

THE EVOLUTIONARY ECOLOGY OF RESISTANCE TO BACTERIAL INFECTION
IN NATURAL POPULATIONS OF *DROSOPHILA MELANOGASTER*

by

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(Under the Direction of Daniel E. L. Promislow)

ABSTRACT

Parasites are often unevenly distributed across host populations. Part of this variation in parasite prevalence is likely due to variation in the host's ability to resist infection. Such variation depends on both evolutionary and ecological factors. The overall purpose of this work is to understand the evolutionary and ecological forces that maintain variation for resistance within and across naturally isolated host populations of the fruit fly, *Drosophila melanogaster*. Through the work presented here, I characterize variation for host fitness following bacterial infection within and among *D. melanogaster* host populations. I then seek to determine why such variation exists by testing two hypotheses. First, I test whether genetic variation for resistance within host populations is due to tradeoffs between resistance and fitness in the absence of infection. Second, I determine whether ambient temperature and the number of bacteria co-occurring with *D. melanogaster* hosts shape among-population differences in resistance to a novel infection. Overall, these experiments address important unanswered questions regarding the evolutionary ecology of resistance in natural *D. melanogaster* populations. Further,

these results provide key insights into the natural history of *D. melanogaster*-bacteria interactions, which will motivate ecologically relevant laboratory models of insect-bacteria interactions in this experimentally tractable system.

INDEX WORDS: *Drosophila melanogaster*, *Enterococcus faecalis*, *Lactococcus lactis*, *Pseudomonas aeruginosa*, resistance, bacteria, genetic variation, geographic variation, tradeoff, temperature, species richness, evolution, ecology

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CHAPTER 1

INTRODUCTION

Studies of variation in immunity across geographic locations often find that parasites are unevenly distributed within and across host populations (Hudson *et al.* 2002). A fundamental question arises, then, of what causes this variation in parasite prevalence. One explanation is that hosts vary in their ability to resist these parasites. However, a central question in evolutionary ecology remains, if resistance to infection increases host fitness, why is some maximum level of resistance not fixed within and across host populations? From studies of resistance in nature, we know that some hosts are able to fight infection and keep their parasite load low, whereas other hosts harbor many parasites (Moller 1990, Roy 1993, Grosholz 1994, Henter and Via 1995, Boulinier 1997, Ebert *et al.* 1998, Kover and Schaal 2002, Lazzaro *et al.* 2004, Price *et al.* 2004). These observed differences in resistance among hosts have motivated many studies that aim to further characterize and explain variation for resistance observed within and across natural host populations.

Variation in host resistance can be due to either evolutionary or ecological forces. From an evolutionary perspective, there are several factors that might maintain variation, including frequency-dependent selection between hosts and parasites (van Valen 1973, Levin 1988, Dybdahl and Lively 1998, Otto and Michalakis 1998), tradeoffs between resistance and fitness in the absence of infection (Antonovics and Thrall 1994,

Kraaijeveld *et al.* 2002, Schmid-Hempel 2003), or stabilizing selection on host resistance alleles (Apanius *et al.* 1997, Thurz *et al.* 1997, Hedrick and Kim 2000, Giese and Hedrick 2003, Schad *et al.* 2004). From an ecological standpoint, factors such as host population density (Barnes and Siva-Jothy 2000, Wilson *et al.* 2002), host social structure (Barnard *et al.* 1996), or host behavior (Rosengaus *et al.* 1998) can give rise to variation in host resistance. These ecological and evolutionary factors, combined with abiotic effects such as temperature (Elliot *et al.* 2002, Stacey and Fellowes 2002, Thomas and Blanford 2003, Mitchell *et al.* 2005, Bensadia *et al.* 2006), interact to determine the resistance profile and corresponding parasite load of a host population.

Despite a recent surge in interest in the evolutionary ecology of the immune response (Rolff and Siva-Jothy 2003, Schmid-Hempel 2003), we know relatively little about the forces that maintain variation in resistance within and between populations, nor how these forces translate to broader geographic scales. Unfortunately, it has proved difficult to determine the exact mechanisms that generate variation (Little 2002), often due to the intractability of the experimental system or confounding factors such as environment and metapopulation dynamics (Little 2002). To understand resistance in nature, we need an experimentally tractable system that can be manipulated so that hypotheses related to the causes of this variation can be explicitly tested. Fortunately, *Drosophila* are highly variable in their level of response to infection (Kraaijeveld and Godfray 1999, Lazzaro *et al.* 2004), and combined with what is already known about their biology, provide us with an outstanding opportunity to study the genetic basis of resistance and the forces that maintain variation in this trait.

Experimental system

The fruit fly, *Drosophila melanogaster*, is a cosmopolitan insect, distributed throughout the United States, Europe, Asia, and South America. These dipterans are prevalent in nature and are easily collected and maintained in the laboratory. Perhaps for this reason, it has been used as a model organism for both genetic and evolutionary studies since early in the last century (Powell 1997), leading to extraordinary insight into the ecology, population biology, systematics, behavior, genetics, and molecular biology of this one organism. In addition, in recent years, there has been a steady stream of information regarding the molecular and genetic pathways underlying the *Drosophila* immune response (Tzou *et al.* 2002, Hoffmann 2003, Hultmark 2003), making it an ideal system in which to study patterns of host resistance in natural populations.

Because bacteria are ubiquitous and because *Drosophila* associate with rotting plant material, it is unsurprising that bacteria associate with *Drosophila* in nature (Leach 1952, Kvasnikov *et al.* 1971, Kimoto and Kitayama 1974, Gilbert 1980) and in laboratory culture (Brummel *et al.* 2004). However, we know almost nothing about which bacteria are actually pathogenic to *Drosophila* in the wild. Given that many of the bacterial species *D. melanogaster* associate with in nature could be pathogenic (Corby-Harris *et al.* 2007), it was important to focus on a handful of these bacterial species for the resistance experiments described here. In the experiments described here, I use three species of bacteria – *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Lactococcus lactis* – to demonstrate variation for resistance among *Drosophila* hosts following infection. The pathology of these bacteria in natural *D. melanogaster* hosts is unknown. However, they are valid bacteria for which to study resistance for several reasons. These bacteria are

commonly found in nature in soil, water, and decomposing vegetation (Forbes *et al.* 2002), and are likely to interact with cosmopolitan insects such as *D. melanogaster*. Two of these species, *E. faecalis* and *L. lactis*, were isolated from natural populations of *Drosophila* (Lazzaro *et al.* 2006). Species closely related to *P. aeruginosa*, *E. faecalis* and *L. lactis* are found in natural populations of flies (Corby-Harris *et al.* 2007). Moreover, several studies have found that these bacteria associate with insects in the wild, and in particular, that they colonize insect guts (Mead *et al.* 1988, Lacey 1997, Reeson *et al.* 2003). Overall, these bacteria increase pathogen-associated mortality in *Drosophila* and are found to naturally co-occur with *D. melanogaster*, and are therefore useful for modeling the immune response of natural *Drosophila* host populations.

The innate immune response

Although they are cognizant of the mechanisms underlying the innate immune response, many evolutionary ecologists adopt a ‘black-box’ approach to studying invertebrate immunity (Sheldon and Verhulst 1996). Therefore, the mechanisms causing the observed phenotypic variation in host resistance is generally unknown (Schmid-Hempel 2003). For example, is the evolutionary and ecologically important variation in resistance due to differences in how hosts recognize pathogens or differences in how the immune effector molecules are produced? Nonetheless, a short discussion of how invertebrates such as *D. melanogaster* resist pathogenic challenge is useful.

Invertebrates protect themselves from parasitic challenge through an innate immune response. This innate immune response is remarkably conserved across both invertebrate and vertebrate species. However, the invertebrate immune response differs

from that of vertebrates because invertebrates do not possess that extra layer of defense, the adaptive immune response, found in vertebrate species. Therefore, invertebrates cannot ‘remember’ past infection through adaptive immune cells such as T-cells. Despite this lack of adaptive immunity, invertebrates are remarkably resistant to infection.

Two main ‘arms’ govern the innate immune response – the cellular and humoral response. The cellular response mainly involves such reactions as clotting, phagocytosis, and encapsulation of foreign bodies. In contrast, the humoral response involves the upregulation of antimicrobial peptides (AMPs). These AMPs are transcriptionally upregulated under the control of the Toll and *imd* pathways (Hoffmann and Reichhart 1997, Hultmark 2003). These effector molecules are potent weapons against broad classes of bacteria and work, for example, by poking holes in the bacterial cell membrane or by altering bacterial cell membrane permeability (Zasloff 2002). The pathogen-associated elicitor molecules that activate the humoral immune response are broadly conserved across many species of bacteria, and involve recognition of cell wall motifs such as peptidoglycan, lipopolysaccharide, and β -1,4-glucan (Ferrandon *et al.* 2004). Because these effector and elicitor molecules are not specific to one species of bacteria, invertebrates are thought to possess a somewhat general immune response to bacterial infection.

Purpose and purview

The overall purpose of this work is to understand the evolutionary and ecological forces that maintain variation for resistance within and across natural insect host populations. To investigate this broad topic, I have conducted a series of experiments

that test explicit hypotheses related to how these forces act within and among *D. melanogaster* host populations. I address six questions over the next four chapters before concluding with a section where I convey several broad conclusions one should draw from these projects.

In chapter two I ask whether bacterial load, a commonly used measure of immunocompetence, is correlated with host fitness in the absence of infection. I infect strains of *D. melanogaster* with a pathogenic bacterium and then measure the correlation between host bacterial load and the ability to survive infection. Despite the presence of genotypic variation for both traits, bacterial load and survival post-infection are not correlated. These results illustrate two main points. First, direct measures of host fitness following infection, such as mortality rates, are more appropriate from in an evolutionary context than indirect measures of immunocompetence, such as bacterial load. Secondly, the observation that survival and bacterial load were not correlated suggests that defense requires hosts to balance the physiological costs of immune system upregulation and the negative effects of infection. I suggest that one way that hosts might cope with the negative effects of bacteria is by tolerating infection, an aspect of defense overlooked in much of the invertebrate literature.

In chapter three, I characterize resistance variation within a naturally isolated population of *D. melanogaster*. I determine whether naturally isolated isofemale lines vary for how well they survive being infected with one of three species of bacteria. Several authors have suggested that one reason resistance to infection is variable within host populations is because evolutionary tradeoffs between resistance and fitness prevent alleles that influence either trait from reaching fixation. I apply this tradeoff theory in

chapter two and ask whether such mechanisms explain the variation for resistance within a single population. Despite significant variation among lines for resistance to all three types of bacterial challenge, tradeoffs between resistance and fitness were not evident. This result suggests that alternative mechanisms may be operating to maintain resistance variation in this host population.

Almost nothing is known about the bacterial communities that associate with *D. melanogaster* in nature. In an effort to understand these communities, in chapter four, I use a sequenced-based approach to determine the diversity and distribution of bacteria that co-occur within natural *D. melanogaster* hosts. I find that these bacterial communities are quite diverse and differ with respect to the hosts' location. Variation in bacterial community structure across the range of *Drosophila* hosts may have implications for host resistance across large spatial scales, a topic I address in the following chapter.

In chapter five, I characterize geographic variation for resistance to bacterial infection across naturally isolated *D. melanogaster* hosts. I find that host populations differ in how well they resist separate infections with one of two species of bacteria. Motivated by previous empirical studies, I then investigate whether differences in host environment – specifically, temperature and bacterial species richness – explain among-population differences in resistance. I find that temperature is a significant predictor of resistance to *L. lactis*, while bacterial species richness explained much of the observed variation in resistance to *P. aeruginosa*.

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CHAPTER 2

ALTERNATIVE MEASURES OF RESPONSE TO *PSEUDOMONAS AERUGINOSA* INFECTION IN *DROSOPHILA MELANOGASTER*¹

¹ Corby-Harris V., K.E. Habel, F.G. Ali, and D.E.L. Promislow. 2007. *Journal of Evolutionary Biology*. 20:526-533. Reprinted here with permission of publisher.

ABSTRACT

Studies of invertebrate immune defense often measure genetic variation either for the fitness cost of infection, or for the ability of the host to clear the parasite. These studies assume that variation in measures of resistance is related to variation in fitness costs of infection. To test this assumption, we infected strains of the fruit fly, *Drosophila melanogaster*, with a pathogenic bacterium. We then measured the correlation between host bacterial load and the ability to survive infection. Despite the presence of genotypic variation for both traits, bacterial load and survival post-infection were not correlated. Our results support previous arguments that individual measures of immune function and the host's ability to survive infection may be decoupled. In light of these results, we suggest that the difference between tolerance and resistance to infection, a distinction commonly found in the plant literature, may also be of value in studies of invertebrate immunity.

INTRODUCTION

Theoretical and empirical work has shown that parasites may play an important role in the evolution of sex (Jaenike 1978, Hamilton 1980), the origin and maintenance of sexually selected traits (Hamilton and Zuk 1982), the regulation of population size (Hudson *et al.* 1998), and host behaviour (Dobson 1988). Evolutionary models of host-parasite interactions typically rest on two critical assumptions: that there is genetic variation within the host population for resistance to infection, and that there is some fitness cost to the host of being infected (Anderson and May 1982, Sorci *et al.* 1997).

In studies of invertebrates, researchers have used a variety of approaches to

identify genetic variation for host defense against pathogens. Comparisons among clonal lines of both *Daphnia* (Ebert *et al.* 1998, Little and Ebert 2000, Carius *et al.* 2001, Haag and Ebert 2004, Mucklow *et al.* 2004) and aphids (Henter and Via 1995, Ferrari *et al.* 2001) have found significant variation in resistance to bacterial and fungal infection as well as to parasitoid attack. Using artificial selection in *Drosophila* (Fellowes *et al.* 1999) and snails (Webster and Woolhouse 1998), researchers have successfully created lines that are highly resistant to parasitoid and parasite attack, respectively. Numerous other studies have estimated genetic variation for other measures that describe immunodefense capabilities, including differences in survival post-infection (Schulenburg and Ewbank 2004), haemocyte counts (Cotter *et al.* 2004), cuticular melanisation (Cotter *et al.* 2004), encapsulation ability (Lambrechts *et al.* 2005), phenoloxidase activity (Cotter *et al.* 2004, Mucklow *et al.* 2004), infectivity (Grosholz 1994, Henter and Via 1995, Ebert *et al.* 1998, Carius *et al.* 2001, Ferrari *et al.* 2001, Little and Ebert 2001), pathogen load (Lazzaro *et al.* 2004, Lambrechts *et al.* 2005), and antimicrobial activity (Cotter *et al.* 2004). Taken together, these studies suggest that there is abundant genetic variation for defense against pathogens in natural populations.

Despite the many examples that demonstrate the presence of genetic variation for host fitness post-infection, it is still unclear just how these immune parameters relate to host fitness post-infection (Adamo 2004b). In fact, many studies have demonstrated that there is not always a positive relationship between immunocompetence parameters and disease resistance (reviewed in Adamo (2004)). For this reason, it is important to understand how different measures of immunocompetence relate to each other and, if there is not a clear relationship, to ask why they are not correlated.

Differences in immune parameters, such as bacterial load, may not translate into differences in host fitness if the host is tolerant to the infection. Plant biologists draw a useful distinction between resistance, a character that reduces the parasite's chances of successful infection or increases the host's rate of parasite clearance (Restif and Koella 2004), and tolerance, the host's ability to experience damage without suffering a reduction in fitness (Mauricio 2000). This distinction between resistance and tolerance has been explored in theoretical treatments of host-parasite interactions in invertebrates (Boots and Bowers 1999, Restif and Koella 2004). However, it remains to be determined how these two different aspects of host defense in invertebrates interact to determine host fitness in the presence of infection.

Many studies make the tacit assumption that a single measure of immunocompetence is a reasonable proxy for the effects of infection on host fitness. Here, we set out to test this assumption explicitly. In particular, we were interested in the relationship between host pathogen load, which we suspect to be related to resistance, and survival, a measure of immunocompetence that is more directly related to overall host fitness in the presence of infection. To compare these two characters, we infected different genotypes of the fruit fly, *D. melanogaster*, with a pathogenic bacterium, *Pseudomonas aeruginosa*, and then measured both pathogen load and survival post-infection. If pathogen load and mortality are strongly and positively correlated, then this single measure of immunocompetence, pathogen load, should be a good predictor of how a particular host population is likely to fight bacterial infection. If we find genotypic variation for both of these traits, but fail to find a correlation between them, this would suggest that invertebrates use processes apart from initial resistance (as defined by Restif

and Koella (2004)) to cope with microbial infections. The distinction made between resistance and tolerance, a common element in the plant literature, is relatively unknown among insect studies. By focusing explicitly on the outcome of these two aspects of immunity, the research described here should increase our understanding of whether these two processes have the potential to occur in invertebrates.

MATERIALS AND METHODS

Fly Strains

We measured response to infection in eleven lines of *D. melanogaster*, six of which were obtained from B.P. Lazzaro (2b, 2c, 3a, 5c, 7b, and 9h), and five isolated from wild populations and inbred for approximately 20 generations. The stocks were maintained on standard cornmeal:molasses:yeast fly medium at 24°C on a 12:12 h light:dark cycle. The six lines donated by B.P. Lazzaro had been shown previously to differ in mean bacterial load after infection with *Serratia marcescens* (Lazzaro *et al.* 2004). Each of these lines contained a different second chromosome, isolated directly from a wild North American population, and then crossed into a common genetic background.

Details of infection

We acquired a strain of *Pseudomonas aeruginosa*, a laboratory stock culture, from B.P. Lazzaro. This culture is a laboratory stock and was not derived from wild flies. However, *P. aeruginosa* is a common insect pathogen (Lacey 1997) and *Pseudomonas* species have been identified in wild *D. melanogaster* (V. Corby-Harris, unpublished

manuscript). Working stocks of bacterial cultures were maintained in nutrient broth at 4°C in a 10^{-2} dilution (of an unmeasured dense culture) until ready for use. The day before the infections, these working stocks were grown for approximately 18 h at 37°C, which corresponds to the log phase of growth (V. Corby-Harris, unpublished data).

For each of the eleven lines, we injected a random sample of approximately 1081 mated and virgin male flies that were between 24 and 48 hours old. This random sample was collected 24 hours prior to the infections, at ages 0-24 hours. Therefore, the individuals that were infected were a random combination of both mated and virgin flies. A subset (N = 120) of the flies was infected with a sterile broth culture as a negative control. Of the 961 flies injected with bacteria, in 536 flies we monitored survival post-infection for 48 h. In the remaining 425 individuals, we assayed for bacterial load at approximately 20 h after infection. The infections were performed across 4 days and all lines were tested each of the four days. Among the control flies, only 1 out of 60 flies monitored for survival died within 48 hours (0.02%), and zero out of the of the 60 control plates exhibited bacterial growth. Consequently, we limit all discussion to infected flies.

Adult flies were manually infected in the lateral thorax using a fine stainless steel needle (Fine Science Tools, Foster City, CA) dipped in bacterial culture (Tzou *et al.* 2002). The bacterial culture was standardized by diluting the overnight culture with sterile nutrient broth until it measured 0.200 ± 0.050 A at 600λ using a UV spectrophotometer (Thermo Electron, Rochester, NY). After standardizing the culture, we diluted it further by adding one part of the standardized bacterial culture to five parts sterile nutrient broth. This technique yielded fewer bacteria per plate while still allowing us to standardize the culture reliably with the spectrophotometer.

Survival

To monitor survival post-infection, the infected male flies were placed into clean, unyeasted vials with 5 ml of standard cornmeal:molasses:yeast fly medium in groups of ten individuals, and monitored for 47 hours at 24°C with a 12:12 h light:dark cycle. We recorded the number of dead flies in each vial at two and seven hours post-infection, hourly from 16 through 34 hours post-infection, and then again at 41 and 47 hours post-infection. In previous assays, we found that flies that appeared to die of infection did so between eight and 47h of being infected (V. Corby-Harris, unpublished data). Flies dying before this period seem to die of the injury itself, and mortality abruptly declines after 47 hours post-infection. While we have not explicitly tested for why mortality abruptly declines after 47 hours, we surmise that those individuals who live past this point have cleared the infection.

We tested for the effect of genotype on host survival using a combination of statistical approaches. First, a Cox Proportional Hazards Model (Parmar and Machin 1995) was used to test for the effect of genotype on an individual's survival time post-infection. Host genotype was treated as a fixed effect and date was treated as a strata variable. Next, we tested for the effect of genotype on host survival within each date separately using the proportional hazards model. Individuals dying before eight hours post-infection (N = 3) were eliminated from further analyses, and flies living past 47 hours (N = 44) were censored. Last, an ANOVA model was used to test for the effect of genotype and date on the proportion of censored individuals within a vial. All predictor

variables were treated as fixed effects in this model. Survival analyses were performed using proc tphreg in SAS version 9.1 (SAS Institute, Cary, NC) and JMP Version 5.0.1a (SAS 2003).

Bacterial load

To measure the number of bacteria present in the fly following infection, surviving flies were anaesthetized under light CO₂ gas and briefly placed singly into 50 ul of 70% ethanol to wash off any surface microbes. This wash kills only surface bacteria and does not affect the bacteria inside of the fly (V. Corby-Harris, unpublished data). Individual flies were then placed into 50 ul of sterile distilled water and homogenized. The homogenate was then manually spread onto selective *Pseudomonas* isolation media (Remel Inc., Lenexa, KS), incubated at 37°C for approximately 20 h, and the number of colonies on the plate was counted manually. Due to the high number of colony forming units (CFUs) per plate, each plate was divided into 27 numbered pie pieces, and CFUs were counted in three randomly chosen sections. To estimate the total number of CFUs per plate, or plate count, we multiplied this number by nine. In 48 cases (11%), growth was quite dense (greater than 8000 CFUs per plate), with colonies overlapping one another. These plates were labeled as having too many to count. All flies were assayed for bacterial load at 20 hours post-infection, which corresponds to the point at which mortality begins to increase relative to the negative control. Assaying bacterial load at this single time point should adequately represent qualitative differences in bacterial load across host genotypes during the course of infection based on previous work showing

that, for flies infected with *Serratia marcescens*, there is no interaction between the time load is assayed and genotype (Lazzaro *et al.* 2004).

A series of nonparametric models was employed to analyze the potential sources of the observed variation in bacterial load. The distribution of the error variances was significantly non-normally distributed despite a variety of transformations. Therefore, we used the rank of the plate count as the response (Conover and Iman 1981) in an ANOVA to analyze the full model where host genotype, date, the interaction between host genotype and date, and individuals nested within genotype were the independent variables. We also analyzed the full model including host genotype and individuals nested within genotype for each date separately. Ties were treated as the average between ranks. An advantage of this approach is that it allowed us to include plates that had either no growth (N = 111; 26%) or too many CFUs to count (N = 48; 11%). However, this method also has drawbacks. Notably, it tends to underestimate the sum of squares for the interaction effect (Salter and Fawcett 1993). Unfortunately, more robust nonparametric models (Sokal and Rohlf 1995), such as the Kruskal-Wallis and Friedman's tests, are not useful for models including a nested effect. Therefore, we began by using the method of Conover and Iman (1981) to test the full model. Because we did not observe a significant effect of individuals nested within genotype when dates were pooled ($F_{99,282} = 1.28$, $P = 0.0635$) or analyzed separately (day 1: $F_{11,88} = 0.33$, $P = 0.9825$; day 2: $F_{11,86} = 1.11$, $P = 0.3621$; day 3: $F_{11,88} = 0.60$, $P = 0.8235$; day 4: $F_{11,86} = 1.74$, $P = 0.0789$), we then analyzed a reduced model using more robust approaches. For the data pooled across dates, we analyzed the effect of host genotype, date, and the interaction between genotype and date on the rank of the plate count using a Scheirer-Ray-Hare extension of

the Kruskal-Wallis test (Sokal and Rohlf 1995). For each date treated separately, the effect of host genotype on the rank of the plate count was analyzed using the Kruskal-Wallis test (Sokal and Rohlf 1995). Again, ties were treated as the average between ranks. All bacterial load analyses were performed using proc glm in SAS version 9.1 (SAS 2005).

Genotypic correlation

In addition to determining whether genotype was a significant predictor of each individual trait, we tested for a positive genotypic correlation between the two traits, for each date separately and pooled across all four days, using Spearman's rank correlation in JMP version 5.0.1 a (SAS 2003). Family means were used to determine the genotypic correlation between the traits (Lynch and Walsh 1997). To estimate the family means for survival post-infection, we used the parameter estimate for the hazard ratio generated under the proportional hazards model. Under this model, a hazard ratio greater than one indicates a greater risk of death post-infection. To estimate the family means for bacterial load post-infection, we used the least-square estimates of the rank of the plate count generated under the nonparametric model. The Z-transform test (Whitlock 2005) was employed to combine the results of the correlation tests performed for separate dates into one overall test for a positive correlation between traits.

RESULTS

Survival

Among the flies treated with *P. aeruginosa* (N = 536), 91.7% died within the 47 hour period when data were collected. Across all eleven genotypes, the mean time to death was 37.03 ± 0.44 hours among those flies that died within the 47 hour period (N = 492). Under the proportional hazards model, host genotype (Wald $\chi^2_{10} = 41.06$, $p < 0.0001$; Figure 2.1) was a significant predictor of mortality post-infection when the dates were pooled. Treating each date separately, there was a significant effect of host genotype on mortality post infection on days two (Wald $\chi^2_{10} = 24.78$, $P = 0.0058$) and four (Wald $\chi^2_{10} = 34.83$, $P = 0.0001$), but not for days one (Wald $\chi^2_{10} = 12.95$, $P = 0.2263$) and three (Wald $\chi^2_{10} = 11.97$, $P = 0.2872$). There was no effect of genotype ($F_{10,30} = 1.55$, $P = 0.1687$) or date ($F_{3,30} = 2.77$, $P = 0.0587$) on the proportion of censored individuals within a vial.

Bacterial load

Across all genotypes, the mean number of CFUs per plate (excluding those that had too many to count) was 1280 ± 1800.1 s.d. among those individuals treated with *P. aeruginosa* (N = 425). Host genotype ($H_{10} = 35.97$, $P < 0.0001$, Figure 2.2), date ($H_3 = 60.64$, $P < 0.0001$), and the interaction between host genotype and date ($H_{30} = 78.16$, $P < 0.0001$) were significant predictors of the rank of the plate count. For each date treated separately, host genotype was a significant predictor of the rank of the plate count (day 1: $\chi^2_{10} = 40.63$; $P < 0.0001$; day 2: $\chi^2_{10} = 33.48$; $P = 0.0002$; day 3: $\chi^2_{10} = 32.45$; $P = 0.0003$; day 4: $\chi^2_{10} = 34.38$; $P = 0.0002$).

Correlation between survival and bacterial load post-infection

Pooled across dates, we did not find a significant positive genotypic correlation between the hazard ratio and rank of the plate count at 20 hours post-infection (Spearman's $\rho = -0.22$; one-tailed $P = 0.26$; Figure 2.3a) among the eleven family lines. When the correlation was analyzed separately for each date, the relationship between mortality and bacterial load post-infection was nonsignificant across three dates (day 1: Spearman's $\rho = 0.42$; one-tailed $P = 0.10$; day 2: Spearman's $\rho = 0.13$; one-tailed $P = 0.35$; day 4: Spearman's $\rho = -0.54$; one-tailed $P = 0.46$; Figure 2.3b), and significant on one (day 3: Spearman's $\rho = 0.56$; one-tailed $P = 0.04$; Figure 3.3b). There was not a significant positive correlation between mortality and bacterial load post-infection ($Z_{s(4)} = 0.639$; $P = 0.52$) after combining the four dates.

DISCUSSION

In the experiments described here, we found that genotype was a significant predictor of the ability to suppress and clear an infection with *P. aeruginosa* and to survive post-infection. However, contrary to our expectation, we did not find a significant genotypic correlation between the two traits, suggesting that pathogen load may be a poor proxy for host fitness post-infection. Our results lend further support to a recent suggestion that in both vertebrates and invertebrates, as in plants, the relationship between measures of immunity and the ability of the host to survive infection may be uncorrelated (Adamo 2004b).

The primary conclusion drawn from these data, that bacterial load is not a reliable predictor of host survival post-infection, has important implications for how researchers assay immunocompetence. First, although we did not observe a correlation between these traits, it is possible that another immune trait is a more reliable predictor of host fitness post-infection. For example, work by Adamo (2004a) in the cricket, *Gryllus texensis*, showed no correlation between total phenoloxidase activity or baseline lysozyme-like activity and an individual's ability to resist bacterial challenge. However, there was a correlation between the magnitude of the increase in lysozyme-like activity post-infection and resistance to bacterial infection, as well as between total hemolymph protein concentration and bacterial resistance (Adamo 2004a). It is also possible that reproduction, not survival, is negatively correlated with bacterial load. If this were true, then bacterial load would be a reliable predictor of host fitness post-infection. Clearly, further studies are necessary to determine what measures of host immunocompetence are reliable predictors of host fitness post-infection.

These findings also have important implications for how biologists envision invertebrate immune defense. Studies that focus on a single measure of immunocompetence might miss the fact that a host's response to pathogens is likely to be far more complex than simply resisting infection. A simple, two-dimensional heuristic diagram illustrates the value of considering multiple measures of immunodefense (Figure 2.4). If we compare different genotypes for their ability to clear bacteria (measured in terms of bacterial load), and for their ability to survive infection, we can identify four distinct patterns that could explain the maintenance of genetic variation for immune function in natural populations. Among genotypes with low survival rates, individuals

may exhibit low pathogen loads but high mortality rates due to the high cost of mounting an immune response (Moret and Schmid-Hempel 2000, Kraaijeveld *et al.* 2002, Zerofsky *et al.* 2005), or they may exhibit high pathogen loads and high mortality rates due to the high fitness cost of harboring a virulent pathogen. In contrast, genotypes with high survival rates may have well-functioning or low-cost immune systems (and consequently low pathogen loads), or may simply be tolerant to the infection, so they need not invest resources in clearing the infection. This distinction between resistance and tolerance has been surprisingly underrepresented in the invertebrate immunology literature (but see Boots and Bowers (1999), Hansen and Koella (2003), and Restif and Koella (2004)). Our results suggest that tolerance to bacterial infection may be an important mode of immune defense in *D. melanogaster*. More and longer-term studies are needed to determine those circumstances under which hosts might be able to evolve tolerance.

Caveats

It is possible, of course, that in some cases the lack of a correlation between survival and bacterial load is due to a type II error. This type II error could be due to significant day-to-day variation in both bacterial load and mortality, which could confound the correlation between the traits. Three possible factors might account for differences across days – variation across days in the bacterial dose that the flies are infected with, variation in temperature, and variation in male mating status. Post hoc analyses show that dose does not predict variation in the rank of the plate count (Spearman's $\rho = 0.20$, $p = 0.80$) or mean time to death (Spearman's $\rho = 0.20$, $p = 0.80$), suggesting that the bacterial treatment was consistent across days. Temperature is

an important regulator of host-parasite interactions in insects (Thomas and Blanford 2003), and in *Drosophila*, temperature affects resistance to parasitoid attack (Fellowes *et al.* 1999), encapsulation ability (Salt 1970), and lifespan (Loeb and Northrop 1916). It is unclear whether the very small ($\pm 1^\circ\text{C}$) fluctuations in temperature that these flies experienced affected either bacterial load or survival post-infection. As previous work in *Drosophila* suggests (McKean and Nunney 2001), male mating status might have affected host immunocompetence in our assays, because we did not assay only virgin males. Clearly, both bacterial load and mortality are sensitive to a variety of environmental effects. It would be of obvious interest to determine if these different measures of immune function respond differently to various environmental factors.

Despite this variation among days, our data suggest that our estimates of bacterial load and survival were reliable. Within days, genotype is a significant predictor of bacterial load on all four dates and of mortality on two of the dates (one and four). Thus, despite the experimental noise, there are differences in both response variables that can be attributed to genotypic variation. On the two dates where there is a significant effect of genotype on both mortality and load (days one and four), there is still no relationship between mortality and bacterial load. These results suggest that the relationship between mortality and bacterial load are robust.

The lack of correlation between pathogen load and fitness could also be due, in part, to the way in which the pathogen entered and attacked the host tissues in our study. Here, we used the standard septic injury protocol (Tzou *et al.* 2002). However, at least among adults, bacteria in nature usually enter the insect's system orally or through the tracheoles (Vodovar *et al.* 2004). It is unlikely that the method of infection accounts for

the lack of correlation, as previous work has shown that there is a significant concordance between genes induced by natural oral infection and those induced by septic injury (Vodovar *et al.* 2005). While these caveats point to the many factors that may influence invertebrate immune response, taken together, our results suggest that we have correctly accepted the null hypothesis of no correlation.

Conclusion

The diagram that we presented in Figure 2.4 suggests a useful framework for future studies, and points to at least three specific problems that should provide a focus in our attempts to understand variation for immune function in natural populations of insects. First, we need to consider which immune assays can provide the most robust measure of how hosts resist infection. Second, following from an already extensive plant literature (reviewed in (Strauss and Agrawal 1999, Mauricio 2000)), studies of invertebrate immunity are needed to determine if tolerance and resistance exist in insects, and whether these two elements of immunity have distinct physiological and genetic bases. Third, if tolerance and resistance do exist in natural host populations, how important are they in determining overall host defense in natural populations? Together, this information could have implications for how we think about the evolutionary ecology of the invertebrate immune response.

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FIGURE LEGENDS

Figure 2.1. Family mean estimate of the hazard ratio \pm 1 s.e. for each genotype.

Individuals with low hazard ratios have higher survival post-infection. Under a proportional hazards model, host genotype (Wald $\chi^2_{10} = 41.06$, $p < 0.0001$) was a significant predictor of time to death post-infection.

Figure 2.2. Least squares estimates of the family mean \pm 1 s.e. for the rank of the plate count at 20 hours post-infection. Using a Scheirer-Ray-Hare extension of the Kruskal-Wallis Test, host genotype ($H_{10} = 35.97$, $p < 0.0001$), date ($H_3 = 60.64$, $p < 0.0001$), and the interaction between host genotype and date ($H_{30} = 78.16$, $p < 0.0001$) were significant predictors of the rank of the plate count.

Figure 2.3. (a) Overall correlation between hazard ratio and pathogen load (Spearman's $\rho = -0.22$; one-tailed $p = 0.26$), generated using the family mean estimates for both the hazard ratio and ranked plate count. (b) Correlation between hazard ratio and pathogen load, separated by date. The positive correlation was nonsignificant across three dates (day 1: Spearman's $\rho = 0.42$; one-tailed $p = 0.10$; day 2: Spearman's $\rho = 0.13$; one-tailed $p = 0.35$; day 4: Spearman's $\rho = -0.54$; one-tailed $p = 0.46$), and significant on one (day 3: Spearman's $\rho = 0.56$; one-tailed $p = 0.04$). Summed across dates, there was not a significant positive relationship between the two traits ($Z_{s(4)} = 0.639$; $p = 0.52$).

Figure 2.4. Schematic diagram classifying genotypes based on survival and bacterial pathogen load. The figure illustrates four possible classes of flies, including ones with high or low bacterial load, and high or low survival to infection. Further details are provided in the Discussion.

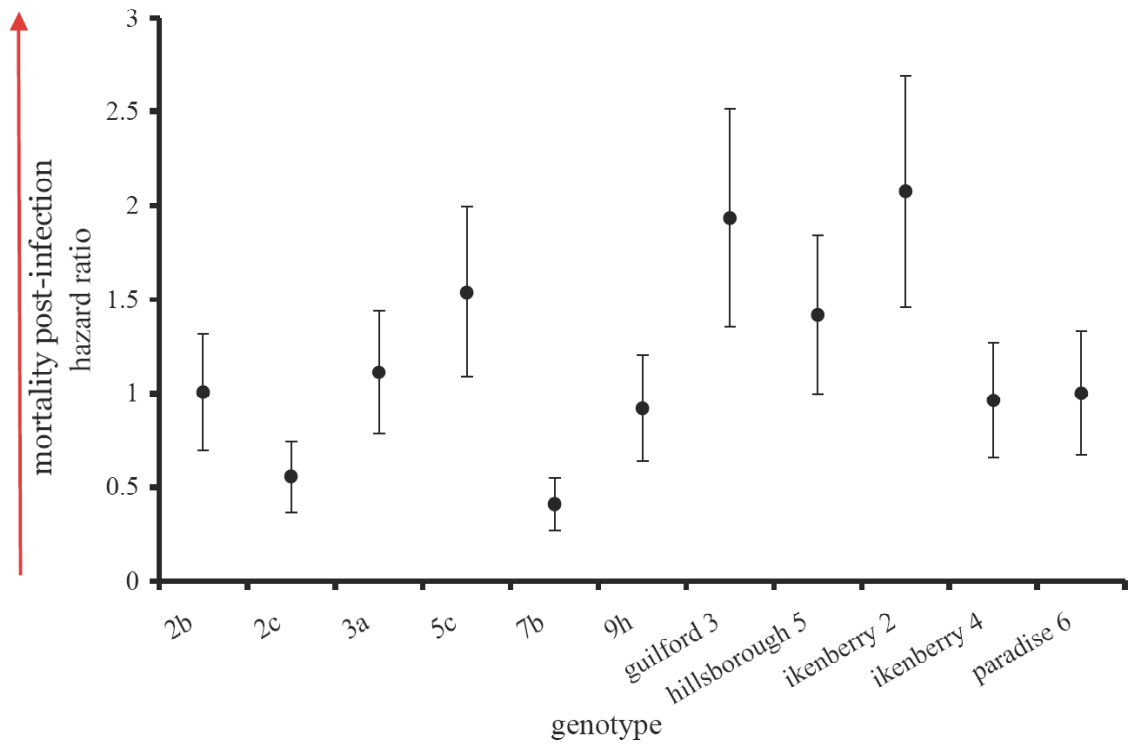


Figure 2.1

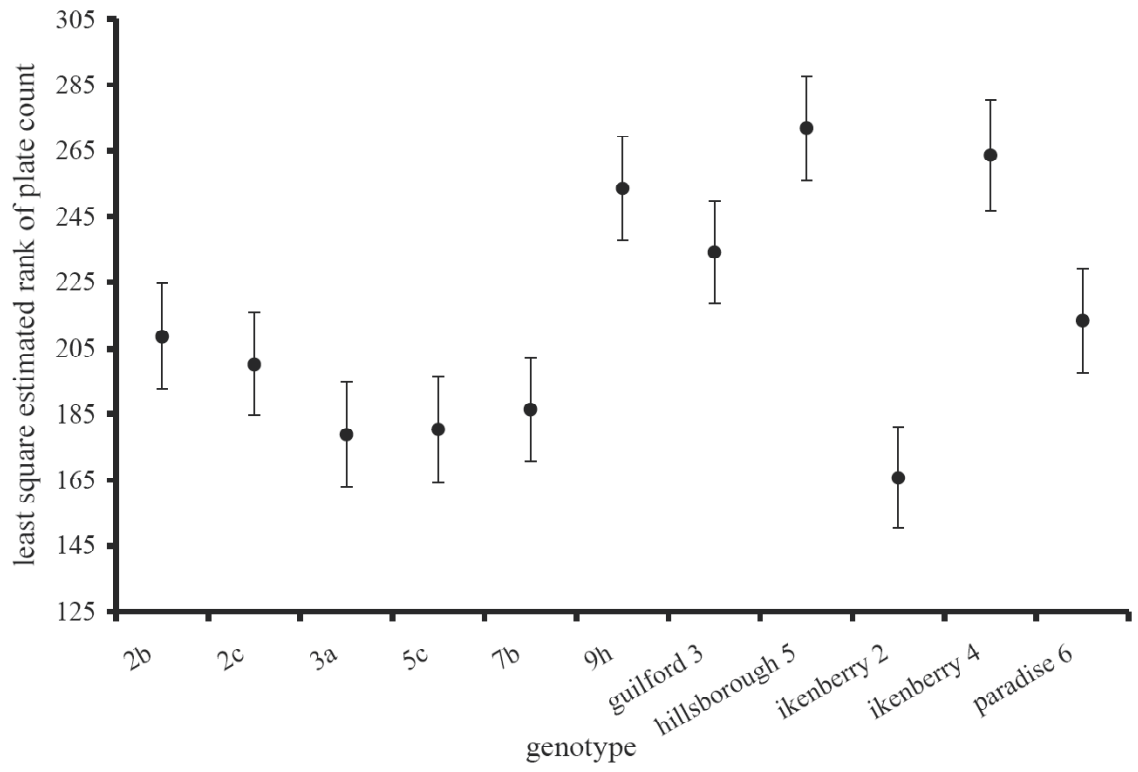


Figure 2.2

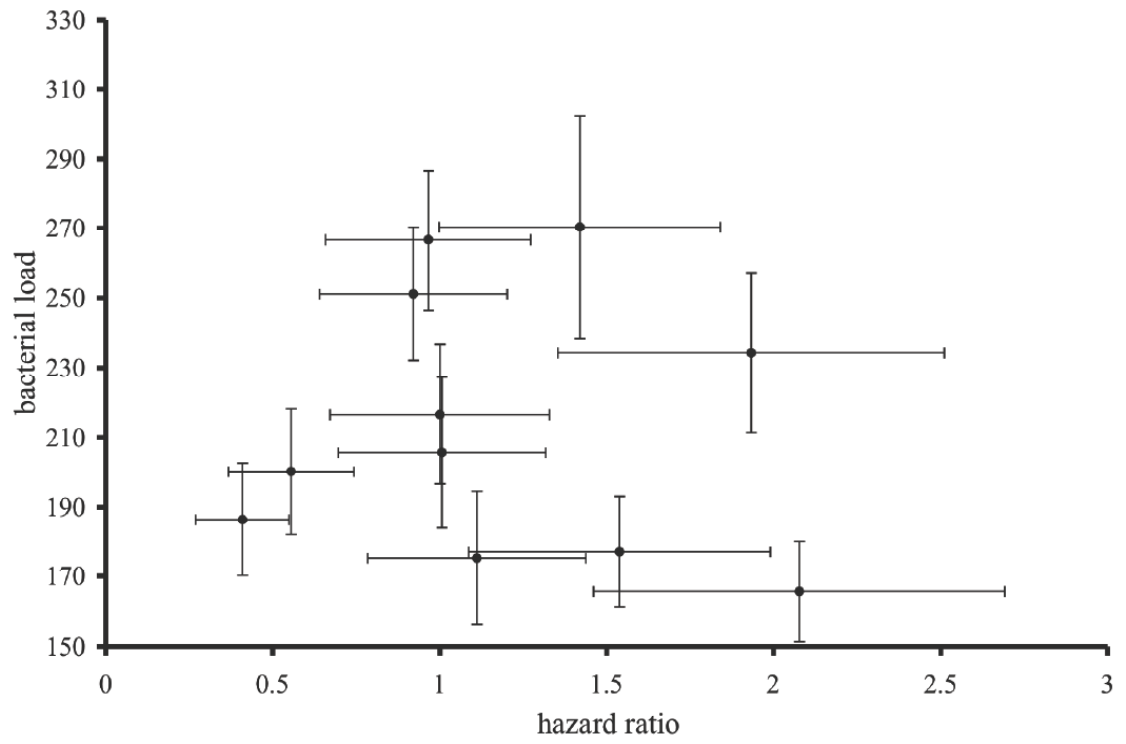


Figure 2.3 (a)

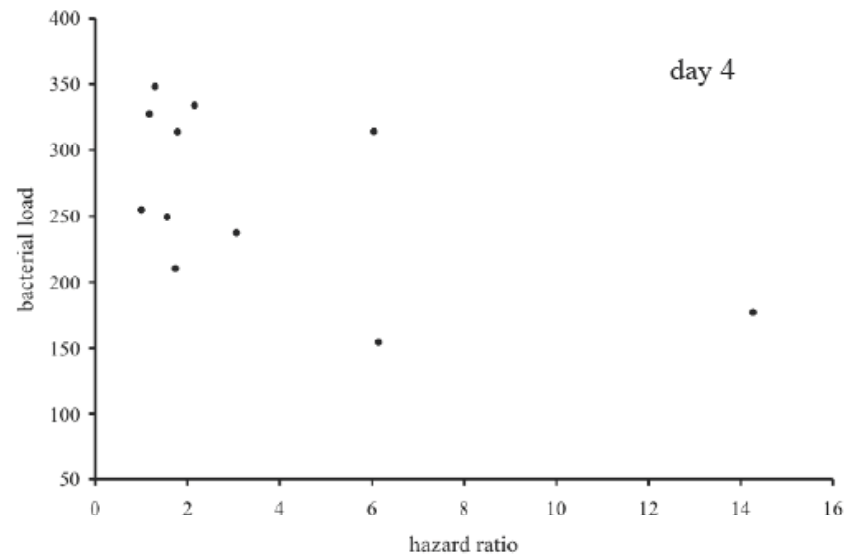
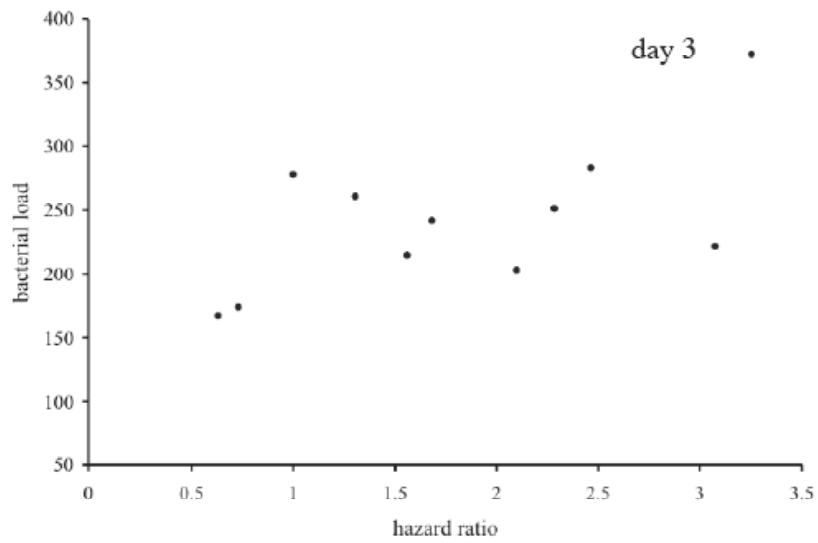
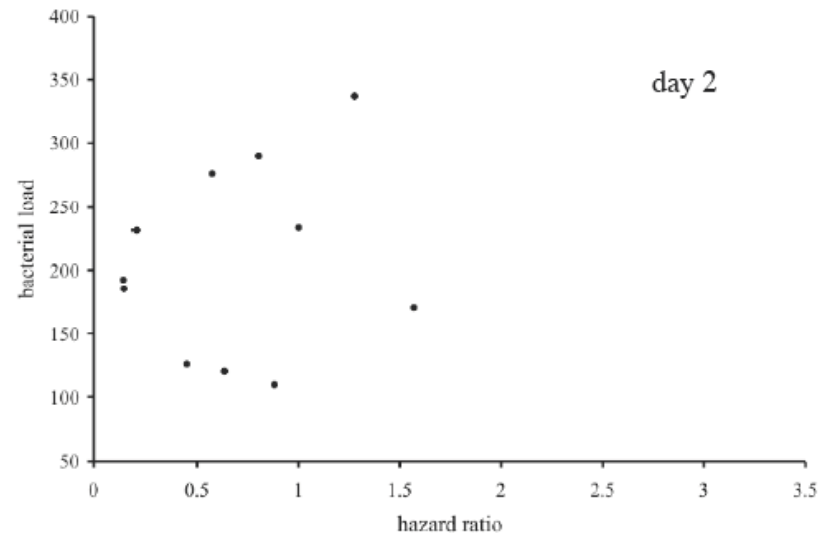
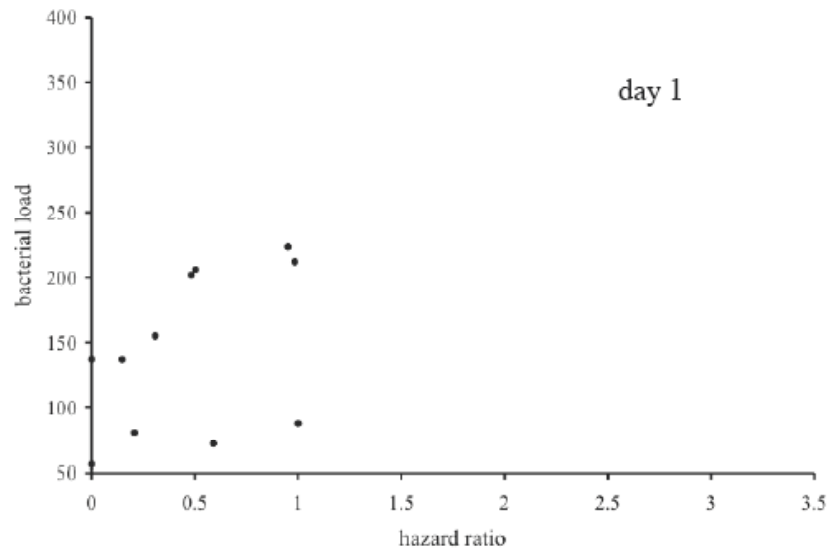


Figure 2.3 (b)

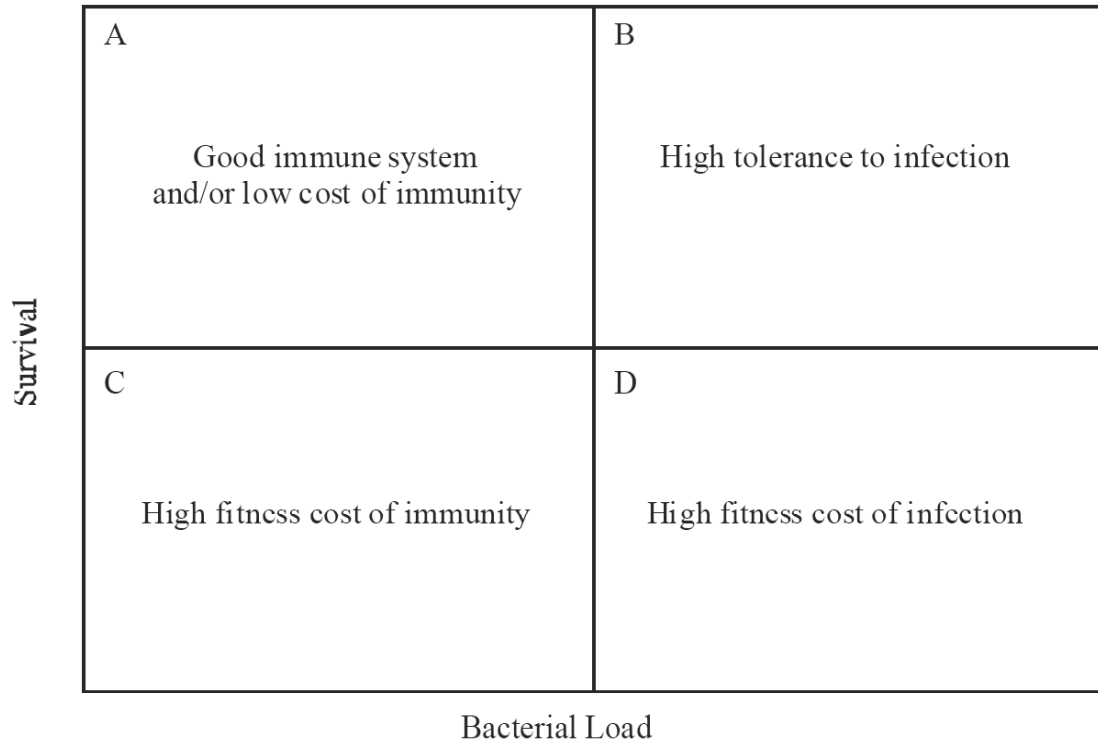


Figure 2.4

CHAPTER 3
GENOTYPIC VARIATION FOR RESISTANCE TO BACTERIAL INFECTION IN
NATURAL POPULATIONS OF *DROSOPHILA MELANOGASTER*²

² Corby-Harris, V. and D.E.L. Promislow. Submitted to *Evolutionary Ecology Research*,
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ABSTRACT

Despite several studies assaying the genetic architecture of resistance in natural populations of *Drosophila melanogaster*, there is currently no definitive evidence showing that fitness in the presence of infection, a requirement for the evolution of resistance, varies across host genotypes. In the experiments described here, we infected sixteen naturally-isolated genotypes of *D. melanogaster* with three different species of bacteria – *Pseudomonas aeruginosa*, *Lactococcus lactis*, and *Enterococcus faecalis*. We found that host genotype was a significant predictor of the ability to survive all three species of bacterial infection. Motivated by previous studies of tradeoffs in *D. melanogaster*, we then asked whether such forces explained differences in bacterial resistance among the genotypes we assayed. Despite the presence of genotypic variation for both resistance and host fitness in the absence of infection, we did not detect negative correlations between the two traits. Overall, these data suggest that there is significant genetic variability for resistance to bacterial infection within natural populations of *D. melanogaster*. However, across these host genotypes, this variation is not explained by evolutionary tradeoffs. We suggest that other factors shape resistance to bacterial infection within natural populations of *D. melanogaster*.

INTRODUCTION

Theoretical and empirical work suggests that parasites may play a role in the evolution of sex (Jaenike 1978, Hamilton 1980) and sexually selected traits (Hamilton and Zuk 1982), regulation of host population size (Hudson *et al.* 1998), and host behavior (Dobson 1988). Evolutionary models of host-parasite interactions typically rest on the

critical assumption that there is genetic variation within the host population for resistance to infection (Anderson and May 1982, Sorci *et al.* 1997). This assumption has motivated numerous empirical studies investigating whether genetic variation for resistance, in fact, exists in natural host populations (Grosholz 1994, Henter and Via 1995, Ebert *et al.* 1998, Little and Ebert 2000, Carius *et al.* 2001, Ferrari *et al.* 2001, Lazzaro *et al.* 2004, Mucklow *et al.* 2004, Schulenburg and Ewbank 2004, Lambrechts *et al.* 2005, Lazzaro *et al.* 2006, Tinsley *et al.* 2006).

In *Drosophila melanogaster*, researchers have used a variety of approaches to assay levels of genetic variation for host defense against pathogens (Fellowes *et al.* 1999, Lazzaro *et al.* 2004, Lazzaro *et al.* 2006, Tinsley *et al.* 2006). There appears to be considerable genetic variation for resistance to bacterial infection, in particular. For example, Lazzaro *et al.* (2004, 2006) showed that different *D. melanogaster* host genotypes exhibit variation for bacterial load post-inoculation (Lazzaro *et al.* 2004) and that the genetic architecture of this response differs with respect to the species of bacteria hosts are challenged with (Lazzaro *et al.* 2006). However, what is important from an evolutionary standpoint is whether these genetic differences translate to fitness differences among hosts. It is questionable whether bacterial load, the measure of resistance employed by Lazzaro *et al.* (2004, 2006), is an appropriate way to assay differences in host fitness following bacterial infection (Corby-Harris *et al.* 2007a). An alternative, more appropriate, way to show that genetic differences lead to fitness differences following infection is to assay host fitness directly by measuring traits such as survival post-infection.

Infection with pathogens can cause considerable negative effects on host fitness. A central question in biology, therefore, is why resistance to infection varies at all within host populations. One mechanism that might maintain such variation is negative genetic covariances, or tradeoffs, between immunity and host fitness in the absence of infection (Kraaijeveld *et al.* 2002, Rolff and Siva-Jothy 2003, Schmid-Hempel 2003). Such tradeoffs between resistance to parasitoid attack and host fitness have been demonstrated in many invertebrate systems (Boots and Begon 1993, Sheldon and Verhulst 1996, Cotter *et al.* 2004, Lambrechts *et al.* 2004), including *D. melanogaster* (Kraaijeveld and Godfray 1997, Fellowes *et al.* 1998). For example, Kraaijeveld and Godfray (1997) selected increased resistance to a larval parasitoid, *Asobara tabida*, and then assayed larval competitive ability, a correlated response to artificial selection. They observed that highly resistant lines were less competitive than susceptible lines, suggesting that evolutionary tradeoffs are operating in *D. melanogaster* larvae (Kraaijeveld and Godfray 1997). Currently, there are no examples of tradeoffs between host fitness and resistance to bacterial infection in natural populations of *D. melanogaster*. However, based on previous work with *D. melanogaster* hosts and their parasitoids, we might expect a negative relationship between resistance to bacteria and host fitness in the absence of infection.

In the experiments described here, we add to the growing body of literature concerning the presence and maintenance of genetic variation for resistance to bacterial infection in *D. melanogaster*. Specifically, we test for differences among host genotypes in survival following infection with three species of bacteria. We then determined whether differences among lines were explained by negative genetic relationships

between resistance and either survival or male fertility in the absence of infection. Our assays suggest that there is a substantial contribution of host genotype to the observed variation in host fitness following infection. However, tradeoffs did not account for the observed resistance variation, suggesting that mechanisms other than evolutionary tradeoffs may operate to maintain genetic variation within this host population.

MATERIALS AND METHODS

Fly Strains

We measured resistance to bacterial infection in sixteen isofemale lines. These isofemale lines were collected from fruit orchards in Northeast Georgia in the summer and fall of 2003 and were inbred for approximately 30 generations before being assayed for resistance and fitness. The stocks were maintained on standard cornmeal, molasses, and yeast fly medium at 24°C on a 12:12 h light:dark cycle.

Bacterial stocks

We acquired one strain each of *Pseudomonas aeruginosa*, *Lactococcus lactis*, and *Enterococcus faecalis* from B.P. Lazzaro in January of 2004. *E. faecalis* and *L. lactis* were isolated from a wild population of *D. melanogaster* in Pennsylvania (B.P. Lazzaro, personal communication). *P. aeruginosa* is a laboratory stock and was not derived from wild flies. However, it is a common insect pathogen (Lacey 1997) and *Pseudomonas* species have been identified in wild *D. melanogaster* (Corby-Harris *et al.* 2007b). Working stocks of bacterial cultures were maintained in nutrient broth at 4°C in a 10⁻²

dilution (of an unmeasured dense culture) until ready for use. The day before the infections, these working stocks were grown for approximately 18 h at 37°C, which corresponds to the log phase of growth (V. Corby-Harris, unpublished data).

Measuring resistance

For each of the sixteen lines, we injected a random sample of 2559 five-day-old mated and virgin male flies with one of the three bacterial treatments (N = 1919) or, as a negative control, sterile broth culture (N = 640). Infections were performed across twenty days. The design was unbalanced: not all genotypes were assayed for resistance each day. Adult flies were manually infected in the lateral thorax using a fine stainless steel needle (Fine Science Tools, Foster City, CA) dipped in bacterial culture (Tzou *et al.* 2002). The morning of the infections, the bacterial cultures were standardized by diluting an overnight culture with sterile nutrient broth until it measured 0.200 ± 0.050 A at 600λ using a UV spectrophotometer (Thermo Electron, Rochester, NY). Resistance to infection was measured by assaying individual survival following the application of each bacterial treatment. To monitor survival post-infection, the infected male flies were placed into clean, unyeasted vials with 5 ml of standard cornmeal, molasses, and yeast fly medium in groups of ten individuals, and monitored every hour for 48 hours at 24°C with a 12:12 h light:dark cycle. In pilot assays, we found that flies that appeared to die of infection did so between twelve and 48h of being infected (V. Corby-Harris, unpublished data). Across the three species of bacterial treatments, four individuals died before twelve hours post-infection (0.0021%). Among the control flies, only 2 died within 48 hours (0.0031%). Consequently, survival analyses were limited to infected flies dying from

bacterial infection, not injury. Additionally, flies living past 48 hours ($N = 104$) were censored in the survival model.

For each species of bacteria, we determined whether lines differed in resistance to infection using a combination of approaches. First, to test for the effect of genotype on host survival time post-infection, we analyzed a Proportional Hazards Model (Cox 1972, Collett 1994, Parmar and Machin 1995, Therneau and Grambsch 2000) where host genotype was treated as a fixed effect and date was treated as a strata variable so that

$$h_{ij}(t | X, Z) = h_{0j}(t) e^{(x_i \beta_i)}$$

for $Z = 1, \dots, j$ dates. The hazard at time t given factor X and on the Z^{th} date for genotype i is represented by $h_i(t | X, Z)$ and $h_{0Z}(t)$ is an underlying hazard function of unknown shape specific to the Z^{th} date. This allowed us to account for the inherent differences in resistance due to date and to normalize the resistance of each genotype relative to the dates that genotype was tested and therefore alleviates many of the negative effects of the unbalanced design. Parameter estimates for each genotype i , $\hat{\beta}_i$, were obtained by maximizing the partial log-likelihood function of the proportional hazards model shown above. We then estimated the hazard ratio for each genotype i by calculating the hazard of that genotype relative to baseline genotype 0

$$hr_i = \frac{e^{(X_i \hat{\beta}_i)}}{e^{(X_0 \hat{\beta}_i)}} = e^{(X_i - X_0) \hat{\beta}_i}$$

This resulting hazard ratio measures how likely genotype i is to die relative to the baseline genotype 0 . Baseline genotype 0 always has a hazard ratio of one, because it is evaluated with respect to itself. We next determined whether host genotype was a significant contributor to the observed variation in host survival time post-infection independent of the date effect. For each date and for each bacterial species, we analyzed a Proportional Hazards Model where host genotype was treated as a fixed effect. For each of the three bacterial species, we performed separate survival analyses for each date and then used two combined probability tests – the Z-transform test (Whitlock 2005) and Fisher’s combined probability test (Sokal and Rohlf 1995) – to combine the results of each separate survival analysis into one overall test for the effect of host genotype on resistance. All survival analyses were performed using proc tphreg in SAS version 9.1 (SAS Institute, Cary, NC).

Mortality in the absence of infection

Male mortality rates in the absence of infection were assayed for each of the sixteen isofemale lines. Virgin males were collected over a 24 hour period and were maintained in demography cages (described in Promislow and Bugbee 2000) with standard cornmeal, molasses, and yeast fly medium. Each of the sixteen genotypes was replicated three times, with approximately 65 males per cage. Every third day, the number of dead flies was recorded, dead individuals were removed, and food was replaced. Cages were monitored over a period of 93 days. At 93 days, all remaining individuals ($N = 4$) were censored.

To test for genetic variation for mortality in the absence of infection, a Proportional Hazards model was analyzed. Days to death was the dependent variable and host genotype and replicate cage nested within host genotype were the fixed independent variables. Maximum likelihood estimates were obtained for the effect of each genotype on mortality (see above) and hazard ratios were estimated (see above) to describe the mean survival of each genotype in the absence of infection. Survival analyses were performed using proc tphreg in SAS version 9.1 (SAS Institute, Cary, NC).

Male fertility in the absence of infection

To gain a more complete picture of the lifetime male fertility of each isofemale line, age-specific male fertility in the absence of infection was measured for each of the sixteen *D. melanogaster* genotypes. Male fertility was assayed by mating males of each genotype to a “focal” female genotype. These focal females were all of the same genotype, and this genotype was not the same genotype as any of the lines assayed for male fertility. Virgin males were collected over a 48 hour period and were maintained in demography cages with standard cornmeal, molasses, and yeast fly medium until they were assayed for fertility. Food was replaced in these cages every three days. At three, ten, 17, 24, 31, 38, and 45 days of age, virgin males were removed from their cage by aspirating and were placed into a yeasted vial with two two-day-old virgin focal females to maximize mating opportunities for the male. After 24 hours of mating, these three flies were transferred to another clean, yeasted vial for an additional 24 hours before being discarded. Eclosed progeny from both vials were removed and counted 13, 18, and 22 days post-mating. Transferring mated adults into a new vial after 24 hours and

periodically counting and removing progeny reduced the densities of larvae and eclosed individuals in the vials. The number of eclosed progeny was summed across all vials and days post-eclosion prior to analysis to yield estimates of male fertility for each replicate. Approximately 15 individuals were assayed per line per age class, yielding a total sample size of 1624 individuals tested.

We next determined whether male fertility differed across host genotypes. The number of progeny per mating remained non-normally distributed despite a variety of transformations. Therefore, these response data were ranked and analyzed using an ANOVA (Conover and Iman 1981, Corby-Harris *et al.* 2007a). Ties were treated as the average between ranks. An advantage of this approach is that it permits nested effects. This contrasts with more robust nonparametric models, such as the Kruskal-Wallis and Friedman's tests, which do not permit nested effects (Sokal and Rohlf 1995). However, this method has drawbacks. Notably, it tends to underestimate the sum of squares for the interaction effect (Salter and Fawcett 1993). Therefore, we began by using the method of Conover and Iman (1981) to test the full model, which tested whether the independent variables host genotype, replicate within host genotype, age, and genotype x age significantly contributed to the observed variation in ranked progeny counts. Replicate within host genotype was nonsignificant ($F_{224,1289} = 1.03$, $P = 0.3771$), and was eliminated from the reduced model. This reduced model that included the host genotype, age, and genotype x age was then investigated using the more robust Scheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal and Rohlf 1995). Last, least squared estimates of the ranked progeny counts were obtained for each of the sixteen genotypes to yield family

mean estimates of male fertility. All male fertility data was analyzed using proc anova in SAS version 9.1 (SAS Institute, Cary, NC).

Tradeoffs between resistance and fitness

To determine whether tradeoffs existed between resistance to any of the three types of bacterial infection and either male mortality or fertility in the absence of infection, we tested for a significantly negative correlation between resistance and fitness across the sixteen isofemale lines (David *et al.* 2005). Across the three types of bacteria, this resulted in six pairwise comparisons. These correlations were estimated using the formula

$$r_M = \frac{\text{cov}_M(X,Y)}{\sqrt{(\text{var}_{M(X)} \text{var}_{M(Y)})}}$$

where $\text{cov}_M(X,Y)$ represents the covariance between the X and Y character family means, and $\text{var}_{M(X)}$ and $\text{var}_{M(Y)}$ are the variances across families for each of the characters (Via 1984).

RESULTS

Resistance to bacterial infection

Among the flies treated with bacteria (N = 1919), 94.3% died within the 48 hour period when data were collected. Across the sixteen genotypes, the mean hours to death was 25.3 ± 4.46 s.d. among those flies that died within the 48 hour period (N = 1810).

Under the proportional hazards model, host genotype was a significant predictor of mortality post-infection for *P. aeruginosa* (Wald $\chi^2_{15} = 30.76$, $P = 0.0095$, Figure 3.1), *L. lactis* (Wald $\chi^2_{15} = 27.66$, $P = 0.0238$, Figure 3.1), and *E. faecalis* (Wald $\chi^2_{15} = 46.57$, $P < 0.0001$, Figure 3.1). After reanalyzing the data for each date separately (separate test statistics are presented in appendix A), there was a significant effect of host genotype on mortality post-infection for some dates but not others. After combining the p-values for the effect of host genotype from each day, host genotype remained a significant predictor of host mortality post-infection for *P. aeruginosa* (Fisher's combined $X^2_{15} = 90.33$, $P < 0.0001$, $Z_{s(15)} = 6.58$, $P < 0.0001$), *L. lactis* (Fisher's combined $X^2_{15} = 81.18$, $P = 0.0001$, $Z_{s(15)} = 6.11$, $P < 0.0001$), and *E. faecalis* (Fisher's combined $X^2_{15} = 96.73$, $P < 0.0001$, $Z_{s(15)} = 6.66$, $P < 0.0001$).

Male mortality in the absence of infection

In the absence of infection, the mean number of days to death for males across all sixteen genotypes was 66.46 ± 3.59 s.d.. Genotype (Wald $\chi^2_{15} = 213.87$, $P < 0.0001$, Figure 3.2) and replicate within genotype (Wald $\chi^2_{32} = 102.65$, $P < 0.0001$) were significant predictors of days to death.

Male fertility in the absence of infection

Genotype ($H_{15} = 37.84$, $P = 0.0009$, Figure 3.3a) and age ($H_6 = 27.42$, $P = 0.0001$, Figure 3.3b) were significant predictors of the ranked number of total progeny produced by matings between one male with two focal females. The genotype x age

interaction ($H_{89} = 44.74$, $P = 0.99$) was not a significant contributor to the observed variation in male fertility.

Tradeoffs between resistance and fitness

We did not observe any significantly negative relationships between host resistance to infection and host fitness in the absence of infection. In contrast, one of the six pairwise comparisons was actually positive: there was a significant positive relationship between resistance to *P. aeruginosa* infection and survival in the absence of infection ($r_{M(16)} = 0.54$, two-tailed $P = 0.031$).

DISCUSSION

In the experiments described here, we found that host genotype was a significant predictor of the ability to survive *P. aeruginosa*, *L. lactis*, and *E. faecalis* infection. Genotypes also differed for the two fitness traits assayed – survival and fertility in the absence of infection. However, despite the presence of genotypic variation for both resistance and host fitness in the absence of infection, we did not detect any negative correlations between resistance to any of the three species of bacterial infections tested and host fitness in the absence of infection. Overall, these data suggest that there is significant genetic variability for resistance to bacterial infection within natural populations of *D. melanogaster*. However, across these host genotypes, this variation could not be explained by evolutionary tradeoffs, suggesting other forces may shape resistance to bacterial infection within natural populations of *D. melanogaster*.

This study is unique in that it is the first to show that naturally derived *D. melanogaster* genotypes vary for an important fitness trait – survival – following various types of bacterial challenges. Previous work by Lazzaro and colleagues (2004, 2006) showed that bacterial loads following infection vary with respect to host genotype (Lazzaro *et al.* 2004) and that the genes controlling resistance differ depending on the species of bacterial challenge (Lazzaro *et al.* 2006). However, this does not necessarily mean that host fitness following infection varies across host genotypes. As we noted in a previous study, bacterial load post-infection is not significantly positively correlated with host survival following infection, suggesting that bacterial load is a poor proxy for host fitness in the presence of infection (Corby-Harris *et al.* 2007a). From an evolutionary standpoint, it is important to show that differences in fitness following infection have a genetic basis. Therefore, this work adds to the body of literature (Lazzaro *et al.* 2004, Lazzaro *et al.* 2006, Tinsley *et al.* 2006) aimed at understanding the genetic architecture of *D. melanogaster* host fitness following bacterial challenge.

It is important to note that, in assaying resistance across isofemale lines, we did not assay additive genetic variation, *per se*. Therefore, it is possible that non-additive effects are contributing to the variation in resistance we observed among genotypes (Falconer and Mackay 1996). Further, inbreeding in the lab over successive generations can increase between-line variance due to genetic drift (Robertson 1952), thereby increasing the likelihood that we observe differences among genotypes. However, founding isofemale lines from wild populations is a basic tool for understanding the genetic architecture of many traits in *Drosophila*, and despite obvious drawbacks, is a valid tool (David *et al.* 2005). We observed highly significant differences among host

genotypes, suggesting that there is some level of additive genetic variation for resistance and that host populations have the genetic material to adaptively respond to infection. However, this level of additive genetic variation may be small if nonadditive effects contribute to most of the among line differences.

We did not observe any negative genetic correlations between resistance and host fitness in the absence of infection, suggesting that evolutionary tradeoffs are not operating across these host genotypes. In contrast, resistant lines were generally robust, with high fitness in the absence of infection also. However, the pattern we observed is certainly not conclusive evidence that tradeoffs do not exist. In order to assay fitness and resistance, individuals were removed from their natural environments and put into stress-free conditions, where food is fed *ad libitum*. Under such stress-free conditions, tradeoffs (Reeson *et al.* 2000), particularly between resistance and fitness (Moret and Schmid-Hempel 2000) may not be evident. Additionally, the strength of genetic tradeoffs could be significantly reduced in response to a novel environment (Service and Rose 1985) such as the laboratory. Overall, failure to observe negative genetic correlations under laboratory conditions does not mean tradeoffs don't exist, and we cannot discount the possibility that they operate in nature. However, failure to observe tradeoffs between resistance and fitness suggests that other forces, such as frequency-dependent selection (van Valen 1973) or environmental heterogeneity (Felsenstein 1976, Hedrick *et al.* 1976), might be more important for maintaining genetic variation in this system despite strong selection by parasites.

The work presented here demonstrates that there are significant differences across host genotypes within a population despite no evidence for tradeoffs between resistance

and fitness. This result is somewhat surprising given the negative fitness effects infection has on the host. Although costs of immune defense are widely acknowledged to be operating in many animal systems, this is not always the case (Sandland and Minchella 2003). Instead, we hypothesize that other mechanisms, frequency-dependent selection (van Valen 1973) or environmental heterogeneity (Felsenstein 1976, Hedrick *et al.* 1976), might operate to maintain genetic variation within *D. melanogaster* host populations. Given the diverse bacterial communities *D. melanogaster* associate with in nature (Corby-Harris *et al.* 2007b), there is great potential for interesting host-parasite interactions to shape resistance variation.

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FIGURE LEGENDS

Figure 3.1. Host genotypes differ in resistance to three species of bacterial infection. For each bacterial treatment and each host genotype, mortality post-infection is plotted as the hazard ratio estimate generated under a proportional hazards model. Error bars represent the standard error around the hazard ratio parameter estimate. Under a proportional hazards model, host genotype was a significant predictor of mortality post-infection for *P. aeruginosa* (black, Wald $\chi^2_{15} = 30.76$, $P = 0.0095$), (b) *L. lactis* (dark grey, Wald $\chi^2_{15} = 27.66$, $P = 0.0238$), and (c) *E. faecalis* (light grey, Wald $\chi^2_{15} = 46.57$, $P < 0.0001$).

Figure 3.2. Male mortality rates in the absence of infection differ among genotypes. For each bacterial treatment and each host genotype, mortality is plotted as the hazard ratio estimate generated under a proportional hazards model. Error bars represent the standard error around the hazard ratio parameter estimate. Genotype (Wald $\chi^2_{15} = 213.87$, $P < 0.0001$) was a significant predictor of the mortality rate in the absence of infection.

Figure 3.3. Male fertility in the absence of infection differs among genotypes and with respect to age. (a) Genotype ($H_{15} = 37.84$, $P = 0.0009$) and (b) age ($H_6 = 27.42$, $P = 0.0001$) were significant predictors of the ranked number of total progeny produced by matings between one male with two focal females. Points plotted are the mean ranked progeny counts. Error bars represent the standard error around the mean ranked counts.

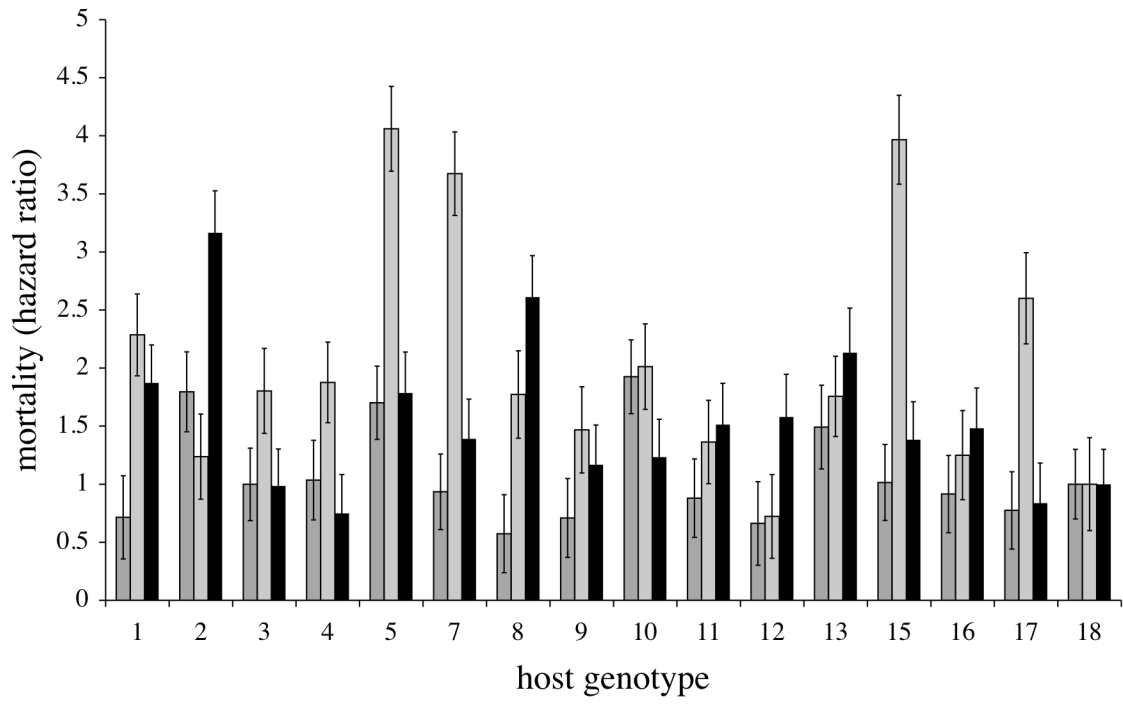


Figure 3.1

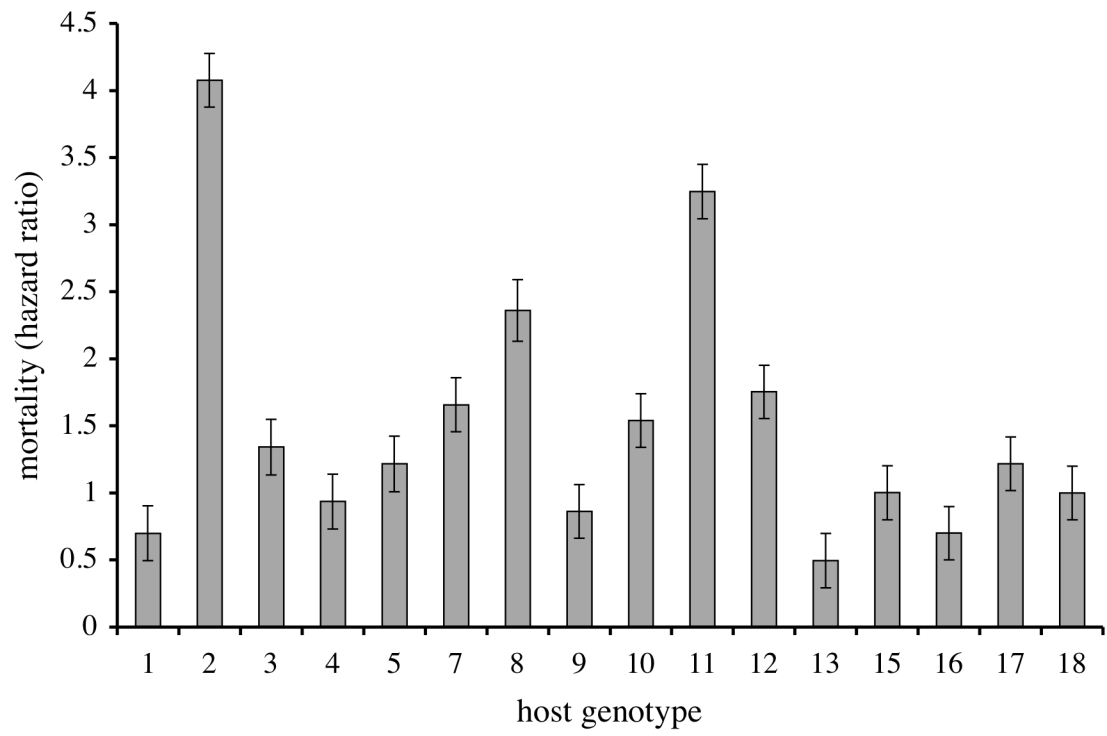


Figure 3.2

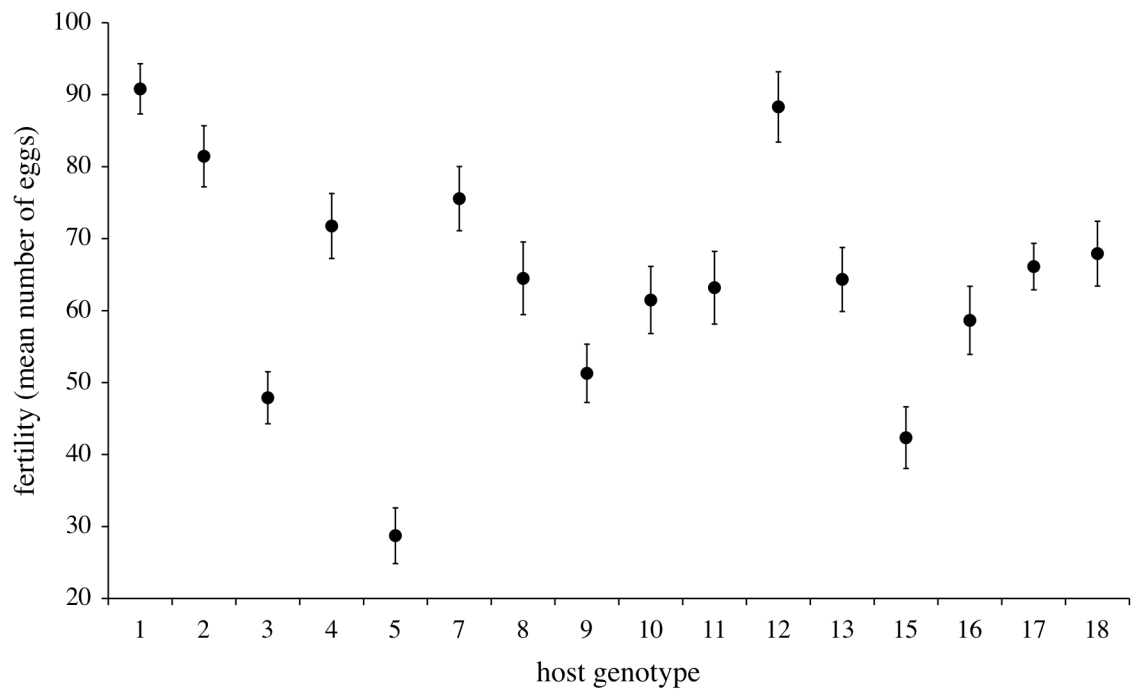


Figure 3.3 (a)

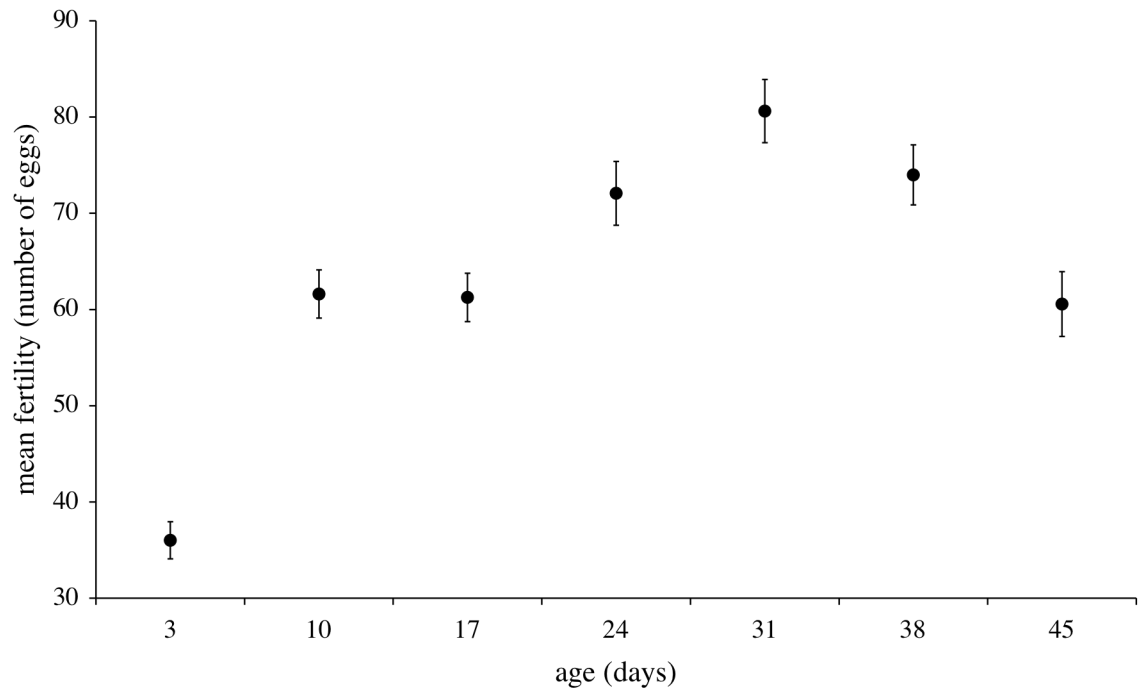


Figure 3.3 (b)

CHAPTER 4
THE GEOGRAPHICAL DISTRIBUTION AND DIVERSITY OF BACTERIA
ASSOCIATED WITH NATURAL POPULATIONS OF *DROSOPHILA*
*MELANOGASTER*³

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Promislow. 2007. *Applied and Environmental Microbiology*. 73:3470-3479.

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ABSTRACT

Drosophila melanogaster is one of the most widely used model systems in biology. However, little is known about its associated bacterial community. As a first step towards understanding these communities, we compared bacterial 16S rRNA gene sequence libraries recovered from eleven natural populations of adult *D. melanogaster*. Bacteria from these sequence libraries were grouped into 74 distinct taxa, spanning the phyla Proteobacteria, Bacteroidetes, and Firmicutes, which were unevenly spread across host populations. Summed across populations, the distribution of abundance of genera was closely fit by a power law. We observed differences among host population locations both in bacterial community richness and composition. Despite this significant spatial variation, no relationship was observed between species richness and a variety of abiotic factors, such as temperature and latitude. Overall, bacterial communities associated with adult *D. melanogaster* hosts are diverse and differ across host populations.

INTRODUCTION

Insects harbor diverse microbial communities (Jeyaprakash *et al.* 2003, Schmitt-Wagner *et al.* 2003, Campbell *et al.* 2004, Hongoh *et al.* 2005), and interactions between hosts and their microbes can range from mutualistic, such as the interaction between termites and their gut microbes (Breznak and Brune 1994, Schmitt-Wagner *et al.* 2003), to parasitic, such as the bacterium *Paenibacillus larvae* (American foulbrood) in honeybees (Schmid-Hempel 1998). Some of these interactions are relatively well characterized, owing to their economic importance or because of their remarkable

biology. However, the exact nature of many other potentially interesting and experimentally tractable insect-microbe interactions, specifically between microbes and the major insect model systems, remains poorly understood.

In addition to the immediate association between insect hosts and the bacterial community they harbor, the bacteria that insects carry can also associate with and affect the fitness of other hosts through vector transmission. The most common vector-borne zoonotic inflammatory disease in the United States, Lyme Disease (caused by *Borrelia burgdorferi*) is transmitted by the deer tick, *Ixodes scapularis* and infected more than 23,000 people in 2002 (Gross 2006). In addition, *Erwinia carotova*, responsible for soft rot in many species of plants and for significant economic losses, can be vector-transmitted by a variety of insects, including *Drosophila melanogaster* (Kloepper *et al.* 1979). Clearly, vector-borne bacterial infections can have large economic and health impacts and are important determinants of fitness for a variety of potential hosts.

It is estimated that approximately 99% of the bacteria in nature are unculturable (Amann *et al.* 1990). With the advent of molecular techniques, such as PCR and genome sequencing, and metagenomic approaches, researchers have uncovered an astonishing level of microbial diversity in natural habitats, ranging from soil (Picard *et al.* 1992, Borneman *et al.* 1996, Tringe *et al.* 2005) and marine environments (Schmidt *et al.* 1991, Fuhrman *et al.* 1993, Venter *et al.* 2004, Tringe *et al.* 2005), to the human gut (Gill *et al.* 2006). These same techniques are currently being applied to understand the microbiota of a range of insects (McKillip *et al.* 1997, Jeyaprakash *et al.* 2003, Peloquin and Greenberg 2003, Reeson *et al.* 2003, Schmitt-Wagner *et al.* 2003, Campbell *et al.* 2004, Pidiyar *et al.* 2004, Hongoh *et al.* 2005). For example, using such sequence-based

approaches, Dunn *et al.* (Dunn and Stabb 2005) found that the ant lion, *Myrmeleon mobilis*, harbors a relatively simple microbial community, represented mostly by *Enterobacteriaceae*- and *Wolbachia*-like microorganisms. In contrast, Campbell *et al.* (2004) found a more diverse microbiota in the biting midge, *Culicoides sonorensis*, comprised of genera from five different bacterial divisions. From the literature on the microbial communities of insects, two salient points emerge. First, these communities differ greatly among host species. Second, researchers are only beginning to understand the taxa comprising these microbial communities and how these microbes interact with their hosts. Considering the estimated 30 million insect species worldwide (Erwin 1983), the potential for uncovering new species of bacteria and for understanding interesting features of these insect-microbe interactions is staggering.

Despite their widespread use in the laboratory, relatively little is known about the interaction between model laboratory insect species and their associated microbial communities. Due to its experimental and genetic tractability, the fruit fly, *Drosophila melanogaster*, provides an ideal system to study these interactions more closely. Recently, there have been numerous studies on *Drosophila* immunity, ranging from understanding the molecular basis of resistance (Hoffmann and Reichhart 1997, Lavine and Strand 2002, Hultmark 2003) to the evolutionary ecology of the immune response (Rolff and Siva-Jothy 2003, Schmid-Hempel 2003, 2005). Some of these studies use bacteria isolated from natural populations of *Drosophila* (Lazzaro *et al.* 2004, Vodovar *et al.* 2005). However, in many other cases, researchers use bacteria that are not yet known to naturally co-occur with or infect *Drosophila*. One plausible reason that naturally occurring bacteria are not used to study immunity is that, to date, there are few

comprehensive studies of the bacteria that associate with natural *D. melanogaster* populations (except see (Cox and Gilmore 2007)). Knowledge of the actual interactions taking place between insect hosts and their microbial communities is critical to those studying insect immunity. However, before characterizing these interactions, we must characterize the bacterial communities and identify taxa that are of potential ecological interest.

Specific species of microbes, as well as traits of the microbial population overall, like community richness or composition, have the potential to greatly affect the ecology and evolution of their *Drosophila* hosts. Here, we characterize this composition and richness across eleven natural populations of *D. melanogaster*. We first identify the bacterial microbes present in host populations collected across a latitudinal cline using a sequence-based approach. We then compare microbial community richness among these host populations, and ask whether richness is associated with latitude or climate. This is the first study to characterize microbial communities associating with *D. melanogaster* hosts within and among natural host populations. These data will provide an important first step in understanding host-microbe interactions in this widely studied model system.

MATERIALS AND METHODS

Fly collections

Flies were collected from eleven sites along a latitudinal transect on the East Coast of the United States between June and September 2005 (Table 3.1) using a combination of sweep netting behind fruit stands and sweep netting over fruit bucket traps. Details of the eleven collection sites are presented in table 1.

Following collection, flies were anesthetized over ice. Male *D. melanogaster* were separated from other Drosophilid species on the basis of morphological characteristics such as size, color, body patterning, wing shape, and genital morphology (Ashburner 1989, Markow and O'Grady 2006). These individuals were set aside and preserved in groups of five in 70% ethanol. After returning to the laboratory from the collection sites, the ethanol-preserved samples were kept at -80°C. In preparation for DNA isolation, the ethanol-preserved flies were shaken before being removed from the ethanol. While this method will not remove all surface-associated bacteria, those that are loosely associated will likely wash off.

DNA isolation

Total DNA from the flies and the bacteria was isolated from one group of five male flies at each of the eleven different locations. Flies were removed from the ethanol and homogenized using a pestle in 200 μ l of STE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 150 mM NaCl) with lysozyme added (final concentration, 4 mg/ml). The sample was then incubated for 30 minutes at 37°C. Following incubation, 20 μ l of 10% sodium dodecyl sulfate (SDS) and proteinase K (final concentration, 0.2 mg/ml) were added. These samples were then vortexed and incubated at 55°C overnight. After the overnight incubation, RNase A was added (final concentration, 0.1 mg/ml) and samples were incubated for one hour at 37°C. The samples were then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) and chloroform:isoamyl alcohol (24:1), and the DNA was ethanol precipitated. The DNA pellets were resuspended in 50 μ l of Tris-EDTA (10mM Tris HCl [pH 8.0], 1mM EDTA [pH 8.0]).

PCR amplification

Bacterial 16S rRNA gene sequences were selectively PCR amplified from the isolated DNA samples for the construction of clone libraries. Each reaction tube contained 50-100 $\mu\text{g/ml}$ of template genomic DNA, forward primer 27f (5'-AGA GTT TGA TCM TGG CTC AG-3'), reverse primer 1522r (5'- AAG GAG GTG ATC CAG CCG CA-3'), and one Ready-to-Go PCR bead (GE Healthcare Life Science). The PCR program was as follows: 9 minutes at 95°C, 15 cycles of 1 minute at 95°C, 1 minute at 55°C, and 2 minutes at 72°C, followed by a final extension step of 60°C for 10 minutes. PCR products were resolved on a 1% agarose gel, and the gel was stained with SYBR green (Invitrogen) in order to visualize the relatively weak bands on a dark reader transilluminator. The approximately 1.5 kb 16S rRNA gene fragment was extracted from the agarose gel using the QiaQuick Gel Extraction Kit (Qiagen) according to manufacturer's directions and was eluted in 30 μl of sterile distilled water.

Construction of clone libraries

Clone libraries of bacterial PCR products were constructed using a TOPO TA Cloning Kit (Invitrogen) with TOPO One Shot[®] Electrocompetent cells. Successful transformants were plated onto Luria-Bertani plates containing kanamycin (final concentration, 50 $\mu\text{g/ml}$). Plasmids were extracted from the bacteria using standard techniques (Sambrook *et al.* 1989).

Sequencing of 16S rRNA genes

Partial length 16S rRNA gene fragments were sequenced in one direction using an ABI 3700 capillary sequencer using T7 primers and ABI BigDye Terminator chemistry. Sequences were examined visually, and vector and low-quality bases (20-bp window with an average quality value PHRED score < 16) were trimmed from the libraries using LUCY (Chou and Holmes 2001). After trimming these regions, the average sequence length was 827 base pairs.

Nucleotide sequence accession numbers

All unique 16S rRNA gene sequences are available in the GenBank database under accession numbers DQ980639 through DQ981381.

Chimera detection

All clones were checked for chimeras using both Chimera Check from the Ribosomal Database Project-II (Cole *et al.* 2005) and Bellerophon (Huber *et al.* 2004). All sequences resembling a chimera using either program were removed from further analyses.

Determination of operational taxonomic units

Sequences were aligned separately for each clone library using ClustalW (Higgins *et al.* 1994) in BioEdit version 7.0.5 under the default settings with a gap-opening penalty of 10.0 and a gap extension penalty of 0.1 or 0.2 for pairwise and multiple alignments, respectively. As points of reference, published sequences for *Escherichia coli* 16S rRNA

(Accession number L10328; base position numbers 131193-132733), *Borrelia burgdorferi* (Accession number X85189), and *Bacillus subtilis* (Accession number AY553095) were used when aligning the sequences. After the alignments were performed, the sequences were truncated at the 5' and 3' ends and ambiguous areas of the alignment were manually removed. The number of common bases for each were as follows: GA 441, 617 base pairs (bp); Hillsborough, 440 bp; Horticulture Farm, 578 bp; Ikenberry, 371 bp; Inwood, 318 bp; Layman, 550 bp; Macon, 502 bp; Oakland, 413 bp; Raleigh, 661 bp; Thomas, 546 bp; Woodstock, 248 bp. Distance matrices were constructed using the DNADIST program within BioEdit version 7.0.5 using the Jukes-Cantor correction for multiple substitutions. Using the DOTUR software package (Schloss and Handelsman 2005) under the default settings, operational taxonomic unit (OTU) groupings were determined using 97% sequence identity. In further phylogenetic analyses, a consensus sequence was generated to represent each OTU.

Phylogenetic analyses of 16S rRNA gene sequences

Using the BLAST (Altschul *et al.* 1997) and Seqmatch (Cole *et al.* 2005) tools, the Ribosomal Database Project-II (RDP-II) (Cole *et al.* 2005) and GenBank sequence databases were screened for published sequences that closely matched the 74 consensus sequences generated to represent each of the OTUs isolated from the host populations. All analyses were performed between June 2006 and January 2007. These published sequences (Figures 2 and 3), along with the 74 OTU consensus sequences were then used in a series of phylogenetic analyses designed to understand the relationship that the 16S rRNA gene sequences we recovered from *D. melanogaster* shared both with published

16S rRNA sequences and with each other. To begin, sequences were aligned using ClustalW (Higgins *et al.* 1994) in BioEdit version 7.0.5 under the default settings with a gap-opening penalty of 10.0 and a gap extension penalty of 0.1 or 0.2 for pairwise and multiple alignments, respectively. After the alignments were performed, ambiguous areas of the alignment were removed using Gblocks (Castresana 2000), yielding 649 common bases. A distance matrix and neighbor-joining tree were constructed under default parameters following 10,000 bootstrap replicates using the PHYLIP software package (Felsenstein 2005). After determining how sequences grouped with each other, they were divided into seven subcategories for further analysis - Wolbachia, Bacteroidetes, Firmicutes, α -Proteobacteria, β -Proteobacteria, γ -Proteobacteria, or ϵ -Proteobacteria. For each of the seven individual categories, the published sequences and OTUs corresponding to that category were compiled, and distance matrices were again constructed using the same methods as described above. Neighbor-joining trees were generated in MEGA 3.1 (Kumar *et al.* 2004) for each subcategory, using the Kimura 2-parameter model for nucleotide substitutions and 10,000 bootstrap replicates. Neighbor-joining trees for the Wolbachia subcategory were unrooted, and trees for the remaining subcategories were rooted with *Synechococcus elongata* (AF132930).

Estimates of species richness

Using DOTUR (Schloss and Handelsman 2005) and a 97% level of sequence identity to define the OTUs, rarefaction curves were generated to ask if our libraries approached a level of taxonomic diversity that represented the true diversity present in these eleven locations and the nonparametric species richness estimators Chao1 (Chao

1984, Chao *et al.* 1993) and ACE (Chao and Lee 1992, Chao *et al.* 1993) were obtained. Library coverage was estimated by calculating the ratio of actual number of OTUs observed to the Chao1 estimate of species richness.

Statistical tests for clinal variation in richness

To determine if there was significant clinal variation for microbial species richness, we tested for significant correlations between latitude and the Chao1 estimate of species richness, and between latitude and ACE, at 97% sequence identity. All data were analyzed using the Spearman non-parametric correlation test in JMP Version 5.0.1a (SAS 2003).

Statistical test for the correlation between richness and climatic factors

Because latitude is not the only factor that may determine differences in microbial community richness, we also tested for a significant correlation between the Chao1 and ACE estimates of richness and several other climatic factors – mean annual temperature, monthly temperature range (defined by the 12-month average of the difference between the monthly mean maximum and minimum temperatures), mean annual precipitation, and mean January low temperature. These climatic data were published by the National Oceanic and Atmospheric Administration, and represent 30 year averages recorded between 1971 and 2000 at various locations across the United States (NOAA 2002a, c, d, b, e). In cases where the exact collection site location was not listed in the report, the listed location that was closest to the collection site was used. All data were analyzed using the Spearman non-parametric correlation test in JMP Version 5.0.1a (SAS 2003).

Species-abundance distribution

Recent studies find that microbial species abundance distributions are well-described by a power-law distribution, $S_N = \zeta(\gamma) \cdot N^\gamma$, where S_N is the number of genera that are found in N sequence samples, γ is the power coefficient, and ζ is Reimann's zeta function, a normalizing constant. When plotted on a log-log scale, if the distribution of microbial genera fits a power-law, then $\log(S_N)$ versus $\log(N)$ should be a straight line with a slope of γ .

The data were fit to a power-law distribution using maximum likelihood (Microsoft Excel program available from DELP upon request). A recent study of species abundance distributions in soil microbes (Gans *et al.* 2005) compared power-law distributions with other related distributions. Given the relatively small size of our dataset, we do not have sufficient statistical power to make these comparisons. Our interest in fitting a power-law distribution to these data is primarily a heuristic one.

RESULTS

A total of eleven libraries were constructed to represent the bacterial community present in hosts at each of the eleven locations. From these eleven libraries, we obtained 992 sequences. Of these 992 sequences, 264 (26.6%) were discarded as chimeric.

Using DOTUR, the 728 remaining sequences from the eleven locations were grouped into 74 operational taxonomic units (OTUs) at the 3% level of sequence divergence. No OTUs were present in all libraries, and there were no populations containing all 74 OTUs. The OTUs were unevenly spread both within and across host populations (Figures 4.1 through 4.4), with many appearing in only one host population

among the eleven sampled. The mean number of OTUs per host population was 13.8 (Table 4.1).

A wide range of bacterial species was present in the gene clone libraries recovered from the eleven locations. One prominent feature of these libraries was the high prevalence of *Wolbachia* (453 of the total 728 sequences; 62.2%). The two most abundant groups of bacteria fell into the α -Proteobacteria (125 of the total 728 sequences; 17.2%) and γ -Proteobacteria (59 of the total 728 sequences; 8.1%) classes, respectively. In addition, these libraries contained a significant proportion (39 out of 728; 5.4%) of sequences with matches in the RDP-II and GenBank databases that were taxonomically unclassified environmental samples. Many of the sequences showed relatively low similarity to published 16S rRNA gene sequences in the RDP-II Database. Across all eleven libraries, 31 of the 728 sequences (4.3%) shared less than 97% sequence identity with published 16S rRNA gene sequences in the RDP Database. The lowest of these, assigned to OTU 46, shared only 59.4% identity with its next closest relative in the database.

Based on sequence separation at the 3% level of sequence divergence, our samples contained 10 OTUs that grouped with *Wolbachia* (Figure 4.1). The neighbor-joining tree had a wide range of bootstrap support values (10%-100%), but qualitatively captured aspects of previous studies (Lo *et al.* 2002, Mateos *et al.* 2006) in the manner taxa grouped into supergroups. All ten *Wolbachia* OTUs formed a monophyletic group with supergroup A (Figure 4.1). Two of the *Wolbachia* OTUs, OTUs 1 and 10, were widespread, while the remaining OTUs were limited to specific populations (Figure 4.1).

The remaining 275 sequences were grouped into 64 OTUs (Figures 3.1 through 3.4). Forty-two OTUs grouped with the Proteobacteria (15 α -Proteobacteria; 5 β -Proteobacteria; 21 γ -Proteobacteria; 1 ϵ -Proteobacteria; Figure 4.2). Seventeen of these OTUs grouped within the Firmicutes (Figure 4.2). The remaining five OTUs grouped within the Bacteroidetes (Figure 4.4). Bootstrap values for the neighbor-joining trees varied widely, with many OTUs assigned to clades defined by a published sequence with high bootstrap support (Figures 4.2, 4.3, and 4.4).

Our analyses suggest that we have not sampled all of the taxa present in these *Drosophila* hosts, because the rarefaction curves do not plateau as more sequences are sampled from the library (Figure 4.5). Using the Chao1 estimator of richness for comparison, the average coverage was $65.7\% \pm 6.06$ SE, across all eleven libraries (Table 4.1). The Oakland population had the highest coverage (100%; Table 4.1), whereas the Hillsborough population had the lowest (26.5%; Table 4.1).

Species richness varied across host locations as measured by both the Chao1 and ACE estimators (Table 4.1). The two estimators of richness were positively correlated with each other (Spearman's $\rho = 0.76$, $P = 0.006$). Despite spatial variation for richness using either of the two estimators, there was no clear relationship between microbial species richness and latitude for the Chao1 (Spearman's $\rho = 0.06$, $P = 0.87$) or the ACE (Spearman's $\rho = -0.05$, $P = 0.89$) estimates of species richness.

Species richness across host populations was not correlated with climate using either of the two richness estimators. There was no correlation between the Chao1 estimate of richness and mean annual temperature (Spearman's $\rho = -0.05$, $P = 0.89$), monthly temperature range (Spearman's $\rho = 0$, $P = 1.0$), mean annual precipitation

(Spearman's rho = -0.21, $P = 0.56$), or mean January low temperature (Spearman's rho = 0, $P = 1.0$). There was also no significant correlation between the ACE estimate of species richness and mean annual temperature (Spearman's rho = 0.13, $P = 0.71$), monthly temperature range (Spearman's rho = 0.02, $P = 0.95$), mean annual precipitation (Spearman's rho = -0.20, $P = 0.59$), or mean January low temperature (Spearman's rho = 0.11, $P = 0.76$).

The frequency distribution of species abundance appeared to approximately fit a power-law distribution, with $\gamma = -1.46$. This value is not far from the range of values for γ reported by Gans *et al.* ($\gamma = -1.96 - -2.11$) (Gans *et al.* 2005), though we used genera as opposed to Gans *et al.*'s use of species.

DISCUSSION

We used a sequence-based approach to study the microbial communities within natural host populations of *Drosophila melanogaster*. Our data suggest that there are many species of bacteria present in these *Drosophila* hosts, including a large number of *Wolbachia*. Most of these species of bacteria were unevenly distributed among the host populations. Bacterial species richness of these microbial communities differed among host populations. However, despite significant spatial variation in microbial community richness, there was not a clear relationship between latitude or climate and microbial species richness.

Our interpretation of these data comes with three caveats. First, our method of DNA extraction does not allow us to discern between bacteria associated with the inside or outside of the host. Second, because microbial DNA was isolated from whole bodies

of flies, we cannot draw conclusions about the tissue-specificity of the microbes observed in these libraries. Last, we observed a high proportion of chimeric sequences in these libraries. This pattern could be due to our thorough methods of identifying chimera, or due to inappropriate concentrations of MgCl₂ or dNTP in the PCR beads used to amplify the DNA. With these three caveats in mind, we highlight several interesting characteristics of these libraries.

Bacterial phyla present

Three phyla – Proteobacteria, Firmicutes, and Bacteroidetes – were present in these samples. OTUs falling within the Proteobacteria phylum were represented considerably more than those OTUs grouping within the Firmicutes or Bacteroidetes, even after subtracting the highly prevalent gram-negative bacterium *Wolbachia*. The overabundance of Proteobacteria we observed could simply be due to the limitations of using lysozyme, which can be ineffective against gram-positive anaerobic cocci (Ezaki and Suzuki 1982), instead of bead beating to lyse the bacterial cells. Alternatively, the overabundance of Proteobacteria in these samples could be due to the ecology of the host. Our findings are consistent with work in the deer tick, *Ixodes scapularis* (Benson *et al.* 2004). Benson *et al.* (2004) noticed a high prevalence of Proteobacteria, even after subtracting intracellular bacteria such as *Wolbachia*. They hypothesized that this pattern could be due to the humid environments that ticks prefer, which are more permissive conditions for desiccation-sensitive microbes such as Proteobacteria. The prevalence of Proteobacteria has also been shown for several other species of insects, including *Culicoides sonorensis*, an orbivirus vector (Campbell *et al.* 2004), the honeybee *Apis*

mellifera (Jeyaprakash *et al.* 2003), and the ant lion, *Myrmeleon mobilis* (Dunn and Stabb 2005). In contrast, bacterial communities associated with certain species of wood- and soil-feeding termites tend to be biased towards gram-positive microorganisms (Schmitt-Wagner *et al.* 2003, Hongoh *et al.* 2005). In *D. melanogaster*, it is possible that abiotic and biotic factors, such as climate or the availability of certain food sources, affect the proportion of Proteobacteria or other bacterial phyla in the host.

Non-Wolbachia genera

Aside from *Wolbachia*, these libraries contained sequences from a diverse bacterial community. Many of these sequences have not been found in a cultured organism and may represent novel genera. A phylogenetic approach was used to classify many of the OTUs isolated from the *D. melanogaster* hosts. For many of the OTUs, low bootstrap support precludes taxonomic identification. However, tentative classifications could be made for many other cases, and some suggest potentially interesting host-bacteria interactions taking place in this system that might be studied more rigorously in future experiments.

Most of the OTUs isolated from *D. melanogaster* host populations belonged to four classes of the Proteobacteria, a diverse phylum containing upwards of 460 genera and 1619 species (Kersters *et al.* 2006a). These OTUs were located primarily within the α - and γ -Proteobacteria, a feature consistent both with their ubiquity in nature (Kersters *et al.* 2006a) and their presence in many species of insect hosts (Jeyaprakash *et al.* 2003, Schmitt-Wagner *et al.* 2003, Benson *et al.* 2004, Campbell *et al.* 2004). Many OTUs were highly similar to taxa that interact with animals and plants in interesting ways,

suggesting a possible role for these bacteria in the ecology of their *D. melanogaster* hosts and for *D. melanogaster* in mediating interactions between these bacteria and alternative hosts. For example, within the α -Proteobacteria, many of the OTUs grouped closely within the *Gluconacetobacter* genus. Species in this genus are found primarily in sugary, acidic, and alcoholic habitats such as flowers, fruits, plant tissues, and plant rhizospheres (Kerstens *et al.* 2006b), and associate with insects such as the pink sugarcane mealybug (Kerstens *et al.* 2006b) and honeybees (Jeyaprakash *et al.* 2003). Three OTUs in the β -Proteobacteria grouped closely with published sequences from the genera *Acidovorax* and *Bordetella*, which are implicated in both plant (Assouline 1996) and animal (Weiss 2002) diseases. OTU 18 closely matched a γ -Proteobacterium isolated from the intestine of the honeybee and could represent a generalist capable of cross-species horizontal transmission. OTUs 51, 68, and 8 grouped closely with the insect pathogens *Providencia rettgeri* (Jackson *et al.* 1995) and *Pseudomonas fluorescens* (Lacey 1997, Schmid-Hempel 1998), and could, therefore, be pathogenic to *D. melanogaster* in nature.

Seventeen of the 74 OTUs were members of the gram positive Firmicutes. Many of these OTUs showed high similarity with published sequences from the *Leuconostoc* genus. As some members of the *Leuconostoc* genus ferment fructose (Ljungdahl 1962), these OTUs may play a role in host digestion of fruits or other plant materials or live commensally in the host gut. OTU 58 was highly similar to the published sequence for members of the *Lactobacillus* genus. Although members of this genus are sometimes pathogenic to plants and animals (Hammes and Hertel 2006), others are part of the normal nonpathogenic flora of plants, insects, and vertebrates (Hammes and Hertel 2006)

and have been shown to increase lifespan in laboratory strains of *Drosophila* (Brummel *et al.* 2004).

Wolbachia

One of the more striking characteristics of these 16S rRNA clone libraries was the large number of *Wolbachia* sequences. Because PCR can be biased, the frequency of *Wolbachia* sequences we observed cannot be a direct measure of the frequency of *Wolbachia* in nature. However, the relatively strong bias towards *Wolbachia* in our libraries probably reflects an abundance of these microbes relative to other bacteria. Although the exact frequency of *Wolbachia* infections in natural *D. melanogaster* populations is unclear and likely variable, it is estimated that approximately 30% to 75% of the *D. melanogaster* stocks housed at Drosophila stock centers are infected with this intracellular parasite (Clark *et al.* 2005, Mateos *et al.* 2006). Further, preliminary studies in our lab suggest that 55-60% of wild-caught *D. melanogaster* isofemale lines, including lines derived from the populations used in this study, are infected with *Wolbachia* (V. Corby-Harris, unpublished data). Studies from other arthropods suggest that within-species infection rates range from 2 to 83% in *Solenopsis invicta* (Shoemaker *et al.* 2003), 5 to 100% in *Acraea* species (Jiggins *et al.* 2001), and 25 to 100% in fig wasps (Shoemaker *et al.* 2002). When sequences are grouped based on 3% sequence divergence, the *Wolbachia* and *Wolbachia*-like sequences were grouped into 10 distinct OTUs that were unevenly distributed across host locations. This pattern suggests distinct species or lineages of *Wolbachia* across host locations, a pattern similar to that

demonstrated for *Wolbachia* in geographically distinct populations of *S. invicta* (Ahrens and Shoemaker 2005).

To understand how the *Wolbachia* sequences we isolated were related to each other and to published *Wolbachia* 16S rDNA gene sequences, we constructed a phylogeny consisting only of *Wolbachia*-like gene sequences we isolated and published *Wolbachia* 16S rRNA gene sequences. The phylogeny we constructed agrees qualitatively with previous work (Lo *et al.* 2002) in that the published sequences formed five distinct supergroups (A through F). However, the bootstrap support values varied widely, with many below 70%, and the relationship of the supergroups relative to each other was inconsistent with previous studies (Lo *et al.* 2002, Mateos *et al.* 2006). Such inconsistencies could be due to the slow-evolving nature of the 16S RNA molecule, which may not provide adequate resolution between the clades (Lo *et al.* 2002). Indeed, Lo *et al.* (Lo *et al.*) suggest that more rapidly evolving sequences, such as *ftsZ*, are more appropriate for understanding the phylogenetics of the *Wolbachia* genus. Nonetheless, the phylogeny we constructed suggests that there are two distinct lineages of *Wolbachia* in natural populations of *D. melanogaster* hosts. Both of these lineages appear to be monophyletic with published sequences from supergroup A, which is found in a variety of arthropod hosts, including Drosophilids, *Tribolium*, and *Nasonia* (Lo *et al.* 2002). This monophyly is consistent with one or few origins of *Wolbachia* in *D. melanogaster* hosts found in nature.

Species richness and composition

Aside from identifying species of bacteria associated with natural populations of *D. melanogaster*, we also aimed to characterize the richness of these microbial communities overall. Here, it is important to point out that we sampled only five flies per location (pooled into one sample), and our estimates of species richness and composition could change with increased sampling effort. In addition, our ability to identify trends based on these data is weakened by the lack of multiple samples within each collection site. With these limitations in mind, however, there are nonetheless some intriguing features of the communities that were sampled.

The shapes of the rarefaction curves suggest that the taxonomic diversity present in these eleven host populations has not been completely sampled. Many taxa were found only once or twice. The fact that we were able to uncover these rare taxa with this sampling effort suggests that many unidentified and ecologically important species of bacteria living in natural populations of *D. melanogaster* have yet to be identified in these host populations.

We observed that microbial species richness varied across host populations. Motivated by previous studies in plants and animals that demonstrate a negative correlation between species richness and latitude (Brown 1995, Rosenzweig 1995), we sought to explain this variation in richness among locations using latitudinal data from each location. In addition, because latitude is only one of the many characteristics of a geographic location, we also tested for a significant relationship between climatic factors and richness. While microbial species richness varied across the host locations that we sampled, there was no evidence of a relationship between richness and latitude. The lack

of clear relationship between richness and latitude in these eleven host populations could simply be due to the fact that the range we sampled (approximately 10 degrees latitude) was not large enough to observe an effect. There was also no evidence for a significant correlation between richness and climatic factors such as mean annual temperature, monthly temperature range, mean annual precipitation, or mean January low temperature using either the Chao1 or ACE richness estimators. One reason for these nonsignificant results is that bacterial populations are responding to climatic factors over relatively short time scales or within microenvironments that the 30 year climate averages cannot adequately represent. Alternatively, as many biotic and abiotic factors account for the distributions of microbes in the environment (Horner-Devine *et al.* 2003), it is possible that other unidentified and ecologically important factors, or interactions between these factors, may account for the differences in richness that we observed across populations. Factors such as the types of fruit present in these habitats, host genetic structure, or the presence of alternative insect hosts could account for such variation and need to be studied empirically.

Cox and Gilmore recently completed a survey of bacteria isolated from wild and laboratory-reared *D. melanogaster* (Cox and Gilmore 2007). Several features of their study agree with the data presented here. First, the authors show that wild *D. melanogaster* harbor a wide range of bacterial species from the Proteobacteria, Firmicutes, and Bacteroidetes phyla (Cox and Gilmore 2007). Additionally, although the γ -Proteobacteria are the most diverse group isolated flies, the α -Proteobacteria are the most abundant, after the highly prevalent *Wolbachia* species are excluded (Cox and Gilmore 2007). Last, Cox and Gilmore's estimates of species richness (Cox and Gilmore

2007), fall well within the range of species richness estimated in the present study.

Together with Cox and Gilmore's work, the data presented here move us towards a better understanding of the bacteria interacting with *Drosophila*.

Our study sheds light on the composition and richness of microbial communities present in natural populations of *D. melanogaster* hosts and highlights several important features of these communities. Although our findings have important consequences for how researchers understand the ecology and evolution of *Drosophila* hosts in nature and the dynamics of insect-associated bacterial communities, more work must be done to explicitly test hypotheses regarding the nature of the interactions between *Drosophila* and the microbes we identified. Fortunately, *D. melanogaster* is an experimentally tractable model organism that lends itself beautifully to such in-depth studies.

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Table 4.1. Characteristics of the eleven collection sites and the 16S rRNA gene sequence clone libraries.

Location ¹	Population name ²	Location	Latitude	Date collected	Ambient temp (°C)	Source ³	Site description ⁴	N ⁵	# OTUs ⁶	Chao1 ⁷	ACE ⁸	Coverage ⁹
A	Oakland	Oakland, New Jersey	41.01	Aug 28, 2005	29.4	Cantaloupe traps	Behind private residence	76	16	15.3 ± 0.78	15.7 ± 0.78	100
B	Inwood	Inwood, West Virginia	39.22	July 13, 2005	29.5	Dumpster	Local fruit market	74	30	56.2 ± 3.2	61.3 ± 3.2	53.4
C	Woodstock	Woodstock, Virginia	38.87	Sept 2, 2005	27	Peaches, tomatoes, cantaloupe	Roadside fruit stand	51	12	15.7 ± 1.8	21.3 ± 1.8	76.4
D	Ikenberry	Daleville, Virginia	37.41	Sept 2, 2005	28	Banana traps	Local fruit market	78	15	18.0 ± 3.2	18.9 ± 3.2	83.3
E	Layman	Daleville, Virginia	37.41	Sept 2, 2005	28	Apples, peaches, tomatoes	Local fruit market	80	12	17.0 ± 1.6	42.9 ± 1.6	70.6
F	Hillsborough	Hillsborough, North Carolina	36.07	July 19, 2005	32.2	Cantaloupe, peaches, tomatoes	Roadside fruit stand	58	13	49.0 ± 9.7	42.9 ± 9.7	26.5
G	Raleigh	Raleigh, North Carolina	35.82	July 20, 2005	36	Peaches, cantaloupe	Open-air farmers market	86	9	14.0 ± 5.8	14.2 ± 5.8	64.3
H	Thomas	Watkinsville, Georgia	33.86	Aug 11, 2005	32.2	Peaches	Roadside fruit stand	76	11	21.0 ± 6.3	17.0 ± 6.3	52.4
I	Horticulture Farm	Watkinsville, Georgia	33.87	Sept 6, 2005	26.7	Cantaloupe traps	Wooded area near fruit orchard	31	7	12.0 ± 5.4	20.8 ± 5.4	58.3
J	GA 441	Bishop, Georgia	33.81	Aug 11, 2005	32.2	Peaches, tomatoes	Roadside fruit stand	46	8	9.5 ± 0.81	11.1 ± 0.81	84.2
K	Macon	Macon, Georgia	32.83	Aug 10, 2005	32.2	Dumpster	Open-air farmers market	69	19	35.5 ± 3.0	47.8 ± 3.0	53.5

Table 4.1 (*continued*).

- ¹ Locations are labeled from A through K in Figures 4.1 through 4.4. Five male flies were sampled from each of the eleven collection sites.
- ² Population names as they are referred to in the text.
- ³ Description of the medium that *Drosophila* hosts were congregating over.
- ⁴ Description of collection sites: roadside fruit stands are small, open-air businesses; local fruit markets are enclosed businesses selling fruit from local farms; open-air farmers markets host large collections of local farmers.
- ⁵ Number of non-chimeric 16S rDNA sequences isolated from that host population.
- ⁶ Number of OTUs identified in that 16S rDNA clone library; OTUs are defined based on 3% sequence divergence.
- ⁷ Chao1 estimate of community richness (Chao 1984, Chao *et al.* 1993), using 3% sequence divergence \pm standard error around the estimate. Calculations for standard error were calculated according to Chao (1984) and Chao *et al.* (1993).
- ⁸ ACE estimate of community richness, using 3% sequence divergence \pm standard error around the estimate (Chao and Lee 1992, Chao *et al.* 1993).
- ⁹ The proportion of OTUs observed out of the estimated total number of OTUs in the population. As an estimate of the total number of OTUs in the population, the Chao1 estimate of community richness was used.

FIGURE LEGENDS

Figure 4.1. Unrooted phylogenetic tree of *Wolbachia* sequences based on 16S rDNA gene sequences showing the positions of members of the six *Wolbachia* supergroups (A-F) as described in Lo *et al.* (2002) and the ten *Wolbachia* OTUs isolated from the eleven *D. melanogaster* host populations. Letters in parentheses to the right of the OTU represent the populations where the OTU was observed. Populations corresponding to each letter are presented in Table 4.1. The neighbor-joining tree is the result of 10,000 bootstrap replicates using 1313 nucleotides. Bootstrap values $\geq 50\%$ are posted above the branch at the node. Branch lengths greater than zero nucleotide substitutions per site are indicated below the corresponding branch. Scientific names correspond to the invertebrate host species where the *Wolbachia* is found and are followed by the GenBank accession numbers. Letters to the right of the phylogeny represent *Wolbachia* supergroup designations Lo *et al.* (2002).

Figure 4.2. Phylogenetic trees representing the taxonomic position of Proteobacterial OTUs isolated from the eleven *D. melanogaster* host populations. (a) α -Proteobacteria, (b) β -Proteobacteria, (c) ϵ -Proteobacteria, and (d) γ -Proteobacteria. Phylogenies were inferred using the neighbor-joining method and were bootstrapped for 10,000 replicates. The number of bases used for each analysis was (a) 670, (b) 427, (c) 253, and (d) 690. Numbers above branch points represent bootstrap values $\geq 50\%$. Numbers below branches indicate branch lengths (nucleotide substitutions per site) greater than zero. Trees are rooted with the 16S rRNA gene sequence for *Synechococcus elongata*

(AF132930), a member of the phylum Cyanobacteria. Letters in parentheses to the right of each OTU indicate the *D. melanogaster* host populations where that OTU was observed. A key for these letters is presented in Table 4.1.

Figure 4.3. Phylogenetic trees representing the taxonomic position of Firmicutes OTUs isolated from the eleven *D. melanogaster* host populations. Phylogenies were inferred using the neighbor-joining method using 371 bases and were bootstrapped for 10,000 replicates. Numbers above branch points represent bootstrap values $\geq 50\%$. Numbers below branches indicate branch lengths (nucleotide substitutions per site) greater than zero. Trees are rooted with the 16S rRNA gene sequence for *Synechococcus elongata* (AF132930), a member of the phylum Cyanobacteria. Letters in parentheses to the right of each OTU indicate the *D. melanogaster* host populations where that OTU was observed. A key for these letters is presented in Table 4.1.

Figure 4.4. Phylogenetic trees representing the taxonomic position of Bacteroidetes OTUs isolated from the eleven *D. melanogaster* host populations. Phylogenies were inferred using the neighbor-joining method using 808 bases and were bootstrapped for 10,000 replicates. Numbers above branch points represent bootstrap values $\geq 50\%$. Numbers below branches indicate branch lengths (nucleotide substitutions per site) greater than zero. Trees are rooted with the 16S rRNA gene sequence for *Synechococcus elongata* (AF132930), a member of the phylum Cyanobacteria. Letters in parentheses to the right of each OTU indicate the *D. melanogaster* host populations where that OTU was observed. A key for these letters is presented in Table 4.1.

Figure 4.5. Rarefaction analysis of bacterial 16S rRNA gene clone libraries recovered from each of eleven *D. melanogaster* host populations. Populations are labeled from A through K. Key to population labels is presented in Table 4.1. The predicted number of operational taxonomic units (OTUs) was calculated from the number of clones analyzed at the 3% level of sequence divergence. The slope of the curve indicates whether the diversity was completely sampled (zero or low slope) or whether new taxa are predicted had additional clones been analyzed (steep slope).

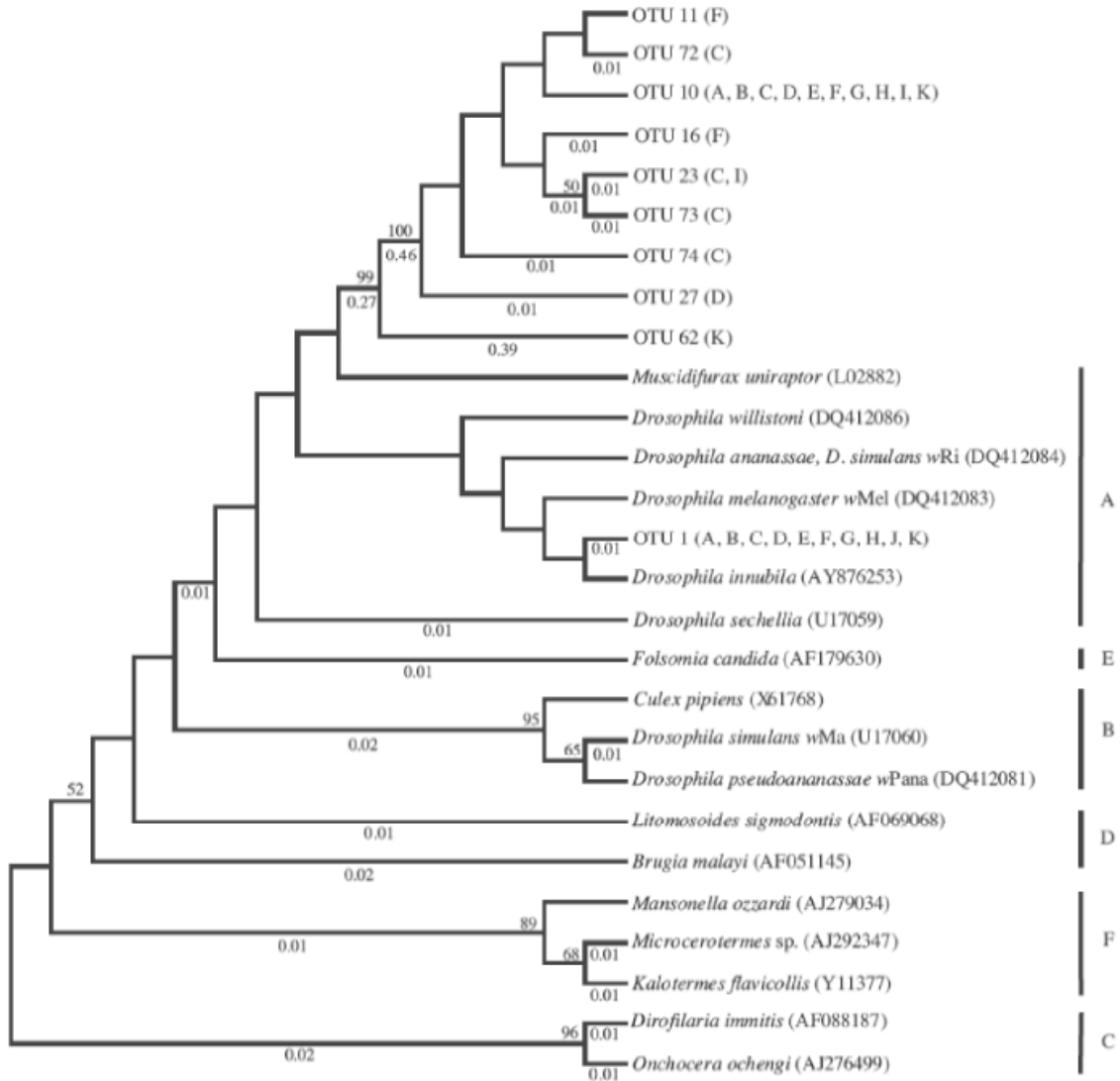


Figure 4.1

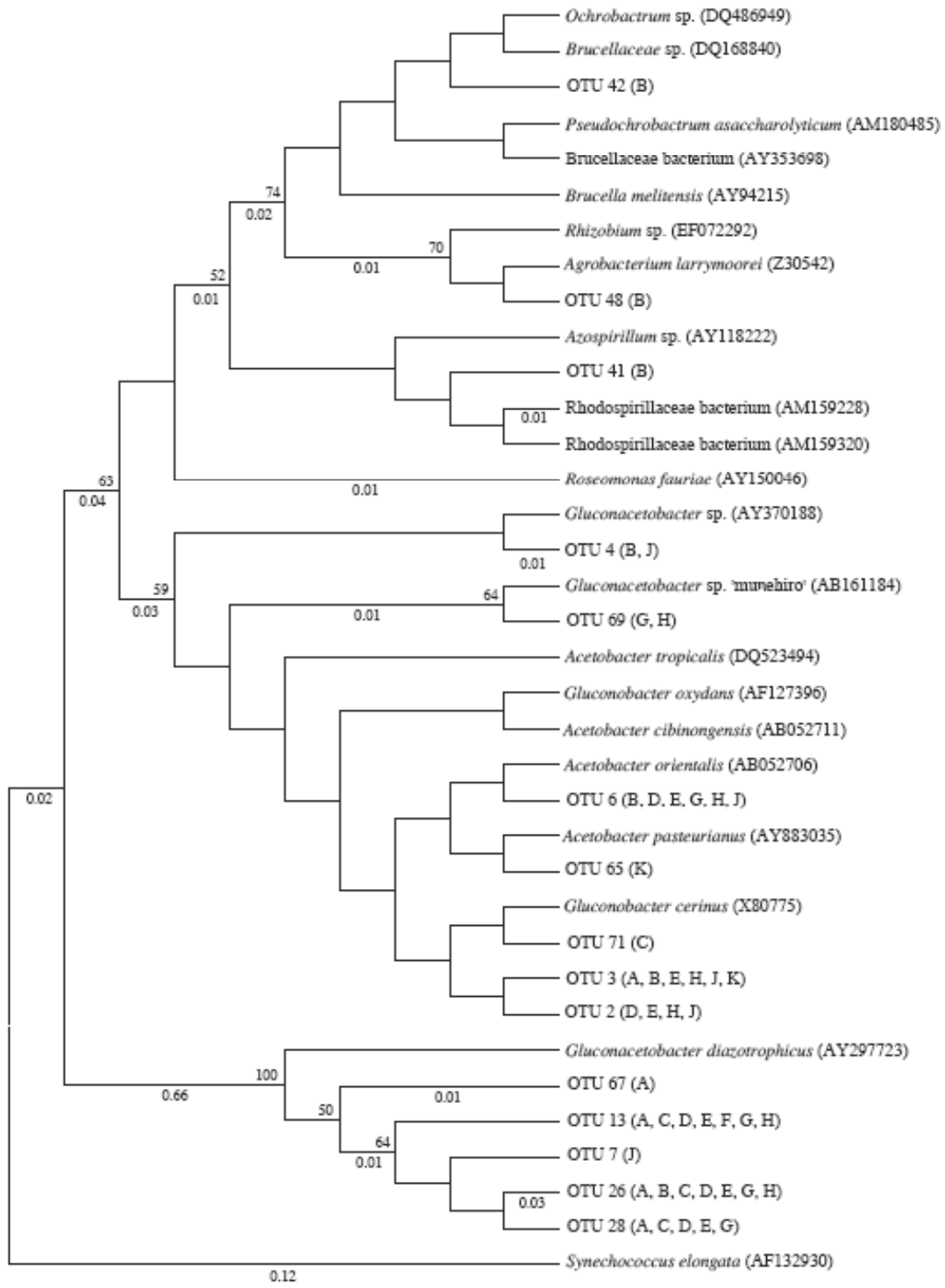


Figure 4.2 (a)

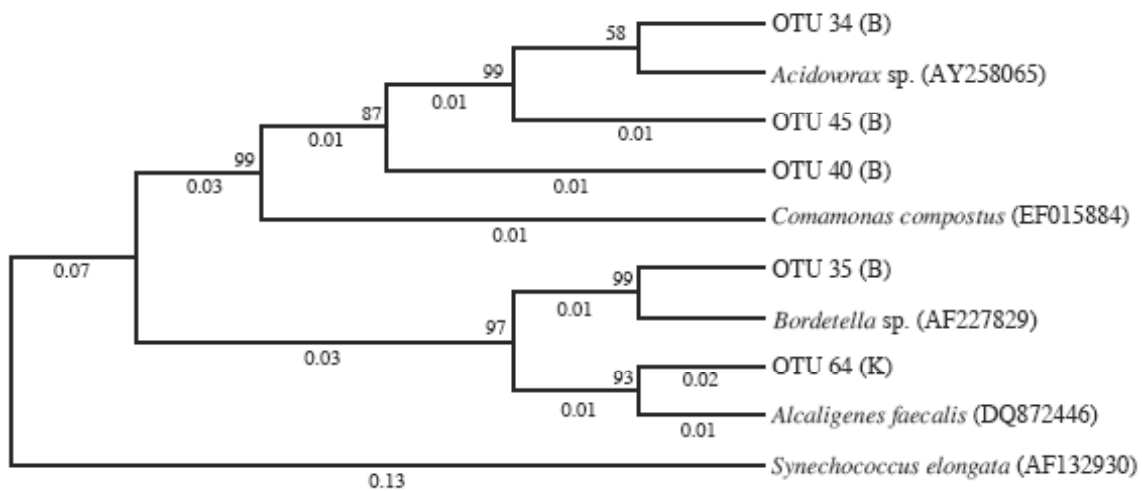


Figure 4.2 (b)

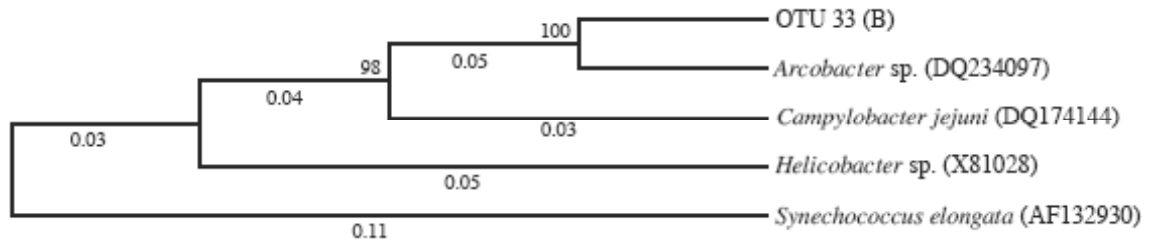


Figure 4.2 (c)

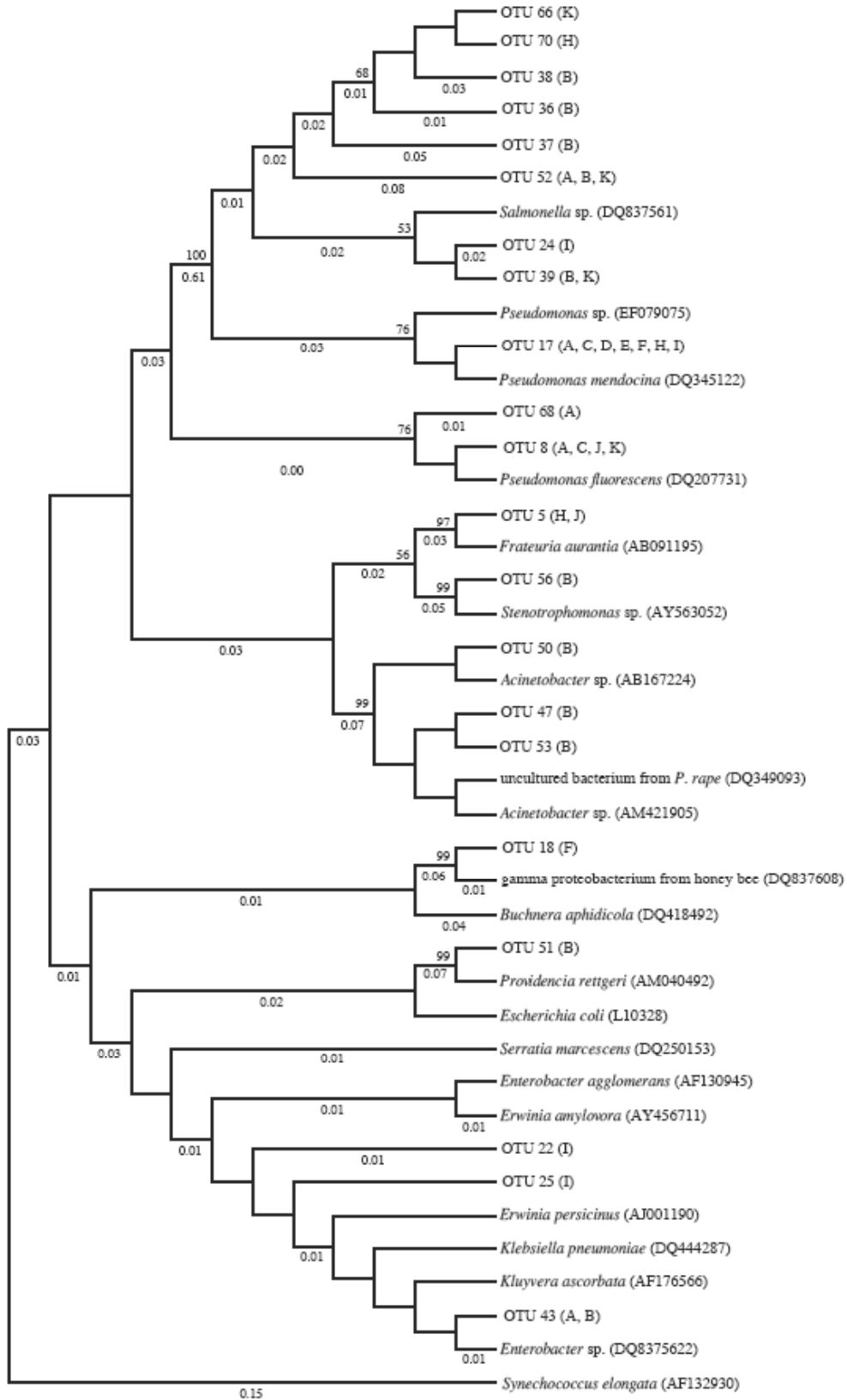


Figure 4.2 (d)

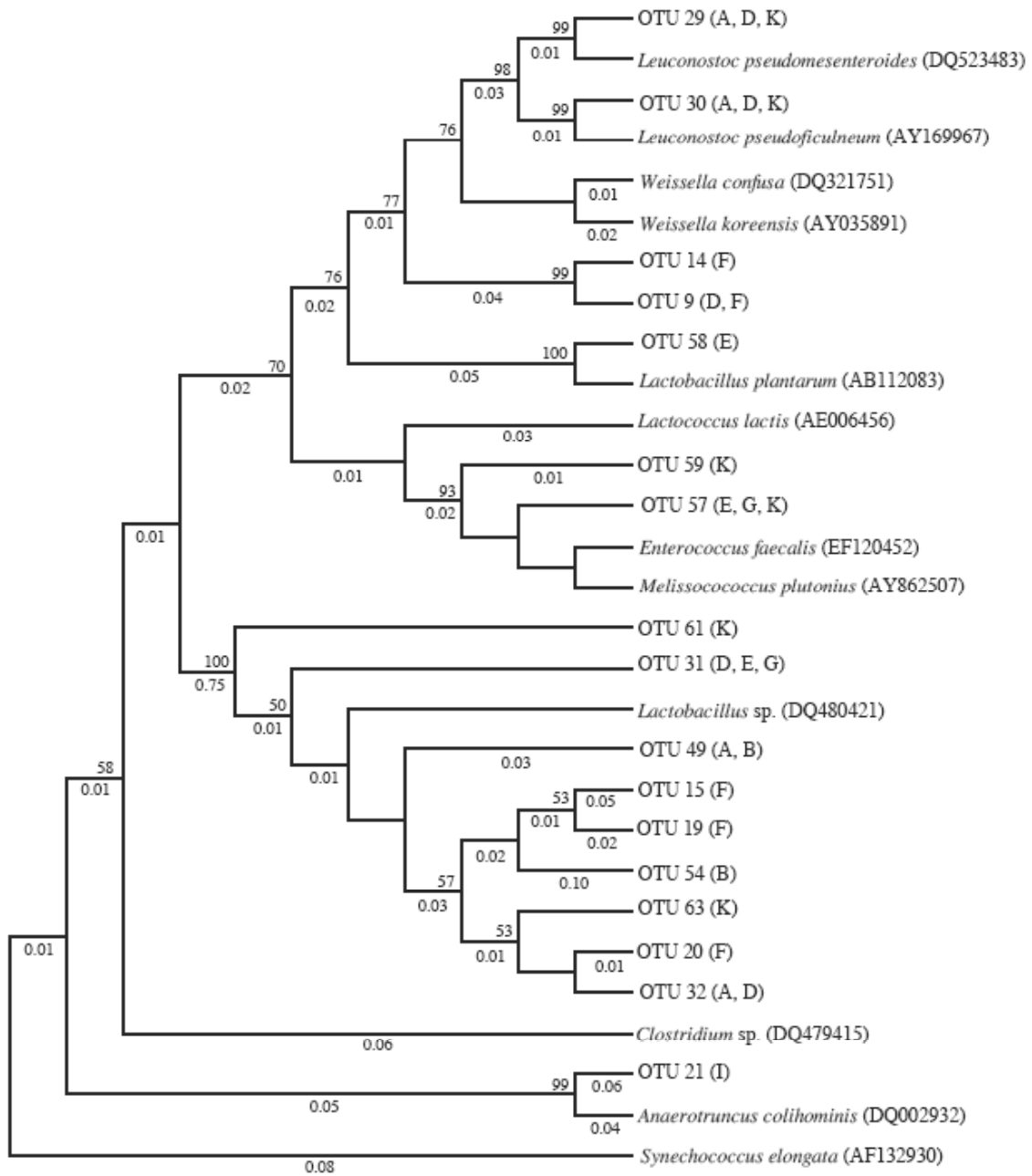


Figure 4.3

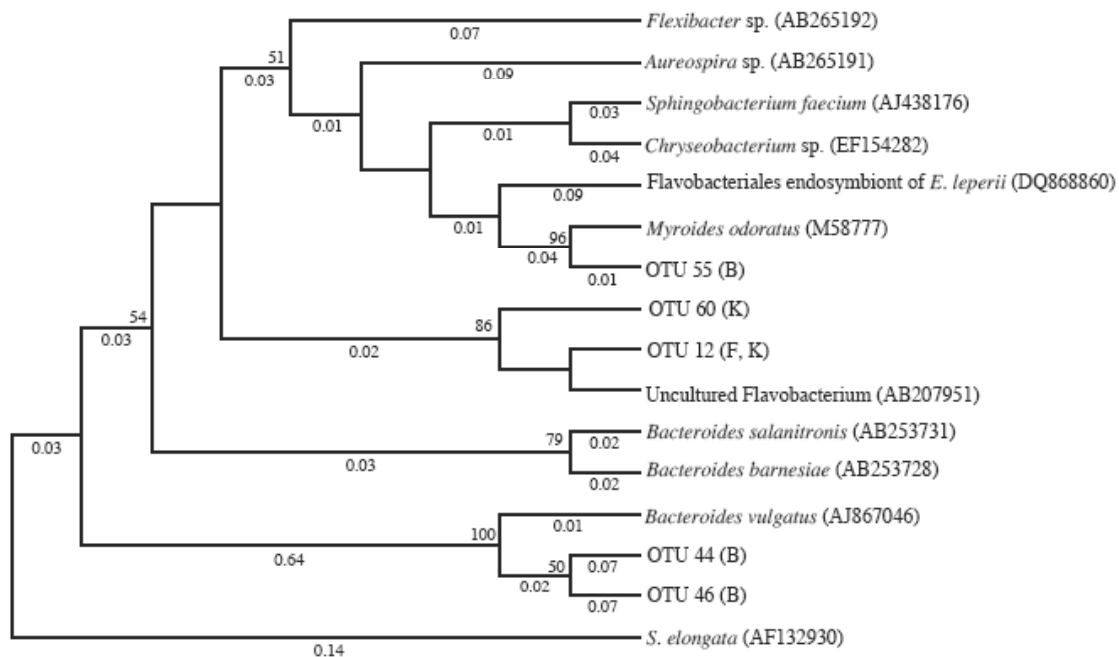


Figure 4.4

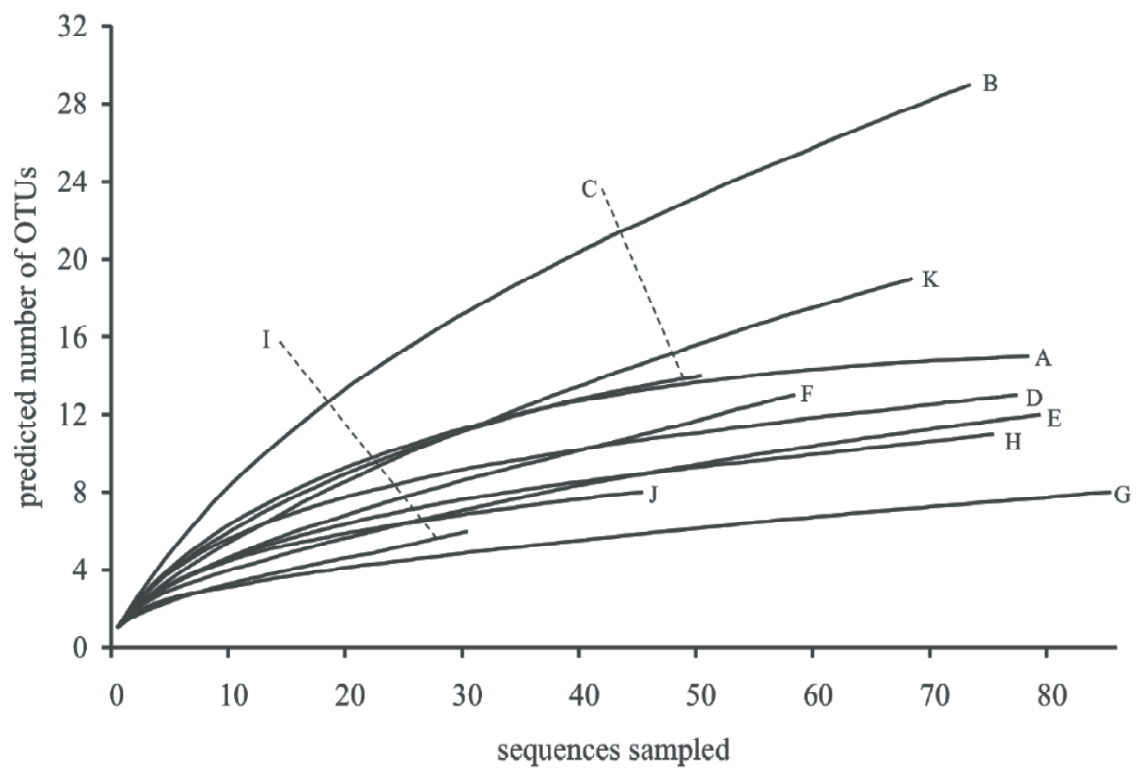


Figure 4.5

CHAPTER 5
GEOGRAPHIC VARIATION FOR RESISTANCE TO BACTERIAL INFECTION IN
*DROSOPHILA MELANOGASTER*⁴

⁴ Corby-Harris, V. and D.E.L. Promislow. Submitted to *Journal of Animal Ecology*,
05/23/2007.

ABSTRACT

Despite numerous studies characterizing patterns of resistance to infection within invertebrate host populations, there are few examples illustrating patterns of resistance variation among natural populations. Here, we determined whether twenty naturally isolated populations of *Drosophila melanogaster* collected along the East Coast of the United States varied for survival after being inoculated with one of two species of bacteria – *Lactococcus lactis* and *Pseudomonas aeruginosa*. We then asked whether latitude or host environment accounted for the observed patterns of resistance. Resistance to both types of infection varied spatially, and only resistance to *L. lactis* was correlated with latitude. The hosts' natural environment was predictive of the observed spatial variation. Hosts exposed to rich bacterial communities were more resistant to a novel *P. aeruginosa* infection. Hosts from locations with lower mean annual temperatures and high summer temperatures were more resistant to *L. lactis* infection. We conclude that biotic and abiotic characteristics of the host environment shape host resistance to bacterial infection. We conclude with a discussion of the evolutionary and ecological mechanisms that might produce such broad spatial patterns.

INTRODUCTION

A variety of life history (Azevedo *et al.* 1996, James *et al.* 1997, Gockel *et al.* 2001, Hoffmann *et al.* 2001, Schmidt *et al.* 2005) and functional traits (Hoffmann and Parsons 1989, Kraaijeveld and Vanalphen 1995, Hoffmann *et al.* 2002, Frydenberg *et al.* 2003) are spatially variable in the fruit fly, *Drosophila melanogaster*. Many of these traits exhibit clear latitudinal clines (Hoffmann and Parsons 1989, Azevedo *et al.* 1996, James

et al. 1997, Gockel *et al.* 2001, Hoffmann *et al.* 2001, Hoffmann *et al.* 2002, Frydenberg *et al.* 2003, Schmidt *et al.* 2005). However, other spatially variable traits do not correlate tightly with latitude. For example, *D. melanogaster* populations collected throughout Europe are spatially, but not clinally, variable, for resistance to the parasitoid *Asobara tabida* (Kraaijeveld and van Alphen 1995). Although it has been suggested that differences in host-parasitoid community structure could cause such differences in resistance (Kraaijeveld and Godfray 1999), to date, we lack a clear understanding of the processes causing spatial patterns in resistance.

The presence and prevalence of host-associated bacteria associating with *D. melanogaster* vary across this host species' range (Corby-Harris *et al.* 2007). Such variation could cause a geographic mosaic (Thompson 1997) of infection risks. Where infection risk is high, there would be positive selection for resistance to a novel infection. Conversely, if resistance is costly (Kraaijeveld *et al.* 2002), resistance to a novel infection would be detrimental to host fitness in areas where the risk of infection is low. It is presently unclear whether the uneven distribution of *D. melanogaster*-associated bacteria contributes to geographic variation for host resistance. However, a recent study by Scharsack *et al.* (2007) in vertebrates provides some clues. Scharsack *et al.* (2007) exposed river-adapted sticklebacks, which normally live with fewer parasites relative to lake-adapted individuals, to parasite-dense lake conditions, and found they had higher parasite loads and reduced immune response relative to lake-adapted fish (Scharsack *et al.* 2007). Such patterns suggest that the risk of encountering parasites shapes host immunocompetence (Scharsack *et al.* 2007). Given the available data for the numbers of bacterial species associating with *D. melanogaster* hosts in nature, we can generate a

testable prediction motivated by Scharsack *et al.*'s (2007) work: hosts that associate with a rich community of bacteria are more likely to resist a novel infection.

Temperature varies across a host species' range and is an important regulator of invertebrate host immunocompetence. Constant fluctuations in ambient temperature can greatly impact host resistance to fungal, bacterial, and viral infections (see Thomas and Blanford (2003) and references within) over short timescales. Temperature also affects long-term host-pathogen dynamics by impacting the presence of bacteria and the impact these bacteria have on host fitness. Mitchell *et al.* (2005) studied whether temperature mediated interactions between *Daphnia magna* and its sterilizing pathogen, *Pasteuria ramosa*. At high temperatures, the pathogen was much more virulent and sterilized more hosts relative to low temperature conditions, suggesting that parasite-mediated selection is temperature-dependent (Mitchell *et al.* 2005). Additionally, when Bensadia *et al.* (2006) tested the ability of aphids at different temperatures to resist parasitoid attack, they found that hosts were less resistant at high temperatures. They suggested this effect could be due to loss of secondary endosymbionts at high temperatures (Bensadia *et al.* 2006). Overall, temperature is an important regulator of invertebrate immunocompetence and plays a role over short and long timescales. As *D. melanogaster* inhabit a wide geographical range, they are likely impacted by temperature shifts. It is therefore possible that temperature is an important contributor to spatial variation in host immunocompetence.

Here, we investigated whether *D. melanogaster* populations collected along the East Coast of the United States varied for resistance to bacterial infection. Motivated by previous empirical studies, we then sought to explain this spatial variation in resistance

by asking whether resistance varied clinally, or whether temperature or bacterial community richness explained variation in resistance among host locations. We tested three hypotheses. First, because many traits show evidence for latitudinal variation in *D. melanogaster* (Hoffmann and Parsons 1989, Azevedo *et al.* 1996, James *et al.* 1997, Gockel *et al.* 2001, Hoffmann *et al.* 2001, Hoffmann *et al.* 2002, Frydenberg *et al.* 2003, Schmidt *et al.* 2005), we predicted that resistance to bacterial infection would correlate with latitude among host populations. Second, if host resistance increases with the risk of infection (Scharsack *et al.* 2007), we predicted that flies from locations with more bacterial species richness would be more likely to resist a novel infection. Third, because previous work (Mitchell *et al.* 2005, Bensadia *et al.* 2006) demonstrates that temperature plays a role in resistance, we hypothesized that temperature would correlate significantly with host resistance to bacterial infection across a broad spatial scale. Interestingly, our data suggest that differences among host locations in bacterial species richness and temperature shape geographic variation for resistance in naturally isolated *D. melanogaster* populations.

MATERIALS AND METHODS

Fly collections

Flies were collected from twenty sites along a latitudinal transect on the East Coast of the United States between June and September 2005 (Figure 5.1, Table 5.1) by sweep netting behind fruit stands and over fruit bucket traps. Following collection, flies were anesthetized over ice. Male *D. melanogaster* were separated from other Drosophilid species on the basis of morphological characteristics (Ashburner 1989, Markow and

O'Grady 2006). These males were set aside and preserved in groups of five in 70% ethanol for subsequent analysis of their bacterial communities. After returning to the laboratory from the collection sites, the ethanol-preserved samples were kept at -80°C. The *D. melanogaster*-like females were separated from the group of field-collected flies and were maintained in individual vials containing standard cornmeal, molasses, and yeast fly medium. After returning to the lab from the field, these isofemale stocks were maintained at 24°C on a 12:12 h light:dark cycle. Females were cleared from their vial after laying eggs. Approximately twelve days later, their male progeny were further examined to determine which lines were *D. melanogaster*. *D. melanogaster* isofemale lines were established by first mating F₁ full- or half-sibs, followed by subsequent generations of full-sib matings.

After ten generations of inbreeding, for each of the twenty populations, ten isofemale lines were randomly selected and combined to form an outbred population. Because only ten *D. melanogaster* isofemale lines were collected from two of the populations, this method allowed us to keep the genetic variance relatively constant among each outbred population while including the maximum number of populations in the study. Each of these randomly selected lines was represented equally in the new outbred population by placing two males and two females from each of these ten isofemale lines in duplicate 17 ml culture bottles containing standard medium under the conditions described above.

Bacterial stocks

We acquired strains of *Pseudomonas aeruginosa* and *Lactococcus lactis* from B.P. Lazzaro in January of 2004. *L. lactis* was isolated from a wild population of *D. melanogaster* in Pennsylvania and has been described previously (Lazzaro *et al.* 2006). *P. aeruginosa* is a laboratory stock and was not derived from wild flies. However, it is a common insect pathogen (Lacey 1997) and *Pseudomonas* species have been identified in wild *D. melanogaster* (Corby-Harris *et al.* 2007). Working stocks of bacterial cultures were maintained in nutrient broth at 4°C in a 10⁻² dilution (of an unmeasured dense culture) until ready for use. The day before the infections, these working stocks were grown for approximately 18 h at 37°C, which corresponds to the log phase of growth (V. Corby-Harris, unpublished data).

Details of infection

Each of the twenty outbred *D. melanogaster* populations was maintained for approximately six to ten non-overlapping generations before being experimentally infected over a period of seven days between January and February of 2006. Adult male flies were manually infected in the lateral thorax using a fine stainless steel needle (Fine Science Tools, Foster City, CA) dipped in bacterial culture (Tzou *et al.* 2002). Bacterial cultures were standardized by diluting an overnight culture with sterile nutrient broth until they measured 0.200 ± 0.050 A at 600 λ using a UV spectrophotometer (Thermo Electron, Rochester, NY). On each of the seven infection days, for each of the twenty outbred host populations, we inoculated a random mixed sample of approximately thirteen mated and virgin male flies that were between 24 and 48 hours old. Five of these flies were inoculated with the *P. aeruginosa* treatment, five with the *L. lactis* treatment,

and three with a negative control (sterile nutrient broth). The inoculated individuals were placed into clean, unyeasted vials with 5 ml of standard cornmeal, molasses, and yeast fly medium in groups of five individuals, and monitored for 48 hours at 24°C with a 12:12 h light:dark cycle. We recorded the number of dead flies in each vial at three hours post-infection, hourly from 16 through 31 hours post-infection, and then again at 48 hours post-infection. Among the control flies, only 8 out of 371 flies died within 48 hours (0.02%). Additionally, previous observations suggest that flies dying from the experimentally applied infection do so within 16 to 48 hours post-inoculation (V. Corby-Harris, unpublished data). Consequently, we limit all analyses and discussion to flies inoculated with the experimental treatments that did not die before 16 hours post-inoculation (N=26). All individuals that did not die within 48 hours of the inoculation were censored in our analyses.

For each of the two bacterial treatments, we asked whether populations varied in how well they resisted infection by analyzing a Cox Proportional Hazards model, where hours to death was the dependent variable and population was the fixed independent variable. Date, representing the seven different dates that resistance was assayed, was treated as a strata variable. All survival analyses were performed using proc tphreg in SAS version 9.1 (SAS Institute, Cary, NC).

Statistical test for clinal variation in resistance

To determine whether resistance to bacterial infection exhibited clinal variation, we tested for a significant correlation between latitude and resistance among the twenty populations assayed. The estimated hazard ratio generated under the proportional hazards

model for the *P. aeruginosa* and *L. lactis* treatment was used to represent each population's mean resistance to *P. aeruginosa* and *L. lactis*, respectively. Latitude and the *L. lactis* hazard ratio were non-normal, so a nonparametric Spearman's rank correlation was tested. All correlation analyses were performed using proc corr in SAS version 9.1 (SAS Institute, Cary, NC).

Microbial richness assays

The species richness of the microbial communities associating with *D. melanogaster* hosts was assayed at ten of the twenty sites where *D. melanogaster* were collected from June to September of 2005. A full description of the methods used to isolate, amplify, and sequence the microbial DNA inside the *Drosophila* hosts is provided in Corby-Harris *et al.* (2007; Chapter 4). Operational taxonomic unit (OTU) groupings were determined using 99% sequence identity and the Chao1 nonparametric species richness estimator (Chao 1984, Chao *et al.* 1993) was obtained. The ACE (abundance-based coverage estimator) estimate of species richness was also obtained for each microbial community, but did not qualitatively differ from the Chao1 estimates of richness (Corby-Harris *et al.* 2007).

Statistical tests to determine environmental factors contributing to resistance variation

Latitude is a variable that describes many different climatic factors and, alone, may not accurately reflect the environmental pressures that hosts experience. Therefore, we investigated whether more specific descriptors of the hosts' environment – temperature and the number of host-associated bacterial species – account for the

observed spatial variation in resistance to *L. lactis* and *P. aeruginosa*. We analyzed a full model that included temperature and bacterial species richness to understand whether these parameters contributed additively to differences in population mean resistance. The full model including resistance to either species of bacteria as the dependent variable, and the independent variables mean annual temperature, mean summer temperature (defined as the average temperatures between the months of July, August, and September), and bacterial species richness at each of the ten collection sites, were analyzed using least squares regression. Temperature data represented 30 year averages recorded by the NOAA at each collection site (NOAA 2002a, c, d, b, e). To represent each population's mean resistance to either species of bacteria, the estimated hazard ratio generated under the proportional hazards model for each treatment was used. For the *L. lactis* treatment, this estimate was square root transformed to improve normality (Shapiro-Wilk $W=0.87$, $P=0.20$). The Chao1 nonparametric estimates of species richness generated from the 16S rRNA gene sequence data were used to estimate the number of bacterial OTUs co-occurring with each of the ten *D. melanogaster* populations. To investigate whether a reduced model with fewer than three predictor variables would best explain the data, the Akaike information criterion (AIC) and Bayesian information criterion (BIC) were employed. These reduced models were then analyzed using least squares regression. The proc reg procedure in SAS version 9.1 (SAS Institute, Cary, NC) was used to perform all model selection procedures and to evaluate the full and reduced regression models.

RESULTS

Geographic variation for resistance

Under both the *P. aeruginosa* and *L. lactis* treatments, there was significant variation for resistance among outbred *D. melanogaster* host populations. Of the 689 male *D. melanogaster* infected with *P. aeruginosa*, 649 (94.2%) died within 48 hours post-inoculation. For the *L. lactis* treatment, 677 (97.4%) out of the 695 males died within 48 hours. Host population (Wald $\chi^2_{19}=57.90$, $P < 0.0001$, Figure 5.2) was a significant predictor of this observed variation in time to death following inoculation with *P. aeruginosa*. The effect of population remained significant in the data sets with fewer populations used for testing the contribution of host environment (N=10 populations, Wald $\chi^2_9=44.56$, $P < 0.0001$) to differences in population mean resistance. Host population was a significant predictor of the observed variation in time to death post-inoculation for the *L. lactis* treatment across the twenty (Wald $\chi^2_{19}=44.41$, $P = 0.0008$, Figure 5.2) and ten (Wald $\chi^2_9=41.88$, $P < 0.0001$) populations assayed for geographic variation and microbial richness, respectively.

Clinical variation for resistance

Across the twenty populations assayed, latitude and mortality following *L. lactis* inoculation were significantly positively correlated ($r_s=0.51$, $P = 0.021$, Figure 5.3). Conversely, there was no significant relationship between latitude and mortality following *P. aeruginosa* inoculation.

Bacterial species richness within D. melanogaster hosts

Microbial richness varied across host populations as measured by the Chao1 estimator (Table 5.1). The mean number of bacterial OTUs associating with *D. melanogaster* hosts was 31.1 ± 13.7 s.d.

Environmental factors contributing to resistance variation

Temperature contributed to the observed geographic variation for resistance to *L. lactis* inoculation. The full model including mean annual temperature, mean summer temperature, and bacterial species richness as predictors provided a relatively poor fit to the data ($F_{3,6}=3.10$, $P = 0.11$) and none of these predictor variables were significant sources of population mean mortality following *L. lactis* inoculation. The AIC and BIC selection methods both yielded a best model including mean annual temperature and mean summer temperature as the predictor variables. This reduced model fit the data better than the full model ($F_{2,7}=4.58$, $P = 0.054$). Both mean annual temperature ($\beta=0.296$, $F_{1,7}=5.92$, $P = 0.045$) and mean summer temperature ($\beta=-0.46$, $F_{1,7}=7.00$, $P = 0.033$) were significant predictors of the among population differences in resistance to *L. lactis*.

Bacterial species richness contributed to the observed among population variation for resistance to *P. aeruginosa* inoculation. A full model including resistance to *P. aeruginosa* inoculation as the dependent variable and mean annual temperature, mean summer temperature, and bacterial species richness as the independent variables provided a somewhat poor fit to the data ($F_{3,6}=3.79$, $P=0.078$). Under this full model, bacterial species richness ($\beta=-0.036$, $F_{2,7}= 9.06$, $P = 0.024$) was a significant predictor of

resistance. The BIC and AIC selection methods both yielded a best model that included the predictor variables bacterial species richness and mean annual temperature. This reduced two variable model provided an improved fit to the data ($F_{2,7}=5.86$, $P = 0.032$). Under this reduced model, only bacterial species richness ($\beta=-0.032$, $F_{1,7}=9.78$, $P = 0.017$) significantly contributed to the among population differences in mortality following *P. aeruginosa* inoculation.

DISCUSSION

In the experiments described here, we observed that naturally isolated populations of *D. melanogaster* differed in how well they resisted both *P. aeruginosa* and *L. lactis* infection. We then sought to explain this variation in resistance by asking if resistance varied clinally and relative to the different environments hosts experienced in nature. Resistance to *L. lactis*, but not *P. aeruginosa*, inoculation varied clinally. Among population differences in *L. lactis* resistance were significantly associated with two climatic factors - mean annual temperature and mean summer temperature. Differences in bacterial species richness accounted for the spatial variation in *P. aeruginosa* resistance. Our results suggest that resistance to bacterial inoculation differs among naturally isolated host populations of *D. melanogaster* and results from hosts' response to their local microenvironments.

The dataset we have analyzed here has both strengths and limitations. A notable strength of this work is that it provides an increased understanding of the ecology of *D. melanogaster*, a model organism whose natural history is not well understood. In addition, as *D. melanogaster* are increasingly used as models for studying the molecular

and genetic basis of the immune response, an understanding of the natural forces shaping *Drosophila* resistance variation is warranted. There are three significant limitations of this study. First, we sampled only five flies per location (pooled into one sample), and our estimates of community richness could change with increased sampling effort. Second, our ability to identify trends in bacterial species richness is potentially weakened by the fact that there are not multiple samples within each collection site. Last, we did not explicitly test whether bacterial richness or temperature results in resistance variation through experimental manipulation of either parameter. However, our results suggest that *D. melanogaster* can be a powerful model organism not only for lab-based studies of immune function, but also to help us predict and understand the ecological forces that shape immunity.

Spatial variation for resistance

There are several examples in the invertebrate immunity literature demonstrating that resistance to bacterial infection varies within host populations (Ebert *et al.* 1998, Little and Ebert 2000, Haag and Ebert 2004, Lazzaro *et al.* 2004, Lazzaro *et al.* 2006). However, this is the first evidence that resistance to bacterial infection varies among *D. melanogaster* populations. Clear spatial patterns exist for resistance to parasitoid attack among naturally isolated populations of *D. melanogaster* (Kraaijeveld and van Alphen 1995, Kraaijeveld and Godfray 1999). Interestingly, there were clear clinal trends for *L. lactis* resistance. Populations from higher latitudes were less likely to resist inoculation with this microbe. A clinal trend for resistance agrees with previous work in *D. melanogaster* (Hoffmann and Parsons 1989, Azevedo *et al.* 1996, James *et al.* 1997,

Gockel *et al.* 2001, Hoffmann *et al.* 2001, Hoffmann *et al.* 2002, Frydenberg *et al.* 2003, Schmidt *et al.* 2005) that suggests both functional and life history traits vary in relation to latitude. In contrast, *P. aeruginosa* resistance did not vary significantly with respect to latitude. This pattern agrees with the patterns of parasitoid resistance in *D. melanogaster* observed by Kraaijeveld and van Alphen (1995). That *L. lactis*, but not *P. aeruginosa*, vary clinally suggests that different microenvironmental factors influence resistance to either type of bacteria.

Relationship between bacterial species richness and resistance

Individuals exposed to a rich bacterial community are more resistant to a novel *P. aeruginosa* infection, suggesting bacterial community structure shapes host immunocompetence. The impact of microbial community structure on host resistance has previously been acknowledged in reference to invertebrate (Kraaijeveld and Godfray 1999, Kurtz and Hammerschmidt 2006) and vertebrate (Scharsack *et al.* 2007) hosts. Several processes could be operating to produce such a pattern. Below, we discuss each of these processes in further detail.

In pathogen-rich environments, where hosts are likely to encounter novel infections, there selection should favor an increased immune response that would decrease the likelihood of novel infection. This might lead, in turn, to among population patterns of in resistance among populations similar to those we observed if hosts adaptively responded to bacterial species richness. Many *Drosophila* immune genes show signs of strong selection (Begun and Whitley 2000, Schlenke and Begun 2003, Lazzaro 2005, Jiggins and Kim 2006, Obbard *et al.* 2006, Jiggins and Kim 2007),

suggesting they coevolve with pathogens (Jiggins and Kim 2007). However, it is unclear whether *Drosophila* hosts respond to traits of individual bacteria (i.e., virulence) or the bacterial community overall (i.e., species richness).

These hosts may be responding to the presence or absence of a particular species of bacteria, and not to bacterial species richness, *per se*. For example, the presence of a particular *Pseudomonas* species could select for increased resistance to subsequent *P. aeruginosa* infection. This possibility remains an open question. Our sequencing effort was not extensive enough to identify all of the bacteria present in these populations, and some rare species likely remained undiscovered (Corby-Harris *et al.* 2007). Without a complete catalog of all bacteria living with these flies in nature, it is difficult to investigate this relationship.

In cases where the host could be confronted with subsequent infections, mechanisms such as adaptive immunity that reduce the likelihood of secondary infection should be selected for (Kurtz 2004, Little and Kraaijeveld 2004). These adaptive immune responses would be activated in pathogen-rich environments, priming the host's immune response towards infection. Kurtz and Hammerschmidt (2006) tested this expectation by subjecting copepod hosts to homogenous or heterogeneous combinations of tapeworm parasites. They then tested their resistance to a novel subsequent tapeworm infection. Contrary to their expectations, however, the heterogeneous environment did not lead to increased resistance towards a subsequent challenge, suggesting that the effect of parasite heterogeneity on host immunity is not straightforward (Kurtz and Hammerschmidt 2006). However, we doubt that the case studied here is an example of such priming because hosts were removed from their natural environments for more than fifteen generations

before being assayed. Current examples demonstrate the positive effects of trans-generational priming over a maximum two generations (Little and Kraaijeveld 2004, Sadd *et al.* 2005, Moret 2006).

Relationship between temperature and resistance

Temperature significantly contributed to differences in resistance to *L. lactis* infection among host populations. Hosts can react to changing temperatures through temperature-associated immune plasticity (Elliot *et al.* 2002, Thomas and Blanford 2003). However, because hosts were removed from their natural environments for many generations before being assayed, we doubt such mechanisms are operating. Instead, we hypothesize that temperature shapes long term resistance dynamics. It is unclear, though, why mean summer temperature and mean annual temperature are positively and negatively associated, respectively, with resistance. This conflicting result points towards temperature's complex role in mediating host resistance.

Flies collected from areas with higher mean summer temperatures were more likely to resist *L. lactis* infection. Such a pattern could be due to temperature-associated shifts in pathogen virulence or the presence of particular pathogenic bacteria. For example, if certain *L. lactis*-like pathogens are more prevalent and exert stronger selection on the host population at high temperatures, resistance to *L. lactis* would increase host fitness in these populations.

Individuals from areas with higher mean annual temperatures were less resistant to *L. lactis* infection. This pattern may reflect the loss of beneficial bacteria at high temperatures (Van Opijnen and Breeuwer 1999, Kyei-Poku *et al.* 2003). Bensadia *et al.*

(2006) argued that such heat-sensitive beneficial endosymbionts were lost from aphid hosts under heat stress, after observing individuals were less resistant to parasitoid attack at high temperatures (Bensadia *et al.* 2006).

Conclusion

Overall, our data are consistent with the hypothesis that, among naturally isolated *D. melanogaster* host populations, geographic variation for resistance to both *L. lactis* and *P. aeruginosa* infection reflects the hosts' response to their abiotic and biotic environment. It is important to realize that factors apart from microbial species richness and temperature are likely to influence resistance. The work described above serves as an important beginning towards understanding how and why resistance to bacterial infection varies among naturally isolated *D. melanogaster* populations.

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Table 5.1. Characteristics of the ten host populations assayed for bacterial species richness.

Population number ¹	Collection location	Latitude	Date collected	Mean annual temperature (°C) ²	Mean summer temperature (°C) ³	Chao1 ⁴
1	Oakland, New Jersey	41.01	August 28, 2005	10.3	25.0	21.8 ± 0.78
6	Inwood, West Virginia	39.22	July 13, 2005	12.6	24.3	52.9 ± 3.2
8	Woodstock, Virginia	38.87	September 2, 2005	11.8	22.7	38.9 ± 1.8
9	Daleville, Virginia	37.41	September 2, 2005	13.5	22.7	34.0 ± 3.2
10	Daleville, Virginia	37.41	September 2, 2005	13.5	22.7	18.3 ± 1.6
12	Hillsborough, North Carolina	36.07	July 19, 2005	15	25.8	43.5 ± 9.7
13	Raleigh, North Carolina	35.82	July 20, 2005	15.3	21.1	17.5 ± 5.8
14	Watkinsville, Georgia	33.86	August 11, 2005	16.4	24.2	28 ± 6.3
16	Bishop, Georgia	33.81	August 11, 2005	16.4	25.1	9.5 ± 0.81
17	Macon, Georgia	32.83	August 10, 2005	17.6	21.8	33.2 ± 3.0

Table 5.1 (*continued*).

- ¹ Population number as referred to in Figure 5.1.
- ² 30-year average of mean annual temperatures (in degrees Celsius) recorded at that location by the NOAA (NOAA 2002a, c, d, b, e).
- ³ 30-year average of mean summer temperatures (in degrees Celsius) recorded at that location by the NOAA (NOAA 2002a, c, d, b, e).
- ⁴ Chao1 estimate of community richness (Chao 1984, Chao *et al.* 1993), using 1% sequence divergence \pm standard error around the estimate. Calculations for standard error were calculated according to (Chao 1984, Chao *et al.* 1993).

FIGURE LEGENDS

Figure 5.1. *D. melanogaster* were collected at twenty different locations along the East Coast of the United States. Numbers on the right side of the figure correspond to the population numbers in Figure 5.2 and in Table 5.1. The grey box (left) indicates the range encompassed by the enlarged map on the right. Single points with two corresponding numbers represent separate locations ≤ 20 km apart. Map courtesy of <http://www.lib.berkeley.edu>.

Figure 5.2. Populations of *D. melanogaster* varied for how well they resisted both *P. aeruginosa* (black) and *L. lactis* (grey) infection. Outbred host populations (x-axis) are arranged from left to right in order of decreasing latitude. Mortality post-inoculation (y-axis) represents each population's hazard ratio post-inoculation under a proportional hazards model. Error bars represent the standard error around this hazard ratio estimate. Host population was a significant predictor of time to death following inoculation for both the *P. aeruginosa* (Wald $\chi^2_{19}=57.90$, $P < 0.0001$) and *L. lactis* (Wald $\chi^2_{19}=44.41$, $P = 0.0008$) treatments.

Figure 5.3. Populations from higher latitudes are less resistant to *L. lactis* infection. Ranked latitude (x-axis) is plotted versus ranked mortality following *L. lactis* inoculation (y-axis). Prior to ranking, mean mortality post-inoculation was estimated for each population using the hazard ratio estimated under a proportional hazards model. Latitude and mortality were significantly positively correlated ($r_s=0.51$, $P = 0.021$) using a Spearman's rank correlation.

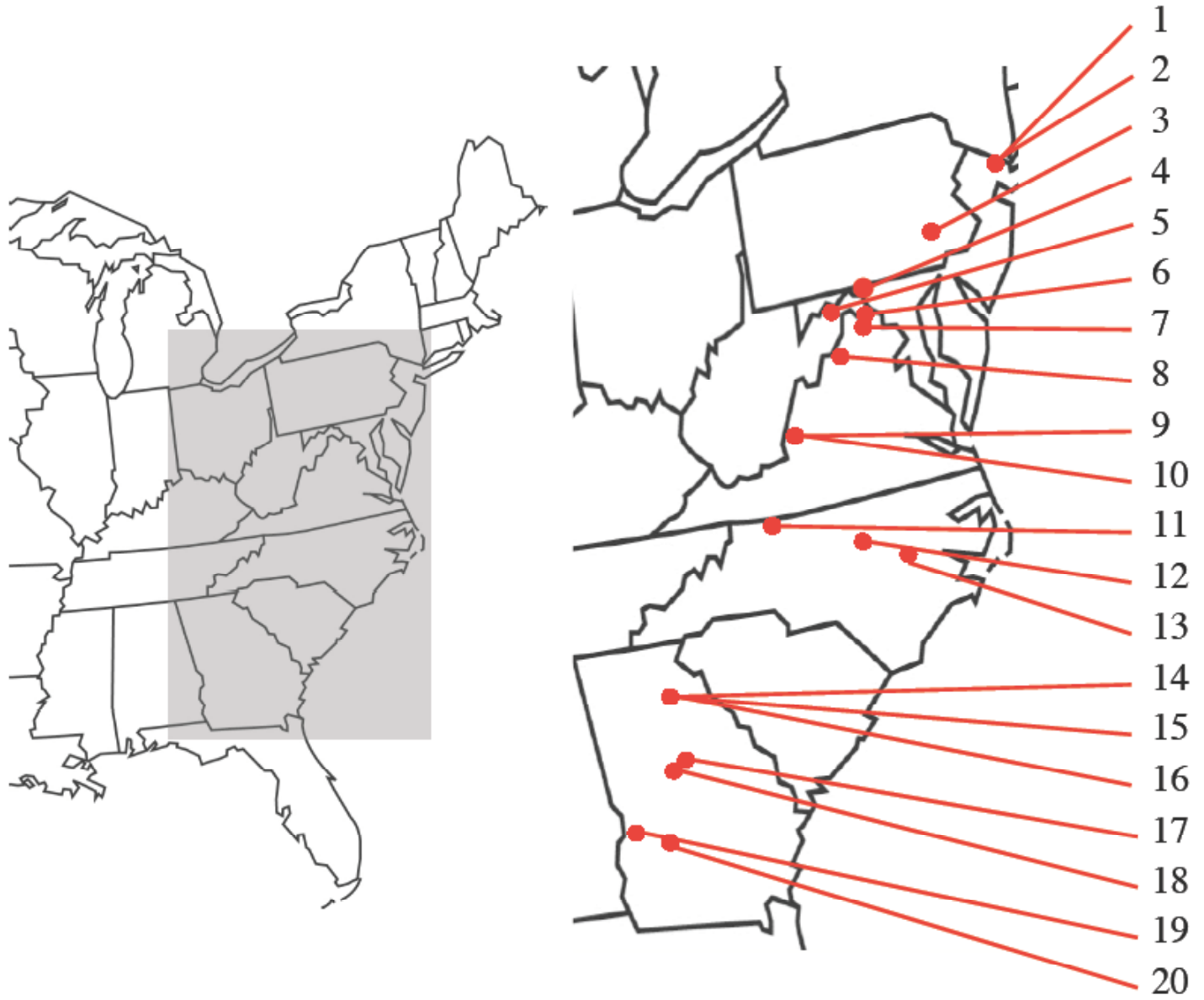


Figure 5.1

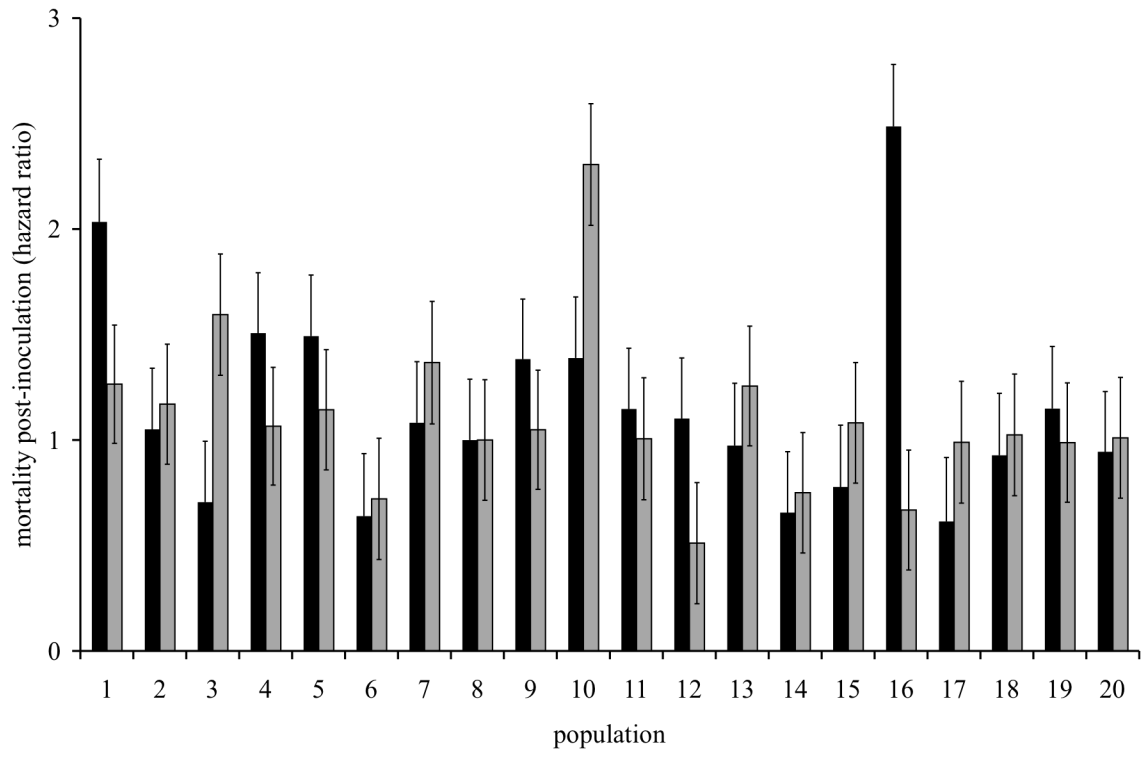


Figure 5.2

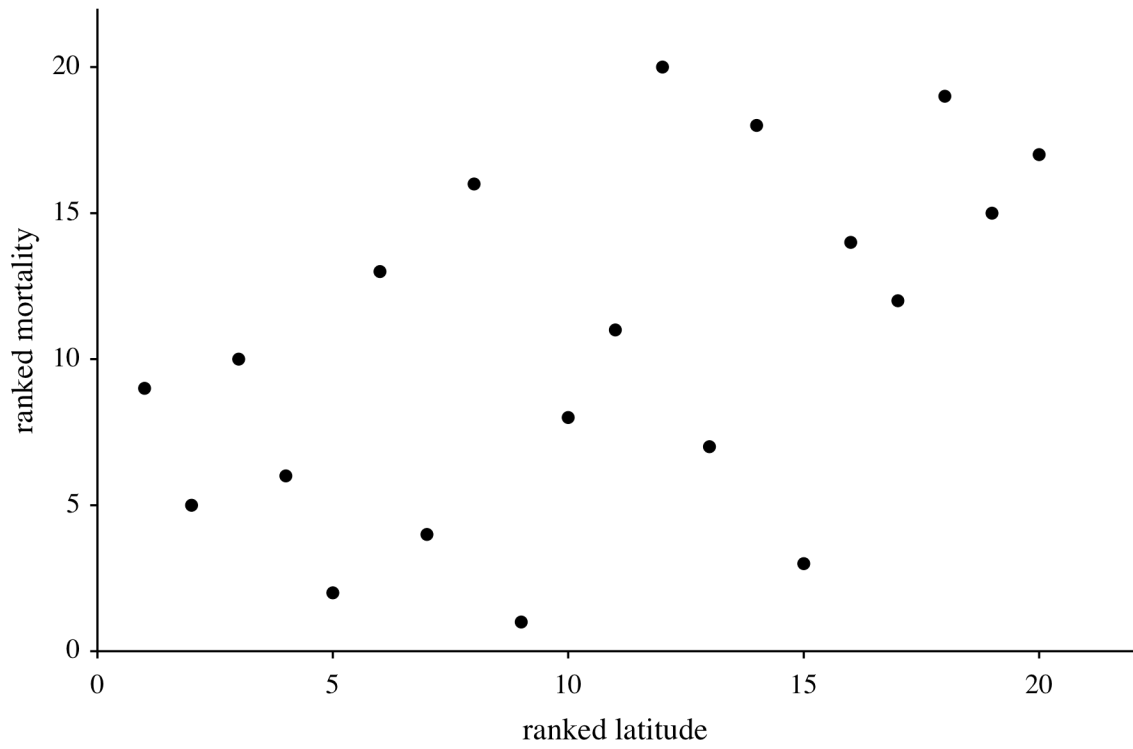


Figure 5.3

CHAPTER 6

CONCLUSION

Bacterial infections lower host fitness. Thus, from the host's perspective, resistance to bacterial infection is an essential trait. Within and across *D. melanogaster* host populations, I observed considerable variation among hosts in how well they survived being singly infected with various species of bacteria. These results suggested not only that there is opportunity for hosts to respond to parasite-mediated selection, but also that resistance is geographically structured across broad spatial scales. However, an important question remained after characterizing this variation: if resistance is so integral to host fitness, what maintains variation in resistance to bacterial infection in natural host populations? I hypothesized that ecological or evolutionary forces were operating to maintain variation among hosts in nature.

In the experiments described here, I tested whether two factors – (1) tradeoffs between resistance and fitness in the absence of infection (Antonovics and Thrall 1994, Kraaijeveld *et al.* 2002, Schmid-Hempel 2003) or (2) interactions between hosts and their environment – led to variation in resistance in natural *D. melanogaster* populations. I found no evidence for evolutionary tradeoffs between resistance and fitness within the *D. melanogaster* population studied. However, differences in resistance among host populations were explained by differences in temperature and bacterial species richness that hosts experienced in nature. These data were consistent with the hypothesis that local environmental differences produce a mosaic of selection pressures (Thompson

1999), and that this mosaic shapes resistance variation across broad spatial scales.

Overall, I conclude that interactions between hosts and their microbial and abiotic environments are a main factor leading to variation in resistance among *D. melanogaster* host populations in nature.

This research is also particularly significant to those studying invertebrate immunity and host-pathogen interactions because it resolves two important issues faced by researchers – (1) what is the best way to assay host fitness post-infection and (2) what bacteria should one use to model host resistance in nature? *D. melanogaster* is currently one of the most widely used systems with which to study invertebrate immunity, and so it was particularly relevant to address these two questions in this model system.

Researchers use direct and indirect measures to assay host fitness following infection.

However, indirect measures, such as bacterial load post-infection, may not actually be correlated with host fitness post-infection. If the researcher's objective is to study host fitness, direct measures, such as survival post-infection, are more appropriate.

Additionally, most studies of resistance in *D. melanogaster* use laboratory cultures of bacteria that hosts are not likely to interact with in nature. Therefore, when we see a response in the host to these laboratory strains of bacteria, it is questionable whether the resistance response being modeled reflects a real world *Drosophila*-bacteria interaction. I characterized the bacterial communities associating with *D. melanogaster* as a first step towards understanding what bacteria these hosts interact with in nature and the bacterial species that might, therefore, shape host resistance. These communities were geographically diverse, demonstrating that *D. melanogaster* interact with many species of bacteria, many of which could be biologically relevant to the host. These data should

be helpful to researchers looking for models of invertebrate immunity that are motivated by the host's natural history.

The *Drosophila*-bacteria system that I developed here has been especially amenable to testing hypotheses related to the maintenance of variation for resistance in nature, as well as hypotheses related to host-parasite coevolution and disease dynamics in general. This system is unique in that it allows researchers to use the wealth of knowledge available on *Drosophila* biology to examine changes in host populations due to parasite pressure. I manipulated this system to explicitly test hypotheses about the evolution and ecology of resistance to bacterial infection in nature. By doing so, I experimentally captured some of the many factors that make host-parasite interactions so variable, exciting, and interesting. This system and the essential question of what maintains variation for resistance in host populations in nature will continue to lead to areas of fruitful future research.

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APPENDIX A

Test statistics produced under a Cox Proportional Hazards Model for each bacterial treatment and each date that host genotypes were infected.

Bacterial species ^a	date tested	<i>d.f.</i> ^b	χ^2 ^c	<i>P</i> ^d
<i>Enterococcus faecalis</i>	6	2	6.39	0.041
	7	2	0.03	0.98
	8	2	1.15	0.56
	9	1	0.04	0.84
	10	3	0.55	0.91
	11	5	13.58	0.018
	12	3	5.87	0.12
	13	3	6.27	0.099
	14	5	17.38	0.0038
	15	5	18.20	0.0027
	16	5	10.78	0.056
	17	2	4.86	0.088
	18	4	2.73	0.60
	19	3	10.07	0.018
	20	2	0.21	0.90
	21	3	11.11	0.011
	23	4	17.02	0.0019
	24	3	8.97	0.030
25	3	0.79	0.85	
<i>Lactococcus lactis</i>	6	3	3.83	0.28
	7	5	9.55	0.089
	8	4	3.76	0.44
	9	9	21.63	0.010
	10	3	2.26	0.52
	11	4	3.94	0.41
	12	3	7.06	0.070
	13	1	0.11	0.74
	14	2	0.28	0.87
	15	3	6.01	0.11
	16	3	12.11	0.007
	17	1	7.03	0.008
	18	3	4.97	0.17

Bacterial species ^a	date tested	<i>d.f.</i> ^b	χ^2 ^c	<i>P</i> ^d
<i>Lactococcus lactis</i>	19	3	0.92	0.82
	20	2	0.58	0.75
	21	3	4.00	0.26
	22	3	9.59	0.022
	23	3	5.65	0.13
	24	1	3.72	0.054
	25	1	3.05	0.081
<i>Pseudomonas aeruginosa</i>	6	3	6.19	0.10
	7	6	13.94	0.030
	8	5	12.68	0.026
	9	1	0.11	0.73
	10	1	0.18	0.67
	11	2	3.14	0.21
	12	2	8.58	0.014
	13	5	20.43	0.001
	14	4	5.28	0.26
	15	2	2.39	0.30
	16	1	1.95	0.16
	17	6	7.73	0.26
	19	3	11.25	0.010
	20	3	5.48	0.14
	21	1	0	0.99
	22	4	7.78	0.10
	23	2	10.31	0.0058
	24	1	2.72	0.61
25	1	8.66	0.12	

^a Species of bacteria that *D. melanogaster* hosts were inoculated with.

^b Degrees of freedom for the Cox Proportional Hazards Model; *d.f.* = *n* – 1, where *n* = number of genotypes tested on that date.

^c χ^2 test statistic produced under the Cox Proportional Hazards Model.

^d *P*-value produced after evaluating the χ^2 test statistic at *n*-1 degrees of freedom.