RESEARCH ARTICLE

Drosophila immune priming against *Pseudomonas aeruginosa* is short-lasting and depends on cellular and humoral immunity [v1; ref status: indexed, http://f1000r.es/p6]

Theodoulakis Christofi, Yiorgos Apidianakis

Department of Biological Sciences, University of Cyprus, Nicosia, 1678, Cyprus

v1 **First Published:** 05 Mar 2013, **2**:76 (doi: 10.12688/f1000research.2-76.v1)
Latest Published: 05 Mar 2013, **2**:76 (doi: 10.12688/f1000research.2-76.v1)

Abstract

Immune responses are traditionally divided into the innate and the adaptive arm, both of which are present in vertebrates, while only the innate arm is found in invertebrates. Immune priming experiments in *Drosophila melanogaster* and other invertebrates during the last decade have challenged this dogma, questioning the boundaries between innate and adaptive immunity. Studies on repeated inoculation of *Drosophila* with microbes reveal a long-lasting cellular immunity adaptation against particular microorganisms. Here we study the lasting effect of immune priming against infection with *Pseudomonas aeruginosa*, an opportunistic human pathogen that is lethal to the common fruit fly. *Drosophila* priming with heat-killed or low in virulence *P. aeruginosa* extends fly survival during a secondary lethal infection with a virulent strain of the same species. The protective immune response can last for more than 10 days after exposure to a persistent low-in-virulence live infection, but it is eliminated 7 days after the host is primed with heat-killed bacteria. Moreover, not only the cellular, but also the systemic NF-κB-mediated immune responses contribute to immune priming. Thus each microbe might elicit different mechanisms of immune priming that may or may not last for long.

Article Status Summary

Referee Responses

Referees	1	2	3	4
v1 published 05 Mar 2013	? report	✓ report	? report	? report

1. **Petros Ligoxygakis**, University of Oxford UK, **Marcus Glittenberg**, University of Oxford UK
2. **Bernard Mathey-Prevot**, Duke University School of Medicine USA
3. **Dimitrios Kontoyiannis**, The University of Texas MD Anderson Cancer Center USA
4. **Bruno Lemaitre**, École Polytechnique Fédérale de Lausanne Switzerland

Latest Comments

No Comments Yet

Corresponding author: Yiorgos Apidianakis (apidiana@ucy.ac.cy)

How to cite this article: Christofi T, Apidianakis Y (2013) *Drosophila* immune priming against *Pseudomonas aeruginosa* is short-lasting and depends on cellular and humoral immunity [v1; ref status: indexed, http://f1000r.es/p6] *F1000Research* 2013, **2**:76 (doi: 10.12688/f1000research.2-76.v1)

Copyright: This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Grant information: Marie Curie PCIG-GA-2011-303586 grant to YA.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: No competing interests were disclosed.

First Published: 05 Mar 2013, **2**:76 (doi: 10.12688/f1000research.2-76.v1)
First Indexed: 20 Mar 2013, **2**:76 (doi: 10.12688/f1000research.2-76.v1)

Introduction

Organisms are targets of various infectious microbes that attack a host by penetrating its body in order to feed and reproduce. To cope with infection, each host has developed physical barriers that inhibit microbial entry and tissue homeostasis factors and immune responses that may increase tolerance or resistance to infection. Immune responses can directly target microbes and are observed in most species, from bacteria to mammals through a variety of versatile mechanisms that may be of a broad or of a very microbe-specific nature. In terms of immediacy and specificity, the immune responses have been traditionally divided into innate and adaptive[1].

"Traditional" vertebrate innate and adaptive immunity

Defence barriers, such as the physical barrier of the skin or insect cuticle, intestinal mucus or insect peritrophic membrane and the low or high acidity in the gastrointestinal tract are the first lines of defence against invading microorganisms[1]. In addition, innate immunity can be elicited as a fast and broad response against pathogens. Specialised immune cells such as macrophages and neutrophils can internalise and digest microbes initiating an inflammatory response at the site of infection or systemically to produce a hostile environment for the intruder. The complement group of proteins can also be activated to fight invading microorganisms[2–5].

Components of the immune system can exhibit further specificity and acquire memory of past infections. This evolutionary step, termed 'adaptive immunity', can only be seen in vertebrates and displays antigenic specificity, diversity, immunologic memory and self/non-self recognition. Adaptive immunity depends on innate immune responses such as phagocytosis and inflammation that trigger the utilisation of specific immune response on the invader[6]. Adaptive immunity can produce a variety of immune responses specific to antigenic challenges through a variety of effectors. Cooperation between lymphocytes and antigen presenting cells is the main mechanism of action, according to which naive B lymphocytes expressing a membrane-bound antibody molecule are activated when they bind to their specific antigen and divide quickly into memory B cells and effector B cells that induce humoral immunity[7]. T lymphocytes recognise cell antigens only from major histocompatibility complex molecules and proliferate into memory and effector T cells. T lymphocytes can be subdivided into T helper (TH) and T cytotoxic (TC) cells that are responsible for the tight regulation of the immune response and cytotoxic T lymphocyte activity (CTL)[8]. During a primary immune response, naive T and B lymphocytes become antigenically committed and expand rapidly in a process called clonal selection[9]. Immunologic memory can be attributed to these memory cells, which have long life spans and exhibit a heightened response during secondary exposure.

The immune response of *Drosophila melanogaster*

Drosophila is the main model organism for studying innate immunity among invertebrate species. *Drosophila* immune defences include physical barriers[10], homeostatic factors[11] and local and systemic immune responses. Three systemic responses have been described in the fly: the humoral response, melanization and the cellular response[12]. Similarly to other arthropods, *Drosophila* contains a circulating hemolymph with blood cells called hemocytes. These can be sub-divided into three cell types with different functions: plasmatocytes, lamellocytes and crystal cells[12]. Plasmatocytes, which comprise the majority of mature hemocytes, can clear unwanted cells and pathogens through phagocytosis[12]. Lamellocytes can only be observed in larvae where they encapsulate and neutralise larger objects, and crystal cells are involved in the melanization process[12]. The synthesis and deposition of melanin in the affected area is thought to play an important role in wound healing, captivation and encapsulation of invading microbes and production of toxic substances for subsequent microbial destruction[12]. Coagulation occurs to prevent hemolymph loss but can also trap microorganisms and facilitate their destruction[13].

The *Drosophila* fat body is analogous to the mammalian liver where humoral response molecules are produced[14]. Bacteria and fungi activate the Toll pathway indirectly via production of a "danger" signal[15]. In addition, bacteria and fungi induce the Toll and Imd pathways directly by the recognition of bacterial peptidoglycan and fungal beta-glucan via peptidoglycan recognition proteins and Gram-negative binding protein 3 respectively[16]. Upon systemic immune response Toll and Imd pathways induce the NF-κB factors Dif and Rel respectively, which in turn induce the expression of several antimicrobial peptides (AMP)[17]. Besides AMPs, plasmatocytes locate and phagocytose bacteria through the help of scavenger receptors Eater and Dscam[18,19]. The epithelial barrier also exhibits local immunity where production of reactive oxygen species (ROS) and AMPs provides a defence mechanism in the gut[20]. In addition to the plasmatocyte-expressed cytokine unpaired 3 (Upd3) induces the JAK/STAT pathway mediating robust responses to bacterial and fungal infection[12]; while the same pathway can be induced upon tissue damage or viral infection[21,22].

Evidence of pathogen specific immunological memory

The aforementioned innate immune responses have not been proven to exhibit adaptive properties such as memory or specificity. However, the classic division between innate and adaptive immunity has recently been brought into question by a number of studies in invertebrate organisms, which challenge the currently defined boundaries of immunological memory[23].

Recent evidence suggests that arthropods can display selected 'specificity' towards particular microorganisms. Pham and colleagues demonstrated that the fruit fly exhibits a specific primed immune response dependent on plasmatocytes[24]. They tested various pathogens including bacteria and fungi and found that flies mount a prolonged protective response against *Streptococcus pneumoniae* after being primed with a sub-lethal or heat-killed dose of the bacterium. *S. pneumoniae* bacteria are killed by the host within 1 day of infection only in primed flies whereas unprimed flies still contained bacteria indicating that survival depends on the elimination rate of *S. pneumoniae*[24]. They also found a similar adaptation with the natural fungal pathogen *Beauveria bassiana*.

Protection against other bacteria was not observed by priming with *S. pneumonia*. Conversely, other heat-killed bacteria – known to be strong immune activators – did not exert a protective response against *S. pneumoniae*. Immune pathway mutants demonstrated that immune priming is due to the activation of the Toll pathway but not due to the expression of AMPs. These findings illustrate

the selective adaptability of the immune system through the activation of Toll pathway and plasmatocytes. However it is important to note that not all pathogens respond in the same way. In this report, we use the example of *Pseudomonas aeruginosa*, a gram-negative bacterium that induces the Imd and the Toll pathways, as well as the cellular immune response.

Immune priming with *P. aeruginosa*

Previous studies show that live *P. aeruginosa* infection with the low-in-virulence CF5 strain primes the immune system and helps to protect *Drosophila* from subsequent lethal infection with the virulent PA14 strain (UCBPP-PA14)[25]. This protection is evident 6, 12 and 24 hours post immune priming and involves the activation of both the Imd and the Toll pathway[25]. Here we assess the duration of this protective response and the involvement of humoral and cellular immune responses. We find that immune priming with heat-killed *P. aeruginosa* CF5 confers protection for less than 7 days and that the Imd and the Toll pathways, as well as phagocytosis, contribute to host protection at 2 and 5 days respectively post-immune priming.

Methods

Fly strains

Wild type Oregon R and Eater mutant flies were a gift from Christine Kocks[18]. Canton S (CS) was obtained from Bloomington stock Center. Imd[1], Rel[E20] and Dif[1] mutant flies were a gift from Bruno Lemaitre.

Bacterial strains and infection assays

P. aeruginosa strains PA14 and CF5 are previously described human isolates[25]. For inoculation with live CF5 cells flies were pricked with a needle previously dipped into a solution of 3 x 10^8 CF5 cells/ml or in PBS as a control as previously described[25,26]. For CF5 colony forming units (CFUs) enumeration, 3 flies per time point, in triplicates, were ground and plated every two days. Using the injection method 9.2 nl of a bacterial solution was introduced into the fly thorax to prime or infect the flies and host survival was measured every hour as previously described[25,26]. 9.2 nl of a solution containing 3 x 10^8 heat-killed CF5 cells/ml or the equivalent volume of PBS was injected to prime flies with heat-killed *P. aeruginosa*. Primed flies were subsequently injected with 9.2 nl of a live bacteria solution containing 3 x 10^7 PA14 cells/ml.

Statistical analysis

Fly survival kinetics were analyzed using the MedCalc software (www.medcalc.org/). Survival curve analyses were performed using the Logrank test of the Kaplan-Meier survival analysis[27]. The supplementary data tables (Table S1–Table S7) accompanying this work provides the actual number of flies per experiment and individual results used for analysis.

Results

To test if long-term protection could be achieved by immune priming, we initially infected wild type male Oregon R flies with 130 colony forming units (CFUs) of the low-in-virulence *P. aeruginosa* strain CF5. Infection was persistent for at least 10 days when >100 CFUs/fly were still present in the flies (Figure 1). Injections with the virulent PA14 strain were performed on the 11th day of priming with CF5 and in control non-primed flies. In primed flies, the 50% survival time was over two hours longer and over 10% of primed flies had survived at 30 hours post-infection (Figure 2). It should be noted that *P. aeruginosa* infection with the PA14 strain is reproducibly 100% lethal under these conditions and short time differences between survival curves are biologically and statistically significant using the Kaplan-Meier survival kinetic analysis[11,26]. Here we observe a protective role against a virulent bacterium when the host is primed with live bacteria of the same species ($P<0.0001$) (Figure 2).

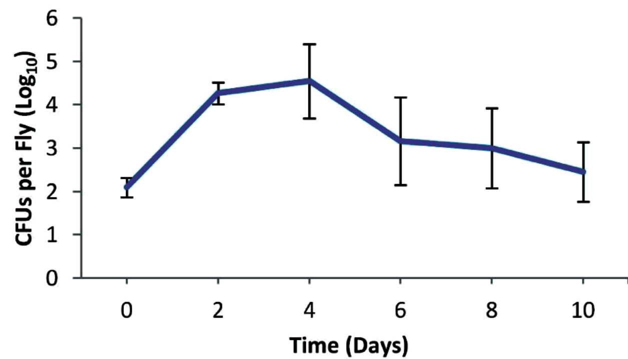

Figure 1. Persistence of *Pseudomonas aeruginosa* CF5 in the fly. Wild type Oregon R male flies were infected with 130 colony forming units (CFUs) of the low virulent *P. aeruginosa* CF5 strain. Infection is persistent for at least 10 days with more than 100 CFUs per fly.

To assess the duration of immune priming when bacteria are not able to replicate and persist in the host, we primed flies with bacteria that were heat-killed for 10 minutes at 60°C. ~3000 heat-killed CF5 cells were injected per fly 2, 5 or 7 days prior to infection with the lethal strain PA14. Under these conditions, no CFUs could be recovered from flies prior to PA14 infection.

Initially wild type Oregon R (OR), Rel mutant (Imd pathway) and Dif mutant (Toll pathway) mutant flies were primed with heat-killed

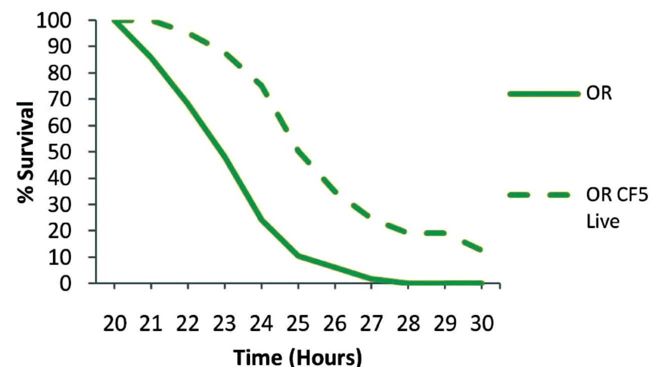

Figure 2. Survival kinetics of wt PA14 infected flies 11 days post priming with live *Pseudomonas aeruginosa* CF5. *P. aeruginosa* PA14 injection in primed (dashed line) and non primed (continuous line) Oregon R flies with live CF5 bacteria showed an extension in the 50% survival time for over of two hours in contrast to controls and more that 10% survived at 30 hours post infection ($P<0.0001$). n=40–49 flies per condition.

(H.K.) CF5 cells and infected with the PA14 strain 2 days later. OR flies showed a prominent extension in survival (P<0.0001) with more than 30% of primed flies surviving the infection at 30 hours post-infection (Figure 3). However the Rel mutant primed and non-primed flies died at similar rates (P=0.2199), suggesting that protective priming responses against *P. aeruginosa* depends on the *rel* gene. Dif mutant flies could elicit a protective response (P=0.0010), which was nevertheless less prominent compared to that of OR flies. Thus *rel* and to a lesser extent *dif*, the 2 main NF-κB immune factors of *Drosophila*, appear to contribute to the protective immune response that lasts for at least 2 days.

To examine if phagocytosis is important for immune priming against *P. aeruginosa* we primed Eater deficient flies for 2 days. Mutant Eater non-primed flies were more susceptible to infection than OR non-primed flies (P<0.0001), and contrary to wild type flies, priming of mutant flies did not lead to any survivors at 30 hours (Figure 4). Nevertheless, primed Eater deficient flies survived longer when primed (P<0.0001), suggesting that additional immune responses contribute to host protection at 2 days post priming. The role of cellular responses in immune priming was nevertheless clear when the PA14 strain was injected 5 days after priming, when primed Eater mutant flies are equally susceptible to non-primed flies (P=0.5616), while wild type flies were still significantly protected by priming (P=0.0037) (Figure 5). This suggests that immune priming at 5 days depends heavily on phagocytosis.

To assess if the priming effect can last for longer, we primed wild type but also Imd and Rel mutant flies 7 days prior to PA14 infection, and we noticed that priming had no significant effect in the survival rates of any of the genotypes tested: Canton S (P=0.0726), Rel (P=0.9163), Imd (P=0.0663) (Figure 6). To assess if 7 day primed flies are incapable of mounting a protective immune

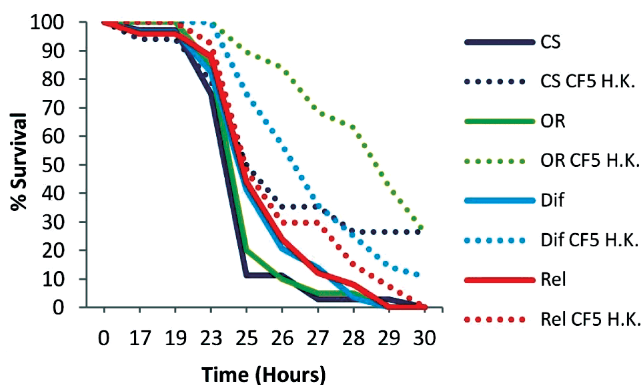

Figure 3. Survival kinetics of wt and humoral response deficient flies 2 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Wild type Oregon R (OR) and mutant flies of the Imd (Rel) and Toll (Dif) pathways were primed with heat-killed (H.K.) CF5 cells (doted lines) 2 days prior to PA14 challenge. Primed OR flies showed extended survival times and 30% survivors (P<0.0001). Rel mutant control and primed flies died at similar rates indicating a protective role of the *rel* gene (Rel P=0.2199). Dif mutant flies exhibited a low but significant protective effect (P=0.001). n=19–29 flies per condition.

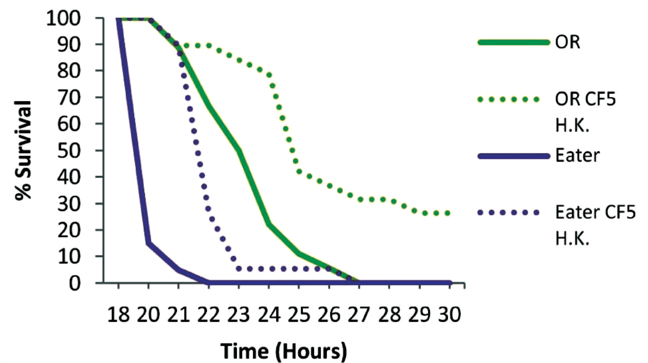

Figure 4. Survival kinetics of wt and cellular response deficient flies 2 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Eater mutant and wild type Oregon R flies (OR) were primed two days prior to PA14 injection to investigate the role of phagocytosis in immune priming. Eater deficient flies were more susceptible to infection than wild type flies (P<0.0001) in the absence of priming. Nevertheless Eater mutants survived longer when primed indicating additional immune responses of the host at 2 days post priming. n=18–20 flies per condition.

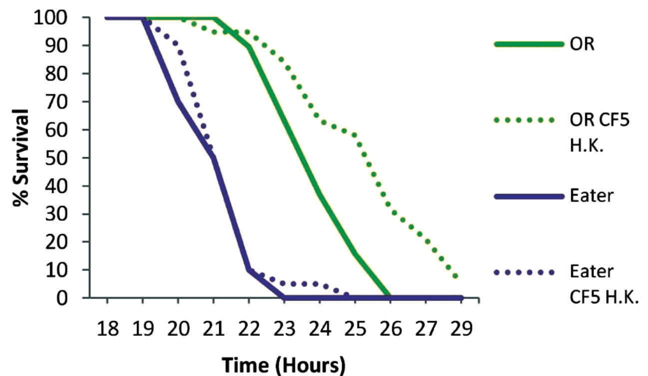

Figure 5. Survival kinetics of wt and cellular response deficient flies 5 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Wild type Oregon R (OR) and Eater mutant flies where challenged with PA14 5 days post injecting with heat-killed CF5. Eater mutants were equally susceptible to non primed flies (P=0.5616) in contrast to control OR flies (P=0.0037). This apparent cellular response suggests that phagocytosis efficacy depends on the priming period prior to challenge. n=19–20 flies per condition.

response or if priming diminishes after 7 days, we double primed wild type flies 5 and 7 days prior to PA14 infection. We noticed that double priming extends the survival of flies (P=0.0007) (Figure 7), thus priming with dead *P. aeruginosa* has a short-lasting protective effect of less than a week.

Discussion

Specific responses to different microbes

Collectively our data indicate that low-in-virulence *P. aeruginosa* can prime the *Drosophila* humoral and cellular immune responses against a subsequent lethal infection with a more virulent strain. Nevertheless, unlike priming with *S. pneumoniae* or *B. Bassiana*,

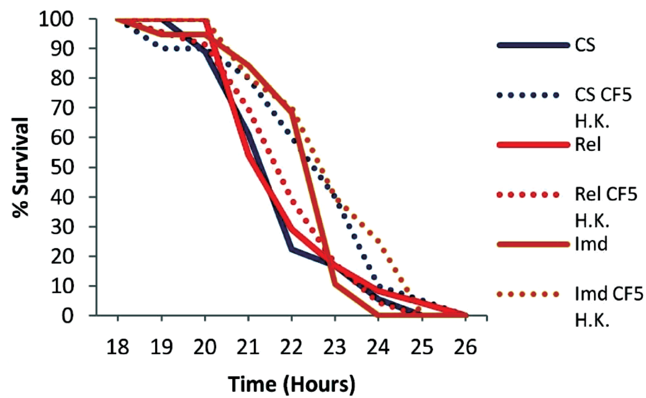

Figure 6. Survival kinetics of wt and humoral response deficient flies 7 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Wild type Canton S (CS), Imd and Rel mutant primed and non primed flies were tested. All fly genotypes exhibited no significant effect, CS (P=0.0726), Rel (P=0.9163), Imd (P=0.0663), indicating that priming is transient. n=18–24 flies per condition.

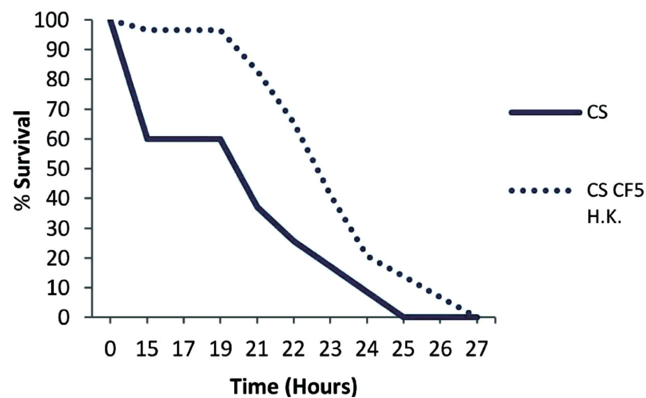

Figure 7. Survival kinetics of wt flies 5 and 7 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Wild type Canton S flies (CS) were primed with heat-killed CF5 cells 5 and 7 days before *P. aeruginosa* PA14 injection. A protective response was observed (P=0.0007) in the primed flies. n=29–35 flies per condition.

this is not a long-lasting effect. It is thus pivotal that future studies assess in detail the differences in the immune responses among many different microbes and in time points that last for many days rather than hours as is customary. Long-term responses to single or repeated challenges of the immune system might pinpoint novel aspects of immunological memory. One aspect of immune responses that might be related to immunologic memory in invertebrates is specificity. A recent breakthrough in the specificity of immune responses in insects came with the discovery of the multi-variable gene *Dscam*[28]. Dong and his team found that different immune elicitors in the mosquito direct the production of pathogen-specific splice variants of the Down Syndrome Cell Adhesion Molecule receptor necessary for the protection of the host from infection with *Plasmodium*. Though no experiments were done to test the duration of this specific response, this work illustrates the adaptability of the insect immune system. There are additional examples of specific immune responses in invertebrates such as the snail *Biomphalaria glabrata*, in which fibrinogen related proteins (FREPS) exhibit a high rate of diversification at a genomic level, and the expression profiles of the scavenger receptor cysteine-rich proteins in the sea urchin, although how these proteins respond to re-challenge is not known[29,30]. Protection against a secondary infection is also seen in the mealworm beetle, *Tenebrio molitor*[31]. Prolonged protection was observed when initial exposure to lipopolysaccharides (LPS) before infection with spores of the entomopathogenic fungus *Metarhizium anisopliae* occurred. This was attributed to a long-lasting antimicrobial response of the LPS-challenged larva, which provided a survival advantage when it was exposed to fungal infection. Thus invertebrate hosts can be further studied to understand the parameters of long-lasting immune responses and their relation to immune specificity and memory.

In conclusion, the area of immunological memory remains elusive in the invertebrate world and only recently have small steps been made to investigate this aspect of the immune system. Specific responses can occur against particular pathogens. Generalisations on the defence mechanisms do not represent the true complexity of the immune system. Therefore, the immune system of invertebrates is still a field that can advance our understanding of how organisms defend themselves from intruders.

Author contributions
YA designed the research and performed experiments; TC analyzed the data; YA and TC wrote the paper. Both authors approved the final manuscript for publication.

Competing interests
No competing interests were disclosed.

Grant information
Marie Curie PCIG-GA-2011-303586 grant to YA.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplementary tables

Table S1. Persistence of *Pseudomonas aeruginosa* CF5 in the fly. Infected flies were ground and plated, 3 individuals per replicate, at days 0, 2, 4, 6, 8 and 10 post infection where the colony forming units (CFU) were measured and calculated per fly. The experiment was performed in triplicates where the average CFU numbers were plotted with the corresponding standard deviation.

Infection with live CF5						
Time (Days)	0 days	2 days	4 days	6 days	8 days	10 days
CFUs per Fly	220	32000	320000	7000	10000	1330
	100	20000	20000	4500	700	300
	87	10000	7000	100	150	56
Average CFUs per Fly	135,67	20666,67	115666,7	3866,67	3616,67	562
Standard deviation	73,33	11015,14	177077,2	3493,33	5534,97	676,2

Table S2. Survival kinetics of wt PA14 infected flies 11 days post priming with live *Pseudomonas aeruginosa* CF5. Oregon R (OR) uninfected flies (n=49) and OR infected with live CF5 bacteria (n=40) for 11 days were injected with the virulent PA14 strain. Top row numbers indicate the time in hours post injection whereas lower rows the corresponding number of surviving flies in naive and primed flies respectively. Sample size is indicated at 20h.

11 Days infected with live CF5											
Time (Hours)	20h	21h	22h	23h	24h	25h	26h	27h	28h	29h	30h
OR	49	42	33	23	11	5	3	1	0	0	0
OR CF5 live	40	40	38	35	30	20	14	10	8	8	5

Table S3. Survival kinetics of wt and humoral response deficient flies 2 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Wild type Oregon R (OR) and mutant Dif and Rel flies were primed with heat-killed (H. K.) CF5 cells. Naive and primed flies were injected with the virulent PA14 strain after 2 days. Top row numbers indicate the time in hours post challenge whereas lower rows indicate the corresponding number of surviving flies in naive and primed flies respectively for each genotype. Sample size is indicated at 0h.

2 Days priming										
Time (Hours)	0h	17h	19h	23h	25h	26h	27h	28h	29h	30h
OR	20	20	20	17	4	2	1	1	0	0
OR CF5 H.K.	19	19	19	19	17	16	13	12	8	5
Dif	29	28	28	24	12	6	4	1	0	0
Dif CF5 H.K.	28	28	28	28	21	16	10	7	4	3
Rel	25	24	24	22	11	6	3	2	0	0
Rel CF5 H.K.	27	27	27	25	13	8	8	4	2	0

Table S4. Survival kinetics of wt and cellular response deficient flies 2 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Oregon R (OR) (n=18) and Eater (n=20) mutant uninfected flies, and primed with heat-killed (H. K.) CF5 bacteria were inoculated at 2 days with the virulent PA14 strain. Top row numbers indicate the time in hours post challenge whereas lower rows indicate the corresponding number of surviving flies in naive and primed flies respectively for each genotype. Sample size is indicated at 18h.

2 Days priming												
Time (Hours)	18h	20h	21h	22h	23h	24h	25h	26h	27h	28h	29h	30h
OR	18	18	16	12	9	4	2	1	0	0	0	0
OR CF5 H.K.	19	19	17	17	16	15	8	7	6	6	5	5
Eater	20	3	1	0	0	0	0	0	0	0	0	0
Eater CF5 H.K.	19	19	17	5	1	1	1	1	0	0	0	0

Table S5. Survival kinetics of wt and cellular response deficient flies 5 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Wild type Oregon R (OR) and Eater mutant uninfected flies (n=19, n=20) and primed with heat-killed (H. K.) CF5 bacteria (n=19, n=20) were inoculated at day 5 with PA14. Top row numbers indicate the time in hours post challenge whereas lower rows indicate the corresponding number of surviving flies in naive and primed flies respectively for each genotype. Sample size is indicated at 18h.

5 Days priming											
Time (Hours)	18h	19h	20h	21h	22h	23h	24h	25h	26h	27h	29h
OR	19	19	19	19	17	12	7	3	0	0	0
OR CF5 H.K.	19	19	19	18	18	16	12	11	6	4	1
Eater	20	20	14	10	2	0	0	0	0	0	0
Eater CF5 H.K.	20	20	18	10	2	1	1	0	0	0	0

Table S6. Survival kinetics of wt and humoral response deficient flies 7 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Wild type Canton S (CS) and mutant Rel and Imd flies were primed with heat-killed (H. K.) CF5 cells. Flies were injected with the virulent PA14 strain 7 days post priming. Top row numbers indicate the time in hours post injection whereas lower rows indicate the corresponding number of surviving flies in naive and primed flies respectively for each genotype. Sample size is indicated at 18h.

7 Days priming									
Time (Hours)	18h	19h	20h	21h	22h	23h	24h	25h	26h
CS	18	18	16	11	4	3	1	0	0
CS CF5 H.K.	20	18	18	16	12	8	2	1	0
Rel	24	24	24	13	7	4	2	1	0
Rel CF5 H.K.	23	22	21	16	9	4	1	0	0
Imd	19	18	18	16	13	2	0	0	0
Imd CF5 H.K.	20	20	20	16	14	8	5	0	0

Table S7. Survival kinetics of wt flies 5 and 7 days post priming with heat-killed *Pseudomonas aeruginosa* CF5. Canton S (CS) wild type flies were primed twice with heat-killed (H. K.) CF5 cells at days 5 and 7 prior to injection with PA14. Naive (n=35) and primed (n=29) flies were inoculated with the virulent PA14 strain 7 days post priming. Top row numbers indicate the time in hours post challenge whereas lower rows indicate the corresponding number of surviving flies in naive and primed flies respectively. Sample size is indicated at 0h.

5+7 Days priming													
Time (Hours)	0h	15h	17h	19h	21h	22h	23h	24h	25h	26h	27h	28h	36h
CS	35	21	21	21	13	9	6	3	0	0	0	0	0
CS H.K. CF5	29	28	28	28	24	19	12	6	4	2	0	0	0

References

1. Kindt TJ, Goldsby RA, Osborne BA, *et al.*: **Kuby Immunology.** W. H. Freeman; 2007.
Reference Source
2. Beutler B: **Innate immunity: An overview.** *Mol Immunol.* 2004; **40**(12): 845–59.
PubMed Abstract | Publisher Full Text
3. Morgan BP, Marchbank KJ, Longhi MP, *et al.*: **Complement: Central to innate immunity and bridging to adaptive responses.** *Immunol Lett.* 2005; **97**(2): 171–9.
PubMed Abstract | Publisher Full Text
4. Carroll MC: **The complement system in regulation of adaptive immunity.** *Nat Immunol.* 2004; **5**(10): 981–6.
PubMed Abstract | Publisher Full Text
5. Akira S, Takeda K, Kaisho T: **Toll-like receptors: Critical proteins linking innate and acquired immunity.** *Nat Immunol.* 2001; **2**(8): 675–80.
PubMed Abstract | Publisher Full Text
6. Jutras I, Desjardins M: **PHAGOCYTOSIS: At the crossroads of innate and adaptive immunity.** *Annu Rev Cell Dev Biol.* 2005; **21**: 511–27.
PubMed Abstract | Publisher Full Text
7. Chaplin DD: **1. overview of the human immune response.** *J Allergy Clin Immunol.* 2006; **117**(2 Suppl Mini-Primer): S430–5.
PubMed Abstract | Publisher Full Text
8. Alam R, Gorska M: **3. lymphocytes.** *J Allergy Clin Immunol.* 2003; **111**(2 Suppl): S476–85.
PubMed Abstract | Publisher Full Text
9. Fekety FR: **The clonal selection theory of acquired immunity.** *Yale J Biol Med.* 1960; **32**(6): 480.
Free Full Text
10. Kuraishi T, Binggeli O, Opota O, *et al.*: **Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in drosophila melanogaster.** *Proc Natl Acad Sci U S A.* 2011; **108**(38): 15966–71.
PubMed Abstract | Publisher Full Text | Free Full Text
11. Apidianakis Y, Mindrinos MN, Xiao W, *et al.*: **Involvement of skeletal muscle gene regulatory network in susceptibility to wound infection following trauma.** *PLoS One.* 2007; **2**(12): e1356.
PubMed Abstract | Publisher Full Text | Free Full Text
12. Lemaitre B, Hoffmann J: **The host defense of drosophila melanogaster.** *Annu Rev Immunol.* 2007; **25**: 697–743.
PubMed Abstract | Publisher Full Text
13. Muta T, Iwanaga S: **The role of hemolymph coagulation in innate immunity.** *Curr Opin Immunol.* 1996; **8**(1): 41–7.
PubMed Abstract | Publisher Full Text
14. Manfruelli P, Reichhart JM, Steward R, *et al.*: **A mosaic analysis in drosophila fat body cells of the control of antimicrobial peptide genes by the rel proteins dorsal and DIF.** *EMBO J.* 1999; **18**(12): 3380–91.
PubMed Abstract | Publisher Full Text | Free Full Text
15. El Chamy L, Leclerc V, Caldelari I, *et al.*: **Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of toll.** *Nat Immunol.* 2008; **9**(10): 1165–70.
PubMed Abstract | Publisher Full Text | Free Full Text
16. Valanne S, Wang J, Rämet M: **The drosophila toll signaling pathway.** *J Immunol.* 2011; **186**(2): 649–56.
PubMed Abstract | Publisher Full Text
17. Weber AN, Tauszig-Delamasure S, Hoffmann JA, *et al.*: **Binding of the drosophila cytokine spatzle to toll is direct and establishes signaling.** *Nat Immunol.* 2003; **4**(8): 794–800.
PubMed Abstract | Publisher Full Text
18. Kocks C, Cho JH, Nehme N, *et al.*: **Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in drosophila.** *Cell.* 2005; **123**(2): 335–46.
PubMed Abstract | Publisher Full Text
19. Watson FL, Puttmann-Holgado R, Thomas F, *et al.*: **Extensive diversity of ig-superfamily proteins in the immune system of insects.** *Science.* 2005; **309**(5742): 1874–8.
PubMed Abstract | Publisher Full Text
20. Meister M, Lemaitre B, Hoffmann JA: **Antimicrobial peptide defense in drosophila.** *Bioessays.* 1997; **19**(11): 1019–26.
PubMed Abstract | Publisher Full Text
21. Jiang H, Patel PH, Kohlmaier A, *et al.*: **Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the drosophila midgut.** *Cell.* 2009; **137**(7): 1343–55.
PubMed Abstract | Publisher Full Text | Free Full Text
22. Dostert C, Jouanguy E, Irving P, *et al.*: **The jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila.** *Nat Immunol.* 2005; **6**(9): 946–53.
PubMed Abstract | Publisher Full Text
23. Ziauddin J, Schneider DS: **Where does innate immunity stop and adaptive immunity begin?** *Cell Host Microbe.* 2012; **12**(4): 394–5.
PubMed Abstract | Publisher Full Text
24. Pham LN, Dionne MS, Shirasu-Hiza M, *et al.*: **A specific primed immune response in drosophila is dependent on phagocytes.** *PLoS Pathog.* 2007; **3**(3): e26.
PubMed Abstract | Publisher Full Text | Free Full Text
25. Apidianakis Y, Mindrinos MN, Xiao W, *et al.*: **Profiling early infection responses: Pseudomonas aeruginosa eludes host defenses by suppressing antimicrobial peptide gene expression.** *Proc Natl Acad Sci U S A.* 2005; **102**(7): 2573–8.
PubMed Abstract | Publisher Full Text | Free Full Text
26. Apidianakis Y, Rahme LG: **Drosophila melanogaster as a model host for studying pseudomonas aeruginosa infection.** *Nat Protoc.* 2009; **4**(9): 1285–94.
PubMed Abstract | Publisher Full Text
27. Kaplan EL, Meier P: **Nonparametric estimation from incomplete observations.** *J Am Stat Assoc.* 1958; **53**(282): 457–81.
Publisher Full Text
28. Dong Y, Cirimotich CM, Pike A, *et al.*: **Anopheles NF-kappaB-regulated splicing factors direct pathogen-specific repertoires of the hypervariable pattern recognition receptor AgDscam.** *Cell Host Microbe.* 2012; **12**(4): 521–30.
PubMed Abstract | Publisher Full Text | Free Full Text
29. Pancer Z: **Dynamic expression of multiple scavenger receptor cysteine-rich genes in coelomocytes of the purple sea urchin.** *Proc Natl Acad Sci U S A.* 2000; **97**(24): 13156–61.
PubMed Abstract | Publisher Full Text | Free Full Text
30. Zhang SM, Adema CM, Kepler TB, *et al.*: **Diversification of ig superfamily genes in an invertebrate.** *Science.* 2004; **305**(5681): 251–4.
PubMed Abstract | Publisher Full Text
31. Moret Y, Siva-Jothy MT: **Adaptive innate immunity? responsive-mode prophylaxis in the mealworm beetle, tenebrio molitor.** *Proc Biol Sci.* 2003; **270**(1532): 2475–80.
PubMed Abstract | Publisher Full Text | Free Full Text

Current Referee Status:

Referee Responses for Version 1

Bruno Lemaitre, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland
Approved with reservations: 22 March 2013

Ref Report: 22 March 2013
This is a short paper on the influence of priming on the *Drosophila* immune response.The results are worthy enough to be published but require major improvements.

1) The paper, especially the introduction, is poorly written. I just mention few problems

*All the parts on the vertebrate adaptive immune should be double checked by an immunologist. Many sentences make no sense.

Ex.: 'Cooperation between lymphocytes and antigen presenting cells is the main mechanism of action, according to which naive B lymphocytes expressing a membrane-bound antibody molecule are activated when they bind to their specific antigen and divide quickly into memory B cells and effector B cells that induce humoral immunity'

This is a short cut. B cells are activated upon direct recognition of antigen and a signal coming from T cell that has been previously activated by a DC This is not a mechanism of action...

• **Intro part on *Drosophila***
- (ref 11) is a paper unrelated to what it is linked ('homeostatic factors').
- Can melanization be considered as a part of the humoral response?
- What does 'captivation' mean?
- The role of dScam in phagocytosis is poorly established compared to NimC1. I am not aware that Eater has been shown to 'locate' bacteria.
- 'also exhibit local immunity': 'Also' seems weird when the part above discusses systemic immunity.

• **Intro part on specificity**
- Specificity has been shown. Flies activate adapted immune response to aggressors (ex. Toll/Imd, encapsulation only against parasites). This is not new. What is possibly new is the high degree of specificity.
- What is the meaning of 'selected specificity' and 'selective adaptability'?
- 'They also found similar adaptation': the term adaptation is unclear.

• Boman, in his famous 1972 Nature paper, has already shown that priming with *E. clocae* could protect fly from an infection by *P. aeruginosa*. This could be mentioned.Figure 4: Eater is not a cellular deficient mutant but a phagocytic mutant. Idem for 'humoral response mutant' in figure 6. This is not precise enough.

2) All the experiments in figures 3, 4, 5, 6, 7 should be repeated at least a second time in a way to have an independent repeat and a higher number of flies.

3) The observed effect is not striking: improvement of only 2h. From my point of view, this actually suggests that priming is not very efficient and could be simply explained by the long-lasting effect of AMPs or other immune factors. This hypothesis should be discussed first before evoking dScam, other complex mechanisms or the concept of memory. The memory hypothesis of dSCAM is clearly not needed to explain the observed results.

4) Mutation should be better described and written in italic. Is there an *eater* mutant or are the authors using a set of deficiencies to generate a mutant?

5) Figure 3: this author has already reported that Toll pathway contributes to host defense against *P. aeruginosa*. In this case, can they explain why *dif* mutant survives better than the the wild-type?

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Dimitrios Kontoyiannis, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
Approved with reservations: 20 March 2013

Ref Report: 20 March 2013
This is an interesting report on a complex and somewhat controversial area (insect immune memory responses) by an investigator who has done nice work in the field. The experimental methods are sufficient, although some methodological issues might create some difficulties in data interpretation;

- The results would be more convincing if isogenic strains of *Pseudomonas* and *Drosophila* were used, as differences are rather small (and possibly biologically non-significant), raising the question of genetic-background influences. In addition, backing up the mortality experiments with bacterial burden data (CFU/gr) or even histopathology in selected differences would make the claims much stronger.
- It would be of interest to have, in addition to heat-killed bacteria, septic injury in the same day as the control. Perhaps priming could occur even by septic injury, in a bacteria-independent factor
- Future experimental direction could be to investigate the effect of different inocula and whether there is a critical minimal threshold of exposure that results in priming.
- Another way to investigate the role, if any, of phagocytosis would be to feed wild flies corticosteroids (see Chamilos G *et al*. PNAS) and evaluate if wild type flies have sluggish priming compared to corticosteroid-unexposed flies.
- I would also analyze survival data of figs 3 and 6 by alternative statistical method in view of the close overlap.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Bernard Mathey-Prevot, Department of Pharmacology & Cancer Biology, Duke University School of Medicine, Durham, NC, USA

Approved: 18 March 2013

Ref Report: 18 March 2013

In this manuscript, Christofi and Apidianakis present an extension of their previous work on immune priming with *Pseudomonas aeruginosa*. They focus on the duration of the protective response elicited against the virulent PA14 strain by pre-treating adult *Drosophila* flies with live or heat-killed low-in virulence CF5 bacteria. There are few new insights into the mechanisms by which the protective effect is mediated (involvement of the Toll, Rel and Imd pathways as well as phagocytosis), but the report makes the important point that the duration and extent of priming will vary greatly with the bacteria or fungi used in the experiments, and strongly advocates that future studies into the adaptive characteristics of insect immunity should be carried out over more than a day rather than a few hours as it is commonly done. Careful and extended time-courses will uncover important differences in how priming can lead to significantly diverse responses to infection with one strain of bacteria or another.

General: I do have some issues and would like the authors to comment on the following:

- The authors present reasonably performed experiments, but I do have some reservation about the conclusions drawn from their experiments. In particular, I was struck that OR and CS strains show some differences in response to priming (Fig. 3). While the end point is similar at 30h for both strains, they show very distinct survival rates between 25 and 29h. This apparent difference might suggest that different genetic background will play an important role in the priming response. In that regard, I wonder why the OR or CS were chosen as controls, rather than strains that are of the same genetic background than the mutants used in this study. What is also surprising is that the authors chose to ignore the apparent discrepancy between the two strains, and furthermore go on to perform additional experiments where they either chose OR (Fig. 4, 5) or CS (Fig. 6, 7) without really justifying or giving a rationale why they included a particular control strain rather than the other. For consistency sake, it would have been better to select one or the other, or include both stains in each experiment.
- I am probably missing something but I don't get the logic in Fig 7. The double priming at 5 and 7 days prior to infection is said to extend the protective effect over that of 7 days, but in reality they are looking at flies which had been primed last at 5 days prior the challenge. To me, the correct comparison should have been doubly primed CS flies vs. 5 day primed CS flies, rather than untreated CS. Untreated flies should have been included, but only to serve as a reference.

- One potential explanation for the modest involvement of plasmatocytes and phagocytosis in mediating protection against a challenge with PA14 might be that the initial priming leads to a transient increase in plasmatocytes. It would be nice to have a sense of whether the number of circulating plasmatocytes is increased after priming. I realize that the protection observed in priming is supposed to be strain specific (no cross protection against other bacteria); however, the protective effect related in this report is rather modest. As such, one wonders whether there are two types of responses: 1) The increase in plasmatocytes, which alone would confer a broad protection but be too weak to have a significant effect on other bacteria, and 2) The humoral and recognition pathways that confer specificity against a particular strain.

Minor: The sentence on page 2, starting with “In addition to the plasmatocyte-expressed…” to the end of the paragraph is awkward and needs to be edited.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Petros Ligoxygakis, Department of Biochemistry, University of Oxford, Oxford, UK
Marcus Glittenberg, Department of Biochemistry, University of Oxford, Oxford, UK
Approved with reservations: 12 March 2013

Ref Report: 12 March 2013
In this manuscript, the authors study a controversial issue in insect immunity: the existence of memory or in other words whether sub-lethal doses of a pathogen may "prime" the insect to respond more efficiently (and survive) a subsequent infection with an otherwise lethal dose of the same pathogen.

Memory in insect immunity has been reported since the field began in the classic work of Metalnikow [Metalnikow S (1929) Immunité d'adaptation et immunité de defense SR. *Soc. Biol.* **101**, 34–67] and more recently in a paper by David Schneider's lab [Pham LN, Dionne MS, Shirasu-Hiza M, Schneider D (2007) A specific primed response in Drosophila is dependent on hemocytes. *PLoS Pathog*. **3**, e26] as well as in a paper published by Siva-Jothy's lab in Sheffield [Moret Y & Siva-Jothy MT (2003). Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*. *Proceedings of the Royal Society of London B*, **270**: 2475-2480].

Therefore, the possible existence of memory (or "priming") is an interesting subject and warrants further investigation to explore the limitations of such a response and its characteristics. The types of experiments that one would use to do this (and are indeed used in this work) are mainly survival experiments following infection. Any differences must be very well documented with appropriate statistical tests.

Regarding statistical analysis one comment that I would like to make (prompted by Figure 6; curves for other figures seem OK) is that crossing survival curves between different treatments/genotypes indicate non-proportional hazards (so one needs to check for crossing hazards to be sure). Such a scenario increases the probability of a Type II error when using the log-rank test (and weighted log-rank tests) i.e. concluding there is no statistical difference when there actually is one. **There are alternative tests to analyse data with crossing hazards (e.g. Renyi-type), and applying one of these to the data set of Figure 6 in addition to their current analysis is a must.**

Regardless of statistics, their wild-type-background result (and the basis of the research) is a shift in survival of 2 – 4 hours if flies are pre-primed: what is the significance of that? For Figures 3 and 4 the OR flies survive at approx. 30% by 30 hours (although I am not clear of how many times these experiments were repeated? See below comment) – **what would be of more interest is whether these flies have recovered from the PA14 infection** i.e. do they continue to survive after 30 hours (and for how long)? Have the flies cleared / controlled the PA14 bacteria. **The authors therefore need to look at survival and CFUs beyond 30 hours.**

Further, the biggest shift at 50%_survival - from all the experiments - between primed and non-primed is 4 hours (Fig. 3, OR): **is this a real difference or is it within the boundaries of variation?** Generally, from what I can tell, experiments have only been performed once, and mostly this is with 19 – 35 flies: **if more flies were used, along with biological repeats, would the same trends consistently be observed?**

Finally, I think the authors really need to show the fly survival with CF5 and heat-killed CF5; but more

importantly, inject live CF5 into the pre-seeded flies as they did with PA14 i.e. if then, the pre-seeded CF5-live flies survive slightly better as they did with PA14, it points to a general mechanism rather than one relating to the virulence of the bacteria. And vice versa, where if a strain is more virulent, then “priming” may be advantageous.

Minor: Generally, data is better presented as Kaplan-Meier curves; since you are dead or not dead, having slopes between time-of-death is misleading (unless the data set is modelled).In figure legends: if error bars are shown, it should be indicated what they are describing. And the test used to derive the P-value should be given with this value.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.