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1 **Title:** Rapid seasonal evolution in innate immunity of wild *Drosophila melanogaster*
2

3 **Authors:** Behrman, Emily L.[1,2], Virginia M. Howick[3,4], Martin Kapun[5], Fabian Staubach[6,7], Alan
4 O. Bergland[6,8], Dmitri A. Petrov[6], Brian P. Lazzaro[3] and Paul S. Schmidt[1].
5
6 **Author Affiliations:**
7 1. Department of Biology, University of Pennsylvania, 433 S. University Ave. Philadelphia, PA
8 19104
9 2. Janelia Farms Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive
10 Ashburn, VA 20147
11 3. Department of Entomology, Cornell University 3125 Comstock Hall Ithaca, NY 14853
12 4. Wellcome Trust Sanger Institute, Hinxton, University of Cambridgeshire, CB10 1AS, UK
13 5. Department of Ecology and Evolution, University of Lausanne, Lausanne 1015
14 6. Department of Biology, Stanford University 371 Serra St, Stanford, CA 94305-5020
15 7. Albert-Ludwigs University, Freiburg, Germany
16 8. Department of Biology, University of Virginia, 409 McCormic Rd Charlottesville, VA 22904
17
18 **Corresponding Author:**
19 Emily L. Behrman
20 Email Address: Behrmane@janelia.hhmi.org
21 Mailing address: 19700 Helix Drive
22 Ashburn, VA 20147
23 Phone: 571-209-4000
24
25
26
27

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31
32
33

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34 **Abstract**

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

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54 **Introduction**

55 The rate at which populations respond to environmental change is a fundamental
56 parameter in the process of adaption. Evolution is historically considered to be an innately slow
57 process that occurs over very long timescales [1], but there are now examples that evolutionary
58 change can occur much faster [2-5]. The limits of how fast populations evolve and the genetic
59 architecture underlying rapid evolution remain unclear [6]. The classical approach to infer
60 adaption through the association of traits and genotypes that co-vary along spatial environmental
61 gradients (e.g., latitude, longitude, altitude) [7] can be expanded across temporal environmental
62 gradients to provide insight to the rate of adaption in the wild.

63 The biotic environment may shape the rate of adaptation through the immune system,
64 which sits at the crucial interface between an organism's external and internal environment.
65 Strong selection imposed by pathogens may result in rapid evolution of immune defense in
66 nature because microbiotic infection directly affects host fitness with consequences ranging from
67 resource reallocation away from other functions to host mortality [8-23]. Comparative studies
68 across a broad range of taxa indicate that genes with immune function are among the most
69 rapidly evolving genes in the genome [24-31]. *Drosophila melanogaster* immune genes show
70 evidence of local adaptation across large spatial gradients with high levels of population
71 differentiation and latitudinal enrichment across multiple continents [32-35]. There is less
72 evidence for differentiation at smaller spatial scales [36,37], although some screens of infection
73 response in *D. melanogaster* indicate continental differences in defense quality [36]. Thus,
74 immune defense in natural populations of *D. melanogaster* is a good system to study the how
75 fast natural populations can evolve and genetics underlying the rapid change.

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76 We predict seasonal variation in *D. melanogaster* immune defense even in the absence of
77 established clinal differences in performance. Seasonal climatic changes produce predictable
78 environmental gradients over a temporal scale that select for different phenotypes [38,39] and
79 allele frequencies [40,41] in multivoltine organisms like *D. melanogaster*. Abiotic variables
80 (e.g., temperature) that cycle across seasons can influence microbial growth, so it is possible that
81 microbial communities and pathogen diversity that vary over spatial gradients [42-49] also
82 change as a function of seasonal time [50-53]. Changes in pathogen diversity and frequency
83 across seasons may select for immune resistance or tolerance in either or both of the primary
84 humoral immune pathways: the Toll pathway that is preferentially activated by Gram-positive
85 bacteria or the IMD pathway that is primarily activated by Gram-negative bacteria [54].
86 We tested whether innate immunity evolves seasonally in mid-Atlantic *D. melanogaster*
87 populations in North America (Massachusetts, Pennsylvania, and Virginia). We found that
88 immune defense changed rapidly and repeatedly from spring to fall, and that seasonally cycling
89 alleles of immune genes determine seasonal variation in resistance to and tolerance of infection.
90 We used reconstructed outbred populations to show that epistatic interactions among seasonally
91 cycling SNPs produced the immune phenotypes observed in natural populations. This rapid,
92 cyclic response to seasonal environmental pressure broadens our understanding of the complex
93 ecological and genetic interactions determining the evolution of immune defense in natural
94 populations.
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99 **Methods**

100 **Experimental Model Details**

101 *Wild Drosophila Samples*

102 Wild *D. melanogaster* were collected by direct aspiration both in early July (Spring
103 population) and late October (Fall population) at three locations spaced evenly along a 4º
104 latitudinal gradient: George Hill Orchard in Lancaster, MA (42.500493ºN, -71.563580ºE),
105 Linvilla Orchards in Media, PA (39.884179ºN, -75.411227ºE) and Carter Mountain Orchard in
106 Charlottesville, VA (37.991851ºN, -78.471630ºE). Collections were repeated across two years.
107 Isofemale lines were established from wild-caught inseminated females and were maintained on
108 standard cornmeal molasses food under controlled laboratory conditions (25ºC, 12L:12D) on a
109 three-week transfer cycle for 6-8 generations before immune assessment.

110

111 *Recombinant outbred population cages*

112 Recombinant outbred populations [55]fixed for specific seasonal allele combinations in a
113 randomized genetic background were constructed using lines from the Drosophila Genetics
114 Reference Panel (DGRP) [56]. Ten gravid females from 15 lines were pooled to lay eggs for 48
115 hours for each combination of seasonal alleles. The offspring were permitted to mate freely for at
116 least 10 subsequent non-overlapping generations before immune assessment. This produced
117 populations fixed for the alleles of interest in a heterogeneous unlinked background. The immune
118 function of the two SNPs in *Thioester-containing protein 3 (Tep3)* was tested using three
119 genotypes that combined *2L*:7703202 and *2L*:7705370 (*D. melanogaster* reference genome
120 v.5.39) spring and fall alleles: (1) *Tep3*^{TG} contained spring alleles for both *2L*:7703202 and
121 *2L*:7705370, (2) *Tep3*^{TT} contained the spring *2L*:7703202 and the fall *2L*:7705370 modifier allele
122 and (3) *Tep3*^{CT} contained fall alleles for both SNPs. The final combination of the fall *2L*:7703202

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123 coding allele and the spring *2L*:7705370 modifier allele was too rare in the DGRP to create the
124 recombinant populations. Two independent biological replicate populations were created for
125 each of the three *Tep3* genotypes. Epistatic interactions between *Tep3* and either *Fas-associated*
126 *death domain (Fadd)* or *Drosomycin-like-6 (Dro6)* were assessed in the same way with
127 recombinant outbred populations fixed for either both spring or both fall *Tep3* alleles and either
128 *Fadd* or *Dro6* alleles.
129
130 *Fly husbandry*
131 Flies were reared in standard laboratory conditions (25ºC, 12:12 L:D) at controlled
132 density in vials. Male flies were collected for infection at 3-5d using light CO_2 anesthesia. Flies
133 were stored in groups of 10 after infection.
134
135 **Method Details**
136 *Immune survival*
137 Quality of immune defense was probed using systemic bacterial infection [57] with
138 Gram-negative *Providencia rettgeri* [58] and Gram-positive *Enterococcus faecalis* [59] strains
139 that were originally isolated from infected wild-caught *D. melanogaster*. Post-infection survival
140 was measured in males over two repeated blocks of five consecutive days after infection.
141 Mortality was highest in the first 24h and plateaued (Figure S2) so the final mortality 5d post
142 infection was analyzed in the model. Flies were infected with cultures started with a single
143 colony grown to saturation in LB media at 37ºC with shaking overnight and diluted to A_{600nm} of
144 1.0. Infections were delivered at a dose of 10^3 to 10^4 bacteria to each CO_2-anesthetized fly by
145 inoculating the lateral thorax with a 0.15 mm minute pin (Fine Scientific Tools) dipped into

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146 bacterial culture [57]. Two controls were used: a sterile wound by a needle disinfected in 95%
147 ethanol and unwounded flies anesthetized on CO_2 for the duration of the infection.
148
149 *Bacterial load*
150 The systemic bacterial load of infected flies was quantified using the same infection
151 method as was described above for survival of infection. When evaluating the natural
152 populations, 20 lines from each of the 3 collection locations were infected during a 9a-12p daily
153 infection window. All infections were repeated over two consecutive days by two infectors and
154 the infector and infection order was randomized daily using a random number system. Twelve
155 males from each line were infected each day and maintained in vials with food at 25ºC,
156 12:12(L:D). The infected flies were measured for bacterial load at 24h after infection. Up to 3
157 replicate groups of 3 flies were homogenized in 500 mL of LB for the 2012 natural populations
158 and up to three single flies were homogenized in 500 mL of PBS for the 2014 natural and
159 recombinant populations. The samples were then plated on LB agar plates at a dilution of 1:100
160 for *P. rettgeri*, 1:10 for *E. faecalis* natural populations and 1:1 for the recombinant populations
161 using a Whitley Automatic Spiral Plater (Don Whitley Scientific, Shipley, UK). The plates were
162 incubated overnight at 37ºC and the number of colony forming units on each plate was counted
163 using the ProtoCOL3 automated plate counter (Synbiosis, Cambridge, UK). The number of
164 colonies was used to calculate the concentration of bacteria in each homogenate.

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165

166 *Expression data*

167 Expression differences were determined using a published dataset of RNA-seq on 192

168 inbred sequenced lines from the DGRP [60]. We extracted the expression levels for *Tep3*, *Dro6*

169 and *Fadd* and used the sequence data from [56] to identify the *Tep3*, *Dro6* and *Fadd* haplotypes.

170

171 **Quantification and Statistical Analysis**

172 *Phenotypic statistical analyses*

173 All statistical analyses were performed using the R software (v 3.2.2; The R core team

174 2012). Post-infection survival was measured daily and the survival 5 days post infection was

175 analyzed using a binomial linear regression. The mean proportion of surviving infected flies was

176 standardized by the survival under sterile wound control treatment and then was evaluated using

177 the following model:

178

179 Survival / Control survival = Year*Population*Season + Line + Replicate

180

181 Population, year and season were considered as fixed effects and the random effects of replicate

182 and line were nested within season within population within year.

183

184 The number of colonies is used to calculate the concentration of bacteria in each

185 homogenate. The concentrations were log transformed and then analyzed using mixed-model

186 ANOVAs as follows:

187

188 $\log_{10}$(count/mL) = Year*Population*Season + Line + Replicate

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189

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

193

194 *Seasonal SNPs*

195 Seasonal immune SNPs were identified by screening for alleles that fluctuate in
196 frequency as a function of seasonal time [61] in 88 genes known to have immune function [62].
197 The seasonal SNPs were cross-referenced with a group of paired spring and fall samples
198 collected from 10 populations along the North American cline by the *Drosophila* Real Time
199 Evolution Consortium (Dros-RTEC 12 unpublished samples; https://sites.sas.upenn.edu/paul-
200 schmidt-lab/pages/opportunities). Additional information was collected on each SNPs including
201 a clinal q-value [61] and a p-value in a genome wide association study to identify SNPs involved
202 with *P. rettgeri* pathogenic infection [63]. Enrichment for immune genes was calculated using
203 customized python scripts that compared proportion of seasonal and non-seasonal immune genes
204 to control genes that were matched for size and position using χ^2 with 10,000 bootstrap
205 iterations.

206 Linkage disequilibrium (LD) among the candidate seasonal immune SNPs was calculated
207 in the DGRP using allelic correlation of physical distances using the LDheatmap package [64] in
208 *R*. The 205 sequenced inbred lines of the DGRP were used to examine LD among all of the
209 candidate SNPs by chromosome [56].

210

211 *Seasonal genotypes*

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212 The genotypes from wild populations were determined using a panel of inbred lines
213 originally collected in Pennsylvania in the spring and autumn of 2012. The lines were inbred by
214 full-sib mating for 20 generations and subsequently sequenced. Genotype deviation was
215 calculated as the difference between observed frequency and a predicted frequency based on the
216 individual alleles. The haplotype distribution of *Tep3* was calculated for SNPs with a minor
217 allele frequency greater than 0.1 using integer joining networks[65] in PopArt vs. 1.7 [66].
218
219 *Expression data*
220 The expression data for *Tep3*, *Dro6* and *Fadd* was extracted from an RNAseq dataset of
221 the DGRP [60]. The lines were sorted by genotype based on the published DGRP data [56]
222 and differences among haplotypes was analyzed using a Welsh t-test in R.
223
224 **Results**
225 *Geographic differences in immunity*
226 The geographic origin of the *D. melanogaster* population across the latitudinal transect
227 determined survival post infection but did not predict systemic bacterial load sustained by flies
228 infected with either pathogen. While survival after *P. rettergi* infection directly depended on the
229 latitude at which the population was collected ($\chi^2_{(2)}$=12.805, p=5.87^{-4}), geographic origin and
230 season of collection had a combined effect on survival after *E. faecalis* infection ($\chi^2_{(2)}$=10.035,
231 p=6.62^{-3}). Survival after *E. faecalis* infection was higher in the lower-latitude Virginia
232 population in the spring but the clinal difference disappeared in the fall (Figure 1 A-B). The
233 high-latitude Massachusetts and Pennsylvania populations had similar load and survival after *P.*

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234 *rettgeri* infection and exhibited a greater seasonal change in both survival and bacterial load
235 compared to the lower-latitude Virginia population (Figure 1 C-D).
236
237 *Immunity changes rapidly within a population over seasonal time*
238 Immune defense changed rapidly across approximately 10 generations in the wild from
239 spring to fall. The relationship between bacterial load and survival varied between source
240 population and seasonal collection in a pathogen-specific way (Figure 1). Spring populations
241 were more resistant to *E. faecalis* bacterial growth ($F_{(1, 219)}$=87.758, p<0.0001) and maintained
242 low load with marginally higher survival rates ($\chi^2_{(1)}$=3.201, p=07.36^{-2}), while the fall populations
243 infected with the same bacteria did not restrict bacterial growth as effectively, resulting in high
244 load and high mortality (Figure 1 A-B). However, the converse relationship occurred when flies
245 were infected with *P. rettgeri*: higher survival in the spring ($\chi^2_{(1)}$=16.145, p=5.87^{-4}) despite
246 higher bacterial load ($F_{(1, 215)}$=4.3404, p<0.0001) and high mortality in the fall even though the
247 bacterial growth was restricted to low levels (Figure 1 C-D).
248
249 *SNPs in immune genes oscillate across seasonal time*
250 Immune genes as a functional category were not enriched among genes carrying
251 polymorphisms that oscillate in frequency over seasonal time in these populations [61] when
252 compared to controls matched for size and position. We identified 24 candidate SNPs (Table 1)
253 that oscillate in frequency across seasonal time in these populations [61] located within or in
254 proximity to 13 genes that are known to be involved in immune function [67]. Candidate
255 immune genes containing seasonal SNPs were distributed across all levels of the humoral innate
256 immune pathway: two genes in recognition receptors involved with the detection of pathogens,

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257 six genes in the signaling cascades and five effector proteins that contribute directly to bacterial
258 killing (Table 1).
259
260 *Seasonally oscillating Tep3 SNPs have functional differences in immunity*
261 Over 1/3 of the seasonally variable SNPs near immune genes were near *Tep* family
262 genes, with *Tep* homologs comprising 1/4 of all of the seasonally variable immune genes. *Tep3*
263 contained numerous seasonally oscillating loci with high LD across the 2.5 kb region in which
264 the seasonal alleles are located in the DGRP (Figure 2B). There were two primary sequence
265 haplotypes carrying spring $Tep3^{TG}$ variants and two sequence haplotypes carrying the fall $Tep3^{CT}$
266 variants in the Pennsylvania orchard (Figure 3F, Table S2). We tested the function of these SNPs
267 using recombinant outbred populations with two loci as markers: the non-synonymous coding
268 change at *2L*:7703202 that is surrounded by five intronic seasonal SNPs and the intronic SNP
269 *2L*:7705370 that is 2 kb downstream from the cluster (*D. melanogaster* reference genome
270 v.5.39). Alleles of the intronic SNP at *2L*: 7703202 were non-randomly distributed with respect
271 to karyotype: in both of the independent DGRP and Pennsylvania populations, we observed that
272 the fall allele (C) was strongly associated with *In(2L)t*. In contrast, the spring allele (T) occurred
273 mostly in a standard arrangement genetic background (Fisher's exact test; $p<0.0001$).
274 *2L*:7705730 had no significant association with either arrangement of *In(2L)t* (Fisher's exact test;
275 $p=0.161$).
276 There was no difference among the *Tep3* recombinant outbred populations in bacterial
277 load, but there was differential survivorship after infection with both Gram-positive and Gram-
278 negative pathogens. Flies containing the spring $Tep3^{TG}$ haplotype had higher survival than those
279 containing the fall $Tep3^{CT}$ or mixed $Tep3^{CG}$ haplotypes when infected with Gram-positive *E.*

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280 *faecalis* ($\chi^2_{(2)}$=6.73, p=0.0346; Figure 3A). The *Tep3* SNPs are associated with an additive effect
281 on survival of Gram-negative *P. rettgeri* infection with higher survival in flies containing the fall
282 haplotype than those containing the spring haplotype and intermediate survival in flies
283 containing the mixed haplotype ($\chi^2_{(2)}$=3.651, p=0.161, Figure 3B). Flies containing the seasonal
284 *Tep3* haplotypes have no difference in *Tep3* expression in the absence of infection ($F_{(3, 360)}$=
285 1.419 p= 0.239, Figure 3C) based on previously published RNAseq expression of the DGRP
286 lines [60].
287
288 *Epistasis among AMP genes involved in rapid seasonal adaptation*
289 We tested whether additional seasonal SNPs in the immune pathways interact with *Tep3*
290 to facilitate rapid immune evolution across seasons. We examined epistasic interactions in
291 immune function between *Tep3* and a seasonally cycling immune SNP (*3L*:3334769, an
292 upstream modifier of *Drosomycin-like 6* (*Dro6*)), that was shown to significantly affect
293 resistance to *P. rettgeri* in a genome-wide association study [63]. We also tested epistasis among
294 the *Tep3* SNPs and *3R*:17861050, a 3' UTR modifier in the signaling gene *Fas-associated death*
295 *domain ortholog* (*Fadd*, also known as *BG4*), which was the only SNP that demonstrated
296 concordant patterns between seasonal change and latitudinal differentiation (Figure 2A, Table 1).
297 There was no difference in immune defense among recombinant populations containing
298 combinations of *Tep3 and Fadd*, but the non-additive interactions among recombinant
299 populations containing *Tep3* and *Dro6* alleles begin to explain more of the complexity of
300 immune defense of natural populations (Figure 4A-D).
301
302

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303 **Discussion**

304 *Natural populations differ in immunity over geographic space and across seasonal time*

305 We show that immune response differs among populations across space and time. Season
306 of collection is a strong predictor of the immune response across the geographic locations that
307 span 4º latitude with a seasonal decline in resistance to *E. faecalis* and a seasonal decline in
308 tolerance of *P. rettgeri* infection. The change in immunity across seasonal time occurs rapidly
309 within each geographic location with approximately 10 generations between the spring and fall
310 collections. The repeated seasonal change in immune defense is consistent with previous
311 findings for other measurements of stress resistance [38,39]. Together this suggests that the harsh
312 winter selects for a suite of traits that produce a robust spring population and that selection on
313 those traits is relaxed during the summer producing a less stress resistant population in the fall.

314 Although the strongest differentiation of immunity occurred across seasonal time, there
315 was also a signal of geography along the sampled spatial gradient. Our results contrast with
316 previous studies that did not detect a robust association between latitude and survival [68] or
317 load [36,62]. The difference may be attributed to the interaction between season and latitude. It is
318 possible that geographical differences in immune response may be even greater across a longer
319 distance that may capture a larger difference in pathogen diversity [42-49].

320 The repeatability of the change in immune defense across replicate years and locations
321 indicate deterministic evolutionary processes. Rearing the lines for multiple generations in a
322 common laboratory environment that is distinct from the external sample sites removes
323 environmental variation and ensures that differences among collections and populations can be
324 attributed to genetic diversity among the source populations. It is possible that gene flow due to
325 migration from other latitudes contributes to the differences between the spring and fall

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326 populations. However, migration is unlikely to be the primary cause underlying seasonal immune
327 differences because the latitudinal differentiation was weak compared to seasonal change.
328 Furthermore, infection with different pathogens resulted in opposing clinal patterns but parallel
329 change across seasons. Additionally, migration alone appears insufficient to explain genome-
330 wide differences in allele frequency profiles that characterize spring and fall populations in
331 Pennsylvania orchard [61]; thus, migration is unlikely to explain the seasonal differences in
332 immune response. Wild *Drosophila* populations live in a heterogeneous environment and evolve
333 rapidly in response to environmental parameters that change with season [38,39], potentially
334 including rapid turn-over in microbial and pathogen communities (Figure S2).
335
336 *SNPs in immune genes oscillate across seasonal time*
337 The changes in immune defense are due to differences in genes with immune function
338 across space and time. Genomic screens show that immune genes are enriched across latitudinal
339 gradients [32-35], but we did not find enrichment among immune genes in SNPs that cycle in
340 frequency with season. Seasonal differences in immunity could arise from variation in genes that
341 are not classically identified as part of the immune system and were not detected from our
342 screen. However, the *D. melanogaster* immune system is well characterized and changes in even
343 a single immune gene could affect the phenotypic response to infection even without enrichment
344 for all immune genes. Alternatively, the immune changes may be controlled by non-additive
345 genetic interactions that would not be identified in the enrichment analysis.
346
347 *Immune survival of flies containing seasonally oscillatingTep3 haplotypes*

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348 The patterns in the recombinant outbred populations were consistent with the seasonal
349 patterns in natural populations: spring populations and flies containing the spring *Tep3* haplotype
350 both had a higher defense against Gram-positive *E. faecalis* whereas fall populations and flies
351 containing the fall *Tep3* haplotype had higher defense against Gram-negative *P. rettgeri*.
352 Opposite survival patterns for flies with spring and fall *Tep3* haplotypes were consistent with
353 antagonistic pleiotropy [69] within the branches of the immune system limiting the host such that
354 improvements in response to one class of pathogens (e.g., Gram-negative bacteria) restrict the
355 ability to respond to other pathogens (e.g., Gram-positive bacteria). Trade-offs within the
356 immune system occur in several insect systems between humoral antimicrobial peptides that
357 combat microbial infections and phenoloxidase that is deployed against eukaryotic parasites
358 [14,70,71] as well as in the T helper cells of the vertebrate immune system (reviewed in [72]).
359 We hypothesize that genetic variation for allocation of either immune activity may be maintained
360 if the risk of pathogenesis changes over space or time. The genotypes have pathogenic-specific
361 genetic effects. Additivity among the loci in response to *P. rettgeri*, but a non-additive response
362 to *E. faecalis*, suggests that the fall allele at *2L*:7705370*,* or genetic variants linked to it, has a
363 dominant effect that decreases survival to *E. faecalis* infection.
364 Our data suggest that these *Tep3* loci are natural variants in immune tolerance because
365 flies containing the haplotypes with the same infection load had differential survivorship. The
366 molecular function of the seasonal loci in *Tep3* remains unclear. *Tep* proteins are α-
367 macroglobulin protease traps that bind to pathogen surface and act as opsonins [73-75]. The
368 polymorphism at *2L*:7703202 produces a nonsynonymous Ala/Val polymorphism at residue 18,
369 but both amino acids produced are hydrophobic. The intronic SNP at *2L*:7705370 is directly
370 upstream of the exon cassette region and may regulate expression, but *Tep3* is constitutively

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371 expressed and not strongly induced by *E. faecalis* or *P. rettgeri* infection [76]; B.P. Lazzaro
372 unpublished data). Therefore, the SNPs we examined may most appropriately be considered as
373 markers for a larger haplotype that contains the causal variants.

374 Pathogen-specific higher survival associated with the spring and fall *Tep3* haplotypes
375 may increase their frequency in the wild compared to flies containing a combination of spring
376 and fall alleles. Inversions could theoretically maintain the LD that preserves the high-fitness
377 spring and fall haplotypes [77,78], but this is unlikely because the *In(2L)t* inversion that contains
378 *Tep3* does not cycle with season [61,79]. Additionally, *Tep3* is not located near a recombination-
379 limiting breakpoint of In(2L)t nor is it in LD with other seasonal immune SNPs within the
380 inversion. However, we found that in two independent populations alleles of the intronic SNP at
381 *2L*: 7703202 were non-randomly distributed with respect to karyotype while *2L*:7705730 had no
382 significant association with either arrangement of *In(2L)t.* LD might be created and maintained
383 by selection against recombinant phenotypes either due to lower immunocompetence or another
384 pleiotropic trait or because of intraspecific genetic incompatibilities. Deleterious
385 incompatibilities maintain distinct haplotypes in *Arabidopsis thaliana* NLR immune receptors
386 [80] and may also explain the near absence of the $Tep3^{CG}$ combination of spring and fall alleles
387 in all populations examined. Flies containing the $Tep3^{CG}$ haplotype appear three times across the
388 haplotype tree constructed from the seasonal Pennsylvania inbred lines, suggesting that the
389 haplotype may form occasionally through recombination but does not proliferate in the
390 population. Thus, it is likely that selection for the immune benefits of the spring and fall
391 haplotypes and against the combination of spring and fall alleles maintains these distinct
392 haplotypes in the wild. While these *Tep3* haplotypes explained some of the seasonal differences

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393 in immune tolerance of natural populations, other seasonally changing genes may also contribute
394 to the observed differences in bacterial resistance in natural populations
395
396 *Epistasis among AMP genes involved in rapid seasonal adaptation*
397 Intergenic epistatic interactions between *Tep3* and *Dro6* suggest that season-specific
398 genotypes have highest fitness. In our experiment, flies having all spring or all fall alleles had
399 higher survival after infection while flies that contained a combination of spring and fall had
400 higher mortality. This suggests that complex genetic interactions shape winter and summer
401 fitness with distinct haplotypes maintained by non-additive epistatic interactions [81-83].
402
403 *Conclusions*
404 With this work, we demonstrate that pathogen-specific innate immunity evolves rapidly
405 in natural populations of *D. melanogaster* across replicate years and geographic locations.
406 Comparative studies across species and among populations have indicated that immune genes
407 evolve faster than other genes in the genome, but the rapid phenotypic and genetic change we
408 observed over approximately 10 generations is a substantially faster rate than previously
409 considered. We tested a small subset of the immune SNPs that oscillate in allele frequency over
410 seasonal time and observed intra- and inter-genic interactions consistent with changes in immune
411 tolerance and resistance across seasons in natural populations, perhaps in response to seasonally
412 changing bacterial communities. Epistatic interactions among seasonally oscillating immune
413 alleles may help facilitate this rapid phenotypic change over a short seasonal timescale. This
414 rapid, cyclic response to biotic variables broadens our understanding of the complex ecological
415 and genetic interactions in the evolutionary dynamics of natural populations.

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416

417 **Author Contributions**

418 ELB, VMH, BPL & PSS designed the project. ELB & PSS collected the wild samples and ELB
419 & VMH performed the infections. FS analyzed the microbial communities and AOB and DAP
420 inbred and sequenced the seasonal lines used for genotypes in natural populations. ELB, MK and
421 PSS did the data analyses. ELB, VMH, MK, FS, AOB, DAP, BPL and PSS wrote the paper.

422

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675 **Figure Legends**
676
677 Figure 1. Immune defense relationship between bacterial load and survival in natural spring and
678 fall populations. Isofemale lines (small, outline) were used to calculate population mean (large,
679 filled) from natural orchard populations collected along a latitudinal gradient in Massachusetts
680 (circle) Pennsylvania (triangle) and Virginia (square) in the spring (blue) and fall (red) for two
681 replicate years: 2012 (A & C) and 2014 (B & D). Immune defense was probed with two natural
682 pathogens: a gram-positive bacterium *Enterococcus faecalis* (A&B) and a gram-negative
683 bacterium *Providencia rettgeri* (C&D). Twenty isofemale lines from each collection were
684 measured for 5-day survival after infection and bacterial load at 24 hours post-infection scaled by
685 average load for the experiment.
686
687 Figure 2. Seasonal changes in immune genes in natural populations. (A) Manhattan plot of SNPs
688 in immune genes that change in frequency as a function of seasonal time with a zoom in on
689 *Tep3*. The red line indicates the seasonal q-value cutoff >0.3[61] and all immune genes that have
690 significant SNPs are labeled by name on the x-axis. The SNPs on which functional analyses were
691 performed are highlighted: *Fadd* (pink square), *Dro6* (yellow circle), *2L*:7703202 (upwards cyan
692 triangle) and *2L*:7705370 (downwards blue triangle). (B) Heat map showing linkage
693 disequilibrium (LD) among SNPs in immune response genes across each chromosome. Linkage
694 disequilibrium calculated as allelic correlation between the physical distances of *2L*:7703202 and
695 *2L*:7705370 in the DGRP is r^2=0.8138. (C) Cycling of seasonal allele frequencies of candidate
696 immune SNPs across three years. (D) Allele frequencies of candidate SNPs across the latitudinal
697 gradient in the eastern United States. Only *Fadd* shows clinal variation with a clinal q-value of
698 0.006.

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700 Figure 3. Functional difference of seasonal *Tep3* alleles as defined by the focal SNPs. Mean +/-
701 SE for bacterial load 24 hours post infection and survival 5 days post infection for the *Tep3*
702 genotypes. (A) Higher survival for the spring genotype than the fall or combination genotypes
703 when infected with *E. faecalis*. (B) Additive effect of alleles when infected with *P. rettgeri* (C)
704 Lower constitutive *Tep3* mRNA expression in the rare *Tep3*^{CG} haplotype in flies from the DGRP.
705 (D-E) Frequency of *Tep3* haplotypes in the Pennsylvania orchard across seasonal time. (F)
706 Minimum spanning network illustrates that linkage disequilibrium among the SNPs is
707 maintained in distinct haplotypes.

708

709 Figure 4. Intergenic interactions among *Tep3, Dro6,* and *Fadd*. Non-additive interaction among
710 *Tep3* and *Dro6* alleles. (A-B). No significant interaction among *Tep3* and *Fadd* SNPs (D-E).

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Tables and Figures

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]. The frequency of the SNPs at each collection date is indicated.

Gene	Position	Effect	Molecular Function	SQ	CQ	GWAS	PA 7.09	PA 11.09	PA 7. 10	PA 11.10	PA 7.1	PA 10.11	PA 11.11
Tep2	2L:2834400	Upstream modifier	effector	0.242	0.956	0.253	0.887	0.746	0.889	0.617	0.846	0.694	0.776
Tep3	**2L:7703202**	**NS coding**	**effector**	**0.243**	**0.159**	**0.420**	**0.657**	**0.356**	**0.515**	**0.361**	**0.500**	**0.424**	**0.590**
Tep3	2L:7703509	Upstream modifier	effector	0.151	0.529	0.084	0.840	0.567	0.813	0.667	0.838	0.677	0.694
Tep3	2L:7703518	Upstream modifier	effector	0.220	0.643	0.084	0.825	0.554	0.803	0.671	0.831	0.710	0.706
Tep3	2L:7703748	Upstream modifier	effector	0.271	0.819	0.114	0.827	0.524	0.700	0.661	0.750	0.569	0.818
Tep3	2L:7703757	Upstream modifier	effector	0.291	0.956	0.632	0.748	0.476	0.488	0.541	0.664	0.367	0.618
Tep3	**2L:7705370**	**Upstream modifier**	**effector**	**0.219**	**0.163**	**0.385**	**0.479**	**0.158**	**0.255**	**0.240**	**0.457**	**0.273**	**0.444**
bsk	2L:10247834	Intron	signaling	0.300	0.822	0.255	0.716	0.680	0.571	0.500	0.826	0.470	0.778
bsk	2L:10252450	Intron	signaling	0.257	0.749	0.962	0.145	0.369	0.261	0.358	0.355	0.497	0.308
Tep1	2L:15887030	Downstream modifier	effector	0.227	0.188	0.089	0.590	0.841	0.647	0.889	0.732	0.846	0.789
Tep1	2L:15888031	Downstream modifier	effector	0.221	0.520	NA	0.000	0.368	0.063	0.360	0.000	0.013	0.012
cact	2L:16309682	Downstream modifier	signaling	0.135	0.782	0.829	0.850	0.667	0.649	0.426	0.704	0.407	0.474
cact	2L:16310896	Downstream modifier	signaling	0.235	0.635	0.375	0.671	0.432	0.700	0.239	0.533	0.441	0.552
cact	2L:16318067	Intron	signaling	0.281	0.719	0.335	0.592	0.474	0.550	0.382	0.551	0.256	0.627
sick	2L:19923496	Intron	signaling	0.232	0.032	0.505	0.096	0.047	0.130	0.048	0.328	0.053	0.269
IM1	2R:14270817	Upstream modifier	effector	0.256	0.695	0.423	0.358	0.075	0.571	0.193	0.213	0.115	0.390
Dro6	**3L:3334769**	**Upstream modifier**	**effector**	**0.201**	**0.427**	**0.000**	**0.770**	**0.613**	**0.814**	**0.612**	**0.798**	**0.489**	**0.625**
Drs-l	3L:3336529	Upstream modifier	effector	0.251	0.975	0.028	0.778	0.483	0.893	0.768	0.783	0.682	0.813
GNBP1	3L:18671289	Downstream modifier	recognition	0.187	0.150	0.729	0.116	0.458	0.240	0.393	0.230	0.315	0.271
GNBP2	3L:18671295	Downstream modifier	recognition	0.218	0.167	0.666	0.144	0.472	0.255	0.407	0.257	0.344	0.294
Fadd	**3R:17861054**	**UTR 3'modifier**	**signaling**	**0.200**	**0.006**	**0.822**	**0.669**	**0.250**	**0.369**	**0.353**	**0.638**	**0.411**	**0.410**
Fadd	3R:17861073	UTR 3'modifier	signaling	0.287	0.425	0.712	0.734	0.351	0.407	0.407	0.613	0.467	0.459
kay	3R:25600668	Intron	signaling	0.200	0.588	0.743	0.686	0.453	0.607	0.464	0.636	0.383	0.475
Tak1	X:20388404	Intron	signaling	0.227	0.326	0.964	0.575	0.032	0.273	0.135	0.271	0.150	0.217

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715 **Supplemental Material**

716

717 Figure S1. Related to Figure 1 Post infection survivorship curves for 5 days post infection across
718 seasonal time. Population mean +/- SE.

719

720 Figure S2. Related to Figure 1 Microbial community associated with wild and F1 *Drosophila*
721 *melanogaster* changes over space and time. *D. melanogaster* samples were collected as part of
722 the *Drosophila* Real Time Evolution Consortium (Dros-RTEC 12 unpublished samples;
723 https://sites.sas.upenn.edu/paul-schmidt-lab/pages/opportunities). DNA was extracted as
724 described in [84]. Analysis was performed using a customized MOTHUR (v.1.36.0) [85] script
725 that is available upon request. *Wolbachia* sequences were removed from the analysis.

726

727 Table S1. Related to Figure 3. Tep3 haplotypes in the 2012 Pennsylvania population. Focal
728 SNPs are highlighted in black and the genotype combinations are highlighted: spring (blue), fall
729 (red), high-frequency combination (purple), rare combination (grey).

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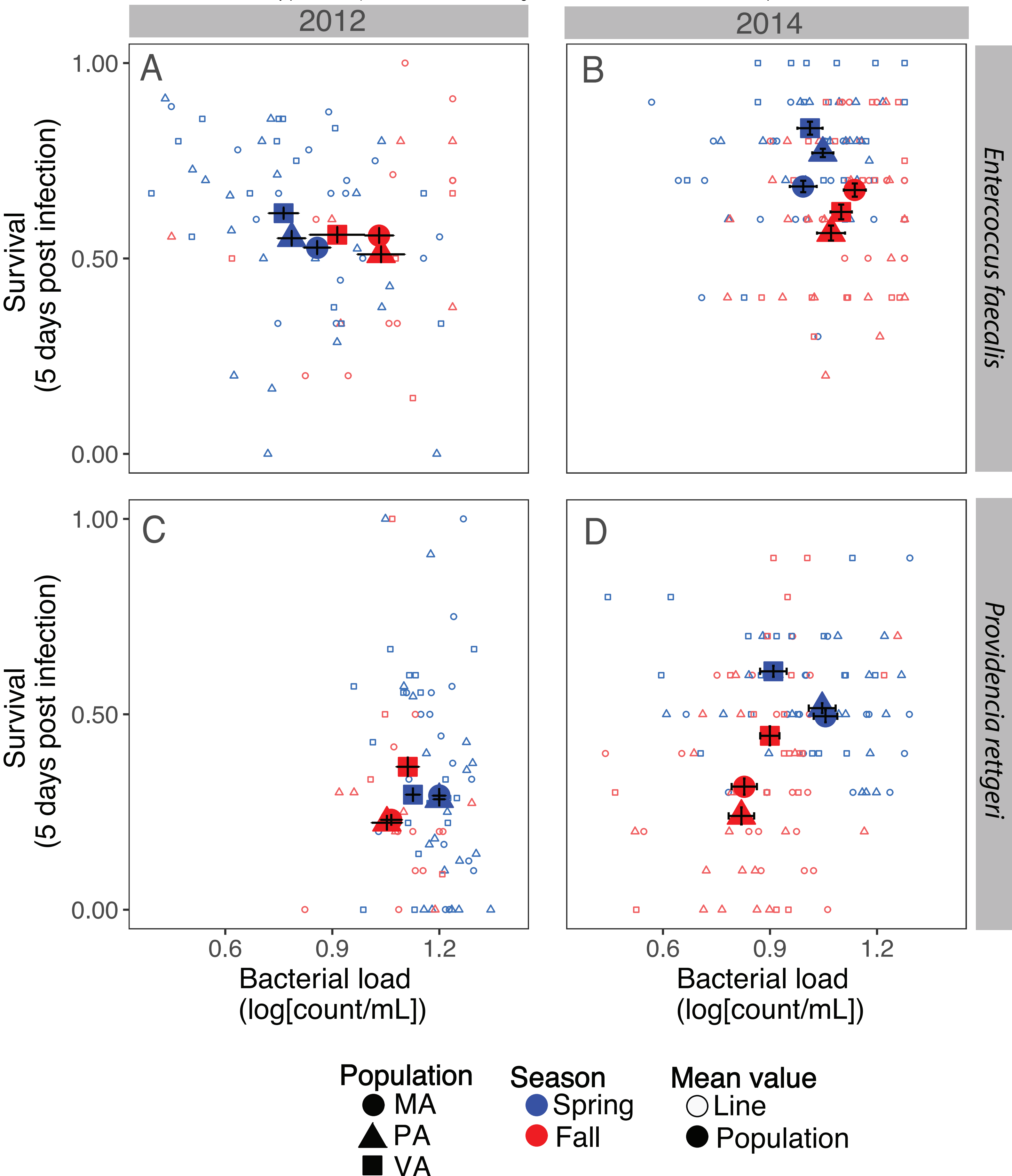

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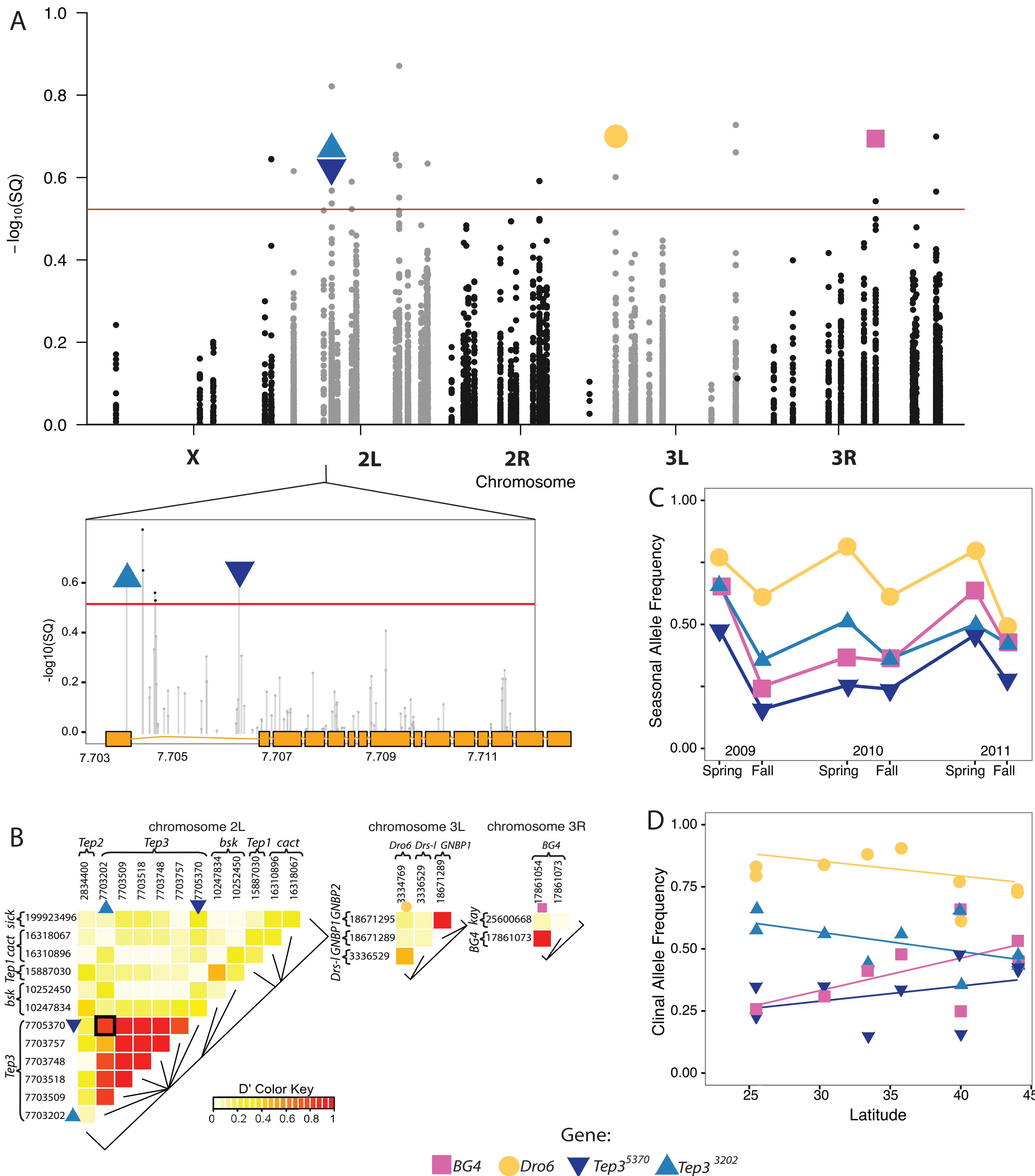

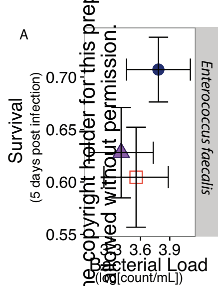

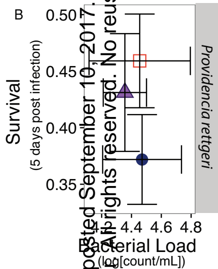

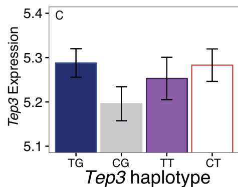

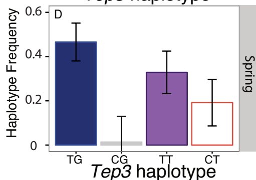

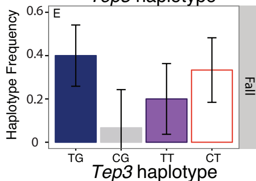

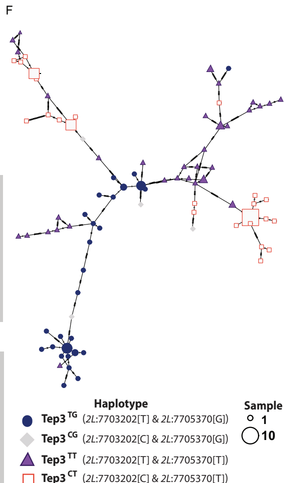

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