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# Genome-wide association study identifies genes and networks that influence innate immune response in an age-specific manner in *Drosophila melanogaster*

**Shonda Campbell**

University of Maryland, Baltimore County

**Isabella Gudino**

University of Maryland, Baltimore County

**Mary Rhee**

University of Maryland, Baltimore County

**Jeff Leips** (✉ [leips@umbc.edu](mailto:leips@umbc.edu))

University of Maryland, Baltimore County

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## Research Article

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# Abstract

## Background

The innate immune response is an evolutionarily conserved process that is essential for survival in multicellular organisms. As individuals age, immune functions decline, a phenomenon known as immunosenescence, reducing one's ability to fight infections. While immunosenescence is a universal feature of aging, the rate at which immune functions decline with age varies greatly among individuals and this variation has a genetic component. However, we have limited knowledge of the actual genes that contribute to this variation.

## Methods

Here, we used 183 genetically distinct genotypes of the *Drosophila* Genetic Reference panel (DGRP) to assess their ability to clear an infection at one and five weeks of age. We then carried out a genome-wide association study (GWAS) to identify candidate genes that contribute to differences in immune responses among genotypes at each age.

## Results

We found that, on average, the ability to clear infection declined by 70% with age. However, the effect of age on clearance ability varied significantly among genotypes. We identified a total of 242 single nucleotide polymorphisms (SNPs) and 107 candidate genes associated with variation in clearance ability. Polymorphisms in 48 genes were associated with clearance in 1 week old flies and fifty-nine genes were associated with clearance ability at 5 weeks of age. Only one gene, a G-coupled protein receptor, *CG31760*, was a candidate at both ages. Of the 107 candidate genes, 25 were mapped to genetic networks.

## Conclusion

Our results identify candidate genes that could be targets for age-appropriate therapeutic treatments to maintain or restore immune function in the elderly.

## Background

The innate immune response is the first line of defense against infection for all multicellular organisms and is critical for survival.<sup>1–4</sup> In natural populations, the effectiveness of the immune response varies among individuals and this variation has a significant genetic component<sup>3, 5–9</sup>. Our previous studies also indicated that the effects of genes that contribute to natural variation in innate immune responses are

age-dependent<sup>5,8</sup>. This has important implications for treatment of age related declines in immune function<sup>1,10–14</sup>, as the genes that should be targeted may depend on the age of the individual. Much of the work in this area has focused on understanding how variation in transcription levels following infection affects immune function<sup>5,8,9,15,16</sup>. Less is known about how variation at the level of the DNA sequence contributes to age-dependent differences in immune function among individuals

Here, we measured the ability of young (1 week) and old (5 week) female virgin flies to clear an infection by *Escherichia coli* (*E.coli*) using 183 genotypes of the *Drosophila* Genetic Reference Panel<sup>17,18</sup>. We then used a genome wide association (GWA) test to identify single nucleotide polymorphisms (SNPs) and candidate genes that contribute to the variation in the clearance ability among genotypes at each age. We found that immune function decreases significantly with age, but also that the effect of age on the ability of flies to clear bacterial infection varies significantly among genotypes. We identified multiple genes associated with this variation at young and old age, however, only one gene, *CG31760*, a G-coupled protein receptor<sup>19</sup>, was significantly associated with bacterial clearance at both ages. Many of the candidate genes encode integral plasma membrane and cytoplasmic proteins. The plasma membrane and cytoplasm are two essential components involved in, and required for, multiple processes within the innate immune response. This includes phagocytosis, cell-cell interactions and signaling, and so these genes are promising candidates to investigate further. Our results aid in our understanding of the genes that contribute to individual differences in age-dependent immune function that, in turn could aid in the development of more effective, personalized treatments for an aging population.

## Results

### Clearance Ability Varies Significantly Among Genotypes and with Age

We first estimated differences in clearance ability among genotypes assessed at each age, separately, using a random-effects ANOVA. We found that clearance ability varied significantly among genotypes at both ages (wk 1:  $F_{154, 3824} = 2.00$ ,  $P < 0.0001$ ; wk 5:  $F_{154, 3728} = 4.94$ ,  $P < 0.0001$ ; Fig. 1), with the average CFU/mL ranging from 178–11,000 CFU/mL at 1 week of age, and 4,300–23,100 CFU/mL at 5 weeks of age. We also found that on average, across all genotypes, clearance ability declined by approximately 70% with age ( $F_{1,7552} = 1549$ ,  $P < 0.0001$ ; **Supp Fig. S1**). There was also a significant age x genotype interaction, with all but three genotypes (DGRP lines 235, 646, and 853) declining with age, but at different rates ( $F_{154, 7552} = 2.98$ ,  $P < 0.0001$ ; Fig. 2).

We then calculated the coefficient of genetic variation,  $CV_G$ , and genetic correlation,  $r_{GA}$ , to determine the correlation among lines in clearance ability across ages. Although the  $CV_G$  decreased with age, from 73 to 20,  $CV_G$  was higher relative to  $CV_E$  at 5 weeks of age. The  $r_{GA}$  across ages was positive but low, 0.221 (Table 1), indicating that there was a positive but low correlation between the ability of genotypes to clear infection across ages.

Table 1  
Statistical genetic analysis for clearance ability of those lines in common at both ages.

Age (Weeks)	Mean colony forming units/mL (CFU/mL) $\pm$ SE	CV <sub>G</sub>	CV <sub>E</sub>	$r_{GA}$
1	3092 $\pm$ 1430	73	217	0.221
5	10677 $\pm$ 1873	20	74	

CV<sub>G</sub>, coefficient of genetic variation; CV<sub>E</sub>, coefficient of residual variation;  $r_{GA}$ , genetic correlation between clearance ability at 1- and 5-weeks of age.

## GWAS Identified Candidate Genes that Influence Immune Response

Using the LSmeans for each line from the random-effects model, we carried out three separate GWAs of the effect of genotype on clearance ability: one GWA on the 1 week old data (175 lines), one on the 5 week old data (183 lines), and a third that used only those lines for which we had data at both ages (155 lines), hereafter referred to as either age-specific GWA or common-line-GWA, respectively.

For the two age-specific GWAs, we found 67 polymorphisms (7 INDELs and 60 SNPs) and 25 genes associated with clearance ability at 1 week of age, and 73 polymorphisms (7 INDELs and 66 SNPs) and 35 genes associated with clearance ability at 5 weeks of age (Table 2). Most of the SNPs identified at 1 week of age were located in introns (34%) or coding regions (33%) of a gene, while only 3% were located in the 3' untranslated region (UTR) (Supp. Fig. S2A/B). Similarly, the 3' UTR was also where the fewest number of SNPs (2.7%) were located at 5 weeks of age, while close to half (44%) were found in introns and 34% in coding regions (Supp Fig. S2A/B). Interestingly, there were not any genes found in common between the two ages (Supp. File S2).

For the common-line-GWA, we found a total of 196 polymorphisms and 89 genes associated with clearance ability (Table 2). Of those, 92 polymorphisms and 41 candidate genes were significantly ( $p \leq 10^{-5}$ ) associated with clearance ability at 1 week of age, and 104 polymorphisms and 48 candidate genes were significantly ( $p \leq 10^{-5}$ ) associated with clearance ability at 5 weeks of age. Similar to the two age-specific GWAs, nearly half (47%) of SNPs were located in introns, 28% in coding regions, and 2% in the 3' UTR (Supp. Fig. S2A/B). Of the 89 candidate genes identified, only one, *CG31760*, was significantly associated with clearance ability at both ages. Compared to the two age-specific GWAs, 47 additional genes were identified as candidates in the common line GWA. The low genetic correlation between the two ages, combined with the lack of overlap in polymorphisms identified across GWAs, suggests that the SNPs contributing to the variation in clearance ability are age-specific. At a minimum, these results imply that the relative contribution of these polymorphisms to the phenotypic variation changes as the organism ages.

We then combined the results from three GWAs, first removing any overlapping/duplicated polymorphisms (e.g., a SNP located on 3R\_26642288\_SNP, in gene *cindr*, was identified in both the 5 week and common GWA), and then removing any overlapping genes. This resulted in a total of 242 polymorphisms and 107 candidate genes. After removing duplicated polymorphisms and genes, 46% (112) of the polymorphisms were located within introns and 30% (73) in coding sequences, with 45 (40%) and 33 (45%) of those, respectively, found on the third (3R) chromosome, along with 32 (30%) of the identified genes (Fig. 3). Candidate genes from all three GWAs were used in the network analysis below.

Table 2  
GWA of effect of genotype on  
clearance ability from all three GWAs  
combined.

Age (Weeks)	Indels	SNPs	Genes
1	15	99	48
5	12	115	60
Total	27	214	107

For complete GWA outputs, see Supp File S2 and Supp. Table 1.

## Network and Gene Ontology Analysis of Immune Response Genes

Although there was a lack of overlap in candidate genes between the two age-specific GWAs, it is possible that many of them belong to the same genetic network or pathways. However, when we analyzed the candidate genes from the two age-specific GWAs, separately, the majority of the genes could not be mapped to a network (See **Supp. Fig. S3** for the individual genetic networks).

To improve the probability of mapping all potential interactions, we combined all of the candidate genes identified (107 total) in the three GWAs for the network analysis. Of the 107 genes analyzed, a network comprised of 25 candidate genes, along with 14 computationally recruited, or missing genes, was revealed. Only those nodes (genes) connected to another node by an edge (connecting line) are shown in Fig. 4, as there were a few single, unconnected genes removed (for complete networks, see Supp. Fig S3). Fourteen genes (*a*, *con*, *EcR*, *fru*, *gpp*, *lim1*, *kirre*, *mbi*, *pnt*, *RhoGef64C*, *NetA*, *Snoo*, *sn* and *ths*), all with known human orthologs, were significantly associated with clearance ability of older flies.

To better understand and interpret the biological and molecular functions of genes in the network, we performed a Gene Ontology (GO) analysis on the 107 candidate genes. The GO analysis identified two functional clusters, and the most significantly represented ( $p < 0.05$ ) GO terms are shown in Fig. 5 (for the full GO report, see **Supp. File S3**). The 5 week old genes were enriched in roles involved in cell adhesion

(*CG42594*, *CG43373*, *DIP-alpha*, *Rh7*, *dpr8*, and *kirre*), transcriptional regulation (*Lim1*, *EcR*, *pnt*, *NelfE*, and *CG9899*), compound eye development (*a*, *cindr*, *Lim1*, and *NetA*), cell/plasma membrane (*atl*, *CG10550*, *CG42269*, *CG42594*, *CG8852*, *CG42594*, *CG43373*, *con*, *DIP-alpha*, *Rh7*, *dpr8*, *Hs6st*, *side-VIII*, and *kirre*), lymph gland plasmatocyte differentiation (*pnt* and *ths*), and cell-cell junction (*kirre* and *rols*) (**Supp. File S3**). Although none of the 5 week old genes were found within a single, significantly overrepresented pathway, three of the 1 week old genes (*app*, *fred*, and *upd3*) are involved in the Hippo signaling pathway in flies, which controls organ growth as well as plays a role in innate immune response<sup>20</sup>.

Although there was no overlap in the candidate genes associated with immune response at young and old age, many of the genes identified at both ages are of particular interest. First, of the 155 genotypes analyzed, age had a significant effect on clearance ability, with all but 3 genotypes declining with age, yet the genes contributing to this variation were different at each age. Second, many of the genes identified at each age are components of the plasma membrane, functioning as receptors or adhesion molecules, that play an integral part in cytoskeletal reorganization and/or regulate transcription. Third, all of the candidate genes revealed within genetic network have known human orthologs, which would allow us to test their roles in the innate immune response and potentially translate that to human health.

## Discussion

In this study, we evaluated how genetic differences impact the age-related ability to clear a bacterial infection using 183 genotypes of *Drosophila melanogaster*. We used a GWAS to identify genes and genetic networks that influence the age-specific immune response, resulting in three key findings.

First, we found significant variation among genotypes at each age (Fig. 1). These results are consistent with previous findings by Lesser et al. (2006) and Felix et al. (2012)<sup>5,8</sup>. We also found a significant effect of age, with clearance ability worsening, on average, by just over 70%. Two previous studies found different overall effects of age on bacterial clearance. Lesser et al. reported a moderate improvement (17%) in bacterial clearance ability with age when averaged across the 25 genotypes used in their study. Felix et al observed no general effect of age on clearance in their study, which used 20 genotypes. Comparisons of the effects of age in our study with those of the previous studies is slightly misleading for two main reasons. First, due to technical issues, we had to use two different methods of injection. If flies were infected with a higher titer at old age in our study, this could bias our results and overestimate the effect of age on clearance ability. Second, these earlier studies used different genotypes and a much smaller number of genotypes than the current study. Of course, one of the main goals of the current and prior studies was to look at the genotype specific effects of age on immune function. When we do this, by comparing the reaction norms of each genotype and the age by genotype interaction terms from the ANOVAs, each study reached the same general conclusion - the effect of age on bacterial clearance ability differed dramatically among genotypes.

Our second key finding is that we identified several polymorphisms and candidate genes that contribute to variation in clearance ability at each age (Table 2; **Supp File S2**), suggesting that clearance ability is

age-specific, as no genes are overlapping between ages. While this could be due to lack of power, additional lines of evidence suggest that this is not the entire explanation. The first is that age affected clearance ability in a genotype dependent manner. This was reflected in the significant age by genotype interaction and the crossing reaction norms of the clearance ability of each line across ages (Fig. 2). If, instead, the polymorphisms had the same relative contribution to clearance ability at both ages, the rank order of clearance ability of the lines would not be expected to change so dramatically with age. The second line of evidence is that the genetic correlation of clearance ability across ages was fairly low (0.221; Table 1). While the correlation is not 0, it is also not close to 1, indicating that the ability of a genotype to clear a bacterial infection at one age is not predictive of its ability to clear infection at the other. If the magnitude of the effect of a polymorphism on clearance ability changes with age, then these two outcomes would be predicted.

The third key finding was that we were able to map many of the genes to a global network (Fig. 4). GO analysis of the candidate genes revealed several different processes, with the most significantly represented GO term enriched with genes involved in cell adhesion, with 6 candidate genes (*CG42594*, *CG43373*, *DIP-alpha*, *Rh7*, *dpr8*, and *kirre*) being of those identified at 5 weeks of age (**Table 3; Supp. File S3**). Other significantly overrepresented GO terms that included 5 week old candidate genes included cellular components such as the cell/plasma membrane (*atl*, *CG10550*, *CG42269*, *CG42594*, *CG8852*, *CG42594*, *CG43373*, *con*, *DIP-alpha*, *Rh7*, *dpr8*, *Hs6st*, *side-VIII*, and *kirre*), or molecular functions such as transcriptional regulation (*Lim1*, *EcR*, *pnt*, *NelfE*, and *CG9899*) and compound eye development (*a*, *cindr*, *Lim1*, and *NetA*) (**Table 3; Supp. File S3**).

This lack of overlap prompted us to compare our GWA results to the list of candidate genes identified by Felix et. al. (2012). Although Felix et. al. did not use the DGRP lines, they did look at differences in clearance ability of 20 different inbred fly lines with age and assessed the association between gene expression in response to infection using 12 of those lines. They measured the expression of more than 1300 genes and found that about half (678) of the genes were upregulated and the other half (665) were downregulated with age<sup>5</sup>. Of those, 139 upregulated genes were associated with a reduced clearance ability, while 105 downregulated genes were associated with an improved, or better, clearance ability with age<sup>5</sup>. Surprisingly, only 10 candidate genes overlapped with those identified by Felix et. al, with 7 of them (*CG13492*, *CG10550*, *CG31087*, *lim1*, *upSET*, *NetA*, and *Nelf-E*) overlapping with our 5 week candidate genes; the other 3 genes, *Wbp2*, *sens-2*, and *CG34417*, overlapped with our 1 week candidate genes. All 10 overlapping genes were of those whose expression was upregulated, with higher expression levels of *NetA*, *Lim1*, and *upSET* being associated with a reduced clearance ability with age<sup>5</sup>.

*lim1*, *Nelf-E* and *upSET* are components of the nucleus involved in regulating transcription, while *NetA* is found within the cytosol and functions as a developmental protein. This lack of overlap in candidate genes affecting clearance ability across ages and between the study mentioned, could be due, in part, to the regulatory control of gene expression declining with age<sup>21-23</sup>. However, we cannot make a definitive statement about the magnitude and direction in which the expression of these genes are regulated as we



did not measure expression levels, and the previous study did not use the DGRP lines. Future studies are needed to test this hypothesis.

It is important to note that our study was conducted using virgin females. Using males and or mated females may have yielded significantly different results. Other studies that have used males<sup>9,24,25</sup> and/or mated females<sup>21,24, 26–29</sup> have observed differences in immune response, suggesting that sex and mating status, also have an effect and underlying genetic contribution on immune response. For example, Short and Lazzaro looked at differences in virgin vs. mated females and identified many differentially expressed immune-response genes in response to infection between the two<sup>27</sup>. Similar findings on age-dependent genetic effects have been observed in other organisms, such as mice<sup>30–32</sup>, zebra fish<sup>33,34</sup>, and even cows<sup>15</sup>, indicating the importance of identifying the underlying mechanisms that contribute to age-related changes, not only in immune response, but other biological processes as well. Future studies using mated flies of both sexes, and/or a different pathogen, will be necessary to gain a more complete understanding of the genes involved in regulating the age-dependent immune response.

## Conclusion

Overall, our results confirm the influence genetic differences and age have on the immune response. The functional connection between the immune response and genes identified in this study remains to be discovered. However, the genes identified are promising candidates that could lead to improved therapeutic treatments in an aging population, providing age-appropriate drug targets to restore the immune function.

## Materials And Methods

### Drosophila Stocks and Maintenance

We used flies from the *Drosophila* Genetic Reference Panel (DGRP) obtained from the *Drosophila* Stock Center in Bloomington Indiana. The DGRP is made up of 205 inbred lines derived from the natural population of *Drosophila* in Raleigh, NC, and each has been fully sequenced<sup>17,18</sup>. Flies were maintained in vials containing a standard cornmeal-molasses-yeast food, in a temperature controlled room set at 24°C and 50% relative humidity, under a 12-h day/night cycle. Virgin females from each line were collected under light CO<sub>2</sub> anesthesia, and aged to either 1 or 5 weeks of age before the bacterial clearance assay.

### Bacterial Clearance Assay and Statistical Analysis

We measured the ability of flies to clear an infection of *Escherichia coli* (*E. coli*) using a standard clearance assay<sup>5</sup>. One and five week old flies were injected with an *E.coli* solution (strain HB101) grown from a single colony of streptomycin-resistant *E. coli*. *E. coli* was grown in LB broth containing 100 µg/mL streptomycin, to an OD<sub>600</sub> of  $1.0 \pm 0.1$  using a spectrophotometer. This corresponds to a concentration of  $\sim 5.5 \times 10^8$  colony forming units per milliliter (CFU/mL)<sup>5</sup>. Before loading the *E. coli*

solution into a pulled-glass capillary needle, we added green food dye to the solution because it allows for visual confirmation that the fly was injected. One week old flies were injected using an Eppendorf Femtojet® 5247 Microinjector (Eppendorf AG, Westbury, NY, USA). Due to technical problems with the Femtojet unit, five week old flies were injected using the Nanoject II® automatic nanoliter injector (Drummond, cat# 3-000-204). This may have resulted in slightly different injection volumes at the different ages so our results should be interpreted with that in mind.

After 24-hours, surviving flies from each line were individually homogenized in 200  $\mu$ L Ringer's solution. Twenty five  $\mu$ L of the homogenate were spread on LB/agar plates containing 100  $\mu$ g/mL streptomycin using sterilized glass beads, and grown overnight at 37°C. The resulting colonies were counted using an automated ProtoCOL SR 92000 Colony Counter (Synoptics Ltd, UK). Colony counts were then converted to colony forming units per milliliter (CFU/mL). The CFU/ml represents the remaining bacteria in each fly and was used as the phenotype for that individual. We measured bacterial clearance on a minimum of 12–19 individuals for 13 of the lines, and on 20–30 individuals for the other 170 of the lines. In total, we measured the ability of 4307 individuals to clear infection at 1 week of age and 4408 individuals at 5 weeks of age.

All statistical analyses were performed in R 4.1.3 (<https://www.r-project.org/>) using the lme4 package. Outliers that fell outside the 0.995% quantile were removed. Due to the size of the experiment, we injected samples of flies from each line/age on different days (time block). We obtained colony count data from a minimum of 2–3 individuals from all DGRP lines at each time block. To account for any variation due to day of infection (which would include variation in infection load, needle bore size, or the time of day in which individuals were injected, i.e., first vs last fly injected), we incorporated the random effect, block, into our statistical models. First, a random-effects analysis of variance (ANOVA) model,  $Y = \mu + \text{genotype} + \text{block}$ , was performed to test for genetic differences in clearance ability, corrected for block (random), among genotypes at each age, separately, and among the lines in common at both ages. We then used a mixed-effect model:  $Y = \mu + \text{age} + \text{genotype} + \text{age} \times \text{genotype} + \text{block}$  to test for overall differences in clearance ability between the two ages (fixed), among genotypes (random), and differences among genotypes in the effect of age on clearance ability (age  $\times$  genotype interaction, a random effect) for those lines in common at both ages. The least squared mean (LSmean) for each line, at each age (corrected for block), was used for the GWAS described below.

Using the variance components from the random-effects models, we calculated the coefficients of genetic variation ( $CV_G$  and  $CV_E$ )<sup>35–37</sup>.  $CV_G$  was used to estimate the proportion of phenotypic variation (clearance ability) explained by genetic differences among lines, and was calculated as  $100(\sqrt{\sigma^2_L})/\text{mean}$ , with the among-line variance component,  $\sigma^2_L$ , standardized by the average CFU/mL for all lines at each age.  $CV_E$ , which estimates the proportion of phenotypic variation that could not be explained by genetic differences among lines, was calculated as  $100(\sqrt{\sigma^2_E})/\text{mean}$ , where  $\sigma^2_E$  is the residual variance, standardized by the average CFU/mL for all lines at each age.

We also calculated the genetic correlation ( $r_{GA}$ )<sup>35</sup> across ages using  $\text{cov}_{1,5} / (\sqrt{\sigma^2_{L1}\sigma^2_{L5}})$ , where  $\text{cov}_{L1,5}$  is the covariance between the clearance phenotype across ages,  $\sigma^2_{L1}$  is the variance among lines at 1 week, and  $\sigma^2_{L5}$  is the variance among lines at 5 weeks.

## Genome-wide Association Test of Immune Response

We carried out three separate GWAS to identify candidate SNPs and genes that contribute to the variation in clearance ability among lines using the available sequence data. The least squared mean of each line at 1 week (175 lines), 5 weeks (183 lines), and of the lines in common at both ages (155 lines) were submitted to the DGRP2 analysis pipeline (<http://dgrp2.gnets.ncsu.edu/>, accessed on October 11, 2022). For each polymorphic marker, the DGRP2 statistical pipeline runs a linear mixed model ANOVA on nearly 1.9 million SNPs, that incorporates any potential cryptic genetic relatedness present in the lines<sup>18,38</sup>, using:  $y = Xb + Zu + e$ , where  $y$  is the phenotypic line mean adjusted for effects of segregated polymorphic inversions and *Wolbachia* infection,  $X$  is the design matrix for the fixed SNP effect  $b$ ,  $Z$  is the incidence matrix for the random polygenic effect  $u$ , and  $e$  is the residual effect. The output includes all candidate SNPs associated with the phenotype at a nominal  $P$ -value  $< 10^{-5}$ .

## Network Analyses and Gene Ontology Analysis of Immune Response

To identify genetic networks that play a role in age-specific immunity, we performed four separate network analyses using those genes identified in the three GWA that were significant at  $p < 10^{-6}$ : one for the genes identified at 1 week of age, one for the genes identified at 5 weeks of age, one for the genes identified from the lines in common at both ages, and one combining the genes from all three GWAs. We identified computationally predicted networks of genetically interacting genes, allowing one missing gene in between the candidate genes (i.e., one gene that was not identified as a candidate gene in our study but that connected two genes that were identified as candidates).

We mapped candidates to physical and genetic interaction databases downloaded from FlyBase release r5.57 using the *igraph* package in R. In this database, genes in the networks are represented as nodes, where edges between the nodes represent interactions. Subnetworks were extracted from the global networks whose edges are either directly connected between candidate genes or were bridged by one or two genes not among the candidate gene list. We used a permutation procedure<sup>39,40</sup> to determine if randomly selected  $n$  genes, where  $n$  is the number of significant genes mapped to the global network, is found significantly greater than expected by chance. The size of the largest subnetwork was then computed. This procedure was repeated 1,000 times, and the  $P$ -value calculated as  $[(A + 1)/1,001]$ , where  $A$  is the number of permutations where the size of the largest subnetwork was equal to or greater than the size of the largest subnetwork with the observed gene list.

A Gene Ontology (GO) enrichment analysis of the candidate genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v2022q1<sup>41,42</sup>, ShinyGO v0.76.3

(<http://bioinformatics.sdstate.edu/go>)<sup>43</sup>, and STRING v11.5 (<http://string-db.org>)<sup>44</sup>. Human orthologs were obtained using the DRSC Integrative Ortholog Prediction Tool with all available prediction tools, excluding low scores of less than 2 (DIOPT, version 9.0; <http://www.flyrnai.org/diopt>)<sup>45</sup>.

## **Declarations**

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### **Author information**

Isabella Gudino and Mary Rhee contributed equally to this work.

### **Authors and Affiliations**

**Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, 21250, USA**

Shonda Campbell, Isabella Gudino, Mary Rhee & Jeff Leips

[campb2@umbc.edu](mailto:campb2@umbc.edu) (S.M.C); [igudino1@umbc.edu](mailto:igudino1@umbc.edu) (I.G); [marhee1@umbc.edu](mailto:marhee1@umbc.edu)

### **Contributions**

S.M.C and J.L wrote the manuscript. All authors read and approved the manuscript.

### **Corresponding author**

Correspondence to Jeff Leips, [leips@umbc.edu](mailto:leips@umbc.edu); Tel.: +410-455-2238

### **Ethics declarations**

Ethics approval and consent

None required.

Consent for publication

All authors have approved the paper for publication.

Competing interests

The authors have no conflicts of interest to declare.

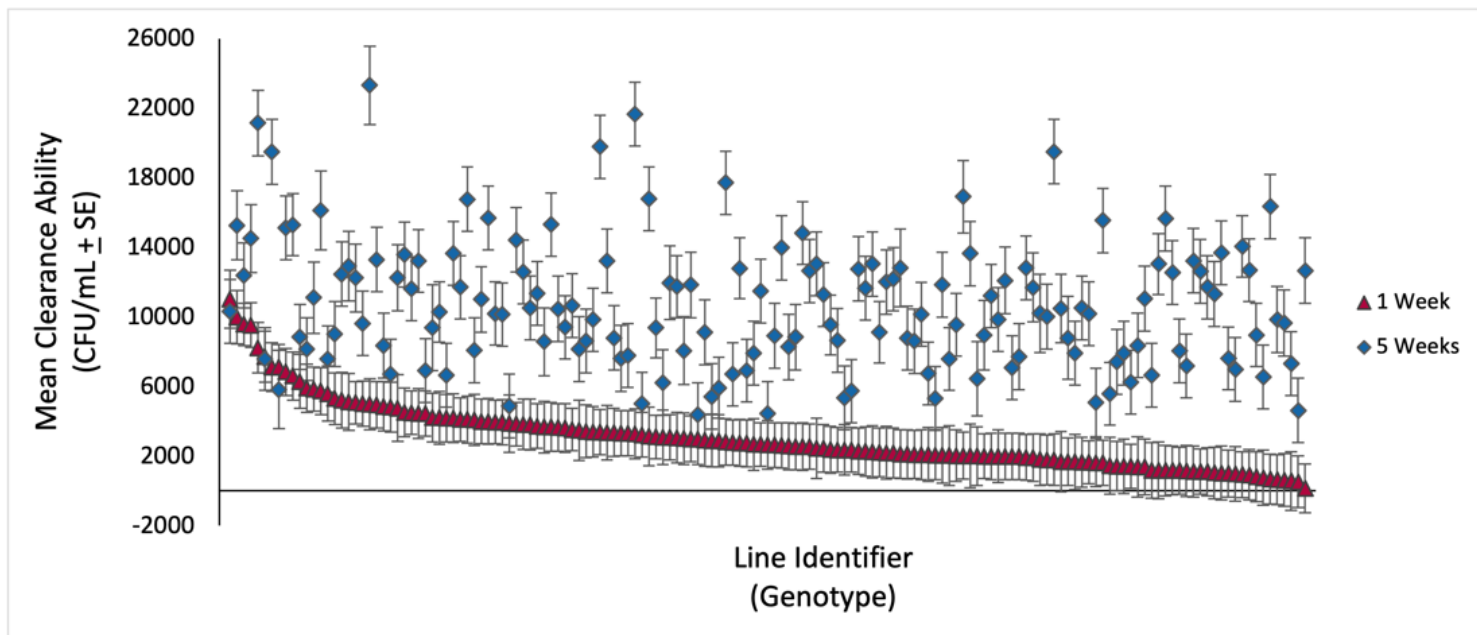
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## Figures



**Figure 1**

Mean clearance ability (CFU/mL) ( $\pm$ SE) of each genotype, ranked from worst to best clearance ability at 1 week (red) of age, maintaining that ranking for 5 week (blue) of age align accordingly. Clearance ability significantly varies among genotypes at each age ( $p < 0.0001$ ). Note that a higher CFU/mL correlates to a reduced clearance ability.  $N_{1,5} = 155$ . Data that fell outside of the 0.995% quartile range were removed. For the raw data, see Supp. File S1.

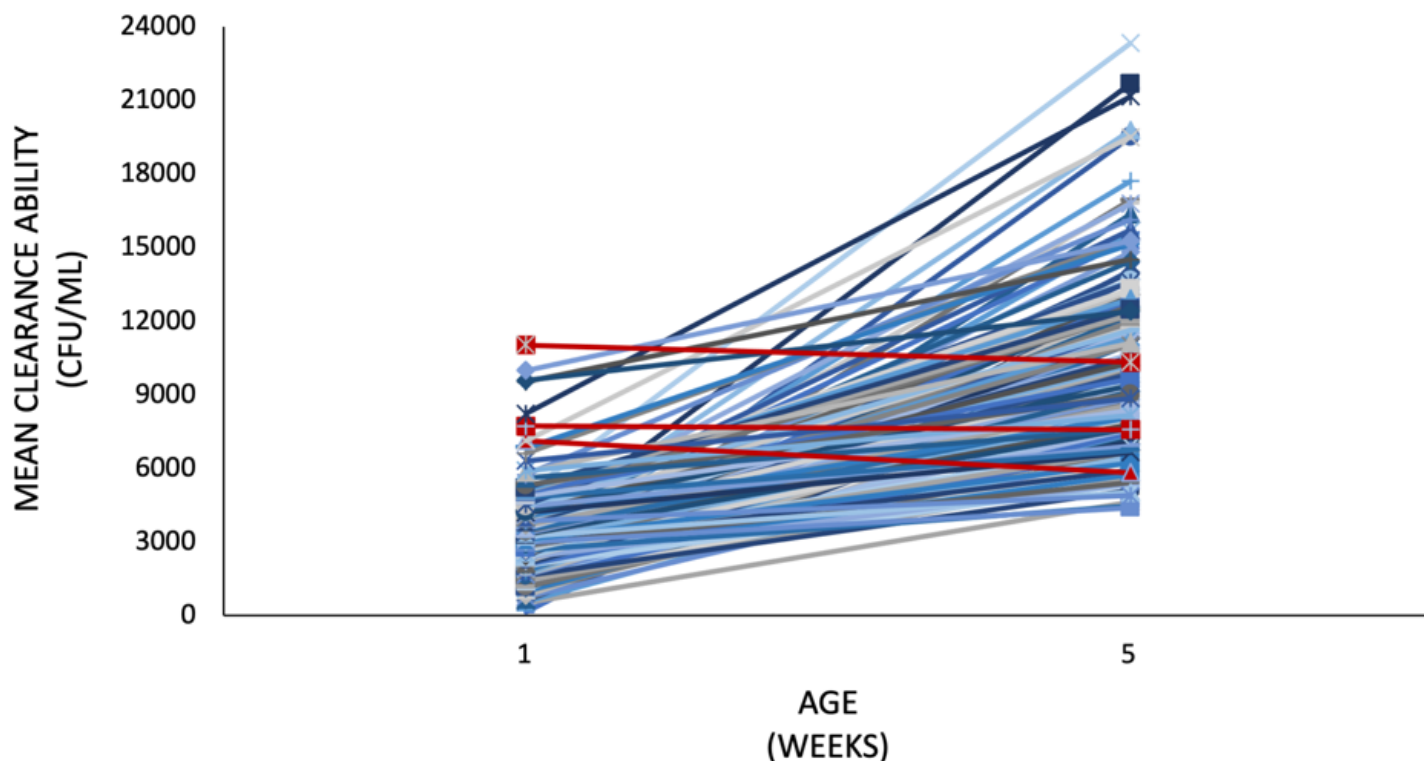




Figure 2

Reaction norms of genotype x age interaction on clearance ability (CFU/mL) for genotypes in common across 1 and 5 weeks of age. Three genotypes (DGRP lines 235, 646, and 853), shown in red, did not worsen with age, indicated by the decrease in CFU/mL. N = 155. For raw data, see Supp. File S1.

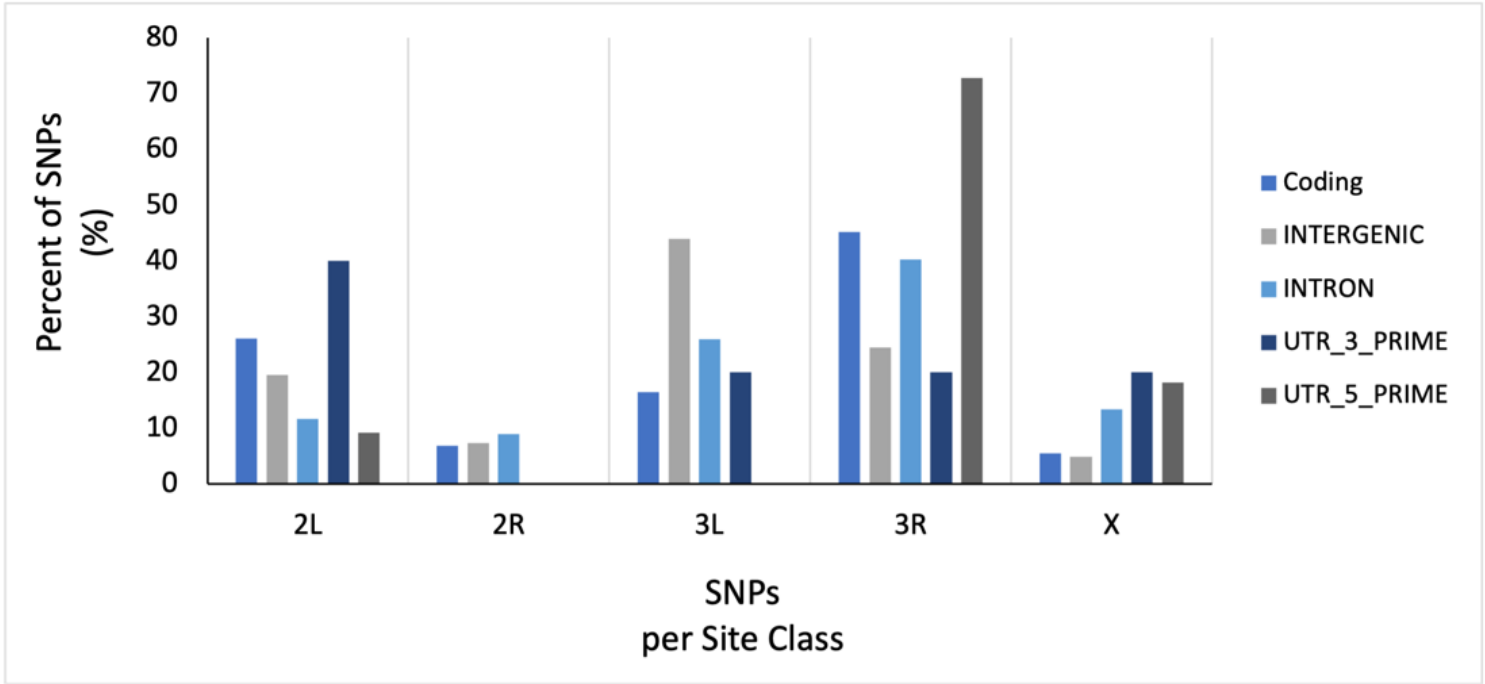
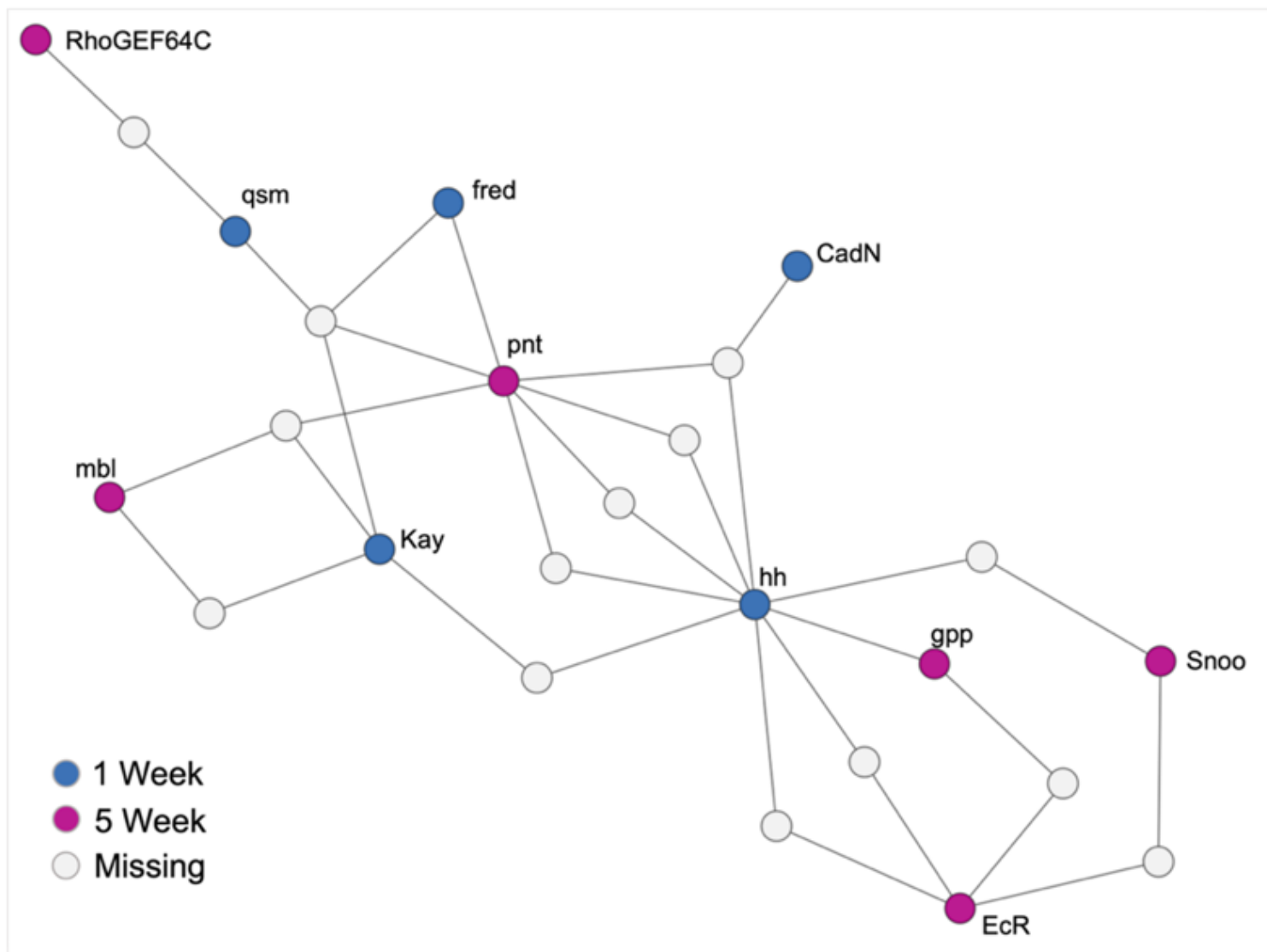


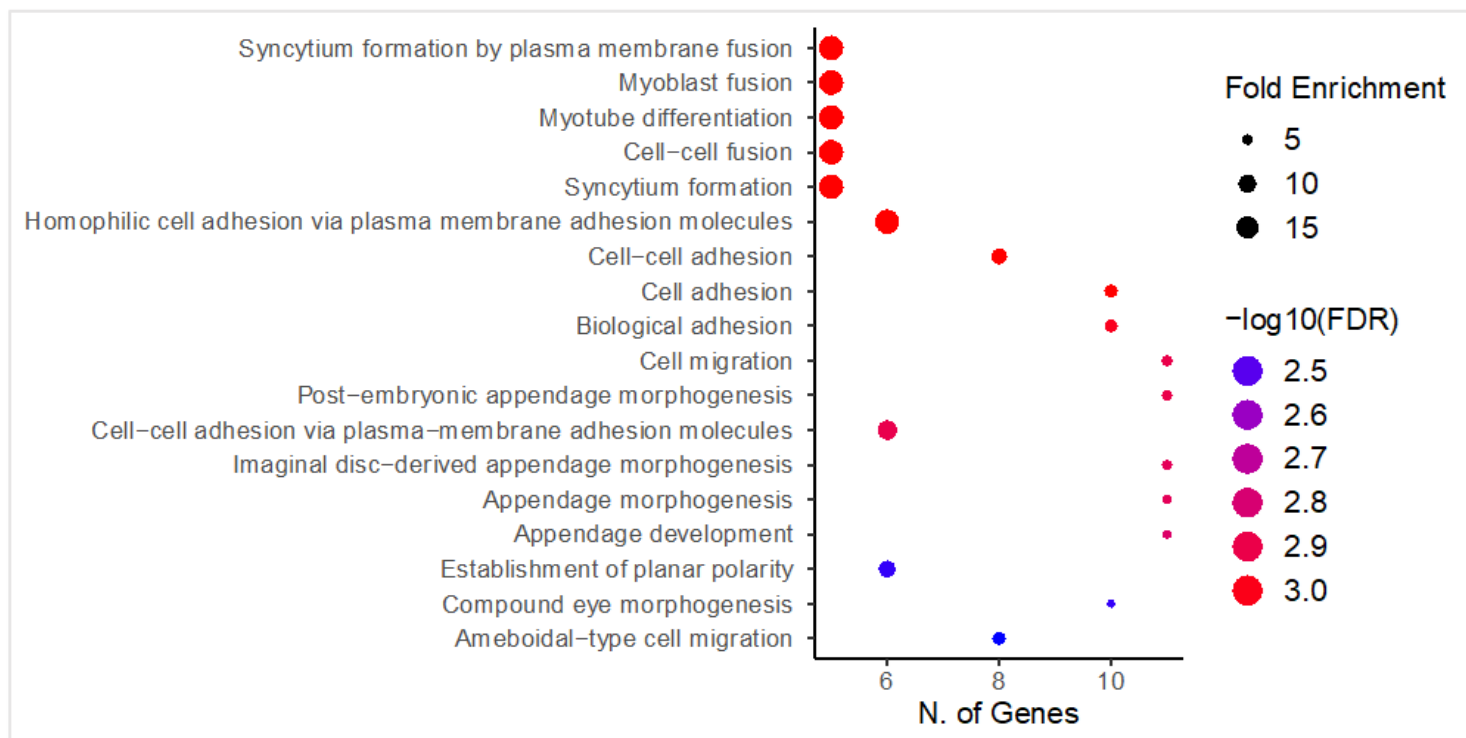
Figure 3

Distribution of significant SNPs per site class across chromosomes, combined across all three GWAs, with any duplicated SNPs removed, as described above.



**Figure 4**

Genetic network analysis of the candidate genes mapped to the global network, along with 14 missing genes (grey). Network was derived from the 107 candidate genes identified within the three GWAs for clearance ability, Those genes significant at 1 week of age are shown in blue, and those significant at 5 weeks of age are shown in pink.



**Figure 5**

Term enrichment analysis via ShinyGO 0.76.3 of the top 20 most significantly enriched GO terms associated with the candidate genes identified in the GWAs of clearance ability. The size of the circles is based on fold enrichments, while the color is the  $-\log_{10}(\text{FDR})$  with blue being low and red being highly enriched by chance. For full GO results, see Supp File S3.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SuppFileS1ClearanceCFUmL.xlsx](#)
- [SuppFileS2GWResults.xlsx](#)
- [SuppFileS3GOTerms.xlsx](#)
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