Rapid seasonal evolution in innate immunity of wild *Drosophila melanogaster*

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Understanding the rate of evolutionary change and the genetic architecture that facilitates rapid adaptation is a current challenge in evolutionary biology. Comparative studies show that genes with immune function are among the most rapidly evolving genes across a range of taxa. Here, we use immune defence in natural populations of *Drosophila melanogaster* to understand the rate of evolution in natural populations and the genetics underlying rapid change. We probed the immune system using the natural pathogens *Enterococcus faecalis* and *Providencia rettgeri* to measure post-infection survival and bacterial load of wild *D. melanogaster* populations collected across seasonal time along a latitudinal transect along eastern North America (Massachusetts, Pennsylvania and Virginia). There are pronounced and repeatable changes in the immune response over the approximately 10 generations between spring and autumn collections, with a significant but less distinct difference observed among geographical locations. Genes with known immune function are not enriched among alleles that cycle with seasonal time, but the immune function of a subset of seasonally cycling alleles in immune genes was tested using reconstructed outbred populations. We find that flies containing seasonal alleles in *Thioester-containing protein 3* (*Tep3*) have different functional responses to infection and that epistatic interactions among seasonal *Tep3* and *Drosomycin-like 6* (*Dro6*) alleles underlie the immune phenotypes observed in natural populations. This rapid, cyclic response to seasonal environmental pressure broadens our understanding of the complex ecological and genetic interactions determining the evolution of immune defence in natural populations.

1. Introduction

The rate at which populations respond to environmental change is a fundamental parameter in the process of adaption. Evolution is historically considered to be an innately slow process that occurs over very long time scales [1], but there are now examples that evolutionary change can occur much faster [2–4]. The limits of how fast populations evolve and the genetic architecture underlying rapid evolution remain unclear [5]. The classical approach to infer adaption through the association of traits and genotypes that covary along spatial environmental gradients (e.g. latitude, longitude and altitude) [6] can be expanded across temporal environmental gradients to provide insights to the rate of adaption in the wild.

Biotic environment may shape the rate of adaptation through the immune system, which sits at the crucial interface between an organism's external and internal environment. Strong selection imposed by pathogens may result in rapid

evolution of immune defence in nature, because microbiotic infection directly affects host fitness, with consequences ranging from resource reallocation to host mortality [7–13]. Comparative studies across a broad range of taxa indicate that genes with immune function are among the most rapidly evolving genes in the genome [14–19]. *Drosophila melanogaster* immune genes show evidence of local adaptation across large spatial gradients with high levels of population differentiation and latitudinal enrichment across multiple continents [20–23]. There is less evidence for immune differentiation at small spatial scales [24,25], although some screens of infection response in *D. melanogaster* indicate continental differences in defence quality [24]. Thus, immune defence in natural populations of *D. melanogaster* represents a tractable system to study the rate of evolution.

We predicted seasonal variation in *D. melanogaster* immune defence even in the absence of established clinal differences in performance. Seasonal climatic changes produce predictable environmental gradients over time that select for different phenotypes [26,27] and allele frequencies [28,29] in multivoltine organisms like *D. melanogaster*. Abiotic variables (e.g. temperature) that cycle across seasons can influence microbial growth, so microbial communities and pathogen diversity that vary over spatial gradients [30–36] may also change across seasonal time [37–40]. Seasonal differences in pathogen diversity and frequency may select for immune resistance or tolerance in either or both of the primary humoral immune pathways: the Toll pathway that is preferentially activated by Gram-positive bacteria or the IMD pathway that is primarily activated by Gram-negative bacteria [41].

We tested whether innate immunity evolves seasonally in mid-Atlantic *D. melanogaster* populations in North America (Massachusetts, Pennsylvania and Virginia). We found that immune defence changes rapidly and repeatedly from spring to autumn, and seasonally cycling alleles of immune genes determine seasonal variation in resistance to and tolerance of infection. We show that epistatic interactions among seasonally cycling SNPs produce the immune phenotypes observed in natural populations. This rapid, cyclic response to seasonal environmental pressure broadens our understanding of the complex ecological and genetic interactions determining the evolution of immune defence in natural populations.

2. Material and methods

(a) *Drosophila* samples

Wild *D. melanogaster* were collected by aspiration in early July (spring population) and late October (autumn population) repeated across 2 years at three locations spaced evenly along a 4° latitudinal gradient: George Hill Orchard in Lancaster, MA (42.500493° N, −71.563580° E), Linvilla Orchards in Media, PA (39.884179° N, −75.411227° E) and Carter Mountain Orchard in Charlottesville, VA (37.991851° N, −78.471630° E). Isofemale lines were established from wild-caught inseminated females and were maintained on standard cornmeal molasses food in standard laboratory conditions (25°C, 12 L : 12 D) on a three-week transfer cycle for six to eight generations before immune assessment.

We screened a published dataset of SNPs that oscillate in these populations across seasonal time [29] for genes with known immune function [42] to identify candidate seasonal immune SNPs. The immune function of these SNPs was assessed using recombinant outbred populations (ROPs) [43] fixed for specific seasonal allele combinations in a randomized genetic background that were constructed using lines from the Drosophila Genetics

Reference Panel (DGRP) [44]. Ten gravid females from 15 lines were pooled to lay eggs for 48 h for each combination of seasonal alleles (electronic supplementary material) and the offspring mated freely for at least 10 non-overlapping generations before immune assessment. The immune function of the two SNPs in *Thioester-containing protein 3* (*Tep3*) was tested using three genotypes that combined 2 L:7703202 and 2 L:7705370 (*D. melanogaster* reference genome v.5.39) spring and autumn alleles: (i) $Tep3^{TG}$ contained spring alleles for both 2 L:7703202 and 2 L:7705370, (ii) $Tep3^{TT}$ contained the spring 2 L:7703202 and the autumn 2 L:7705370 modifier allele, and (iii) $Tep3^{CT}$ contained autumn alleles for both SNPs. The final combination of the autumn 2 L:7703202 coding allele and the spring 2 L:7705370 modifier allele was too rare in the DGRP to create ROP. Two independent biological replicate populations were created for each of the three *Tep3* genotypes. Epistatic interactions between *Tep3* and either *Fas-associated death domain* (*Fadd*) or *Drosomycin-like-6* (*Dro6*) were assessed in the same way with ROP fixed for either both spring or autumn *Tep3* alleles and either *Fadd* or *Dro6* alleles.

(b) Immune measurements

Quality of immune defence was probed using systemic bacterial infection [45] with Gram-negative *Providencia rettgeri* [46] and Gram-positive *Enterococcus faecalis* [47] strains that were originally isolated from infected wild-caught *D. melanogaster*. Post-infection survival was measured in males 3–5 days over two repeated blocks of five consecutive days after infection. Mortality was highest in the first 24 h and plateaued (electronic supplementary material, figure S1), so the final mortality 5 days post-infection was analysed in the model. Flies were infected with cultures started with a single colony grown to saturation in LB media at 37°C with shaking overnight and diluted to A_{600nm} of 1.0. Infections were delivered at a dose of 10^3–10^4 bacteria to each CO_2-anaesthetized fly by inoculating the lateral thorax with a 0.15 mm minutien pin (Fine Scientific Tools) dipped into bacterial culture [45]. Two controls were used: a sterile wound by a needle disinfected in 95% ethanol and unwounded flies anaesthetized on CO_2.

Systemic bacterial load of infected flies was quantified using the same infection method described above for infection survival. When evaluating the natural populations, 20 lines from each of the collection were infected during a daily infection window (09.00–12.00). All infections were repeated over two consecutive days by two infectors with infector and infection order randomized daily. Twelve males from each line were infected and maintained with food at 25°C, 12 L : 12 D for 24 h post-infection. Up to three replicate groups of three flies were homogenized in 500 ml of LB for the 2012 natural populations, and up to three single flies were homogenized in 500 ml of PBS for the 2014 natural and ROP. The samples were then plated on LB agar plates at 1 : 100 dilution for *P. rettgeri*, 1 : 10 for *E. faecalis* in natural populations and 1 : 1 for both bacteria in ROP using the Whitley Automatic Spiral Plater (Don Whitley Scientific, Shipley, UK). The plates were incubated overnight at 37°C, and the number of colony-forming units was counted using the ProtoCOL3 automated plate counter (Synbiosis, Cambridge, UK) and used to calculate the concentration of bacteria in each homogenate.

(c) Gene expression

Expression levels of *Tep3*, *Dro6* and *Fadd* were determined using a published dataset of RNAseq on 185 inbred sequenced lines from the DGRP [48]. We extracted expression of *Tep3*, *Dro6* and *Fadd* for each inbred line and used sequence data [44] to identify *Tep3*, *Dro6* and *Fadd* haplotype.

(d) Statistical analyses

Statistical analyses were performed using R software (v. 3.2.2; R Core Team). Post-infection survival was measured daily and

survival 5 days post-infection was analysed using a binomial linear regression. Survival post-infection was evaluated using the model:

$$survival = year \times population \times season + line + replicate.$$

Population, year and season were considered fixed effects, and replicate and line were random effects nested within season, population and year. Mean survival post-infection was standardized by survival under sterile wound control.

Concentration of bacteria in each homogenate was calculated using the number of colonies log-transformed and analysed using mixed-model ANOVAs:

$$\log_{10}(count/ml) = year \times population \times season + line$$
$$+ replicate.$$

Population, year and season were fixed effects, and replicate and line were random effects nested within season, population and year. Infector and infection order were initially included in model but had no significant effect and were removed.

(e) Seasonal SNPs

Seasonal immune SNPs were identified by screening a published dataset for alleles that fluctuate in frequency as a function of seasonal time [29] in 88 genes known to have immune function [49]. Seasonal SNPs were cross-referenced with a group of paired spring and autumn samples collected from 10 populations along the North American cline by the Drosophila Real Time Evolution Consortium (NCBI SRA BioProject PRJNA308584 [29,50]). Additional information collected on each SNPs included clinal q-value [29] and p-value in a genome-wide association study to identify SNPs involved with *P. rettgeri* pathogenic infection [51]. Enrichment for immune genes was calculated using customized python scripts that compared the proportion of seasonal and non-seasonal immune genes with control genes that were matched for size and position using χ^2 with 10 000 bootstrap iterations.

Linkage disequilibrium (LD) among the candidate seasonal immune SNPs was calculated in the DGRP using allelic correlation of physical distances using the LDheatmap package [52] in R. The 205 sequenced inbred lines of the DGRP were used to examine LD among all of the candidate SNPs by chromosome [44].

(f) Seasonal genotypes

Genotypes from wild populations were determined using inbred lines originally collected in Pennsylvania in the spring and autumn of 2012. The lines were inbred by full-sib mating for 20 generations and subsequently sequenced (NCBI SRA BioProject PRJNA383555). Genotype deviation was calculated as the difference between observed frequency and a predicted frequency based on the individual alleles. Haplotype distribution of *Tep3* was calculated for SNPs with a minor allele frequency greater than 0.1 using integer joining networks [53] in PopArt v. 1.7 [42].

3. Results

(a) Geographical differences in immunity

Geographical origin across the latitudinal transect determined survival post-infection, but did not predict systemic bacterial load sustained by flies infected with either pathogen. While survival after *P. rettergi* infection directly depended on the latitude ($\chi^2_{(2)} = 12.805, p = 5.87 \times 10^{-4}$), the interaction between geographical origin and season of collection affected survival after *E. faecalis* infection ($\chi^2_{(2)} = 10.035, p = 6.62 \times 10^{-3}$). The lower-latitude Virginia population had higher survival after *E. faecalis* infection in the spring but no difference in the autumn (figure 1a,b). High-latitude Massachusetts and

Pennsylvania populations had similar load and survival after *P. rettgeri* infection and exhibited a greater seasonal change in both survival and bacterial load compared with the lower-latitude Virginia population (figure 1c,d).

(b) Immunity changes rapidly within a population over seasonal time

Immune defence changed rapidly across approximately 10 generations in the wild from spring to autumn with a pathogen-specific relationship between bacterial load and survival (figure 1). Spring populations were more resistant to *E. faecalis* bacterial growth ($F_{1,219} = 87.758, p < 0.0001$) and maintained low load with marginally higher survival rates ($\chi^2_{(1)} = 3.201, p = 7.36 \times 10^{-2}$), while the autumn populations infected with the same bacteria did not restrict bacterial growth as effectively, resulting in high load and high mortality (figure 1a,b). The converse relationship occurred when flies were infected with *P. rettgeri*: higher survival in spring ($\chi^2_{(1)} = 16.145, p = 5.87 \times 10^{-4}$) despite higher bacterial load ($F_{1,215} = 7.88, p < 0.0001$) and high mortality in autumn even though the bacterial growth was restricted (figure 1c,d). There is a general consistency of the patterns from spring to autumn, but the annual variation in the environment results in year and year-by-month interaction as significant factors contributing to survival after infection with *E. faecalis* (year: $\chi^2_{(1)} = 33.80, p = 6.10 \times 10^{-9}$; year × month: $\chi^2_{(1)} = 7.05, p = 7.94 \times 10^{-3}$) and *P. rettgeri* (year: $\chi^2_{(1)} = 39.98, p = 2.57 \times 10^{-10}$; year × month: $\chi^2_{(1)} = 11.70, p = 6.23 \times 10^{-4}$).

(c) SNPs in immune genes oscillate across seasonal time

Immune genes as a functional category were not enriched among genes carrying polymorphisms that oscillate in frequency over seasonal time in these populations [29] when compared with controls matched for size and position using previously published data. Twenty-four candidate SNPs oscillated in frequency across seasonal time in these populations [29] located within or in proximity to 13 genes with known involvement in immune function [54] (electronic supplementary material, table S1; table 1 and figure 2a).

(d) Seasonally oscillating Tep3 SNPs have functional differences in immunity

Over one-third of the seasonally variable SNPs near immune genes were near *Tep* family genes, with *Tep* homologues comprising one-fourth of all of the seasonally variable immune genes. *Tep3* contained numerous seasonally oscillating loci with high LD across the 2.5 kb region in which the seasonal alleles are located in the DGRP (figure 2a,b,c). We tested the function using ROP with two loci as markers: the non-synonymous-coding change at *2 L*:7703202 that is surrounded by five intronic seasonal SNPs and the intronic SNP *2 L*:7705370 that is 2 kb downstream from the cluster (*D. melanogaster* reference genome v. 5.39). These markers are in LD in the DGRP ($r^2 = 0.8138$) and cycle independently across seasonal time (electronic supplementary material, table S1; figure 2c), but neither allele varies along a cline (electronic supplementary material, table S1). Alleles at *2 L*:7703202 were non-randomly distributed with respect to karyotype: in both DGRP and Pennsylvania populations that the autumn allele (C) was strongly associated with
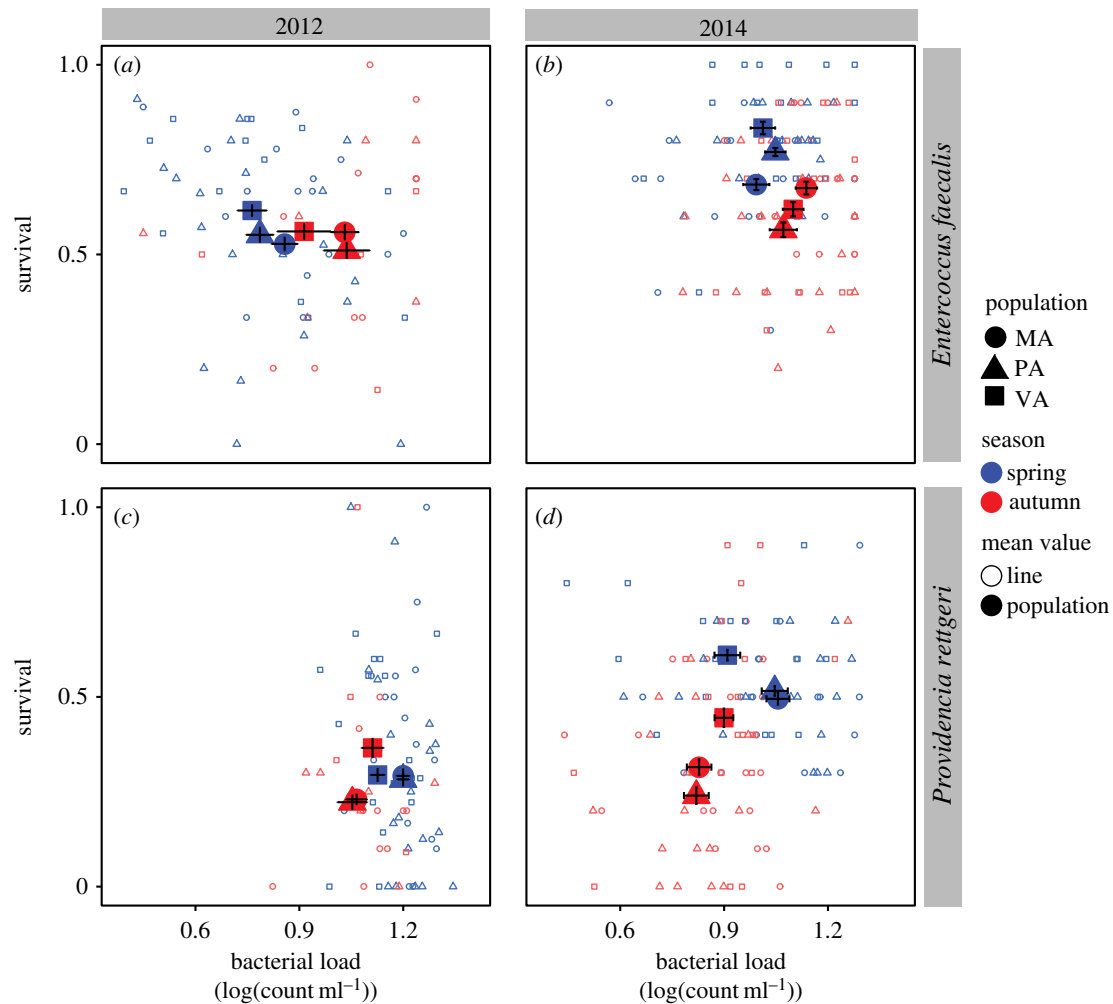
Figure 1. Immune defence relationship between bacterial load and survival in natural spring and autumn populations. Isofemale lines (small, outline) were used to calculate population mean $\pm$ s.e. (large, filled) from natural orchard populations collected along a latitudinal gradient in Massachusetts Pennsylvania and Virginia in the spring and autumn for two replicate years: 2012 (*a,c*) and 2014 (*b,d*). Immune defence was probed with two natural pathogens: a Gram-positive bacterium *Enterococcus faecalis* (*a,b*) and a Gram-negative bacterium *Providencia rettgeri* (*c,d*). Twenty isofemale lines from each collection were measured for 5-day survival after infection and bacterial load at 24 h post-infection scaled by average load for the experiment.

In(2 L)t. By contrast, the spring allele (T) occurred mostly in a standard arrangement genetic background (Fisher's exact test; $p < 0.0001$). *2 L*:7705730 had no significant association with either arrangement of *In(2 L)t* (Fisher's exact test; $p = 0.161$).

There was no difference among the *Tep3* ROP in bacterial load, but there was differential survivorship after infection with both Gram-positive and Gram-negative pathogens. Flies containing the spring *Tep3*^{TG} haplotype had higher survival than those containing the autumn *Tep3*^{CT} or mixed *Tep3*^{CG} haplotypes when infected with Gram-positive *E. faecalis* ($\chi^2_{(2)} = 6.73$, $p = 0.0346$; figure 3*a*).The *Tep3* SNPs are associated with an additive effect on survival of Gram-negative *P. rettgeri* infection with higher survival in flies containing the autumn haplotype than those containing the spring haplotype and intermediate survival in flies containing the mixed haplotype ($\chi^2_{(2)} = 3.651$, $p = 0.161$; figure 3*b*). Flies containing the seasonal *Tep3* haplotypes have no difference in *Tep3* expression in the absence of infection ($F_{3,360} = 1.419$, $p = 0.239$, figure 3*c*) based on previously published RNAseq expression of the DGRP lines [48]. The *Tep3*^{TG} haplotype containing spring alleles occurred at higher in the spring Pennsylvania population compared with the autumn, while the *Tep3*^{CT} haplotype containing autumn alleles increased in frequency from spring to autumn (figure 3*d,e*). There were two primary sequence haplotypes carrying spring *Tep3*^{TG} variants and two sequence haplotypes carrying the autumn *Tep3*^{CT} variants in the Pennsylvania orchard (figure 3*f*; electronic supplementary material, table S1).

(e) Epistasis among AMP genes involved in rapid seasonal adaptation

We tested whether additional seasonal SNPs in the immune pathways interact with *Tep3* to facilitate rapid immune evolution across seasons: *3 L*:3334769, an upstream modifier of *Drosomycin-like 6* (*Dro6*), that was shown to significantly affect resistance to *P. rettgeri* in a genome-wide association study [51] and *3R*:17861054, a 3′-UTR modifier in the signalling gene *Fas-associated death domain orthologue* (*Fadd*), which was the only SNP with concordant patterns between season and latitude (figure 2*a* and table 1). There was no difference in immune defence among ROP combinations of *Tep3 and Fadd*, but the non-additive interactions among ROP containing *Tep3* and *Dro6* alleles begin to explain the complexity of immune defence of natural populations (figure 4).
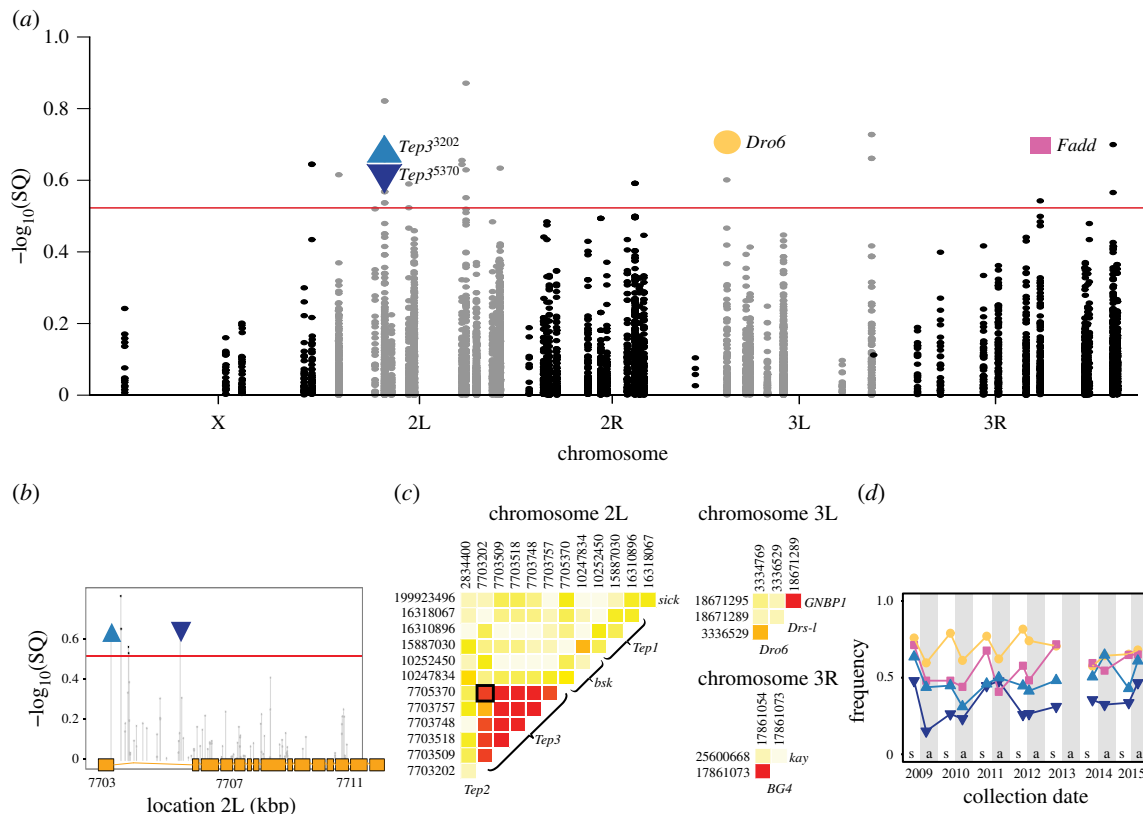
Figure 2. Seasonal changes in immune genes in natural populations. (*a*) Manhattan plot of SNPs in immune genes that change in frequency across seasonal time [29]. The red line indicates the seasonal *q*-value cutoff > 0.3 [29]. The SNPs on which functional analyses were performed are highlighted: *Fadd*, *Dro6*, *Tep3*^{3202} and *Tep3*^{5370}. (*b*) Manhattan plot of SNPs in immune genes within *Tep3* with genic structure along the *x* axis. Exons indicated in orange boxes. (*c*) Heat map showing linkage disequilibrium among SNPs in immune genes across each chromosome using DGRP. (*d*) Cycling of seasonal allele frequencies of candidate immune SNPs in Pennsylvania in the spring (s) and the autumn (a) from 2009 to 2015. (Online version in colour.)

4. Discussion

(a) Natural populations differ in immunity over geographical space and across seasonal time

Immune response differs among populations across space and time. Season of collection is a strong predictor of the immune response across geographical locations that span 4° latitude with a seasonal decline in resistance to *E. faecalis* and a seasonal decline in tolerance of *P. rettgeri*. The change in immunity across seasonal time occurs rapidly within each geographical location with approximately 10 generations between spring and autumn collections. Repeated seasonal change in immune defence is consistent with previous findings for other measurements of stress resistance [26,27]. Taken together, this suggests that a harsh winter selects for a suite of traits that produce a robust spring population and selection on those traits is relaxed during summer producing less stress resistant populations in autumn.

Although the strongest differentiation of immunity occurred across seasonal time, there was also a signal of geography along the sampled spatial gradient. Our results contrast with previous studies that did not detect a robust association between latitude and survival [55] or load [24,49]. The difference may be attributed to the interaction between season and latitude. It is possible that geographical differences in immune response may be even greater across a longer distance that may capture a larger difference in pathogen diversity [30–36,56]. In addition, there was secular change in post-infection survival but not in bacterial load, which may be caused by year-to-year differences in the microbial community of the environment that result in different allele frequencies in genes with immune function.

The repeatability of the general patterns of change in immune defence across space and time indicates deterministic evolutionary processes. Rearing lines for multiple generations in a common laboratory environment distinct from external sample sites removes environmental variation and ensures that differences among collections and populations can be attributed to genetic diversity among source populations. It is possible that gene flow due to migration from other latitudes contributes to the differences between spring and autumn populations. However, migration is unlikely to be the primary cause underlying seasonal immune differences, because latitudinal differentiation was weak compared to seasonal change. Furthermore, infection with different pathogens resulted in opposing clinal patterns but parallel change across seasons. Additionally, migration alone appears insufficient to explain genome-wide differences in allele frequency profiles that characterize spring and autumn populations in Pennsylvania orchard [29]; thus, migration is unlikely to explain the seasonal differences in immune response. Wild *Drosophila* populations live in a heterogeneous environment and evolve rapidly in response to environmental parameters that change with season [26,27], potentially including rapid turn-over in microbial and pathogen communities (electronic supplementary material, figure S2).

(b) SNPs in immune genes oscillate across seasonal time

Changes in immune defence are at least in part due to differences in genes with immune function across space and
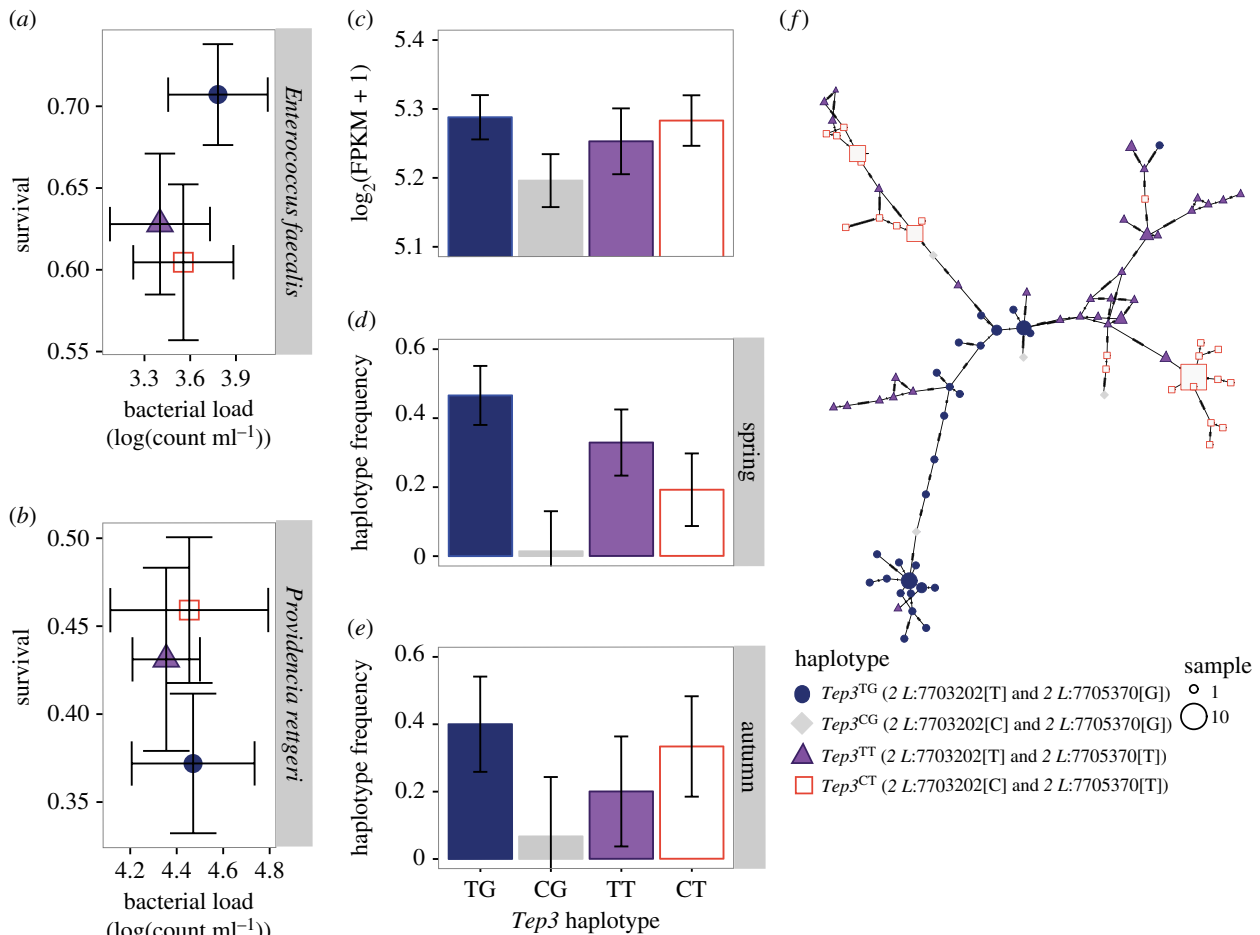
Figure 3. Functional difference of seasonal *Tep3* alleles as defined by the focal SNPs. Mean $\pm$ s.e. for bacterial load 24 h post-infection and survival 5 days post-infection for the *Tep3* genotypes. (a) Higher survival for the spring genotype than the autumn or combination genotypes when infected with *E. faecalis*. (b) Additive effect of alleles when infected with *P. rettgeri*. (c) Lower constitutive *Tep3* mRNA expression in the rare *Tep3*^{CG} haplotype from the published dataset of DGRP flies [48]. (d,e) Frequency of *Tep3* haplotypes in the Pennsylvania orchard across seasonal time. (f) Minimum spanning network illustrates that LD among the SNPs is maintained in distinct haplotypes. (Online version in colour.)

time. Genomic screens show that immune genes are enriched across latitudinal gradients [20–23], but we did not find enrichment among immune genes in SNPs that cycle in frequency with season. Seasonal differences in immunity could arise from variation in genes not classically identified as part of the immune system and were not detected from our screen. However, the *D. melanogaster* immune system is well characterized and changes in even a single immune gene could affect phenotypic response to infection even without enrichment for all immune genes. Alternatively, immune changes may be controlled by non-additive genetic interactions that would not be identified in enrichment analysis.

(c) Immune survival of flies containing seasonally oscillating Tep3 haplotypes

Immune responses in the ROP were consistent with the seasonal patterns in natural populations: spring populations and flies containing the spring *Tep3* haplotype had a higher defence against Gram-positive *E. faecalis*, whereas autumn populations and flies containing the autumn *Tep3* haplotype had higher defence against Gram-negative *P. rettgeri*. Opposite survival patterns for flies with spring and autumn *Tep3* haplotypes are consistent with antagonistic pleiotropy [57] operating across distinct branches of the immune system, limiting the host such that improvements in response to one class of pathogens (e.g.

Gram-negative bacteria) restrict the ability to respond to other pathogens (e.g. Gram-positive bacteria). Trade-offs within the immune system occur in several insect systems between humoral antimicrobial peptides that combat microbial infections and phenoloxidase that is deployed against eukaryotic parasites [58–60] as well as in the T-helper cells of the vertebrate immune system (reviewed in [61]). We hypothesize that genetic variation for allocation to either immune activity may be maintained if the risk of pathogenesis changes over space or time. Additivity among the loci in response to *P. rettgeri*, but a non-additive response to *E. faecalis*, suggests that the autumn allele at 2 L:7705370, or genetic variants linked to it, has a dominant effect that decreases survival to *E. faecalis* infection.

Our data suggest that these *Tep3* loci are natural variants in immune tolerance, because flies containing the haplotypes with the same infection load had differential survivorship. The molecular function of the seasonal loci in *Tep3* remains unclear. *Tep* proteins are α-macroglobulin protease traps that bind to pathogen surface and act as opsonins [62–64]. The polymorphism at 2 L:7703202 produces a non-synonymous Ala/Val polymorphism at residue 18, but both amino acids are hydrophobic. The intronic SNP at 2 L:7705370 is directly upstream of the exon cassette region and may regulate expression, but *Tep3* is constitutively expressed and not strongly induced by *E. faecalis* or *P. rettgeri* infection [65,66]. Therefore, the SNPs we examined may most appropriately be

Table 1. Seasonal immune SNPs identified using whole-genome resequencing of the Pennsylvania spring and autumn populations across three consecutive years. SNPs with a seasonal q-value (SQ) <0.3 are classified as seasonal and the SNPs investigated here are in bold. Most of seasonal SNPs do not have significant clinal q-values (CQ) and were not significant in a genome wide association study (GWAS) for response to *P. rettgeri* pathogenic infection [52].

gene	position	effect	molecular function	SQ	CQ	GWAS
Tep2	2 *L*:2834400	upstream modifier	effector	0.242	0.956	0.253
Tep3	**2 L:7703202**	**NS coding**	**effector**	**0.243**	**0.159**	**0.420**
Tep3	2 *L*:7703509	upstream modifier	effector	0.151	0.529	0.084
Tep3	2 *L*:7703518	upstream modifier	effector	0.220	0.643	0.084
Tep3	2 *L*:7703748	upstream modifier	effector	0.271	0.819	0.114
Tep3	2 *L*:7703757	upstream modifier	effector	0.291	0.956	0.632
Tep3	**2 L:7705370**	**upstream modifier**	**effector**	**0.219**	**0.163**	**0.385**
bsk	2 *L*:10247834	intron	signalling	0.300	0.822	0.255
bsk	2 *L*:10252450	intron	signalling	0.257	0.749	0.962
Tep1	2 *L*:15887030	downstream modifier	effector	0.227	0.188	0.089
Tep1	2 *L*:15888031	downstream modifier	effector	0.221	0.520	NA
cact	2 *L*:16309682	downstream modifier	signalling	0.135	0.782	0.829
cact	2 *L*:16310896	downstream modifier	signalling	0.235	0.635	0.375
cact	2 *L*:16318067	intron	signalling	0.281	0.719	0.335
sick	2 *L*:19923496	intron	signalling	0.232	0.032	0.505
IM1	2 *R*:14270817	upstream modifier	effector	0.256	0.695	0.423
Dro6	**3 L:3334769**	**upstream modifier**	**effector**	**0.201**	**0.427**	**0.000**
Drs-l	3 *L*:3336529	upstream modifier	effector	0.251	0.975	0.028
GNBP1	3 *L*:18671289	downstream modifier	recognition	0.187	0.150	0.729
GNBP2	3 *L*:18671295	downstream modifier	recognition	0.218	0.167	0.666
Fadd	**3 R:17861054**	**UTR 3′modifier**	**signalling**	**0.200**	**0.006**	**0.822**
Fadd	3 *R*:17861073	UTR 3′modifier	signalling	0.287	0.425	0.712
kay	3 *R*:25600668	intron	signalling	0.200	0.588	0.743
Tak1	X:20388404	intron	signalling	0.227	0.326	0.964

considered as markers for a larger haplotype that contains causal variants.

Pathogen-specific higher survival associated with spring and autumn *Tep3* haplotypes may increase their frequency in the wild compared with flies containing a combination of spring and autumn alleles. Inversions could theoretically maintain LD that preserves the high-fitness spring and autumn haplotypes [67,68], but this is unlikely because the *In(2 L)t* inversion that contains *Tep3* does not cycle with season [29,50]. Additionally, *Tep3* is not located near a recombination-limiting breakpoint of In(2 L)t nor is it in LD with other seasonal immune SNPs within the inversion. However, we found that in two independent populations, alleles of the intronic SNP at *2 L*: 7703202 were non-randomly distributed with respect to karyotype, while *2 L*:7705730 had no significant association with either arrangement of *In(2 L)t.* LD might be created and maintained by selection against recombinant phenotypes either due to lower immunocompetence or another pleiotropic trait or because of intraspecific genetic incompatibilities. Deleterious incompatibilities maintain distinct haplotypes in *Arabidopsis thaliana* NLR immune receptors [69] and may also explain the near absence of the $Tep3^{CG}$ combination of spring and autumn alleles in all populations examined. Flies containing the $Tep3^{CG}$ haplotype appear three times across the haplotype tree constructed

from the seasonal Pennsylvania inbred lines, suggesting that the haplotype may form occasionally through recombination but does not proliferate in the population. Thus, it is likely that selection for the immune benefits of spring and autumn haplotypes and against combination of spring and autumn alleles maintains these distinct haplotypes in the wild. While these *Tep3* haplotypes explained some of the seasonal differences in immune tolerance of natural populations, other seasonally changing genes may also contribute to the observed differences in bacterial resistance in natural populations.

(d) Epistasis among AMP genes involved in rapid seasonal adaptation

Intergenic epistatic interactions between *Tep3* and *Dro6* suggest that season-specific genotypes have highest fitness. In our experiment, flies having all spring or all autumn alleles had higher survival after infection, while flies that contained a combination of spring and autumn had higher mortality. This suggests that complex genetic interactions shape winter and summer fitness, with distinct haplotypes maintained by non-additive epistatic interactions [70–72].
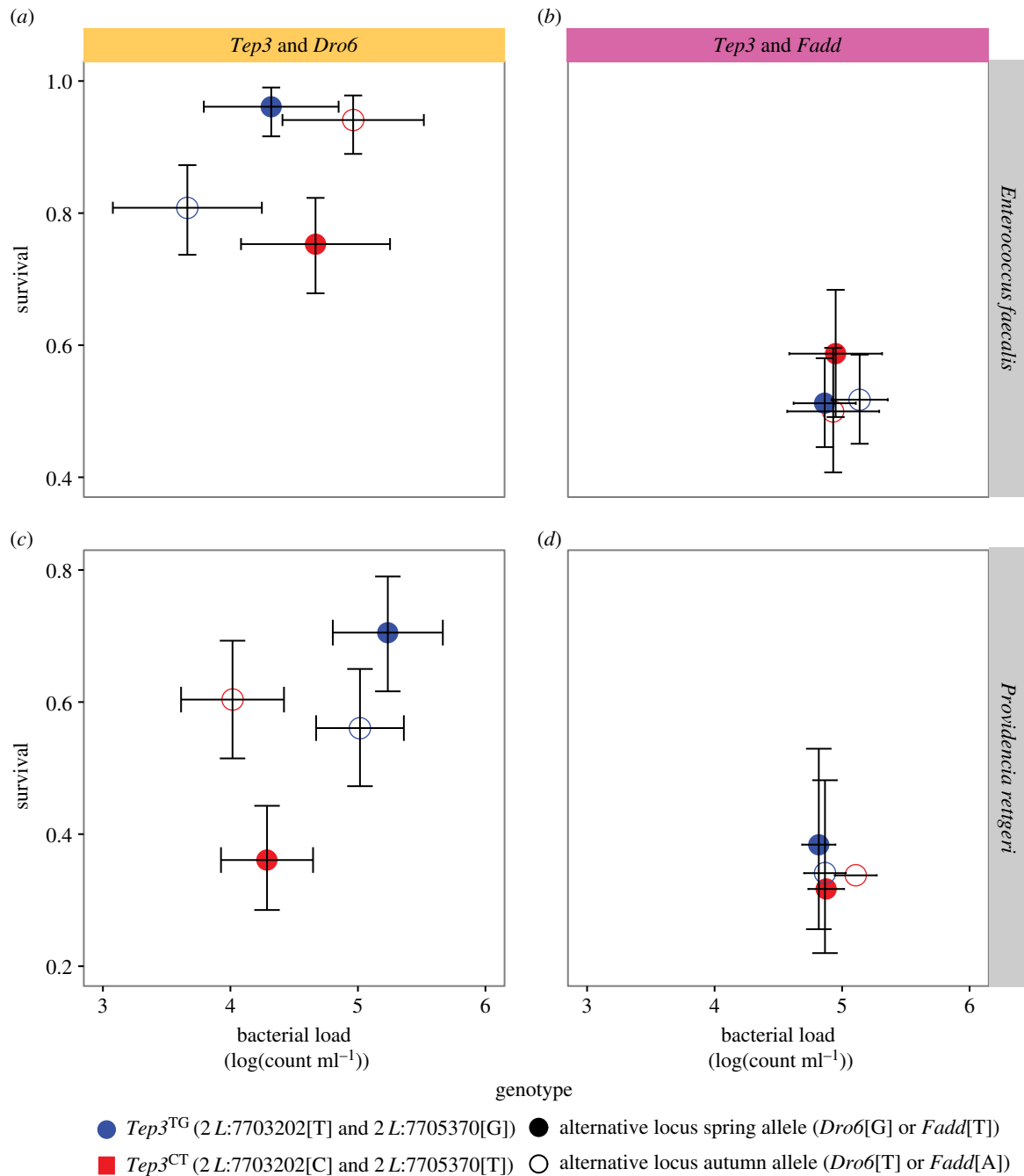
Figure 4. Intergenic interactions among *Tep3*, *Dro6* and *Fadd*. Non-additive interaction among *Tep3* and *Dro6* alleles. No significant interaction among *Tep3* and *Fadd* SNPs.

5. Conclusion

We demonstrate that pathogen-specific innate immunity evolves rapidly in natural populations of *D. melanogaster* across replicate years and geographical locations. Comparative studies across species and among populations have indicated that immune genes evolve faster than other genes in the genome, but the rapid phenotypic and genetic change we observed over approximately 10 generations is a substantially faster rate than previously considered. We tested a small subset of the immune SNPs that oscillate in allele frequency over seasonal time and observed intra- and intergenic interactions consistent with changes in immune tolerance and resistance across seasons in natural populations, perhaps in response to seasonally changing bacterial communities. Epistatic interactions among seasonally oscillating immune alleles may help facilitate this rapid phenotypic change over a short seasonal time scale. This rapid, cyclic response to biotic variables broadens our understanding of the complex ecological and genetic interactions in the evolutionary dynamics of natural populations.

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