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Rapid recruitment of innate immunity regulates variation of intracellular pathogen resistance in *Drosophila*

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ABSTRACT

Genetic variation in susceptibility to pathogens is a central concern both to medicine and agriculture and to the evolution of animals. Here, we have investigated the link between such natural genetic variation and the immune response in wild-type *Drosophila melanogaster*, a major model organism for immunological research. We found that within nine wild-type strains, different *Drosophila* genotypes show wide-ranging variation in their ability to survive infection from the pathogenic bacteria *Listeria monocytogenes*. *Canton-S*, a resistant strain, showed increased capacity to induce stronger innate immune activities (antimicrobial peptides (AMPs), phenol oxidase activity, and phagocytosis) compared to the susceptible strain (*white*) at early time points during bacterial infection. Moreover, PGRP-LE-induced innate immune activation immediately after infection greatly improves survival of the susceptible strain strongly suggesting a mechanism behind the natural genetic variation of these two strains. Taken together we provide the first experimental evidence to suggest that differences in innate immune activity at early time points during infection likely mediates infection susceptibility in *Drosophila*.

There is a well-documented genetic variation in resistance to infection in the animal kingdom including humans [1]. Pathogens are capable of causing considerable morbidity in host populations therefore these invaders exert potent selective forces thereby creating the potential to drive the evolution of various genetic traits in their hosts. Included among these traits, the efficacy of the immune response is a critical determinant of fitness, subsequently higher eukaryotes have evolved elaborate mechanisms for eradicating pathogen infection. For invertebrates, this immune response is mediated by highly conserved innate immune pathways that control both cellular and humoral responses including phagocytosis by scavenging plasmatocytes (*Drosophila* macrophages) and the secretion of antimicrobial peptides (AMPs) [2,3]. The Toll pathway responds to Gram-positive and fungal infections while the Imd pathway is required for proper immune responses to Gram-negative bacteria; mutation to either pathway results in severely immunocompromised flies [2].

The fruit fly *Drosophila melanogaster* is an interesting model to study genetic variation of resistance to infection due to the relative ease in assessing functional variation within natural populations. Studies of population-based variation suggest that immunity genes in *Drosophila* evolve under positive natural selection [4] however, phenotypic effects of naturally occurring genetic variation in the ability to activate innate immune mechanisms in invertebrates (e.g., production of AMPs) remain largely unknown. *Drosophila* rely largely on their hardwired host defense mechanisms that are constructed along broadly similar lines to the innate immune systems of humans and mice [5]. Furthermore, *Drosophila* has been well characterized in terms of response to human pathogenic organisms, especially *Listeria monocytogenes*, a Gram-positive, intracellular bacterial pathogen especially in food-borne infections in immunocompromised individuals and pregnant women. Making it of particular interest, *L. monocytogenes* infection of *Drosophila* shares numerous features with mammalian infection [6].

In this study, nine wild-type *Drosophila* strains were examined for their susceptibility to *L. monocytogenes* infection. Consistent with antimicrobial peptide (AMPs) expression, *Canton-S* was found to be most resistant while *white* was particularly susceptible; *Canton-S* showed high AMP expression at early time points (~24 h) but was level with the susceptible *white* by later time points (1 day~). Taken together, our results indicate that variation in the immune-activating ability of flies during the early phase post-infection could drive variation in the susceptibility of host invertebrates.

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Materials and methods

Fly stocks: Flies were raised on standard *Drosophila* medium at indicated temperatures. *white* (w^{1118}), *da-GAL4*, *UAS-GFP*, and *tub-GAL80^{ts}* were obtained from the Bloomington Stock Center. The *UAS-PGRP-LE* line was a gift from Sho-ichiro Kurata [7]. *Amherst*, *Canton-S*, *Harwich*, *Hikone*, *Oregon-R*, *Samarkand*, *Sevelen*, and *Swedish-C* strains were gifts from Daisuke Kageyama and Takema Fukatsu.

Bacterial strains. *Salmonella typhimurium* (SL1344) and *L. monocytogenes* (10403S) were gifts from David Schneider [8,9]. *Staphylococcus aureus* was provided from Hiroshi Hamamoto and Kazuhisa Sekimizu [10]. SL1344 was cultured at 37 °C in the dark without agitation in LB broth medium with 100 μg/ml streptomycin. 10403S was cultured at 37 °C in the dark without agitation in BHI broth medium with 100 μg/ml streptomycin. *S. aureus* was cultured at 37 °C in the dark with agitation in LB broth medium.

Fly infection. Bacteria-containing medium was adjusted to appropriate concentrations using Gene Quant *pro* (Amersham) (SL1344; 0.1 OD, 10403S; 0.01 OD, and *S. aureus*; 0.005 OD). Flies were anesthetized with CO_2 and injected in the abdomen, close to the junction with the thorax ventral to the junction between the ventral and dorsal cuticles with each strain of bacteria in 65 nl of medium. Injection was carried out by using an individually calibrated pulled glass needle attached to an IM-300 microinjector (Narishige). Injected flies were transferred to fresh vials once a week [11].

Bacterial load. Bacterial colony forming units (CFU) within infected flies were determined by grinding 30 living infected flies in 1.5 ml tubes with 100 μl of 10 mM $MgSO_4$ with a pestle as previously described [6]. Serial dilutions were prepared from each fly homogenate and aliquots were plated onto BHI agar with streptomycin and incubated overnight before counting within 24 h.

Northern blot analysis. Total RNAs were isolated from flies using TRIzol reagent (Invitrogen). mRNA expression of antimicrobial peptide (AMP) genes (*diptericin*, *attacin*, *cecropin A1*, *drosocin*, and *drosomycin*) and *rp49* was analyzed by RNA blot as previously described [12]. AMP expressions were analyzed with the STORM Bioimager (Amersham) and quantified using ImageJ (NIH) after normalizing lane loading differences using *rp49* as a control.

Phenoloxidase activity assay. Phenoloxidase (PO) assays were performed as previously described [14]. Briefly, adults were dissected in 100 mM Tris–HCl (pH 7.2) to collect hemolymph that was subsequently incubated at room temperature for 30 min. Twenty microliters of aliquots of the supernatant was added to the PO assay mixture (1 ml of 100 mM phosphate buffer (pH 6.0), 200 μl of 50 mM 4-hydroxy proline ethyl ester, and 50 μl of 100 mM 4-methyl catechol as substrate), incubated for 10 min at room temperature before reaction termination by addition of 20 μl of 1 M thiourea. Phenoloxidase activity was quantified by measuring absorbency at 520 nm.

In vivo phagocytosis assay. Phagocytosis assays were performed essentially as previously reported [9] with minor modifications. Briefly, flies were injected with 65 nl of FITC-labeled dead *L. monocytogenes* [13] and incubated for 1 h at 25 °C to permit phagocytosis of bacteria. After 1 h 130 nl of trypan blue (0.2%) was injected to quench fluorescence of extracellular FITC-labeled bacteria while leaving phagocytosed bacteria unaffected.

Control of GAL4 induction. The *GAL80^{ts}/GAL4* system [15] provides a general method for temporal gene expression using the conventional GAL4-upstream activator sequence (UAS) system and a temperature-sensitive *GAL80* molecule, a repressor of *GAL4* transcriptional activity at permissive temperatures. For combined expression of *GAL80* and *GAL4* by ubiquitous promoters, *da-GAL4* and *tub-GAL80^{ts}* transgenes were located within the same line and then crossed to the *UAS-PGRP-LE* line. Development was allowed to proceed at 18 °C until adulthood. At the permissive temperature (18 °C), the *GAL80* protein is active and represses induction of *PGRP-LE* expression by inhibiting *GAL4* (Fig. 3A, 18 °C). At the restrictive temperature (29 °C), *GAL80* is inactivated, permitting expression of *PGRP-LE* (Fig. 3A, 29 °C).

Table 1
Survival rate of wild-type *Drosophila*. Survival of wild-type flies infected with *L. monocytogenes*, *S. aureus* and *S. typhimurium* was estimated. The mean day to 50% deaths indicates the time (days) required to reach 50% mortality as calculated from survival curves (Fig. 1A and Supplementary Fig. 1). The results of *Canton-S* and *white* are highlighted as bold and underlined.

Rank order of resistance	Wild-type *D. melanogaster* (mean day to 50% death)		
	L. monocytogenes	*S. aureus*	*S. typhimurium*
1	***Canton-S*** (8.12)	*Oregon-R* (4.00)	*Harwich* (14.2)
2	*Oregon-R* (7.36)	***white*** (3.58)	*Samarkand* (13.0)
3	*Swedish-C* (7.26)	*Swedish-C* (2.83)	*Oregon-R* (12.8)
4	*Harwich* (7.11)	*Harwich* (2.68)	*Swedish-C* (12.6)
5	*Samarkand* (7.09)	*Hikone* (2.59)	*Amherst* (10.9)
6	*Hikone* (7.00)	*Amherst* (2.55)	*Hikone* (11.0)
7	*Amherst* (6.66)	***Canton-S*** (2.38)	*Sevelen* (9.05)
8	*Sevelen* (6.34)	*Samarkand* (2.10)	***Canton-S*** (9.00)
9	***white*** (5.08)	*Sevelen* (2.05)	***white*** (8.97)

Results and discussions

Different susceptibility of wild-type Drosophila strains in infection of intracellular bacteria, L. monocytogenes

A set of nine wild-type *D. melanogaster* strains (*Amherst, Canton-S, Harwich, Hikone, Oregon-R, Samarkand, Sevelen, Swedish-C,* and *white*) was examined for their responses to the bacterial pathogens, *L. monocytogenes*, *S. aureus*, and *S. typhimurium*. Given that genetic variation in wild-type lines should be the result of fixation of particular genetic variants occurring among natural populations, these wild-type lines represent practical models for studying the genetic basis for resistance to bacterial infection. Importantly, these bacterial pathogens can infect both humans and *Drosophila* [6,9,16]. Susceptibilities of wild-type lines of *Drosophila* to the pathogenicity of each bacterial species were markedly varied (Table 1 and Supplementary Fig. 1). Canton-S was the most resistant line (8.12 days to 50% death) while white was especially susceptible to infection with *L. monocytogenes* (5.08 days) (Table 1 and Fig. 1A) while in the case of *S. aureus* infection, the phenotypes of these two strains were reversed (Canton-S; 2.38 days, white; 3.58 days). Moreover, these lines showed the same susceptibility to *S. typhimurium* infection (Canton-S; 9.00 days, white; 8.97 days) (Table 1 and Supplementary Fig. 1), suggesting that fluctuation of susceptibility likely reflects genetic variation in biological characteristics of each host–pathogen interaction rather than in core components of the Toll- or Imd-mediated innate immunity pathways. To further investigate genetic variation-mediated infection resistance in detail, we decided to focus on infection of Canton-S and white wild-type strains with *L. monocytogenes*.

In order to determine if the increased survival of *Canton-S* lines was a result of reduced pathogen load, suspected to be related to resistance, or due to tolerance, more directly related to overall host fitness in the presence of infection (Shinzawa et al., manuscript in preparation), the bacterial load of *L. monocytogenes* was determined. Growth curves of *L. monocytogenes* in *Canton-S* and *white* showed that numbers of bacteria in *Canton-S* increased more slowly than in *white* in 1 day after infection (Fig. 1B), indicating that resistant of *Canton-S* depends on differences in the number of virulent bacteria within their bodies. While there is not always a positive relationship between survival and bacterial load [17] in this study there was a significant correlation between those

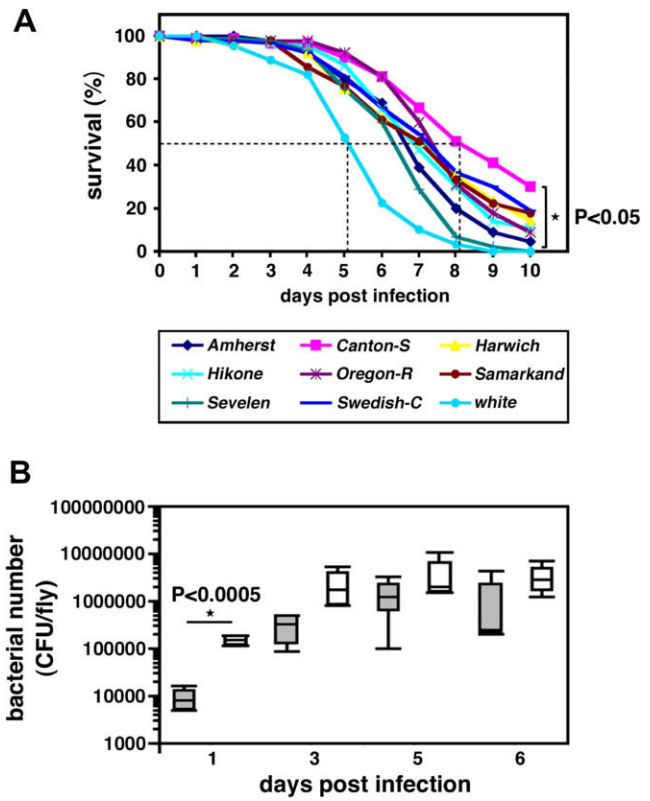

Fig. 1. Different susceptibility of wild-type *Drosophila* strains to infection of intracellular bacteria, *Listeria monocytogenes*. (A) Survival of wild-type flies injected with *L. monocytogenes*. Groups of 30-adults (aged 0–7 days) from each wild-type *Drosophila* strain (*Amherst*, *Canton-S*, *Harwich*, *Hikone*, *Oregon-R*, *Samarkand*, *Sevelen*, *Swedish-C*, and *white*) were injected with *L. monocytogenes* (OD = 0.01). The values are the averages of three independent experiments. The dotted line represents the day required to reach 50% mortality in *Canton-S* and *white*. Asterisk, $P < 0.05$ (Wilcoxon–Mann–Whitney test), for *Canton-S* versus *white*. (B) *Canton-S* inhibits the growth of *L. monocytogenes* compare with *white*. The number of *L. monocytogenes* was measured in *Canton-S* (resistant strain) and *white* (susceptible strain). Data are plotted as box plots with whiskers. Gray bars indicate the *Canton-S*. White bars indicate the *white*. Asterisk, $P < 0.0005$ (Student's *t*-test), for *Canton-S* versus *white*.

two parameters. This suggests two possible components contributing to suppression of bacteria load in resistant strains: environmental factors (e.g., nutrition) within the host fly that could affect proliferation of bacteria and/or immune components in the Toll or Imd cascades that work effectively to eradicate invading bacteria.

Different abilities of wild-type Drosophila strains in the induction of innate immune responses

Invertebrates possess a potent innate immune system, functioning via an integrated response of both humoral and cellular components. *Drosophila* relies almost entirely on innate immunity to combat specific pathogen classes [18] including the well-studied production of circulating antimicrobial peptides (AMP). These peptides are produced rapidly (within a few hours of infection) in response to activation of one of three NF-κB-related transcription factors (Dif, Dorsal, and Relish) [11]. In order to examine AMP production in response to *L. monocytogenes* infection, we monitored a time course of AMP expression (*attacin*, *cecropin A1*, *diptericin*, *drosocin*, and *drosomycin*) via Northern blotting. All examined AMPs were induced in both *Canton-S* and *white* in response to infection with *L. monocytogenes*, however, *Canton-S* showed increased AMP expression at early time points (6 and 9 h post-infection) (Fig. 2A).

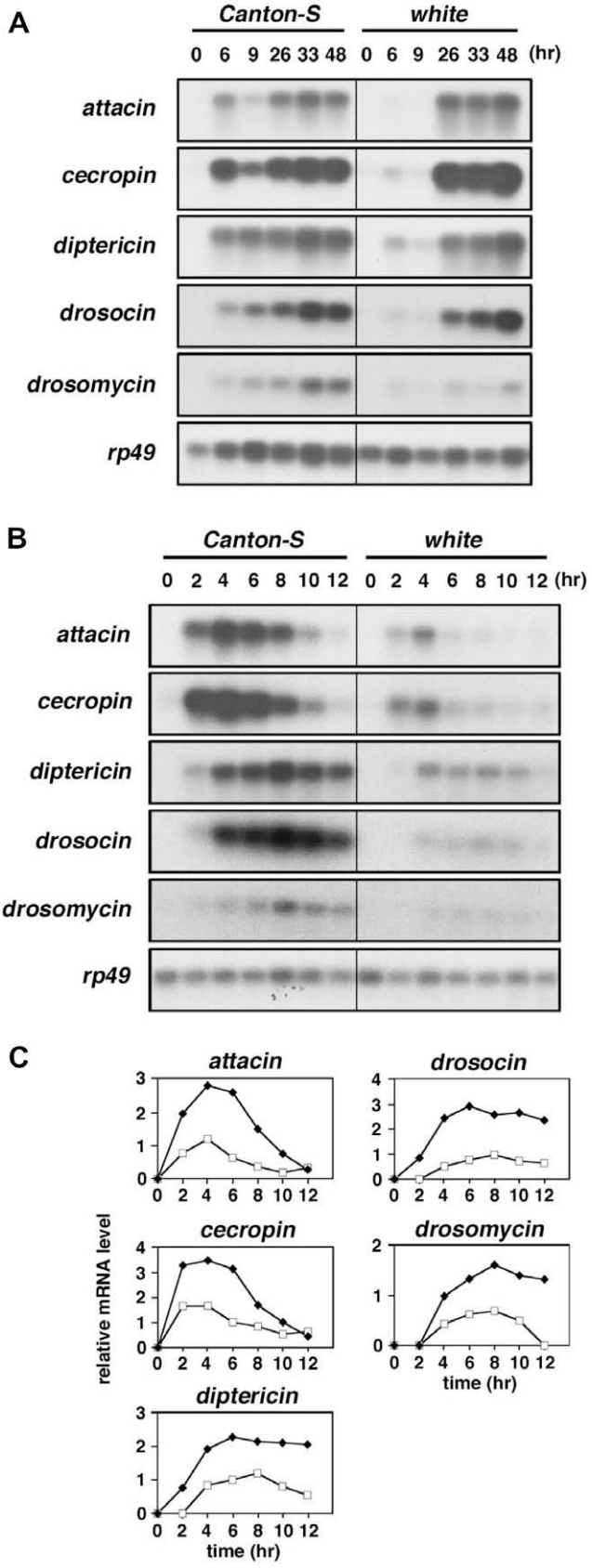

Fig. 2. *Canton-S* induces AMP expression earlier and higher than *white*. *Attacin*, *cecropin A1*, *diptericin*, *drosocin*, and *drosomycin* levels were determined by northern blot analysis from 0 to 48 h (A) and from 0 to 12 h (B) post-infection. (C) The relative induction levels of AMP mRNA in *Canton-S* (filled diamond) and *white* (open square). Expression levels shown in (B) were quantified. The levels of AMP gene expression were normalized using *rp49* as a control.

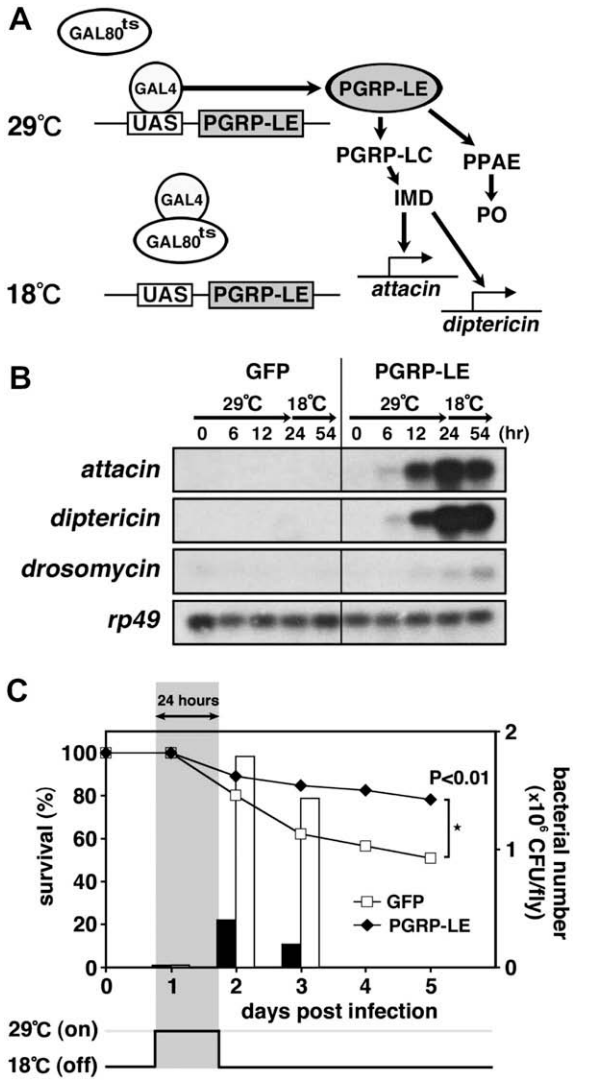

Fig. 3. Activation of innate immunity during the early stages of infection confers resistance to susceptible lines. (A) The *GAL80^{ts}/GAL4* system was used to temporally control induction of innate immunity. The *GAL80* repressor is active thereby inhibiting expression of *PGRP-LE* at 18 °C. Upon temperature shift to 29 °C the repressor is inactivated allowing GAL4-driven expression of *PGRP-LE*. (B) Expression of *PGRP-LE* induces *attacin* and *diptericin* at 29 °C in the absence of microbial challenge. (C) Survival rate of susceptible lines is improved and growth of *L. monocytogenes* is inhibited in flies artificially expressing *PGRP-LE* at early time points of infection. Adult flies (aged 10–20 days at 18 °C) were injected with *L. monocytogenes* (OD = 0.04). At 18 h post-infection flies were transferred to 29 °C for 24 h. The survival rate (line chart) and number of bacteria (bar graph) were examined in *PGRP-LE*-expressing flies (filled columns) and control flies (open columns). Asterisk, $P < 0.01$ (Wilcoxon–Mann–Whitney test), for *GFP* versus *PGRP-LE*.

Because differences were seen at early time points we more closely examined AMP expression during that phase of infection. Consistently, *Canton-S* showed increased AMP expression during the early phase of infection (Fig. 2B and C). In addition to AMP production, the humoral response also includes the melanization reaction whose activity can be monitored through measurement of phenoloxidase (PO) activity [18,19]. Cellular responses to infection come in the form of phagocytosis of microbes and were also measured in the two wild-type lines [20]. As seen with AMP production, both melanization and phagocytosis were both highly evoked in the resistant *Canton-S* (Supplementary Figs. 2 and 3), consistent with the notion that differences in general innate immune activity correlate to diversity of susceptibility to *L. monocytogenes* infection.

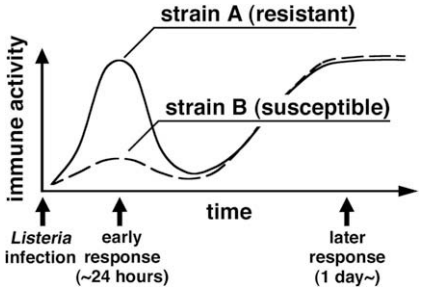

Fig. 4. A model for driving variability of resistance among wild-type *Drosophila* strains. Immune activity induced by infection of *L. monocytogenes* could be divided into early (~24 h) and later (1 day~) response in *Drosophila*. Strain A (resistant) can activate stronger early immune reactions than strain B (susceptible).

From this, an attractive hypothesis emerged whereby early activation of innate immunity alters the susceptibility of *Drosophila* to *L. monocytogenes* infection.

Activation of innate immunity at early time point of infection results in resistance in Drosophila

Since the resistant line reduced pathogen load while highly expressing AMPs we hypothesized that early activation of innate immunity during infection might be responsible for the resistance seen in the resistant *Canton-S*. If this were true then resistance of the *white* line, susceptible to *L. monocytogenes* infection and lacking a strong early immune response, might be improved by ectopic activation of the immune response. AMP gene expression is regulated through the Imd and Toll pathways when they become activated by bacterial peptidoglycans that trigger these pathways through peptidoglycan-recognition proteins (PGRPs). Despite being Gram-positive, *L. monocytogenes* contain DAP-type peptidoglycans capable of activating the Imd pathway via PGRP-LE [21]. We therefore employed PGRP-LE to stimulate an innate immune response using the GAL80^{ts}/GAL4 system capable of temporal control of gene expression and consequently temporal control over generation of an immune response (see Materials and methods). *UAS-PGRP-LE* transgenic flies in the *white* genetic background expressing both *GAL80^{ts}* and *GAL4* proteins were allowed to develop at 18 °C. At this temperature the *GAL80^{ts}* repressor is active and expression of *PGRP-LE* is subsequently inhibited (Fig. 3A). Flies were then shifted to 29 °C, resulting in inactivation of the *GAL80^{ts}* repressor and consequent *GAL4*-driven expression of *PGRP-LE* (Fig. 3A). Consistent with previous reports transient activation of *PGRP-LE* induced *attacin* and *diptericin* at 29 °C without induction of *drosomycin*, a Toll pathway reporter (Fig. 3B) [7,22] confirming activation of an Imd pathway-mediated immune response through expression of *PGRP-LE*. In order to determine if resistance of *white* to *L. monocytogenes* could simultaneously be raised *PGRP-LE* was expressed for 24 h 18 h post-infection. Indeed, survival rate was significantly improved in *PGRP-LE*-expressing flies compared with control flies (Fig. 3C). Furthermore, bacterial load was remarkably reduced after temporal expression of *PGRP-LE* (Fig. 3C). Taken together our results clearly demonstrate that transient activation of innate immunity at early time points during infection improves resistance of flies.

The expression levels of *attacin* and *cecropin* increased immediately after infection before decreasing by 12 h with maximum expression levels 4 h post-infection while *diptericin*, *drosocin*, and *drosomycin* diverged showing maximum expression levels by 6–8 h post-infection (Fig. 2A–C). From this it appears that immune activity induced by infection with *L. monocytognes* can be divided into early (~24 h) and later (1 day~) responses both in *Canton-S* and *white* (Fig. 4). The early response, conventionally referred to

as acute-phase kinetics that responds to a combination of injury and the presence of microorganisms, exhibits striking similarities to the vertebrate acute-phase response [12,23]. The later response, however, appears to respond to increasing bacterial load. *L. monocytogenes* was found to grow exponentially in infected flies, increasing approximately 10-fold in *Canton-S* and 150-fold in *white* just 1 day after infection (data not shown). The later response appears to respond to growing *L. monocytogenes* that escaped early responses of host innate immunity. Considering activation levels of immunity are equal between wild-type lines by the later response, our results provide the first experimental evidence that variation in immune activity during early responses could regulate variation in susceptibility of wild-type *Drosophila* (Fig. 4).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.097.

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