Immunoparasitology series

Nitric oxide: an antiparasitic molecule of invertebrates

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Since Furchgott, Ignarro and Murad won the Nobel prize in 1998 for their work on the role of nitric oxide (NO) as a signaling molecule, many reports have shown the seemingly limitless range of body functions controlled by this compound. In vertebrates, the role of NO as a defense against infection caused by viruses, bacteria, and protozoan and metazoan parasites has been known for several years. New evidence, however, shows that NO is also important in defending invertebrates against parasites. This discovery is a breakthrough in the understanding of how the invertebrate immune system works, and it has implications for the emerging field of invertebrate ecological immunology.

Ecological immunology

In recent years, knowledge of the immune mechanisms in invertebrates has taken a giant leap forward, much of which has been motivated by the importance of invertebrate vectors and intermediate hosts in the transmission of serious diseases, such as malaria, yellow fever, trypanosomiasis and filariasis. Descriptions of the mode of action of the different invertebrate immune mechanisms have given way to studies focusing on the role that the immune system plays in determining the fitness of organisms in the wild: what creates and maintains variation in immune defense in hosts and what are the correlated coevolutionary responses in pathogens? This discipline, so-called ecological immunology, has become one of the most dynamic and fastest growing areas in biology [1,2].

Broadly speaking, three invertebrate immune effector mechanisms have been the subject of these studies: phagocytosis (largely aimed at small pathogens, such as virus, bacteria, and fungi), antimicrobial peptides (aimed at bacteria and fungi), and encapsulation (aimed mostly at larger pathogens, such as protozoan and metazoan parasites) [3]. Recently, a fourth effector mechanism has been discovered, a free radical called nitric oxide (NO), which has been shown to be inducibly synthesized in response to parasite infection in several species of insects [4–7].

Inducible NO has two defining characteristics that set it apart from most other invertebrate immune mechanisms. First, it is an ubiquitous pathogen-killing

mechanism in nature. Inducible NO was described around a decade ago as a component of the vertebrate immune system [8], and since then, it has been described not only in invertebrates, but also in plants, where it provides effective protection against bacterial infections [9]. Second, it is a truly generalist (non-specific) response to infection. *In vitro* and *in vivo* studies have demonstrated the direct toxicity of inducible NO towards virtually every tested pathogen, from viruses to metazoan parasites, such as the filarial nematode *Brugia* and the trematode *Schistosoma* [8]. No other invertebrate immune mechanism has such a broad spectrum of action.

What is nitric oxide?

Most of what we know about NO comes from studies of vertebrates. It is a highly reactive and unstable free-radical gas that is produced by the oxidation of L-arginine to citrulline mediated by the enzyme NO synthase (NOS; Figure 1). In vertebrates, two main types of the enzyme have been found: constitutive (cNOS) and inducible (iNOS) [10] (Table 1).

The cNOS is part of the basal metabolism of cells and has been found in two different isoforms: neuronal (nNOS) and endothelial (eNOS) (Table 1). The rapid activation (and inactivation) of nNOS and eNOS through changes in intracellular calcium levels, and the facility with which NO crosses cellular membranes, enables a very efficient response that is ideal for the transmission of cellular signals. By contrast, the iNOS isoform is absent in resting cells but is rapidly synthesized by a wide array of cells and tissues in response to the pro-inflammatory cytokines produced in acute infectious diseases. Regulation of NO production via iNOS probably occurs at the transcriptional and translational levels because, once present, iNOS catalyzes NO synthesis until the substrate is depleted [10]. During this time, iNOS typically synthesizes 100–1000 times more NO than do the constitutive enzymes (nNOS and eNOS). The high toxicity of inducible NO comes from its high concentration and from its reactivity with oxygen and oxygen-related reactive intermediates, which yield numerous toxic species that have enzymatic and DNA-damaging properties [11]. Inducible NO is toxic to many kinds of pathogens, including viruses, fungi, bacteria, and parasites; the latter include intracellular and extracellular protozoa as well as some metazoan parasites [8,12].
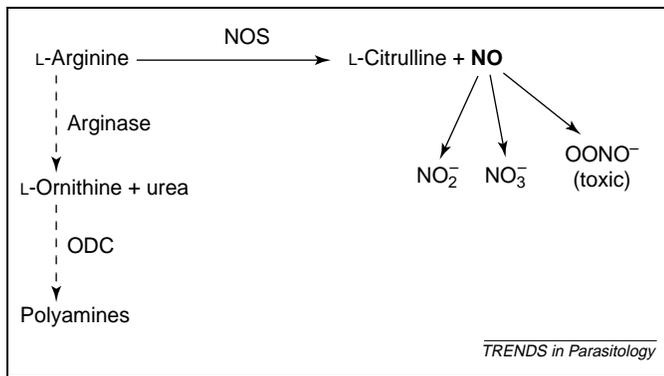
Figure 1. The NO synthetic pathway. NO is synthesized through the oxidation of L-arginine to L-citrulline, mediated by the enzyme NOS (solid arrows). NO is a highly unstable radical that rapidly reacts with other oxygen species to form stable products, such as nitrites (NO_2^-) and nitrates (NO_3^-), and some highly toxic radicals, such as peroxynitrite ($OONO^-$). Arginases compete with NOS for the same substrate (dashed arrows). As a result they produce L-ornithine, which in turn can be decarboxylated by the ornithine decarboxylase (ODC) to produce polyamines.

Nitric oxide in vertebrates

There is evidence to suggest that NO could be an adaptive host defense mechanism in humans. African children have a mutation in the iNOS promoter that seems to be associated both to increased amounts of NO production and to a significant protection against malaria [13]. There is also evidence that NO exerts an important selective pressure for parasites. Most parasites seem to have evolved mechanisms to protect themselves against the damaging effects of NO, and one of the most widespread of these is the manipulation of the host's arginase levels [14]. An increase in the levels of this enzyme, whose role is to break down L-arginine into L-ornithine and urea, depletes the substrate of the NO synthase and produces poly-amines, which are essential for parasite growth and differentiation (Figure 1). This strategy, which takes advantage of the relationship between L-arginine concentration and amount of NO produced, is used by bacteria, *Trypanosoma*, *Leishmania* and *Schistosoma* [14]. Recently, it has been suggested that parasites also defend themselves against NO by preferentially colonizing certain tissues that are particularly rich in NO-scavenging molecules, such as hemoglobin and myoglobin [15].

Nitric oxide in invertebrates

In the early 1990s, NOS was characterized in the brains of several insects. This NOS was found to share many of the characteristics of the constitutive NOS of vertebrates: it had a signaling role, synthesizing NO in minute quantities following activation of the enzyme through changes in calcium levels [16]. The NO produced was implicated in chemosensory and visual information processing and in the formation of long term memory [16]. Since then, NO has also been found to have a role in the induction of the insect cellular and humoral immune responses [17–19] (Table 1).

Recently, however, it has been shown that insects also produce an inducible form of NOS. Luckhart and collaborators have shown that *Anopheles stephensi* mosquitoes limit *Plasmodium berghei* development via inducible synthesis of NO [5]. Using primers originally designed against *Drosophila* NOS, they identified and sequenced the *An. stephensi* NOS (AsNOS) and showed that AsNOS was strongly expressed when mosquitoes were fed on *Plasmodium berghei* infected blood (Figure 2a). AsNOS expression was highest during the first 3 days after infection, coincident with parasite invasion and early oocyst development, suggesting that NO may be the first barrier against infection in mosquitoes. Boosting NO production reduced the percentage of mosquitoes infected by almost 30% (Figure 2b), whereas blocking NO production increased the number of

Table 1. The functional roles of the different NOS isoforms in vertebrates and in invertebrates

	NOS isoform	Role	Site of production	Mode of action	Refs
Signaling					
Vertebrates	Constitutive: eNOS (NOS I); nNOS (NOS III)	Neurotransmitter (nNOS), cardiovascular homeostasis (eNOS)	Central and peripheral nervous system (nNOS), endothelium (eNOS)	Enzymes constitutively present inside the cells; Rapid activation and deactivation of enzymes by in response to Ca^{2+} levels; NO produced in minute quantities	[65]
Invertebrates	NOS[a]	Modulation of chemosensory signals, long term memory, facilitation of blood feeding and induction of humoral and cellular immune responses	Central nervous system, antennal lobe, visual system, salivary glands	Same as vertebrate constitutive NOS	[16–19]
Defense against infection					
Vertebrates	Inducible: iNOS (NOS II)	Direct elimination of pathogens	Phagocytic cells (macrophages)	Enzyme is synthesized in response to infection (the trigger is proinflammatory cytokines); enzyme is active from the moment it is synthesized until it runs out of substrate (activation is independent of Ca^{2+}); NO is produced in large quantities ($100-1000 \times$ more than constitutive NOS)	[66,67]
Invertebrates	NOS[a]	Direct elimination of pathogens	Midgut cells, hemocytes and fat body	Same as vertebrate iNOS (the trigger for synthesis is unknown)	[4,5,16,19, 20,68]

[a]To date, only a single NOS isoform has been found in each invertebrate species, with either a constitutive (signaling) [16–19] or an inducible (toxic) role [4,5]; in some cases the same isoform has been found to have both roles [19]. In insects, NOS is often named according to the species from which it has been sequenced: *d*NOS (*D.melanogaster*) [69], *As*NOS (*A.stephensi*) [5], *Ag*NOS (*A. gambiae*) [4], *Ap*NOS (*Anopheles pseudopunctipennis*) [20], *Bm*NOS (*Bombyx mori*) [19] and *Ms*NOS (*Manduca sexta*) [70].
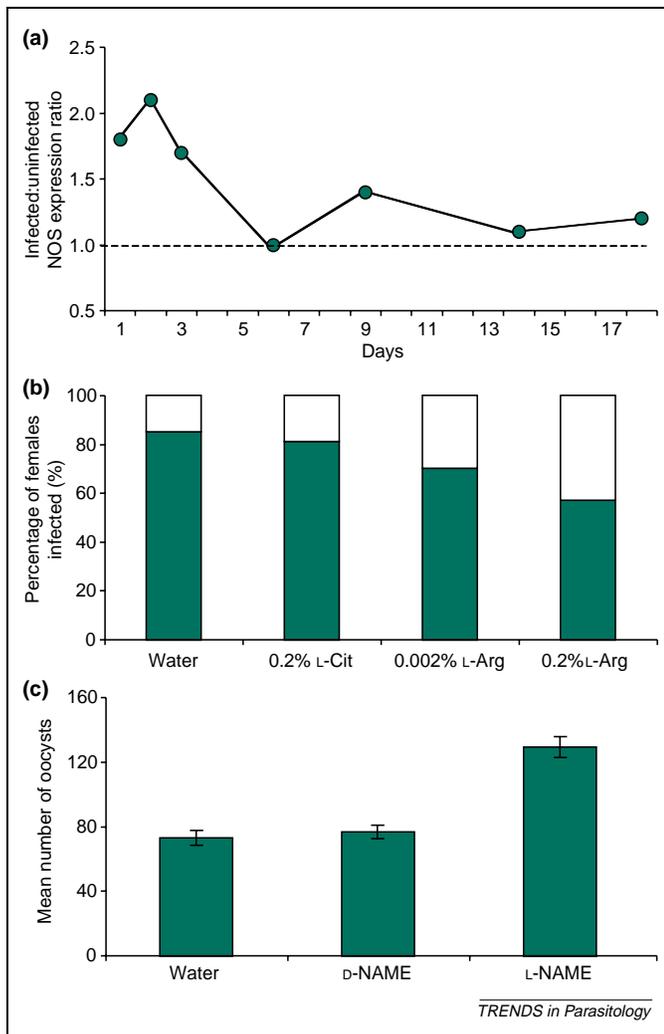
Figure 2. NO as an efficient defense mechanism in *An. stephensi* infected with *Plasmodium berghei*. (**a**) NOS expression. The solid line represents the ratio of NOS expression in infected females relative to uninfected ones. The dashed line (ratio = 1) is shown as a reference. Points above the dashed line show a higher NOS expression in infected females than in uninfected ones. (**b**) The percentage of females infected when supplied with different diets. Arg, arginine; Cit, citrulline. (**c**) The effects of L-NAME (an L-arginine analog) and D-NAME (its inert enantiomer) on the mean number of oocysts produced (bars represent standard errors). Data from Ref. [5].

oocysts in the gut by over 75% (Figure 2c). Recent studies have confirmed the role of inducible NO in the fight against *Plasmodium* parasites in other species of *Anopheles* [4,20] and in the response of *Rhodnius prolixus* to infection by *Trypanosoma rangeli* [6,21]. In addition, NO is involved in the killing of *Schistosoma* parasites in the snail *Biomphalaria glabrata* [7].

The NOS genes of several invertebrate species have already been sequenced [19]. In two mosquito species, the gene has been found to be polymorphic [22,23]. Although the functional significance of these polymorphisms is not yet well established, an interesting association has been found in *Anopheles gambiae* between the frequency of *Plasmodium* infection in a Kenyan population and the frequencies of certain alleles [23].

Two main questions remain regarding the origin and mode of action of the inducible form of NOS in invertebrates. First, we do not know what the cellular signals are that trigger the inducible synthesis of NOS in invertebrates. In *An. stephensi,* however, a putative

LPS- and cytokine-responsive transcription factor binding site has been discovered [22], and invertebrates have been found to have cytokine-like proteins similar to the interleukins and tumor necrosis factors of vertebrates [24]. Second, we do not know whether invertebrates, like vertebrates, have several isoforms of NOS as, thus far, only a single copy gene has been discovered in each species investigated (Table 1). Preliminary data, however, seem to suggest that AsNOS produce both constitutive and *Plasmodium-* inducible transcripts [22].

Interestingly, a great degree of sequence similarity has been found between the different insect NOS proteins sequenced so far (up to 84%) [19] and between the insect and vertebrate NOS proteins (up to 49%) [22], which points to the great degree of conservation of structure and function of these enzymes across taxa and suggests that they could all have derived from a single ancestral type. As the search for inducible forms of the NOS in invertebrates continues, it is likely that this highly conserved mechanism of defense against parasites will be discovered in most, if not all, species.

Manipulation and quantification of NO production

The NO system provides opportunities for (i) experimentally enhancing or blocking the production of NO and (ii) quantifying the amount of NO subsequently produced. NO production can be manipulated by at least three different mechanisms. First, NO production can be increased through the addition of L-arginine to the diet of the insect, thereby increasing the amount of substrate for the enzyme. This technique, widely used in vertebrates and *in vitro* studies [10], takes advantage of the mode of action of the inducible form of the enzyme. In *Anopheles*, a 100-fold increase in L-arginine concentration brought about a 18% decrease in the number of *Plasmodium* oocysts produced (Figure 2b). Second, NO production can be decreased through the addition of an inert L-arginine analog, such as L-NAME, to the diet of the insect; the analog competes with the L-arginine for the site of action of the enzyme [10] (Figure 2c). Third, NO production can be silenced at the transcription level by using RNA interference (RNAi) [25]. The synthesis of NOS from a single copy gene (in contrast to other invertebrate immune mechanisms [26]), and the great degree of sequence similarity between the genes from different insect species sequenced thus far (which greatly simplifies the search for primers), makes this technique a very promising tool for manipulating NO levels in infected insects.

The quantification of nitrites and nitrates using the Griess reaction is a standard procedure for indirect measurement of NO production [27]. Nitrites and nitrates are produced as a result of the high reactivity of NO with different oxygen species (Figure 1) but they are also common by-products of many metabolic reactions of the organism, and thus will be produced in considerable quantities even in uninfected individuals. Comparison of nitrite and nitrate levels of infected and uninfected individuals, however, provides a simple and inexpensive way of quantifying the amount of NO produced. A more accurate measure of NO production is through the direct quantification of NOS activity [28]. This technique

measures the rate of conversion of [3]H- or ^{14}C-labeled arginine into labeled L-citrulline (see Figure 1). It has the advantage of being specific for the NOS pathway and of being much more sensitive than the Griess reaction, allowing the detection of picomole activities of NOS.

A final advantage to working with NO is that the susceptibility of parasites to NO can be experimentally tested *in vitro* using NO-releasing compounds such as S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) [29]. Caution must, however, be exercised when extrapolating the results of these tests – in which high concentrations of NO can be released – to the natural levels of NO produced by organisms.

A new molecule for invertebrate ecological immunology

The two defining characteristics of inducible synthesis of NO, its ubiquity and its generality, coupled with the wide range of techniques available for quantifying and/or manipulating inducible NO production, make it a potentially key molecule for future ecological immunology studies. Here, I explore the potential for quantifying the costs associated with the NO immune response, one of the mainstays of evolutionary ecologists [1,2], and discuss its lack of specificity and ubiquity. I suggest the potential role of NO in two emerging areas in invertebrate ecological immunology: (i) the immune-mediated interactions between parasites in mixed infections [30,31], and (ii) the immune-mediated interactions between vertebrate and invertebrate hosts in parasites whose life cycle alternates between them [32].

Costs of NO production

Many studies of ecological immunology have found that immune responses are costly to the host. These costs are expressed as trade-offs with other life history traits, such as reproduction and survival, or as trade-offs between different immune mechanisms, such that a host which is efficient at defending itself against a specific parasite or parasite strain may be at a disadvantage to defend itself against other parasite strains or against a general immune insult.

The costs of immunity can arise in two different, although not mutually exclusive, ways. Inducible costs are the costs of mounting the immune response after parasite infection [2]. The ease with which the encapsulation response can be experimentally triggered and quantified has made it the classical system for quantifying the costs associated with mounting immune responses in insects. Several studies have demonstrated trade-offs between the induced encapsulation response and other life history traits, such as fecundity [33] and longevity [34]. These types of costs are assumed to arise from the competition for a limited amount of resources between the encapsulation response and the life history trait in question. Although this seems to be supported by studies correlating the magnitude of the immune response to the nutritional status of the insect [35], the limiting resource or physiological mechanism underlying the cost has been difficult to identify, a problem that is common to many phenotypic trade-offs [36].

One potential physiological mechanism through which inducible costs of NO are likely to be expressed is the common demand for arginine between the NO synthetic pathway and other key metabolic pathways in the insect. Arginine is an essential amino acid for egg production in insects [37], and it could have an important role in sperm maturation [38]. Insects, however, appear to have lost the ability to synthesize arginine, which must be obtained from the diet and is thus likely to be limiting. An obvious way to start looking for costs of NO induction, therefore, would be to check for trade-offs between NO production and insect reproductive output by varying the amount of arginine supplied with the diet.

Another potential cost of NO induction is autoimmunity, which arises when the immune mechanism is toxic to the organism that produces it. In insects, the phenoloxidase cascade responsible for the melanotic encapsulation (formation of a melanized capsule around the parasite) produces some toxic intermediates called quinones, which are cytotoxic to the individual [39]. In vertebrates, the NO released during viral and helminthic infections results in a localized tissue damage, which contributes to the pathogenesis of the disease [40]. Given the high toxicity and wide spectrum of action of NO, the autoimmune costs of NO in invertebrates are not to be underestimated.

The second type of immunity costs are constitutive costs. Constitutive costs are the costs of evolving a particular immune response and arise through negative genetic correlations between the immune response and other life history traits [2]. Selection for an increased encapsulation ability against parasitoids has been shown to result in constitutive costs in the form of a reduced competitive ability [41] and a lower survival rate [42] of the host. Looking for constitutive costs of NO production will require either the exploitation of natural (e.g. geographic) variation in NO production between different insect populations that have evolved under different parasite pressures, or the artificial selection of lines for high and low NO production. The recent discovery of polymorphisms in the mosquito NOS gene (see above) may provide a good starting point in the search for constitutive costs of the NO defence system.

NO and mixed infections

Although most immunological studies tend to consider the interaction between one pathogen and one species of host, concomitant infections by two or more parasite species or genotypes are thought to be the rule rather than the exception in natural situations [30,31]. A common outcome of mixed infections is that the immune response invoked by one parasite species reduces the parasitemia caused by a different species that concurrently infects the host. In vertebrates, iNOS is widely acknowledged as important in mediating such antagonistic interactions between parasites in mixed infections [43,44]. NO, which is known to be toxic to the blood stages of malaria parasites [45], has been held responsible for the antagonistic interactions between *Plasmodium* and other concomitant infections [46–48]. NO has also been invoked as a likely candidate regulating total parasite densities in mixed-genotype malaria infections [49,50].

There is increasing evidence that mixed infections are common in invertebrate hosts in nature, between closely

related parasite species [51,52] or parasite genotypes [53,54], as well as between phylogenetically unrelated pathogens, such as viruses and bacteria [55], viruses and fungi [56], and fungi and protozoan parasites [57]. The interactions between parasite species in experimentally induced mixed infections are often attributed to competition for space and/or resources between the different parasites [51,52] or to the production of substances that are directly toxic to the competitor [58]. By contrast, the role of non-specific immune effectors in mediating such interactions has been little explored.

This could, however, be relevant for understanding better some puzzling empirical results. Immunological priming with bacteria or bacterial products (LPS) has been shown to trigger unidentified generalist immune mechanisms that protect the insects against a subsequent infection by heterologous parasites [59,60]. Work with *Aedes aegypti* and *A. gambiae* has demonstrated that mosquitoes infected by bacteria are significantly less susceptible to concomitant infections by *Plasmodium gallinaceum* and *P. berghei* parasites [60]. This study excluded antibacterial peptides as a cause and attributed the observed results to an unspecified, generalist, fast-acting, 'killing molecule' that would act on the first hours after *Plasmodium* infection, before the parasite traverses the midgut wall. Future experiments are necessary to determine the potential role of NO in this interaction, although a priori it seems to fulfil the criteria for such a killing molecule: NO is synthesized in the first few hours after infection in response to either a bacterial or a *Plasmodium* challenge [5].

As invertebrate ecological immunology moves into studying the complex interactions involved in mixed infections, the role of non-specific immune mechanisms such as NO should be explored further. The nature of NO as a fast acting molecule of low persistence might, however, limit its role in mixed infections to concomitant, rather than sequential, infections. NO production is unlikely to provide an explanation for why beetles exposed to a bacterial challenge are protected against a subsequent fungal challenge for up to 7 days afterwards [61]. It could, however, be a relevant mechanism for hematophagous insects that simultaneously acquire different types of pathogens during a single blood meal.

NO in parasites with complex life cycles
Many parasites have complex life cycles that involve different types of host, most frequently a vertebrate and an invertebrate host. Similarities between the invertebrate and the vertebrate immune systems will favor the existence of a positive correlation between the ability to exploit the two different hosts [62]. It follows that the evolution of pathogen resistance in the invertebrate host will inevitably have consequences for the virulence of the pathogen in the vertebrate host, and vice versa.

There has been much recent discussion about the degree of similarity between the vertebrate and invertebrate immune systems [32,63,64]. Although functionally homologous innate immune effector mechanisms exist in vertebrates and invertebrates [32], by and large, molecular data seems to argue against a high degree of conservation between vertebrate and invertebrate effector mechanisms [63]. The discovery of the high degree of conservation of sequence, structure and function between the insect and the vertebrate NOS [22] is a breakthrough in our understanding of the evolutionary link between the vertebrate and the invertebrate immune systems.

The identification of NOS as a key defense mechanism in some of the most important invertebrate vectors and intermediate hosts of pathogens begs the question of the potential consequences of the evolution of parasite resistance against NO in the vertebrate host. A particularly poignant example, not least because of its epidemiological implications, is malaria: humans [40] and mosquitoes [5] are both now known to protect themselves from the *Plasmodium* parasite with inducible levels of NO. Although no NO-resistant *Plasmodium* strains have yet been detected, there is ample evidence that other pathogens with complex life cycles have evolved sophisticated resistance mechanisms to protect themselves against the damaging effects of NO (see above). Vertebrate NO-resistant strains of *Trypanosoma*, *Leishmania* and *Schistosoma* [14] should be tested in their invertebrate vector or intermediate host to determine whether there is a correlation between the ability to exploit the two different types of host. The existence of such positive correlations between traits in the different hosts could be a key factor driving the evolution of virulence and the evolution of transmission routes among different hosts [62].

Concluding remarks
The discovery of inducible NOS in insects has been a breakthrough in our understanding of how the invertebrate immune system works and of the similarities between the vertebrate and invertebrate immune systems. The evolution of mechanisms of resistance against the damaging effects of NO is evidence that NO exerts a potent selection pressure on parasites, comparable to that imposed by more widely studied invertebrate immune mechanisms such as encapsulation and antimicrobial peptides. More work is needed, however, on the role of NO in different species of invertebrates and against different species of parasites. This should ideally combine *in vitro* experiments using susceptibility to NO of the parasite stages transmitted by the vectors with *in vivo* studies determining NO production in response to parasite infection. In addition, the range of techniques available to manipulate and quantify NO production will help determine the efficiency of NO as an immune mechanism in specific host–parasite interactions. Efforts should also be directed at detecting polymorphisms in the invertebrate inducible NOS gene and to determine whether, as in humans, these are associated with increased NO levels and better protection against parasites.

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