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Antimicrobial Defense and Persistent Infection in Insects

Eleanor R. Haine,¹ Yannick Moret,² Michael T. Siva-Jothy,¹ Jens Rolff^{1*}

During 400 million years of existence, insects have rarely succumbed to the evolution of microbial resistance against their potent antimicrobial immune defenses. We found that microbial clearance after infection is extremely fast and that induced antimicrobial activity starts to increase only when most of the bacteria (99.5%) have been removed. Our experiments showed that those bacteria that survived exposure to the insect's constitutive immune response were subsequently more resistant to it. These results imply that induced antimicrobial compounds function primarily to protect the insect against the bacteria that persist within their body, rather than to clear microbial infections. These findings suggest that understanding of the management of antimicrobial peptides in natural systems might inform medical treatment strategies that avoid the risk of drug resistance.

By contrast with the clinical use of antibiotics, resistance to natural antibiotics appears to be rare (1, 2). Possibly, natural antibiotics play a different role in the wild than in medical applications (3), and our lack of understanding of

their natural role results in unforeseen problems when they are used therapeutically, such as the rapid emergence of antibiotic-resistant pathogens.

Insects rely on a suite of systemic responses to combat infection (4) that can be classified into

two main types. "Constitutive" defenses are always present and ready to act; they rely on the response of insect immune cells (haemocytes) and several rapidly activated enzyme cascades such as phenoloxidase (5, 6) to defend against pathogens. Coupled with this line of defense is the "induced" response, which consists mainly of a suite of antimicrobial peptides (7). This component of the antimicrobial response takes at least 1 to 3 hours to generate (8) and 12 to 48 hours to reach peak levels (9). The induced response persists for weeks in a variety of insects: for example, at least 14 days in bumble bees (10) and mealworm beetles (9), and up to 44 days in dragonflies (11). Because immune responses bear costs [e.g., antagonistic pleiotropy (12), metabolic costs (13), and self-harm (14)], these slow and long-lasting antimicrobial responses, which are

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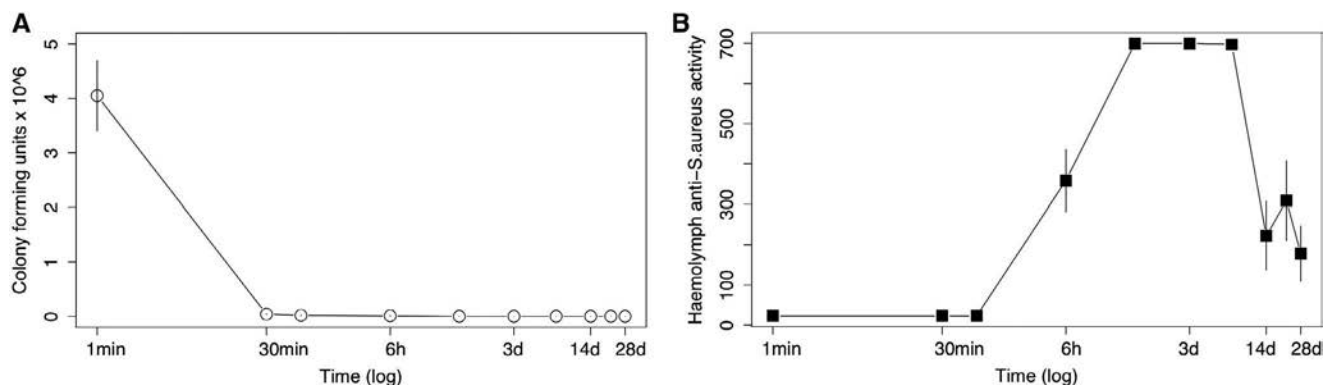


Fig. 1. The number of colony-forming units (CFU) recovered from *T. molitor* haemolymph over 28 days (A), and the haemolymph anti-*S. aureus* activity from the same individuals (B). Induced haemolymph anti-*S. aureus* activity

was measured as the number of *S. aureus* CFUs killed during 2 hours of exposure to *T. molitor* haemolymph and is shown as CFU × 10³. Each point represents the mean number of CFUs from 7 to 10 beetles (±1 SEM).

under selection in the wild (15), must have an as yet undetermined adaptive benefit. Insects may maintain heightened defenses after an insult to deal with reinfection by the same agent (16, 17). However, recent work on *Drosophila melanogaster* showed that haemocytes, rather than antimicrobial peptides, are responsible for the protection against secondary infection (18).

Alternatively, we propose that long-lasting antimicrobial responses serve to “mop up” bacteria that survive the constitutive immune response and eliminate or control these potentially resistant bacteria from the haemocoel. At the level of an individual host, this would prevent the reappearance of infection that is refractory to the host’s constitutive defenses. A consequence of this process would be a reduction in the emergence of resistant bacteria that at least provides a means of managing persistent infections, especially because most insects use a variety of antimicrobial peptides (7).

We made three predictions from this basis. (i) Most bacteria will be eliminated before the induced antimicrobial response occurs. (ii) Some bacteria will survive the initial haemocyte-mediated immune response and persist in the haemolymph for at least as long as the induced antimicrobial response persists. (iii) When exposed to a naïve insect, these surviving bacteria will be more resistant to the insect’s constitutive immune response than the original pathogen strain.

We conducted two studies to test our predictions. First, we examined how quickly a dose of bacteria is cleared from insect haemolymph by the constitutive immune system, while measuring

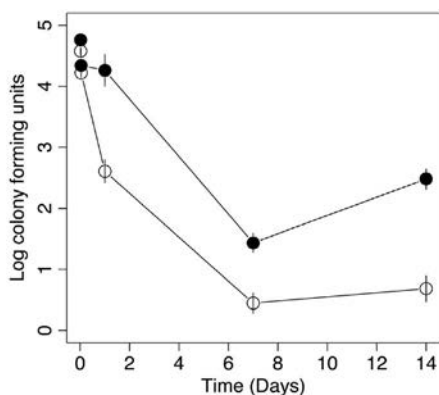


Fig. 2. The \log_{10} of CFUs recovered from *T. molitor* haemolymph after injection of the host-naïve strain of *S. aureus* (open circles) and after injection of surviving bacteria into naïve individuals (closed circles). Briefly, glycerol stocks were made from bacterial colonies recovered at five different time points after injection of the original *S. aureus* strain; these were then used to immunize naïve beetles, and the number of surviving CFUs was counted at the same time points, i.e., bacteria recovered after 1 day of exposure to the beetle immune response were injected into naïve individuals, and the number of CFUs surviving was counted after 1 day. Each data point represents the mean log CFUs counted from 10 individuals \pm 1 SEM.

induced haemolymph antimicrobial activity in the same insects [Supporting Online Material (SOM)]. Second, we conducted a selection experiment in which we compared the survivorship of bacteria within hosts that had been exposed to insect immune responses for different times with the naïve population from which they were drawn.

We measured the ability of the beetle *Tenebrio molitor* immune system to clear bacteria from its hemocoel by injecting a large dose [4×10^6 colony-forming units (CFUs)] of *Staphylococcus aureus* (19, 20) into the body cavity of individual adult female beetles. We used stationary-phase bacteria to ensure that there was sufficient genetic variation in the overnight culture for our selection experiment and because naturally acquired bacteria in an insect’s environment are likely to be in stationary phase. We used a high dose to maximize the likelihood of detecting downstream effects in the experiment. Haemolymph was harvested at 10 time points (between 0 and 28 days) after injection and used to determine the number of surviving bacteria and the induced antimicrobial activity against *S. aureus* in the haemolymph. There was very rapid clearance of *S. aureus* from naïve host haemolymph. More than 99.5% of injected *S. aureus* had been cleared in less than an hour (Fig. 1). Recovery of live bacteria continued to fall until 14 days after injection, after which ~50 CFUs per host remained. It is possible that rather than being killed, bacteria were inaccessible to our sampling technique. Although our data showed that bacteria were attached to insect tissues (SOM), they also revealed that the pattern of *S. aureus* clearance from tissue homogenates was identical to that from the haemolymph samples (SOM), indicating that these bacteria were still attacked by the host’s immune system.

Cell-free haemolymph was tested for its ability to kill *S. aureus* by means of an in vitro killing reaction (SOM). Induced antimicrobial activity only started to increase in the haemolymph 30 min after most bacteria were cleared (Fig. 1). This activity peaked at 24 hours after challenge, long after most bacterial clearance had occurred, and remained elevated until 28 days after challenge, when there were few recovered CFUs (Fig. 1).

Constitutive defenses, including haemocytes and cytotoxic enzyme cascades, are responsible for “frontline” physiological defense against microbial insults (21). Our observations imply that induced antimicrobial effectors do not function to clear bacteria, but rather “mop up” those that have survived selection via the host’s constitutive defenses (22). We propose that “surviving” bacteria exhibit some resistance to the initial beetle immune response and that the function of the late, and prolonged, peak in induced antimicrobial peptide activity is to prevent the enrichment of resistant bacteria. This argument relies on the assumption that the surviving bacteria are more resistant to the host’s defenses than were those bacteria that were killed. We tested this assumption by harvesting surviving bacteria from

beetles after five different periods of immune exposure within the host (30 min, 1 hour, 1 day, 7 days, and 14 days). Surviving bacteria were then grown overnight in vitro, after which we compared survival of persisting bacteria against the survival of naïve bacteria within naïve beetle hosts. The results are shown in Fig. 2. Separate analyses of covariance testing for the effects of “strain” (“survivors” versus “naïve”) and beetle size on \log_{10} CFUs recovered at each time point revealed significant effects of strain at 30 min ($F_{1,18} = 9.97$, $P = 0.0054$), 1 day ($F_{1,15} = 58.63$, $P < 0.0001$), 7 days ($F_{1,17} = 12.62$, $P = 0.0025$), and 14 days ($F_{1,24} = 23.33$, $P < 0.0001$). Host size had no effect. Significantly more “survivor” *S. aureus* survived after injection into naïve beetles compared with “naïve” bacteria (Fig. 2). Bacteria that survived longer exposure to a beetle’s immune system were better survivors upon exposure to a naïve beetle’s immune system.

Two arguments favor our idea that long-lasting antimicrobial activity has evolved as part of a two-stage process, preventing resistance evolution in bacteria and/or managing persistent infections. First, bacteria readily evolve resistance against individual antimicrobial peptides in isolation (23), and recent work (18) suggests that phagocytic haemocytes are responsible for the immune reaction against secondary infections in insects. Moreover, our explanation for long-lasting induced immunity functions in the context of a single infection, whereas the prophylactic explanation requires at least two infections.

Our results have two important implications. First, antimicrobial peptides could well be the last line of defense dealing with persistent infections and the prevention of the evolution of resistant mutants. Second, at the functional level, our interpretation is analogous to human antibiotic therapies. The current focus on the development of insect and other natural antimicrobial peptide-based drugs (2) should take into account functional studies of these compounds in their ecological context (3).

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