Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses

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Abstract | The innate immune system is present in all animals and is a crucial first line of defence against pathogens. However, animals also harbour large numbers of beneficial microorganisms that can be housed in the digestive tract, in specialized organs or on tissue surfaces. Although invertebrates lack conventional antibody-based immunity, they are capable of eliminating pathogens and, perhaps more importantly, discriminating them from other microorganisms. This Review examines the interactions between the innate immune systems of several model invertebrates and the symbionts of these organisms, and addresses the central question of how these long-lived and specific associations are established and maintained.

Symbiont
As used in this Review: a microorganism that forms a specific, stable and beneficial association with a particular host. Although this is now a commonly accepted definition, the original definition of symbionts, by Anton de Bary, included pathogenic, commensal and mutualistic symbionts.

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Multicellular life arose in a world dominated by microorganisms, and interactions between animals and their microbial companions have profoundly influenced animal evolution. Perhaps the best surveyed habitat for microorganisms in animal hosts is the digestive tract, where the microbial community carries out many crucial functions such as aiding in digestion, providing essential nutrients, protecting against colonization by pathogens and stimulating the immune response[1–3]. In recognition of the importance of these organisms, the term microbiome was introduced to describe the collective genome of the indigenous microbiota of an animal[4]. 16S rRNA gene surveys indicate that the specificity of the microorganisms colonizing mammalian digestive tracts is not conserved at the species or strain level, but rather there is conservation at the level of functional genes among diverse microbiota[5–8]. However, some invertebrate–symbiont model systems that lend themselves to experimental manipulation have revealed a higher degree of specificity that can even be achieved at the species or strain level[9–11].

This specificity is multifactorial in origin, and there are some general requirements that quickly reduce the number of organisms capable of establishing a foothold in a particular animal host. Basic physiological requirements include an ability to grow and outcompete other microorganisms at the temperature, redox potential, pH and osmolarity that are found in the host. General nutritional conditions are often set by the host ingesting or providing nutrients at defined intervals and of particular nutritional quality[12,13]. Many of these factors can also be modified to some degree by the metabolism of the microbial community[2,12,14,15]. Furthermore, specific organs might help to foster these associations and create microenvironments that are conducive to symbiont growth but inhibit the growth of other microbial competitors. This sequestration at specific sites could serve to protect other environments in the body from colonization.

In addition to these constraints, interactions between symbionts and the host immune system have a crucial role in the establishment and regulation of these microbial communities[16–22]. For example, in the healthy human gut, immune defence mechanisms are modulated in response to the microbiota, an effect that can be referred to as the development of tolerance[21,23]. Animal immune systems are often classified based on either the broad and nonspecific innate immune response or the highly specific antibody-based adaptive immune response. Whereas jawed vertebrates use both types of immune response, all invertebrates combat potential pathogens and also foster the formation of mutualistic symbioses in the absence of conventional antibodies. Given that many of these invertebrate host–microorganism partnerships require a high degree of specificity, how can such associations form without obvious mechanisms for distinguishing between specific microorganisms?

In this Review, we explore the interactions between the innate immune system of invertebrates and the symbionts of these organisms, and discuss the role that these interactions have in establishing and maintaining specific

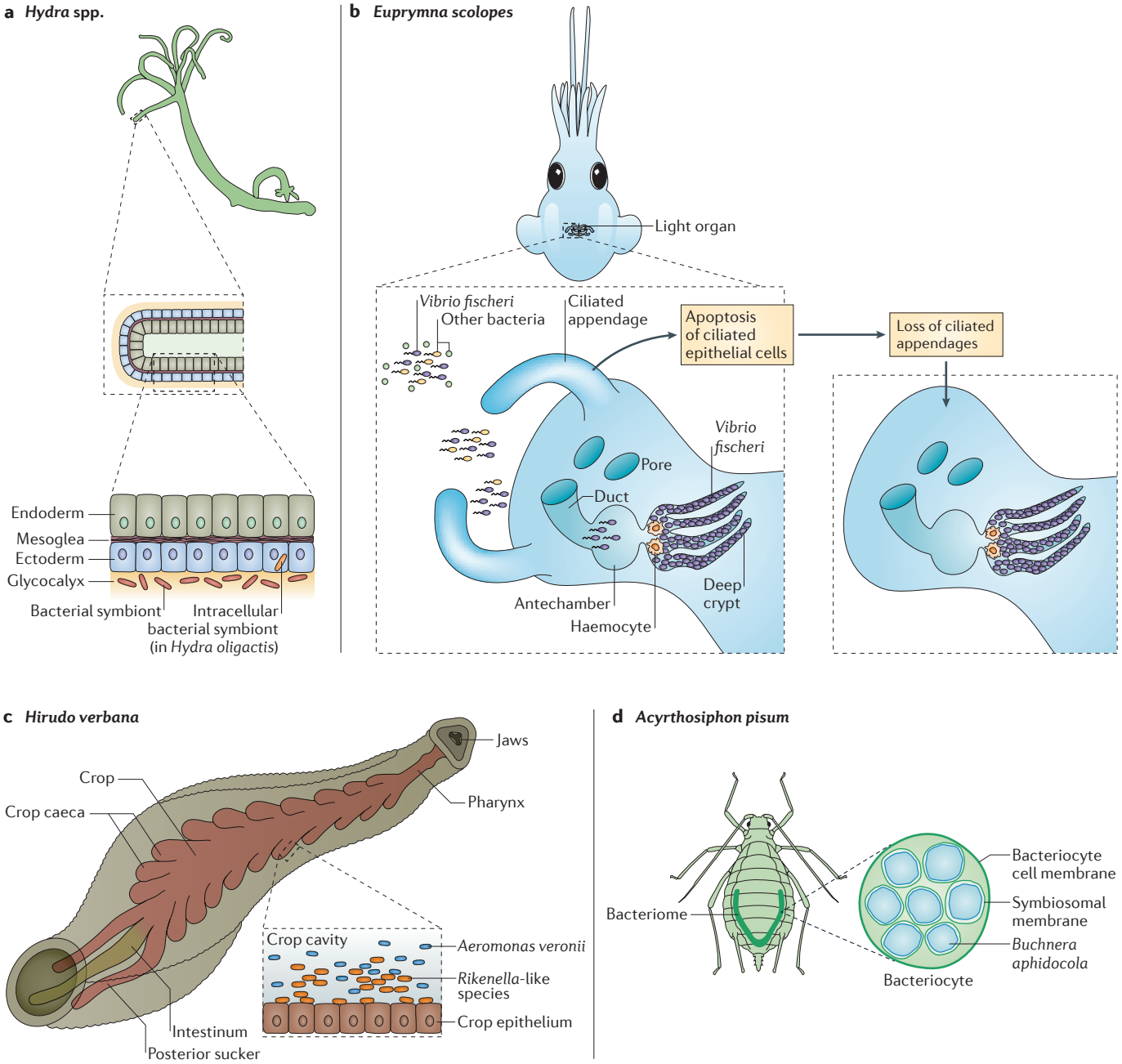

Figure 1 | **Model systems of invertebrate symbioses.** Four model hosts and their symbionts are discussed in this Review. **a** | *Hydra* spp. are composed of two epithelial cell layers and house bacterial symbionts on the outermost cell type (the ectoderm), in the glycocalyx. *Hydra oligactis* also harbors an intracellular symbiont in its ectoderm. **b** | *Euprymna scolopes* forms a binary association with the bacterium *Vibrio fischeri* in the crypts of the light organ. After the squid hatches, *V. fischeri* is selected from among all other bacteria in the environment and colonizes the juvenile light organ; this colonization initiates a developmental programme that results in morphogenesis of the light organ. **c** | The leech *Hirudo verbana* carries two dominant symbionts in the crop (the largest compartment of the digestive tract), *Aeromonas veronii* and a *Rikenella*-like species. **d** | The pea aphid, *Acyrthosiphon pisum*, houses the obligate intracellular symbiont *Buchnera aphidicola* in cells known as bacteriocytes, which are located in an organ-like structure known as the bacteriome. Part **b** image is modified, with permission, from REF. 30

microbial communities. This outcome is in stark contrast to the traditional view of the immune system and its role in eliminating pathogenic bacteria, and it is particularly interesting because the innate immune system reportedly lacks robust immunological memory. Given the abundance of model systems, we have selected only a few invertebrate species (FIG. 1) that represent different phylogenetic lineages and for which experimental models have revealed clues about how the innate immune system helps in shaping the microbiota.

Box 1 | **Alternative anticipatory and memory-like immune mechanisms of invertebrates**

Although invertebrates lack a conventional antibody-based and recombination-activating gene (RAG) protein-mediated adaptive immune system, some invertebrates do possess the ability to generate highly diverse and polymorphic proteins or peptides in response to microorganisms and might have the ability to recognize self versus non-self tissues[24–26,145]. However, the role of such mechanisms in the establishment of the microbiota has not yet been addressed. These mechanisms, along with the discovery of an additional and alternative adaptive immune system with variable lymphocyte receptors in jawless fish[146], suggests that other types of anticipatory and memory-like immunity remain to be discovered. Below are some examples of adaptive immune effectors found in invertebrates.

Fibrinogen-related proteins
Fibrinogen-related proteins (FREPs) contain a fibrinogen and one or two immunoglobulin superfamily domains, and are secreted into the haemolymph of the snail *Biomphalaria glabrata* in response to trematode parasites[25]. Although a detailed mechanism of FREP function has not been described, these proteins can bind to parasites and bacteria, and the host can generate a larger FREP repertoire through somatic diversification in response to infection.

Down's syndrome cell adhesion molecules
Down's syndrome cell adhesion molecules (DSCAMs) were discovered in *Drosophila melanogaster*[26]. By means of differential RNA processing, thousands of protein variants are generated, some of which seem to adhere to and mediate phagocytosis of bacteria.

Immune priming
Examples of memory-like innate immune responses have been described in insects[27,42]. Hosts that are challenged with specific pathogens will occasionally exhibit a more efficient immune response after a second exposure. In the mosquito *Anopheles gambiae*, this primed response might be partially mediated by the gut microbiota, as well as by haemocyte differentiation[27].

Pattern recognition receptors
Eukaryotic proteins that bind to microorganism-associated molecular patterns, activating downstream signalling and, ultimately, innate immunity effector responses. Examples include Toll-like receptors, peptidoglycan recognition proteins and Gram-negative bacteria-binding proteins.

Peptidoglycan
The polymer that makes up the bacterial cell wall, and consists of β-(1-4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid linked to small peptide chains.

Lipopolysaccharide
A major component of the outer membrane of Gram-negative bacteria. It is composed of an O antigen, a core oligosaccharide and lipid A.

Outer-membrane proteins
Proteins that are common to the outer membrane of Gram-negative bacteria.

Flagellins
Bacterial major flagellar proteins.

Type III secretion system
A needle-like secretion system that allows Gram-negative bacteria to inject toxins directly into eukaryotic cells.

Microorganism-associated molecular patterns
Molecular motifs that are unique to microorganisms (both pathogens and non-pathogens) and are often recognized by components of the innate immune system.

Toll-like receptors
(TLRs). A diverse group of evolutionarily conserved pattern recognition receptors that are characterized by extracellular leucine-rich repeats and a cytoplasmic Toll–interleukin-1 receptor motif. Binding of microorganism-associated molecular patterns to TLRs often leads to downstream signalling via the nuclear factor-κB pathway or related pathways and results in the regulation of immune effectors.

The immune system of invertebrates

When early multicellular life first associated with microorganisms, hosts would have required sophisticated systems for recognizing and also differentiating beneficial and pathogenic microorganisms. However, unlike vertebrates, invertebrates lack classical antibody-based adaptive immunity, and key molecular and cellular players such as recombination-activating genes (RAGs), B lymphocytes and T lymphocytes are absent[24]. Although there are indications that some invertebrates have alternative adaptive or anticipatory immune functions and memory-like responses[24–27] (BOX 1), to date none of these functions and responses has been shown to be widely evolutionarily conserved among invertebrate phyla. Despite this perceived limitation, invertebrates have mounted efficient defence responses to pathogens for hundreds of millions of years. In addition, growing evidence suggests that the innate immune system interacts differently with symbionts than with pathogens. Although there are many factors associated with invertebrate innate immunity, we focus on three main aspects here: the recognition of microorganisms by pattern recognition receptors (PRRs), cellular immunity in the form of the phagocyte response, and humoral components of the immune system, comprising acellular and biochemical factors (FIG. 2).

Pattern recognition. Cell–cell signalling between microorganisms and their hosts is largely mediated through interactions between host receptors and microorganism-derived ligands[23,28–30]. Hosts detect specific bacterial components such as peptidoglycan (PGN), lipopolysaccharide (LPS), outer-membrane proteins (Omps), flagellins and other molecules that are expressed by a wide range of bacteria. These molecules were originally termed pathogen-associated molecular patterns (PAMPs) by Janeway in 1989, and this term was popularized by Medzhitov and Janeway in 1997 (REFS 31–33). This concept was based on the idea that the recognition of a molecule such as LPS, which is found in all Gram-negative pathogens but absent in animals, allows receptors of the immune system to recognize the presence of pathogens and launch an immune response. However, PGN, LPS, Omps and flagellin are not pathogen specific, instead being common to many different types of microorganism, including those that form beneficial associations with animals and are generally benign to the host. Indeed, even classical virulence factors such as the type III secretion system (T3SS)[34] are required by some beneficial microorganisms for interactions with animal hosts and are therefore not pathogen specific[16,35]. Thus, the more general term microorganism-associated molecular patterns (MAMPs) was introduced by McFall-Ngai and colleagues[36]. Some of the best known examples of PRRs are the Toll-like receptors (TLRs) and peptidoglycan recognition proteins (PGRPs), which trigger downstream signalling pathways, often activating cellular and humoral innate immunity effectors such as phagocytes, antimicrobial peptides (AMPs) and reactive oxygen species (ROS) (reviewed in REFS 30,37) (FIG. 2). Because most animal hosts encounter MAMPs from both pathogenic and non-pathogenic microorganisms, additional regulatory levels must be in place that allow for discrimination between beneficial and pathogenic associations.

Phagocytic cells. Phagocytic cells are common to all eukaryotes, and some of the earliest endosymbioses were probably the result of single-celled organisms engulfing bacteria that eventually evolved into organelles such as mitochondria and chloroplasts[38,39]. As metazoans evolved, circulating phagocytic haemocytes became ubiquitous to most groups except those with only tissue level organization, such as cnidarians; certain organisms, such as the nematode *Caenorhabditis elegans*,

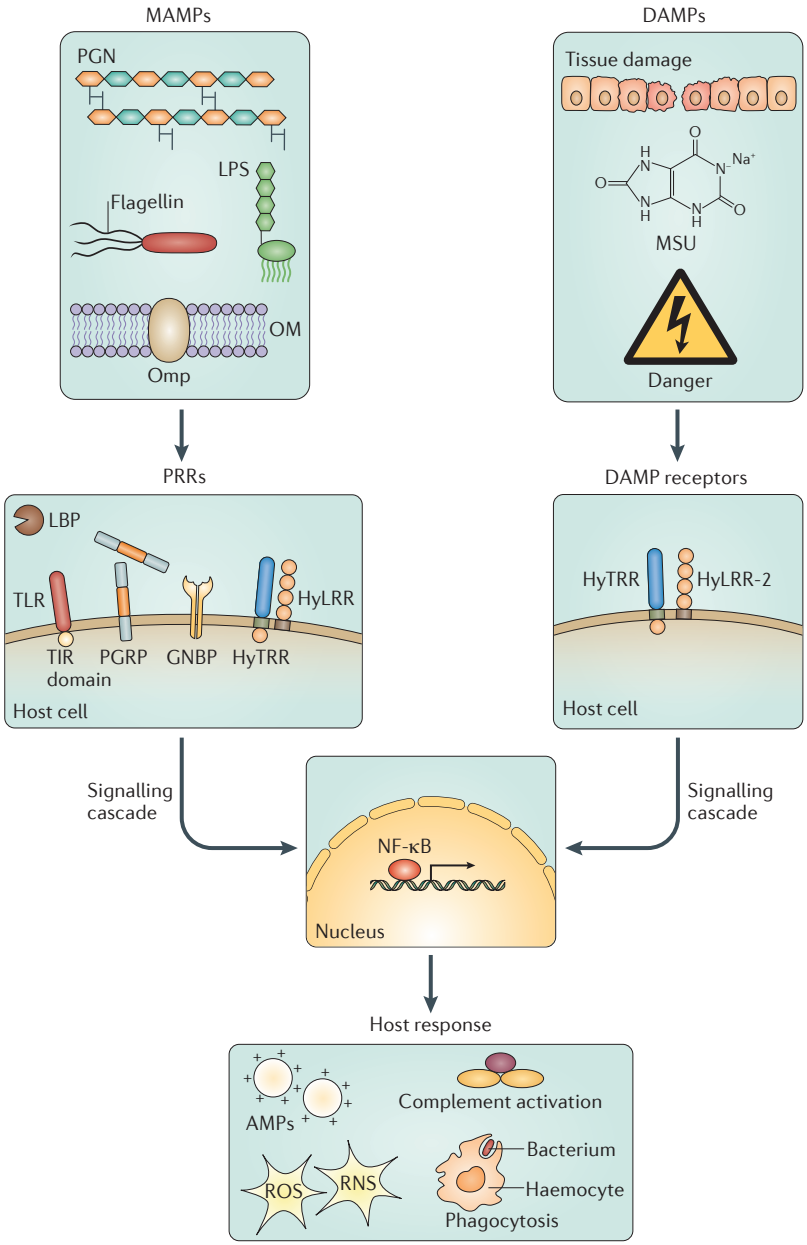

Figure 2 | **Proposed pathways of innate immune response activation in invertebrates.** Microorganism-associated molecular patterns (MAMPs) can be recognized as non-self by the host and include compounds such as lipopolysaccharide (LPS), peptidoglycan (PGN), bacterial flagellin and bacterial outer-membrane proteins (Omps). MAMPs can initiate signalling cascades by first interacting with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and other Toll–interleukin 1 receptor (TIR) domain-containing proteins, peptidoglycan recognition proteins (PGRPs), Gram-negative bacteria-binding proteins (GNBPs) and LPS-binding proteins (LBPs). *Hydra* spp. also possess *Hydra* leucine-rich-repeat proteins (HyLRRs), which function with *Hydra* TIR domain-containing proteins (HyTRRs) to recognize MAMPs (although the specific family members that function as receptors in this pathway have not yet been determined). Other signals that can trigger immune responses are damage-associated molecular patterns (DAMPs), which might result from physical tissue damage and the release of compounds such as monosodium urate (MSU). In *Hydra* spp., DAMPs can interact with HyLRR-2, which functions with a HyTRR to initiate immune signalling. Both MAMP- and DAMP-mediated signalling can initiate signalling cascades, usually through the nuclear factor-κB (NF-κB) or similar pathway, that lead to the activation of immune responses such as haemocyte trafficking and phagocytosis; induction of the complement pathway; and production of antimicrobial peptides (AMPs), reactive oxygen species (ROS) and reactive nitrogen species (RNS). OM, outer membrane.

subsequently lost this cell type[40,41]. Recent evidence suggests that interactions between microorganisms and invertebrate haemocytes can be highly specific and are important for the establishment and maintenance of symbioses in invertebrates[18]. Furthermore, studies with different fly and mosquito species have shown that haemocytes are likely to be involved with priming the immune system to bacterial challenge[27,42]. These studies have also shown that haematopoiesis and the development of other components of the innate immune system might be influenced by the presence of bacterial symbionts[43]. These data, along with the studies of symbioses described below, suggest that haemocytes are capable of highly specific interactions with bacteria.

Humoral defences. In addition to the cellular immune response, animals possess acellular, chemical-based mechanisms that constitute the humoral response and also shape the microbial community. This response includes the production of AMPs, which lyse cells, and ROS, which oxidize lipids and proteins and also damage DNA. Both pathogens and symbionts must overcome these insults to thrive in the specific niche of the host. AMPs and ROS are among the most widely used immune effector mechanisms among both vertebrates and invertebrates, and they appear to have been conserved throughout evolution[44–46]. In addition, many invertebrates possess components of the complement system, which assists in the detection of microorganisms and MAMPs using opsins and lectin–ligand interactions[47].

Invertebrate models of symbioses

Below, we describe four model invertebrates, from diverse taxa, that form associations ranging in complexity from involving a single symbiotic species to diverse consortia (FIG. 1). These symbionts are localized in or on specialized cells, tissues or organs. The first of these model systems is cnidarians of the genus *Hydra*, which represent basal metazoans that have a simple tissue organization[48–50]. Owing to their basal position in the animal lineage, these organisms provide an important model for understanding microbial interactions with hosts. This basal position also means that the immune system processes which these cnidarians use to shape the composition of their microbial community are likely to be present in more complex animals. Two lophotrochozoan models are also discussed: *Euprymna scolopes* (the Hawaiian bobtail squid), which houses a single bacterial species, *Vibrio fischeri*, in a specialized light organ[51–53]; and *Hirudo verbana* (a medicinal leech), which has a simple microbial community in its gut that is dominated by two bacterial species, *Aeromonas veronii* and a *Rikenella*-like bacterium[54–56]. Of the ecdysozoan insects, *Drosophila melanogaster* has a moderately complex gut microbiota comprising 5–20 species and is the best characterized invertebrate model system[57]. However, the features of the innate immune response of *D. melanogaster* have been reviewed elsewhere recently[30,58,59]. Instead of this system, for our fourth model we discuss the association between *Acyrthosiphon pisum* (the pea aphid) and its primary endosymbiont, *Buchnera aphidicola*[60,61].

We explore these systems with regard to, first, whether the symbionts possess specific capabilities to escape or neutralize the immune response; second, common mechanisms by which the innate immune system of the host detects and responds to bacterial symbionts (TABLE 1) and third, how the immune response is influenced by spatial and temporal factors, and whether additional signals such as tissue damage are also required for immune stimulation.

Bacterial symbiosis in *Hydra* spp.

Hydra vulgaris and related species of the phylum Cnidaria consist of two main cell layers, the ectoderm and the endoderm[50] (FIG. 1a). A feature that distinguishes the members of this phylum from the other animal models described in this Review is the lack of motile phagocytic cells. Thus, *Hydra* spp. provide the opportunity to study the immune response of ectodermal and endodermal cells[62]. General features of the cnidarian immune response include AMPs, PRRs and a signalling cascade related to the nuclear factor-κB pathway (NF-κB pathway)[63]. Another generic protective feature of cnidarians that is also widespread among animals is a mucus layer known as the glycocalyx, which covers the ectoderm (FIG. 1a).

The microbial consortia of* Hydra *spp. A ground-breaking study revealed that *H. vulgaris* and the closely related species *Hydra oligactis* are capable of shaping their epidermal microbiota[64], which might aid in the defence against pathogenic fungi (T. Bosch, personal communication). A comparison of *H. vulgaris* and *H. oligactis* isolates collected from two lakes — including some isolates of both species from the same lake —with strains that had been maintained for more than 30 years in a laboratory revealed two interesting results[64]. One intracellular symbiont, an alphaproteobacterium related to *Rickettsia* and *Ehrlichia* spp., had been retained in laboratory cultures of *H. oligactis* for more than 30 years but was absent in the *H. vulgaris* laboratory strain. Furthermore, the diversity and composition of the extracellular bacteria associated with the epithelium differed between the two species. Both laboratory and wild isolates of *H. vulgaris* harboured substantially more species than *H. oligactis* from either source, suggesting that *H. vulgaris* is permissive to a wider range of microorganisms than *H. oligactis*. Analysis of 16S rRNA gene sequence data showed that although several bacterial species are common to both the wild *H. vulgaris* and the wild *H. oligactis* isolates, as a whole each species possesses a distinct microbiota and might thus use different mechanisms to shape these microbial communities. Presumably, these mechanisms must include recognizing specific microorganisms and/or fostering an environment that leads to the establishment of the community.

A key follow-up study discovered that *Hydra* spp. can mount a powerful immune response in the absence of motile phagocytic cells[65]. This response is contingent on two independent components: the presence of bacterial flagellin (a classic example of a MAMP) and the secretion of a tissue damage or danger signal, known as a damage-associated molecular pattern (DAMP) (FIG. 2). Interestingly, *Hydra* spp. do not appear to encode typical TLR-like proteins. Instead, two proteins are required for MAMP recognition and signal transduction: a *Hydra* Toll–interleukin-1 receptor domain (TIR domain)-containing protein (HyTRR) and *Hydra* leucine-rich-repeat protein 2 (HyLRR-2), containing a transmembrane domain. HyLRR-2 interacts with the HyTRR protein in response to flagellin. When exposed to a DAMP, such as monosodium urate (which is released from injured cells), an even stronger induction of defence genes is observed. For example, there is increased production of AMPs such as periculin-1. It is tempting to speculate that the requirement for both a MAMP and a DAMP to launch an effective immune response constitutes a mechanism that allows *Hydra* spp. to distinguish pathogens from symbionts.

This study has several important implications[65]. First, it demonstrates that an ancient lineage of multicellular life can recognize broad categories of microorganisms based on their MAMPs and can integrate DAMPs (which are induced only by organisms that harm the host) to induce an immune response (FIG. 2). This ability to integrate the two signals (MAMPs and DAMPs) may contribute to shaping of the microbiota, but further mechanistic studies are needed to clarify this. A further study also demonstrated that periculins are not only important in mounting an immune response in developing *H. vulgaris* embryos but also instrumental in establishing the bacterial community, suggesting that the innate immune system is crucial for both the establishment and long-term maintenance of host-associated bacterial communities in *Hydra* spp.[20].

The *Euprymna scolopes–Vibrio fischeri* symbiosis

Cephalopods are among the most complex invertebrates. They have a closed circulatory system with a highly developed vasculature that allows circulating phagocytic haemocytes to migrate between tissues and interact with both symbiotic and non-symbiotic microorganisms[18,66–68]. These invertebrates also express PRRs that can recognize MAMPs in a number of tissues, and they are capable of mounting potent antimicrobial responses using ROS as well as other biochemical mechanisms[19,68,69].

The association between the Hawaiian bobtail squid, *E. scolopes*, and the bioluminescent bacterium *V. fischeri* has served as a model for understanding how beneficial bacteria influence animal development, colonize host epithelia and interact with the host innate immune system[19,51–53,70–72]. In contrast to the other examples discussed in this Review, this association is binary, involving a single host and a single symbiont. Thus, this system offers an opportunity to study the involvement of the immune system in establishing and maintaining such high specificity.

Within the squid, *V. fischeri* is housed in a vascularized light organ that is responsible for a behaviour known as counter-illumination; the squid uses light produced by the symbiont to mask itself from predators

Peptidoglycan recognition proteins
Eukaryotic proteins that can bind and sometimes degrade bacterial peptidoglycan, and are expressed in various locations within a cell.

Endosymbioses
As used in this Review: associations between hosts and intracellular symbionts (endosymbionts). These symbionts fall into two groups: obligate or primary symbionts, which are always found associated with the host, and secondary symbionts, which are not always present.

Complement system
A humoral component of the innate immune system; the complement pathway leads to the opsonization of microorganisms. There are three types of complement pathway: classical, alternative and lectin.

Nuclear factor-κB pathway
An evolutionarily conserved signalling cascade involving multiple protein complexes that control the transcription of immunity genes.

Damage-associated molecular pattern
A host-derived non-microbial factor that is released following tissue damage or necrosis and can activate the immune system in a similar way to microorganism-associated molecular patterns.

Toll–interleukin-1 receptor domain
A cytosolic domain that is common to a diverse group of receptors, including Toll and Toll-like receptors, and is found in proteins of the basal *Hydra* spp. discussed in this Review.

Table 1 | **Innate immune effectors in selected invertebrates that harbour associations with bacterial symbionts**

Organisms	PRR–MAMP signalling			Humoral components		
	PRRs	NF-κB and/or other immune signalling pathways	Haemocyte response	AMPs	Complement-like factors	ROS and RNS
Hydra spp.	HyTRRs and HyLRRs	Present (NF-κB pathway)	Absent	Present	Some present	Present
Euprymna scolopes	PGRPs, TLR and LBPs	Present (NF-κB pathway)	Present	Unknown	Present	Present
Hirudo verbana	Predicted PRRs	Mostly present (NF-κB pathway)	Present	Present	Present	Present
Acyrthosiphon pisum	Toll and GNBPs, but no PGRPs	Present (Toll and JAK–STAT pathways; reduced and incomplete Imd pathway)	Present	Reduced	Present	Present

AMPs, antimicrobial peptides; GNBPs, Gram-negative bacteria-binding proteins; HyLRRs, *Hydra* leucine-rich repeat proteins; HyTRRs, *Hydra* Toll–interleukin-1 receptor domain-containing proteins; Imd, immune deficiency; JAK–STAT, janus kinase–signal transducer and activator of transcription; LBPs, lipopolysaccharide-binding proteins; MAMP, microorganism-associated molecular pattern; NF-κB, nuclear factor-κB; PGRPs, peptidoglycan recognition proteins; PRR, pattern recognition receptor; RNS, reactive nitrogen species; ROS, reactive oxygen species; TLR, Toll-like receptor.

when it is foraging at night[73]. The bacteria are contained within epithelium-lined crypt spaces that are directly connected to the environment via ducts which terminate in the host's mantle cavity (FIG. 1b). The association is transmitted environmentally: after hatching, the juvenile squid obtain bacteria from the surrounding sea water, selecting *V. fischeri*, which constitutes less than 0.1% of the 10^6 bacteria that are found per ml of sea water[74,75]. There are many mechanisms by which the partners ensure this specificity, and the innate immune system of the host seems to play a central part in this process[18,19,44,52,70,72,76–78].

Pattern recognition in the squid. Host recognition of bacterial MAMPs is crucial for many colonization events in the *E. scolopes*–*V. fischeri* association. For example, one of the earliest host responses is copious secretion of mucus following exposure to the external environment when the juvenile squid emerges from the safety of its egg case[79,80]. The presence of bacterial PGN is detected, and in response, the superficial ciliated fields of the juvenile light organ secrete mucus, which aids in the development of a *V. fischeri* biofilm outside the nascent light organ. Interestingly, *V. fischeri* dominates this biofilm over other environmental bacteria[81]. The nature of this dominance is unknown, but this aggregation serves as one of the first sites of specificity in this association. During the first days of the association, *V. fischeri* MAMPs, including LPS, PGN and PGN derivatives, play an important part in mediating specificity and initiating the onset of colonization[36,82]. For example, LPS and the PGN derivative trachaeal cytotoxin (TCT) work synergistically to induce normal development of the light organ by initiating apoptosis and regression of the juvenile ciliated fields within days of colonization by *V. fischeri*[36] (FIG. 1b). Although MAMPS such as PGN, LPS and TCT are often implicated as virulence factors among pathogens, in this symbiotic relationship these MAMPs actually carry out a beneficial function by inducing proper tissue development in the host.

Several *E. scolopes* PRRs have been characterized, including a TLR, five PGRPs and three LPS-binding proteins (LBPs)[44,70,76–78]. The *E. scolopes* PGRPs (EsPGRPs) have cell- and tissue-specific expression patterns, which may confer a tissue-specific ability to respond to PGN and TCT depending on the MAMPs that are encountered in different microenvironments. For example, EsPGRP1 is expressed in the nuclei of host epithelial cells but is lost from late-stage apoptotic cells during *V. fischeri*-induced morphogenesis[78]. A *V. fischeri* mutant that is deficient in lytic transglycosylase activity (and is therefore defective for TCT release)[83] prevents this loss of EsPGRP1 (REF. 78), suggesting that *V. fischeri* influences the expression of host PRRs. EsPGRP2 is secreted in host-derived mucus during colonization and is also localized in the light organ crypts after the onset of *V. fischeri* colonization[76]. EsPGRP2 has also been shown to have TCT-degrading amidase activity[76] that might ameliorate the deleterious effect of this powerful toxin, which in pathogenic associations is cytotoxic to host mucosal and epithelial surfaces[84,85]. The ability to degrade TCT might be especially important during periods when symbiont concentrations, and presumably TCT concentrations, are high. The roles of EsPGRP3, EsPGRP4 and EsPGRP5 are currently unknown, but these proteins have been detected in haemocytes[70] (B. Rader and S.V.N., unpublished observations). Although the three *E. scolopes* LBPs (EsLBPs) have yet to be characterized in detail, EsLBP1 has been detected in the crypt spaces, and its expression is upregulated in symbiotic hosts[44,86].

Phagocytosis in the squid. Host haemocytes migrate from the vascular system through the epithelium of the light organ crypt to interact with the symbionts[66,67,87], and recent work has focused on understanding whether these cells can differentiate between symbiotic and non-symbiotic bacteria[18]. Haemocytes have been shown to recognize and bind members of the family Vibrionaceae to different degrees, and *V. fischeri* has low binding activity[18]. When the symbionts are eliminated from the light organ of the adult squid (using antibiotics for a period of 5 days), haemocytes from these cured hosts bind significantly more *V. fischeri* cells than haemocytes obtained from untreated or fully colonized animals. The increase in adhesion is specific to *V. fischeri* cells, as binding of non-symbiotic bacteria remains unchanged. Thus, variation among related bacterial species in their ability to avoid adhesion to haemocytes, coupled with an altered

haemocyte response to colonization, might contribute to the specificity of this symbiotic interaction. As the haemocytes migrate into the crypts and probably sample these microenvironments, the ability of *V. fischeri* to avoid adherence could contribute to long-term persistence of the organism at these sites. This change in the binding affinity of haemocytes is a striking example of symbiont-induced host tolerance. The mechanism (or mechanisms) by which *V. fischeri* mediates this immune tolerance in the host remains unknown, but a bacterial Omp might be involved. Deletion of the *V. fischeri* gene encoding OmpU leads to a significant increase in binding of the bacterium to squid haemocytes, suggesting that this protein helps *V. fischeri* to avoid phagocytosis[18]. Ongoing research is focusing on an analysis of the transcriptomes and proteomes of haemocytes from symbiotic and cured hosts, and a recent study has shown that EsPGRP5, nitric oxide synthase (NOS) and a squid orthologue of complement component C3 are all differentially expressed in these cells depending on the colonization state[70].

These findings in the adult squid host raise questions about how the immune system of the juvenile host develops in response to colonization. In many animals, the immune system undergoes a maturation and development period in response to microbial and environmental challenges, and this often leads to tolerance or homeostasis[43,88–90]. Although haemocytes with internalized bacteria have been observed within the crypts of colonized juvenile hosts, no engulfed bacteria have been observed in the light organ haemocytes of adults, despite the fact that these cells are exposed to high concentrations of *V. fischeri*[67]. Another study also revealed that there is an infiltration of haemocytes in the superficial ciliated field of the nascent light organ within 2 hours of exposure to *V. fischeri*[66]. These observations suggest that the cellular immune response of the host is altered by the symbionts and might mature in response to the persistent colonization by *V. fischeri*. Although the development of the immune system in *E. scolopes* has not been characterized in detail, these studies suggest that symbiosis with *V. fischeri* is one factor that contributes to immune system maturation.

Chemical factors. Chemical components of the innate immune system have also been implicated in the *E. scolopes*–*V. fischeri* symbiosis, and several studies have shown that ROS and reactive nitrogen species (RNS) have an important role in this association[91–93]. *E. scolopes* produces squid halide peroxidase (sHPO), a protein similar to vertebrate myeloperoxidase. This enzyme converts H_2O_2 into hypohalous acids (for example, hypochlorous acid), which are microbiocidal compounds[91]. sHPO is expressed in tissues that directly contact bacteria, including the light organ, gills and the accessory nidamental gland (a female reproductive organ that harbours a consortium of bacteria)[94]. In the light organ, *V. fischeri* can presumably overcome this challenge owing to its expression of a periplasmic catalase that degrades H_2O_2 (REF. 95).

The enzyme NOS is necessary for the production of nitric oxide, which can react with oxygen or superoxide anions to generate antimicrobial RNS such as peroxynitirite and dinitrogen trioxide. Previous studies have shown that both NOS and nitric oxide are active during initiation of the *E. scolopes*–*V. fischeri* symbiosis[92,96,97]. Specifically, NOS and nitric oxide have been detected in host-derived vesicles found within the mucous secretions in which *V. fischeri* aggregates before entry into the light organ, and they have also been reported in the ciliated epithelium, ducts and antechambers (FIG. 1b). Successful colonization leads to an irreversible attenuation of the NOS and nitric oxide levels in the light organ, and LPS and TCT seem to be required to induce this response, suggesting that the symbiont and/or host can adjust the innate immune response to facilitate colonization[92,97]. *V. fischeri* also contains a haem nitric oxide–oxygen (H-NOX)-encoding gene, which probably acts as a nitric oxide sensor, along with a number of potential nitric oxide detoxification genes, including *hmp* (encoding flavohaemoprotein), *norVW* (encoding the anaerobic nitric oxide reductase flavorubredoxin (NorV) and the associated NorV oxidoreductase (NorW)) and the *nrf* operon (encoding cytochrome *c* nitrite reductase) (reviewed in REF. 96).

Other innate immune factors. Recent analyses of the *E. scolopes*–*V. fischeri* system have also identified a number of putative members of the complement cascade that might play a part in modulating symbiosis[19,98]. An orthologue of complement component C3, one of the crucial components of the cascade, has been localized to the apical surfaces of the crypt epithelium, although the role of this orthologue in the symbiosis has yet to be analysed in detail[98]. Quantitative PCR was carried out to compare expression of the C3-encoding gene in haemocytes from symbiotic and cured hosts, revealing that transcript levels in haemocytes from naive or cured squid are significantly higher than the levels in haemocytes isolated from a fully colonized light organ, suggesting that *V. fischeri* dampens the complement response in these cells[70]. Recent analyses of the light organ and haemocyte proteomes also found several thioester-containing proteins[70,93]. In invertebrates, these proteins have an important role in the innate immune response as members of a complement-like system or as protease inhibitors; future studies will focus on investigating the role of C3 and other members of the complement response in this symbiosis[99–101].

The leech–bacteria symbiosis

Compared to vertebrates, some invertebrates house relatively simple microbial communities in their gut. Although in some cases this is probably due to the extremely alkaline conditions in the invertebrate gut[13], other invertebrates lack such an obvious barrier to colonization. One example of these other invertebrates is the Hungarian medicinal leech, *Hirudo verbana*, in which the microbial community in the largest compartment of the digestive tract, the crop, is dominated by two bacterial species, *Aeromonas veronii* and a *Rikenella*-like bacterium[54–56,102] (FIG. 1c). The digestive-tract symbionts are thought to provide the host with vitamin B, which

is in low abundance in blood[103]. So how is this simple microbial community established and maintained, and what is the role of the leech immune system in these processes[104]?

Immunity in leeches is mediated by haemocytes (also known as amoebocytes) that circulate in the haemolymph and in the lumen of the digestive tract[105]. In addition, several AMPs have been identified in leech species, including theromacin, theromyzin, lumbricin and neuromacin. These peptides are found in large fat cells, neurons and microglia, but their presence in other regions of the leech body has not been excluded[106]. Expressed sequence tag (EST) data also indicate that proteins with some similarity to PRRs might also be present in *Hirudo medicinalis* and *H. verbana*[107].

Phagocytosis in the leech. The crop is a storage compartment for the ingested blood meal and, analogous to the human colon, the site where water and osmolytes are absorbed[105]. In a single feeding, a leech can consume more than five times its body weight in blood. The ingested erythrocytes are concentrated by the leech absorbing water from the blood meal, and the removal of osmolytes makes the intraluminal fluid isosmotic with the leech haemolymph[105]. Interestingly, the complement system of ingested vertebrate blood remains active for one or two days and kills sensitive bacteria in the gut[108]. Although this is an important barrier to microbial colonization of the gut, it is only transitory and not sufficient to explain the low diversity of microorganisms.

Another mechanism that contributes to specificity in the leech is phagocytosis of sensitive bacteria by haemocytes that patrol the crop[16,104]. The importance of haemocytes was discovered by a signature-tagged-transposon mutagenesis screen, which obtained *A. veronii* mutants that had lost the ability to colonize the medicinal leech gut[16,109]. One of these mutants had a defective T3SS and was found to be phagocytosed by haemocytes, whereas the parent strain adhered to haemocytes but was not phagocytosed. These data suggest that the leech haemocytes did not actively differentiate between mutant and parental *A. veronii* cells but that the parental cells possess protective mechanisms that inhibit phagocytosis. When animals were colonized with a mixture of the T3SS mutant and the parent strain, the mutant was still cleared from the leech, indicating that the T3SS of the wild-type strain does not interfere with phagocytosis of other bacteria (such as the mutant strain). This lack of cross-protection for cells that are susceptible to phagocytosis (in this case, the T3SS mutant) is likely to be important for maintaining the specificity of the association. Taken together, these data suggest that specific features of the bacterial symbiont promote avoidance of the innate immune system in the digestive tract of the leech.

Because *A. veronii* is also a pathogen of mammals, it was possible to assess whether the same T3SS mutant could cause disease in mice, and to test for cytotoxicity: the T3SS mutant had reduced virulence in mice and was significantly less cytotoxic to mouse macrophages[16]. *A. veronii* possesses at least two type III effectors, AexT and AexU[110,111]. AexT had previously been identified in *Aeromonas salmonicida*, an important fish pathogen[112,113], and AexU is a novel toxin that was discovered in an *Aeromonas hydrophila* diarrhoeal isolate, as well as in the leech symbiont *A. veronii*[110,111]. The role of the T3SS in causing cytotoxicity to mouse macrophages while protecting the bacterium against phagocytosis in the leech suggests that this system has two very different roles in these two animals.

Analysis of the genome of the second dominant leech symbiont, the *Rikenella*-like bacterium, did not reveal the presence of a T3SS, but these bacteria are either exclusively associated with the host mucus or present in microcolonies that are surrounded by a polysaccharide matrix, which could serve as a protective barrier against phagocytosis[102]. Thus, both of these *H. verbana* symbionts (*A. veronii* and the *Rikenella*-like bacterium) possess protective mechanisms that do not directly interfere with the activity of haemocytes. These data suggest that symbiosis in *H. verbana* is at least partially mediated by the ability of the symbionts to avoid phagocytosis, in contrast to symbiosis in *E. scolopes*, for which both symbiont avoidance of phagocytosis by haemocytes and the ability of haemocytes to discriminate between symbionts and non-symbionts might be important for specificity.

Chemical factors and pattern recognition. *In vivo* transcriptomes of the digestive-tract symbionts, in combination with analyses of A. *veronii* mutants, suggest that the bacteria encounter membrane stress inside the leech crop[109,114]. A potential source of this stress is AMPs; for example, lumbricin and neuromacin were found in the central nervous system of the closely related leech *H. medicinalis*[106]. In the distantly related leech *Theromyzon tessulatum*, AMPs were also detected in the leech intestinal epithelium[115–117], raising the possibility that AMPs are active in the leech crop. How the expression of AMPs is regulated in leeches is currently unknown. Furthermore, 12 different transcripts that are predicted to encode PRRs have also been detected in EST libraries from *H. medicinalis* and *H. verbana*[107]. Some of the predicted PRRs are only detected in libraries constructed from either embryos or the nervous system, which suggests that the leech exhibits tissue-specific expression of PRRs, although functional studies have not yet been carried out.

Acyrthosiphon pisum–Buchnera aphidicola

The pea aphid, *A. pisum*, and its symbiotic bacterium, *Buchnera aphidicola*, have co-evolved to form an association that is both highly specific and essential for viability of both the host and the symbiont[118]. Aphids are dependent on *B. aphidicola* for essential amino acids, and the vertically transmitted bacterium is an obligate endosymbiont that has an extremely reduced genome. *B. aphidicola* resides within specific host cells (bacteriocytes) and obtains essential nutrients from the host[119] (FIG. 1d). A number of studies have described the genes that are expressed in host bacteriocytes, and in addition to bacterial proteins involved in the delivery

Expressed sequence tag (EST). A cDNA sequence that has been obtained from a reverse-transcribed mRNA.

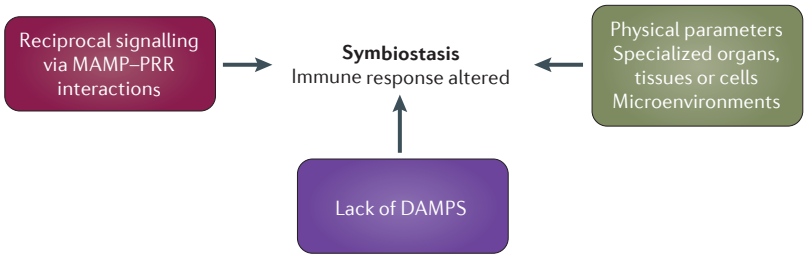

Figure 3 | **Model for the establishment of symbiostasis in host–microorganism associations.** Multiple mechanisms can lead to the successful establishment and maintenance of beneficial symbioses. Here, we propose three possibilities. The first possible mechanism is reciprocal signalling between the partners, often mediated through microorganism-associated molecular pattern (MAMP)–pattern recognition receptor (PRR) interactions, which can lead to an altered immune response and host tolerance to the symbiont. The second possible mechanism is sequestration of the symbionts in specialized tissues, organs or cells, where the molecular dialogue leading to stasis can be regulated locally. The third potential mechanism is the avoidance of combined MAMP–damage-associated molecular pattern (DAMP) signalling, allowing the symbiont to persist in the host at the specialized site; if the symbiont or another microorganism (that is, an invasive pathogen) infiltrate other areas of the host and/or causes tissue damage, then signalling events mediated through DAMPS and/or MAMPs can lead to a heightened immune response and clearance by the host.

of essential amino acids and vitamins to insect hosts, *A. pisum* innate immunity factors have also been detected in these cells, suggesting that components of the innate immune system are important in interfacing with *B. aphidicola*[120–122].

However, unlike other insects, the innate immune system of *A. pisum* lacks PGRPs and defensins, and has a non-functioning immune deficiency pathway (Imd pathway)[123]. It has been suggested that the symbiosis with *B. aphidicola* contributed to the evolution of reduced immune capabilities in the aphid host, and this hypothesis was recently examined[122]. Specifically, when *D. melanogaster* Schneider 2 cells were challenged with *B. aphidicola*, an increase in the expression of a PGRP as well as components of the Imd pathway was observed, and there was a subsequent production of AMPs, which led to the clearance of *B. aphidicola* within 2 days. These data suggest that *B. aphidicola* would face a serious threat from a fully functional immune system in aphids and/or that a reduced immune response facilitated the establishment of the *B. aphidicola* symbiosis.

One interesting question is whether, and how, hosts adapt differently to bacterial endosymbionts that have reduced genomes and are vertically transmitted (such as *B. aphidicola*), as opposed to bacterial endosymbionts that have large genomes and are environmentally transmitted, and whether obligate endosymbionts lead to reduced immune capabilities in other systems. Aphids are not, however, defenceless; for example, they produce lysozyme, and their haemolymph contains up to five different types of immune cell, at least two of which are capable of phagocytosing bacteria[120,124,125]. Pea aphids often harbour facultative secondary bacterial symbionts[126] (*Serratia symbiotica*, '*Candidatus* Hamiltonella defensa' or '*Candidatus* Regiella insecticola'), and aphids appear to have differing numbers of haemocytes depending on the type of secondary symbiont that they carry, suggesting that colonization by specific symbionts can influence host cellular immunity[124]. Haemocytes from aphids also seem to be capable of phagocytosing both *B. aphidicola* and secondary symbionts, but the role of these cells in mediating symbioses remains to be determined. Pea aphids possess components of the Toll and janus kinase–signal transducer and activator of transcription (JAK–STAT) signalling pathways, as well as prophenoloxidase, a key component of the insect melanization defence response, and nitric oxide synthase[123]. Secondary symbionts such as '*Ca*. Hamiltonella defensa' and '*Ca*. Regiella insecticola' also seem to be able to induce pea aphid resistance to parasitic wasps and fungi, respectively[127,128]. These observations, along with the finding that the reproductive parasites *Wolbachia* spp. can confer resistance to viruses in flies and mosquitoes[129–131], suggest that insects can supplement their innate immune system by exploiting the defence mechanisms of their symbionts.

Integrating mechanisms towards symbiostasis

The examples described above show that, in addition to the classical mechanisms of responding to pathogens, invertebrates are capable of using the innate immune system to interact with symbionts in order to promote the establishment and maintenance of symbiotic associations. The use of invertebrate models gives researchers the opportunity to discover additional regulatory pathways that are likely to be ancient and might reveal novel immunological mechanisms that confer high microbial specificity (outside of the conventional antibody-based adaptive response attributed to vertebrates).

We propose three possible mechanisms by which hosts differentiate between symbionts and pathogens, and these mechanisms would not be mutually exclusive (FIG. 3). First, reciprocal signalling between the partners (mediated by MAMP–PRR interactions) could induce an altered immune response that would lead to host tolerance of the symbiont. Second, symbionts might be sequestered in specialized tissues, organs or cells, where the molecular dialogue leading to stasis could be regulated locally. Last, if the symbiont or other microorganism (such as an invasive pathogen) infiltrates other areas of the host and/or causes tissue damage, signalling events mediated through DAMPS and MAMPs could lead to heightened immune responses and clearance by the host. A lack of such damage signals could also promote maintenance of the symbiont. The outcome of one or all three of these processes would be a healthy and stable association referred to as symbiostasis[132].

The first mechanism requires the sequential recognition of signals from both partners, for example, mediated by the interaction between MAMPs and PRRs. In the *E. scolopes*–*V. fischeri* symbiosis and in the classical symbiosis between leguminous plants and rhizobial bacteria, intricate reciprocal signalling is required to establish these associations[19,52,133]. Multiple signalling events allow colonization by the appropriate microorganisms and modify the immune defence responses accordingly. The pea aphid has apparently taken a

Immune deficiency pathway
An immune pathway that is found in most insects and responds to bacteria via peptidoglycan recognition proteins binding to diaminopimelic acid-containing peptidoglycan. Subsequent signalling pathways can lead to AMP production.

different strategy by limiting the number of interactions between MAMPs and PRRs, in addition to having a reduced repertoire of signalling and immune effectors.

The second mechanism relies on tissue-specific conditions that select for particular microorganisms. Whereas some organs and tissues of metazoans are continuously exposed to microorganisms, others are normally sterile. Spatial or even temporal differences in the host immune response, physiology and/or anatomy can create zones of tolerance where the symbionts thrive. For example, in both the leech crop and the squid light organ, physical conditions exist that promote specificity of the symbionts and sequester them to specific tissues[52,134]. *B. aphidicola* and certain other intracellular symbionts are confined to bacteriocytes, which may limit symbiont exposure to the host immune system. Recent evidence from the weevil suggests that host AMPs are crucial for containing endosymbionts within bacteriocytes and that bacteria escape this niche when AMP pressure is removed[135]. Temporal differences can also occur during development, such that specific stages of the animal are conducive to colonization, whereas other stages are not. For example, the squid light organ briefly allows entry of non-symbiotic bacteria for 30 minutes after hatching[52], although these interlopers are promptly removed by mechanisms that might involve the innate immune response. Changes in the composition of the gut microbiota are also well described in a number of systems[136,137]. These microbial communities can vary widely depending on a number of parameters, including the age, health and diet of the host, and the presence or absence of antibiotics[138]. In the event of symbiont translocation from such specialized sites — for example, owing to tissue damage in a diseased host — the symbiont can become pathogenic at the new site and elicit an immune response[139,140].

The third mechanism requires the avoidance of stimulating both MAMP- and DAMP-mediated signalling. The requirement for the integration of a MAMP and a DAMP to provoke an immune response was originally proposed by Matzinger[141]. An excellent example of this can be found in *Hydra* spp., in which the combination of two signals — bacterial flagellin and tissue damage — is required for the strongest induction of an immune response[65]. In *D. melanogaster*, low levels of bacterial PGN negatively regulate NF-κB signalling and subsequent AMP production, as well as ROS pathways (reviewed in REF. 142). During a systemic infection or following tissue damage, such downregulation is inhibited, allowing for the initiation of innate immunity effector mechanisms. In vertebrates, tissue damage is often followed by the activation of several signalling pathways that are induced by the release of cytokines. Although these cytokine-induced pathways are not as well characterized among the invertebrates, cytokine homologues have been described and probably cause similar responses in these hosts[143]. The requirement for tissue damage in order to trigger an immune response would presumably prevent symbionts (which do not damage the tissue) from stimulating the innate immune system, unless the interaction shifted from beneficial to pathogenic. The generality of this signal integration is currently unknown, and further mechanistic studies on animals from different lineages are required to establish whether it is a common pathway for differentiating between symbionts and non-symbionts.

Conclusions

Since the first ground-breaking phagocytosis experiments by Metchnikoff more than 100 years ago, it has been apparent that the innate immune system is crucial for the response to pathogens and for preventing disease. Here, we present findings from four model symbioses that highlight the importance of the invertebrate innate immune system in establishing and maintaining symbiotic bacteria. Key aspects of an ideal model system for studying symbioses and the immune system include the ability to culture the host and symbiont separately, the availability of genetic tools to manipulate each partner, the availability of genomic information for both partners and experimental tractability of the system[144]. Each of the systems described here is experimentally tractable and has some genomic information available, although currently only ESTs are available for *E. scolopes* and *H. verbana*; furthermore, most of the symbionts discussed, with the notable exception of *B. aphidicola*, have been cultured. Genetic manipulation of the host is limited to RNAi in *Hydra* spp., the pea aphid and the leech. Although none of these four models currently fulfils all the criteria of an ideal model system, the study of these models and other invertebrate systems nonetheless provides broad insight into symbiotic relationships and the range of immune interactions that shape such associations.

The combined evidence suggests that the innate immune system can distinguish between and respond differently to symbionts and pathogens, although the mechanisms behind this ability are not fully elucidated. This differential response might be regulated by several mechanisms (FIG. 3), including specific recognition (or lack thereof) of symbionts and MAMPS, and spatial and temporal factors that might prevent a general immune response from being triggered by MAMPs and/or DAMPs. Microbial recognition is at least partially mediated by PRRs that are conserved among metazoans, although the likely crucial role of downstream signal transduction pathways remains to be resolved, but a role for the ancient NF-κB pathway has been implied. Future research should focus on examining an even more diverse array of invertebrate systems for the identification of evolutionarily conserved mechanisms. The exciting explosion of omics techniques has made a plethora of new information available, but these tools often suggest new hypotheses on the basis of only the presence or absence of specific sequences, so these hypotheses will require experimental testing. In addition, bioinformatic analyses are challenging, especially when the identified sequences are not functionally characterized or when the characterized gene comes from a distantly related organism. Thus the use of omics in parallel with the more challenging functional studies is crucial to advance this exciting field. More effort

should also be put into understanding how the immune system develops in these diverse invertebrate groups and whether interactions with the microbiota influence its maturation. Each model system has its own benefits and limitations, but only by applying a number of methods to a variety of associations will researchers reveal the breadth of interactions that exist in nature. We predict that these studies will lead to novel discoveries about the role of the innate immune system in fostering symbiotic relationships with microorganisms.

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