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Report

Insect Immunity Shows Specificity in Protection upon Secondary Pathogen Exposure

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Summary

Immunological memory in vertebrates, conferring lasting specific protection after an initial pathogen exposure, has implications for a broad spectrum of evolutionary, epidemiological, and medical phenomena [1]. However, the existence of specificity in protection upon secondary pathogen exposure in invertebrates remains controversial [1–6]. To separate this functional phenomenon from a particular mechanism, we refer to it as specific immune priming. We investigate the presence of specific immune priming in workers of the social insect *Bombus terrestris*. Using three bacterial pathogens, we test whether a prior homologous pathogen exposure gives a benefit in terms of long-term protection against a later challenge, over and above a heterologous combination. With a reciprocally designed initial and second-exposure protocol (i.e., all combinations of bacteria were tested), we demonstrate, even several weeks after the clearance of a first exposure, increased protection and narrow specificity upon secondary exposure. This demonstrates that the invertebrate immune system is functionally capable of unexpectedly specific and durable induced protection. Ultimately, despite general broad differences between vertebrates and invertebrates, the ability of both immune systems to show specificity in protection suggests that their immune defenses have found comparable solutions to similar selective pressures over evolutionary time.

Results and Discussion

If one wishes to understand the invertebrate immune system in terms of both evolution and ecology, a whole-organism functional approach is a promising complement to the search for proximate mechanisms [7]. Here we investigate the occurrence of long-term specific immune priming in workers of the bumblebee *Bombus terrestris*. Social insects such as *B. terrestris* should be particularly dependent on immune priming because their social life makes them prone to repeated exposure to the same pathogens established in a colony. We use the gram-negative bacterium *Pseudomonas fluorescens* [8], and two closely related gram-positive bacteria, *Paenibacillus alvei* and *P. larvae*, that are known to be pathogens of insects. *P. alvei* and *P. larvae* cause similar diseases in honeybees, *Apis* spp [9]. These three bacteria were chosen because they were found to be clearable at low doses by all immune-naïve bumblebees tested, whereas higher doses had a similar probability of killing bumblebees tested in preliminary trials (see Table S1 in the Supplemental Data available with this article online). This was supported by the fact that in the full experiments reported in this paper there was no effect of bacterial type on the survival probability ($p > 0.7$ for all tests). Our experiments were designed to answer the question of whether a prior homologous pathogen exposure gives a benefit in terms of greater response and survival upon a second exposure, over and above that given by a prior heterologous exposure. Therefore, for the question being asked, functionally relevant groups were assigned in the experiment concerning the relatedness of an initial injection to the secondary pathogen exposure that an individual bumblebee worker had received: homologous; related heterologous; heterologous; and control saline.

We carried out experiments to investigate the ability of the bumblebee's immune defense to show protection, specificity, or both upon exposure to a pathogen when the bee had previously been exposed to a rapidly clearable homologous (i.e., same bacterium), related heterologous (congeric bacteria), heterologous (different bacteria), or control (saline) challenge. We carried out two experiments with time lags of 8 and 22 days between initial and secondary exposure to investigate the effects of time on any observed immune-system priming (see Figure S1). We observed the survival of age-controlled bumblebee workers after secondary exposures of a high bacterial dose (2 μl of 2.5×10^6 cells/ml). We first checked that the results wee not due to selection imposed by the treatment regime. Indeed, we found no effect of the first injection on survival ($LR\chi_2 = 1.62$ [= the change in the −2 log-likelihood if the term is removed from the model], d.f. = 3, $p = 0.655$). Considering the effect of the type of previous exposure, we found that when the lag between exposures was 8 days there was a significant effect of exposure combination on survival. However, individuals receiving a homologous combination were only longer lived than saline-injected individuals receiving a bacterial challenge for the first time (Figure 1A and Table 1A). In contrast, when the lag between exposures was 22 days, individuals exposed to a homologous combination showed significantly greater survival than individuals exposed to any other exposure combination (Figure 1B and Table 1B). The lag of 22 days meant the second exposure took place 27 days after emergence. Priming of this duration would compare with an average life span of around 4 weeks for adult workers in the field. Our experiment was designed to determine whether a prior homologous pathogen exposure gives a benefit in terms of greater response and survival after a second exposure, over and above that given by a prior heterologous exposure, and not to investigate particular combinations of

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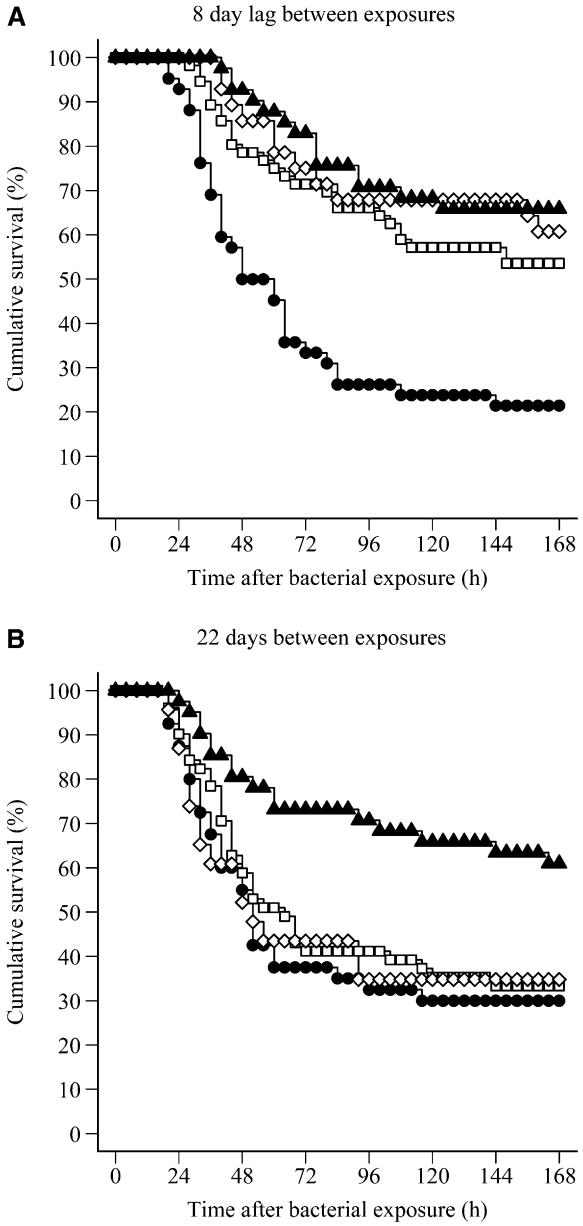

Figure 1. The Survival of Worker Bumblebees after Secondary Exposure to Bacteria

Survival curves of workers after exposure to lethal bacterial doses either 8 (A) or 22 (B) days earlier. Groups refer to the initial injection and its relatedness to the secondary bacterial exposure: individuals only receiving a Ringer saline injection previously (circle, n = 42 for the 8 day lag, n = 40 for the 22 day lag) or individuals having received a heterologous (square, n = 56, n = 51), related heterologous (i.e., congeneric bacteria; diamond, n = 28, n = 23), or homologous (triangle, n = 41, n = 41) prior bacterial exposure. See Table 1 for statistics on the predictors of survival.

bacterial species. However, when the experimental results are separated by the second bacterial challenge, the same trends can be seen in every case (see Figure S2).

In addition to being checked for survival, a second group of bumblebee workers was checked for levels of bacterial clearance 24 hr after receiving secondary exposures of intermediate bacterial doses (2 μl of 1.5×10^6 cells/ml). The proportion of individuals having cleared this intermediate bacterial dose was significantly greater in the group exposed to a homologous combination than in individuals exposed to any other exposure combination at both 8 and 22 days after initial exposure (Figure 2 and Table 2).

The above results demonstrate that bees having been exposed to a previous bacterial challenge are more likely to clear a subsequent homologous infection and to survive this challenge better than when exposed to a heterologous second infection. This response is specific even to the level of differences between congeneric bacteria (*Paenibacillus*). Specificity is expressed in terms of 24 hr clearance of bacterial cells from the hemolymph with 8 or 22 days between the first and second exposures, and in increased survival after a lag of 22 days. The discrepancy between cumulative survival and rapid clearance on day 8 could be due to the presence of passive protection conferred by lingering factors involved in a more general immune response [10]. It is plausible that such factors take a longer time to act than the factors responsible for specific priming. Because of such a difference in response time, we may expect to see the observed discrepancy in the level of specificity between the results of 24 hr clearance and those for longer-term survival at this time point. However, the protection and specificity we see at 22 days is more reminiscent of the functional outcome of immune memory in vertebrates.

Induced antibacterial peptides that persist in the hemolymph as soluble molecules are unlikely to be the reason for the observed protection. In insects, transcription of antibacterial peptides starts hours after the challenge but subsides after 24–36 hr, with the peptide products degrading after this time [11]. Furthermore, previous studies suggest that antibacterial activity lingers for up to 14 days in bumblebee workers [12]. Additionally, zone-of-inhibition assays [12] were carried out with the hemolymph of 104 individuals collected 8 or 22 days after a single challenge of Ringer saline or one of the three bacteria used in this study. These samples were probed on plates seeded with the three bacterial species. In none of the cases did we find any residual activity of soluble hemolymph peptides, whereas positive controls (1 mg/ml Tetracycline) produced inhibition on all plates. Therefore, the effects on day 22, long after transcription and the lingering of antimicrobial peptides have ceased, are suggestive of a mechanism other than lasting protection conferred by the passive lingering of soluble peptides. Recent molecular work has uncovered novel mechanisms by which invertebrates may diversify their repertoire of pathogen-recognizing molecules and thereby achieve a higher level of specificity [13, 14]. These mechanisms appear to be conserved across the major insect orders [13] and may play a key role in the specific immune priming in insects.

The demonstration of durable specific immune priming in an invertebrate reinforces suggestive results of previous studies in the fields of ecological immunology, immunology, and host-parasite interactions. Prior to the recent discovery of recognition-molecule diversity [13], studies with invertebrates uncovered genotype-genotype interactions between hosts and parasites that suggest a high degree of specificity in the host response

Table 1. Results of the Cox Regression Model for Survival after a Second Bacterial Exposure

Time Lag between Exposures	Factors	b[a]	S.E.[b]	Wald[c]	df	p[d]	Odds[e]
(A) 8 days	Body size	1.11	0.48	5.39	1	0.02	
	Injection combination			28.65	3	<0.001	
	Homologous versus initial Ringer saline	1.47	0.33	19.89	1	<0.001	4.35
	Homologous versus heterologous	0.50	0.34	2.18	1	0.139	1.65
	Homologous versus related heterologous	0.20	0.41	0.23	1	0.630	1.22
(B) 22 days	Body size	0.94	0.53	3.17	1	0.075	
	Injection combination			10.51	3	0.015	
	Homologous versus initial Ringer saline	1.00	0.32	9.90	1	0.002	2.71
	Homologous versus heterologous	0.77	0.30	6.46	1	0.011	2.16
	Homologous versus Related heterologous	0.81	0.36	5.03	1	0.025	2.25

[a] Regression coefficient of overall survival function for variable.
[b] Standard error of regression coefficient.
[c] Wald statistic for variable.
[d] Significance level for Wald statistics.
[e] Odds ratio of relative survival for variable.

[15–18] . Further studies have shown induced responses that give future protection [19, 20]; some such responses can only be induced by appropriate proteins [21, 22]. However, functional studies showing both specificity and protection in tandem are rare and often involve various caveats. Bacterial-strain-specific resistance was clearly demonstrated as a maternal effect in *Daphnia* [23], but there was no study within individuals of the same generation. Early work on American cockroaches claimed to demonstrate specificity and protection [24, 25]. However, either these studies did not explicitly implicate immunity over tolerance and had no measure of clearance [24], or sequential challenges were not truly homologous because initial exposures were heat-killed [25]. In addition, with the lack of a reciprocal design [25], these findings could have been equally explained by one bacterial challenge eliciting a stronger, but not specific, immune response. A more recent study carried out on copepods [26] suggested specific priming in invertebrates, but this study was over a very short time frame (the second infection was only 3 days after the first challenge), did not explicitly implicate immunity, and could have been confounded by kin-mediated competition among parasites [26].

The present work overcomes the above issues by using truly homologous exposures and a reciprocal design. The active clearance of bacterial cells means that we can rule out the possibility that we are merely observing non-immune-related tolerance of the bacteria after secondary exposure. The results clearly show that the insect immune system is capable of, at least, lasting bacterial species-specific protection upon secondary exposure. Specificity at this level is still coarse grained compared to the recognition ability of the adaptive immune system of vertebrates. However, this level of specificity is greater than that of which the invertebrate immune system was previously thought to be capable [27]. Because truly homologous bacterial strains were used for each species, the result does not exclude the possibility that the invertebrate immune system could show a still finer grain of specific protection. An immune mechanism conferring both protection and specificity after secondary exposure to a pathogen type is likely to be especially useful when individuals are exposed to the same pathogens repeatedly. Such scenarios are likely when hosts and pathogen types are spatially linked, especially in the case of social living. For example, many social insects, such as the bumblebee *Bombus terrestris,* are faced with such a scenario when a particular pathogen type becomes frequent once it has gained entry into the colony [9].

The functional demonstration of specific protection and its persistence over a period of weeks in insects is important for understanding the evolution, ecology, and epidemiology of diseases [28, 29] and should be relevant for measures of pest control. We again emphasize that our experiment does not and could not address the mechanisms underlying the specific immune priming and protection. But we also stress that showing such a function is a crucial first step in defining the search for molecular and cellular mechanisms that generate such specific immune priming in invertebrates. We would also remind the reader that the demonstration of an immunological memory in vertebrates (e.g., by vaccination) pre-dated knowledge of antibodies and memory cells by decades or even centuries. Hence, the search for the corresponding mechanisms in invertebrates is timely and, according to our results, should be rewarding.

Conclusion

In this study we demonstrate that the immune system of insects is capable of responding in a specific manner to a previously encountered pathogen type. Such a lasting specific response, shown here over a period of three weeks, is also functionally protective upon secondary exposure. The functional similarity between the outcome of immunological memory in vertebrates and the specific protection demonstrated here suggests that, despite general differences between these lineages, their immune systems have been selected to cope with similar tasks.

Experimental Procedures

Insects

All animals used in this study were of the species *Bombus terrestris*. Workers were sourced from healthy colonies set up from queens collected in northwestern Switzerland in the spring of 2005. Callow

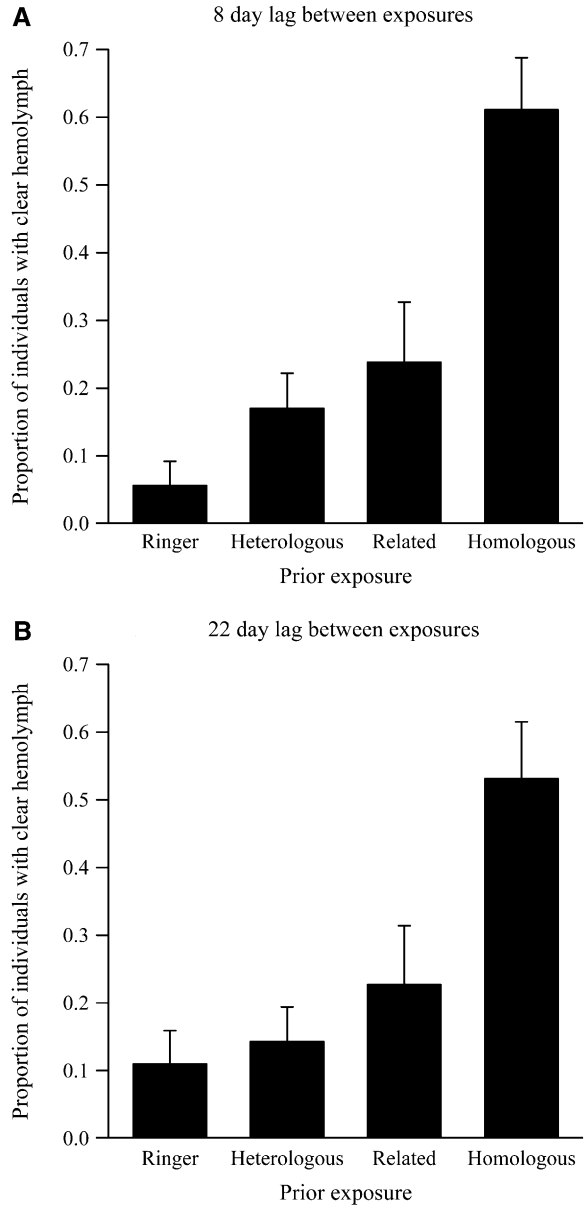

Figure 2. The Clearance of Bacterial Cells 24 hr after Their Injection into the Hemolymph of Bumblebee Workers

The proportion of workers having cleared a bacterial exposure after 24 hr after having previously been exposed to initial injections 8(A) or 22(B) days earlier. Groups refer to the relatedness of the two exposures, being initial injections of only a Ringer saline solution (n = 36 for the 8 day lag, n = 36 for the 22 day lag) or heterologous (n = 47, n = 42), related heterologous (n = 21, n = 21), or homologous (n = 36, n = 32) bacterial injections. See Table 2 for statistics on the predictors for the clearance of bacteria from the hemocoel. Error bars refer to 95% confidence intervals for binomial distributions.

workers were removed, isolated, and randomly assigned to one of the experimental groups 5 days after emergence. For each experiment, treatments were fully repeated in each colony used. After experimentation, the length of the radial cell of the forewing was taken as a measure of body size and included in analyses when appropriate. All colonies and individually isolated bees were kept at 28ºC ± 2ºC under red-light illumination, with pollen and sugar water (ApiInvert) provided ad libitum. All injections took place between 1400 hr and 1800 hr, and treatments carried out on a particular day were randomly assigned.

Bacteria

Pseudomonas fluorescens (DSM 50090), *Paenibacillus alvei* (DSM 29), and *Paenibacillus larvae* (DSM 3615) were used. These bacteria were cultured in the appropriate medium (www.dsmz.de) at 30ºC prior to use. Immediately before use, bacterial cells were washed three times by centrifuging (1300 × g, 4ºC, 10 min), removal of the supernatant, and resuspension in Ringer saline solution. We determined cell concentrations and adjusted them to achieve one of three dosage levels to be injected into worker bumblebees in 2 μl amounts. Cell concentration was adjusted to 5×10^4 cells/ml for an initial exposure dose that all naïve individuals could clear within 48 hr of injection. An intermediate dose was adjusted to 1.5×10^6 cells/ml. A high dose was adjusted to 2.5×10^6 cells/ml. We injected bacteria or saline solutions by chilling worker bees on ice and inserting a sterile pulled-glass micro-capillary needle between the first and second abdominal tergites.

Exposure Combination and Survival

Five days after emergence, individuals received an initial dose of either one of the three bacterial species or saline. At 8 or 22 days after initial exposure, we injected high doses of the three bacteria to achieve the four exposure combinations. The "Ringer" group contained individuals previously only receiving a saline injection. The "heterologous" group received combinations of *P. fluorescens* with either *P. alvei* or *P. larvae*. The "related heterologous" group received combinations of *P. alvei* with *P. larvae*. The "homologous" group contained individuals receiving the same bacterial species twice. After the second exposure, survival was recorded every 4 hr for 7 days, at which point surviving individuals were censored in the analysis.

Exposure Combination and Bacterial Clearance

Injection combinations were carried out as above, except that second bacterial challenges consisted of the intermediate dose. Twenty-four hours after the second exposure, individuals were chilled on ice, and all haemolymph was removed with a chilled glass micro-capillary needle. The removed haemolymph was added to 100 μl of chilled bacterial medium, and this solution was then further diluted by two sequential 10× dilutions. Ten microliters of each of the solutions was spread onto plates containing agar medium and incubated for 24 hr. We counted bacterial colonies and calculated the number of colonies per bee by taking into account the dilution factor and the initial volume of haemolymph collected. It was assumed that each differentiated bacterial colony originated from a single cell.

Statistical Analysis

In all experiments there was no effect or trend in responses due to the bacterial species, and thus groupings remained as the functionally relevant "Ringer," "heterologous," "related heterologous," and "homologous." Analysis of predictor variables for survival upto the secondary exposure was carried out with a Binary logistic regression. Body size and initial injection type were entered, and a backward stepwise procedure was used to determine the best model. Analysis of survival data was carried out via the Cox regression with a backward stepwise procedure including body size and then injection combination as predictor variables. For analysis of the bacterial cell counts, a large number of zeros and non-normal distribution required the dataset to be recoded for analysis. Codes of 0 or 1, respectively, were given to individuals that had not cleared the bacteria from their haemolymph and to those that had. These data were analyzed by a binary logistic regression with a backward stepwise procedure including body size and then injection combination as predictor variables. All analyses were carried out with SPSS 11 for Mac OSX.

Supplemental Data

Supplemental figures are available with this article online at http://www.current-biology.com/cgi/content/full/16/12/1206/DC1/.

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Table 2. Results of the Binary Logistic Model for Clearance of a Second Bacterial Exposure

Time Lag between Exposures	Factors	b[a]	S.E.[b]	Wald[c]	df	p[d]	Odds[e]
(A) 8 days	Body size	−0.07	1.21	0.003	1	0.955	
	Injection combination			26.27	3	<0.001	
	Homologous versus initial Ringer saline	−3.88	0.88	19.36	1	<0.001	0.021
	Homologous versus heterologous	−2.508	0.61	16.84	1	<0.001	0.081
	Homologous versus related heterologous	−2.06	0.71	8.34	1	0.004	0.127
(B) 22 days	Body size	3.40	1.59	4.58	1	0.032	
	Injection combination			19.61	3	<0.001	
	Homologous versus initial Ringer saline	−2.73	0.75	13.23	1	<0.001	0.065
	Homologous versus heterologous	−2.87	0.73	15.46	1	<0.001	0.057
	Homologous versus related heterologous	−1.90	0.76	6.23	1	0.013	0.150

[a] Regression coefficient of overall survival function for variable.
[b] Standard error of regression coefficient.
[c] Wald statistic for variable.
[d] Significance level for Wald statistics.
[e] Odds ratio of relative clearance for variable.

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