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The genetic architecture of defence as resistance to and tolerance of bacterial infection in *Drosophila melanogaster*

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Abstract

Defence against pathogenic infection can take two forms: resistance and tolerance. Resistance is the ability of the host to limit a pathogen burden, whereas tolerance is the ability to limit the negative consequences of infection at a given level of infection intensity. Evolutionarily, a tolerance strategy that is independent of resistance could allow the host to avoid mounting a costly immune response and, theoretically, to avoid a co-evolutionary arms race between pathogen virulence and host resistance. Biomedically, understanding the mechanisms of tolerance and how they relate to resistance could potentially yield treatment strategies that focus on health improvement instead of pathogen elimination. To understand the impact of tolerance on host defence and identify genetic variants that determine host tolerance, we defined genetic variation in tolerance as the residual deviation from a binomial regression of fitness under infection against infection intensity. We then performed a genomewide association study to map the genetic basis of variation in resistance to and tolerance of infection by the bacterium *Providencia rettgeri*. We found a positive genetic correlation between resistance and tolerance, and we demonstrated that the level of resistance is highly predictive of tolerance. We identified 30 loci that predict tolerance, many of which are in genes involved in the regulation of immunity and metabolism. We used RNAi to confirm that a subset of mapped genes have a role in defence, including putative wound repair genes *grainy head* and *debris buster*. Our results indicate that tolerance is not an independent strategy from resistance, but that defence arises from a collection of physiological processes intertwined with canonical immunity and resistance.

Keywords: *Drosophila*, *DGRP*, genomewide association study, host-pathogen interactions, immunity, resistance, tolerance

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Introduction

To deal with infection, a host must control pathogen burden while maintaining health and fitness. The collection of these strategies is known as defence. Pathogen control strategies that either kill the pathogen or prevent it from proliferating are known as resistance, while processes that reduce the decline of health or fitness per parasite burden are known as tolerance (Strauss & Agrawal 1999; Råberg *et al.* 2009). Host resistance mechanisms may apply selective pressure on the pathogen to overcome this defence, whereas tolerance strategies are predicted to have a neutral or positive impact on pathogen fitness and thereby potentially avoid a co-evolutionary arms race (Boots & Bowers 1999; Roy & Kirchner 2000; Miller *et al.* 2006). Understanding the patterns of natural genetic variation in tolerance, as well as the underlying biological processes that promote tolerance, will provide a better understanding of the evolutionary trajectories of host–pathogen interactions and could

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yield novel sustainable therapeutic strategies. In this study, we performed a genomewide association study (GWAS) using 172 inbred lines from the *Drosophila melanogaster* Genetic Reference Panel (DGRP) to quantify resistance and tolerance and to establish the genetic architecture of these traits.

To fully understand the contributions of resistance and tolerance to defence, the two strategies should be measured independently from one another. Resistance is measured as either immune system activity or pathogen burden after infection (Baucom & de Roode 2011). Tolerance can be estimated as either 'point tolerance' or 'slope tolerance'. Slope tolerance is measured as a reaction-norm or slope, where pathogen burden is plotted on the *x*-axis and health or fitness is plotted on the *y*-axis (Hochwender *et al.* 2000; Råberg *et al.* 2007, 2009). Point tolerance is defined as host health or fitness at a given pathogen burden (Ayres & Schneider 2008; Baucom & de Roode 2011). Slope tolerance estimates are conceptually pleasing because experimental manipulation of pathogen burden could potentially allow tolerance to be estimated independently of resistance, although the two traits may be linearly related across only a limited range of infection severities (Louie *et al.* 2016). In this study, we define a new measure to estimate genetic variation in tolerance as a point estimate of genotypic deviation in tolerance across a population of *D. melanogaster* and compare that to slope tolerance in a subset of genotypes.

Tolerance and resistance are predicted to have divergent impacts on the evolution of host–pathogen interactions. In theory, costly resistance alleles that negatively impact pathogen fitness may be maintained as balanced polymorphisms, oscillating in a frequency-dependent manner with pathogen prevalence or virulence alleles (e.g. Stahl *et al.* 1999). Alternatively, tolerance alleles may drive an increase in pathogen prevalence, resulting in rapid fixation of these alleles (Boots & Bowers 1999; Roy & Kirchner 2000; Miller *et al.* 2006). These models assume that tolerance and resistance act independently. However, any genetic trade-off between resistance and tolerance could constrain the evolutionary trajectories of defence alleles. Interest in tolerance by both plant and animal biologists was piqued by empirical studies demonstrating a negative relationship between resistance and tolerance in morning glory and in mouse (Fineblum & Rausher 1995; Råberg *et al.* 2007). However, a meta-analysis of tolerance and resistance in plants found that a negative relationship may not be a general phenomenon (Leimu & Koricheva 2006).

Although previous work has shown the genetic basis of natural variation in resistance to bacterial pathogens in *Drosophila* (e.g. Lazzaro *et al.* 2004, 2006; Felix *et al.* 2012; Unckless *et al.* 2015), little is known about the underlying genetic basis of variation in tolerance. Weinig *et al.* (2003) measured resistance and tolerance to rabbit herbivory in *Arabidopsis*, and although several resistance QTL were identified, no tolerance QTL were found despite having power to detect alleles that explained >5% of variance (Weinig *et al.* 2003). Additionally, a recent study on tolerance of chronic HIV infection in humans did not identify any loci in a genomewide association study after multiple test corrections, although the authors did find that the HLA-B genotype influenced tolerance using a candidate gene approach (Regoes *et al.* 2014). The lack of QTL at a genomewide level has led to the hypothesis that the genetic architecture of tolerance is composed of many loci with individually small effects (Weinig *et al.* 2003).

Functional studies using mutant and RNAi knockdown *Drosophila* have shown that genes involved in protection of tissues and regulation of immunity and metabolic processes can mechanistically determine tolerance of infection, but it is unknown whether these genes harbour polymorphisms that contribute to natural variation in tolerance. Laboratory mutations in immune genes involved in melanization (Ayres & Schneider 2008), phagocytic encapsulation (Shinzawa *et al.* 2009), insulin signalling (Dionne *et al.* 2006), feeding behaviour (Ayres & Schneider 2009) and regulation of JAK-STAT (Merkling *et al.* 2015) alter tolerance in *D. melanogaster*. Based on these studies, we hypothesized that polymorphisms in genes involved in regulation of immunity and metabolic processes would predict tolerance of infection. For instance, regulatory genes may influence tolerance by tightly regulating the immune response to avoid immunopathology while simultaneously regulating resource allocation and tissue repair, thus allowing for adequate pathogen control while maintaining and returning to homoeostasis.

In this study, we measured tolerance and resistance across a single population of inbred *D. melanogaster* and used a genomewide mapping approach to identify single-nucleotide polymorphisms (SNPs) that predict variation in tolerance. We mapped polymorphisms in genes involved in regulation of gene expression, metabolism, immunity and other processes as predictive of phenotypic variation in tolerance. We showed that tolerance and resistance are positively correlated and that tolerance estimates are dependent on host resistance across varying levels of infection severity. We found that resistance and tolerance are nonindependent traits and that they may be linked through shared biological processes. We have identified novel tolerance genes and confirmed their effects using RNAi.

Material and Methods

Drosophila and bacterial stocks

One hundred and seventy-two inbred lines from the *Drosophila* Genetic Reference Panel (DGRP) were

phenotyped for the genomewide association study. The DGRP is a set of fully sequenced, inbred lines collected from a single population in Raleigh, NC, USA (Mackay *et al.* 2012; Huang *et al.* 2014). A list of lines included in the GWAS for each trait can be found in Table S1 (Supporting information). Six lines that spanned low, medium or high levels of resistance and had phenotypically extreme (high or low) genotypic deviation in tolerance were chosen for further investigation (RAL-801, RAL-26, RAL-882, RAL-359, RAL-138 and RAL-714; Fig. S1, Supporting information). RNAi stocks from the Vienna Drosophila Resource Center KK (phiC31 generated) and GD (P-element generated) libraries (Dietzl *et al.* 2007) were used in the functional testing of candidate genes implicated in the GWAS (Table S2, Supporting information). All flies were maintained at 24 °C on a 12:12 hour light:dark cycle on a rearing medium of 8.3% glucose, 8.3% Brewer's yeast, 1% agar, with 0.04% phosphoric acid and 0.004% propionic acid added to prevent microbial growth in the diet.

Bacterial Infection

Five- to nine-day-old mated female flies were infected with the Gram-negative bacterium *Providencia rettgeri* (strain Dmel). This strain of *P. rettgeri* was isolated from the hemolymph of wild-caught *D. melanogaster* and hence can be consider a natural pathogen (Juneja & Lazzaro 2009; Galac & Lazzaro 2011). Infection into the thorax of *D. melanogaster* results in bacterial proliferation for at least 24 h postinoculation. Some individuals die from the high bacterial loads sustained, corresponding to mortality from acute infection within 72 h. Bacterial load decreases in surviving individuals, resulting in a chronically persistent infection. For this study, all bacterial load measurements were taken at 20 h postinfection, prior to any mortality (Howick & Lazzaro 2014). For each day of infection, an overnight culture was started from a single bacterial colony and was grown overnight in liquid LB at 37 °C with shaking. For the primary mapping experiment and the RNAi knockdown experiments, the overnight culture was diluted in LB to A_{600} of 1.0 ± 0.1. Female flies were infected by pricking the thorax with a 0.15 mm dissecting pin dipped in the dilute overnight culture of *P. rettgeri* (Khalil *et al.* 2015). For experiments that measured defence across infection doses, flies were injected in the thorax with 23 nL of a dilute overnight culture at A_{600} of 0.1, 0.01 and 0.001 (Khalil *et al.* 2015). As a handling and treatment control, flies were sterilely wounded. When the pinprick method was used for infection, sterile wounding was performed by pricking with an aseptic needle. For the multidose experiment using the injector, the wounding control was injected with 23 nL of sterile PBS. In all experiments, 40 flies were infected for each infection treatment on each experimental day and housed in groups of 20.

Survival and bacterial load assay

Approximately 20 h after infection, groups of three flies were homogenized in 1 mL of PBS using a FastPrep-24 homogenizer (MP Biomedicals). Homogenates were diluted 1:100 or 1:1000 in PBS prior to plating. These diluted homogenates were then plated onto LB agar using a WASP 2 spiral plater (Microbiology International, Bethesda, MD, USA). Plates were incubated overnight at 37 °C. Resulting colonies (colony forming units, CFU) were counted using the ProtoCOL plate counting system (Microbiology International) to estimate the number of viable bacteria per pool of flies.

In the primary mapping experiment, survival of infection was estimated for each DGRP line at a single time point at 2 days after inoculation. The number of dead and living flies was counted to estimate the proportion of flies surviving the infection. This time point corresponded to the greatest mortality from the acute phase of *P. rettgeri* infection (Howick & Lazzaro 2014). In subsequent experiments, including the multiple-dose experiment and the RNAi knockdown experiments, survival was measured once a day for 5 days after inoculation, with the flies transferred to fresh medium every 2–3 days. On the fifth day, the flies still alive were counted and censored from the experiment. As a control, 20 females were wounded and survival was monitored in the same fashion. Three independent biological replicates were performed for each experiment.

Genomewide association study

In total, approximately 19 000 flies from 172 lines of the DGRP were infected for phenotyping. Data were collected across three independent experimental blocks, each of which includes the entire set of lines infected over a span of 8 days. On each day, 20–24 DGRP lines were randomly assigned without replacement to be infected. For each DGRP line on each day, 40 female flies were infected and housed in groups of 20. At the time of infection, one group of 20 was arbitrarily assigned to the bacterial load assay, while the other was used for the survival assay.

To estimate the genotypic deviation in tolerance, a mixed model was built using the proportion of flies surviving the infection as the response variable and the bacterial load as a covariate in the model to control for the level of resistance for each *Drosophila* genotype. For the three traits mapped (bacterial load sustained, host survival of infection and genotypic deviation in

tolerance), the data were corrected for experimental variables (random factors: Block: k = 1–3; Day: l = 1–24) and whether the lines carried the endosymbiotic bacteria *Wolbachia pipientis* (fixed factor: Wolbachia: j = 1, 2) using the LME4 package in R (Bates *et al.* 2015; R Core Team 2016). The models for each trait are given below:

Bacterial load:

$$\log_e(CFU)_{jkl} = \mu + \text{Wolbachia}_j + \text{Block}_k + \text{Day}(\text{Block}_k)_l + \varepsilon_{jkl}.$$

Host survival:

$$\text{ProportionAlive}_{jkl} = \mu + \text{Wolbachia}_j + \text{Block}_k + \text{Day}(\text{Block}_k)_l + \varepsilon_{jkl},\ \text{Family=binomial}.$$

Genotypic deviation in tolerance:

$$\text{ProportionAlive}_{jkl} = \mu + \text{Load} + \text{Wolbachia}_j + \text{Block}_k + \text{Day}(\text{Block}_k)_l + \varepsilon_{jkl},\ \text{Family=binomial}.$$

The residuals were extracted from each model, and the predicted mean from each line was used for association testing. These values can be found in Table S1 (Supporting information). Mapping was performed in PLINK version 1.9 (Purcell *et al.* 2007) using the publically available genome sequences of the 172 DGRP lines we measured (Mackay *et al.* 2012; Huang *et al.* 2014). Approximately 2.5 million SNPs were used in the study with a minimum minor allele frequency of 0.05 required for inclusion in the study. A nominal P-value of $P < 10^{-5}$ was used as an initial significance threshold.

Gene ontology analysis

To test for enrichment of functional groups of genes among our mapped hits, gene ontology was performed using GOWINDA, which accounts for an unequal probability of sampling genes as a consequence of gene length (Kofler & Schlötterer 2012). Analysis was performed using both the GOslim gene set (Adams *et al.* 2000) and the FuncAssociate2 gene set (Berriz *et al.* 2009). The GOslim set contained fewer and broader terms than the FuncAssociate2 set. GOWINDA was run using version 5.46 of the *D. melanogaster* genome annotation with 100 000 simulations in gene mode that conservatively assumes complete linkage disequilibrium of all SNPs, where a 'gene' was defined as all SNPs within 2000 bp of an annotated gene. The FuncAssociate2 set was run with a minimum gene set for each category of 10. SNPs that we identified as significantly associated with mapped phenotypes at $P < 10^{-5}$ were included in the analysis.

Functional testing of candidate genes

To unbiasedly test whether the genes bearing the mapped SNPs played a functional role in defence, we used RNAi to ubiquitously knockdown all candidate tolerance genes containing a SNP that mapped with $P < 10^{-5}$ within coding or intronic regions. To test the proportion of arbitrary genes that would alter defence when knocked down, we randomly selected 10 control genes from the annotated list of all *Drosophila* genes and ubiquitously knocked down these genes. To knockdown each gene, females carrying the RNAi construct (Table S2, Supporting information) were crossed to males from the driver line (Actin5C-Gal4/CyO), which ubiquitously expresses Gal4. A small number of genes were also tested using the c564-Gal4 driver line, which drives expression primarily in the fat body and hemocytes. A list of all genes tested can be found in Table S2 (Supporting information). Bacterial load and survival of the knockdown genotypes were compared to the background genotype for that knockdown line crossed the driver line. Progeny were sorted 2–3 days prior to infection and kept in groups of 20 females and five males of the same genotype. Males were discarded at the time of infection.

Results

Defining tolerance as a genotypic deviation

Existing conceptual definitions of tolerance are not suitable for mapping genetic variation because they are either biologically unrealistic (e.g. assume a linear relationship between pathogen burden and fitness) or are experimentally untenable (e.g. require multiple measurements of each genotype across a range of infection doses). To overcome these limitations, we created a new measure of genetic variability in tolerance, which we call 'genotypic deviation in tolerance'. The premise of this approach is that the entire data set is used to predict a general, nonlinear relationship between host fitness and sustained pathogen load after injection of a uniform initial dose. The degree to which individual genotypes depart from this relationship is the genotypic deviation in tolerance.

To estimate genotypic deviations in tolerance, we first determined the pathogen load sustained (Fig. 1A) and proportion of flies surviving the infection (Fig. 1B) for each genotype in the study. We found these estimates to be highly repeatable across biological replicates

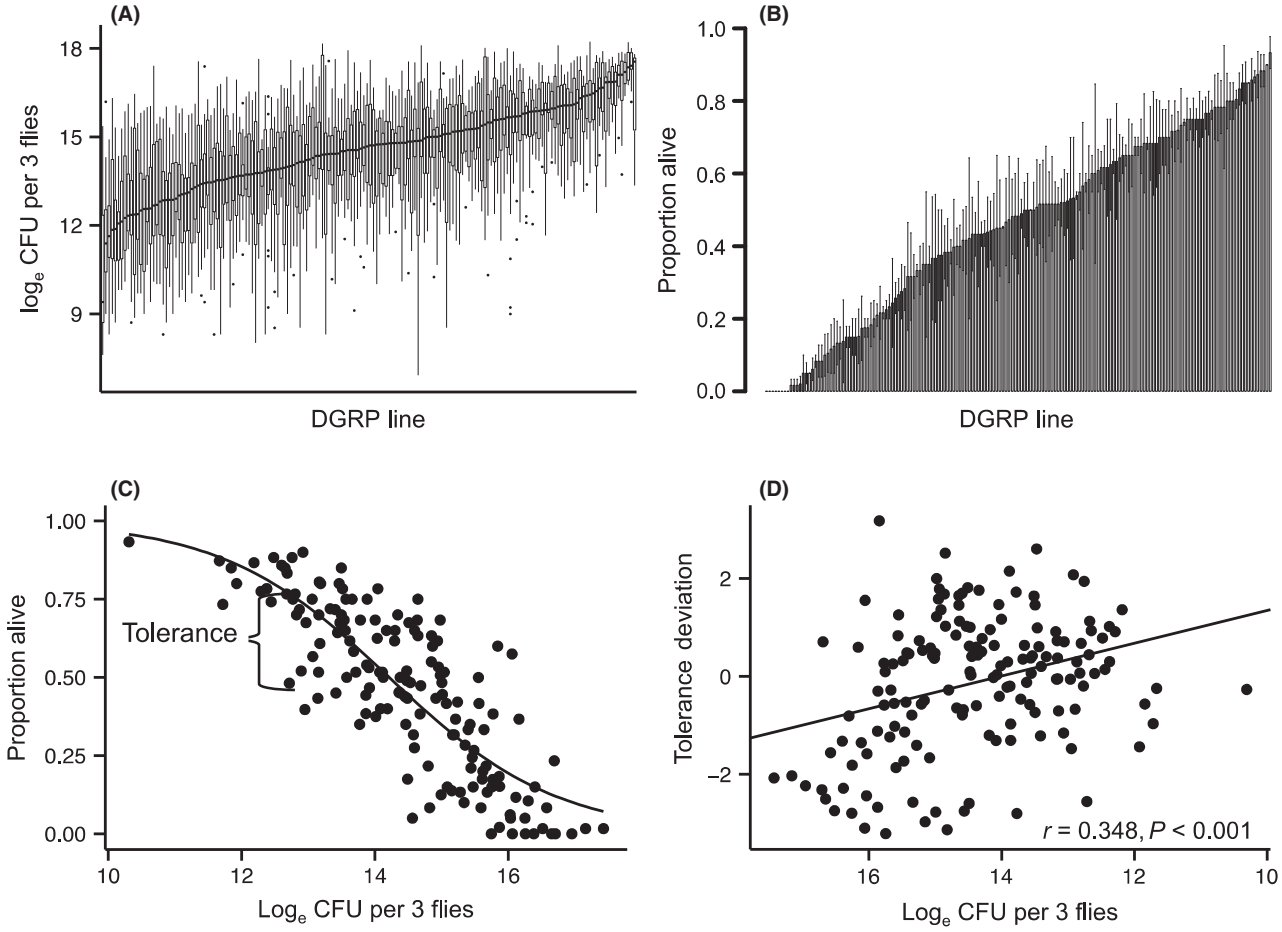

Fig. 1 Distribution of bacterial load (A) and survival (B) across the DGRP; lines are sorted based on phenotypic value for each trait. The bacterial load in A is represented as a box and whiskers plot for each DGRP line where the box represents the first and third quantile, and the solid line represents the median. The error bars in B represent one standard error from the mean. (C) The mean value of survival for each line plotted against the mean bacterial load. The curve represents the fitted model with a binomial distribution, representing the species tolerance across pathogen burdens. The vertical distance of each point from the function is the genotypic deviation in tolerance. (D) Genotypic deviation in tolerance plotted against mean bacterial load for each line.

($P < 1 \times 10^{-9}$, Fig. S2, Supporting information). We then estimated a function describing the expected survival of *D. melanogaster* for *P. rettgeri* infection across the range of pathogen burdens experienced, incorporating the bacterial load and survival data from all genotypes measured. The vertical deviation of each measured genotype from that inferred relationship is our estimate of variation in tolerance. This definition allowed us to measure the departure of a given genotype from the overall population tolerance curve (Fig. 1C).

We found significant genetic variation for survival, resistance and the genotypic deviation in tolerance ($P < 0.0001$). Bacterial loads ranged from a mean of 3.00×10^4 bacteria per pool of three flies in the most resistant line to 3.69×10^7 bacteria per three flies in the least resistant line (Fig. 1A). Eight lines had no flies surviving the infection, whereas the line with highest survival had 93% of flies surviving (Fig. 1B). There was a strong negative relationship between bacterial load and proportion of hosts surviving the infection ($r = -0.852$, $P < 0.0001$, Fig. 1C). There was a positive relationship between the tolerance deviation and the proportion of hosts surviving the infection ($r = 0.772$, $P < 0.0001$, Fig. S3, Supporting information). There was a positive relationship between resistance and genotypic deviation in tolerance ($r = 0.348$, $P < 0.0001$: Fig. 1D). This positive relationship remained significant when the analysis was restricted to lines that fell at an intermediate level of resistance ($r = 0.191$, $P = 0.029$, Fig. S4, Supporting information), suggesting this phenomenon was not driven by lines that fell at the phenotypic extremes. This observed positive relationship suggests that we have not fully

accounted for the effect of resistance in our estimation and this is likely an issue with our statistical estimation as well as the underlying biology of the system.

To understand whether our genotypic deviation in tolerance accurately represented tolerance across infection severity, we infected six DGRP lines that had high or low genotypic deviation in tolerance and experienced a range of bacterial loads (Fig. S1A, Supporting information). We infected these six lines with three inoculation doses, introducing approximately 1×10^1, 5×10^2 or 1×10^4 bacteria per fly – a 1000-fold range in inoculation dose (Fig. S1B, Supporting information). Across these treatments, bacterial loads at 20 h after inoculation ranged from 6.43×10^4 to 6.94×10^7 (Fig. 2B) and survival of infection at 5 days postinoculation ranged from 89% to 0% (Fig. 2A). We found that resistance level was a stronger predictor of survival of infection than inoculation dose or genotype (bacterial load: $P < 2.2 \times 10^{-16}$, dose: $P = 0.042$, genotype: $P = 3.03 \times 10^{-7}$) and that bacterial load was determined much more strongly by genotype ($P < 2.2 \times 10^{-16}$) than by initial infection dose ($P = 6.295 \times 10^{-9}$). In other words, the lines sustained an approximate pathogen burden that was stereotypical for that genotype, regardless of initial infection dose, and the genetically fixed pathogen burden was in turn predictive of survival (Fig. 2C). We note that this pattern renders tolerance estimates based on infection dose irrelevant and probably inaccurate (Fig. 2D), a point we return to in 'Discussion'.

The genetic architecture of resistance and tolerance of infection

To identify candidate genes that predict defence against bacterial infection, we performed a genomewide association study on our three traits of interest (survival of infection, bacterial load and genotypic deviation in tolerance) (Fig. S5, Supporting information). We identified 63 SNPs in 49 genes that predicted survival of infection (Table S3, Supporting information), 25 SNPs in 20 genes that predicted pathogen load (Table S4, Supporting information) and 30 SNPs in 25 genes that predicted genotypic deviation in tolerance (Table 1). SNPs that explained variation in survival of infection are much more likely than random to fall in or around genes. We find that 24.36% of the SNPs mapped for survival fall within coding regions compared to only 9.71% of the of SNPs genomewide ($\chi^2 = 12.56$, $P = 3.94 \times 10^{-4}$) and 25.64% lie within 5000 bp of an annotated gene relative to 14.98% of genomewide SNPs ($\chi^2 = 4.08$, $P = 0.04345$). SNPs that explained variation in bacterial load were slightly enriched in coding regions ($\chi^2 = 3.0373$, $P = 0.08137$). There was no significant enrichment of any site class for SNPs that explained variation in tolerance relative to the rest of the genome.

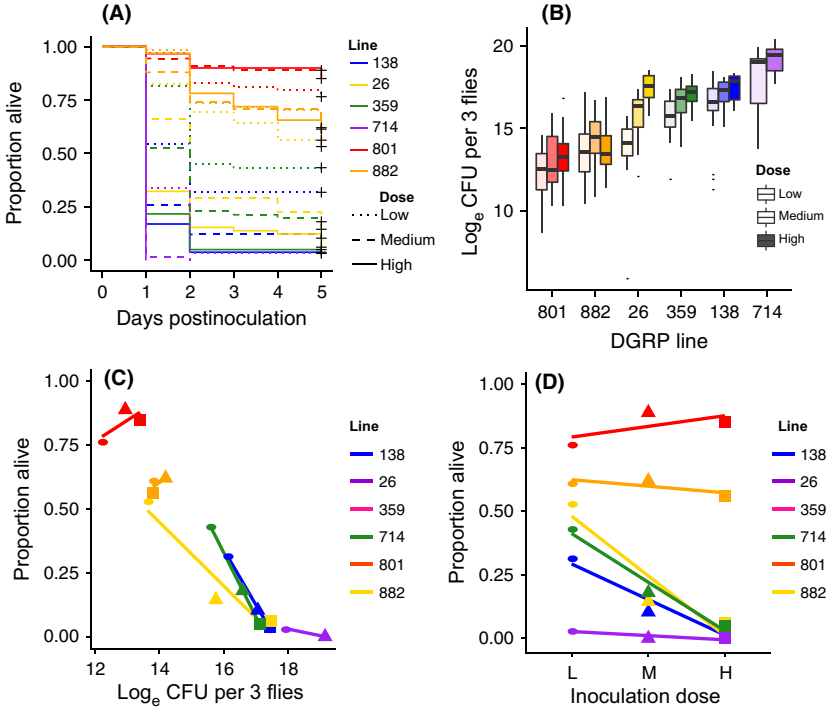

Fig. 2 Genotype and resistance predict tolerance. Six DGRP lines were infected with three inoculation doses low (L), medium (M) and high (H), corresponding to approximately 10^1, $10^{2.5}$ or 10^4 bacteria per fly. (A) Survival was estimated daily for 5 days after inoculation for each line at each dose. Thickness of line dash corresponds to strength of dose, with solid lines representing the highest dose and dotted lines represented the lowest dose. (B) Bacterial load for each line at each dose measured 20 h after inoculation. Shading of boxes represents the strength of the dose with the highest dose being the darkest box. No data were obtained for line 714 at the high dose because all flies were dead by 20 h postinoculation. (C) The proportion surviving 5 days after inoculation plotted against the mean bacterial load 20 h after infection at low (circle), medium (triangle) and high (square) infection doses. (D) Proportion surviving the infection plotted against initial infection dose.

Table 1 SNPs that predicted variation in tolerance to infection. There were 30 SNPs that predicted variation in tolerance at a *P*-value of $<10^{-5}$. At a nominal significant threshold, several of these also appeared to predict survival of infection, but most did not alter bacterial load

SNP	Gene	Site class	Tolerance *P*-value	Survival *P*-value	Load *P*-value
X:21,581,423	none annotated	NA	7.81E-07	4.94E-04	2.00E-01
2R:10,563,254	psq	Intron	9.72E-07	1.14E-02	9.16E-01
2R:5,128,972	CR43256	Splice site acceptor	1.39E-06	1.21E-04	9.70E-02
X:12,140,511	Ten-a	Intron	1.85E-06	1.33E-03	1.78E-01
2R:5,128,902	CR43256	Intron	2.38E-06	2.35E-04	1.15E-01
2L:533,659	ush	Intron	2.50E-06	1.49E-05	2.19E-02
2R:17,840,479	grh	Synonymous	3.51E-06	3.59E-04	5.77E-02
2R:5,332,144	sxc	Downstream	3.72E-06	1.01E-05	3.32E-03
2L:16,628,423	CG42389	Intron	4.22E-06	6.90E-05	1.60E-02
2R:21,323,134	CG30394/dom	5′ UTR/Upstream	4.88E-06	8.25E-05	3.56E-02
3R:21,510,537	C15	Intron	4.96E-06	9.43E-05	1.68E-02
2L:17,229,914	beat-IIIc	Intron	5.14E-06	1.69E-04	7.50E-02
3L:5,645,490	Blimp-1	Intron	5.32E-06	2.45E-05	5.09E-03
2R:17,988,140	dpr13	Intron	5.66E-06	2.44E-04	4.39E-02
2R:14,648,549	CG10139	Downstream	5.90E-06	3.47E-06	1.53E-03
3L:18,601,807	CG4174	Nonsynonymous	5.91E-06	2.88E-03	5.30E-01
2R:15,517,660	Khc-73	3′ UTR	6.15E-06	4.96E-06	1.92E-03
2L:16,578,056	CG42389	Intron	6.52E-06	1.12E-02	8.89E-01
2R:14,681,641	mspo	Intron	6.57E-06	1.15E-03	1.30E-01
2R:5,126,594	gus	Intron	6.58E-06	8.98E-04	2.25E-01
2L:1,766,281	none annotated	NA	8.31E-06	1.70E-04	4.38E-02
2L:8,896,128	CG42713	Downstream	8.49E-06	8.95E-04	4.16E-01
2L:8,896,128	CG34398	Upstream	8.49E-06	8.95E-04	4.16E-01
2L:15,772,825	CG31826	Intron	8.66E-06	8.57E-03	7.67E-01
2L:20,997,569	CG42238	Intron	8.98E-06	1.87E-03	2.61E-01
3R:7,586,053	CG34127	Intron	9.00E-06	8.93E-06	2.17E-03
2L:1,766,283	none annotated	NA	9.17E-06	3.60E-04	8.94E-02
2L:8,895,939	CG34398/CG42713	Upstream/Downstream	9.44E-06	5.36E-04	3.37E-01
3L:8,771,126	Fhos	Intron	9.54E-06	8.71E-04	1.42E-01

Although only five SNPs overlapped between our mapped phenotypes (Table S5, Supporting information), there was a positive correlation between effect sizes of significant SNPs across the traits. This was seen most strongly in SNPs that predicted survival of infection. The effect sizes of the alleles that significantly predicted survival were positively correlated in magnitude and direction with the effect size of those alleles on both resistance and the genotypic deviation in tolerance (resistance: $r = 0.993$, $P < 2.2 \times 10^{-16}$; tolerance deviation; $r = 0.990$, $P < 2.2 \times 10^{-16}$; Fig. 3). This was also seen to a slightly lesser degree for the effect sizes of the SNPs which significantly predicted resistance or the genotypic deviation in tolerance when compared to mapped effect sizes on the other traits (resistance SNPs vs. survival: $r = 0.980$, $P < 2.2 \times 10^{-16}$, vs. tolerance: $r = 0.626$, $P = 8.2 \times 10^{-4}$; tolerance SNPs vs. survival: $r = 0.966$, $P < 2.2 \times 10^{-16}$, vs. resistance: $r = 0.700$ $P = 3.32 \times 10^{-5}$; Fig. 3). This general positive correlation in effects implies that shared SNPs influence other defence traits even when the nominal significance threshold is not met, either because of pleiotropy or because the measured traits are inherently interdependent.

Genes that harbour tolerance SNPs are involved in regulation of gene expression

We performed gene ontology analysis to understand whether the genes we identified as harbouring allelic variation for our mapped traits at $P < 10^{-5}$ were enriched for specific biological processes. Genes that harbour SNPs that explained variation in survival were enriched for the GO categories 'defence response' and 'response to stress'. We found enrichment for genes involved in 'defence response', 'protein kinase activity' and 'structural molecule activity' in predicting bacterial load. In contrast to the survival and load enrichments, we found enrichment in genes involved in the 'nucleus', 'proteinaceous extracellular matrix' and 'endoplasmic reticulum' (Table 2) among those that harbour

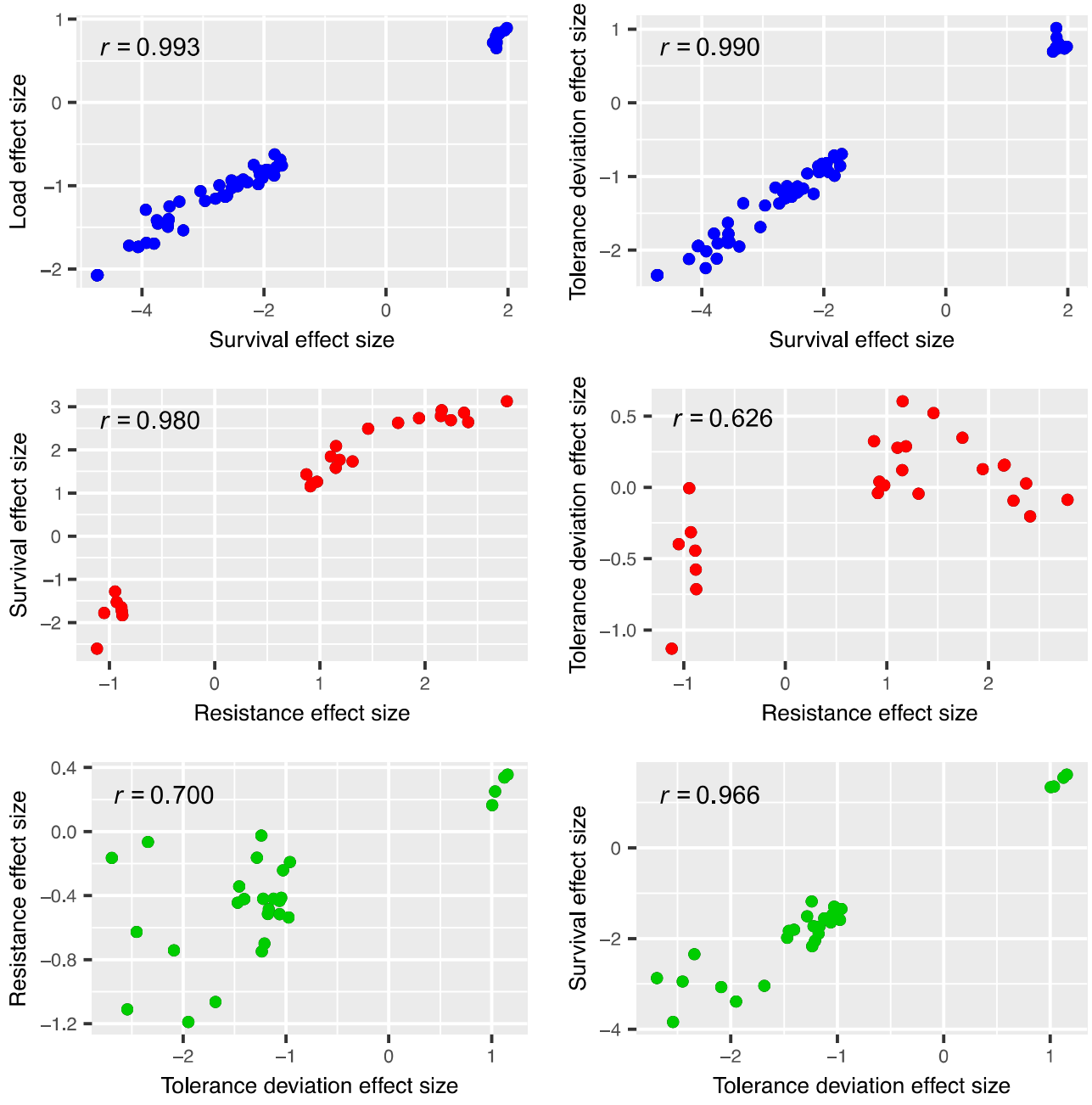

Fig. 3 Positive correlation between effect sizes across traits. The effect sizes of each SNP that mapped significantly for each trait are plotted against the absolute value of the effect size of that SNP (regardless of significance level) on the other two mapped traits. The blue points represent SNPs that significantly predicted survival. The red points represent SNPs that significantly predicted resistance. The green points represent SNPs that significantly predicted the genotypic tolerance deviation.

tolerance SNPs. When the analysis was repeated using a more refined set of GO terms, these genes were enriched 'negative regulation of gene expression' and 'negative regulation of immune system process', as well as other categories. In contrast, candidate resistance and survival genes were involved in the 'antibacterial humoral response' and other categories (Tables S6–S8, Supporting information). Importantly, none of these terms survived FDR correction for multiple tests. Relaxing the *P*-value threshold of the GWAS to $P = 10^{-4}$ only slightly changed the GO results and did not provide further biological insight (data not shown). The lack of significance in GO enrichment is probably because GO analysis of GWAS results assumes an infinitesimal model of quantitative genetics, where many genes in each relevant GO category each make small but

Table 2 Nominally significant GOslim Gene Ontology terms from for survival of infection, bacterial load and tolerance

Trait	GO term	*P*-value
Survival	Defence response	0.0027
	Response to stress	0.0061
Bacterial load	Protein kinase activity	0.0058
	Defence response	0.0125
	Structural molecule activity	0.0410
	Protein modification process	0.0482
Tolerance	Proteinaceous extracellular matrix	0.0208
	Endoplasmic reticulum	0.0344
	Nucleus	0.0423

detectable contributions to overall phenotypic variation. This model is unlikely to hold in experimental practice because (i) trait variation is likely to be determined by a finite number of genes, with very few causal genes representing each functional category, and (ii) if the observed phenotypic variance were distributed among very many causal genes in few GO categories, the proportion of variance explained by each individual gene would become so small as to be undetectable in a study the size of ours and the GO analysis would be underpowered.

Genetic variants that alter tolerance not transcriptionally induced under infection

To test whether any of the genes identified in the GWAS were induced under infection conditions, we compared each gene list with published transcriptomic data from *D. melanogaster* Canton-S mated females infected with *P. rettgeri* (Short & Lazzaro 2013). We found meaningful overlap between genes that contained SNPs that predicted survival and bacterial load and the 186 genes that had altered expression under infection conditions in the microarray study. We found that six of 49 genes that harboured SNPs that predicted survival of infection were induced under infection conditions (*Dpt*, *DptB*, *CG30098*, *TrpA1*, *IM23* and *IM1*). Three of 20 genes that contained resistance SNPs had altered expression under infection. *Dpt* and *DptB* had increased expression, and *dsb* was repressed after infection. Many of the genes that were modulated under infection are previously characterized immune genes. This is not surprising because, historically, the major humoral immune response pathways in *Drosophila* have been defined by genes that transcriptionally respond to infection (De Gregorio *et al.* 2002). We found that none of our 25 genes that contained SNPs predicting genotypic deviation in tolerance were transcriptionally altered by infection. The lack of transcriptional modulation of candidate tolerance genes suggests that tolerance is not determined by induced expression of effector molecules in the same manner as resistance, but may instead be determined by the state of the host at the time of infection. Alternatively, mapped genetic variation in transcription factors could alter tolerance by regulating responsive genes that do not themselves harbour tolerance-altering polymorphisms.

Functional testing of candidate defence genes

Functional studies have characterized the main resistance pathways in *Drosophila* (Lemaitre & Hoffmann 2007); however, we are just beginning to understand how a host tolerates an infection. To confirm that the candidate genes mapped for variation in tolerance played a functional role in defence, we ubiquitously knocked down expression of each gene using RNAi. Out of the 25 tolerance genes tested, 10 of the knocked down genotypes produced viable offspring. Out of those 10 genes, five altered defence by either changing survival or load after infection relative to the control. To test the proportion of arbitrary genes that would alter defence when knocked down, we randomly selected 10 genes from the annotated list of all *Drosophila* genes and measured bacterial load and host survival. Out of these 10 genes test, five produced viable offspring when ubiquitously knocked down and only one (*Rbp9*) significantly altered survival of infection (Cox proportional hazard model: $P = 0.012$, Fig. 4). Our mapped genes that gave defence phenotypes when disrupted with RNAi were *mspo*, *fhos*, *CG4174*, *gus* and *beat-IIIc* (Fig. 4, Fig. S6, Supporting information). Knockdown of all five of these genes resulted in a change in tolerance: survival of infection or bacterial load was altered without the corresponding change in the other trait. Knockdown of *mspo* and *fhos* decreased survival of infection (*mspo*: $P = 0.022$, *fhos*: $P = 0.009$). Knockdown of *beat-IIIc* increased survival of infection ($P = 0.005$). Knockdown of *CG4174* and *gus* decreased bacterial load (increased resistance) (*CG4174*: $P = 0.003$, *gus*: $P = 0.015$). This demonstrates that the genes we have identified can alter tolerance through perturbation of resistance levels without a corresponding change in survival or through altering survival of infection without a change in resistance.

We additionally selected a small number of mapped genes for further investigation because we hypothesized they might play a role in wound healing or defence based on previous studies (Mace *et al.* 2005; Muratoglu *et al.* 2006; Gordon *et al.* 2008; Han *et al.* 2014). We knocked down expression of *u-shaped* (*ush*), *grainyhead* (*grh*), *debris buster* (*dsb*) and *CG30098* using the c564 driver, which is expressed primarily in the fat body and

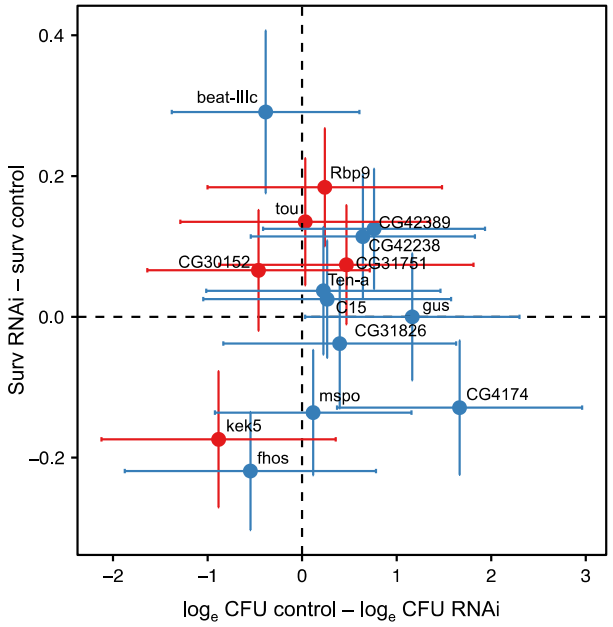

Fig. 4 Functional testing of candidate tolerance genes identified in GWAS. Ten genes that had SNPs that explained genetic variation in tolerance to infection were tested to determine whether they functionally altered defence. We found that five of these 10 genes (*beat-IIIc*, *CG4174*, *fhos*, *mspo* and *gus*) altered either survival or bacterial load. One of five randomly selected control genes also altered survival of infection (*Rbp9*). Blue points are the candidate tolerance genes, and red points are the control genes. The *x*-axis is the natural log of the bacterial load from the knockdown line subtracted from the control for that line (driver crossed to background). A higher value represents an increase in resistance with knocked down expression of the targeted gene. The *y*-axis represents the difference in survival between the knockdown line and the control. A high value represents higher survival in the knockdown flies. The dashed lines represent the level of survival and bacterial load of knockdown control (background genotype without the RNAi construct crossed to the driver line). The error bars represent one standard error from the mean.

hemocytes. *ush* and *grh* were tolerance GWAS hits, *dsb* was a resistance GWAS hit, and CG30098 was a survival of infection GWAS hit. Out of these four genes, three displayed tolerance phenotypes (*ush*, *grh* and *dsb*). Knockdown primarily in the fat body and hemocytes caused a major decrease in survival of the knockdown flies ($P < 0.001$), but no significant change in bacterial load ($P > 0.05$, Fig. 5).

Discussion

We found that bacterial load was strongly predictive of survival of infection. This was seen across all the *D. melanogaster* lines tested and within the phenotypically extremes lines, where fitness under infection was much more strongly predicted by genetically determined resistance level than by infection dose. Lines that fell at the phenotypic extremes stayed at those extreme levels of resistance regardless of dose, while those at intermediate levels of resistance spanned a greater range of bacterial loads based on infection dose (Fig. 2C). The strong impact of resistance on the outcome of infection may have prevented our ability to fully separate resistance and tolerance. Methodologically, these results stress the importance of estimating slope tolerance from fitness plotted against pathogen burden after replication within the host (Strauss & Agrawal 1999). If fitness is plotted against initial infection dose, inferred differences in tolerance may actually be the result of different levels of resistance (Fig. 2D). Additionally, in systems where the pathogen replicates within the host, the use of multiple doses does not estimate or account for endogenous host resistance level. In such cases, only comparison of genotypes that have similar levels of resistance can allow differences in tolerance to be observed without being confounded by heterogeneity in infection severity.

The genetic correlations between the three traits measured were reflected in the association study where we found that the direction and magnitude of effect sizes of the mapped SNPs was positively correlated across traits (Fig. 3). A positive correlation between traits could, in principle, be driven by differences in general vigour in the inbred lines used. However, we did not find any positive correlations between our estimates of tolerance, bacterial load or survival of infection and with measures of fecundity or lifespan obtained from a study performed by Durham *et al.* (2014) (Fig. S7, Supporting information). Biologically, both the positive relationship between resistance and genotypic deviation in tolerance seen across the DGRP as well as the inability to separate the two traits using multiple infection doses implies that we must consider the evolution of tolerance and resistance together. In systems like ours, tolerance will not evolve independently of resistance. These two traits are tangled.

Despite this nonindependence between traits, we have identified candidate genes that alter tolerance but not resistance via our genomewide association study using a nominal critical value of $P < 10^{-5}$. None of the mapped SNPs identified survived a much more stringent genomewide significance level determined by permutation analysis under the assumption of a single primary causal locus (Fig. S8, Supporting information) (Churchill & Doerge 1994). However, we expect a complex trait such as tolerance to be multi-allelic. While the DGRP has been used to successfully identify large-effect alleles in traits with a simple genetic basis, such as resistance to viral infection (e.g. Magwire *et al.* 2012),

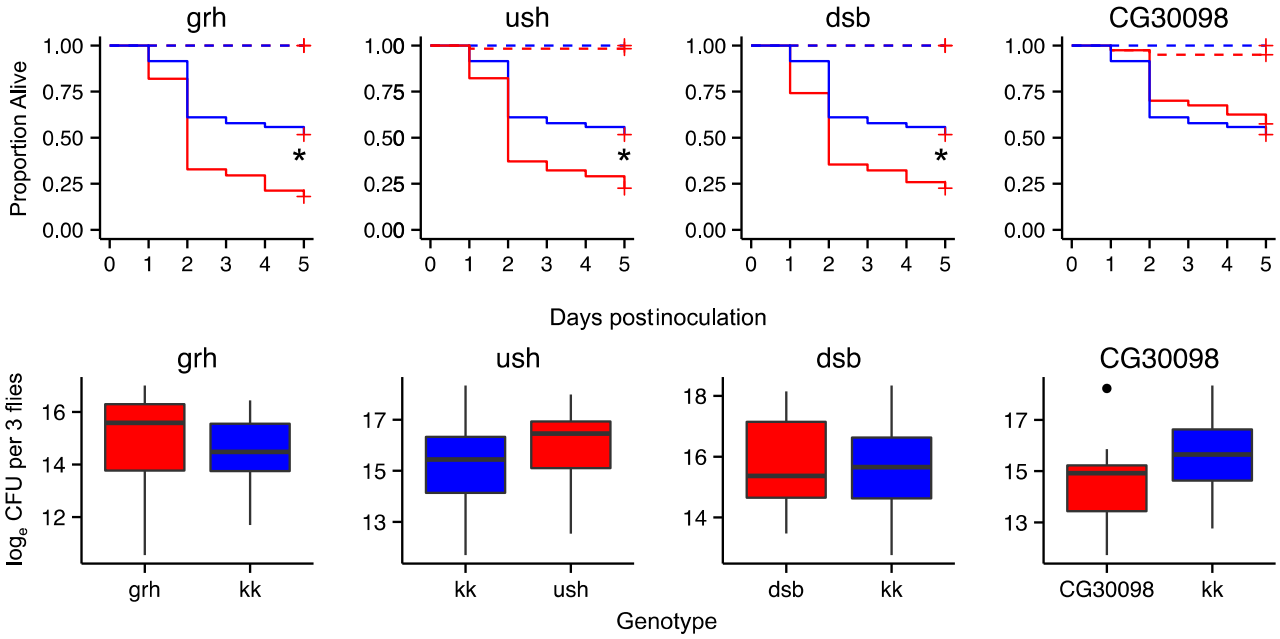

Fig. 5 Survival and bacterial load after RNAi knockdown of four genes primarily in the fat body and hemocytes paired with the matched control (background genotype without the RNAi construct crossed to the knockdown driver). The red line and box represent the knocked down genotype, and the blue represents the control (background genotype without the RNAi construct crossed to the driver line). A star represents a P-value < 0.05. The dashed lines in the survival plots represent the wounded controls, and the solid lines represent the infected treatments.

the resource is known to lack power to detect small-effect alleles at genomewide significance (Vaisnav *et al.* 2014). Our nominal P-value threshold allows for hypothesis generation, but requires follow-up studies to confirm the role of the loci in the mapped traits and to exclude false positives.

Despite the limitations described above, we still have evaluated the set of genes carrying SNPs that predicted genotypic deviation in tolerance. These genes were nominally enriched for the GO term 'nucleus' and are involved in regulation of gene expression, metabolism or immunity included the transcription factors *grainyhead*, *pipsqueak*, *domino*, *Blimp-1* and *C15*. *grainyhead* is involved in developmental processes and wounding healing via ERK signalling in embryo (Mace *et al.* 2005; Kim & McGinnis 2011). *pipsqueak* is involved in embryonic patterning and regulation of chromatin silencing. *domino* is involved in regulation of hemocyte proliferation and defence (Braun *et al.* 1998; Evans *et al.* 2003). *Blimp-1* regulates development through response to ecdysone (Agawa *et al.* 2007). *C15* is involved in regulation of development including notch signalling (Campbell 2005). Based on this enrichment, we hypothesize that variation in tolerance may be determined by differential regulation of gene expression in essential biological processes, potentially at multiple stages of development and not just in response to infection. This is in contrast to survival and resistance, which are largely determined by variation in previously characterized immune and stress responses including the previously characterized nonsynonymous SNP in the antimicrobial peptide gene, *Diptericin* (Unckless *et al.* 2015, 2016).

Using ubiquitous RNAi knockdown, we were able to confirm the role of five mapped tolerance genes in defence: *mspo*, *beat-IIIc*, *fhos*, *gus* and *CG4174*. Previous predictions, expression data or loss-of-function studies have shown *mspo*, *beat-IIIc* and *fhos* may be involved in immune processes or wound healing. *mspo* has been shown to be induced in cell culture infected with *Escherichia coli* (Kleino *et al.* 2008), and beat-IIIc has an immunoglobin-like fold which can be involved in immune function (Watson *et al.* 2005). *fhos* is involved in wound healing (Lammel *et al.* 2014). *gus* and *CG4174* have not been implicated in canonical immunity. *gus* is involved in developmental processes including axis specification, appendage formation and regulation of catabolic processes (Styhler *et al.* 2002; Kugler *et al.* 2010). *CG4174* is predicted to be involved in oxidation–reduction processes including iron ion and ascorbic acid binding (FlyBase Curators *et al.* 2004).

Using tissue-specific knockdown in the fat body and hemocytes, we were able to confirm the role *grainy head*, *u-shaped* and *debris buster* in defence. *grainy head* is a

transcription factor that is involved in embryonic wound healing via epithelial repair (Mace *et al.* 2005) and is predicted to bind the promoters of characterized immune genes (Dobson *et al.* 2016). *debris buster* is known to be involved in autophagy of dendritic debris by fusion of the phaogosome and lysosome (Han *et al.* 2014). Autophagy plays an important role in resistance to some bacterial infection and immunogenic tolerance to symbiotic organisms (Voronin *et al.* 2012; Moy & Cherry 2013), and we hypothesize that both processes may be associated with infection tolerance. *u-shaped* is involved in lymph gland development and crystal cell differentiation as well as regulation of antimicrobial peptide biosynthetic processes (Evans *et al.* 2003; Muratoglu *et al.* 2006; Valanne *et al.* 2010). These functional studies provide further support that the architecture of tolerance is composed of polymorphisms in the regulators of immune and stress responses. Future work can be done to identify the specific actions and timing of these elements in defence tolerance.

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V.M.H. and B.P.L. designed the experiments, V.M.H. conducted the research, V.M.H. analyzed the data, V.M.H. and BP.L. wrote the manuscript.

Data accessibility

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.3p8j3.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 (A) Six lines were chosen that varied in tolerance and resistance in the initial measurements of the DGRP. These lines are represented by large red points. (B) The initial bacterial burden introduced in the low, medium, or high inoculation doses.

Fig. S2 The correlations of survival and load phenotypes across three biological replicates.

Fig. S3 Correlation between genotypic deviation in tolerance and the proportion of flies surviving the infection.

Fig. S4 The positive relationship between resistance and the genotypic deviation in tolerance remained significant ($r = 0.191$, $P = 0.029$) even when the analysis was restricted to lines that fell at intermediate levels of resistance ($\log_e$ CFU between 12 and 16).

Fig. S5 Quantile-quantile plots for survival (A), load (B), and genotypic deviation in tolerance (C).

Fig. S6 Ubiquitous RNAi knock-down of candidate tolerance genes.

Fig. S7 To understand how tolerance and resistance relate to other life-history traits we compared our data with that of Durham (2014), which measured biweekly fecundity at one, three, five, and seven weeks post-eclosion, as well as lifespan. We found no positive correlations between the two studies.

Fig. S8 The distribution of the lowest *P*-values from the permutation analysis for (A) survival, (B) bacterial load, and (C) tolerance.

Table S1 Proportion surviving, bacterial load and estimates of genotypic deviation in tolerance for DGRP lines used in this study.

Table S2 List of VDRC RNAi lines tested.

Table S3 SNPs that predicted variation in survival of infection.

Table S4 SNPs that predicted variation in resistance to infection.

Table S5 Overlapping SNPs between mapped traits.

Table S6 Gene ontology for survival of infection using the functional association gene set.

Table S7 Gene ontology for bacterial load using the functional association gene set.

Table S8 Gene ontology for tolerance of infection using the functional association gene set.