

Commissioned Review Article

Secreted effectors in *Toxoplasma gondii* and related species: determinants of host range and pathogenesis?

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SUMMARY

Recent years have witnessed the discovery of a number of secreted proteins in *Toxoplasma gondii* that play important roles in host–pathogen interactions and parasite virulence, particularly in the mouse model. However, the role that these proteins play in driving the unique features of *T. gondii* compared to some of its nearest apicomplexan relatives (*Hammondia hammondi* and *Neospora caninum*) is unknown. These unique features include distinct dissemination characteristics *in vivo* and a vast host range. In this review we comprehensively survey what is known about disease outcome, the host response and host range for *T. gondii*, *H. hammondi*, and *N. caninum*. We then review what is presently known about recently identified secreted virulence effectors in these three genetically related, but phenotypically distinct, species. Finally we exploit the existence of genome sequences for these three organisms and discuss what is known about the presence, and functionality, of key *T. gondii* effectors in these three species.

Keywords comparative genomics, host range expansion, virulence

INTRODUCTION

In recent years, significant progress has been made in our understanding of secreted *Toxoplasma gondii* effectors that have significant impacts on virulence in the mouse. To date, these effectors have all been found to be secreted from two specialized secretory organelles that are unique to the phylum Apicomplexa to which *T. gondii* belongs:

the rhoptries and the dense granules (Figure 1a). This work was greatly facilitated by pioneering early studies using proteomics to identify some of the constituents of these organelles (1, 2), identifying literally hundreds of putative rhoptry and dense granule proteins. Subsequent work has shown that while some rhoptry and dense granule proteins are constituents of the organelles themselves, others are secreted from these organelles, and can be found in multiple locations post-secretion including the host nucleus (e.g. protein phosphatase 2C (3) and rhoptry protein 16; ROP16; (4)) and the parasitophorous vacuole/vacuolar membrane (e.g. ROP5 and ROP18; (5–9)).

What is not known, however, is if these and other secreted effectors play any role in determining the unique phenotypic characteristics of *T. gondii* compared to its closest Apicomplexan relatives (summarized in Table 1). Specifically, the nearest sequenced relatives of *T. gondii* are *Hammondia hammondi* (10) and *Neospora caninum* (11). In contrast to these other organisms *T. gondii* has an incredibly vast host range, being capable of infecting nearly all mammals (including humans) and birds. Moreover *T. gondii* is not an obligate sexual parasite. Infected intermediate hosts harbour tissue cysts that are infective to both the definitive host (members of the family felidae) and other intermediate hosts (12, 13). Finally *T. gondii* is highly virulent in mice, while *H. hammondi* and *N. caninum* are not (see Figure 2 for an illustration of this phenotype in *N. caninum*).

While it is almost certain that we have just begun to understand the molecular mechanisms of virulence in *T. gondii*, genome sequences of *H. hammondi* and *N. caninum* (10, 11) provide a unique opportunity to conduct a preliminary analysis of whether these effectors can explain the phenotypic differences between these species. Therefore in this review we (i) compare and contrast what is known about host responses to these three organisms; (ii) review the recent literature on the molecular mechanisms, and impact on pathogenesis, of select *T. gondii* virulence fac-

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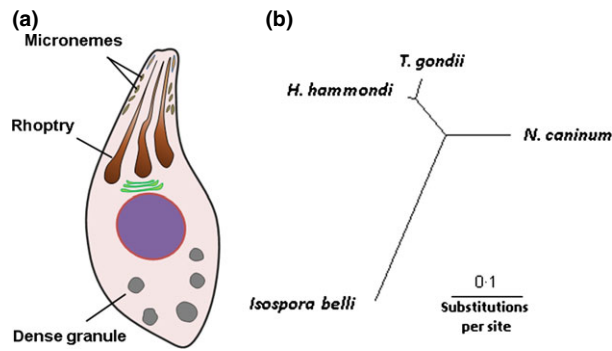


Figure 1 (a) Schematic of *Toxoplasma* (and related apicomplexans *Hammondia hammondi* and *Neospora caninum*) highlighting the three major secretory organelles involved in secreting host-targeting and/or host-modulating effectors. (b) Phylogram based on publicly deposited internal transcribed spacer (ITS1) sequences for *Toxoplasma gondii*, *H. hammondi* and *N. caninum* illustrating the degree of relatedness among these species. *Isospora belli* is used as an out-group.

tors; and (iii) provide new data and review recent work on the conservation of these effectors across these three species. In our conclusion we then speculate on how such a comparative analysis can inform our understanding of the selective evolution of virulence and host range in *T. gondii* compared to *H. hammondi* and *N. caninum*.

HOST RESPONSES TO TOXOPLASMA AND CLOSELY RELATED SPECIES DIFFER GREATLY

Despite a remarkable degree of genomic conservation between *T. gondii* and *H. hammondi* (10, 12, 14) and *Neospora caninum* (11) (Figure 1b), there are a number of striking differences in host response and host range among these species. Here we will discuss differences in host range and

pathogenicity among these species, as well as known differences or similarities in the host response in experimentally infected animals. Additional closely related species, such as *Neospora hughesi*, *Besnotia besnotii*, *Hammondia triffittae* and *Hammondia heydorni* will not be included in this review, as comparatively little is known about these parasites, and currently there are no available genome sequences for any of these species.

Variation in host range between *T. gondii* and closely related species

Toxoplasma gondii has a life cycle typical of tissue-dwelling coccidian parasites. Sexual reproduction occurs exclusively in the definitive host, members of the family felidae (15), while asexual reproduction occurs in a variety of intermediate hosts. Strikingly, *T. gondii* is capable of infecting virtually any warm-blooded animal, from birds to humans (16), and this broad intermediate host range is not only unique in comparison with closely related species such as *H. hammondi* and *N. caninum*, but also with respect to most eukaryotic parasites. An estimated one-third of the world's human population is currently infected with *T. gondii*, and while healthy individuals are able to control infection, those with compromised immune systems are at risk for developing life-threatening symptoms (17–20). In addition, some *T. gondii* strains have been found to cause severe, and even fatal, disease in immunocompetent adults (21–23). Acute infection during pregnancy often results in foetal loss, blindness, hearing loss, or severe cognitive disabilities (24, 25). *T. gondii* is also the cause of foetal loss in a number of domestic animals including sheep, goats, and pigs (26–29). Virulence in humans has never been observed for *H. hammondi*, which is the most closely related extant relative to

Table 1 A summary of the known host range and virulence properties of *Toxoplasma gondii* and the closely related species *Hammondia hammondi* and *Neospora caninum*

	<i>T. gondii</i>			<i>H. hammondi</i>	<i>N. caninum</i>
	Type I	Type II	Type III		
Definitive host		Felids		Felids	Canids
Intermediate host range		Mammals, birds		Rodents	Bovids, horses
Disease associated with infection in intermediate hosts?		Yes		No	Yes
Transmission dynamics between hosts					
Infects humans?		Yes		Unlikely	Unlikely
Disease associated with human infection?		Yes		Unlikely	Unlikely
Virulence phenotype in mice	LD ₁₀₀ = 1	LD ₅₀ = 10 ² –10 ⁴	LD ₅₀ = 10 ⁵ –10 ⁶	Avirulent	Avirulent

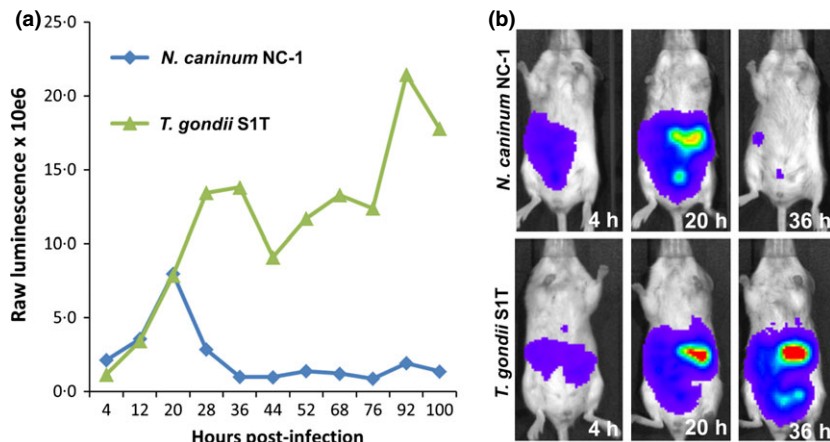


Figure 2 Head-to-head comparison of luciferase-tagged *Neospora caninum* (strain NC-1) and *Toxoplasma gondii* (strain S1T) in mice. Balb/c mice (three per strain) were intraperitoneally infected with 1×10^6 tachyzoites, and *in vivo* bioluminescence imaging was used to quantify parasite burden over the first 96 h of infection. (a) Average total flux (photons/s) indicating parasite burden over the course of infection. For the first 20 h of infection both species proliferate at similar rates, but *N. caninum* is then cleared by 44 h p.i. while *T. gondii* S1T continues to proliferate. All mice survived the infection. (b) Representative images of *N. caninum* and *T. gondii* infections represented in (a), showing the rapid clearance of *N. caninum* from the peritoneal cavity in comparison with *T. gondii*.

T. gondii and shares the same definitive host (12, 30). While it is also assumed that *H. Hammondii* is incapable of infecting humans, it is worth noting that given the antigenic similarity between these species (31) and that the most commonly used serological test for *T. gondii* infection is based on immunoreactivity to *T. gondii* surface antigen 1 (p30; SAG1), it is certain that if *H. Hammondii* is capable of infecting humans such an infection would be misidentified only as a *T. gondii* infection. Development of a serum-based diagnostic test that could distinguish *T. gondii* from *H. Hammondii* would allow for a direct test of the infectivity of *H. Hammondii* in humans.

While the host range of *T. gondii* and *N. caninum* have been extensively studied, less is known about the host range in *H. Hammondii*. Most *H. Hammondii* isolates have been obtained from infected cats, but a wide variety of animals have been experimentally infected with this parasite, including cats, mice, rats, hamsters and monkeys (32, 33). Importantly, however, birds appear to be refractory to *H. Hammondii* infection (34). Another important distinction between *T. gondii* and *H. Hammondii* is the inability of *H. Hammondii* to be transmitted (at least experimentally) from one intermediate host to the other, and from one definitive host to another (12). In fact this is a key diagnostic feature to distinguish isolates of these parasites in the laboratory (12, 35).

Neospora caninum does not share the cat as a definitive host, but rather utilizes canines for sexual reproduction (36). Dogs also appear to be intermediate hosts, as *N. caninum* infection in dogs causes a variety of neurological symptoms including encephalitis and ascending paralysis, often resulting in death (37, 38). The known

intermediate host range of *N. caninum* is more restricted than that of *T. gondii* and consists of dogs, cattle, water buffalo, sheep, goats and horses (38–43). With the exception of dogs and horses, all of these intermediate hosts are members of the Bovidae family. *N. caninum* causes abortion in cattle (44, 45), much like *T. gondii* infection in sheep or goats. Unlike *T. gondii*, and similar to *H. Hammondii*, there is no evidence that *N. caninum* infects humans (46); however, as with *H. Hammondii* the antigenic similarity between *T. gondii* and *N. caninum* makes it difficult to rule out the possibility of *N. caninum* infections in humans (47).

These overlapping, yet distinct host ranges for *T. gondii*, *H. Hammondii*, and *N. caninum* (see Table 1), have been observed for quite some time, yet the genes (both parasite and host) responsible for these differences remain unknown. It should also be noted that while the host ranges of these parasites overlap, the pathologies and host response in overlapping intermediate hosts are not always the same. We review these data below.

Experimental models reveal both similarities and differences in host response to parasite infection in *T. gondii*, *H. Hammondii* and *N. caninum*

As both *T. gondii* and *N. caninum* can cause spontaneous abortion in livestock, experimental infections of sheep, goats and cattle have been used to understand the pathology and modes of transmission of these parasites. Rodent models have also been developed to study infection, dissemination and transmission of *T. gondii*, *H. Hammondii*, and *N. caninum*.

Cattle

The prevalence of *T. gondii* vs. *N. caninum* infection in cattle is variable by region or herd. In some areas, such as southern Vietnam and western Thailand, *T. gondii* is more prevalent than *N. caninum* (48, 49); however, in southern China the prevalence of *N. caninum* infection is slightly higher than that of *T. gondii* (50). Natural infection of cattle by *T. gondii* does occur (51, 52), but is not associated with abortion (53). Experimental infection suggests that there is a low rate of abortion in cattle upon *T. gondii* infection (54) and that this rate increases with *T. gondii* strains that are typically more virulent in mice (55). Surveys of aborted calves show a strong association with *N. caninum* infection, but no association with *T. gondii* infection (56), and herds with high abortion rates tend to have a high rate of *N. caninum* infection (57). Experimental infection of pregnant cattle shows that *N. caninum* infection during early gestation is likely to cause abortion (58); however, virulence among isolates does vary, and less virulent isolates do not appear to cause abortion (59). While some earlier studies suggested that infection late during pregnancy facilitates vertical transmission, but does not cause abortion (60), more recent studies show that infection with *N. caninum* late in gestation can cause abortion (61). There have also been conflicting studies suggesting that horizontal transfer of *N. caninum* infection and abortion does not occur in subsequent pregnancies after initial infection (62), while other studies suggest that chronic infection can lead to recurrent abortions (63). Clearly more experimental work is needed to clarify these conflicting data and to take into account both the genetics of the parasite and the host. Experimental infections of cattle reveal that *N. caninum* disseminates to a variety of tissues including the heart, lung, kidney, skeletal muscle and perhaps most importantly the brain (47). In fact, in one study they detected *N. caninum* in the brain and spinal cord, but in no other surveyed location, including the gastrointestinal tract, liver, kidney, heart, lung and skeletal muscle (64).

The genetic differences between *T. gondii* and *N. caninum* responsible for the differences in virulence in a bovine host have not yet been identified. It is interesting to note that *N. caninum* appears more virulent in cattle than *T. gondii*, whereas in most other shared intermediate hosts it appears that *T. gondii* is more virulent than *N. caninum*. This could be due to the fact that cattle are the natural, and most common, intermediate host for *N. caninum*, and it has evolved specialized methods for evading the bovine immune response. Further studies are required to determine why *N. caninum* is so successful in the bovine host, whereas *T. gondii* is not.

Sheep

Surveys of the prevalence of *N. caninum* and *T. gondii* in sheep herds show a significantly higher proportion of sheep infected with *T. gondii* than *N. caninum* (65–68). A combination of serological studies and experimental infections demonstrate the ability of both *T. gondii* and *N. caninum* to cause abortion in sheep, particularly when infected during early pregnancy (69–73). There is also experimental evidence that both *T. gondii* and *N. caninum* cause recurrent abortions in chronically infected ewes (74, 75). Histological studies of aborted fetuses, weak lambs, congenitally infected healthy lambs and experimentally infected ewes show that *T. gondii* and *N. caninum* dissemination patterns are quite similar (69–71, 76, 77). Aborted *T. gondii*-infected fetuses have lesions primarily in the brain, with some specificity for regions such as the optic tract and rostral margin of the pons (77). Experimental infection in male sheep (rams) has shown that *T. gondii* does infect the male reproductive organs (78), and *T. gondii* infection can be sexually transmitted from infected rams to uninfected ewes (79, 80), but this has not been examined with *N. caninum* infection.

Taken together this suggests that although seroprevalence of *T. gondii* is higher than that of *N. caninum* in domestic sheep, both species are successful parasites of sheep and clearly cause similar pathology. This is in stark contrast to experimental infection in cattle, (described above), where *N. caninum* is clearly much more virulent than *T. gondii*.

Goats

The seroprevalence of *T. gondii* infection in goats is generally much higher than that of *N. caninum* (81–83). Surveys of aborted goat fetuses suggest that *T. gondii* infection contributes to a number of these abortions (84, 85), and experimental infection confirms that *T. gondii* is capable of causing abortions in goats (86, 87). There are very few studies examining *N. caninum* infection in goats, compared to the number of studies done in sheep and cattle. Experimental infection of pygmy goats during pregnancy suggests that *N. caninum* infection in goats does cause abortion when infection occurs early during gestation, and that abortion in these goats does not recur with subsequent pregnancies (88). As with infection in sheep, *T. gondii* does infect the male reproductive organs (89), and infection can be sexually transmitted (90).

As with cattle and sheep, the genetics underlying the pathology differences between *N. caninum* and *T. gondii* in goats are not known. Additional studies in each of these intermediate hosts with genetically engineered parasites may help to uncover the genes responsible for both

similarities, and differences, in host range and host response in *T. gondii* and *N. caninum*.

Rodents

Several rodent models have been developed for studying *T. gondii*, *N. caninum*, and *H. hammondi* infections. These are particularly relevant as rodents are a natural intermediate host for both *T. gondii* and *H. hammondi*, and likely play an important role in the evolutionary history of these parasites.

Multiple mouse strains have been utilized in developing models of *T. gondii* infection, including both outbred (CD-1) and inbred (Balb/c, Cba/j, C57BL6) mouse strains (91, 92), and most recently, the house mouse (93). Pregnant mouse models have also been developed to better understand why *T. gondii* infection causes abortion (94–97). Mice infected with *T. gondii* have enlarged spleens and lymph nodes, caused in part by an increase in mononuclear phagocytes and CD8⁺ T cells, which produce interferon- γ (IFN- γ) (98). Production of innate immune effectors such as interleukin-12 (IL-12) and IFN- γ increases shortly after infection and is required for host survival and control of parasite growth (97, 99, 100). Immune-compromised mice (lacking both B cells and T cells) that have a larger population of natural killer cells, and are therefore able to produce higher levels of IFN- γ , have a lower parasite burden (94), once again providing evidence that IFN- γ production is essential for mouse survival following *T. gondii* infection. Neutralization of IFN- γ increases parasite burden in these mice, but decreases transmission of *T. gondii* infection to offspring (94). Symptoms of acute infection by *T. gondii* generally decrease after several weeks, when the adaptive immune system has had time to respond and produce antibodies and effector cells to combat *T. gondii* (96).

The population structure of *T. gondii* isolates has been studied extensively in an effort to better understand parasite virulence and host interaction. The majority of North American and European *T. gondii* isolates can be grouped into three main lineages that vary in virulence, as well as host responses (8, 101). *T. gondii* strains exhibit a broad range of virulence in mice (Table 1), with the most virulent type I strains being capable of killing a mouse after infection with a single parasite (8, 102). Less virulent type II and type III strains of *T. gondii* have 50% lethal doses of $>10^3$ and 10^5 parasites, respectively (102). Some 'atypical' *T. gondii* strains from South America, which do not belong to any of the three major lineages, have also been shown to be highly virulent in mice (103). Comparisons of these strains and differences in host response following infection have facilitated the discovery of many parasite

factors responsible for virulence and/or interaction with the host, including rhoptry proteins 5, 16 and 18 (ROPs) (4, 5, 7–9, 104–106) and dense granule proteins 15, 24, 25 and MAF1 (107–110).

In general, immunocompetent mice experimentally infected by intraperitoneal injection of *N. caninum* tachyzoites exhibit no signs of disease; however, immunosuppression of mice using methylprednisolone acetate (MPA) results in a range of neurological symptoms, from a slight head tilt to paralysis and death, depending on the dose of immunosuppressant (111). It also appears that subcutaneous injection of tachyzoites in inbred Balb/c mice results in a number of neurological symptoms without the use of MPA immunosuppression (112). IFN- γ -deficient mice, as well as mice lacking Toll-like receptor 4 (TLR-4) and a functional IL-12 receptor, are also susceptible to *N. caninum* infection by intraperitoneal injection of tachyzoites (113–116). In these mice, parasites can be found in the pancreas, liver, lung, intestine, heart and brain, while parasites are not detectable in these organs in immunocompetent mice (116). Infection of dendritic cells is likely important for the dissemination of *N. caninum* within the host, as adoptive transfer of *N. caninum*-infected dendritic cells increases parasite load as well as vertical transmission in pregnant mice (117). As head-to-head comparisons between *T. gondii* and *N. caninum* have not been conducted in mice, we tagged *N. caninum* strain NC-1 (38) with luciferase and compared its proliferation *in vivo* to a highly avirulent strain of *T. gondii*, S1T. S1T is an F1 progeny clone derived from a cross between a *T. gondii* type II and type III strain and contains avirulent alleles of all five identified *T. gondii* virulence factors (118, 119). Mice eventually control parasite proliferation and are able to survive infection with up to 1×10^6 tachyzoites of this parasite clone. As shown in Figure 2, both species proliferate at a similar rate during the first 20 h post-infection, but then *N. caninum* is rapidly controlled while *T. gondii* S1T continues to proliferate. This suggests that the inability of *N. caninum* to be virulent in wild-type mice does not have to do with an inability to replicate within mouse cells *in vivo*, but rather an inability to disrupt host innate immune defences that rapidly control this parasite. It will be interesting in future studies to compare host responses to these two species during the early stages of infection.

Given this attenuated phenotype in mice, several genetically altered mouse models have been developed for *N. caninum* infection with tachyzoites (described above). For infections with other life stages, such as sporulated oocysts, both interferon-gamma knockout mice (120) and gerbil models of infection (*Meriones unguiculatus*) have been used effectively (121).

Similar to *T. gondii*, virulence differs among *N. caninum* isolates, which has been revealed by a number of comparisons (112, 122, 123). The NC-Liverpool strain, isolated from the brain tissue of a young dog euthanized after presenting with severe neurological symptoms, is a more pathogenic strain than the NC-SweB1 strain, isolated from a stillborn calf (122). NC-Nowra, isolated from a congenitally infected calf, is also less pathogenic than LC-Liverpool, but does cause some disease in a small portion of infected mice (124). NC-1 and NC-3 were both isolated from the tissues of congenitally infected dogs, and NC-1 is much more pathogenic than NC-3 (112). No studies have been done to compare the pathogenicity of all *N. caninum* isolates; however, these studies suggest a wide range in ability to cause neurological disease when injected subcutaneously in Balb/c mice. Genetic crosses in the definitive canine host between these strains with distinct phenotypes could potentially lead to the identification of the virulence factors responsible. However it is not known whether they would be relevant to natural *N. caninum* infections as, in contrast to *T. gondii*, rodents do not appear to be a relevant host for *N. caninum* in the wild.

Relatively little work has been carried out in *H. hammondi*-infected mice, as there is currently no way to grow *H. hammondi* parasites in cell culture to perform the same types of experiments that have been done with *T. gondii* and *N. caninum*. Much of the work has been carried out in IFN- γ knockout mice. In parenteral infections in both wild-type and IFN- γ knockout mice *H. hammondi* is benign, resulting in chronically infected mice that show almost no symptoms of infection (based on behavioural responses to hyperinflammation or adverse neurological symptoms). However oral infections with large numbers of *H. hammondi* oocysts can cause severe disease (12) and even mortality (30) in Swiss-Webster mice. It is important to note that IFN- γ KO mice that are chronically infected with *H. hammondi* are infective to the definitive host, and rodents have been found to harbour *H. hammondi* in the wild (12). Given that IFN- γ is required for control of both *T. gondii* and *N. caninum*, it is intriguing that this cytokine is not required for control of *H. hammondi*. This could be due to as yet unidentified host innate immune responses, or it could be due to a hard-wired developmental programme. In *H. hammondi* that results in the spontaneous conversion from rapidly growing tachyzoites to slow-growing, encysted bradyzoites. Consistent with this latter explanation, *H. hammondi*-infected mice have orally infective tissue cysts in muscle and other non-CNS tissues in both wild-type and IFN- γ KO mice, and multiple groups have observed the spontaneous conversion of *H. hammondi* tachyzoites to infectious cysts during cultivation *in vitro* (12, 125). Further analyses will be necessary to more fully

characterize the differences in parasite development between *H. hammondi* and particularly *T. gondii*. Regardless of the root cause overall the existing work on *H. hammondi* indicates that it is unique compared to both *T. gondii* and *N. caninum* in terms of its behaviour in immune-deficient mice.

SECRETED *T. GONDII* EFFECTORS DRIVE STRAIN-SPECIFIC VIRULENCE DIFFERENCES IN MICE

Multiple effector proteins have been identified in *T. gondii* that play key roles in the interaction of this parasite with its host, particularly the mouse. Without exception, these effectors are secreted from either the rhoptries or the dense granules, and some have now been shown to interact with host cell proteins. A number of reviews have been written on the subject of these effectors and the host signalling pathways that they interface with (126–130). Here we present recent data on these effectors and their mechanism of action and discuss how they determine *T. gondii* strain-specific virulence phenotypes. We will then briefly speculate based on the level of conservation at the sequence and functional levels whether the absence of certain key effectors can explain some of the differences in pathogenesis between *T. gondii* and its near relatives that we have just outlined above.

Co-evolution of *T. gondii* effector proteins and host innate immune defence mechanisms

Based on gene knockout and forward genetic studies the most potent mouse virulence factors identified to date in *T. gondii* are rhoptry proteins 5 and 18 (ROP5/ROP18). These loci were identified using genetic crosses between canonical *T. gondii* strain types that differ in their virulence phenotypes in mice (5, 7–9), and both belong to the rhoptry 2 kinase family protein superfamily. At least 30 family members can be found throughout the *T. gondii* genome, and many have undergone local tandem duplication and locus expansion events (including ROP5; (5, 131)). All members of the ROP2 superfamily encode putative proteins with an N-terminal domain encoding membrane-interacting amphipathic helices, and a C-terminal domain encoding either a functional kinase domain or a pseudokinase domain. The amphipathic helix domain is crucial for interaction of the rhoptry kinase with host membranes (particularly the host-derived parasitophorous vacuole (PV); (6)). Importantly, knocking out the entire ROP5 locus in a highly virulent type I strain (RH) renders this parasite completely avirulent: while wild-type strains cause 100% mortality at a dose as low as 10 parasites, the

ROP5 knockout parasites are completely avirulent at doses as high as 1×10^6 tachyzoites (5, 7). *ROP18* knockout parasites are also attenuated in mice compared to wild type, but are still capable of killing mice at doses as low as 1000 tachyzoites (132). These data further suggest that *ROP5* is the more potent of the two loci in terms of impact on parasite virulence.

As the discovery of *ROP5* and 18 as virulence effectors, multiple groups have demonstrated that they target the same host defence mechanism, namely immunity-related GTPases (IRGs; (104)). Multiple lines of evidence indicate that *ROP5* and *ROP18* act in close collaboration, particularly with respect to host IRG proteins. Specifically, members of the *ROP5* family (which have kinase-like folds but lack catalytic activity) bind to IRG proteins which, at least in one study, blocked IRG oligomerization and activation. Subsequently, *ROP18*-driven phosphorylation of *ROP5*-tethered IRG proteins renders them functionally inactive (104, 133). Additionally it has been shown that the kinase activity of *ROP18* depends on the presence of *ROP5*, further emphasizing the important interactions between these distinct gene products (134). Interestingly this all happens on the PV membrane, where in the absence of either *ROP5* or *ROP18* these proteins are loaded on the PV where they lead to its disruption and eventual parasite destruction. Recent work has also indicated that *ROP5* and *ROP18* may be a part of a much larger complex, which includes the secreted rhoptry kinase *ROP17* (135). *ROP17* knockout parasites have reduced virulence in mice, and *ROP17* and *ROP18* have preferences for distinct phosphorylation sites on distinct IRG proteins (135). Additionally, *ROP17* has a particularly strong preference for oligomerized IRG proteins, which again distinguishes it from *ROP18* which has a preference for monomeric IRG proteins (134, 135). These data provide strong support for the idea that the *ROP2* superfamily of secreted kinases and pseudokinases have expanded due to strong selective pressure to interact with difference components of the mouse IRG repertoire. Genes encoding IRG proteins can be found in tandem arrays on multiple chromosomes of the mouse, and the overall IRG gene content varies significantly across even very closely related mouse species (93). IRGs are not conserved in humans, suggesting either that (i) the *ROP5/ROP18* complex (and any other *T. gondii* effectors that target the IRG host resistance pathway) do not play a role in human infection; or (ii) *ROP5* and *ROP18* have additional targets that are conserved across multiple species (including mice and humans). Support for the former hypothesis can be found in the fact that *ROP5* and *ROP18*-dependent resistance to IFN- γ stimulation of host cells only occurs in mouse cells, and not human cells (136). However other work suggests that the *ROP2*-related

effectors may have other targets in the host cell, including ATF6 β (132) and guanylate binding proteins (137, 138).

In addition to *ROP5* and *ROP18*, effectors are consistently being identified that directly modulate host cell signalling. These include *ROP16*, a non-*ROP2*-family rhoptry protein which is secreted into the host cell and traffics to the host cell nucleus due to the presence of a canonical mammalian nuclear localization signal. In the host cell cytoplasm (prior to nuclear translocation) it directly phosphorylates both STAT3 and STAT6 (139–141), leading to dramatic changes in innate immune signalling at the transcriptional and translational level (4, 139). The result of this activation of innate immune signalling is a slight decrease in parasite virulence (8), possibly through an attenuation of IL12 signalling that reduces the hyperinflammation that is known to be at least partially responsible for mouse mortality in *T. gondii* infections (142). Another is *GRA15* (108), which, depending on the allele, is capable of activating nuclear factor κ B (NF κ B). Importantly ectopic expression in host cells of the type II allele of *GRA15* can activate NF κ B, although the mechanism for this activation is not yet clear. Other dense granule proteins modulate host cell signalling, including *GRA25* (110), *MAF1* (107), *GRA6* (143) and *GRA24* (109) (see Table 2).

Each of the three major *T. gondii* clonal lineages has a distinct repertoire of virulence factor alleles

It is important to note that most of the effectors described above were identified based on strain-specific differences in gene sequence and/or phenotypic effect. *Toxoplasma* is a sexual species, and what this work clearly demonstrates is the significant impact that sexual recombination can have on parasite pathogenesis. Specifically, the major European and North American clonotypes used in these studies (clonotypes I, II and III) appear to be siblings (144) based on polymorphism analyses. All three clonotypes have regions of their genomes (and sometimes entire chromosomes) that are shared between two of the clonotypes. For example, type II and type III strains share a nearly identical copy of chromosome IX, while types I and II strains share a nearly identical copy of chromosome IV. All three clonotypes share a nearly identical copy of chromosome Ia (144–146). Consistent with this, each of these strain types harbour a different complement of the virulence effectors described above, and in most cases 2 of the 3 strains will have nearly identical alleles compared to a divergent 3rd clonotype (e.g. the type II alleles for *ROP16* and *GRA15* are divergent compared to types I and III, while type III has the most divergent allele for *ROP18*; Figure 3). As shown in Table 2, both type I and

Table 2 A sampling of known secreted virulence factors in each of the three major European and North American *Toxoplasma gondii* clonotypes, their mechanism(s) of action and their degree of conservation in *Hammondia hammondi* and *Neospora caninum*

Gene	Mechanism	Strain-specificity			<i>H. hammondi</i> ortholog?	<i>N. caninum</i> ortholog?
		I	II	III		
<i>ROP18</i>	IRG phosphorylation ^a	Active	Active	Inactive (low expression)	Yes	No (pseudogene)
<i>ROP5</i>	IRG binding ^b	Active	Less active	Active	Yes	Yes
<i>ROP16</i>	STAT3/6 phosphorylation (I,III) ^c	Active	Inactive	Active	Yes	Yes
<i>GRA15</i>	NFκB activation (II) ^d	Inactive	Active	Inactive	Yes	No (pseudogene)
<i>GRA25</i>	CCL2/CXCL1 induction ^e	ND	Active	Less active (low protein expression)	Yes	Yes
<i>MAF1</i>	Host mitochondrial association ^f	Active	Inactive (low expression)	Active	Yes	Yes
<i>GRA24</i>	P38α MAP kinase activation ^g	None			Yes	No (undetectable by BLAST)

^a(156); ^b(104); ^c(140, 141); ^d(108); ^e(110); ^f(107); ^g(109).

type II parasites harbour a 'virulent' allele of *ROP18* (8, 9), while both types I and III strains harbour the 'virulent' allele of *ROP5* (5, 7). This fact might be surprising given that type III strains are typically less virulent in mice than types I and II (147), but we now know that the sexual recombination event(s) that generated the progenitors of these lineages led to random (or possibly nonrandom via selection of the progeny for a particular virulence phenotype) re-assortment of the virulent and avirulent (or less virulent) versions of these alleles, and therefore virulence in each strain type is driven by what complement of alleles they harbour (see Table 2 for a list of the virulence genes harboured by types I, II and III). As an example, type III strains have an allele of *ROP18* that is essentially null due to a 107 bp deletion in the *ROP18* promoter (10), but when either the type I or type II allele of *ROP18* (which both have a functional promoter) is introduced into this strain it increases virulence in mice by up to 4 logs (8, 9). These data demonstrate the enormous impact that even single genetic crosses can have on the fitness and virulence phenotype of haploid parasites like *T. gondii*.

Thus far the ability to use strain type (and therefore the presence or absence of certain key virulence effectors) to predict disease outcome in humans, or a significant link between strain type and host range, has been elusive. However it is clear from the work described above that lacking any number of virulence effectors (or having the 'avirulent' allele) does not necessarily render a parasite unsuccessful. For example, type III strains are highly prevalent in the USA and North America, and it has been postulated that they are the most prominent *T. gondii* isolate in the world (148). Perhaps this is due to reduced vir-

ulence and therefore increased transmission from host to host, but this has not been directly tested. Also, type I and II strains are also highly dominant in Europe and North America, suggesting that while they each harbour a different complement of alleles and key virulence loci, this has not had a significant impact on their ability to become so dominant in these regions. In addition, this suggests that there exist other virulence effectors in the *T. gondii* genome that are shared across most *T. gondii* lineages that could be termed 'core' effectors that enable *T. gondii* to evade host defences. We anticipate the recent advances in genetic manipulation of *T. gondii* and related species (149, 150) may allow for these core effectors to be identified in a more unbiased way through the generation of strain collections where every putative secretory protein has been deleted.

CONSERVATION OF KNOWN *T. GONDII* SECRETED EFFECTORS IN *H. HAMMONDI* AND *N. CANINUM*

The identification of key host-interacting effectors (like *ROP5/18* and others) begs the question as to whether or not they play a role in determining the dramatic life cycle, host range and virulence differences between *T. gondii* and its near relatives. With the recent sequencing of both the *H. hammondi* (10) and *N. caninum* (11) genomes, one can begin to make comparisons across these three closely related species to begin to address this question. Our recent sequencing of the *H. hammondi* genome showed that *T. gondii* and *H. hammondi* are over 99% syntenic (10), while estimates of synteny between *T. gondii* and

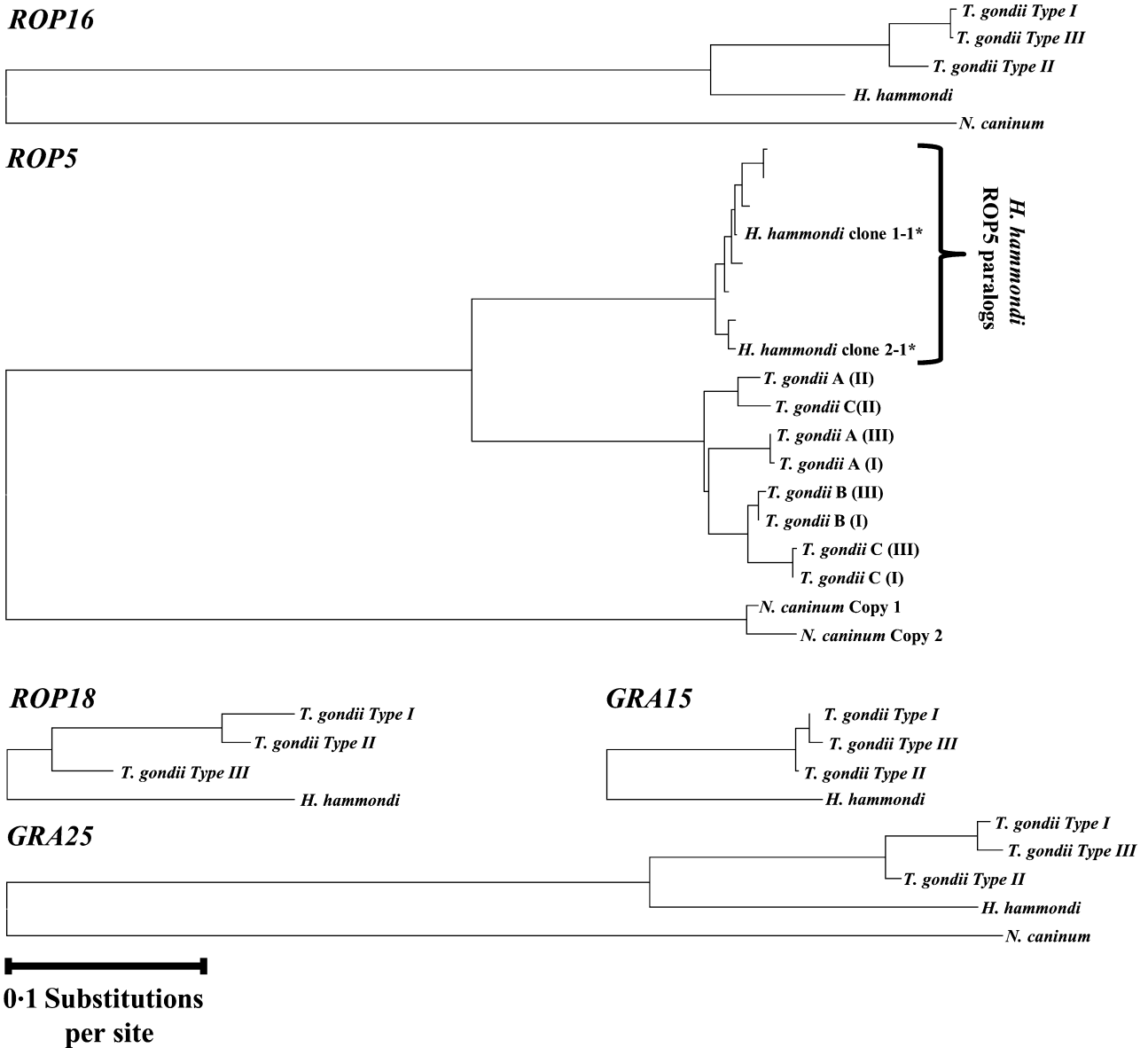


Figure 3 Neighbour-joining trees of known *Toxoplasma gondii* secreted effectors in the three canonical *T. gondii* lineages (types, I, II and III), *Hammondia hammondi* and *Neospora caninum*. All trees are scaled identically, and the bar indicates sequence distance (0.1 substitutions/site). In *N. caninum*, orthologs of ROP18 (11) and GRA15 are pseudogenes and therefore were omitted from the alignments.

N. caninum are closer to 85% (10, 11). When looking at the known *T. gondii* virulence effectors in *H. hammondi* and *N. caninum*, a different story emerges depending on the comparison. Starting with *H. hammondi*, this species harbours clear orthologs of multiple *T. gondii* effectors (ROP18, ROP5, ROP16, GRA15, GRA25, MAF1 and GRA24) while *N. caninum* appears to lack orthologs of ROP18 and GRA15 due to pseudogenization and appears to be missing GRA24 entirely ((11) and Boyle, unpublished; Table 2 and Figure 3). Interestingly, it was recently

reported that complementation of *N. caninum* strain NC-1 with the ROP18 allele from *T. gondii* type I dramatically increased the virulence of *N. caninum* in mice (151). This is an interesting, and entirely surprising result, given what is now known about the coordination between ROP18 and ROP5 in *T. gondii* virulence. It will be interesting to dissect this complementation phenotype further.

Clearly then, the presence of a given virulence effector in the *H. hammondi* genome is not sufficient to explain its highly avirulent phenotype in mice (12). We have also

shown that *H. hammondi* ROP18, ROP5, ROP16 and GRA15 are *functional* effectors when expressed in relevant *T. gondii* genetic backgrounds (10, 152). Specifically, *H. hammondi* has a functional ROP18 ortholog that is highly effective at increasing the virulence of essentially ROP18-null *T. gondii* strains (such as the type III strain CTG; (10)). Through our analysis of the *H. hammondi* genome we found that there was a 107 bp deletion in the type III ROP18 promoter that is responsible for this difference. The ROP18 genes in type I and type II *T. gondii* strains, as well as the *H. hammondi* isolate HhGerCat041, harbour this 107 bp sequence, and we found it to be sufficient to 'resurrect' the type III *T. gondii* ROP18 promoter when inserted into the proper location just upstream of the transcriptional start site (10). As for *ROP5*, this locus is duplicated and expanded in the HhCatGer041 *H. hammondi* isolate, containing approximately 10 copies (10). Importantly, complementing a *T. gondii* ROP5 knockout with *H. hammondi* ROP5 paralogs (1-1 and 2-1; Figure 3) dramatically increased virulence of this attenuated strain, clearly demonstrating that the *H. hammondi* ROP5 orthologs are functional as virulence genes. In contrast, the ROP5 locus has not significantly expanded in *N. caninum*, having only two copies. Moreover, the one ROP5 ortholog that has a complete sequence uninterrupted by a sequence gap is highly divergent from both *H. hammondi* and *T. gondii* ROP5 isoforms (Figure 3). It is not known, however, if *N. caninum* ROP5 could complement virulence defects in *T. gondii* ROP5 knockout strains as for those from *H. hammondi*.

Hammondia hammondi ROP16 and GRA15 are also functional effectors. Specifically, expressing *H. hammondi* ROP16 in type II *T. gondii* significantly increased STAT6 phosphorylation and translocation to the nucleus (152), which is similar to the type I and III *T. gondii* ROP16 alleles (4, 139–141, 153). Importantly, the STAT6 induction was significantly higher than type II *T. gondii* expressing an additional copy of a type II ROP16 allele, providing strong evidence that the 'active' allele of ROP16 with respect to STAT6 activation is ancestral to the *T. gondii*/*H. hammondi* split. Interestingly, however, the *H. hammondi* ROP16 gene promoter had a 16 bp deletion compared to *T. gondii* ROP16, and this 16 bp was essential for ROP16 promoter function (152, 154). It is therefore possible that in *H. hammondi* ROP16 is fully functional with respect to STAT6 activation, but is poorly expressed. Similar to ROP16, expressing *H. hammondi* GRA15 in a type I strain significantly increased NF κ B translocation to the nucleus (152, 154), and this is similar to what has been shown previously for *T. gondii* GRA15 alleles from type II strains. This also

suggests that GRA15-driven NF κ B activation is also an ancestral phenotype. In addition based on our analyses the GRA15 promoter appears to be fully functional (152, 154).

One caveat of these *H. hammondi* heterologous expression studies is that we do not yet know if any of the effectors in question are actually expressed in this species. Determining this is hampered by the fact that long-term cultures of *H. hammondi* (in contrast to *T. gondii* and *N. caninum*) cannot be generated. However next generation ultra-deep sequencing of the *H. hammondi* transcriptome from short-term cultures (which can only be generated from cat-derived oocysts; (12, 155)) could provide new insights into the importance of gene content vs. gene deployment in the relatively avirulent phenotype of *H. hammondi* compared to *T. gondii*.

CONCLUSIONS AND FUTURE PROSPECTS

Comparative analyses between *T. gondii* and its near relatives represent an exciting approach to identify 'core' virulence factors in *T. gondii*. The advent of rapid genome sequencing technologies and high-throughput genetic manipulation techniques in this organism will not only facilitate genome-by-genome comparisons but will also open the door to cross-species complementation experiments to determine the role of individual loci in *T. gondii* virulence and host range. While the present review focused exclusively on studies in whole animals, *in vitro* studies, such as those recently conducted by Beiting et al. (156), will also be crucial in identifying the host responses that may be differentially suppressed/targeted by distinct species like *T. gondii* and *N. caninum*. RNAseq will provide a robust tool to neutralize the problems with growing large numbers of *H. hammondi* tachyzoites, as small-scale infections can be used to make RNAseq libraries (including host and parasite RNA) suitable for ultra-deep sequencing. As a clearer picture of the differences in both gene content (e.g. gene gain/loss) and gene deployment (e.g. expression) across the local phylogeny of *T. gondii* emerges, new hypotheses regarding the evolutionary events that led to the emergence of a species of parasite with the capacity for near global dominance will be generated and tested.

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