Perspectives on the evolutionary ecology of arthropod antimicrobial peptides

Jens Rolff1,2 and Paul Schmid-Hempel3

1Evolutionary Biology, Institute of Biology, Freie Universität Berlin, Königin-Luise-Strasse 1–3, 14195 Berlin, Germany
2Berlin-Brandenburg Institute of Advanced Biodiversity Research (BBIB), 14195 Berlin, Germany
3ETH Zürich, Institute of Integrative Biology (IBZ), ETH-Zentrum CHN, Universitätstrasse 16, 8092 Zürich, Switzerland


Accepted: 23 March 2016

One contribution of 13 to a theme issue ‘Evolutionary ecology of arthropod antimicrobial peptides’.

Subject Areas:
- evolution
- immunology
- genetics

Keywords:
- antimicrobial peptides
- ecological immunology

Author for correspondence:
Paul Schmid-Hempel
e-mail: psh@env.ethz.ch

Antimicrobial peptides (AMPs) are important elements of the innate immune defence in multicellular organisms that target and kill microbes. Here, we reflect on the various points that are raised by the authors of the 11 contributions to a special issue of Philosophical Transactions on the ‘evolutionary ecology of arthropod antimicrobial peptides’. We see five interesting topics emerging. (i) AMP genes in insects, and perhaps in arthropods more generally, evolve much slower than most other immune genes. One explanation refers to the constraints set by AMPs being part of a finely tuned defence system. A new view argues that AMPs are under strong stabilizing selection. Regardless, this striking observation still invites many more questions than have been answered so far. (ii) AMPs almost always are expressed in combinations and sometimes show expression patterns that are dependent on the infectious agent. While it is often assumed that this can be explained by synergistic interactions, such interactions have rarely been demonstrated and need to be studied further. Moreover, how to define synergy in the first place remains difficult and needs to be addressed. (iii) AMPs play a very important role in mediating the interaction between a host and its mutualistic or commensal microbes. This has only been studied in a very small number of (insect) species. It has become clear that the very same AMPs play different roles in different situations and hence are under concurrent selection. (iv) Different environments shape the physiology of organisms; especially the host-associated microbial communities should impact on the evolution of AMPs. Studies in social insects and some organisms from extreme environments seem to support this notion, but, overall, the evidence for adaptation of AMPs to a given environment is scant. (v) AMPs are considered or already developed as new drugs in medicine. However, bacteria can evolve resistance to AMPs. Therefore, in the light of our limited understanding of AMP evolution in the natural context, and also the very limited understanding of the evolution of resistance against AMPs in bacteria in particular, caution is recommended. What is clear though is that study of the ecology and evolution of AMPs in natural systems could inform many of these outstanding questions, including those related to medical applications and pathogen control.

This article is part of the themed issue ‘Evolutionary ecology of arthropod antimicrobial peptides’.

1. Introduction

Scientific interest in insects has a long History, for example the first issue of Philosophical Transactions, published in 1665, included two letters on the breeding of silkworms (Bombyx mori). Reports of insect diseases are even older—more than 2500 years ago, Chinese breeders noted ‘abnormalities’ in their silkworm stocks. Today, these abnormalities are known to result from infections by fungi (typically Cordyceps). However, the formal study of insect diseases had to await pioneers such as Agostino Bassi (1773–1856) and Louis Pasteur (1822–1895), who paved
the way for the field of insect pathology [1]. Starting with Ilia Metschnikow’s classic observation on the cellular response of starfish against foreign objects, the interest in immune defences also turned towards how invertebrates, and insects in particular, might defend themselves against infections. As knowledge accumulated, it became clear that the immune system of invertebrates has both a cellular (based on cells) and a humoral (based on soluble components) component, like the much better studied defences of vertebrates [2].

Immune defences based on humoral components include evolutionarily ancient systems, such as complement or antimicrobial peptides (AMPs). The first AMP of a multicellular organism to be characterized was cecropin [3]: first identified in another silk moth, *Hyalophora cecropia*, this ultimately paved the way for the discovery of the role of the Toll-pathway in innate immunity [4]. AMPs are potent effectors against a wide range of microorganisms. Over the course of an infection, AMPs are rapidly expressed in epithelial surfaces [5] and the humoral defences [6,7]. This expression can last for weeks and hence AMPs may also serve to clear bacteria that have survived the initial response [8]. Moreover, AMPs have other functions such as fostering the right conditions for mutualists and acting as modulators of the immune response [9–11]. A large number of studies and reviews on AMPs have been published to date, but the majority of studies on arthropod AMPs have focused on their biochemical and molecular properties [12–14].

This issue focuses on AMPs in insects and related taxa. Insects are the major taxon within the arthropods, and constitute approximately 70% of named animal species [15]. To date, 256 AMPs from insects (http://aps.unmc.edu/ap/) have been described. Given the diversity of insects, this is almost certainly a tiny fraction of all existing AMPs. Even in such a well-studied organism such as *Drosophila* a new group of AMPs, the bomanins, was only discovered last year [16]. While the discovery of new AMPs is considered economically and medically important as a potential source for new antimicrobials (see also [9] and [17]), using AMPs as drugs is also controversial because of the risks of cross-resistance to host AMPs (see below; [18]). Questions such as why such a diversity of AMPs exists, or how and why it is maintained are of fundamental interest for understanding their biology. These questions have rarely been addressed, but point to several conundrums. In this review, we discuss a number of features that make AMPs special and which, therefore, deserve attention— in the light of the contributions to this volume. We expand the traditional focus on molecular mechanisms by considering the role of AMPs at the interface with the microbial world and in an evolutionary ecology framework.

(a) Antimicrobial peptides evolve slowly compared with other immune genes

Immune genes evolve rapidly in comparison with most other genes [19–21]. One common explanation for this difference is the strong selective pressure exerted by pathogens. In fact, pathogens are ubiquitous, numerically abundant and often have shorter generation times than hosts; thus, pathogens should evolve faster than their hosts. An example illustrating the latter is the fast pace at which virulence can evolve in experimental settings as was for example shown in a co-evolutionary experiment using *Caenorhabditis elegans* as a host and *Bacillus thuringiensis* as a pathogen [22]. There is considerable evidence supporting the view that fast adaptation by pathogens drives the fast evolution of immune genes. This is most obvious in the co-evolution of hosts with their viruses. Host antiviral RNAi genes of *Drosophila*, for example, evolve rapidly, including duplication and loss of genes over longer time scales [20]. But so do the suppressors of these elements on the side of the viruses [21], a situation that is indicative of a fast host–parasite co-evolutionary race. Genetically, this kind of co-evolution can be based on a rapid dynamic of selective sweeps, where favourable resistance alleles rise in frequency, but decline again as the parasites adapt and the alleles lose their selective advantage [23]. As detailed studies have suggested, the effects are not simply owing to immune genes being subject to fewer evolutionary constraints but are a reflection of the selective pressures on them [24].

When looking more closely at the immune system of arthropods in general and insects in particular, it becomes clear that patterns of evolutionary change are more complex than the simple scenario above would suggest. For example, in *Drosophila*, receptor molecules that serve the cellular arm of the defence, such as thioester-containing proteins and class C scavenger receptors, show high rates ofadaptive evolution—but not in the domains that ensure the binding to the foreign surface. Rather, changes occur in domains that ensure the activation of the proteins [25,26]. Similar complexity is evident in the canonical pathways of insect immune defence, such as the Toll or Imd pathways. These pathways are evolutionarily conserved across large taxa (e.g. within the arthropods). Similarly, genes for pattern recognition receptors—which are involved in activating the humoral defences—are conserved. However, some genes within recognition pathways change rapidly and show signs of adaptive evolution. In the *Drosophila* group, this is the case for extracellular (serpins or serine proteases) and intracellular signalling molecules (*Dredd*, *Imdl*, transcription factors such as *Dorsal* or *Relish*), or for transmembrane receptors [24,26,27].

Against this background, it is remarkable that AMPs do not follow this pattern of fast evolution in immune genes. Although they are important effector molecules, interact tightly with microbial surfaces, and are ubiquitous [28,29], their sequence (i.e. at the amino acid level) seems to be surprisingly conserved, at least in insects [20,24,30,31]. The case for vertebrates seems less clear as signatures of selection on AMPs are documented [32,33]. In *Drosophila*, the best-investigated group of insects to date, individual genes in all classes of AMPs show very low rates of evolution or even no signs of selection [20,24,30,31]. The families of AMP genes, in contrast, show evolutionary dynamics in that gene duplications and deletions are observed [34]. A similar pattern of gene duplication of AMP genes is found in other crustaceans such as shrimps [35]. Even though evidence for adaptive evolution in AMPs in arthropods and insects is therefore not completely absent [30,36–38], the pattern is not as clear as for other genes of the immune system. It thus remains puzzling that, at the level of individual genes, AMPs are well conserved and yet have not lost their effectiveness against a wide range of potentially rapidly co-evolving parasites.

Unckless & Lazzaro [39] now discuss an alternative view of the evolutionary dynamics of AMPs. They argue that in a number of cases—especially in the vertebrates—where no signs of directional selection on AMPs are seen, there is, nevertheless, evidence for polymorphism in AMPs, i.e. the presence of more than one allelic variant, as reported for frogs [40], passerine birds [33], humans [32] or Atlantic cod [41]. A single-site
amino acid polymorphism in defensin, likely showing signs of historical selection and adaptation, has also recently been discovered in mussels [42].

Polymorphisms can be maintained by a number of different processes, most importantly, by balancing selection. In their re-analysis of AMPs from various species of *Drosophila*, Unckless & Lazzaro [39], indeed, find the telltale signs of balancing selection. If this were a general pattern, then this result would open up new avenues of research and pose the important question under which ecological conditions a particular variant of the same AMP becomes favourable and when the selection regime would turn itself against a frequent allele. In fact, balancing selection implies an evolutionary dynamic that unfolds over short time scales, pushing back and forth the frequency of different alleles in the population—with the longer-term effect of maintaining several alleles in a population over time. Pathogens, with their rapid rate of adaptation to changes in the host population, would be prime candidates to create such a selection regime. Selection resulting from the interactions of the immune system with mutualistic microbes also likely plays a role here. Such interactions are arguably more frequent than encounters with parasites and, therefore, could even be the most important selective force.

(b) Antimicrobial peptides act in combination and can relate to specificity

As individual genes, AMPs may be rather conserved, even if they show some degree of polymorphism [39]. However, despite this limited variation at the level of genes, the deployment of AMPs after infection can be regulated differentially. AMPs can be used in a variety of ways, for example, by expressing a different kind of AMP cocktail depending on the kind of infecting parasite strain. This would generate different effects—depending on how the AMPs act in combination. The possible interactions can be synergistic, additive and antagonistic, i.e. the effect of two AMPs combined is stronger, equal and weaker than that of the individual AMP, respectively. Several contributions to this volume emphasize the role of possible synergistic action of AMPs. Mylonakis et al. [9] refer to synergisms of AMPs with added antibiotics against challenging and drug-resistant bacterial pathogens such as *Staphylococcus aureus*. Such interactions of AMPs with ‘foreign’ antibiotics or other agents have been studied repeatedly [43] because of their obvious importance to medical applications: the effect of an administered drug can be enhanced by adding a second compound, such as an AMP. From an evolutionary ecological perspective, however, the interaction between different AMPs produced by the host itself is of prime interest.

A number of studies, including transcriptomic data on AMPs, show that native AMPs are typically expressed in combination [44–49]. Often these expression patterns are assumed to support synergism between co-expressed AMPs—even though experimental evidence by knockouts is still lacking. This pattern of co-expression of AMPs is also observed in many insects, including damsells [50], firebrats [51], Lepidoptera [52], Hymenoptera [53], Coleoptera [54] or Diptera [55]. A parallel extending to the situation in insects is found in another group of ecdyzoa, Hymenoptera [56], Coleoptera [57] or Diptera [58]. A parallel based on the fact that AMPs in pairwise combinations, against different strains of a common trypanosomatid pathogen of bees, under controlled laboratory conditions. Note that the majority of studies to date have focused on the effect of AMPs against bacteria or fungi. By contrast, Marxer et al. [59] focus on the effects against protozoa, in particular, a trypanosome infecting the bumblebee gut. AMPs are known to work against trypanosomes, for example, against *T. brucei* (the agent of human sleeping sickness) or against *Leishmania* [68–70]. By applying the toolbox
of Baeder et al. [64], Marxer et al. [67] found that both the Loewe and Bliss model can be used to describe the combined effects of AMPs. Moreover, the most effective combinations seem not to require maximum doses for each of the tested AMPs. Rather, the best effect in combination typically appears at intermediate doses—a result similar to the findings by Yu et al. [71]. One cautionary note should be added here, as the significance of effects at these intermediate doses when compared with maximum doses is not yet thoroughly investigated. Despite these shortcomings, these studies suggest that analysing interactions among AMPs with the explicit tools of Baeder et al. [64] is a fruitful avenue to quantify synergism; moreover, Baeder et al., provide a protocol for the systematic screening of the combined effects of AMPs.

Typically, the interaction between hosts and parasites is specific, that is, a given variant of the parasite can only infect a limited range of host variants, and, vice versa, a host variant is only resistant to a limited range of parasites [72]. Specificity has a number of important ecological and evolutionary consequences. For example, specificity ensures the maintenance of polymorphisms in both host resistance and parasite infectivity. At the same time, specificity is considered to ultimately result from the balance between costs and benefits of a defence [73]. This is because a generalized response may be beneficial to eliminate or control a wide range of pathogens, but at the same time brings the risk of misdirected actions and is therefore assumed to be more costly.

Given that the classically defined ‘acquired immune system’—based on expanding lymphocyte populations with high degrees of specificity—is restricted to the vertebrates, specificity in the immune response was traditionally considered only to be relevant to vertebrates. However, as numerous studies have shown, this is not true. Specificity is also found in the invertebrate hosts studied to date [73]. The underlying mechanisms and mode of action are often still unclear. For example, the post-transcriptional processing of the Dsca-m cassette—the Down syndrome adhesion molecule, a complex gene encoding for a member of the immunoglobulin superfamily—can generate many thousands of variants of a receptor, but the exact function is still under debate [74–77].

One way to achieve specificity with an innate immune system is through combinations of immune effectors. If so, the same ‘mixture’ of expressed AMPs should affect different parasites or different strains of the same parasite in different ways. In addition, hosts should vary in their expressed response to the strains of a given parasite and, moreover, should respond differently to different parasites. For example, variation in the suppression of a bacterial pathogen among different D. melanogaster lines can be traced back to natural genetic differences in AMPs [78], and vice versa when the same lines are exposed to different bacteria, albeit with weak effects in this case [79]. Sackton et al. [80] studied the expression of candidate genes in infected Drosophila—AMPs among them. They found that expression—depending on which bacteria infected which host genetic line—did not differ much, resulting in only a weak signal of a specific host–parasite interaction. However, several other studies have found variation in AMP expression among host lines responding to the same parasite [81], or the same host responding to different parasites [56], or even different strains [82]. Similar to [57] using AMPs as candidate genes, Barribeau et al. [53], in a genomic study, showed that different host–parasite pairings resulted in significant variation especially in the expression of various AMPs, suggesting that hosts respond to different parasite variants by producing different ‘cocktails’ of AMPs. At the same time, different ‘cocktails’ have been shown to have different effects on the pathogens as in the case of the wax moth [83] or bumblebee [67]. Hence, this work suggests that specific interactions between hosts and parasites can be generated with innate immunity, and that these specific effects are based on the differential use of otherwise rather conserved effectors.

(c) Antimicrobial peptides have several roles and thus pleiotropic effects

As discussed above, AMP genes—especially in the arthropods—typically change more slowly than other genes of the immune system. One possible explanation for this conservation is stabilizing selection [39], which among other things, might be mediated by the action of AMPs in concert with other AMPs and other immune effectors. Yet AMPs do not act only as immune effectors; therefore, AMPs are presumably under concurrent, pleiotropic selection for being able to fulfill their different roles. In vertebrates [11] and to a lesser extent in crustaceans [17], AMPs are also immune-modulators and have anti-inflammatory properties or contribute to wound healing. Thus, while research on AMPs has mostly focused on their antimicrobial properties in the context of pathogens (a fact that might partly be ascribed to the history of their discovery [4]), in many multicellular organisms, AMPs have other functions. They can be important mediators of interactions with commensal or mutualistic bacteria, on both an ecological as well as evolutionary scale. This aspect has not been sufficiently studied, but informative work has been done in the tsetse fly, Glossina [84], mosquitoes [85] or in Rhodnius, the vector of Chagas disease [86]. AMPs are part of the regulatory chain that prepares, activates or supports the immune response, and thereby interactions with the microbiota seem essential.

The gut harbours a microbiota whose number—at least judging by evidence from humans—might rival that of the host’s cells [87]. One of the main uses of the microbiota by the host is the processing of food [88]. In addition, with any uptake of food new microbes [89] are sampled from the environment whereas the host needs to maintain a certain degree of homeostasis in the gut. Microbes entering the gut can potentially become pathogenic, but perhaps could also be considered as competitors for food (in the sense of Janzen’s inter-kingdom competition between microbes and, in the original version, scavengers [90]). In many invertebrates, AMPs play an important role interacting with the gut microbiota [91] or co-evolved symbionts [10,92]. Studies in C. elegans have demonstrated that the combination of AMPs expressed in the gut differs from the composition of AMPs expressed on the exterior cuticle [56], which would add to the interaction scenario.

Gut immunity in insects has been studied in Drosophila [91], an organism that mostly feeds on substrates very rich in microbes, resulting in a close resemblance of the gut microbiota with the microbial food community [93]. At the same time, the gut is constantly changing, both because of the influx of microbes and because of the replacement of host cells by intestinal stem cells. It appears that the composition of the microbiota in the gut results from a coordinated host response based on reactive oxygen species, mediated by Duox, and the expression of AMPs induced through the Imd pathway [94,95]. The activation of these pathways and
other host responses that are involved in gut homeostasis is elevated if pathogenic bacteria enter the gut. This close interaction between microbes and the host immune system in the gut has led to the suggestion that digestion and immune function have a shared history in multicellular life [96] and that concurrent, pleiotropic selection on digestion and immune defence shapes the immune system.

While the microbiota in the gut is often a reflection of what is available in their environment, many organisms harbour co-evolved bacteria in their guts and have often evolved special structures to host and maintain their symbionts [92,97,98]. Using the weevil, Sitophilus oryzae, Login et al. [10] showed that AMPs are crucial players for the maintenance of the symbiotic bacterium Candidatus Sodalis pientonius. The weevils have evolved bacteriomes comprised bacteriocytes, in which the symbionts reside. The expression of AMPs in the bacteriocytes differs markedly from the gut. Only one AMP, a colocepertcin (ColA), is expressed in the bacteriocytes; this AMP induces filamentous growth and cell gigantism not only in Candidatus Sodalis pientonius, but also in E. coli. Login et al. [10] show that ColA is responsible for restricting the symbionts to the bacteriomes. ColA displays a high sequence similarity with another colocepertcin (ColB), which is employed in the weevil’s immune defence. This constitutes a clear signature of selection on an AMP driven by host–symbiont co-evolution. During development of Sitophilus oryzae, the bacterial populations in the bacteriomes undergo dynamic changes. Most AMP responses with the exception of ColA are suppressed and mainly cellular processes regulate bacterial numbers.

Intracellular, co-evolved symbionts seem to be common, but so are free-living symbionts in the guts of insects. The classical example being the lower termites that harbour symbionts helping in the digestion of cellulose [99]. Gut symbionts become an issue during development of holometabolous organisms, such as bees, beetles or flies, where the renewal of the gut is an integral part of metamorphosis, i.e. is completely shed or rebuilt. Metamorphosis thus poses a challenge to the maintenance of symbionts and for the homeostasis of the microbiota. Endosymbionts that reside inside bacteriocytes can actually multiply during metamorphosis where they are protected, as has been shown in the ant Camponotus floridanus [97]. The situation is more difficult for symbionts that are free living and situated in the gut lumen. A recent study of metamorphosis in the wax moth, Galleria mellonella, showed that the host—via upregulation of the immune response (mostly, lysozyme and AMPs) and in combination with the symbiont E. mundtii—ensures that the resulting adult gut microbiota after pupation is dominated by the symbiont [100].

If as reasoned above, AMPs play an important role in mediating beneficial host–microbe interactions, it could be expected that symbiotic bacteria have evolved resistance against AMPs. This has rarely been studied, but a recent work on stinkbugs (Riptortus pedestris) investigated this question. Stinkbugs have evolved a sorting organ for symbionts in their midgut and basically use the hindgut as a breeding vessel for symbiotic Burkholderia. At the same time, food and other microbes cannot enter the hindgut [98]. Stinkbugs express a number of AMPs in their midgut-sorting organ. The expression is minimal in the section of the midgut where the symbionts reside (103). Furthermore, during every moult, the symbiont population is downregulated by the host’s immune system [101]. Interestingly, Burkholderia, when exposed to the host midgut are highly sensitive to AMPs and for example killed by physiological concentrations of a Riptortus defensin. The increase in the susceptibility of Burkholderia to AMPs seems to be a phenotypic response to the within-host conditions, as Burkholderia cultured in vitro display resistance to AMPs [102].

Another explicit example where bacterial resistance to AMPs has been studied is Sodalis inglossinus, which is a maternally inherited symbiont of tsetse flies [103]. In S. inglossinus the PhoQ sensor kinase, part of the PhoP–PhoQ two-component system, has evolved to enable bacteria to infect hosts. This adaptation results in a constitutive expression of AMP resistance in symbiotic S. inglossinus, allowing them to survive within their hosts. More generally, the PhoPQ system is an important regulator for AMP resistance in Gram-negative bacteria and has also been described as a relevant AMP resistance mechanism in patient-derived bacterial strains [104]. Other resistance mechanisms against AMPs that have been described are efflux pumps, modification of the cell surface and biofilm formation.

Given that many bacterial resistance mechanisms have been described in detail at the molecular level [104], the false view that AMP resistance in bacteria rarely if at all evolves still seems to prevail [11]. However, experimental evolution studies have repeatedly shown that AMP resistance evolves readily in vitro [105,106]. Interestingly, the evolution of resistance to AMPs seems more difficult if these are acting in combination, that is, in synergy as discussed above [107]. However, this finding deserves more attention. For example, in the case where a first AMP opens the membrane for a second AMP to enter and kill the cell, the microbe would only need to evolve resistance to the first AMP to neutralize the second, too. In cases where AMPs can substitute one another—at least partly—pathogens need to cope with both effectors at the same time, which may make the evolution of resistance, indeed, more difficult. Finally, if the synergistic interaction among AMPs is quantitative, such that a ‘best cocktail’ works most effectively against a microbe, then the evolution of resistance may or may not be more difficult than evolution of resistance against a single peptide. If anything, the difference should depend on the actual doses and degree of substitution. Regardless of the details, the notion that AMP resistance is impossible to evolve is wrong.

(d) Antimicrobial peptides should be adapted to the organism’s environment

Insects occupy a huge variety of ecological niches. This exposes different species to different sets and abundances of microbes. The hover fly, Eristalis pertinax, for example can successfully develop in manure and sewage, and is thereby probably exposed to a rich community of microbes, which is not the insect’s own microbiota [108]. Socially living species, owing to their typical family structure and dense aggregations in nests, are similarly supposed to be exposed to many pathogens that can readily spread between individuals [109]. Given that AMPs play an important role in fighting infections or in maintaining the microbiota, it is expected that the repertoire or the expression of host AMPs found in such particular habitats would match these local conditions.

Detecting adaptation to a particular environment requires that (i) signs of selection are present and (ii) the actual characteristics of the AMPs that are present should vary with the environment. Directional selection would reflect more persistent
environmental forces, for example, physical–chemical conditions such as temperature or water chemistry. Changing and fluctuating selection regimes, such as those exerted by rapidly co-evolving parasites, could result in signs of balancing selection that contribute to the maintenance of AMP polymorphisms (see above). Trans-species polymorphisms exist, too, which either have survived the speciation process or could result from hybridization and introgression, or from independent mutations. The AMPs of *Drosophila* contain many such trans-species polymorphisms in AMPs and, these trans-species polymorphisms seem to be more convergent than polymorphisms restricted to a single species: the same kind of polymorphism occurs repeatedly in various species as shown by [39]. Cases of trans-species polymorphism in AMPs have also been described for other insects [108], fish (cathelicidins, [41]) or birds (defensins, [33]).

To illustrate habitat-dependent variation, we would predict, everything else being equal, that organisms living in a parasite-rich environment should have a different and in this case richer AMP repertoire than those living in a parasite-poor habitat. Organisms living in habitats with similar characteristics should show convergence in their AMP repertoire or AMP effects, regardless of their phylogenetic relationship. As an example, a cathelicidin from the common toad (*Bufo bufo*) appears to show higher activity against bacteria prevalent in its habitat than against pathogens that it never encounters [110]. Ideally, the effects against pathogens from the native and foreign habitats relative to a random set of bacteria should be known and would be more informative. So far, these kinds of tests are lacking.

Do AMPs in the groups discussed here show the same patterns? Barring polymorphisms, for the insects, the evidence to date is not clear. One of the few studies addressing this issue [111] found that some antifungal peptides (termicins) of Australian termites of the genus *Nasutitermes* have duplicated repeatedly and show the signs of directional selection. However, this process seems to have occurred independently and in the same manner while the various lineages radiated. Hence, the same pattern is found for species living in rainforests or dry savannahs, and for subterranean or arboreal nesting habits—all of which are putative factors that define a relevant environment for the evolution of immune defences, provided the termites, indeed, encounter the respective microbes at all. If so, there is no clear sign that AMPs are specific for some habitats rather than others. Again using termicin and comparing two *Reticulitermes* species, Bulmer et al. [112] suggest a selective sweep has reduced the polymorphism in termicin—probably through selection for favourable nucleotides that provide antifungal activity. However, in this and virtually all other cases, we still lack an understanding of what exactly has selected for these differences. There is also no clear evidence that similar environments select for the same repertoire of AMPs, or that the same AMP allelic variants are maintained, even though there is now a good case for convergent polymorphism [39]. It seems feasible that varying microbial loads encountered in different environments select for differences in the suites of AMPs. Xu et al. [113] report that a richer repertoire of AMPs (termicins) is expressed in *Odontotermes*, a fungus-growing termite living in subterranean nests in wet habitats when compared with its counterpart, *Reticulitermes*, living in drier places and not cultivating fungi. Yet the environmental conditions have not been assessed and are subject to speculation. Whereas the signs of positive selection at the sequence level, as well as for gene duplication are reported [35,114], the relationship to the environmental conditions is typically not investigated. As an aside, we note here that a field like ‘chemical ecology’ strives to find new antimicrobial drugs in a wide range of organisms [115]. This exploration also includes AMPs, which thus come from a variety of organisms with different lifestyles and ecologies. Exploring this knowledge to address the question of whether AMPs vary in relation to the ecology of the organism might be promising but has not yet been addressed.

As contributions to this issue highlight [64,67], the deployment of AMPs can also be differentially regulated according to the particular challenge of infections. In other words, the expression of AMPs rather than the genes themselves could be an adaptation to a particular environment. In this context, it is interesting to note that amino acid substitutions are disproportionally often found in the signal peptide sequence of the AMP [116]. The signal sequence is known to affect the efficiency of secretion from the cell. Evidence for an adaptive use of expression is provided by comparing different life stages, sexes or forms of the same organisms, such as between sexes, and summer or winter workers of the honeybee [117]. Several typical characteristics of AMPs, also discussed in this issue, lend some support to the scenario of environmentally dependent AMP expression. For example, AMPs, in general, are effective against a wide range of pathogens. Because of their mechanism of action, AMP structure may be constrained by the requirements for activity against conserved membrane structures, or by the need to be resistant to counter-defences. As Unckless et al. [39] discuss, even small changes in the amino acid sequence can dramatically alter functionality. Hence, the primary sequence and structure of AMPs would reflect general properties that are hard to modify rather than an actual adaptation, a suggestion that would fit the observations.

Looking at the available data on AMPs in an ecological context we conclude at this point that there is an open field for studies that identify the causes of adaptation, that is, the selective factors that lead to some AMPs being present rather than others. To date, we sorely lack sufficient and solid knowledge to answer these important questions.

(e) The application of antimicrobial peptides should be informed by ecology and evolution

Given the immense diversity of arthropods, especially the insects, we expect a high diversity of AMPs to exist in this group [9,17]. Finding new AMPs and studying their properties in arthropods thus would hold promise, and could ameliorate the dearth of potent new antibiotics that currently are under development in the biomedical industry [118]. In fact, the diversity of arthropod AMPs is also reflected in the diversity of different organisms that can be targeted, which includes bacteria such as medically relevant *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter* species (ESKAPE bacteria), viruses, a number of protists, fungi, and even cancer cells [119]. This search is currently underway and represents an exciting research field [9].

Whereas the need for new drugs is widely recognized, there are serious risks to consider. As with any other drug, bacterial resistance against single AMPs evolves in vitro ([105], see above), and AMP-resistant strains have already been recovered from patients [104]. Some new antibiotics
that target the cell membrane, which bears parallels with the mode of action of many AMPs have been hailed as evolution-proof [120]. Yet as the examples from the current state of research on AMPs discussed above show, caution would be highly advisable. Decisive evolutionary experiments are often lacking (for example on teixobactin [120]). Another risk is related to the conserved bacterial resistance mechanisms against AMPs [104], which could result in the acquisition of cross-resistance against host-derived AMPs. This problem has been highlighted by Bell & Gouyon [18], who pointedly asked if we are going to ‘arm the enemy’ by developing AMPs as drugs. In fact, S. aureus experimentally evolved to become resistant against AMPs from pigs, also showed much higher survival in an insect host [121] highlighting that phylogenetic distance between hosts might not be a safeguard. The same study also found that when S. aureus is selected for resistance against the insect AMP melitin, it also showed slightly elevated resistance against the antibiotic vancomycin—considered to be a last-resort drug when other antibiotics fail.

We therefore feel that the investigation of AMPs with respect to the mechanisms of action, effects or their distribution among organisms, when studied in the natural context, can provide important knowledge in addition and beyond the simple discovery of new drugs. This could also add the issue of sustainable usage of AMPs as drugs and perhaps even other antimicrobials. AMPs employed in drugs will probably interact with AMPs that are already deployed by the host to control or eliminate pathogens. Whereas it is probably a truism that effectors are always deployed in some combinations, it is a demanding empirical task to systematically test for the associated effects. For example, the study of how combinations of AMPs can kill medically important protozoans [67] promises avenues of future research, especially as the active components of AMPs could be synthesized and put to use [122]. Such use also carries the risk of accelerated resistance evolution in the targeted microbes. In addition, as discussed above, AMPs have other important roles for the organism and are important players mediating host–microbiota interactions. The role of the microbiota in infections and the possibility of harnessing the microbiota in treating infections is currently actively researched [123], and AMPs could be part of this toolbox.

Another area of application from the understanding of the AMP–microbiota interactions is the production of food and feed, and hence the improvement of food security. In this volume, this was especially discussed for aquaculture [17], and has also been considered for the mass rearing of insects [124]. Antimicrobial compounds can leak into the environment and enter the human food chain and here AMPs have the advantage of reduced stability of AMPs in the environment [17]. As means of producing protein for consumption, aquaculture and mass rearing of insects not only depend on improving ‘classical’ pest control, but would also offer an opportunity for the application of AMPs to manage the microbiota, which is a natural system that ensures homeostasis and defense against pathogens, without the need for ‘direct interference’. This can save costs and efforts in pest and pathogen management. Learning from such semi-natural systems as used in production, and thereby understanding the workings of AMPs conceptually as well as in the natural defence context, holds the promise of contributing to the sustainable use of antimicrobials. Rather than just blindly adopt a strategy of AMP use similar to traditional usage of anti-biotics, the potential ramifications of how the increased or targeted use of AMPs changes the microbial communities around the organism is a key issue that needs to be carefully addressed. This, essentially, means finding a strategy and the means to slow down the evolution of resistance to AMPs [125].

2. Outlook

Since Hans Boman’s discoveries in the 1970s [4], a vast and growing literature has unravelled many of the secrets of AMPs. We now have a much better understanding of their role and function. For example, we now better understand how AMPs become activated [126], the tight connections between immune defences, and the neuronal system, which includes AMPs [127], the very critical function of AMPs on surfaces [128] or in maintaining gut homeostasis [129]. At the same time, AMPs are explored as new antibiotics [130], and synthetic AMPs with improved properties are developed [131].

The contributions to this issue approach the subject of AMPs, from the perspective of ecology and evolution; this highlights two important issues. First, compared with the research efforts in the medical field that deals with mice and humans, research done in the vast group of arthropods, including insects, is still rather limited. Second, compared with the research efforts going into the study of mechanisms and the molecular bases, research into the ecology and evolution of AMPs is similarly limited. However, why should these aspects be important? We think there are several points that are worth mentioning.

Arthropods are arguably one of the largest and most diverse group of organisms. The implication is that the kinds of AMPs found in this group should be most numerous and diverse, and this is generally agreed upon [9,17]. Despite this agreement, it is remarkable that there is no overwhelming evidence that the organism’s ecology, its habitat and biology, influences the kind of AMPs we expect to find. AMPs typically are effective against a wide spectrum of microbial pathogens, well beyond those that the organism normally encounters [17]. The observation that—in contrast to many other immune genes—AMPs do not evolve rapidly, but instead seem to have a polymorphism of variants [39] adds to this scenario. A stable polymorphism is of course especially exciting in this respect, as it can be maintained by some degree of specificity in the co-evolving hosts and parasites, as reflected in the signature of balancing selection in AMPs. Nevertheless, it is conceivable that the phylogenetic heritage and the constraints on AMP evolution imposed by the integration within the immune system may be equally or perhaps more relevant for the kind of AMPs an organism produces.

AMPs present a puzzle. They are generally small molecules that evolve slowly. At the same time, pathogenic bacteria [104]—and surely many other kinds of parasites, too [132]—have evolved mechanisms to manipulate, evade or neutralize the effects of AMPs. Yet, despite these formidable obstacles, AMPs have remained effective and their function has not really been outpaced by the typically faster-evolving parasites [28]. This as yet unsolved puzzle is instructive for the general problem of how to design drugs that resist evolutionary pressures as much as possible [133]. As to AMPs, their mode of action—typically targeting key characteristics, such as membrane structure, which are hard to change is one suggested
answer to the puzzle [9,104]. However, as resistance against AMPs can evolve readily in vitro [105], this shows that by targeting basic structural characteristics of the bacterial membranes, AMPs do not necessarily have a universal advantage. Two other findings therefore come to mind. Polymorphism in host populations resulting from antagonistic, fluctuating selection by rapidly co-evolving parasites [134] is a distinct possibility that can explain the continued effectiveness of AMPs. Cases of trans-specific polymorphisms [33,39] would suggest that such polymorphisms can persist over long evolutionary times and even survive speciation events.

At the same time, synergisms could be another key to counter the selective pressures by co-evolving parasites. The existence and importance of AMP synergism has been known for some time and is highlighted by several authors of this volume [7,56,64,67]. The combined expression of different AMPs that are most effective against a certain parasite or parasite variant requires less peptide over all, is likely to be less costly and also lowers the risk of side effects and self-damage. Although Marxer et al. [67] go one step towards elucidating these possibilities, the conjectured effects are still unproven, and it is a mystery how insect hosts can sense the kind of infecting parasite variant and respond by a tailored cocktail—even though expression profiles do vary with parasite variant [53]. Most importantly for this discussion, synergistically expressed AMPs may be evolutionarily harder to evade by the parasite, as expression profiles are rapidly moving targets that can quickly and flexibly respond to changing parasite strategies. We have no proof yet for whether this hypothesis is a good explanation or not. But given that the evolution of expression systems—especially in the eukaryotes—may be a key element of adaptive phenomena [135], the idea of an adaptive, flexible AMP expression system is plausible and deserves further study [136,137].

Finally, there is yet another dimension of AMP functionality that can add to the persistence of their effects—the interaction with symbiotic microorganisms of the host [92], especially the bacterial microbiota in the host gut [56,91]. The microbiota has emerged as an important third party in host–parasite interactions in almost all organisms studied to date [138,139]. Even small changes in how AMPs are expressed could affect the composition or effectiveness of the microbiota as a defence system against infections as also discussed here [91]. Again, while this scenario is very plausible and accumulating evidence strongly supports the idea that AMPs mediate interactions with the microbiota, we still largely lack an understanding of how this interaction can also keep rapidly co-evolving parasites at bay and keep the AMPs effective as primary tools of the host.

We propose that we will not understand why AMPs remain effective, are so widespread, and a core element of the innate immune defences unless their ecology and evolutionary dynamics are also considered. Ecology and evolution will also be crucial to answer one of the greatest remaining puzzles—why are such small and conserved molecules still effective after aeons of ongoing co-evolution with parasites and pathogens? An answer to this question will not only be fascinating for biologists but also relevant for the search for new means to combat infections in humans, livestock or managed populations more generally. We hope that this issue will carry this research into the future.

Competing interests. We declare we have no competing interests.

Funding. We were supported by an ERC grant to J.R. (no. 260986, EVORESIN) and an ERC Advanced Grant to P.S.H. (no. 268853 RESIST).

Acknowledgements. We thank Olivia Judson and David S. Schneider for helpful comments on this manuscript and Paul Johnston for final proof reading. Helen Eaton was a supportive and patient editor of the volume.

References


76. Kim JK et al. 2015 Insect gut symbiont susceptibility to host antimicrobial peptides caused by alteration of the bacterial cell envelope. J. Biol. Chem. 290, 21, 042 – 21, 053. (doi:10.1074/jbc.M115.651158)


