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Non-immunological defense in an evolutionary framework

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After parasite infection, invertebrates activate immune system-based defenses such as encapsulation and the signaling pathways of the innate immune system. However, hosts are often able to defend against parasites without using these mechanisms. The non-immunological defenses, such as behaviors that prevent or combat infection, symbiont-mediated defense, and fecundity compensation, are often ignored but can be important in host–parasite dynamics. We review recent studies showing that heritable variation in these traits exists among individuals, and that they are costly to activate and maintain. We also discuss findings from genome annotation and expression studies to show how immune system-based and non-immunological defenses interact. Placing these studies into an evolutionary framework emphasizes their importance for future studies of host–parasite coevolution.

Expanding ecological immunology

Studying the immune system in the context of natural environments has been critical for the understanding of host–parasite coevolution [1,2]. Fundamental to this approach is the recognition that immunity is costly to organisms in terms of evolving defense [3,4], and in terms of using and maintaining immune systems [5–7]. From this research field (which is often termed 'ecological immunology'), we have learned that internal constraints caused by the high costs of immunity have frequently led to measurable variation among individuals in parasite defense, and analyses of polymorphism in genes of the immune system show clear traces of natural selection [8–10]. The complexity of host defenses is of interest not just because of the devastating effects of parasites on host populations which by definition are deleterious for their hosts but also for understanding a major class of interspecific interactions.

Activation of the immune system after parasite infection is, however, only one part of a complete picture of host defense. There are also several mechanisms of defense that fall outside of what is traditionally considered to be the immune system (Box 1). These non-immunological defenses are ignored or, if acknowledged, are often treated as static traits that do not contribute to existing variation in host defense among individuals. We highlight the importance of these defenses for host–parasite coevolution by

focusing on invertebrates because of their increasing importance for the study of ecological immunology [11]. To drive coevolutionary dynamics, we expect these traits to meet three necessary conditions. First, these defenses contribute significantly to host defense against parasites. Second, they vary among individuals, and this variation is heritable. Third, this variation is generated by measurable activation and maintenance costs or by interactions between defense traits and parasite genotype (similar to genotype-by-genotype (GxG) interactions). We place recent studies of non-immunological defenses in this framework, and note the similarities between selective forces shaping immune system-based and non-immunological defenses. We also discuss how immune system-based and non-immunological defenses interact during an infection and over evolutionary time, and how they play an important part in host–parasite coevolution.

Non-immunological mechanisms contribute to host defense

Host defense is usually studied in the context of immune system-based defenses. These are characterized by the recognition of components of the invading parasite and a sequence of signaling cascades that culminate in the production and activation of immune cells or effector molecules that attack invasive organisms [12]. However, without using these mechanisms, hosts are often able to reduce their likelihood of infection, reduce parasite growth once infected, and compensate for the fitness lost from infection (Figure 1).

Hosts can reduce the likelihood of infection by reducing the probability that they are exposed to a parasite. For example, the honey bee (*Apis mellifera*) displays a social grooming behavior that actively removes *Varroa* ectoparasitic mites from colony members, a behavior that appears to be a main mechanism of resistance to the parasite [13]. Hosts can also reduce their likelihood of infection by sequestering compounds that reduce infection probability – a form of 'prophylactic self-medication'. By mixing resin (a plant material rich in volatile compounds) with nest material, wood ants (*Formica paralugubris*) can increase their survival rates if challenged with bacterial and fungal parasites [14]. Hosts can also reduce the growth or burden of parasites once infection has occurred. For example, parasitoid-infected arctiid caterpillars (*Grammia incorrupta*) can increase the

Box 1. How do we define immunity?

Traditionally, an organism is thought to respond to parasites largely via its immune system: the series of molecular mechanisms that recognize an invader and respond through encapsulation, melanization, and signaling cascades that produce effectors such as antimicrobial peptides and lysozymes. Through this framework, immunologists have accumulated a wealth of information about how these immune system-based defenses work to protect hosts from infection, most often using laboratory models, and frequently using non-natural pathogens. Immune systems are often explored in new species based on the identification of genes identified as orthologs of immune genes from model systems.

Recently there has been a call for the consideration of immune-system-based defenses in an environmental context. The field of ecological immunology has tried to place the molecular mechanisms of the immune system into an ecological framework, specifically by focusing on how variation among individuals in these effector systems contributes to host fitness. This includes studies of how defense can be costly for hosts. In addition, researchers are beginning to acknowledge the role of environmental factors in parasite defense. For example, abiotic conditions such as the temperature at which an infection occurs can affect susceptibility to parasites [81,82].

Here we discuss several defense mechanisms that exist outside of what is traditionally considered to be the immune system but which clearly contribute to an organism's defense against parasites. We use the term 'non-immunological defenses' to describe these mechanisms, which are generally ignored in studies of parasite defense. A complete picture of immunity, however, requires a holistic approach to studying parasite defense, especially because in many ways non-immunological and immune system-based defenses are not different. Recent studies have shown that non-immunological and immune system-based defenses interact (e.g. [64,68]), and that there is a strong environmental context to parasite defense as a whole. Future studies of host–parasite interactions will therefore need to consider immune system-based and non-immunological defenses, the ecological context of infection, and the interactions that occur between these factors to gain a complete picture of an organism's defense against parasites.

Behavioral Avoidance: Organisms reduce their risk of exposure to parasites by avoiding infectious materials. For example, gypsy moth larvae preferentially consume foliage uncontaminated by a nucleopolyhedrovirus [25].

Social Immune Mechanisms: Social insects have evolved collective resistance traits, which include behavioral and physiological adaptations. For example, honey bees employ a hygienic behavior that involves the detection and removal of dead larvae in order to prevent disease spread in the colony [26].

Self-Medication: Organisms sequester compounds that provide no nutritional benefit to the host but do prevent infection (**prophylactic self-medication**) or reduce pathogen growth once infected (**therapeutic self-medication**). For example, parasite-infected monarch butterflies preferentially lay their eggs on toxic plants, which reduces parasite growth in their offspring [16, 50].

Behavioral Thermoregulation: Ectotherms can reduce parasite success by behaviorally altering their temperature. This is commonly called behavioral fever but can also result in behaviorally lowered temperature. For example, bumblebees prefer cooler temperatures when parasitized by conopidflies, which stunts parasite growth and extends host lifespan [84].

Anorexia: A common, if apparently counterintuitive, response to infection is reduced feeding, leading to reduced caloric intake that can increase resistance. In the cricket *Gryllus texensis* this appears to be due to a tradeoff between the use of lipoproteins for digestion or for immune function [85].

Symbiont-Mediated Immunity: Many invertebrates harbor vertically transmitted microorganisms that protect them from parasites either directly or indirectly. For example, pea aphids often harbor one of a number of facultative bacteria that have been shown to protect their hosts from wasps [19] and fungi [18].

Fecundity Compensation: Some organisms can compensate for potential fitness losses due to infection by changing the timing of their reproductive efforts in response to a parasite cue [86]. For example, the waterflea, *Daphnia magna*, produces more offspring early in life when exposed to a microsporidian parasite compared to uninfected controls [21].

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Figure 1. **Examples of non-immunological defense.** The extent to which non-immunological defense influences host–parasite dynamics, varies among individuals, and imposes costs differs among defenses and study systems. In general, it is important to demonstrate that a trait is adaptive for a host and is not a mechanism of parasite manipulation [83] before it can be considered to be a non-immunological defense. Other physiological traits, such as feeding rate [23], might evolve as a consequence of parasite pressure, but are not the focus here because they are not active responses of individuals to parasitism. Photographs, from top to bottom, by A. Hunter, R. Naylor, E. Sternberg, P. Schmid-Hempel, S. Adamo, T. Barribeau, and T. Little.

uptake of anti-parasitic chemicals from their food, which reduces parasitoid success [15]. Also, parasite-infected monarch butterflies (*Danaus plexippus*) preferentially lay their eggs on plants that reduce parasite growth in their offspring [16]. These are two forms of therapeutic medication. The microbial symbiont community of a host can also influence parasite growth after infection. Invertebrates are often protected from parasites by symbiotic bacteria (e.g. [17–19]), which can strongly influence population dynamics. For example, a recent study suggested the spread of symbiont-carrying *Drosophila neotestacea* through North America [20]. Lastly, even if they cannot reduce the probability of infection or reduce parasite growth, some hosts can compensate for the fitness lost from infection through 'fecundity compensation'. This is where an individual changes the timing of her reproductive efforts in response to parasitism. The water-flea (*Daphnia magna)* produces more offspring early in life when exposed to the microsporidian parasite *Glugoides intestinalis* compared with uninfected controls [21]. Together these examples and those discussed below show that non-immunological defenses are pervasive in invertebrate–parasite interactions, and can have an important impact on short-term host–parasite dynamics. It might be difficult to measure the benefit of a defense mechanism empirically, e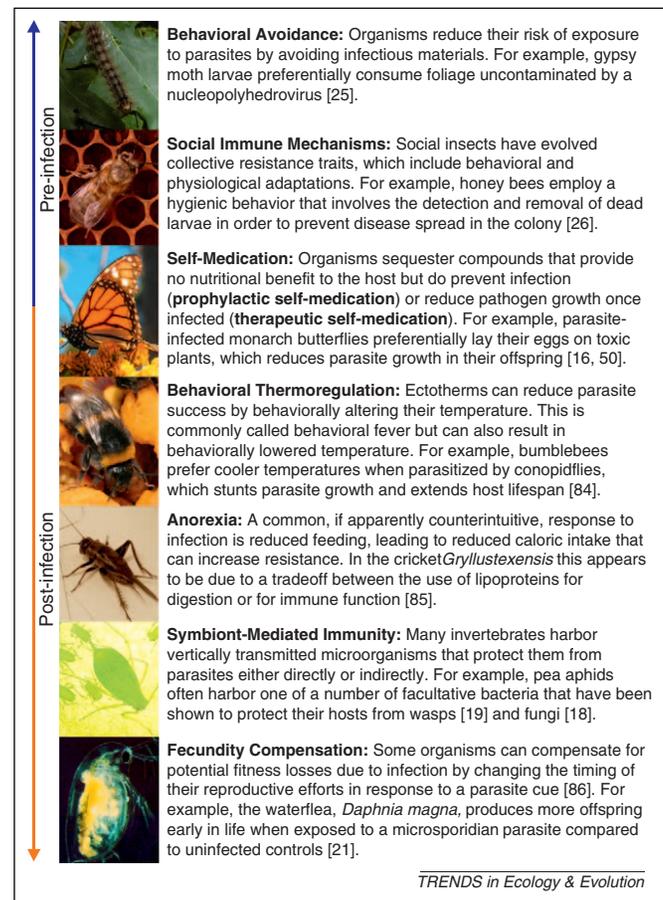specially under certain conditions. Traits that appear as signs of infection might be evolved mechanisms of defense, and should be considered even if their benefit has not been demonstrated.

There are several other physiological properties of invertebrates that have been shown to influence susceptibility (reviewed in [22]). For example, variation in feeding rate among clones of *Daphnia dentifera* was recently shown to contribute to variation in susceptibility to a fungal parasite [23]. Other physiological traits, just as feeding rate, might evolve as a consequence of parasite pressure, but are not the focus here because they are not active responses of individuals to parasitism.

Heritable variation in non-immunological defense

For natural selection to act on defense against parasites, individuals must vary in heritable traits that determine susceptibility to disease [24]. The heritability of a trait has traditionally been shown using experiments that determine the proportion of variation in phenotype that can be explained by genetic lineage *vs.* environmental factors. For example, it was recently shown that gypsy moth larvae (*Lymantria dispar*), which avoid virus-contaminated foliage, show heritable variation among families in avoidance

behavior [25]. The implication behind these studies is that genetics underlie the heritability, but this approach does not lead to a mechanistic understanding of the relevant traits.

The use of genetic and genomic techniques is leading to a different approach. Because genes are passed from parents to offspring, a trait can be shown to be heritable if the underlying genetic basis of the trait can be demonstrated. Extensive research has focused on understanding the genetic basis of hygienic behavior in honey bees, which decreases the transmission of disease and the susceptibility of individual hosts [26]. One such hygienic behavior involves uncapping brood cells containing dead or diseased larvae and then removing the larvae from the colony. This behavior has been shown to be dependent upon homozygosity at two loci, with heterozygosity at either allele resulting in individuals who either uncap or remove, but who do not carry out both tasks [26]. Recent work has identified several additional loci associated with these behaviors [27], confirming a strong genetic component. Wilson-Rich *et al.* [26] note that the genetic differences between colonies determine how quickly disease is detected, and thus how effective the behavior is at reducing disease risk. Artificial selection for increased behavioral resistance to mites has been demonstrated experimentally in honey bees [28]. Also, it was recently shown that behaviorally linked genes are differentially expressed among resistant and susceptible bee colonies [29]. The expression of genes involved with olfaction and neuronal sensitivity were especially different among these colonies, which can be important for the detection of infected brood cells. Hygienic bees have better olfactory sensitivity than nonhygienic bees because they can distinguish between the odors of healthy and diseased brood at a lower level of detection [30–32].

There are also some examples of heritable variation that is not genetic. Studies of variation in susceptibility to parasitoid wasps in pea aphids (*Acyrthosiphon pisum*) falsely concluded that large amounts of genetic variation in resistance existed in wild populations [33,34] until it was discovered that most of this variation is due to bacterial symbionts and not host genotype [35]. Aphids often harbor symbiotic bacteria that have been shown to confer strong protection against parasitic wasps [19,35–39] and fungi [18]. These bacteria are referred to as 'facultative' symbionts because not all individuals maintain them [40–42]. In addition, these symbionts are vertically transmitted from parent to offspring, meaning that this symbiont-mediated protection is heritable, but not via a traditional mendelian genetic mechanism. Thus, such defenses can be considered epigenetic in nature.

What maintains variation in non-immunological defenses?

Given the ubiquity of parasites in nature, why do we find variation among individuals in the non-immunological defenses described here and in immune system-based defenses? Ecological immunology has offered two main explanations (Figure 2). First, variation can occur because host defense is costly and trades off with other host life-history traits. Because an organism's resources are limited, they must be allocated in such a way as to maximize

fitness. Maximizing costly parasite defenses might not be the best evolutionary strategy for an organism if this comes at the expense of growth, maintenance, and fecundity. Second, variation can occur because hosts face many different parasite species and strains. Hence, protection to one parasite does not necessarily provide protection to another. Many studies have found evidence for host genotype by parasite genotype (GxG) interactions, where host genotypes that are resistant to one parasite genotype are susceptible to another [43–45]. GxG interactions can result in negative frequency-dependent selection, which maintains variation in a population [46–49]. The internal constraints caused by costs, combined with the variation in parasite prevalence found in nature, leads to variation among individuals in defense traits.

Although it is often difficult to measure, recent work has identified costs for several non-immunological defenses. Lefèvre *et al.* identified a cost of medication behavior in monarch butterflies infected with a virulent protozoan parasite [16]. Parasite reproduction in monarch hosts is reduced if the larvae feed on certain species of milkweed plant, potentially because of the anti-parasitic effects of secondary plant chemicals [50]. Infected adult monarchs exhibit an oviposition preference for the protective host plant. However, in the absence of the parasite, uninfected monarchs that fed on these protective plants had reduced adult longevity compared with those that fed on a host plant with reduced parasite protection, representing a cost to medication behavior [16].

Another example comes from research on the costs of harboring protective symbiotic microbes. Infection with the facultative symbiont *Serratia symbiotica* has a negative effect on the lifespan of its pea aphid host, potentially because this facultative symbiont competes with the aphid's primary symbiont *Buchnera aphidicola* [51]. Symbionts are also expected to have their own requirements for energy, which they must obtain from their hosts [52]. Although such energetic costs have not been measured directly, they have been measured indirectly. Oliver *et al.* [53] conducted cage experiments that monitored the population dynamics of aphids infected with *Hamiltonella defensa* (a facultative symbiont that has been found to offer protection against parasitoid wasps) [19,35]. The frequency of aphids harboring *H. defensa* increased in the presence of parasitic wasps. However, the frequency of aphids harboring the symbiont decreased in the absence of parasites, suggesting that harboring symbionts is costly.

Recent studies have also shown the equivalent of GxG interactions in non-immunological defenses. For example, resistance of insects to nucleopolyhedrosis viruses is often affected by the plant species on which these insects feed [54]. Hodgson *et al.* [55] showed a strong interaction between plant species and parasite genotype in which pine beauty moths (*Panolis flammea*) were more resistant to one parasite genotype on one plant species, but more resistant to the other on another plant species [55]. Similarly, Vorburger *et al.* (2009) found that aphid parasitoid wasp genotypes differed in their ability to overcome a protective symbiont in black bean aphids (*Aphis fabae*) [38]. Thus, non-immunological defenses can be subject to interactions with parasite genotypes, which provide a
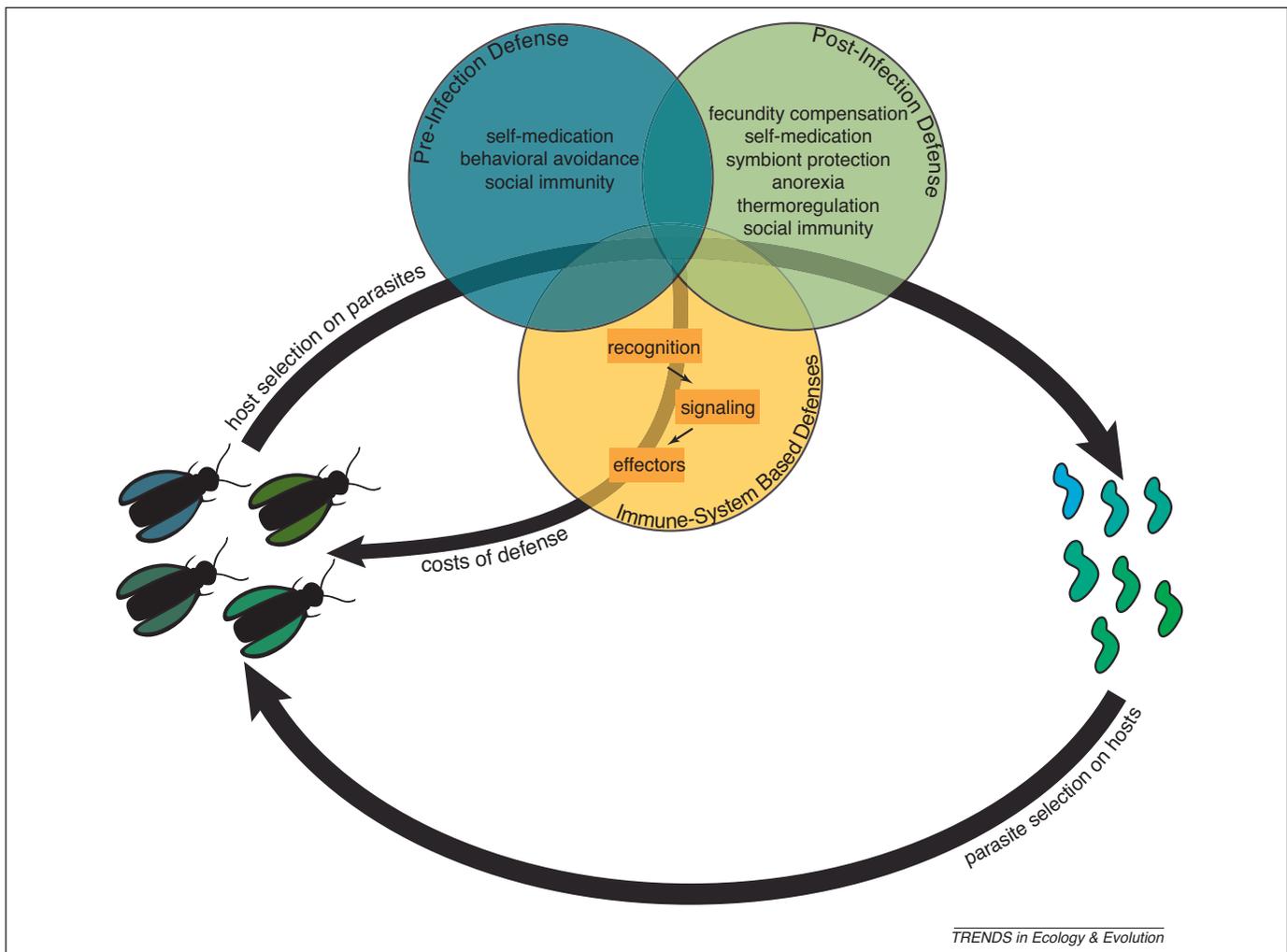
Figure 2. **An expanded view of host–parasite coevolution.** Host–parasite coevolution is driven by parasites selecting for host defense (bottom arrow) and host defense selecting for parasites that can overcome this defense (top arrow). Host selection on parasites is mediated not only through evolution of immune system-based pathways and processes, but also by pre-infection and post-infection mechanisms of non-immunological defense. Non-immunological and immune system-based defenses in turn influence the evolution of each other as well as other host processes due to the cost of defense (middle arrow).

necessary condition for negative frequency-dependent selection and the maintenance of variation.

How do defense traits evolve in the absence of parasites?

Some authors have argued from a theoretical standpoint that non-immunological defenses are a low-cost alternative to immune system-based defenses, and would therefore disappear more slowly in the absence of parasite-mediated selection [56–59]. However, we have highlighted above that non-immunological defenses can be costly and can therefore decrease in frequency in the absence of parasites (e.g. some protective symbionts in the absence of parasites [53]). Additionally, costs are not solely associated with activation, but can also come from evolving and maintaining defense mechanisms [11]. A non-immunological defense can therefore appear to be a low-cost alternative to immune system-based defense if no direct energetic costs of the trait are measured, when in fact the trait has a high maintenance cost that would influence evolutionary dynamics. Therefore, given our current understanding, we cannot make predictions about the relative costs of various defense mechanisms, and mechanisms should not be declared to be 'low-cost' until empirical studies are conducted.

Nevertheless, we can gain some insight into how the costs of defense influence evolutionary dynamics from a comparison with immune system-based defenses. Immune traits can be constitutively expressed or upregulated as needed to combat infection. Constitutively expressed genes are transcribed continuously in the absence of a repressor (e.g. [60]), although environmental conditions can modulate the level of transcription. Other genes are upregulated after parasite challenge as part of an induced response (e.g. [61]). Constitutively expressed genes therefore have an energetic cost regardless of the presence of a parasite, and should be selected against if parasites are absent or at very low frequencies (i.e. if the costs of having the trait outweigh the benefits). Genes that are upregulated only in response to a threat might still be subject to maintenance costs, (e.g. the recognition molecules and signaling pathways necessary for activation), but they themselves do not have direct activation costs in the absence of parasites, and might be lost due to drift in the absence of selection.

A similar dichotomy might exist in non-immunological defenses. Within an individual's lifetime, symbionts are 'constitutively expressed' in the sense that they are always present in their hosts, and their metabolic requirements must be met regardless of parasite densities. Other non-

immunological defenses such as therapeutic self-medication bear similarities to inducible traits in the sense that they are activated in response to an infection. Natural selection might therefore act differently on varying types of non-immunological defenses in the absence of parasite-mediated selection. In general, studies designed to determine how selection acts in the presence and absence of parasites on a diverse array of defense strategies would provide valuable insight into how evolution acts in a broad ecological context.

Interactions between non-immunological and immune system-based defenses

Non-immunological defenses might also have long-term impacts on the evolution of immune system-based defenses. Annotation of the immune system of the honey bee revealed that they have approximately one-third as many immune-system genes as *Drosophila* or *Anopheles* mosquitoes, and that expected genes are missing from multiple pathways [62,63]. Evans *et al*. [63] suggest that social defenses such as the hygienic and grooming behaviors described above are effective in reducing parasite pressure, and as a result honey bees are not as dependent as non-social insects upon immune system-based defenses. While these differences can also be explained by bees relying on different, yet-to-be-discovered immune system-based defenses, this hypothesis is supported by studies showing that not only do non-immunological defenses reduce parasite pressures, they also decrease the use of inducible immune system-based defenses. Two recent studies have shown how prophylactic self-medication can reduce the expression of immune genes. Honey bees that were experimentally exposed to resin as part of an artificial nest showed lower expression of two immune genes compared with non-resin controls [59]. Nests with resin had lower overall bacterial loads, suggesting that resin reduces the expression of these genes by reducing the parasite threat. Similarly, wood ants (*Formica paralugubris*) that are kept in resin-rich experimental nests showed reduced antibacterial and lytic activities [64].

A similar story came from the annotation of an aphid genome, which showed that pea aphids are missing much of the immune deficiency (IMD) pathway, are lacking peptidoglycan recognition proteins (PGRPs) [65,66], and produce no homologs of known antibacterial peptides [67]. Functional studies found a surprisingly reduced upregulation of transcripts and proteins upon infection in aphids compared with other insects [66,67]. While alternative immune-system based defenses might still be uncovered through additional functional assays, one explanation for this reduction is that the benefits afforded to the aphid by obligate and facultative symbionts have selected for reduced use of immune system-based defenses in the host to facilitate these symbiotic relationships. Alternatively, symbiont-conferred resistance might cause immune system-based defenses to become redundant [67]. Support for the effects of non-immunological defenses such as social immunity and symbionts on the evolution of immune system-based defenses will be dependent upon characterizing other organisms that are dependent upon non-immunological defenses.

At most, these findings suggest that with particularly effective non-immunological defenses, immune system-based defenses are lost through drift or selection because organisms are not as dependent upon them for survival. At the very least, these studies highlight the potential for interaction in an evolutionary context between these types of defense. Regardless, the traditional view of invertebrate immunity is clearly not applicable to every invertebrate. Also, as sequencing technology becomes cheaper and more non-model organisms are sequenced (including those with more specialized lifestyles), we will learn more about how life-history strategies are reflected in the genome.

Non-immunological defenses might also stimulate an immune system-based defense, and *vice versa*. It was recently shown that infection with the bacterial symbiont *Wolbachia* in *D. melanogaster* renders hosts more resistant to several RNA viruses [68], and increases resistance to the entomopathogenic fungus *Beauveria bassiana* [69]. Because *Wolbachia* slightly increases the expression of immune-system genes [70], it is possible that symbiont-conferred protection in this system stems from the pre-activation of the host's immune system-based defenses (although more studies are needed). Similarly, non-immunological defenses such as self-medication behavior might be triggered as a result of the activation of immune system-based recognition genes.

Implications for parasite coevolution

Non-immunological defenses to parasites will probably have consequences for parasite evolution that mirror immune system-based defenses. For instance, behavioral avoidance of parasites requires that the potential host can identify parasites, parasite-rich environments, or already infected individuals to avoid them. Similarly, immune system-based defenses are dependent upon recognition of invasive organisms to mount an immune response. Some parasites in turn evolve evasion mechanisms to prevent detection from the immune system, which leads to increasing pressure on the hosts to evolve greater detection ability (e.g. the unusual level of polymorphism at the major histocompatibility complex in the vertebrate immune system [71]).

We can also expect non-immunological defenses to create selection pressures on the virulence and transmission of parasites in the same way as immune system-based defenses. For example, theoretical studies have suggested that anti-growth resistance, through which a host reduces parasite growth instead of infection probability, can select for more virulent parasites [72,73]. This expectation is based on the assumption that parasites evolve optimal growth rates at which the costs of parasite growth (reduced transmission opportunities due to host death) trade-off with the benefits (between-host-transmission) [74,75]. Hence, by reducing parasite growth, anti-growth resistance might select for parasites with higher levels of intrinsic growth and virulence. Although these models have traditionally focused on immune system-based defenses, it has recently been shown that non-immunological defenses such as self-medication can have similar effects [76].

Non-immunological defenses can also have important implications for understanding local adaptation of hosts and parasites. Local adaptation is the phenomenon by which

organisms have higher fitness in their local than foreign environments [77], and has been extensively studied in host–parasite interactions [78]. Although many studies have found evidence for local adaptation of parasites to their hosts or hosts to their parasites, there are also many studies that have not found such evidence [78]. Many of these studies are undertaken by bringing hosts and parasites from multiple populations into the laboratory and assaying them for resistance and infectivity without reproducing the environmental conditions under which hosts and parasites interact in nature [79]. Hence, these experiments might not be able to detect local adaptation of hosts through non-immunological defenses. For example, if hosts in some populations protect themselves against parasites predominantly through self-medication, studies that do not provide the medicinal compounds that these species use would not find evidence for local adaptation. Thus, considering non-immunological defenses might be necessary to reveal the adaptation of hosts to their naturally occurring parasites.

Conclusions

Non-immunological defenses are an important part of many host-defense strategies. They likely influence host–parasite coevolution, and are therefore an important consideration for future studies of the evolutionary dynamics of host–parasite interactions. Previous examples have shown that overlooking non-immunological defenses can lead to false conclusions [33–35]. Addressing these mechanisms can also explain ambiguous results. For example, studies might fail to measure immune costs in some organisms because only immune system-based defenses are considered when hosts are also dependent upon non-immunological defenses. Multiple measures of immunity need to be studied to avoid missing costs of defense [80], and this should also be extended to include non-immunological defenses. Similarly, many studies do not find evidence for local adaptation of host or parasites because only GxG interactions are studied and the environmental factors that can confer non-immunological defense are ignored. A complete picture of host defense therefore relies on a holistic approach which considers immune system-based and non-immunological defenses, the ecological context of infection, and the interactions that occur between these factors. The costs of genome sequencing are declining rapidly, and we are beginning to study the immune systems of more non-model organisms. As we analyze these genomic data and explore how host–parasite coevolution has played a part in shaping these genomes, a diverse set of researchers will need to recognize host defense in its broad context.

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