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Strain-specific priming of resistance in the red flour beetle, *Tribolium castaneum*

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As invertebrates lack the molecular machinery employed by the vertebrate adaptive immune system, it was thought that they consequently lack the ability to produce lasting and specific immunity. However, in recent years, it has been demonstrated that the immune defence of invertebrates is by far more complicated and specific than previously envisioned. Lasting immunity following an initial exposure that proves protection on a secondary exposure has been shown in several species of invertebrates. This phenomenon has become known as immune priming. In the cases where it is explicitly tested, this priming can also be highly specific. In this study, we used survival assays to test for specific priming of resistance in the red flour beetle, *Tribolium castaneum*, using bacteria of different degrees of relatedness. Our results suggest an unexpected degree of specificity that even allows for differentiation between different strains of the same bacterium. However, our findings also demonstrate that specific priming of resistance in insects may not be ubiquitous across all bacteria.

Keywords: immune priming; specificity; adaptive; immune defence; invertebrate; *Tribolium castaneum*

1. INTRODUCTION

Immune specificity is the ability to react against one type of pathogen without concurrent cross-reactivity against other pathogens (Frank 2002; Kurtz 2005). When coupled with immune priming (an immune-mediated increase in protection to a secondary exposure following an initial exposure, relative to naive individuals), the phenomenon of specific immune priming can be achieved. This is the ability, once primed with a particular immune elicitor, to mount a more pronounced and/or faster response on a secondary exposure to this same immune elicitor, than to a distinct elicitor (Agaisse 2007; Pham *et al.* 2007). The presence of specific immune priming is the basis behind vaccination, and allows organisms to plastically adapt to the prevailing pathogen environment.

Vertebrate hosts possess both an innate and adaptive immune system, the latter being characterized by a high degree of specificity and a form of specific immune priming, better known as immune memory. However, extensive homology between vertebrates and invertebrates has only been found for the innate arm of the immune system (Kush *et al.* 2002; Tzou *et al.* 2002; Little *et al.* 2005). Therefore, due to the lack of potential molecular mechanisms, invertebrates were considered to lack both specificity and immune priming functionally similar to that found in vertebrates (Klein 1989).

Recently, molecular work in fruitflies and mosquitoes has begun to uncover the potential for a large diversity of immune receptors in invertebrates (Watson *et al.* 2005; Dong *et al.* 2006). This work coupled with experimental data showing an astonishing degree of specificity (Schmid-Hempel & Ebert 2003) and immune priming (Kurtz & Franz 2003; Little *et al.* 2003; Sadd & Schmid-Hempel 2006; Pham *et al.* 2007) within invertebrate systems, suggesting that a phenomenon of specific immune priming, functionally analogous to vertebrate immune memory, is present in invertebrates (Little & Kraaijeveld 2004; Schmid-Hempel 2005), too.

Specific immune priming has been demonstrated over an adult's lifetime in bumble-bees exposed to bacterial pathogens (Sadd & Schmid-Hempel 2006), and also to strains of tapeworm parasites, *Schistocephalus solidus*, in copepods (Kurtz & Franz 2003), albeit the latter study covered only a short time period (see Rowley & Powell 2007). Similar results have been demonstrated in *Drosophila melanogaster* for particular pathogen types in a study that also reported that this specific immune priming is mediated by phagocytosis (Pham *et al.* 2007). Furthermore, transfer of immunity to offspring depending on the mother's or nest-mate's own experience (trans-generational immune priming) has also been shown in invertebrates (Little *et al.* 2003; Sadd *et al.* 2005; Sadd & Schmid-Hempel 2007). In *Daphnia magna*, this was even demonstrated to be bacterial strain specific (Little *et al.* 2003). While these studies have advanced our understanding of the abilities of invertebrate immune systems, the potential for lasting immune priming that is specific to different strains or genotypes of the same parasite species, thus functionally matching the abilities of the vertebrate immune system, is still unknown.

Outcomes of specific immune priming are differences in resistance, probably based on different immune defences after a primary and a secondary exposure to a pathogen. Such resistance can be subsequently measured as a consequence for survival. Using the model system of the red flour beetle, *Tribolium castaneum*, and bacteria of different degrees of phylogenetic relatedness, we tested for specific priming of resistance in a survival experiment. The bacteria used were either related to one another as defined

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by Gram type (Gram-positive versus Gram-negative), different species within the same genus or different strains within the same species. Larvae were primed with heat-killed bacteria, and eight days later challenged with a potentially lethal (high) dose of live bacteria in a reciprocal design. We used heat-killed bacteria for priming to exclude any confounding effect of harm caused by an initial infection and to guarantee that no live bacteria were present in the animal at the time of the second challenge. Using this set-up, our aim was to investigate the level at which invertebrates show specific priming of resistance.

2. MATERIAL AND METHODS

(a) *The model system*

Owing to its size, short generation time and ease of maintenance and manipulation, *T. castaneum* has already been used for a long time as a model organism for the investigation of the ecology, behaviour and genetics of host–parasite interactions (Park 1948; Sweeney & Becnel 1991). Recently, *T. castaneum* has been further developed into a model system for embryonic development and pesticide resistance (Lorenzen *et al.* 2005; Shippy & Brown 2005), population genetics (Zhong *et al.* 2004; Demuth & Wade 2007), mate choice (Bernasconi & Keller 2001; Pai & Yan 2002; Pai *et al.* 2007; Pai & Bernasconi 2008) and for the study of host–parasite coevolution (Pai & Yan 2003; Fischer & Schmid-Hempel 2005). As its genome sequence has been completed, it is likely that other fields of biology will adopt this model system as well (Richards *et al.* 2008; http://www.hgsc.bcm.tmc.edu/projects/tribolium/).

Tribolium castaneum is known to naturally harbour a range of protozoan and other parasites (West 1958, 1960; Sokoloff 1974; Padin *et al.* 2002; Blaser & Schmid-Hempel 2005; Fischer & Schmid-Hempel 2005). These beetles, nowadays mainly living in mills, grain stores and bird nests, are very likely to be exposed repeatedly to similar infections. In our experiment, we carried out controlled immune priming (first exposure) and challenges (second exposure) using the following bacteria: *Escherichia coli* (DSM no. 498); *Bacillus thuringiensis* 1 (DSM no. 2046, isolated from a Mediterranean flour moth); *B. thuringiensis* 2 (DSM no. 6073); and *Bacillus subtilis* (DSM no. 1088). All bacteria were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Bacillus thuringiensis* is a natural pathogen of *T. castaneum* known to affect beetle fitness negatively (Abdel-Razek *et al.* 1999; Hou *et al.* 2004). *Escherichia coli* was chosen as a very widely distributed bacterium. Neither *E.coli* nor *B. subtilis* are known to be pathogenic to *T. castaneum*. The main goal of our experiment was to study the potential ability of the immune system of an insect to raise a specific immune response, i.e. to discriminate among antigens used for priming and challenge, rather than to work with naturally pathogenic, infectious bacteria (this is also the reason why we heat killed our bacteria for the priming). However, we took advantage of the system by taking one natural pathogen and two bacteria that occur in the natural environment of *T. castaneum* but are so far not known to have a negative impact on them. This design made it possible to test for specific priming of resistance in natural and non-natural host–bacteria interactions.

(b) *Experiment*

Prior to the experiment, a new outcrossed line of *T. castaneum* was produced as follows, to ensure higher genetic variability and to facilitate generalization of the results. Adults of 10 *Tribolium* existing stock lines (coming from different localities around the world) were singly distributed to 30 vials with 3 g of flour each, such that every vial contained 10 individuals (five females and five males). With this design, we forced the animals to outbreed as only mating partners from other lines were offered to them. After two weeks, the adults were taken away and the offspring in the 30 vials were pooled to start the new outbred population. The populations were then allowed to grow and the main experiment started six weeks after the start of generating the outbreeding population. Five 400 ml glass jars were filled with 150 g of flour each. To every glass jar, approximately 200 adult *T. castaneum* were added, and the animals were kept for 48 hours at a temperature of 30°C and 70 per cent humidity in the dark. Subsequently, all adult *T. castaneum* were sieved out of the jars, so that only the eggs were left in the flour. Five days later, young larvae were separated from the flour with a 270 µm mesh size sieve and the larvae were allocated individually to wells of 96-well plates filled with flour. After a further 10 days, the isolated larvae were exposed to priming with heat-killed bacteria. For this purpose, *E. coli*, *B. thuringiensis* strain 1, *B. thuringiensis* strain 2, and *B. subtilis* were grown overnight in medium (5 g peptone, 3 g meat extract, 1000 ml distilled H_2O, pH = 7) at 33°C, then heat killed in a heat block at 90°C for 20 min, centrifuged and counted in a Thoma counting chamber to adjust the concentration to 10^9 cells ml^{-1} in insect Ringer's solution. The animals were exposed to bacteria by dipping a 0.05 mm diameter needle into the bacteria solution and pricking the animal between the last and penultimate segments at a horizontal angle to prevent puncturing the gut (Roth & Kurtz 2008). As controls, we included animals pricked with a needle dipped into insect Ringer's solution (wounding control) and naive animals. After eight days, their survival was checked and they were exposed to a challenge with live bacteria, which were grown as described above and adjusted to a cell concentration of 10^{11} ml^{-1} in insect Ringer's solution. One hundred and fifty-six animals died between priming and challenge (corresponding to 20% mortality), but the dead animals were distributed among all treatments and no significant differences in survival were found between the treatment groups (numbers of dead animals between priming and challenge: *B. subtilis*, 27; *B. thuringiensis* 1, 26; *B. thuringiensis* 2, 25; *E. coli*, 27; naive, 23; Ringer, 28). Challenge treatments were performed in a fully reciprocal design, such that all priming treatments were combined with challenge treatments of *B. subtilis*, *B. thuringiensis* 1, *B. thuringiensis* 2 and *E.coli* for a total of 6×4 bacteria treatment combinations; additionally, the combinations of Ringer–Ringer, naive–Ringer and naive–naive (priming–challenge) were performed, with 23 replicates each, yielding a total of 621 animals. After challenging, animals were randomly distributed into 96-well plates with flour, and survival was checked daily for the next 10 days and every second day thereafter. Following 17 days, the experiment ceased and all animals were sacrificed.

(c) *Statistics and analyses*

The three different control treatments (naive–naive, Ringer–Ringer and naive–Ringer) did not differ from each other (proportional hazards fits, effect likelihood

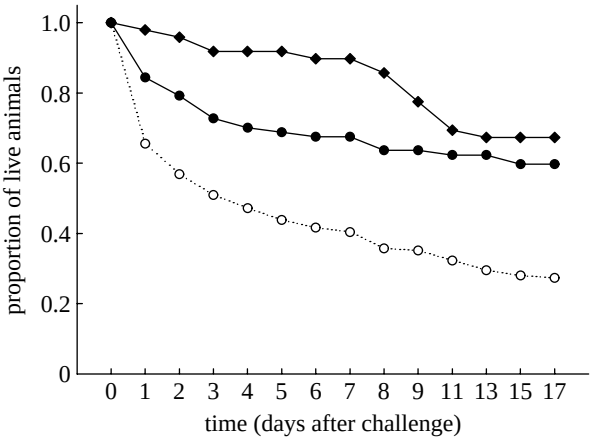

Figure 1. The proportion of individuals surviving following a challenge when they had been previously primed with either a homologous or heterologous bacteria. Homologous: filled circles, Bt1–Bt1, Bs–Bs, Ec–Ec, Bt2–Bt2. Heterologous: open circles, Bt2–Bt1, Bt1–Bt2; Bt2–Bs, Bs–Bt2, Bt1–Bs, Bs–Bt1; Ec–Bt2, Bt2–Ec, Bt1–Ec, Ec–Bt1, Bs–Ec, Ec–Bs; Rin–Ec, Rin–Bt1, Rin–Bt2, Rin–Bs; naive–Bt1, naive–Ec, naive–Bs, naive–Bt2. Controls: filled diamonds, naive–naive, Rin–Rin, naive–Rin. Bt, *B. thuringiensis*; Bs, *B. subtilis*; Ec, *E. coli*; Rin, Ringer.

ratio test, $\chi^2 = 353$; $p = 0.8382$), suggesting that wounding did not affect survival. The control treatments were thus pooled in further analyses.

All other results were analysed on three different levels to answer our main questions (see §3). Initially, we looked at functional categories relating to the priming (first exposure) and the challenge (second exposure). That is, we tested whether those beetles receiving the same bacterial strain twice (homologous) showed a difference in survival compared with those animals that experienced two different exposures (heterologous). All homologous and heterologous bacteria combinations were combined here. The second analysis was to test whether there is a significant priming×challenge interaction, which would suggest that some priming×challenge combinations lead to different effects on survival. We here performed a two-way proportional hazard analysis with priming and challenge as fixed factors and days surviving as the response variable. This analysis clarified whether the interaction was mainly driven by differences among homologous (the same bacteria exposure twice) and heterologous (exposure to two different bacteria) treatment combinations. Furthermore, we could also investigate whether a difference in the level of relatedness of bacteria used for priming and then challenge influenced the probability of survival. In detail, we wanted to know whether different Gram types (priming with Gram-negative–challenge with Gram-positive, or vice versa), different bacterial species (priming with one bacterial species–challenge with another bacterial species within the same genus), different strains (genotypes, i.e. priming with one strain of *B. thuringiensis* and challenge with the other strain of *B. thuringiensis*) or a homologous combination (identical bacteria for the priming and challenge) had different effects on survival. In this analysis, the control treatments were excluded to perform a more balanced analysis.

In the third analysis, the combinations of bacteria were not pooled, but every possible combination was analysed in a full model, such that we could see whether the bacterial type matters for immune priming. For all analyses, a proportional hazard survival test was used and the analyses were performed in JMP 6 (SAS Institute Inc.) and R (R Development Core Team).

3. RESULTS

(a) *Can we find specific priming of resistance in* T. castaneum*?*

Animals experiencing a homologous challenge (the same bacteria twice) survived significantly longer than those experiencing a heterologous challenge. Control animals (either left naive or treated with Ringer's solution to test for the effect of pricking) did not differ from homologous combinations, but survived significantly longer than the heterologous combinations (proportional hazards fits, effect likelihood ratio test, $\chi^2 = 46.13$; $p < 0.001$, the significant difference is revealed by the non-overlapping confidence intervals (heterologous, 0.466–0.907; homologous, −0.488–0.1) (figure 1).

(b) *How specific is the priming of resistance in* T. castaneum*?*

Homologously challenged animals had a greater probability of survival than any of the heterologous combinations. All the different heterologous combinations (different Gram types, different species and different strains) show the same pattern. Hence, the significant priming×challenge interaction appears to be largely driven by the differences between homologous combinations and heterologous ones. This suggests that the immune defence of *T. castaneum* can differentiate even at the species level among very closely related bacteria (table 1).

(c) *Does specific priming vary among bacteria?*

Here, we tested whether every homologous combination would give a survival advantage or whether the outcome of specific priming of resistance varies among bacterial species, as suggested by Pham *et al.* (2007). For example, natural pathogens may induce a more specific response than other non-pathogenic bacteria that may be encountered in an environment.

In the host–bacteria combinations challenged with *B. thuringiensis* 1, those receiving homologous combinations had a greater probability of survival than all heterologous combinations (figure 2*a*; table 2*a*), i.e. priming of resistance was highly specific for *B. thuringiensis* 1. In the animals challenged with *B. thuringiensis* 2, there was no significant difference, but we found a clear trend suggesting increased survival resulting from homologous exposure (figure 2*b*; table 2*b*). Beetles challenged with *B. subtilis* homologously survived significantly longer than those heterologously treated with *B. thuringiensis* 2–*B. subtilis* and those with *E. coli*–*B. subtilis*. Those treated with naive–*B. subtilis* and *B. thuringiensis* 1–*B. subtilis* differed from neither the homologous combinations, nor the other heterologous combinations (figure 2*c*; table 2*c*), yet again there was a trend for increased survival after homologous exposure. Within animals challenged with *E. coli*, there were no significant differences or trends between priming and challenge combinations (figure 2*d*; table 2*d*). These results suggest that, while present in the response to all *Bacillus* species, specific priming of resistance is absent with regard to defence against *E.coli*.

Table 1. A two-way proportional hazard analysis testing for the effects of bacteria priming (first exposure) and bacterial challenge (secondary exposure) on beetle survival. (The confidence intervals show all performed treatment combinations. The highly significant priming×challenge effect emerges mainly from survival differences among heterologous and homologous pathogen exposures. Asterisks indicate significant values; Nparm, number of parameters.)

source	Nparm	d.f.	χ^2	*p*-value
priming	3	3	7.587	0.0554
challenge	3	3	10.054	0.0181*
priming×challenge	9	9	26.282	0.0018*
	lower CL	upper CL		
priming Bs	−0.435	0.146		
priming Bt1	−0.565	0.006		
priming Bt2	−0.164	0.37		
challenge Bs	−0.65	−0.052		
challenge Bt1	0.115	0.64		
challenge Bt2	−0.371	0.173		
priming Bs×challenge Bs	−1.39	−0.205		
priming Bs×challenge Bt1	−0.07	0.833		
priming Bs×challenge Bt2	−0.32	0.647		
priming Bt1×challenge Bs	−0.49	0.583		
priming Bt1×challenge Bt1	−1.34	−0.306		
priming Bt1×challenge Bt2	−0.01	0.893		
priming Bt2×challenge Bs	0.006	0.955		
priming Bt2×challenge Bt1	−0.228	0.629		
priming Bt2×challenge Bt2	−1.29	−0.263		

Table 2. The results of effect likelihood ratio tests (proportional hazards) for survival among the different treatments to investigate how specific the priming of *T. castaneum* is. (The four analyses are for animals challenged with (*a*) Bt1, (*b*) Bt2, (*c*) Bs and (*d*) Ec. Asterisks indicate significant values; Nparm, number of parameters.)

source	Nparm	d.f.	χ^2	*p*-value
(*a*) Bt 1 challenge				
treatment	5	5	14.726	0.0116*
	lower CL	upper CL	different	
Bs–Bt1	−0.411	0.594	A	
Bt1–Bt1	−1.805	−0.472	B	
B2–Bt1	−0.359	0.615	A	
Ec–Bt1	−0.152	0.754	A	
naive–Bt1	−0.183	0.705	A	
(*b*) Bt 2 challenge				
treatment	5	5	6.174	0.2896
	lower CL	upper CL	different	
Bt1–Bt2	−0.639	0.506	A	
Bt1–Bt2	−0.452	0.579	A	
Bt2–Bt2	−1.422	−0.088	A	
Ec–Bt2	−0.246	0.815	A	
naive–Bt2	−0.286	0.718	A	
(*c*) Bt 3 challenge				
treatment	5	5	12.056	0.034*
	lower CL	upper CL	different	
Bs–Bs	−1.754	−0.183	A	
Bt1–Bs	−0.918	0.435	AB	
Bt2–Bs	0.075	1.207	B	
Ec–Bs	−0.075	1.053	B	
naive–Bs	−0.692	0.434	AB	
(*d*) Bt 4 challenge				
treatment	5	5	1.283	0.937
	lower CL	upper CL	different	
Bs–Ec	−0.602	0.579	A	
Bt1–Ec	−0.643	0.486	A	
Bt2–Ec	−0.453	0.562	A	
Ec–Ec	−0.855	0.229	A	
naive–Ec	−0.389	0.596	A	

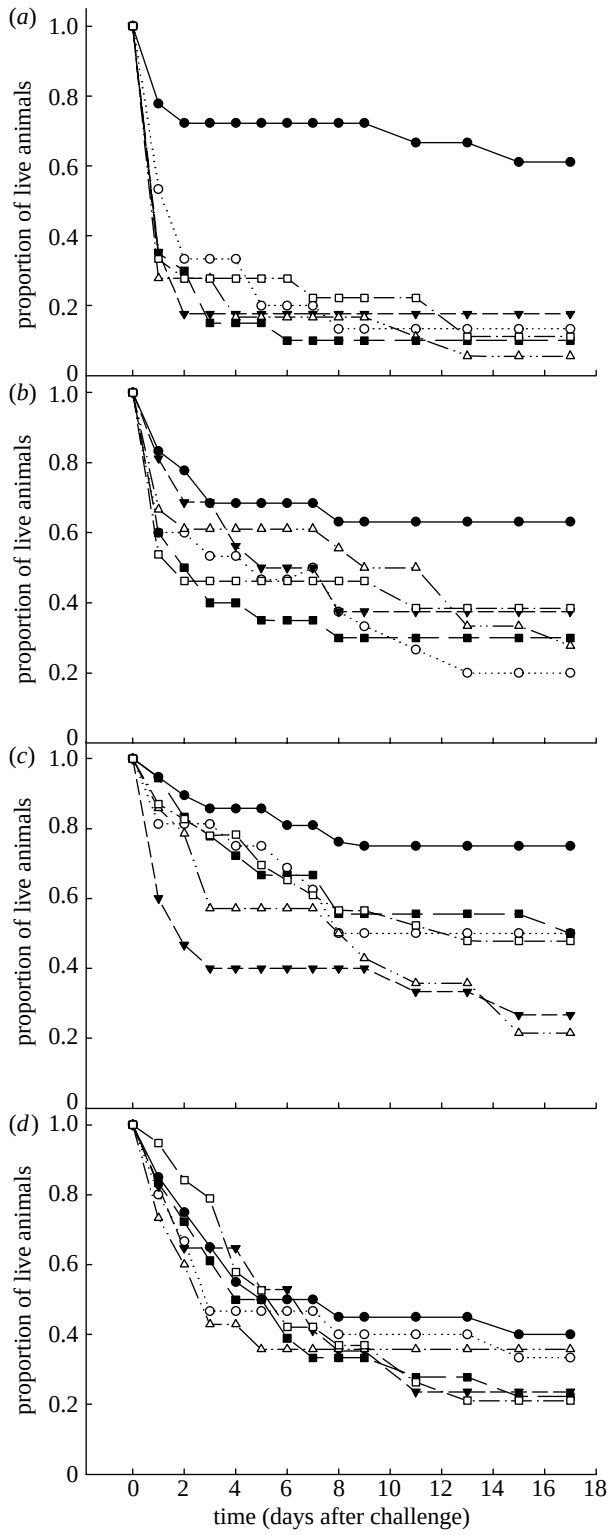

Figure 2. The proportion of individuals surviving following a challenge ((*a*) challenge with Bt1, (*b*) challenge with Bt2, (*c*) challenge with Bs, (*d*) challenge with Ec) when they had been previously primed with either a homologous (filled circles: (*a*) Bt1–Bt1, (*b*) Bt2–Bt2, (*c*) Bs–Bs, (*d*) Ec–Ec) or different levels of heterologous bacteria (open circles: (*a*) Bs–Bt1, (*b*) Ec–Bt2, (*c*) Bt1–Bs, (*d*) Bt1–Ec; filled down triangles: (*a*) Bt2–Bt1, (*b*) Bs–Bt2, (*c*) Bt2–Bs, (*d*) Bt2–Ec; open up triangles: (*a*) Ec–Bt1, (*b*) Bt1–Bt2, (*c*) Ec–Bs, (*d*) Bs–Ec). Filled squares represent animals that were left naive at the priming; open squares represent animals that were treated with Ringer's solution at the priming.

4. DISCUSSION

(a) *Specific priming of resistance in* T. castaneum

Our results demonstrate that beetles exposed to previous priming with heat-killed bacteria are more likely to survive a subsequent exposure to live bacteria that is homologous to the priming, than a heterologous exposure. This supports previous data, which revealed that invertebrates are capable of some degree of specific resistance against pathogens on a secondary exposure (Kurtz & Franz 2003; Little *et al.* 2003; Sadd & Schmid-Hempel 2006). Furthermore, it shows that protection can be induced by heat-killed bacteria, thus resembling the phenomenon of vaccination. Red flour beetles are relatively long-lived insects with a maximum lifespan of approximately 2 years (Sokoloff 1974). Therefore, they have a high probability of encountering the same parasite strain repeatedly. This may select for mechanisms that reduce the impact of a secondary exposure and also reduce the costs of induction of defences from a naive level, such as specific immune priming (Little & Kraaijeveld 2004; Rowley & Powell 2007). Thus, the specific priming of resistance we observed in this study is likely to be adaptive in the case of *Tribolium*.

While the immunological mechanisms that are involved in specific priming could not be investigated in our study, Pham *et al.* (2007) have demonstrated that phagocytosis may mediate the high specificity in insect immune defence. We have preliminary data suggesting that phagocytosis is also involved in specificity in *Tribolium* and in the woodlouse, *Porcellio scaber* (O. Roth 2007 and 2008, unpublished data). As far as is known, insects lack somatic rearrangement of immunological receptors as found in vertebrates, and therefore other mechanisms are likely to be involved in creating specific receptors. One recent emerging possibility is the alternative splicing of recognition genes, for example in the *Dscam* gene (Watson *et al.* 2005; Dong *et al.* 2006). This process has the potential to create a sufficient amount of receptor diversity to discriminate between a variety of different pathogen types (Watson *et al.* 2005; Dong *et al.* 2006; Kurtz & Armitage 2006).

Clearly, more research on the immunological background of specific priming of resistance, as demonstrated here, is needed to substantiate a relationship between survival after subsequent homologous bacterial challenge, the immune defence and the proposed molecular mechanisms.

(b) *How specific is priming of resistance in* T. castaneum*?*

The only studies that tested for a long-lasting specific protection on a secondary exposure within individuals did not test for specificity against different strains of the same pathogen (Sadd & Schmid-Hempel 2006; Pham *et al.* 2007). Studies looking at specificity on the level of strains have either used only a short period between the second and first exposure (Kurtz & Franz 2003) or considered only trans-generational effects (Little *et al.* 2003). Here, we demonstrate that in some combinations of bacteria, the defence of *T. castaneum* shows high specificity at the strain level of the ubiquitous pathogen *B. thuringiensis*. This hints to a defence system that is capable of a high degree of specificity, with limited cross-reactivity against similar but novel pathogens.

(c) *Does specific priming vary among bacteria?*

In *Drosophila*, specific priming was tested for four different pathogens, but specific protection was only shown for *Streptococcus pneumoniae* (Pham *et al.* 2007). The results of our study also suggest that the phenomenon of specific priming depends on the type of pathogen involved. One out of four bacteria gives significant results in terms of specific priming (*B. thuringiensis* 1), while two others show a trend towards this (*B. thuringiensis* 2 and *B. subtilis*). For *E. coli*, we demonstrate that, under our experimental conditions, the animals cannot be primed. There are various reasons why priming might not be observed against all bacteria, including the possibility that the animals commonly encounter a given set of bacteria, and thus are already primed or have high constitutive defences for which priming is not active. In our study, the use of one natural bacterium could have an impact on the results, as *B. thuringiensis* is known to decrease *T. castaneum* fitness (Abdel-Razak *et al.* 1999). We found stronger specific priming of resistance in *T. castaneum* against *B. thuringiensis*, than against *B. subtilis* and *E. coli*. To only react with specific priming of resistance against natural pathogens may make sense, as too much variety of specific immune defences may come at enormous costs, for example, due to the expense of recruiting specific cell populations.

5. CONCLUSION

The innate immune defence of invertebrates shows many functional and mechanistic homologies with vertebrate immune defence. The phenomenon of specificity in immune defence may have evolved several times, as selection for mechanisms of specific immune defence may arise due to similar pressures from parasites and pathogens across different taxa (Schmid-Hempel & Ebert 2003; Zhang *et al.* 2004; Watson *et al.* 2005; Dong *et al.* 2006; Terwilliger *et al.* 2006; Buckley & Smith 2007; Litman *et al.* 2007; Brites *et al.* 2008). The adaptive immune defence of vertebrates, with its somatic recombination, is probably mechanistically unique, but functionally not as exceptional as was traditionally supposed.

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