Alice in microbes' land : adaptations and counter-adaptations of vector-borne parasitic protozoa and their hosts

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REVIEW ARTICLE

Alice in microbes' land: adaptations and counter-adaptations of vector-borne parasitic protozoa and their hosts

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One sentence summary: Using a few pathogen models, the authors reviewed the different facets of the arms race between pathogens and hosts (vertebrate and invertebrate), with an emphasis on the evolutionary aspects of it. The authors discussed the impact of human interventions on these adaptations.
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ABSTRACT

In the present review, we aim to provide a general introduction to different facets of the arms race between pathogens and their hosts/environment, emphasizing its evolutionary aspects. We focus on vector-borne parasitic protozoa, which have to adapt to both invertebrate and vertebrate hosts. Using *Leishmania*, *Trypanosoma* and *Plasmodium* as main models, we review successively (i) the adaptations and counter-adaptations of parasites and their invertebrate host, (ii) the adaptations and counter-adaptations of parasites and their vertebrate host and (iii) the impact of human interventions (chemotherapy, vaccination, vector control and environmental changes) on these adaptations. We conclude by discussing the practical impact this knowledge can have on translational research and public health.

Keywords: parasites; escape; drug resistance; (co-)evolution; insect vector

INTRODUCTION

Infectious diseases are the result of an evolutionary arms race between pathogens and their hosts. As the Red Queen who taught Alice to run ever faster in order to stay in the same place, most infectious microorganisms have developed intricate strategies to survive as colonists of their hosts, whereas the latter have evolved by equally clever adaptations to destroy or fence off these intruders (also known as the Red Queen hypothesis; Van Valen 1973). In many cases, millennia of arms race have led to a biological equilibrium favorable for both pathogens and the host. Understanding these survival strategies and underlying interspecies interactions offers a unique insight in the fundaments of the biology of pathogens and their hosts. This knowledge can lead to the development of new tools to cure or control infectious diseases. However, as shown by the rapid development of drug resistance and the emergence of new diseases, medical interventions and other ecological interferences will inevitably lead to

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a new episode in the competition between pathogens and their hosts.

In this context, vector-borne parasitic protozoa represent unique models for studies on the evolution of parasitism. There is a debate on the origin of parasitism among these primordial eukaryotes: Did they first parasitize insects or vertebrate hosts? While the 'out of insect' origin might seem more likely (Simpson, Stevens and Lukeš 2006), this debate highlights the importance of considering both the invertebrate and the vertebrate, when studying the adaptive skills of parasitic protozoa. In this review, we provide a general introduction to different facets of the arms race between pathogens and their hosts/environment, with an emphasis on its evolutionary aspects. On one hand, although multiple hematophagous arthropods might be involved in the mechanical transmission of some of these parasites, only a limited number of species are capable of biologically transmitting a particular parasite, despite their sympatry with other arthropods (Cohuet *et al.* 2010; Lefèvre *et al.* 2013). This specific relationship between a parasite and an invertebrate host is characterized by the pathogen's ability of colonizing various tissues in the invertebrate host, which provides the pathogen with an efficient mode of transmission and spread into the mammalian host population, as well as possibly a mode of transfer to the arthropod offspring by vertical (e.g. transovarial) transmission. On the other hand, the interaction between a pathogen and its vertebrate host is defined by a complex balance that is driven by the pathogen's necessity to proliferate and spread in order to complete its life cycle, and the requirement for the infected host to preserve its integrity (Beschin *et al.* 2014; Cecilio *et al.* 2014). The relationship between host and pathogen is therefore shaped by the evolution of both organisms adapting to each other (Capewell *et al.* 2015). Last but not least, humans have tried to fight off pathogens for decades, either by attempting to kill them directly through medicines or by preventing their transmission through quarantine of diseased individuals, vaccination or vector control. In efforts to better protect ourselves, medicinal and vector control strategies have been significantly optimized into highly efficient pathogen control measures. Logically, this also has major consequences for the evolution of pathogens (Vanaerschot *et al.* 2014b), which are now forced to adapt further and further again, like Alice.

Using a few parasitic protozoa as main models (essentially trypanosomes, *Leishmania* and *Plasmodium*), we review successively the adaptations and counter-adaptations of pathogens and their invertebrate and their vertebrate host and the impact of human interventions on these adaptations (Fig. 1). We conclude by discussing the practical impact this knowledge can have on translational research and public health.

ADAPTATION AND COUNTER-ADAPTATIONS OF PROTOZOAN PARASITES TO THEIR INVERTEBRATE HOST

During their development in the invertebrate host, parasites have to cope with the generally harsh physiological conditions (pH, proteolytic and other hydrolytic activities) they encounter in the arthropod, have to cross physical barriers and have to survive local and systemic innate immune responses in order to accomplish infection of the tissue (e.g. gut, mouthparts, salivary glands and reproductive organs) that will ensure further transmission (summarized in Fig. 2). Microbiome and symbiotic associations have been documented to play pivotal roles in determining the vector competence of disease vectors with certain bacterial communities rendering disease vectors resistant to infection (Cirimotich, Ramirez and Dimopoulos 2011; Weiss and Aksoy 2011; Lefèvre *et al.* 2013). While harboring symbiotic organisms is beneficial to the invertebrate host, infection with a pathogen can cause some deleterious effects. The molecular determinants underlying the parasite/host interactions are subjected to strong reciprocal selective pressures. The parasite and the invertebrate host undergo an antagonistic co-evolution or arms race that will drive natural selection for adaptation and counter-adaptation thereby accelerating molecular evolution (Obbard *et al.* 2009; Cohuet *et al.* 2010; Lefevre *et al.* 2013). As a result, there are at present various degrees of compatibility of parasite/invertebrate combinations relying on genetic, epigenetic and environmental factors.

Parasite's escape strategies

Escaping insect's physiological and physical barriers

Upon transfer of the parasite from the vertebrate to the invertebrate host, the environmental conditions strongly change, especially when the blood meal bolus moves into the digestive part of the alimentary tract. This environment has a pH outside the physiological range of vertebrate blood and is supplemented with an array of proteolytic, nucleolytic and other hydrolytic enzymatic activities. The parasite has evolved to adapt rapidly to this new environment, with sensing of changes in composition, temperature and pH as important triggers for differentiation (Billker *et al.* 1998; Fang and McCutchan 2002; Dean *et al.* 2009; Bauer, Morris and Morris 2013). For instance, *Plasmodium* responds to the presence of xanthurenic acid, a product of tryptophan catabolism, in the mosquito gut, which induces gametogenesis through calcium and cGMP-dependent protein kinases (Billker *et al.* 2004; McRobert *et al.* 2008; Guttery *et al.* 2012). In *Trypanosoma brucei*, the expression of a surface transporter for citrate is upregulated upon transfer from 37°C to the relative lower temperature encountered in the tsetse fly host (Dean *et al.* 2009). As a result, *T. brucei* deviates its energy source from glucose to proline and starts expressing a new set of surface glycoproteins (procyclins) that are resistant to proteolytic degradation in the midgut. *Leishmania* promastigotes express surface proteophosphoglycans (PPG) and *Plasmodium* utilizes a set of glycosyl phosphatidyl inositol (GPI)-anchored membrane proteins (P25 and P28) that are believed to play protective roles in the digestive conditions of the midgut (Saxena *et al.* 2006; Secundino *et al.* 2010). Rapid changes are essential in the parasite life cycle but are yet poorly understood (Matthews 2011) and seem mostly regulated at the post-transcriptional level by storing stabilized silent mRNAs in cytoplasmic ribonucleoprotein granules, a feature described for *Plasmodium* and American and African trypanosomes, by extensive regulation by RNA-binding proteins and by autophagy-based protein/organelle turnover during cellular remodeling (Cassola, De Gaudenzi and Frasch 2007; Herman *et al.* 2008; Mair *et al.* 2010; Subota *et al.* 2011; Kolev *et al.* 2012).

Several physical barriers including the peritrophic matrix (PM), epithelial layers and basal membranes are the first lines of defense to prevent invasion and systemic dissemination of parasites. Also intestinal stem cell self-renewal plays an important role in protection of the invertebrate host against the effects of epithelial damage caused by the ingested protozoa. Especially the PM, a sheath composed of chitin, proteins and proteoglycans that separates the blood meal in the midgut lumen from the midgut epithelium, represents an important first barrier for ingested parasites (Lehane 1997; Rotureau and Van Den

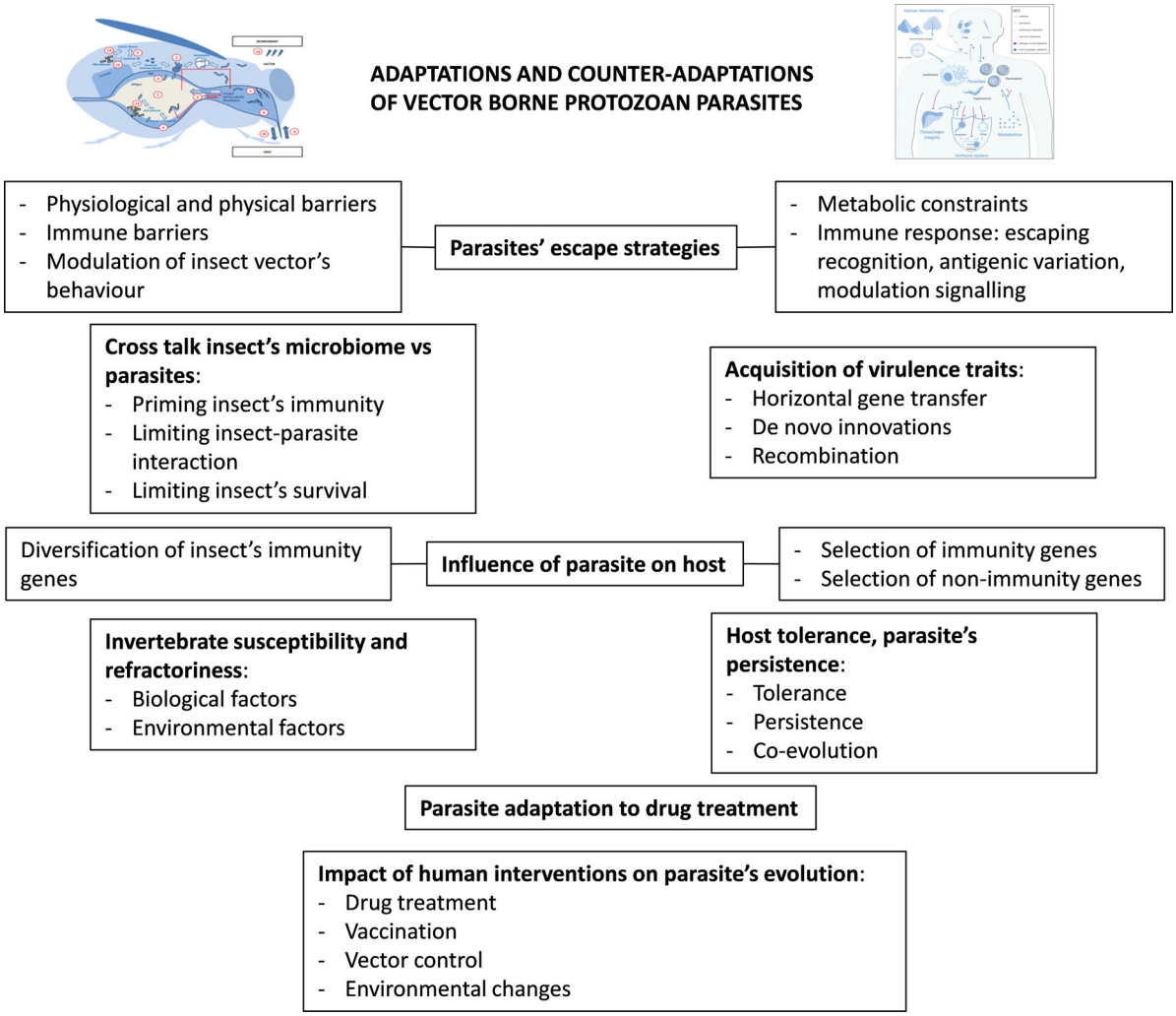

Figure 1. Adaptations and counter-adaptations of vector-borne protozoan parasites in their invertebrate and vertebrate hosts: structure of the paper.

Abbeele 2013). The post-eclosion age (i.e. the emergence of the adult phase) of tsetse flies essentially determines the length of the PM, which provides the parasite with a 24–48 h window of facilitated access to the ectoperitrophic space, thereafter tsetse flies become much more refractory to trypanosome infection (Walshe, Lehane and Haines 2011). Anopheline mosquitoes and sand flies rely on feeding-induced production of a PM by the midgut epithelial cells. In order to overcome this barrier and to facilitate invasion, *Plasmodium* and *Leishmania* have evolved to express enzymes such as proteases and chitinases that target the integrity of the PM (Rogers *et al.* 2008; Li *et al.* 2010). After accessing the ectoperitrophic space, *Leishmania* requires a stage-dependent attachment to the midgut epithelium to avoid being expelled, which involves the abundant surface lipophosphoglycan. In the case of *Plasmodium*, parasites have to transmigrate the midgut barrier, for which the ookinete parasite stage expresses specific ligands for adherence to the epithelium and uses a perforin-like protein and activates the mammalian plasminogen from the blood meal into plasmin for invasion (Kadota *et al.* 2004; Ghosh *et al.* 2011). Entry of *Plasmodium* into the salivary gland relies on the specific interaction of the sporozoite with this tissue and invasion relies on the induction of a mosquito proteolytic activity that facilitates the transmigration process (Rodrigues *et al.* 2012).

Motility is another crucial aspect in several parasite life cycles and various parasite differentiation morphotypes are recognized based on changes in the position and size of the flagellum. A long flagellum provides the long *T. brucei* epimastigote with high motility to migrate upstream inside the insects alimentary tract against the peristaltic flow of the incoming blood meal. It has been shown that motility mutants that are deficient in an axonemal dynein are unable to make this upstream migration (Rotureau *et al.* 2014). Short epimastigotes have a short flagellum, where outgrowths of the flagellar membrane engage in parasite attachment to the salivary gland epithelium (Dyer *et al.* 2013). For *T. brucei*, one RNA-binding protein is upregulated in the insect proventriculus that seems to be an essential master regulator for triggering parasite differentiation into this highly motile long epimastigote (Kolev *et al.* 2012). Also for *Leishmania* amastigotes it is imperative to differentiate into motile promastigotes with a prolonged flagellum that engages in a key interaction with the sand fly midgut epithelium. In the case of *Plasmodium*, male gametes in the midgut lumen have to undergo a process of exflagellation, yielding flagellated microgametes, and after fertilization of the female macrogamete, the zygote has to develop into a motile ookinete (but without flagellum) that can cross the PM, invade the midgut epithelium and reach the basal lamina to form an oocyst. From there, motile sporozoites are

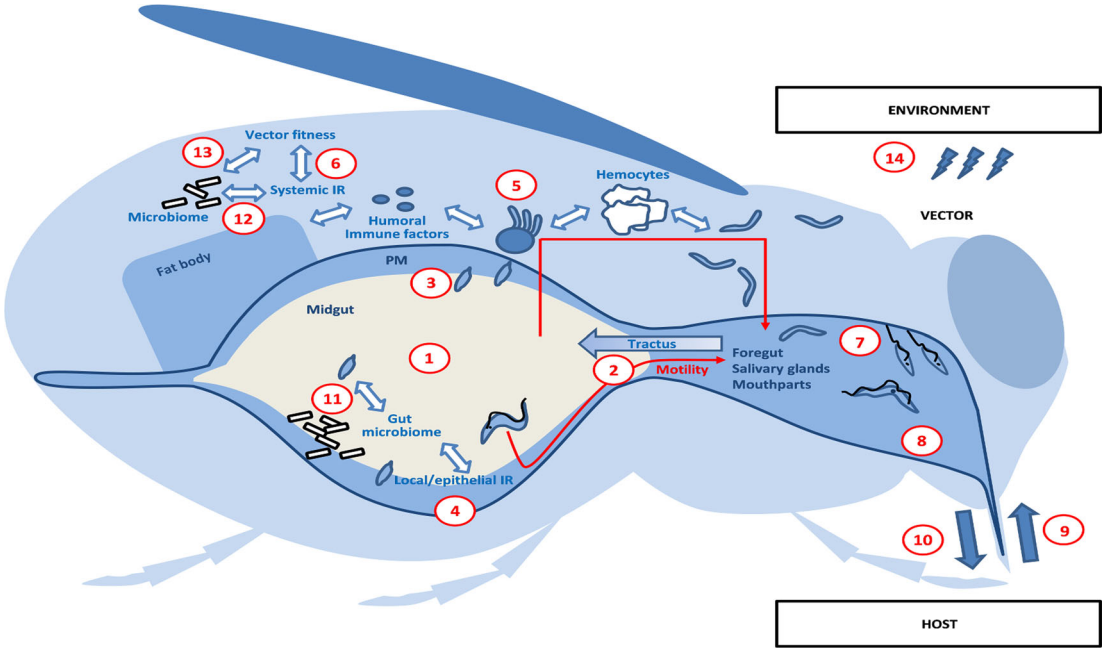

Figure 2. A parasite has to overcome several bottlenecks to complete its life cycle in its invertebrate host. Upon entering the invertebrate host parasites sense changes in composition, temperature and pH as triggers for differentiation (1). These changes convey the parasite properties that allow them to survive the harsh environmental conditions in the digestive environment, to colonize the midgut. Parasite motility allows migration against the peristaltic flow in the gut and avoid being expelled (2). Parasites that enter the hemocoel rely on attachment to the midgut epithelium and on secreted and co-opted proteases to transmigrate the midgut wall (3). This transmigration process triggers local (epithelial) immune responses, including RNS and ROS production and enzymes that catalyze parasite nitration and induce intestinal stem cell renewal (4). Parasites entering the hemocoel are exposed to phagocytic hemocytes and humoral factors including pattern recognition molecules, AMPs, complement factors, lectins and proteases (5). The induction of innate immunity could also impact vector fitness (6). Parasites adhere in the anterior midgut, foregut, mouthparts or salivary glands and can eventually differentiate into vertebrate infective forms or gain access to the salivary gland as infective forms from the hemocoel (7). By mechanical obstruction, binding to sensory structures and by affecting the levels of antihemostatic compounds in the saliva, parasites can influence the probing, persistence and engorgement rate of infected vectors on the vertebrate host, thereby increasing the likelihood of parasite transmission (8). Some parasites induce neurophysiological changes, affecting the vector's locomotion behavior and olfactory system rendering them more responsive to human odors (9). In the opposite direction, physiological changes in the parasite-infected vertebrate host, including an increase in body temperature and altered body odor, enhance the probability of pathogen uptake by the vector (10). The microbiome can also have an important impact by inhibiting pathogen development through the production of antimicrobial factors (11). As there is a functional overlap in the antibacterial, antiparasitic and antiviral innate immune responses of insect vectors, the microbiome can prime the immune system that affects pathogen development (12). It has also been shown that the larval microbiome is important in the development of a competent immune system and in determining vector lifespan (13). The refractoriness/susceptibility phenotype is also influenced by various external/environmental conditions (e.g. temperature and conditions of starvation) that can inflict stress onto the invertebrate host and modify its physiology and immune status (14).

released into the hemocoel (i.e. cavities between the organs of arthropods) and transmigrate into the salivary gland to ensure further transmission.

Escaping insect's immune barriers

In their invertebrate hosts, parasites have to overcome various immunological barriers that are in place to protect against blood-borne pathogens and to control midgut bacterial communities. Transcriptome meta-analyses revealed a strikingly extensive overlap of immune responsive genes to various classes of pathogens spanning various mosquito species (Bartholomay *et al.* 2010). Pattern recognition molecules, antimicrobial peptide (AMP) production, reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been suggested to play significant roles in the epithelial or systemic immune responses against parasites such as *Trypanosoma, Leishmania* and *Plasmodium*, thereby determining the susceptibility of the respective vectors for these pathogens. Interestingly, significant differences in epithelial RNS/ROS responses were observed when comparing different combinations of mosquito and *Plasmodium* strains, indicating that the arms race has resulted in combination-specific immune responsiveness that impacts the infection outcome (Molina-Cruz *et al.* 2012, 2013). It is supposed that parasites have adapted to susceptible hosts by avoiding the induction of immune responses that are functionally important in elimination. Parasites could potentially achieve this by deceiving non-self-recognition or by inhibiting the induction or the effector activity of the defense reactions.

Examples of immune evasion were recently found for African *Plasmodium falciparum* lines that are able to avoid lysis by the complement-like system of *Anopheles gambiae* through a mechanism that involves the surface expressed parasite protein Pfs47 (Molina-Cruz *et al.* 2013). This surface protein is able to inhibit the nitration response that occurs in Anopheline midgut epithelium upon cell damage induced by the parasite invasion process. *Plasmodium* triggers nitric oxide synthase (NOS) in the midgut by their GPI, which adversely affects parasite development. Beside NOS, the nitration process depends on two other essential enzymes, heme peroxidase 2 and NADPH oxidase 5 that are induced through a cell death pathway involving the c-Jun-N-terminal kinase (JNK) and caspases, a pathway that is inhibited by Pfs47 (Ramphul *et al.* 2015). This nitration reaction marks transmigrating ookinetes for complement-mediated lysis from the moment that they appear at the basal side of the midgut. Interestingly, Pfs47 knockout *P. falciparum* parasites were unable to inhibit this nitration response and were efficiently eliminated by the complement system in an otherwise susceptible mosquito strain. Moreover, the same knockout parasite

strain was eliminated and encapsulated in a resistant African mosquito strain, a phenotype that can be reverted by silencing of the mosquito complement-like thioester-containing protein-1 (TEP1). This TEP1, together with leucine-rich repeat proteins, is expressed as a result of activation of the immune deficiency (IMD) pathway. Activation of the IMD pathway by overexpression of the nuclear factor-κB (NF-κB) transcriptional factor REL2 increases resistance of *Anopheles* and *Aedes* mosquitoes to *Plasmodium* (Antonova *et al.* 2009; Dong *et al.* 2011). Also in tsetse flies, the IMD plays a role in controlling trypanosome infection establishment in the tsetse midgut (Hu and Aksoy 2006). In addition to the IMD pathway, also the Toll pathway contributes to pathogen control as overactivation of the Toll pathway renders *An. gambiae* refractory to *P. berghei* (Frolet *et al.* 2006). This experimental evidence shows that various immune pathways can contribute to parasite control. Hence, the parasite needs to overcome the activation or effects of those pathways to persist in a susceptible invertebrate host.

Parasites also have to cope with ROS, which represent important antimicrobial effectors in both the invertebrate and vertebrate host. In insects, dual oxidase (DUOX) plays an important role in the infection-induced production of hydrogen peroxide, which is a substrate for various peroxidases. DUOX is regulated at the transcriptional and post-translational level, allowing for (i) the maintaining of a basal ROS level in normal conditions and (ii) the upregulation of ROS production upon increased bacterial burden in the gut (Ha *et al.* 2009; Charroux and Royet 2012). ROS can exert a direct antimicrobial activity and can also trigger the Toll pathway thereby further enhancing the immune response by stimulating AMP production (Pan *et al.* 2012). Important increases in oxidative responses are detected in disease vectors upon exposure to pathogens, while antioxidants or silencing of detoxifying enzymes exacerbate pathogen establishment in the invertebrate host (Kumar *et al.* 2003; MacLeod *et al.* 2007; Molina-Cruz *et al.* 2008). Kinetoplastids such as *Trypanosoma* and *Leishmania* were shown to possess trypanothione, a low-molecular-weight dithiol, which is kept reduced by the trypanothione reductase. A thioredoxin-like protein is an important mediator in the trypanonothione-dependent redox reactions that play a pivotal role in detoxification of hydroperoxides and peroxynitrites and protect trypanosomes from oxidative stress (Comini, Krauth-Siegel and Flohé 2007). *Plasmodium* makes use of a glutathione/thioredoxin system, which was shown essential in the parasite life cycle for resisting oxidative stress and overcoming the midgut barrier in the mosquito (Vega-Rodríguez *et al.* 2009). ROS were also suggested to modify the gut permeability of disease vectors, by acting as cofactors for the blood meal-induced immunomodulatory peroxidase (IMPer), which catalyzes protein cross-linking of mucins in the ectoperitrophic space. As a result, this modifies the gut permeability and level of exposure of parasites and bacteria to the insect's immune system (Kumar *et al.* 2010). It is suggested that IMPer allows *Plasmodium* parasites to develop in the midgut without activating the immune pathways that regulate NOS expression and contributing to a lower nitration response when parasites invade the gut epithelium. In addition, it is known that NO released in the gut can trigger AMP production in hemocytes and the fat body, which is an important aspect of inter-tissue immunological communication (Wu *et al.* 2012). As such, inhibition of epithelial NOS expression can be expected to affect systemic immune responses as well. Surprisingly, feeding of tsetse flies with L-NAME, a NOS inhibitor, disturbed the procyclic midgut trypanosomes in their upstream maturation process, suggesting that NO could potentially act as an agonist for differentiation in the tsetse fly proventriculus (i.e. muscular dilatation of the foregut) (MacLeod *et al.* 2007).

It is also worth noting that there is a potential trade-off between immunity and life history traits. In mosquitoes, artificial induction of innate immunity resulted in a significant reduction of fitness (Frolet *et al.* 2006; Garver, Dong and Dimopoulos 2009; Corby-Harris *et al.* 2010). As such, immunity can be considered as a double-edged sword that is important for the invertebrate host to control the pathogen infection, but that could also contribute to pathogenic bystander effects. As such, the recognition, elimination, control or toleration of parasites relies on complex and tightly regulated immune responses that need to balance between mounting antipathogen effectors and controlling pathogenicity.

Modulation of the insect vector's behavior to increase transmission probability

Mathematical modeling has shown that alteration of the arthropod feeding behavior by pathogens can easily cause a doubling in the lifetime number of infectious bites (Cator *et al.* 2014). It is therefore not surprising that the general physiology of the invertebrate host is an important target for the parasite. By modulating or manipulating the behavior of an infected arthropod, the parasite can increase the contact between the vector and its vertebrate host and thereby enhance pathogen transmission. The fact that many pathways underlying particular behavioral traits are highly conserved in vertebrates and invertebrates renders them suitable targets for manipulation by parasites (van Houte, Ros and van Oers 2013).

Attracting more insect vectors to infectious hosts is a first way for increasing the transmission potential. Increased attractiveness has been observed for vertebrates infected with *Plasmodium*, *Leishmania* and *Trypanosoma* (Baylis and Mbwabi 1995; O'Shea *et al.* 2002; Lacroix *et al.* 2005; Batista, Costa and Silva 2014). Even more, it was shown that the presence of infectious stages of *P. vivax* or *P. falciparum* (i.e. gametocytes) renders both symptomatic (Batista, Costa and Silva 2014) and asymptomatic individuals (Lacroix *et al.* 2005) more attractive to malaria vectors. This phenomenon seems to be related to an increased body temperature by inducing fever simultaneously to vector biting peaks (Nacher 2005) or changes in body odor as shown for *Plasmodium*-infected mice (De Moraes *et al.* 2014) and *Leishmania*-infected hamsters (O'Shea *et al.* 2002). Moreover, the probability of successfully obtaining a blood meal as well as the actual size of the blood meal is higher when vectors feed on *Plasmodium* or *Trypanosoma*-infected hosts (Baylis and Mbwabi 1995; Taylor and Hurd 2001; Ferguson and Read 2004), favoring uptake of the parasite by the vector.

After increasing the probability of a vector to become infected, the parasite needs to complete its extrinsic incubation period (i.e. the period required to develop into the infectious stage) before the vector dies. Given that the extrinsic incubation period can extend to days or even weeks depending on the parasite (Johansson *et al.* 2010; Blanford *et al.* 2013; Tjaden *et al.* 2013), vector survival is a vulnerable point in the parasite's cycle. As such, it would be beneficial for the parasite to increase the survival of its vector, which is in contrast with the general believe that most of the vector-borne pathogens negatively affect the fitness and survival of their arthropod host. However, for *Plasmodium* this has been debated (Ferguson and Read 2002) and recently it was shown that oocyst stage *Plasmodium* increases the survival of experimentally infected mosquitoes in conditions of starvation (Zhao *et al.* 2012). Moreover, vectors carrying *Plasmodium* oocysts or sporozoites show increased plant attraction and

nectar feeding, both critical in vector survival (Nyasembe *et al.* 2014). Accumulation of glycogen by higher uptake of sucrose (Zhao *et al.* 2012; Nyasembe *et al.* 2014) decreased energy use by influencing metabolic pathways (Zhao *et al.* 2012), such as the reproduction pathway (Vézilier *et al.* 2012; Fellet *et al.* 2014), and favor the survival during the extrinsic incubation period of infected malaria vectors.

Upon reaching its infective stage in the vector, parasite transmission would benefit from an infectious vector that is more attracted to its vertebrate host and that feeds on multiple hosts. Some parasites alter their vector's locomotory behavior, probably through modulating the expression of chemosensory-related molecules (Sim, Ramirez and Dimopoulos 2012) or proteins in the insect's central nervous system (van Houte, Ros and van Oers 2013). This phenomenon has been described for many insect parasites of non-medical importance (van Houte, Ros and van Oers 2013), as well as for DENV-2 infections in experimentally infected *Aedes aegypti* females (Lima-Camara *et al.* 2011). Increased transcripts encoding odorant binding proteins in the saliva and antennae and odorant receptor/coreceptor in the antennae were observed in DENV-infected *Ae. aegypti* mosquitoes, implying that infection might facilitate mosquito host-seeking and/or probing behavior (Sim, Ramirez and Dimopoulos 2012). Similarly, *An. gambiae* infected with *P. falciparum* were more responsive to human odors as compared to non-infected *An. gambiae* (Smallegange *et al.* 2013).

By influencing the probing, persistence and engorgement rate, the parasites additionally manipulate the number of blood meals and the amount of blood taken. This phenomenon has been widely studied for a variety of vector-borne parasites. Increasing the number of feeding attempts can occur by mechanical obstruction of the anterior midgut either with the pathogen itself or with a filamentous PPG gel (e.g. in *Leishmania mexicana* and *L. infantum*-infected sand flies) (Rogers and Bates 2007). Other mechanisms rely on affecting the levels of antihemostatic compounds in the saliva (*T. brucei*, *T. rangeli*, *P. gallinaceum*) or binding to sensory structures (sensillae) in the mouthparts (*T. congolese*, *T. vivax*, *L. major*) (Molyneux, Lavin and Elce 1979; Rossignol, Ribeiro and Spielman 1984; Van Den Abbeele *et al.* 2010). A reduced expression of the anticoagulant and antiplatelet aggregation salivary repertoire seems to mainly extend the probing time required to locate a suitable blood vessel or to generate a blood pool (Rossignol, Ribeiro and Spielman 1984; Van Den Abbeele *et al.* 2010). As the deposition of saliva with parasites during this probing interaction is sufficient to provoke infection, this will increase the likelihood of parasite transmission. Infection of the mouth parts, such as the proboscis and the cibarium, occurs primarily around sensillae that are playing a role in the detection of an incoming blood flux (Molyneux, Lavin and Elce 1979; Beach, Kiilu and Leeuwenburg 1985). The presence of parasites is thought to reduce the diameter of the proboscis and this combined with interference with the sensory structures negatively affects the blood feeding performance in favor of parasite transmission. Increased foraging and feeding was also observed for infected malaria vectors during the infectious sporozoite stage, both in laboratory and field conditions (Koella, Sørensen and Anderson 1998; Cator *et al.* 2012), and seems to be related to the parasite stage. As reviewed by Cator *et al.* (2012), female mosquitoes infected with oocyst-stage malaria parasites are less persistent at blood feeding and less likely to resume feeding if interrupted, while sporozoite-infected females probed more frequently than uninfected controls and were more persistent at attempts to obtain a blood meal. Recent data show that behavioral and neurophysiological changes in the arthropod similar to the ones caused by *Plasmodium* can also be induced by immune challenge with heat-killed *Escherichia coli* (Cator *et al.* 2013), challenging the paradigm of specificity of this manipulation response in the vector.

Crosstalk between parasites and the insect's microbiome

The arthropod gut is inhabited by complex microbial communities (Dillon and Dillon 2004; Weiss and Aksoy 2011). Recently, it was shown that Anophelines' salivary glands display an even higher microbial diversity than the gut (Sharma *et al.* 2014). These bacterial communities consist of primary symbionts, which are obligatory present (e.g. *Wigglesworthia* in the tsetse fly), and secondary symbionts or commensal bacteria, which are acquired throughout the insect life (Weiss and Aksoy 2011; Boissière *et al.* 2012; Aksoy *et al.* 2014). As such, both primary and secondary symbionts have an impact on nutrient supplementation, reproduction and degree of resistance to pathogens (Weiss and Aksoy 2011; Sharma *et al.* 2014). In the context of the latter, the insect microbiome and symbiotic associations have been documented to play pivotal roles in determining vector competence of disease vectors with certain bacterial communities rendering disease vectors resistant to infection (Cirimotich, Ramirez and Dimopoulos 2011; Weiss and Aksoy 2011; Lefèvre *et al.* 2013). Understanding these relationships are of applied interest as they can lead to the development of new vector control strategies (Weiss and Aksoy 2011). Although the mechanisms through which the microbiome acts upon the insect parasites are not completely elucidated, three mechanisms are considered to impact the vector competence. (i) As there is a functional overlap in the antibacterial, antiparasitic and antiviral innate immune responses of insect vectors, the microbiome can prime the immune system that affects parasite development (Cirimotich, Ramirez and Dimopoulos 2011; Weiss and Aksoy 2011). (ii) The microbiome itself can inhibit development of vector-borne parasites by the production of microbial factors with antiparasite activity (Azambuja, Garcia and Ratcliffe 2005). (iii) The microbiome can have an effect on vector lifespan and therefore also vector density (Aksoy *et al.* 2014).

Microbiome priming insect's immunity

The innate immune response pathways activated against the natural gut microbiota are also effective against insect vectored parasites (e.g. *Plasmodium*, *Trypanosoma*, *Leishmania*) (summarized in Weiss and Aksoy 2011). For parasites, these responses are mostly mediated by the IMD pathway through the receptor protein peptidoglycan recognition protein-LC (PGRP-LC) (Cirimotich, Ramirez and Dimopoulos 2011). In laboratory experiments, microbiome-carrying insects displayed a higher activation of the basal immune system as opposed to microbiome-free insects (antibiotic treated) with for example elevated expression of AMP in microbiome-carrying *An. gambiae* (Dong, Manfredini and Dimopoulos 2009), as well as resistance to trypanosome infection in the tsetse fly through the induction of PGRP-LC by *Wigglesworthia* (Aksoy *et al.* 2014). Bacterial-induced ROS leads to loss of *Leishmania* infection in the sand fly (Sant'Anna *et al.* 2014). As such, many studies confirm that the insect microbiome primes the immune system and influences the development of vectored pathogens. Even more, the absence of symbiotic microbiota in the larval stage of tsetse flies results in an impaired immune function development (Weiss and Aksoy 2011; Weiss, Wang and Aksoy 2011), resulting in increased susceptibility of the emerged

adult flies to develop a trypanosome infection. However, the reciprocal effect is also possible; some symbionts could possibly have an exacerbative effect on parasite proliferation. For example, *Sodalis glossinidius* has been positively associated with trypanosome infection in the tsetse fly (Azambuja, Garcia and Ratcliffe 2005; Aksoy *et al.* 2014).

Microbiome limiting insect–parasite interaction

Gut microbiota also prevent colonization of the insect gut by parasites, by limiting interactions between the pathogen and the vector epithelium (reviewed in Azambuja, Garcia and Ratcliffe 2005). In *An. gambiae*, the presence of the natural microbial flora results in the overexpression of a PM protein gene, indicating an enhanced protection of the gut endothelium by an enforced PM (Dong, Manfredini and Dimopoulos 2009). Recent observations have indicated that also the larval microbiome contributes to the integrity of the PM in the emerging adult tsetse fly (Weiss *et al.* 2013). Direct interactions between bacteria and parasites can also inhibit parasite development, which is illustrated by *Serratia marcescens,* which adheres to and lyses *T. cruzi* in triatomines (reviewed in Garcia *et al.* 2010). Moreover, the microbiome can produce several microbial factors, such as prodigiosin, cytotoxic metalloproteases, hemolysins, antibiotics and hemagglutinins, that can target parasites in the vector (reviewed in Azambuja, Garcia and Ratcliffe 2005). *Trypanosoma cruzi,* for example, is lysed *in vitro* by *Pseudomonas fluorescens* (Azambuja, Garcia and Ratcliffe 2005), and an *Enterobacter* bacterium isolated from wild mosquito populations in Zambia confers refractoriness to *P. falciparum* infection through bacterial production of ROS directly targeting the *Plasmodium* parasites in the midgut (Cirimotich *et al.* 2011). Such effects are probably *Enterobacter* species specific, as more recently it was shown that *P. falciparum* positive field-collected Anophelines were hosting more *Enterobacteriaceae* bacteria (Boissière *et al.* 2012). On the other hand, it has been shown that the sand fly is protected from other insect pathogens when its gut is colonized by *Leishmania*. As such, *Leishmania* can also promote the survival of its vector, suggesting a possible mutually beneficial association between *Leishmania* and sand flies (Sant'Anna *et al.* 2014).

Microbiome limiting insect vector's survival

As older adult vectors contribute more to disease transmission, the microbiome can also affect vectorial capacity by its effect on vector survival (Aksoy *et al.* 2014). It has indeed been shown that despite a higher parasitic load, antibiotic-treated (microbiome-free) *An. gambiae* mosquitoes infected with *P. falciparum* have a higher survival rate as compared to microbiome-carrying *An. gambiae* mosquitoes infected with *P. falciparum* (Dong, Manfredini and Dimopoulos 2009). This difference was not observed when these mosquitoes were not infected with *P. falciparum* (Dong, Manfredini and Dimopoulos 2009). As such, the microbiome can have a marked effect on vectorial capacity by reducing vector survival. A notable example of lifespan-shortening bacteria are the endosymbionts of the genus *Wolbachia*. Maternally transmitted, they cause cytoplasmic incompatibility, and some strains can reduce the lifespan of insect vectors and interfere with pathogen replication (Iturbe-Ormaetxe, Walker and O' Neill 2011; Weiss and Aksoy 2011). Given these features, *Wolbachia* has been proposed as a novel strategy to control several disease vectors. However, the effect of *Wolbachia* should be considered carefully, given that both *Plasmodium* parasites and arboviruses such as West Nile Virus are prone to *Wolbachia*-mediated pathogen enhancements (Dodson *et al.* 2014; Hughes, Rivero and Rasgon 2014).

Influence of parasites on the invertebrate host: diversification of insect's immunity genes

Co-evolution of invertebrate hosts and parasites implies that the fitness of both are linked and could drive the fixation of advantageous alleles. In addition, processes of speciation underlying specialization for different breeding sites, ecological niche differentiation and reproductive isolation within disease vector populations can result in changes of the vectorial capacity. A prominent example is *An. gambiae* s.s., which is currently undergoing speciation, as it is being split into two molecular forms named M and S, of which the selection is mainly mediated by larval predation and competition resulting in divergence between larval habitats (Lehmann and Diabate 2008). Genetic differentiation between the molecular forms has been extensively studied. SNP analysis revealed extensive diversification between the M and S *An. gambiae* populations from Mali in genomic regions that contain genes of importance for pathogen transmission, including loci influencing reproduction, longevity, insecticide resistance, aridity tolerance and larval habitat (Neafsey *et al.* 2010). Genome scans revealed a significant peak of M-S divergence on chromosome 3L, implicating the TEP1 locus, which is an important complement factor involved in parasite lysis (see above). Interestingly, variation in the *An. gambiae* mosquito TEP1 alleles was linked to susceptibility/refractoriness of the mosquito to *P. falciparum* (White *et al.* 2011). Variation in resistant and sensitive TEP1 (TEP1*R and TEP1*S) alleles was found to be most prominent in three hypervariable loops within the thioester domain that seems to convey the cleaved TEP1*R protein with a higher stability and longer retention of the active thioester and hence could possibly achieve a more efficient opsonization and subsequent lysis of the *Plasmodium* parasite (Le *et al.* 2012). This indicates that insect speciation can result in the fixation of alleles, which can provide an enhanced level of refractoriness to a pathogen. The TEP1-dependent lytic pathway is downstream of the nitration response in the mosquito midgut during the parasite transmigration process, which is inhibited by the parasite surface protein Pfs47. *Pfs47* is important for *Plasmodium* to overcome this Anopheline midgut nitration responses and was found to be a highly polymorphic gene when comparing *P. falciparum* lines that are resistant and sensitive to elimination in the mosquito. Complementation studies in Pfs47 knockout *P. falciparum* were able to demonstrate that the evasion of immune elimination is directly linked to the expression of a resistant or susceptible *pfs47* allele (Molina-Cruz *et al.* 2013). Moreover, comparison of Pfs47 genotypes of S × R genetic crosses retrieved from resistant versus susceptible mosquitoes revealed that the susceptible Pfs47 genotype is under strong negative selection in resistant but not in susceptible mosquitoes. This indicates that the midgut nitration response and the TEP1 lytic pathway in *Anopheles* versus *Plasmodium* escape mechanisms are subject of the arms race that determines the compatibility of specific mosquito–parasite strain combinations. In *Ae. aegypti* populations that differ in susceptibility to dengue infection, an SNP analysis revealed sequence variation in the open reading frames of a number of peroxidases, serpins and Clip-domain serine proteases suggesting diversification of functional specificity of these components with potential impact on epidemiologically important traits. Also sequence variation in 3′UTRs of immune-related genes associated with autophagy and pattern recognition were recorded, suggesting adaption to different regulatory domains (Bonizzoni *et al.* 2013). In *Drosophila*, immunity genes with most elevated rates of amino acid substitutions were encoding two serine proteases involved in Toll immune

signaling (persephone and spirit) and a homolog of the *Anopheles* serpin SRPN6 (Jiggins and Kim 2007). As these are implicated in immune signaling, this suggests that this is an important target for host–parasite co-evolution. Also comparative phylogenomic analysis based on the published genomes of several mosquito species and *Drosophila* indicated that the immune gene repertoire is amplified in *Culex quinquefasciatus* with expanded gene families recruited into pathogen-responsive defense pathways with an apparent diversification in immune surveillance and immune signal amplification processes [exemplified by an increased number of C-type lectins, fibrinogen-related proteins and serine protease inhibitors (SRPNs)] (Bartholomay *et al*. 2010). Interestingly, when bottlenecks are overcome in insect vectors, this process can enable the enrichment of specific parasite genotypes for further transmission (Oberle *et al*. 2010). As a result, the bottlenecks negatively affect the genetic diversity while possibly also favoring the enrichment of rare variants in individual insect vectors for further dissemination, by which variants with a selective advantage in the vertebrate host (e.g. host range or increased resistance to drugs) might be able to propagate more efficiently.

Invertebrate susceptibility and refractoriness to parasite infection

Biological factors influencing susceptibility and refractoriness

The susceptibility or refractoriness of an invertebrate host to infection with a pathogenic organism depends on many parameters as outlined above, which includes host and pathogen factors (summarized in Fig. 2). From the host side, physical, physiological and local and systemic immunological barriers and microbial associations were shown to be key in determining the susceptibility phenotype. From the parasite side, some specific genes and allelic variants play key roles in overcoming those barriers and in avoiding eliciting immunological reactions that drive parasite elimination. As overall outcome, infection efficiencies in the invertebrate host are very variable, with often high percentages of refractoriness in field as well as under laboratory conditions. For example, natural infection rates in tsetse fly salivary glands of *T. brucei* ssp, the causative agents of human sleeping sickness and *nagana* in cattle, are in the range of 0.1%–1% based on a number of field studies. Prevalence in India of *L. donovani* in the main vector species *Phlebotomus argentipes* was calculated to range between 5% and 17% as elucidated by molecular parasite detection in insect pools (Tiwary *et al*. 2012). Based on the Nigeria *Anopheles* Vector database, mean *P. falciparum* sporozoite rates in various anopheline species in nature vary between 0% and 19% with *An. gambiae* s.s. (19%) and *An. funestus* (12%) identified as highly permissive, while other species seem more resistant to parasite establishment in the salivary glands (Okorie *et al*. 2011).

Influence of environmental factors on susceptibility and refractoriness

Beside the direct invertebrate host–parasite interaction, the refractoriness/susceptibility phenotype is also influenced by various external/environmental conditions. For instance, for tsetse flies it was shown that adult blood-fed tsetse flies are highly refractory to trypanosome infection, a phenotype that can be reverted by starvation (Akoda *et al*. 2009a,b). The condition of starvation was associated with reduced fat body lipid reserves and reduced expression of AMP that exert an antitrypanosomal activity (Akoda *et al*. 2009a,b). Susceptibility of disease vectors can also vary geographically and can be influenced by climatic factors such as temperature, which can have an impact on the incidence, seasonality and distribution of vector-borne diseases. In *An. gambiae*, genes involved in environmental stress responses are involved in determining the level of susceptibility to *Plasmodium* parasites. Indeed, RNAi-based suppression of a desiccation and temperature-sensitive trehalose transporter reduces the numbers of *P. falciparum* oocycts in the anopheline midgut (Liu *et al*. 2013). These examples clearly indicate a direct link between stress adaptation to external triggers and susceptibility to parasite infection.

Collectively, several field observations indicate a highly efficient transmission where a limited number of infected vectors are responsible for a significant disease burden. This optimization of transmission and spread into the mammalian host population illustrates the beneficial trade-off for a pathogen of vector-borne biological transmission despite the stringent physical and immunological bottlenecks to conquer in the invertebrate host. As such, despite the continuous arms race, parasites find shelter in a susceptible invertebrate host, which provides them with opportunities to replicate, to exchange genetic material, to possibly be conditioned to resist innate immune responses and to be transmitted to a range of vertebrate hosts during the natural blood feeding of the vector.

ADAPTATIONS AND COUNTER-ADAPTATIONS OF PARASITIC PROTOZOA TO THEIR VERTEBRATE HOST

Parasites have evolved a wide range of strategies to overcome the defense system of their host and eventually colonize it. Whether intracellular or extracellular, they have established ways of passively escaping the attacks of their host, as well as the capability to actively modulate the host immune responses to create a parasite growth-favorable environment. To maintain these capabilities, parasites are forced to continuously adapt to the evolving immune responses and genetic environment of their hosts, both at the individual and population level.

In vertebrate hosts, adaptation happens at two different time scales. Short-term adaptation reflects the almost instant and recurring functions of the adaptive immune system during the lifetime of a single host, while long-term adaptation reflects natural selection and survival of entire host populations over periods of thousands or millions of years. The adaptive immune system competes with pathogen diversity and evolution at the level of a single infected individual. T cells and B cells apply real-time gene rearrangements to create nearly infinite numbers of specific T-cell receptors and antibodies required for targeting the enormous variety of pathogens. Long-term strategies of host populations to cope with pathogen evolution rely on host genetic variation or gene duplication events followed by positive or balancing selection mechanisms acting on them.

In this section (Fig. 3), we first summarize strategies developed by parasites, with the objective of illustrating the variety of tactics they employ to evade and subvert the defense mechanisms of the vertebrate host. Possible evolutionary mechanisms for the acquisition of such traits by parasites are also presented. We then describe the evolutionary pressures mediated by parasites on host genes and finally discuss their potential for co-evolution.

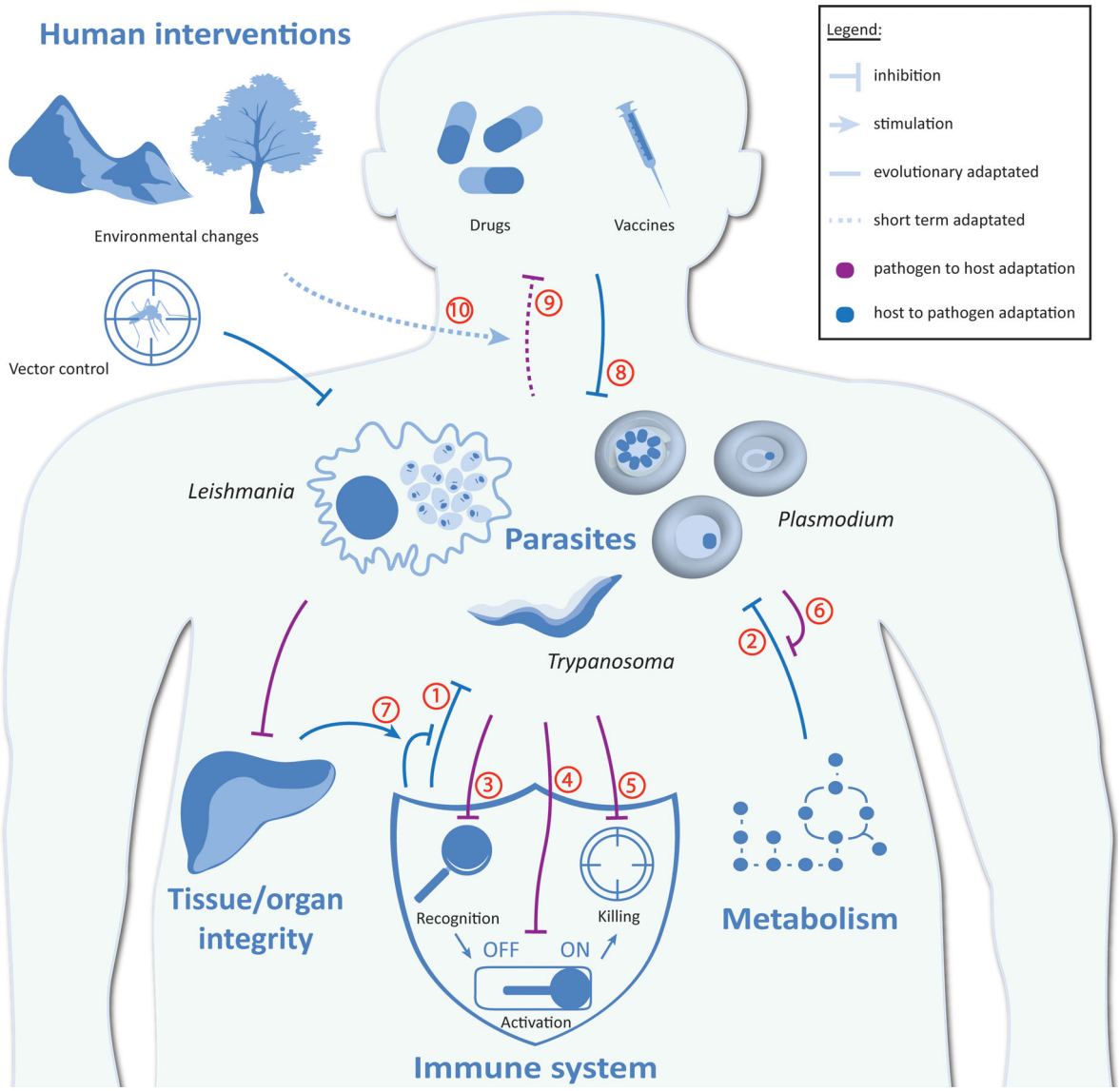

Figure 3. Adaptations and counter-adaptions of parasitic protozoa and their hosts. The immune system of the host evolved so that pathogens can be recognized and eliminated (1) and several host metabolites may negatively impact pathogen development (2). Pathogens have evolved to avoid recognition by the immune system (3), manipulate it to prevent activation (4) and protect against immune effector mechanisms such as oxidative and nitrosative stress (5). Pathogens also adapted to the metabolic stress from the host (6), either by modulating the host's metabolism or compartmentalizing harmful metabolites. Since the interactions between host and parasites often lead to severe tissue damage, hosts may develop a 'tolerance' strategy whereby the immune response is adapted (7). The never-ending arms race between humans and their pathogens has intensified with the development of vaccines, drugs and vector control strategies (8). But pathogens may develop resistance and/or an increased virulence to overcome these human interventions, posing a major threat to pathogen control (9). This can even be exacerbated through environmental changes such as heavy metal pollution that can prime pathogens to develop drug resistance (10).

Parasite's escape strategies

Escaping metabolic constraints in the host

Mammalian hosts have the ability to restrict parasite growth by limiting their access to essential nutrients. In particular, regulation of iron availability is a well-described defense mechanism against invading pathogens (Cassat and Skaar 2013). Iron homeostasis is critical since it is indispensable for both host and parasites but also generates high cellular toxicity. Iron is present in heme and iron–sulfur clusters, and serves as a redox catalyst essential for a broad range of biological processes. On the other hand, in the presence of ROS, iron catalyzes the Fenton reaction to produce hydroxyl radicals that damage lipids, DNA and proteins. In mammals, the amount of free extracellular iron is therefore low, most iron is complexed within hemoglobin inside erythrocytes and extracellular iron binds to transferrin with high affinity. The extracellular parasite *Trypanosoma brucei* develops in the bloodstream of its host; thus, to fulfill its iron and heme requirements, high-affinity receptors for both hemoglobin and transferrin are expressed. The receptor TbHpHbR binds the haptoglobin–hemoglobin complex from the host and is essential for heme supply (Vanhollebeke *et al.* 2008). The heterodimeric transferrin receptor is encoded by the expression site-associated genes (ESAG) 6 and 7, which are part of the variant surface glycoprotein expression sites (VSG-ES) (Salmon *et al.* 1994). Of note, the different copies of ESAG6/7 present in the genome are polymorphic (there are about 15 copies of the VSG-ES in the

genome), affecting the affinity of the receptor for a given transferrin and potentially contributing to rapid adaptation of the parasite to different hosts (Bitter *et al.* 1998; Steverding 2003; Stijlemans *et al.* 2015). Activation of phagocytes at the site of infection also limits iron availability to intracellular pathogens: proinflammatory cytokines decrease the expression of transferrin receptors at the surface of phagocytes and enhance expression of the intracellular iron transporter Nramp 1 (natural resistance-associated macrophage protein 1). This efflux pump has been proposed to reduce iron content in early endosomes, hence limiting iron availability in this compartment. Mutations in Nramp 1 impair the ability of phagocytes to control growth of intracellular pathogens linking iron levels to pathogen proliferation (Jabado *et al.* 2000). The intracellular parasite *Leishmania,* which resides in the endosome-derived parasitophorus vacuole of phagocytic cells, has been shown to express an NADPH-dependent iron reductase (LFR1) that converts ferric Fe^{3+} into Fe^{2+} as well as a ferrous iron transporter (LIT1), which subsequently transports Fe^{2+} into the parasite's cytosol (Huynh, Sacks and Andrews 2006; Flannery *et al.* 2011). LFR1 and LIT1 thus compete with mammalian transporters for the same substrates and allow intracellular *Leishmania* to cope with low levels of iron (Flannery, Renberg and Andrews 2013). As reviewed by Schaible and Kaufmann (2005), parasites are well adapted to their niche within the host ecosystem and developed adaptations to successfully exploit existing nutritive sources: the intracellular parasite *T. cruzi* exploit the host cell cytoplasm for survival, while *Leishmania* amastigotes are adapted to the acidic, hydrolase-rich lysosomal compartment, and the apicomplexan *Toxoplasma gondii* associates with endoplasmic reticulum membranes and mitochondria.

Escaping the immune response of the host

Escaping recognition. Parasites developed diverse strategies to escape recognition by the immune system of their host. For instance, a surprising number of infectious agents have the ability to survive and multiply within host cells, including cells of the macrophage–dendritic cell lineage. Although pathogen multiplication within macrophages may appear counterintuitive given the potent antimicrobial and antigen-presenting activities of these cells, it has been suggested to be a way of avoiding immune recognition by reducing surface antigen presentation. The obligate intracellular protozoan parasite *Leishmania* sp., for instance, infects phagocytic cells. Entry of the infectious promastigote parasites into phagocytes occurs following serum opsonization via complement receptor 1 (CR1) or 3 (CR3) (Sibley 2011). This hijacking of cellular components ensures a minimal respiratory burst, which contributes to parasite intracellular survival and 'silences' its presence. *Leishmania* surface protease gp63 has been shown to facilitate the deposition of opsonic complement C3 on the parasites and enhance their binding to human complement receptors. In addition, the proteolytic activity of gp63 decreases fixation of the terminal complement components thereby increasing resistance to complement-mediated lysis (Brittingham *et al.* 1995). It has also been hypothesized that silent uptake of parasites by macrophages could be mediated by apoptotic infected neutrophils ('Trojan horse' model). Neutrophils have indeed been shown to be recruited at the site of a sand fly bite at early stages of *Leishmania major* infection and to be the first infected cells; they gradually become apoptotic and are subsequently cleared by macrophages. The Trojan horse model hypothesizes that this response to apoptotic cells silences the antimicrobial response allowing viable parasites transferred from apoptotic neutrophils to macrophages to survive and multiply (van Zandbergen, Solbach and Laskay 2007; Peters *et al.* 2008).

Antigenic variation. The process of antigenic variation, by which the immunogenic epitopes exposed to the immune system of the host are altered, has been independently developed by several very diverse pathogens: viral, bacterial, fungal and protozoan (Deitsch, Lukehart and Stringer 2009). A clear example of this is presented by the African trypanosomes. The surface of the extracellular bloodstream form of these parasites is covered by millions of copies of a single protein, the VSG. The VSG is a clear target for the host immune system, but a fraction of the trypanosome population is repeatedly switching to a different VSG, thereby escaping antibody-mediating killing. This fraction of trypanosomes is then able to repopulate the host, resulting in the development of long-lasting chronic infections (Pays, Vanhamme and Perez-Morga 2004). Similarly, during infection with *Plasmodium falciparum*, the major parasite antigen expressed on the surface of infected red blood cells is a highly variable protein called *P. falciparum* erythrocyte membrane protein 1 (Kirkman and Deitsch 2012). Antigenic variation processes have also been observed for the surface protein of the gastrointestinal parasite *Giardia lamblia* (variant-specific surface protein) (Prucca *et al.* 2008).

The molecular mechanisms underlying antigenic variation are not always completely understood, but involve genetic and epigenetic processes, as reviewed by Deitsch, Lukehart and Stringer (2009). In most of the species where antigenic variation occurs, the variable antigens are encoded by large, multicopy gene families, from which only one is expressed at a time (mutually exclusive expression). Antigenic variation can arise following gene conversion, by which the expressed sequence is altered through DNA recombination with one of the multiple genomic copies in the repertoire, leading to a genetic modification. Control of the expression of the active gene and silencing of the other copies frequently depends on epigenetic modifications, including histone modifications, modified nucleotides, changes in chromatin structure or nuclear organization. In addition, translational regulation has also been shown to support antigenic variation. In *G. lamblia*, for instance, multiple members of the variant gene family are expressed but most of the transcripts are degraded through the RNAi pathway, leading to the expression of a single surface protein at a time (Prucca *et al.* 2008).

Modulation of host cell signaling. Parasites have developed the capability to actively modulate the response of their host by interfering with host cell signaling in various ways, with the consequence of attenuating the antimicrobial response and generating an environment favorable for pathogen growth. Several intracellular pathogens have been shown to hinder NF-κB signaling leading to macrophage unresponsiveness. The kinetoplastid parasite *T. cruzi* infects and multiplies in macrophages and parasite survival and replication has been shown to be dependent on delay in type 1 macrophage activation. The expression of IL-12, which induces a type 1 response, was shown to be inhibited following proteolytic cleavage of NF-κB P65 by *T. cruzi* cysteine protease cruzain (Doyle *et al.* 2011). Cysteine protease-dependent degradation of NF-kB in macrophages has also been shown to prevent macrophage activation during *Leishmania mexicana* infection (Cameron *et al.* 2004), and similar strategies to suppress immune responses have been found in the coccidian parasite *Theileria* (Heussler *et al.* 2002).

The metabolism of L-arginine has also been shown to play an important role in the immune response to several parasites.

L-Arginine can be degraded by inducible nitric oxide synthase (iNOS) into citrulline and the cytotoxic nitric oxide (NO), or by arginase into ornithine and urea. Both enzymes compete for the same substrate, and the availability of L-arginine has been supposed to be a limiting factor for NO synthesis. While the iNOS pathway is induced in proinflammatory type 1 macrophages, the arginase pathway is activated in type 2 macrophages. Various pathogens are able to modulate the arginase pathway in macrophages and as a consequence regulate NO production. Arginase is induced during *T. cruzi*, *T. brucei* and *Leishmania* infections in mice; this enhanced arginase is thought to promote parasite growth by providing polyamines through the ornithine decarboxylase pathway and limiting NO-mediated parasite killing (Iniesta *et al.* 2005; Stempin *et al.* 2008; De Muylder *et al.* 2013). The balance between inflammatory and anti-inflammatory responses is therefore critical to determine tolerance or susceptibility to infections, and pathogen factors actively influence it (Raes *et al.* 2007).

Acquisition of virulence traits by parasites

Horizontal gene transfer

The mechanisms by which organisms become pathogens are not completely clear, but general principles are emerging. Horizontal gene transfer, a mechanism that enables acquisition of genes from other organisms, has been widely described in bacteria and is also thought to be responsible for the acquisition of new genetic material in protozoans (Alsmark *et al.* 2013). The origin of these new genetic sequences could be diverse, ranging from endosymbionts, bacteria sharing the same environment or the eukaryotic host. In the apicomplexan parasite *Plasmodium*, several acquisitions through horizontal gene transfer have been hypothesized based on phylogenetic studies following genome sequencing (Kishore, Stiller and Deitsch 2013). The ApiAP2 family (the primary class of transcription factors) is thought to come from the algal endosymbiont that later degenerated into the apicoplast (Balaji *et al.* 2005) and protein domains involved in cytoadhesion and O-linked glycosylation as well as an epigenetic machinery were acquired through horizontal transfer from the animal host (Kishore, Stiller and Deitsch 2013). In *Leishmania* and *Trypanosoma* genomes, lateral gene transfer from prokaryotic donors has been suggested to provide novel genetic material (Alsmark *et al.* 2013). In this case, transfer could occur when parasites are in close contact with bacteria in the gut of the insect vector for instance.

Genetic acquisitions that are maintained through successive generations are assumed to confer significant advantage to the parasites and be key for a successful parasitic lifestyle: for instance, the ApiAP2 transcription factors are important for gene expression and regulate progression of the developmental cycle of *Plasmodium*, and protein domains involved in cytoadhesion and O-linked glycosylation are essential for successful colonization of the host (Templeton 2007; Kishore *et al.* 2009; Wijayawardena, Minchella and DeWoody 2013).

Acquisition of novel genetic material could also occur through mobile genetic elements or viruses when integrating into genomes. Some *Leishmania* strains are infected by the double-stranded RNA virus LRV (*Leishmania* RNA virus), and long-term association as well as co-evolution between *Leishmania* and LRV has been demonstrated (Widmer and Dooley 1995). The presence of the virus is linked to severity of leishmaniasis symptoms, parasite persistence and a greater tendency of cutaneous lesions to evolve into mucocutaneous forms (Hartley *et al.* 2013). It was also recently shown to be associated with treatment failure in cutaneous leishmaniasis due to *L. braziliensis* (Adaui *et al.* 2016) and *L. guyanensis* (Bourreau *et al.* 2016). However, although integration of double-stranded RNA virus and subsequent lateral gene transfer into eukaryotic genomes has been previously shown, integration of LRV sequences into *Leishmania* genome has not yet been observed.

De novo innovations

Besides acquisition of foreign genetic material, mechanisms of changes at the individual level include point mutations, base substitutions and recombination. The rate of appearance of mutations varies between pathogen species and is subject to natural selection (Earl and Deem 2004). It has been shown that pathogens display higher mutation rates *in vivo* than *in vitro*, suggesting that the host–pathogen interaction itself contributes to the mutation pressure on pathogens eventually leading to novel genetic traits important for the pathogen's lifestyle (Brown *et al.* 2006).

De novo innovations via gene and/or genome plasticity have also been described as a possible mechanism for generation of diversity and acquisition of novel properties. Genome plasticity is well described in *Leishmania* where gene content can be amplified at the whole chromosome level (aneuploidy) or at the level of gene arrays (episomes). The copy number of chromosomes does not only seem to differ between different parasite strains of the same or different species (Downing *et al.* 2011; Rogers *et al.* 2011) but also between different cells from the same strain (Sterkers *et al.* 2011). To amplify genes to an even greater extent, *Leishmania* is also able to generate extrachromosomal amplicons of gene arrays through DNA rearrangements involving homologous repeated sequences that are widespread throughout its genome (Ubeda *et al.* 2014). These types of copy number variation in *Leishmania* likely have a major impact on parasite adaptation, as this parasite shows polycistronic transcription and therefore does not dispose of transcriptional regulation. This is well exemplified by phylogenetic studies performed on whole genome sequencing data of more than 200 clinical *L. donovani* isolates from the endemic region in the Indian subcontinent, which clustered these isolates into several populations differing in their genomic structure (mostly copy number variations), but showing a low level of nucleotide polymorphism. The emergence and expansion of these populations could be associated with historical facts (reported visceral leishmaniasis outbreaks; massive antileishmanial drug campaigns or the antimalaria vector DDT campaign) linking the wide capacity of adaptation of these parasites to their extensive genomic variation (Downing *et al.* 2011).

Recombination

Sexual cycles exist for several unicellular parasites (apicomplexa and kinetoplastid parasites for instance), allowing for recombination between genomes and generation of genetic diversity (Tait 1980). Genetic exchange between different strains and generation of hybrid parasites has been experimentally demonstrated for the kinetopalstids *Leishmania* and *Trypanosoma* (Akopyants *et al.* 2009). In both cases, the inheritance pattern of nuclear DNA fitted a model of meiosis followed by fusion of haploid cells. These mating events specifically occurred in the insect vector allowing for transmission of hybrid parasites to the mammalian host. These experimental observations have been confirmed in field isolates in which *Leishmania* hybrids have been identified after whole genome sequencing of strains isolated from sand flies (Rogers *et al.* 2014).

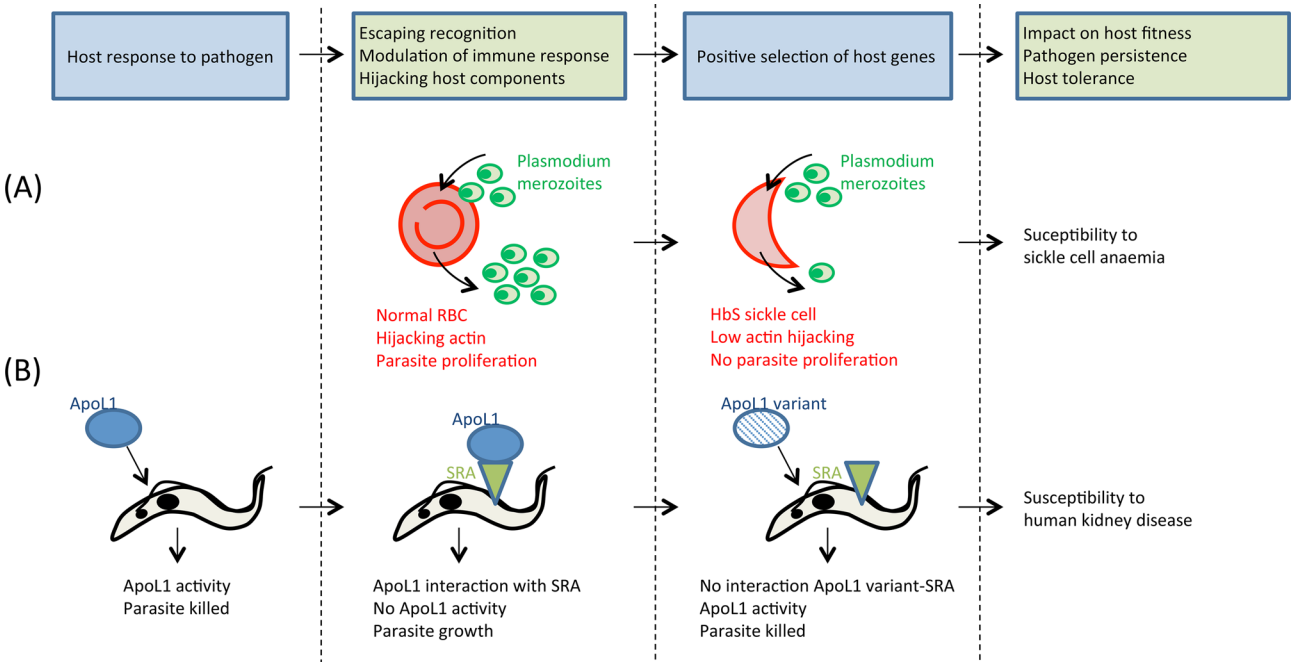

Figure 4. Examples of arms race in different pathogen-vertebrate host models at a trade-off for host fitness. (**A**) Malaria: *Plasmodium* merozoites reproduce in red blood cells by hijacking actin filaments. Individuals carrying hemoglobin genetic variants HbS, HbC or HbE develop sickle cell red blood cells which are relatively resistant to *Plasmodium* infection because of a lower availability of actin to the parasite. These individuals suffer from higher rates of sickle cell anemia-related morbidity. (**B**) African trypanosomiasis: most African trypanosome species are killed by ApoL1, the lytic factor of human serum. Human pathogenic species like *T. brucei rhodesiense* express the SRA protein that interacts with ApoL1, thereby preventing its trypanolytic activity. Individuals from West African descent acquired ApoL-1 variants with restored activity against *T. brucei rhodesiense*, but associated with higher rates of renal disease.

Parasite-mediated selection of host immunity and non-immunity genes

Immunity genes

Pathogens causing significant host morbidity and mortality often directly affect the reproductive success of diseased individuals, thereby selecting for genetic variants in the host population with increased resistance to infection or disease. Genome-wide scans of positive selection, which look for genes or genetic regions that show rates of evolution faster than average, have shown that this process has particularly acted on immunity genes (Barreiro and Quintana-Murci 2010; Meyerson and Sawyer 2011). Interestingly, selection of immune system genes only began relatively recent in human evolution, approximately 10 000 years ago (Barreiro and Quintana-Murci 2010). A plausible trigger for this was the start of agriculture around the same time, resulting in increased exposure to new zoonoses due to the domestication of animals and increased population sizes facilitating the spread of infectious diseases. Genetic variation in the HLA region probably constitutes the best studied example of pathogen-mediated selection pressure on host immunity (Spurgin and Richardson 2010). HLA class I genes, which specifically regulate T- and NK-cell responses to viruses and intracellular bacteria and protozoa, comprise more than 7000 allelic variants discovered in human populations to date (Robinson *et al.* 2013). This extraordinary genetic diversity is believed to be driven by balancing selection under the influence of the wide variety and fast evolution of human pathogens, as evidenced by a positive correlation between pathogen diversity and HLA class I variability in different populations around the world (Prugnolle *et al.* 2005). HLA genes also show specific signatures of positive selection resulting from single pathogens. The HLA*B53:01 allele, for instance, is associated with a significantly reduced risk of malaria in children, and as a result reached high population frequencies in several West African countries (Cooke and Hill 2001).

Non-immunity genes

Positive selection can also occur for genetic mutations in non-immune response genes that confer protection against pathogens by interrupting or hindering certain steps in the pathogen replication cycle. The most compelling examples of this are the hemoglobin genetic variants (e.g. HbS, HbC, HbE) that cause resistance to *P. falciparum* malaria. These variants have reached high population frequencies in endemic areas around the world, despite the increased risks of sickle-cell disease conferred by them (Cooke and Hill 2001). Likewise, FY*0, a null mutation of the Duffy antigen erythrocyte receptor for *P. vivax*, nearly reached fixation in many sub-Saharan African populations. Another example of human selection acting on a non-immunity gene is given by apolipoprotein L-1 (ApoL-1), a high-density lipoprotein component present in human serum that is capable of killing most trypanosome parasites (Vanhollebeke *et al.* 2007). However, the two trypanosome species that cause sleeping sickness in humans, *T. brucei gambiense* and *T. brucei rhodesiense*, are able to resist to ApoL-1 activity. For *T. brucei rhodesiense*, the mechanism of resistance has been shown to be dependent on the expression of the serum-resistance associated (SRA) protein, which neutralizes ApoL-1 through coiled-coil interactions. Recent findings show that individuals from West African descent acquired ApoL-1 variants with restored activity against *T. brucei rhodesiense* (Genovese *et al.* 2010). Interestingly, these ApoL-1 variants were found to be associated with higher rates of renal disease, illustrating the powerful influence that pathogens can have on shaping human genetic evolution (Pays *et al.* 2014). Figure 4 illustrates these examples of arms race between hosts and parasites.

Host tolerance to infection and persistent pathogens

Tolerance

The outcome of infections ranges from death of the host to clearance of the parasite. However, these two extreme situations are often not optimal: wiping out one player does not necessarily improve survival of the other one. A wide range of disease symptoms are therefore actually observed, all consequences of different interactions between host and pathogen. On the host side, disease symptoms often result from damage to cells, tissues or organs (Casadevall and Pirofski 1999). Damage can be mediated either directly by the pathogen or by an excessive immune response of the host. Therefore, in addition to developing resistance to pathogens (i.e. reducing pathogen burden via the immune system), the host can develop a 'tolerance' strategy whereby it protects itself by reducing the negative impact of infection on its fitness without affecting pathogen burden (Medzhitov, Schneider and Soares 2012). Indeed, immunopathology and pathogen load are independent traits, as witnessed by various infections where severe pathology arises in the presence of low levels of pathogen or vice versa. *Plasmodium* infections, for instance, are associated with hemolysis and release of hemoglobin, inducing tissue damage and organ failure. *Plasmodium*-infected hosts have been shown to prevent the cytotoxic effects of free heme via the expression of the heme-catabolyzing enzyme heme oxygenase-1. This cytoprotective response prevents the development of hepatic failure without affecting pathogen burden (Seixas *et al.* 2009). Similarly in experimental and natural African trypanosomiasis, the pathogenicity developed during infection has been shown to be dependent on damage inflicted by the inflammatory immune response rather than by the parasite itself (Magez *et al.* 2002).

Persistence

Some pathogens, on the other hand, have acquired the ability to persist for long periods in their mammalian host, either in a non-replicative dormant state (*To. gondii or P. vivax* hypnozoites) or still replicating but being maintained at very low levels (*T. cruzi*). Persistence is again dependent on the interaction between the pathogen and its host. Following the acute phase of Chagas disease, characterized by a high parasitemia and an inflammatory response, the chronic phase of the disease is in most cases asymptomatic and associated with seropositivity for antibodies against *T. cruzi* without pathological symptoms (Nagajyothi *et al.* 2012). This situation results from the host innate and adaptive immune responses that control parasite levels without achieving complete eradication (Tarleton 2007; Kayama and Takeda 2010). Infections by *Leishmania* are also often characterized by pathogen persistence. Chronic dermal leishmaniasis, for instance, shows low levels but persistent parasites at the lesion site, which have been associated with immunological hyperreactivity and overproduction of pro- and anti-inflammatory cytokines (Navas *et al.* 2014). Persistent parasites could also be involved in post kala-azar dermal leishmaniasis, a cutaneous disease caused by *L. donovani* that develops after apparent successful cure from visceral leishmaniasis (Mukhopadhyay *et al.* 2014).

Co-evolution

The balance between a protective immune response and pathogen survival during persistent infections is therefore crucial for both the host and the pathogen, with the host reducing the immune response when it becomes more harmful than the presence of the pathogen. Persistence, on the other hand, increases the transmission potential of a pathogen. Such a 'compromise' between host and pathogen typically occurs only after long periods of mutual adaptation, a process also termed co-evolution. This was recently reviewed for *Plasmodium* (Deroost *et al.* 2016) and trypanosomes (Berthier *et al.* 2015). In the former paper, the authors discuss among others how the balance between antiparasite immunity versus immunomodulation and escape mechanisms of the parasite can lead to parasite clearance or chronic infection without major symptoms, while imbalances can cause damage to both parasite and host (Deroost *et al.* 2016). Infection by trypanosomes could reach a status, called trypanotolerance, where the proliferation of parasites is controlled and the pathological consequences of infection are limited (reviewed by Berthier *et al.* 2015). Trypanotolerance has been well described in West African bovines that are able to survive, reproduce and produce in testse-trypanosome infested areas without the use of chemotherapy. The heritable nature of trypanotolerance is established and Berthier *et al.* (2015) review the candidate genes in the host; however, experimental and epidemiological studies (including mathematical modeling) integrating hosts and pathogens are needed (Tibayrenc 2010) to fully understand this co-evolution. A major product of this phenomenon can be asymptomatism (or paucisymptomatism), which is increasingly documented (Berthier *et al.* 2015). In visceral leishmaniasis, for instance, up to 90% of the infections may remain asymptomatic in the Indian subcontinent (Ostyn *et al.* 2011), hereby representing a major threat for control programs (Das *et al.* 2014).

Parasite adaptations to drug treatment

The strategies described above, used by parasites to escape the defenses of the vertebrate host, also apply in a context of drug treatment and contribute to the generation of drug-resistant parasites. Horizontal gene transfer or *de novo* innovations, dormancy and modulation of the host immune system have shown to be mechanisms that allow parasites to overcome chemotherapy.

The genome plasticity of *Leishmania,* for instance, has a major impact on parasite adaptations and this is well exemplified by *in vitro* induction of antimonial resistance in *L. donovani* showing that episome copy numbers vary dramatically when drug pressure is applied or alleviated (Leprohon *et al.* 2009). Also in natural antimonial-resistant strains, some genes showed to be present at higher copies than in antimonial-sensitive strains, and this even in absence of the drug (Imamura *et al.* 2016).

Alteration of the cell metabolic rate may also help avoiding drug action. In *P. falciparum*, decelerated development of early ring stages has been linked to delayed artemisinin responsiveness of malaria patients (reviewed by O'Brien *et al.* 2011). This phenotype could be clearly correlated with specific mutations in the propeller domain of the K13 gene of *P. falciparum* (Straimer *et al.* 2015), which relate to an upregulation of the parasite's unfolded protein response and increased levels of phosphatidylinositol-3-phosphate to cope with artemisinin-induced damage (Mbengue *et al.* 2015; Mok *et al.* 2015). Although the exact mechanism of the decelerated development of young rings of *P. falciparum* is not known, it does seem to provide the parasite the necessary time to get the defensive actions mentioned above into place and prevent artemisinin-induced death during drug exposure.

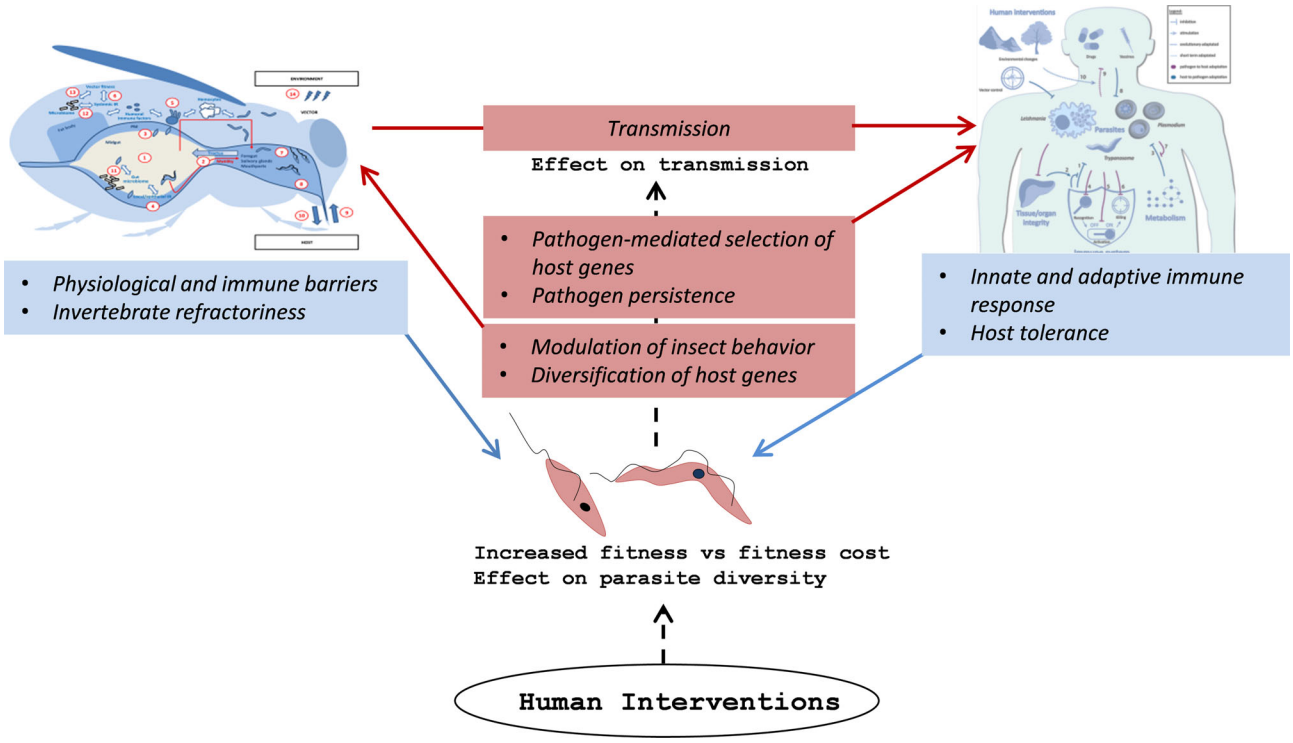

Figure 5. Summary of the interactions between vector-borne parasites and their invertebrate and vertebrate hosts and impact of human interventions (treatment, vaccination, vector control or environmental changes) on these interactions.

Many pathogens also have multiple life stages within a host, some of which are in a dormant stage that is often significantly less susceptible to drug treatment, e.g. liver hypnozoites of *P. vivax* and *P. ovale*. These are specific pathogen traits that have developed throughout thousands of years of evolution and also prove useful to these pathogens at times that their hosts (humans) are trying to step up in the host–pathogen arms race by developing drug treatment. Life cycle specificities may thus significantly affect treatment efficacy and drug susceptibility. This has been reviewed in detail for *Plasmodium*, *Leishmania* and *Schistosoma* elsewhere (Vanaerschot *et al.* 2014a).

In addition, the host immune response to pathogens plays a crucial role in treatment outcome. Many drugs succeed in significantly reducing the pathogen burden but still rely on the immune system to control or eliminate the remaining pathogen load and hereby achieve treatment cure in the long term. This is for example well described in case of visceral leishmaniasis treatment where a competent immune system is generally a prerequisite for successful treatment outcome, avoiding relapse. It is thus conceivable that pathogens may also try to better adapt to, or even manipulate, the host immune system in order to better resist to drug treatment, especially in case of drugs that actively interact with the host immune system to (better) kill pathogens. SSG-resistant (SSG-R) *L. donovani* carry a specific surface glycoconjugate that interacts with Toll-like receptors of the host cell, leading to higher levels of IL-10 produced by SSG-R *L. donovani*-infected host cells compared to SSG-S *L. donovani*-infected host cells (Mukherjee *et al.* 2013). This IL-10 not only further paralyzes the host immune system to mount an effective immune response, but also upregulates a transporter that exports the drug away from the host cell, limiting exposure of the parasite to the drug.

IMPACT OF HUMAN INTERVENTIONS ON PARASITE'S EVOLUTION

Although vector-borne diseases have plagued human kind for several thousands of years, parasite evolution has especially been challenged in the last few decades through the design of more efficient strategies to either cure infection (drug treatment) or prevent it altogether (vaccination and vector control). In addition to interventions that specifically target parasitic diseases, we also exert a large impact on our environment through agriculture, mining and etcetera. To better understand how human interventions may affect parasite evolution, it is important to assess which geno- and phenotypes can be selected for and whether or not they can be fixated in the parasite population in the long term. In this section, we will discuss more broadly how human interventions such as drug treatment, vaccination, vector control and human-induced environmental changes may impact the natural evolution of pathogen survival skills (Fig. 5). Despite knowing that these interventions most often resulted in a significant reduction of parasite populations, there are also examples in which we seem to have unwillingly speeded up parasite evolution towards more successful pathogens.

Impact of drug treatment

The battery of drugs that pathogens are exposed and adapted to leave permanent traces in their genomes. Depending on how these changes impact pathogen reproduction, transmission and survival—all encapsulated in the term 'pathogen fitness'—they may or may not affect pathogen evolution in the longer term.

The acquisition of the drug-resistant trait often comes at a price referred to as fitness cost: a reduced capacity to

persist or survive in the pathogen population once drug pressure is alleviated (e.g. due to a change in treatment policy). This evolutionary trade-off can manifest itself at any point in the pathogens' life cycle, from a reduced capacity to procreate to defects in virulence or transmission. There are ample examples of parasites such as *Plasmodium* or *Schistosoma* that displayed a clear fitness cost after acquiring drug resistance (Dykes and Demeter 2007; Quiñones-Mateu *et al.* 2008; Babiker, Hastings and Swedberg 2009; Borrell and Gagneux 2009). As long as drug pressure is maintained, these pathogens will remain dominant in the pathogen population, repressing drug-sensitive pathogens. However, when drug pressure is relieved, their fitness cost becomes more apparent and may cause drug-resistant parasites to be outselected by the fitter drug-sensitive wild-type parasites. This is what took place in Malawi, for example: few years after chloroquine pressure was relieved as a result of changes in malaria treatment policies, chloroquine-resistant *Plasmodium falciparum* disappeared and wild-type drug-sensitive parasites were again the norm (Laufer *et al.* 2010). These examples highlight that the effect of drugs on parasite evolution is often minimal on the longer term because the pathogen population, or at least the phenotypes that are present, can revert back to normal when drug pressure is alleviated.

There is one, so far, unique situation where a drug-resistant parasite displays traits of a higher fitness or survival capacity compared to its wild-type drug-sensitive counterparts. In the Indian subcontinent, *Leishmania donovani* has been exposed to SSG, a drug causing general oxidative stress to the parasite, for several decades and too often in non-ideal conditions (reviewed in Vanaerschot *et al.* 2013). Although SSG resistance itself is not yet fully understood, phenotypic studies revealed that SSG-R *L. donovani* of certain genetic subpopulations display greater infectivity and *in vivo* survival skills. These phenotypes are probably due to the acquired immunomodulatory capacity of SSG-R strains mentioned above, leading to a higher parasite load in the patient and a more aggravated disease (reviewed in Vanaerschot *et al.* 2013). Whether this increased survival capacity is induced by the drug resistance mechanism or due to a selection of previously present *L. donovani* strains that were inherently more fit is not yet clear. Interestingly, similar observations were also made for the recently observed MIL-treatment failure in the same region: strain infectivity and not *in vitro* drug susceptibility proved to correlate with MIL treatment outcome of the patient (Rai *et al.* 2013). These results suggest that an increased parasite fitness seems to be a driving factor for treatment failure of *L. donovani* infections, while drug resistance as measured *in vitro* might be only of little relevance. Whatever the driving factor, however, the fact that drug treatment of the host apparently selects for fitter parasites has a major impact on the evolution of *L. donovani* in the region. Pathogen to host adaption is a process that normally takes thousands of years. Now, drug treatment of *L. donovani* seems to have forced the parasite to evolve towards a fitter, and possible also more virulent, form in a matter of decades.

Impact of vaccination

Vaccines are the best examples of a human intervention that modulates the host–pathogen relationship, and more specifically the human immune response. Smallpox was the first and so far only disease to be eradicated through worldwide coverage of vaccination campaigns in the late 1960s–1970s, making it a singular example of the capacity of effective vaccination strategies to completely block the evolution of pathogens. However, there are as yet no equally effective vaccines for vector-borne parasites available, meaning that such vaccines will rather shape parasite evolution than block it. For instance, deep sequencing of the male gamete fertilization factor Pfs48/45, a candidate for a malaria transmission blocking vaccine, indicated that several genotypes likely resistant to this vaccine candidate already pre-exist and may be selected for once the vaccine is actually deployed in the field (Juliano *et al.* 2015). In addition, *in vivo* lab studies have shown that more virulent *P. chabaudi* strains (rodent malaria) are less well controlled by vaccination with AMA-1, a candidate antigen part of several human vaccines against malaria, compared to less virulent strains (Barclay *et al.* 2012). Although the AMA-1 locus itself was not mutated, several serial passaging rounds through AMA-1 vaccinated mice resulted in the selection of parasites with a greater virulence compared to control mice. This strongly suggests that vaccines, just like immunotherapy as described above, indeed have the potential to drive the evolution of more virulent parasites.

Impact of vector control

Human interventions against pathogens that are vector-borne usually also include vector control measures such as insecticide spraying or the use of insecticide-treated bed nets. During the Global Malaria Eradication Programme in the 1950s–1960s, DDT was used to kill mosquitos and stop transmission of the parasite (Mendis *et al.* 2009). Although not documented, this will likely have had a massive impact on the genetic diversity of the *Plasmodium* parasite. This DDT campaign also killed sand flies, the vector of *L. donovani*, leading to a significant reduction of visceral leishmaniasis cases in the region. The whole genome study on over 200 clinical strains of *L. donovani* showed that this DDT campaign had a major impact on the genetic diversity of *L. donovani* present in the Indian subcontinent (Imamura *et al.* 2016). Only few genetic lines, likely those in regions not affected by (adequate) DDT spraying, were able to survive this intervention and recolonized the human population in the decades to come. Since the pathogen has no way to adapt to this type of stress—it is the sand fly host that is killed—this deleterious effect on parasite diversity is random from a pathogen fitness point of view. It can swipe away developing phenotypic traits that were previously under selection and can give the chance to previously less successful variants to take the upper hand and recolonize endemic regions if the fitter parasites were eliminated during the intervention. Less intuitively, pathogen survival can also be more directly affected by insecticide spraying. For instance, spraying with neonicotinoid clothianidin to protect crops has shown to negatively affect the immunity of honey bees, promoting replication of viral pathogens in these insects (Di Prisco *et al.* 2013). However, we have as yet not found any proof of introduced (epi-) genetic changes at the parasite level due to insecticide spraying. Nevertheless, one might expect that substandard insecticide spraying could also result in pathogen exposure, and perhaps adaptation, to insecticides and hereby affect their evolution.

Impact of environmental changes

In addition to pathogen or insect control measures, humans also affect their environment through their waste, both from personal and industrial origin (mining, etcetera). Individuals under antibiotic treatment, both humans and (agricultural) animals, excrete low levels of antibiotics into their environment, hereby applying a low but effective selection pressure for resistant bacteria (Kümmerer 2009). Industrial mining, and even

the use of water tube wells, are known causes of widespread heavy metal pollution, with arsenic being one of the main contaminants. Since heavy metal resistance genes are often located on the same plasmids as drug resistance genes in bacteria, low levels of heavy metals in the environment promote survival of (multi-)resistant bacteria (Gullberg *et al.* 2014). Similarly, many (old) drugs against neglected tropical diseases are based on the toxic action of heavy metals, making that environmental pollution can also contribute to the emergence of drug resistance in several vector-borne parasites. Indeed, arsenic pollution of the ground water has been linked to high levels of antimonial treatment failure in the Bihar state in India and neighboring regions (Perry *et al.* 2011). This link has been further substantiated by inducing *L. donovani* resistance to SSG by exposing infected mice to arsenic through their drinking water (Perry *et al.* 2013). Human-induced environmental changes might thus very well have contributed to the emergence and spread of the SSG-R *L. donovani* strains with their specific immunomodulatory traits that contribute to their higher fitness (as described in earlier sections).

CONCLUDING REMARKS: PARASITE SURVIVAL SKILLS AND DEVELOPMENT OF NEW TOOLS TO CURE AND CONTROL INFECTIOUS DISEASES

Understanding pathogen adaptations to their final or intermediate host is crucial for the discovery of appropriate therapeutics. In addition, the benefit of studying host–pathogen interactions from an evolutionary perspective lies in identifying defense mechanisms that are fundamentally important, safe and that can stand the test of time. Novel therapeutic strategies relying on host–pathogen interactions aim at targeting host factors or pathways important for pathogen development instead of directly targeting a pathogen function. This strategy has recently received increasing attention for antiviral therapy but also for other intracellular pathogens (Prussia *et al.* 2011). The identification of host candidate targets has been facilitated by the development of experimental approaches such as systems-wide screens for host factors essential for pathogen replication as well as advances in bioinformatics for data mining (Jayaswal *et al.* 2010; König *et al.* 2010). Immunotherapy, for example, is a strategy by which compounds modulate the immune system to achieve a protective response and pathogen elimination. This approach has received considerable interest for the treatment of leishmaniasis (Roatt *et al.* 2014; Singh and Sundar 2014). For instance, combination therapy with recombinant human IFN-γ and pentavalent antimonials has shown stronger parasitological and clinical cure compared to antimonials alone in patients with visceral leishmaniasis. However, the example of the association between resistance to antimonials (a drug known to target host immune factors, among others) in *Leishmania* and increased virulence is a warning for prudence, as targeting host factors could also lead to the selection of pathogens that are better adapted to the host immune system (Vanaerschot *et al.* 2013). Therefore, new intervention strategies that prevent the emergence of fitter/drug-resistant pathogens are urgently needed. Antimalarial drugs with transmission-blocking activity such as atovaquone that target gametocyte development (the stage transmitted to mosquitoes) have proven their capacity to eliminate malaria from laboratory populations (Blagborough *et al.* 2013). Although atovaquone was rapidly deemed unsuitable as antimalarial treatment in the field due to high rates of recrudescence after development of secondary resistance (Chiodini *et al.* 1995), combination treatments that contain other drugs with transmission-blocking activity have great potential in the fight against vector-borne diseases. Another way to prevent resistant pathogens to be transmitted is the use of evolutionary traps that steer parasite populations into an evolutionary path that will be detrimental for their future development or transmission, a strategy that was recently shown to be a promising concept for the treatment of *Candida albicans* infections and possibly also glioblastoma (Chen *et al.* 2015).

Co-evolution and asymptomatism, its main outcome, deserve a particular attention. From an anthropocentric perspective, asymptomatism may represent an ideal evolutionary path for the parasite and most of all for human health. On the other hand, however, it represents a threat for the most susceptible subjects in the population (due to malnutrition, immunodeficiency, etcetera), especially if asymptomatic infections are important contributors for transmission of the parasites. There is thus urgently more research required into the role of asymptomatism to optimize disease control programs (including both drug treatment and vector control).

Recognition of the importance of the invertebrate host in pathogen transmission highlights that intervention strategies targeting the vector could offer a number of opportunities for disease control. Current vector control strategies, such as the use of insecticide-treated nets against malaria or killing tsetse fly traps against sleeping sickness, are mainly based on reducing vector survival, man-vector contact and vector densities, and do not affect vector–pathogen interactions. The natural bottlenecks that are to be overcome in the invertebrate host by the pathogen are very stringent, resulting in often high degrees of refractoriness that are observed in natural vector populations. These bottlenecks involve physiological barriers and immune barriers and are influenced by the microbiome. Plausible pathogen control strategies could aim at narrowing the existing bottlenecks by increasing the immune barriers or by modifying the microbiome to increase the number of communities with potential antipathogen activity. This can be achieved using naturally occurring bacterial communities (Cirimotich, Ramirez and Dimopoulos 2011) or genetically modified microbial agents that can potentially be introduced in vector populations (Fang *et al.* 2011; Wang *et al.* 2012). As the vector longevity and reproduction are important parameters that determine the lifetime number of infectious bites, life-shortening strategies using endosymbiotic organisms such as *Wolbachia* could be of interest to be explored (Kambris *et al.* 2009). Given the high rate of pathogen–invertebrate host co-evolution and the potential detrimental effects of invertebrate host speciation under pressure from changing environmental conditions, it will be important to continuously and proactively monitor the vectorial competence and capacity of arthropods. Also behavioral changes of infected vectors have to be carefully monitored in order to optimize and adapt vector control strategies over time and have a maximal effect on those infected arthropods.

The evolutionary solutions developed by vertebrate as well as invertebrate host species fighting off pathogens are like a treasure trove waiting to be uncovered. While it is clear that investments in their discovery will be paid off by the leads and solutions they will provide to the development of modern and effective medicines, it is equally clear that the ongoing adaptations of pathogens under drug pressure should be carefully surveilled to avoid development of resistant and better adapted pathogens.

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