treatments (unexposed controls, exposed to *L. boulardi* (Lb17), and exposed to *L. heterotoma* (Lh14)) that were on the 6% ethanol side at the end of a 24 h choice experiment after starting on the 0% ethanol side (a) or the 6% ethanol side (b) of bisected Petri dishes (3 replicates per wasp exposure and starting side combination, ~70 larvae per replicate, error bars: ± 1 SEM).

Additional adult ethanol oviposition preference experiments

The results below are from our first set of oviposition preference experiments, in which we maintained constant 24 h overhead light and did not measure temperature or humidity. We later decided that it was necessary to run a 15 h light: 9 h dark cycle and measure ambient temperature and humidity conditions in the lab, so we repeated these experiments. Otherwise, we used the same population cage protocol described in the Materials and Methods in the main text.

We conducted ethanol oviposition preference experiments in large population cages using two methods of preparing the 6% ethanol food. In the trials with well-mixed food, we observed no significant preference for either 0% or 6% ethanol oviposition sites (Fig. S2a; $t_{23} = 1.22$, $P = 0.234$) and no effect of

wasp treatment ($F_{2,21}$ = 1.80, P = 0.194). In the trials with ethanol-on-top food, flies showed a significant oviposition preference for 6% ethanol (Fig. S2b; t_{23} = 7.45, P < 0.0001) and there was no effect of wasp treatment ($F_{2,21}$ = 0.148, P = 0.864). For both experiments, data were pooled across the two choice periods (0-24 and 24-48 h) because there was no effect of choice period on oviposition preference ($F_{1,20}$ < 0.225, P > 0.640) and no significant interaction between choice period and wasp treatment ($F_{2,18}$ < 0.635, P > 0.540).

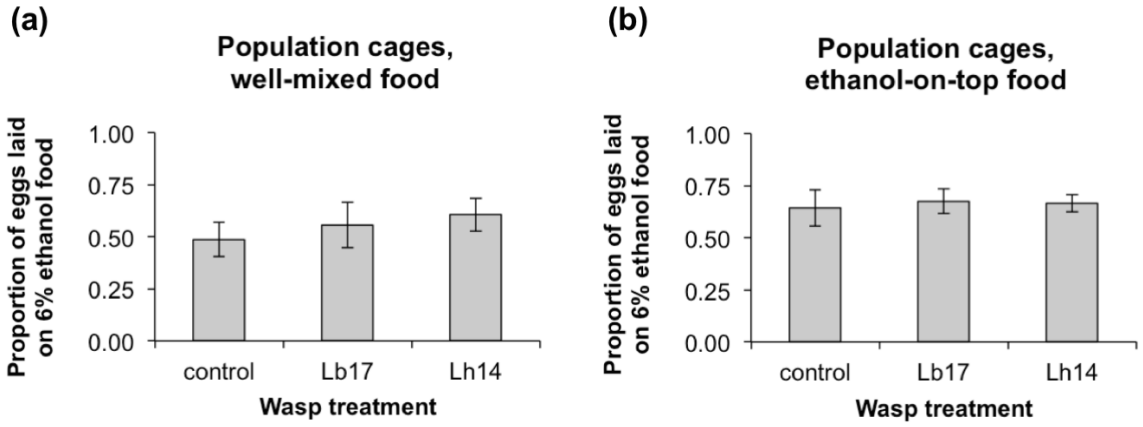

Figure S2. Additional adult ethanol oviposition preference experiments. Proportion of eggs laid on 6% ethanol food when female flies were allowed to choose between 0% and 6% ethanol food in the absence of wasps (control) or in the presence of female *L. boulardi* (Lb17) or *L. heterotoma* (Lh14) using population cages. Two methods of preparing the 6% ethanol dishes were compared: thoroughly mixing ethanol into the food **(a)** and pipetting 1 mL 95% ethanol over the food after 15 mL red 0% ethanol solution had been added **(b)**. N = 8 per treatment, error bars: ± 1 SEM.

Chapter 4

Turnover in local parasite populations favors host outcrossing over self-fertilization during experimental evolution

Zachary R. Lynch, McKenna J. Penley, and Levi T. Morran

Abstract

The ubiquity of outcrossing in plants and animals is difficult to explain given its costs relative to self-fertilization. Despite these costs, exposure to novel environmental conditions can temporarily favor outcrossing over selfing. Therefore, recurring episodes of environmental change are predicted to favor the long-term maintenance of outcrossing. Studies of natural and experimental host–parasite coevolution have provided strong support for this hypothesis. However, it is unclear if recurring exposure to novel parasite genotypes, in the absence of coevolution, is sufficient to favor outcrossing. Using the nematode *Caenorhabditis elegans* and the pathogenic bacterium *Serratia marcescens*, we studied host responses to turnover in local parasite populations. We created several replicates of a host population that was well-adapted to the parasite strain Sm2170 and passaged them with either Sm2170 or one of three novel parasite strains for 18 generations. Hosts exposed to the most virulent novel parasite exhibited the highest outcrossing rates and the most rapid adaptation. Overall, novel parasites induced higher rates of host outcrossing and adaptation than Sm2170. Host populations that were passaged with novel parasites did not suffer

increased mortality against Sm2170, suggesting that there were no cross-resistance trade-offs. Thus, turnover in local parasite populations can favor increased host outcrossing and drive host adaptation, suggesting that recurring episodes of parasite turnover could favor the long-term maintenance of host outcrossing.

Introduction

One of the central mysteries in evolutionary biology is the overwhelming prevalence of sexual reproduction via outcrossing in plant and animal species. Compared to self-fertilization, outcrossing entails substantial costs, including the two-fold cost of males or the cost of meiosis (Williams, 1975; Maynard Smith, 1978; Lively & Lloyd, 1990). The ubiquity of outcrossing in the face of such costs suggests that outcrossing lineages enjoy significant benefits over evolutionary time, relative to selfing (Goldberg *et al.*, 2010) or asexual lineages (Maynard Smith, 1978; Bell, 1982). The Red Queen hypothesis predicts that host–parasite coevolution can favor the long-term maintenance of outcrossing (Jaenike, 1978a; Hamilton, 1980; Bell, 1982). Parasites incur selection to infect the most common host genotypes, which imposes negative frequency-dependent selection on host populations. Outcrossing hosts can produce genetically diverse offspring by exchanging genetic material across lineages and recombining beneficial mutations with different origins into novel or rare genotypes. Conversely, self-fertilizing hosts are more likely to produce offspring with common genotypes that will suffer disproportionately from parasite infection. The fitness advantage that outcrossed offspring gain from reduced parasitism will fluctuate over time; if this

advantage periodically outweighs the costs of sex, then outcrossing will be maintained in the long term (Vergara *et al.*, 2014). Many studies of natural and experimental host–parasite coevolution have supported the Red Queen hypothesis (Lively & Morran, 2014).

Although the Red Queen hypothesis invokes host–parasite coevolution as a mechanism that can favor the long-term maintenance of outcrossing, other sources of recurring environmental change could have similar effects. If adaptive landscapes are constantly shifting over time, outcrossing lineages may gain substantial fitness advantages by producing genetically diverse offspring and assembling beneficial mutations from multiple lineages into novel genotypes. Theory predicts that natural selection will favor increased outcrossing and recombination if the sign of epistasis for fitness changes every two to five generations, such that genotypes with high fitness in any given generation become unfit a few generations later (Barton, 1995; Peters & Lively, 1999; Gandon & Otto, 2007). Conversely, populations evolving under relatively consistent environmental conditions will reach an adaptive peak, after which outcrossing is likely to break up adaptive gene complexes and epistatic relationships. This may lead to outbreeding depression and the re-emergence of self-fertilization or asexual reproduction (Lynch, 1991; Lynch & Deng, 1994). Therefore, it is critical to gain a better understanding of different environmental factors that may change periodically and determine whether they are likely to create population genetic conditions that favor outcrossing.

When spatial heterogeneity in selective pressures is coupled with frequent migration between environments, interactions between epistasis and selection

will determine whether sex and recombination are favored. Sex and recombination can break down linkages between beneficial and deleterious alleles (Hill–Robertson interference) and promote the incorporation of incoming beneficial mutations into highly fit genotypes, but they may also break apart locally adapted genotypes (Hill & Robertson, 1966). These opposing effects may combine to favor sex and recombination if offspring from local × migrant crosses have higher average fitness than offspring from local × local and migrant × migrant crosses (Agrawal, 2009; Otto, 2009). Recent experimental evolution studies have demonstrated that sex can be favored when populations migrate between heterogeneous environments or adapt to new environments. Gray and Goddard (2012) passaged sexual and asexual yeast populations in two different selective environments with varying levels of migration between the environments. Only the sexual populations that experienced migration exhibited simultaneous adaptation to both environments. Becks and Agrawal (2010) used a facultatively sexual rotifer to study the effects of periodic migration between subpopulations in homogeneous and heterogeneous environments. They observed greater responsiveness to a sex-inducing stimulus and higher frequencies of sexually derived offspring in heterogeneous environments. However, production of sexual offspring declined throughout the experiment in all treatments, suggesting that selective pressures in the experiment were insufficient to favor the long-term maintenance of high levels of sex. Becks and Agrawal (2012) tracked rotifer populations adapting to new environments; immediately after the transitions, population densities declined while production of sexual offspring increased, and sexual offspring eventually exhibited higher

fitness. However, as the transitioning populations reached new fitness plateaus, they began to resemble control populations, with stable population densities and fewer, less fit sexual offspring. Therefore, long-term maintenance of high levels of sex and recombination may require frequently changing environmental conditions that impose strong selective pressures on local populations.

Experimental evolution studies using the nematode *Caenorhabditis elegans* have shown that outcrossing can be favored over selfing as populations respond to various selective pressures. *C. elegans* populations consist of males and hermaphrodites. Hermaphrodites cannot mate with each other but can self-fertilize or outcross with males (Brenner, 1974). Mutations at the mating system loci *xol-1* and *fog-2* can be exploited to generate obligately selfing and obligately outcrossing populations (Miller *et al.*, 1988; Schedl & Kimble, 1988). Morran *et al.* (2009) exposed nematode populations to two different selection environments during 40- to 50-generation evolution experiments: (1) a chemical mutagen coupled with a migration barrier, and (2) the pathogenic bacterium *Serratia marcescens*. Obligately outcrossing populations showed stronger adaptation to the challenging environmental conditions than wild-type and obligately selfing populations. Wild-type populations evolved higher levels of outcrossing and exhibited stronger adaptation than obligately selfing populations. Morran *et al.* (2011) conducted a 30-generation evolution experiment in which nematode populations were exposed to either a fixed strain of *S. marcescens* or a potentially coevolving *S. marcescens* population that was isolated from nematode carcasses every generation. Both parasite treatments led to significant increases in host outcrossing rates over the first eight generations,

but only coevolving parasites selected for the maintenance of elevated outcrossing rates throughout the experiment. Masri *et al.* (2013) did not find elevated host outcrossing rates during 48 generations of coevolution between *C. elegans* and the pathogenic bacterium *Bacillus thuringiensis*, but their results still supported the Red Queen hypothesis. Although males were more susceptible to the pathogen, outcrossing was maintained throughout the experiment and outcrossed offspring exhibited stronger resistance to the pathogen.

The primary goal of our study was to determine whether host outcrossing would be favored following turnover in local parasite populations. We hypothesized that local parasite turnover would represent a substantial environmental change that would favor the production of more genetically diverse, outcrossed offspring during adaptation to the new conditions. Starting with a *C. elegans* population that had previously adapted to a non-evolving *S. marcescens* population (strain Sm2170) during a 30-generation evolution experiment (Morran *et al.*, 2011), we established an initial outcrossing rate of ~0.5 by manipulating the ratio of hermaphrodites to males. We made five replicates of this ancestral host population and passaged each of them with the *S. marcescens* strains CoSm, ES1, Rec320, and Sm2170 for 18 generations. The novel parasites (CoSm, ES1, and Rec320) were derived from Sm2170 and experienced different selective pressures during previous evolution experiments: ES1 was selected to cause higher host mortality, Rec320 was selected to cause non-lethal infections, and CoSm was passaged outside of the host (Morran *et al.*, 2011; Gibson *et al.*, 2015). Therefore, we expect our parasite strains to have different initial levels of virulence, resulting in different host evolutionary

trajectories. We measured outcrossing rates every six generations and compared how they changed during adaptation to the novel parasites versus a parasite strain to which the ancestral population was well-adapted (Sm2170). We also investigated whether hosts would evolve greater competitive fitness in the context of the novel parasites and whether resistance to different parasite strains would be subject to trade-offs.

Materials and Methods

Study system

C. elegans is a free-living nematode that colonizes ephemeral bacterial blooms in rotting fruits and herbaceous stems. Conditions such as extreme temperatures, scarce food, and high population density cause *C. elegans* to enter a non-feeding life stage known as dauer, in which larvae are resistant to environmental stresses and starvation. Dauer larvae actively seek invertebrate vectors for long-distance dispersal to new bacterial blooms through nictation behavior, in which they stand on their tails and wave their heads (Felix & Braendle, 2010; Cutter, 2015; Frezal & Felix, 2015). *C. elegans* has an androdioecious mating system with males and self-fertilizing hermaphrodites. The hermaphrodites cannot outcross with each other but may outcross with males (Brenner, 1974). All known natural strains predominantly self-fertilize, although natural outcrossing rates are variable (Teotonio *et al.*, 2006). Given the characteristics of its mating system and its need for frequent migrations that may result in exposure to new parasites, *C. elegans* seems to be an appropriate model for studying how parasite turnover affects host outcrossing rates and adaptive

potential. For our parasite, we used *S. marcescens*, a virulent bacterium that infects many plant and animal species (Grimont & Grimont, 1978). The *C. elegans–S. marcescens* interaction has been used to study the genetics of parasite infectivity, host resistance, and host avoidance behavior (Mallo *et al.*, 2002; Kurz *et al.*, 2003; Schulenburg & Ewbank, 2004; Pradel *et al.*, 2007).

Host and parasite populations

C. elegans stock populations were maintained at 20 °C in 10 cm diameter Petri dishes filled with 30 mL of autoclaved nematode growth medium lite (NGM) (US Biological, Swampscott, MA, USA). These dishes were seeded with 200 μL of *Escherichia coli* strain OP50 culture that was grown overnight at 28 °C in Luria-Bertani broth (LB). After the *E. coli* lawns grew overnight at 28 °C, the dishes were stored at 4 °C for future use. *C. elegans* stock strains are derived from single wild-caught individuals. We obtained the wild-type strain CB4856 (from Hawaii, USA) and the GFP-marked strain JK2735 from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA). Our ancestral *C. elegans* host population, EW2-30, was derived from PX382, a systematically inbred variant of CB4856 (Morran *et al.*, 2009). EW2-30 resulted from a previous evolution experiment in which hosts were passaged with a non-evolving *S. marcescens* population (strain Sm2170) for 30 generations; the complete protocol is published in Morran *et al.* (2011). Briefly, a population of PX382 (the ancestral population of EW2-30) was mutagenized with ethyl methanesulfonate and passaged on *Serratia* selection plates (SSPs), which required nematodes to migrate through live *S. marcescens* and ampicillin to reach their food source, *E.*

coli strain OP50. Under these conditions, naïve host populations can suffer mortality rates up to 80% (Schulenburg & Ewbank, 2004; Morran *et al.*, 2009). Only the offspring of nematodes that reached the food source proceeded to a new SSP to begin the next generation. At the end of the 30-generation evolution experiment, EW2-30 was frozen at -80 °C for future use.

Our novel *S. marcescens* strains (ES1, Rec320, and CoSm) were derived from Sm2170 during previous evolution experiments. ES1 underwent selection for increased infectivity and virulence as it was passaged with a static CB4856 host population for 30 generations. Bacteria that killed nematodes after 24 h of exposure were harvested every generation and used to infect the next generation of hosts; see Morran *et al.* (2011) for further details. Host and parasite populations can be copassaged to allow for coevolution; in this case, parasites are harvested from dead nematodes every generation and used to infect the offspring of surviving nematodes. Rec320 was selected for reduced antagonism as it was copassaged with hosts carrying mild upper intestine infections that were not cleared but did not cause death or prevent reproduction. During a 20-generation evolution experiment, Gibson *et al.* (2015) copassaged offspring from infected parents with bacteria that caused those mild infections, resulting in the Rec320 strain. CoSm was passaged for 20 generations on SSPs without nematodes; see Gibson *et al.* (2015) for further details regarding CoSm and Rec320.

Experimental evolution

Before starting our evolution experiment, we manipulated our ancestral host population (EW2-30) to establish initial male frequencies of ~0.25 in each of

the experimental populations. Groups of 20 L4 nematodes were transferred to NGM dishes seeded with OP50 and allowed to produce offspring; five dishes had a 1:1 ratio of hermaphrodites to males and five dishes had all hermaphrodites. Matings between hermaphrodites and males result in 50% male offspring, whereas selfing hermaphrodites produce ~0.02% male offspring, a frequency that approximates the rate of spontaneous X chromosome non-disjunction (Anderson *et al.*, 2010). Therefore, mixing equal quantities of offspring from those ten dishes resulted in a population with ~25% males. We created five replicates from this mix, transferring ~1000 offspring to each replicate population, and passaged each of them for 18 generations on SSPs with four parasite strain treatments: CoSm, ES1, Rec320, and Sm2170. SSP construction and nematode transfers were performed using published protocols (Morran *et al.*, 2011). Briefly, groups of ~1000 L3-L4 nematodes were washed into M9 buffer and transferred to live *S. marcescens* lawns. Only the offspring of nematodes that successfully migrated through the parasite and a streak of ampicillin to reach their food source (*E. coli* strain OP50) were transferred to a new SSP to begin the next generation. Each of the five replicate ancestral populations were separately passaged with CoSm, ES1, Rec320, and Sm2170 for 18 generations, resulting in 20 evolved host populations.

Host mortality rate assays

Host mortality rates were assayed in 10 cm diameter Petri dishes filled with 30 mL of NGM and seeded with 200 µL of *S. marcescens* culture that was grown overnight at 28 °C in LB. The *S. marcescens* lawns grew overnight at 28

°C, then groups of 200 L4 nematodes were transferred into the dishes. Dead nematodes were counted after 24 h of parasite exposure and 24 h host mortality rates were calculated as the number of dead nematodes divided by 200 transferred nematodes. Before starting our evolution experiment, we used these assays to compare the virulence levels of our four *S. marcescens* strains (CoSm, ES1, Rec320, and Sm2170) towards the ancestral host population. For the ancestral host mortality data, the effects of parasite strain on 24 h mortality rates were analyzed using a generalized linear model (GLM) with quasi-binomial error distribution and logit link function; pairwise differences were assessed using Tukey's honest significant difference (HSD) tests. At the end of our evolution experiment, we used these assays to compare resistance to Sm2170 (to which our ancestral hosts were well-adapted) across replicate host populations that had been passaged with each parasite strain for 18 generations. For the generation 18 host mortality data, the effects of host evolution treatment on 24 h mortality rates were analyzed using a GLM with quasi-binomial error distribution and logit link function. These analyses were performed in R version 3.2.3 (R Development Core Team, 2014) using the *multcomp* package (Hothorn *et al.*, 2008).

Measuring host outcrossing rates

Male frequencies were measured in each host population at the beginning of the experiment and after every six generations of experimental evolution. A transect of each experimental population was counted on the OP50 portion of the SSP every six generations. Approximately 200 L4 offspring were counted and sexed prior to passage to the next round of selection. The outcrossing rate for

each host population was calculated by multiplying the male frequency by two after correcting for the number of males that typically result from spontaneous X chromosome non-disjunction (Stewart & Phillips, 2002). These data are presented in terms of outcrossing rates in the Results but were analyzed in terms of male frequencies to enable the use of binomial GLMs. The effects of parasite strain, host generation, and their interaction on male frequencies in our host populations were analyzed using a GLM with quasi-binomial error distribution and logit link function. For each of the four host evolution treatments (groups of host populations passaged with a given parasite strain for 18 generations), we analyzed the effects of host generation on male frequencies using separate quasi-binomial GLMs. We then performed pairwise comparisons using Tukey's HSD tests and tabulated the key differences: ancestor – generation 6, ancestor – generation 12, and ancestor – generation 18. These analyses were performed in R version 3.2.3 (R Development Core Team, 2014) using the *multcomp* package (Hothorn *et al.*, 2008).

Competitive fitness assays

We measured the competitive fitness of each ancestral and generation 18 host population relative to a common tester strain in our selective environment. In each assay, 100 nematodes from the focal population and 100 nematodes from the GFP-marked strain JK2735 were transferred to the *S. marcescens* side of an SSP. Four days later, approximately 200 of the offspring on the OP50 side of the SSP were counted and assessed for GFP expression. The frequency of focal individuals in the offspring was then calculated as [1 – GFP frequency]; values

above 0.5 indicate that the focal hosts out-competed the tester strain. This measurement may underestimate the fitness of the focal hosts because any cross-progeny of focal and tester individuals will express the dominant GFP marker (Morran *et al.*, 2009; Morran *et al.*, 2014). For the ancestral hosts, we conducted 3 to 5 replicate assays with each parasite strain and calculated the mean frequency of focal offspring ($FO_{Ancestor}$). For the generation 18 hosts, each replicate population was tested against the parasite strain it was passaged with and we conducted 2 to 4 replicate assays per combination. We calculated percent changes in mean fitness by comparing the frequency of focal offspring from each generation 18 assay (FO_{Gen18}) to the $FO_{Ancestor}$ value corresponding to the same parasite strain: $[(FO_{Gen18} - FO_{Ancestor}) \div FO_{Ancestor}]$. These data violated the ANOVA assumptions of normality and homogeneity of variances. Therefore, we used a non-parametric Kruskal–Wallis H test to assess the effects of host evolution treatment (the parasite strain each host population was passaged with) on percent changes in mean fitness. Pairwise differences were assessed using Steel–Dwass tests. These analyses were performed in JMP 12.0 (SAS Institute, Cary, NC).

Results

Before starting our evolution experiment, we conducted host mortality rate assays to measure the virulence of our chosen *S. marcescens* strains towards the ancestral *C. elegans* population, which had previously adapted to Sm2170 during a 30-generation evolution experiment (Morran *et al.*, 2011). Our chosen

parasite strains varied significantly in virulence towards the ancestral host population (Fig. 1; $F_{3,16} = 17.6$, $P < 0.0001$); ES1 caused the greatest host mortality, Sm2170 caused intermediate host mortality, and CoSm and Rec320 caused the lowest host mortality (Tukey's HSD tests, $P < 0.03$). These results match our predictions based on the parasite strains' evolutionary histories: Sm2170 was the ancestor, ES1 was selected to cause higher host mortality, Rec320 was selected to cause non-lethal infections, and CoSm was passaged outside of the host (Morran *et al.*, 2011; Gibson *et al.*, 2015).

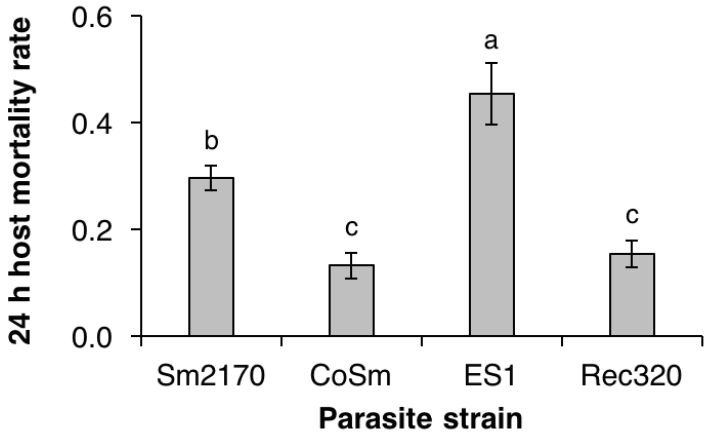

Figure 1. Mortality rates of ancestral hosts when exposed to the four parasite strains. Mortality rates suffered by the ancestral *C. elegans* population after 24 h of exposure to the four *S. marcescens* strains we used for experimental evolution (± 1 SEM). Different letters indicate significant differences between parasite strains (Tukey's HSD tests, $P < 0.03$). Each of the five replicate ancestral host populations was tested against each parasite strain in three replicates.

Five replicates of the ancestral host population were passaged with each of the four parasite strains for 18 generations. We measured outcrossing rates in each host population every six generations to compare how they changed during adaptation to novel parasites (CoSm, ES1, and Rec320) versus a parasite strain to which the ancestral population was well-adapted (Sm2170). There were significant effects of parasite strain (Fig. 2; $F_{3,73} = 60.4$, $P < 0.0001$) and host generation ($F_{3,76} = 15.9$, $P < 0.0001$) on outcrossing rates. Changes in outcrossing rate over time differed across parasite strain treatments (parasite strain × host generation effect: $F_{9,64} = 7.7$, $P < 0.0001$); differences in outcrossing rates between ancestral hosts and generation 6, 12, and 18 hosts are presented in Table 1. In host populations passaged with ES1, outcrossing rates peaked at generations 6 and 12, then decreased back to ancestral levels by generation 18 (Fig. 2; Table 1). In host populations passaged with Rec320, outcrossing rates were not significantly different between generation 0 and generation 6, 12, or 18. In host populations passaged with CoSm, outcrossing rates were not significantly different between generation 0 and generation 6 or 12, but were significantly lower at generation 18. In host populations passaged with Sm2170, outcrossing rates decreased significantly over time.

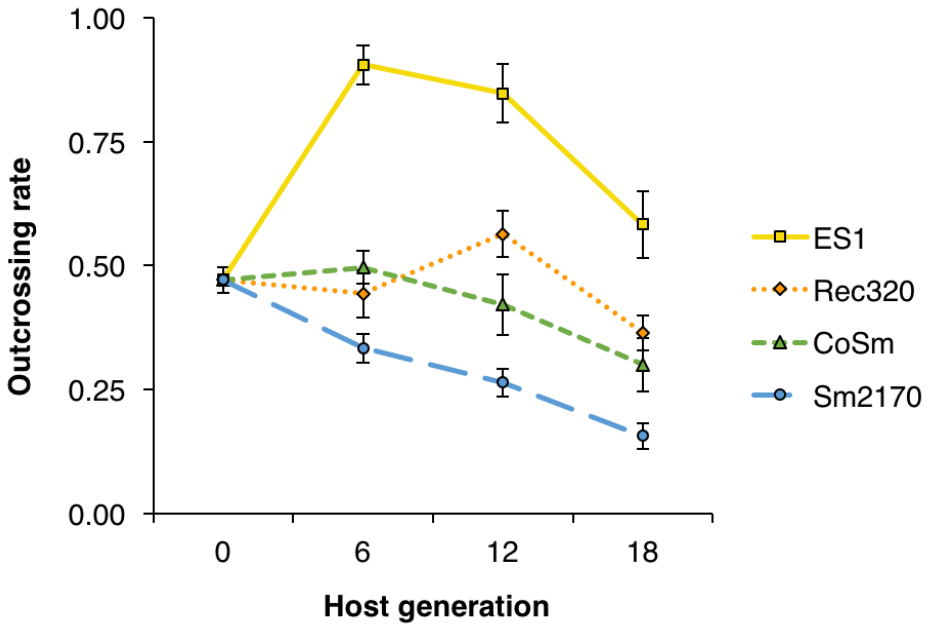

Figure 2. Changes in host outcrossing rates during experimental evolution. Changes in outcrossing rate over time as *C. elegans* populations were passaged with four different *S. marcescens* strains for 18 generations (± 1 SEM). N = 5 replicate host populations per parasite strain treatment.

Table 1. Outcrossing rate contrasts. Differences in outcrossing rates between ancestral hosts (Gen. 0) and host populations that were passaged with four different parasite strains for 6, 12, or 18 generations (Tukey's HSD tests). Outcrossing rates are shown in Fig. 2.

	Gen. 0 – Gen. 6	**Gen. 0 – Gen. 12**	**Gen. 0 – Gen. 18**
ES1	$z = -5.7$, $P < 0.001$	$z = -5.0$, $P < 0.001$	$z = -1.7$, $P = 0.32$
Rec320	$z = 0.49$, $P = 0.96$	$z = -1.5$, $P = 0.41$	$z = 2.0$, $P = 0.19$
CoSm	$z = -0.38$, $P = 0.98$	$z = 0.80$, $P = 0.86$	$z = 2.6$, $P = 0.041$
Sm2170	$z = 3.0$, $P = 0.015$	$z = 4.6$, $P < 0.001$	$z = 7.4$, $P < 0.001$

We evaluated the degree of host adaptation to each parasite strain over 18 generations of repeated exposure by assaying the competitive fitness of ancestral and generation 18 host populations. In each assay, equal numbers of nematodes from the focal host population and a GFP-marked tester strain were mixed and

exposed to the parasite strain the focal host was passaged with, using the same selective environment. After four days of exposure, we calculated the frequency of GFP-marked offspring; frequencies below the starting level of 50% indicated that the focal hosts had greater relative fitness. The change in mean fitness over time differed significantly across host evolution treatments (Fig. 3; $\chi^2_3 = 13.7$, $P = 0.0034$). Specifically, host populations that were passaged with ES1 exhibited the greatest rates of adaptation (Steel–Dwass tests, $P < 0.03$), but there were no significant pairwise differences between the CoSm, Rec320, and Sm2170 treatments ($P > 0.8$).

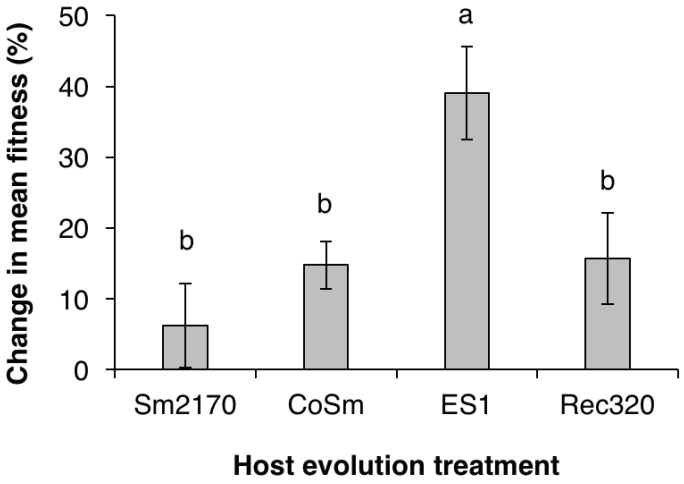

Figure 3. Host adaptation to parasites. Change in mean fitness for ancestral hosts relative to generation 18 hosts during exposure to the parasite strain each host was passaged with, as determined by competitive fitness assays against a GFP-marked tester strain (± 1 SEM). Different letters indicate significant differences between host evolution treatments (Steel–Dwass tests, $P < 0.03$). For ancestral hosts, N = 3–5 replicates per parasite strain; for generation 18 hosts, N = 5 replicate populations per treatment x 2–4 replicates per population.

We conducted host mortality rate assays to compare susceptibility to Sm2170 across our generation 18 host populations. Because the ancestral host population had previously adapted to Sm2170, decreased resistance against Sm2170 in host populations that were passaged with novel parasites would suggest that resistance to different *S. marcescens* strains is subject to trade-offs. However, there were no significant differences among generation 18 host populations in mortality rate following exposure to Sm2170 (Fig. 4; $F_{3,16} = 0.829$, $P = 0.497$), suggesting a lack of cross-resistance trade-offs.

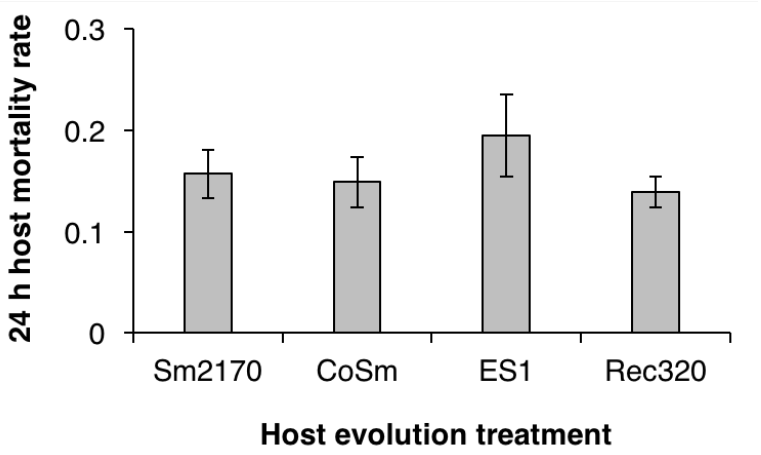

Figure 4. Mortality rates of evolved hosts when exposed to Sm2170. Mortality rates suffered by evolved *C. elegans* hosts after 24 h of exposure to Sm2170 (± 1 SEM). The host populations had been passaged with four different *S. marcescens* strains for 18 generations. N = 5 replicate host populations per treatment x 2 replicates per population.

Discussion

To study the effects of turnover in local parasite populations on host adaptation and outcrossing rates, we passaged replicate *C. elegans* populations that had previously adapted to the *S. marcescens* strain Sm2170 with three novel

parasite strains and Sm2170 for 18 generations. Our novel parasites were derived from Sm2170 during previous evolution experiments: ES1 was selected to cause higher host mortality, Rec320 was selected to cause non-lethal infections, and CoSm was passaged outside of the host (Morran *et al.*, 2011; Gibson *et al.*, 2015). Their levels of virulence towards the ancestral host population aligned well with their evolutionary histories; ES1 caused the greatest host mortality whereas CoSm and Rec320 caused lower host mortality than Sm2170 (Fig. 1). Changes in outcrossing rates over time were strongly influenced by host evolutionary history and parasite virulence. Only the most virulent novel parasite strain, ES1, triggered a significant initial increase in outcrossing rates (Fig. 2; Table 1). Host populations passaged with CoSm and Rec320 generally maintained their outcrossing rates throughout the experiment. Conversely, outcrossing rates decreased throughout the experiment as host populations were passaged with Sm2170, to which the ancestral hosts were well-adapted. Host populations showed the strongest adaptation to ES1 (Fig. 3), demonstrating a link between host outcrossing rates and adaptation. Hosts did not lose resistance to Sm2170 as they adapted to the novel parasites, suggesting that trade-offs in resistance to different parasite strains did not occur (Fig. 4).

The patterns we observed in host outcrossing rates over time broadly agree with theoretical predictions and previous empirical results. Although outcrossing can increase the efficacy of selection during adaptation to novel environmental conditions (Agrawal, 2009; Otto, 2009), it is likely to be disfavored after a population reaches an adaptive peak because recombination and segregation will disassemble adaptive genotypes (Lynch, 1991; Lynch & Deng, 1994). Previous

studies in which *C. elegans* populations were passaged with non-evolving *S. marcescens* found that outcrossing rates increased initially but decreased back to control levels by the end of the experiment; peak outcrossing rates were observed at generation 20 of 40 (Morran *et al.*, 2009) and generation 8 of 30 (Morran *et al.*, 2011). Similar results were found in populations of facultatively sexual rotifers that were adapting to new environments; sexual offspring were produced more frequently and had higher fitness during the initial stages of adaptation, but asexual offspring were favored after the populations reached new fitness plateaus (Becks & Agrawal, 2012). Therefore, it seems unlikely that single episodes of environmental change can favor the long-term maintenance of high levels of outcrossing in species that normally self-fertilize or reproduce asexually.

Host–parasite coevolution can generate the constantly shifting adaptive landscapes that are necessary for the long-term maintenance of outcrossing, as shown in natural (Jokela *et al.*, 2009; Vergara *et al.*, 2014) and experimental (Morran *et al.*, 2011; Masri *et al.*, 2013) systems. Future studies should investigate other mechanisms that may produce similar results. Although they observed higher rates of sex in rotifer populations that evolved in heterogeneous environments, Becks and Agrawal (2010) noted that equilibrium rates of sex were much lower in their experimental populations than in natural populations, perhaps because environmental heterogeneity is much greater in nature. To resolve this issue, experimental designs involving migrations between subpopulations in two different environments (Becks & Agrawal, 2010; Gray & Goddard, 2012) could be expanded to include several environments that differ in parasite presence and/or resource availability. We found short-term adaptive

increases in outcrossing rates following single parasite turnover events, suggesting that recurring turnover in local parasite populations could favor the long-term maintenance of high levels of outcrossing. Regardless of the mechanism employed, theory predicts that outcrossing is most likely to be favored if selective pressures change substantially every two to five generations (Barton, 1995; Peters & Lively, 1999; Gandon & Otto, 2007).

Limited additive genetic variation in host populations may impede the response to selection and make it unlikely that outcrossing will facilitate adaptation to parasites. Large-scale surveys of wild-caught *C. elegans* strains have found low microsatellite polymorphism (Sivasundar & Hey, 2003) and low genomic diversity (Andersen *et al.*, 2012). However, wild isolates can be systematically crossed to generate populations with abundant naturally derived genetic variation (Teotonio *et al.*, 2012), and such populations have exhibited significant adaptation during host–parasite coevolution experiments (Schulte *et al.*, 2010; Masri *et al.*, 2013). Alternatively, isogenic *C. elegans* populations can be chemically mutagenized to introduce novel genetic variation before starting evolution experiments. This approach has revealed that: (1) outcrossing can facilitate adaptation to challenging environmental conditions (Morran *et al.*, 2009; Morran *et al.*, 2011), (2) local adaptation to parasites occurs more rapidly in obligately outcrossing hosts than partially selfing hosts (Morran *et al.*, 2013; Morran *et al.*, 2014), and (3) highly inbred populations that do not experience mutagenesis evolve reduced outcrossing rates and do not adapt to parasites (Parrish *et al.*, 2016). Our ancestral host population was chemically mutagenized before being passaged with the non-evolving *S. marcescens* strain Sm2170 for 30

generations (Morran *et al.*, 2011). After 18 generations of exposure to novel parasite strains, our experimental host populations exhibited higher competitive fitness than their ancestors in the presence of their sympatric parasites (Fig. 3). Overall, increased outcrossing rates (Fig. 2) appear to have driven adaptation to parasite strains that were recently derived from an ancestor to which our starting host population was well-adapted. Therefore, future experiments that impose recurring episodes of parasite turnover using more diverse ranges of parasites may select for the maintenance of outcrossing, provided that ancestral host populations have sufficient standing genetic variation or a means of acquiring variation via gene flow.

Host–parasite coevolution remains the best-supported mechanism for generating constantly shifting adaptive landscapes that may favor outcrossing over selfing despite the inherent costs of sex (Lively & Morran, 2014). However, our results suggest that periodic parasite turnover events could have similar effects in the absence of host–parasite coevolution. It is unclear whether this is a plausible scenario in natural host populations. Outcrossing may be advantageous when populations are migrating between environments with different abiotic conditions (Becks & Agrawal, 2010; Gray & Goddard, 2012). Given that hosts may encounter novel parasites along with changing abiotic conditions as they migrate, or vector parasites into conspecific populations in their new environments, future studies should investigate the collective effects of these selective pressures on host outcrossing rates. Although models that only consider host–parasite coevolution have predicted that parasite virulence must be very high to favor outcrossing (May & Anderson, 1983), pluralistic models have shown that

moderately virulent parasites can select for sex when hosts suffer reasonable deleterious mutation rates (Howard & Lively, 1994; West *et al.*, 1999). The parameter space in which outcrossing is evolutionarily stable further expands if parasite infection exacerbates the effects of deleterious mutations (Cooper *et al.*, 2005; Park *et al.*, 2010). Those studies highlight the importance of considering alternative hypotheses and their potential interactions when attempting to explain complicated phenomena such as the maintenance of sex. *Caenorhabditis* nematodes, which proliferate in ephemeral bacterial blooms, actively seek invertebrate vectors for dispersal to new habitats, and have diverse mating systems (Felix & Braendle, 2010; Cutter, 2015; Frezal & Felix, 2015), may represent ecologically relevant models for studying the combined effects of host migration and parasite turnover on the maintenance of outcrossing.

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Chapter 5

Conclusion

A central prediction of ecological immunology theory is that immune responses are often costly to deploy and maintain; therefore, investment in defenses will be subject to trade-offs (Sheldon & Verhulst, 1996; Rolff & Siva-Jothy, 2003; Sadd & Schmid-Hempel, 2009; Schulenburg *et al.*, 2009; Parker *et al.*, 2011). However, comparative studies of this hypothesis are lacking. In Chapter 2, I measured the strengths of cellular immunity (melanotic encapsulation of wasp eggs by fly larvae) and behavioral immunity (reduced oviposition in the forced presence of wasps) across eight *Drosophila* species (seven from the *D. melanogaster* subgroup plus the more distantly related *D. suzukii*) and two parasitoid wasp species in the genus *Leptopilina*. Cellular immune responses were weaker against the generalist wasp *L. heterotoma* than the specialist wasp *L. boulardi*. These results make sense because *L. boulardi* usually parasitizes *D. melanogaster* and *D. simulans* in nature, whereas *L. heterotoma* has a much broader host range (Carton *et al.*, 1986; Fleury *et al.*, 2004). Furthermore, *L. heterotoma* has a destructive virulence strategy that involves lysing host lamellocytes (Rizki & Rizki, 1984) and broadly suppressing up-regulation of innate immune genes (Schlenke *et al.*, 2007), whereas *L. boulardi* primarily hides in host tissues to evade encapsulation (Rizki *et al.*, 1990). Only *D. suzukii* survived infection by both wasps more than 50% of the time, which aligns with previous findings that its high constitutive hemocyte load enables particularly effective immune responses (Kacsoh & Schlenke, 2012; Poyet *et al.*, 2013).

Lefèvre *et al.* (2012b) originally reported that the behavioral response I tested, laying fewer eggs when forced to live with wasps, was stronger in *D. melanogaster* than *D. simulans*, whereas *D. simulans* had stronger cellular immunity. My results agree with both of those findings, but this potential immune trade-off did not extend across eight fly species. In general, each fly species exhibited similar behavioral responses towards both wasps and strong cellular immunity was not associated with weak behavioral avoidance. To evaluate oviposition reduction behavior as a potential offspring quality vs. quantity trade-off, I tested whether wasp-exposed mothers produced offspring with stronger cellular immune responses, but found no support for this hypothesis. Therefore, it remains unclear whether behavioral and cellular immune responses trade off across *Drosophila* species.

Future studies could benefit from evaluating the costs and benefits of possible behavioral defenses before using them to test trade-off hypotheses. Although oviposition reduction behavior does not appear to be an offspring quality vs. quantity trade-off, there are alternative hypotheses for its adaptive value. First, this behavior could be associated with avoiding wasp-infested oviposition sites when given a choice, suggesting that oviposition preference experiments could be informative (Lefèvre *et al.*, 2012b). Alternatively, the offspring of wasp-exposed mothers might exhibit stronger wasp avoidance behaviors, such as rolling to disrupt ovipositor penetration (Hwang *et al.*, 2007) and fleeing from wasp odors (Ebrahim *et al.*, 2015). Little is currently known about the trans-generational effects of wasp exposure, but a recent study showed that female *D. melanogaster* produced a higher frequency of offspring with

recombinant genotypes after surviving infection by the relatively avirulent wasp *Leptopilina clavipes* (Singh *et al.*, 2015).

Another reason why I might not have observed trade-offs between behavioral and cellular immunity is that my host–parasite combinations were mostly allopatric. *D. melanogaster* and *D. simulans* are human commensals with broad global distributions, but other species in the subgroup are found only in small regions of Africa or on islands (Lachaise *et al.*, 1988; David *et al.*, 2007). *D. suzukii* is native to east Asia but has recently invaded Europe and North America (Adrion *et al.*, 2014), and both *Leptopilina* strains came from California, USA (Schlenke *et al.*, 2007). Although fly species in the *D. melanogaster* subgroup likely share coevolutionary history with both *Leptopilina* species given their shared African origins (Allemand *et al.*, 2002; Fleury *et al.*, 2009), recent local adaptation to sympatric parasite strains might have limited my ability to detect immune trade-offs using allopatric host–parasite combinations. Future studies could address this issue by collecting host and parasite populations at the same field sites, ideally using multiple geographically isolated sites.

D. melanogaster exhibited the weakest cellular immunity and the strongest behavioral avoidance among the species tested in Chapter 2. In Chapter 3, I tested medication with ethanol as a possible behavioral defense (Milan *et al.*, 2012; Kacsoh *et al.*, 2013; Kacsoh *et al.*, 2015). Theory predicts that specialist parasites will be more likely to evolve counter-defenses than generalist parasites (Kawecki, 1998); therefore, I hypothesized that ethanol would be more effective against the generalist wasp *L. heterotoma* than the specialist wasp *L. boulardi*. Indeed, the strongest protective effects were observed when fly larvae consumed

ethanol during exposure to the generalist wasp; fly larvae experienced a two-fold reduction in parasitization intensity and a 24-fold increase in survival to adulthood. Ethanol was substantially less effective against the specialist wasp, in agreement with the results of Milan *et al.* (2012). Weaker protection was observed when fly larvae consumed ethanol 24 hours before exposure to wasps and there were no effects of post-infection ethanol consumption.

If the latter result is also true in nature, then it seems unlikely that *D. melanogaster* larvae would seek ethanol after being parasitized; even if they did, ethanol might not confer any medicinal benefit. I allowed larvae to migrate between 0% and 6% ethanol food in bisected Petri dishes. The vast majority of larvae stayed on their starting side (0% or 6% ethanol) regardless of whether they had been parasitized. My results disagree with previous studies in which uninfected larvae preferred ethanol food (Parsons, 1977; Parsons & King, 1977) and larval migration towards ethanol increased following wasp parasitization (Milan *et al.*, 2012). I found that female flies exhibited significant oviposition preferences for ethanol food regardless of whether they had been exposed to wasps. It seems likely that this behavior protects fly larvae from wasps, but previous studies have disagreed on whether ethanol oviposition preference is innate (Richmond & Gerking, 1979; Siegal & Hartl, 1999; Azanchi *et al.*, 2013; Zhu & Fry, 2015) or induced by wasp exposure (Kacsoh *et al.*, 2013; Kacsoh *et al.*, 2015).

Previous studies that have attempted different assays (Richmond & Gerking, 1979) or analyzed conflicting results from several studies (Siegal & Hartl, 1999) have concluded that ethanol oviposition is a labile behavior in the

laboratory but have not explained this lability beyond suggesting that unknown, uncontrolled environmental factors are responsible. However, lack of ecological relevance is also a serious problem with many behavior studies. Labile results could be a consequence of not allowing animals to demonstrate their natural behaviors. Furthermore, behaviors observed in laboratory environments with artificial food sources may not translate to nature (de Roode *et al.*, 2013). For example, different fruit and yeast species could affect fly–wasp interactions in ways that cannot be observed with artificial diets. Ovipositing *D. melanogaster* females are attracted to volatile terpenes in citrus fruit rinds but these chemicals repel the specialist wasp *L. boulardi* (Dweck *et al.*, 2013). Infected fly larvae have shown feeding preferences for yeast species that enhance their cellular immune responses (Anagnostou *et al.*, 2010). Future studies of fruit fly behavioral defenses against parasitoid wasps should construct environments that better approximate natural conditions while incorporating trophic levels missing from many previous studies: fruits and yeasts.

The Red Queen hypothesis predicts that outcrossing can be maintained despite its costs if adaptive landscapes are constantly shifting and outcrossed offspring periodically gain fitness advantages that outweigh the costs of sex (Jaenike, 1978a; Maynard Smith, 1978; Bell, 1982). Multiple mechanisms can cause adaptive landscapes to shift over time and favor outcrossing, including host–parasite coevolution (Jokela *et al.*, 2009), migration (Becks & Agrawal, 2010; Gray & Goddard, 2012), and environmental change (Becks & Agrawal, 2012). However, the latter two mechanisms have not been conclusively shown to favor the long-term maintenance of outcrossing.

In Chapter 4, I tested whether host populations that had previously adapted to an ancestral parasite strain would display elevated outcrossing rates when exposed to novel parasite strains derived from that ancestor. In an 18-generation evolution experiment with the nematode *Caenorhabditis elegans* and the pathogenic bacterium *Serratia marcescens*, I found that host populations passaged with novel parasites had higher outcrossing rates, which facilitated host adaptation. Specifically, nematode populations passaged with the most virulent novel parasite exhibited the highest levels of outcrossing and the most rapid adaptation. Previous evolution experiments in this system have reported similar results with naïve ancestral host populations (Morran *et al.*, 2009; Morran *et al.*, 2011). Therefore, it is interesting that our ancestral host populations, which had previously adapted to the ancestor of the novel parasite strains we used, maintained sufficient standing genetic variation to respond to selection. My results suggest that recurring episodes of parasite turnover could favor the long-term maintenance of host outcrossing even if there is no opportunity for sustained host–parasite coevolution. Future studies should use a more diverse range of parasites than I used here and consider incorporating host migration along with parasite turnover.

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