Bulletin of Entomological Research (2015) **105**, 523–532
doi:10.1017/S0007485315000504

Immune defence mechanisms of triatomines against bacteria, viruses, fungi and parasites

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Abstract

Triatomines are vectors that transmit the protozoan haemoflagellate *Trypanosoma cruzi*, the causative agent of Chagas disease. The aim of the current review is to provide a synthesis of the immune mechanisms of triatomines against bacteria, viruses, fungi and parasites to provide clues for areas of further research including biological control. Regarding bacteria, the triatomine immune response includes antimicrobial peptides (AMPs) such as defensins, lysozymes, attacins and cecropins, whose sites of synthesis are principally the fat body and haemocytes. These peptides are used against pathogenic bacteria (especially during ecdysis and feeding), and also attack symbiotic bacteria. In relation to viruses, Triatoma virus is the only one known to attack and kill triatomines. Although the immune response to this virus is unknown, we hypothesize that haemocytes, phenoloxidase (PO) and nitric oxide (NO) could be activated. Different fungal species have been described in a few triatomines and some immune components against these pathogens are PO and proPO. In relation to parasites, triatomines respond with AMPs, including PO, NO and lectin. In the case of *T. cruzi* this may be effective, but *Trypanosoma rangeli* seems to evade and suppress PO response. Although it is clear that three parasite-killing processes are used by triatomines – phagocytosis, nodule formation and encapsulation – the precise immune mechanisms of triatomines against invading agents, including trypanosomes, are as yet unknown. The signalling processes used in triatomine immune response are IMD, Toll and Jak-STAT. Based on the information compiled, we propose some lines of research that include strategic approaches of biological control.

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Keywords: Triatomine, antimicrobial peptides, phenoloxidase, nitric oxide, haemocyte, pathogens

(Accepted 12 May 2015; First published online 17 June 2015)

Introduction

Insects have a complex immune machinery that has evolved as a response to their interaction in all their life-cycle stages with parasites and pathogens. Classic scholarly work has divided the insect immune response to invasive agents into humoral plasma-borne factors and cellular or haemocyte-linked molecules. These two types of response, which may act separately or in unison (Gillespie *et al.*, 1997; Beckage, 2011), have received increasing attention among researchers in the last two decades.

One reason for the intense research on insect immunity is that insects are key players in diseases that directly or indirectly affect humans. For example, triatomines are vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease (Rassi *et al.*, 2010). Triatomines include approximately 130 species that are haematophagous, feeding on vertebrate blood, especially that of small terrestrial and arboreal mammals (Schofield & Galvao, 2009). This feeding habit and their preference for colonizing human dwellings make triatomines key agents in transmitting *T. cruzi* to humans and domestic animals.

As any other insect, triatomines are attacked by a variety of parasites and pathogens. Our understanding of how triatomines react to such agents can be used in a variety of contexts, including biological control. To our knowledge, critical information about immune mechanisms used by triatomines against attacking agents has not been gathered in a systematic fashion. Thus, in the present review, we summarize the still scarce information about how triatomines make use of their immune mechanisms against bacteria, virus, fungi and parasites (for a summary, see supplementary material, fig. 1). Our intention is twofold: (a) to provide a concise update of triatomine immune mechanisms during infection against these pathogens, and (b) to suggest potential areas of future research. Despite the fact that studies of triatomine immunity are scarce, we try to gain some insights from these studies to put forward some ideas of biological control. For a better reading of the present review, the immune response of triatomines is considered separately for each of their distinct attacking agents.

General factors involved in the immune response of insects

Insect immune machinery is composed by cells, molecules and reactions aimed to resist and/or ameliorate the cost of pathogens. A first defensive line is a tough one: the exoskeleton cuticle, epidermic physical and chemical properties, gut epithelium and reproductive accessory glands (Gillespie *et al.*, 1997; Casteels, 1998). These components are capable of secreting lysozymes and cytotoxic compounds. A key feature in this immune machinery is the recognition of non-self-parts via pattern recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs) (Gillespie *et al.*, 1997). Recent research has shown that recognition mechanisms like this, allow the insect to recognize and respond to pathogens more effectively in secondary encounters in an analogous fashion to that of vertebrate memory (e.g. Dong *et al.*, 2006; Cisarovsky *et al.*, 2012; Nava-Sánchez *et al.*, 2015). Once such recognition has taken place, then cellular and humoral immune responses are activated. Typical cellular responses are phagocytosis, nodulation and encapsulation, while humoral include antimicrobial systemic molecules, nitric oxide (NO) production, lysozymes (Gillespie *et al.*, 1997). However, both cellular and humoral responses frequently act together. A key player here in immune response is the phenoloxidase (PO) cascade whose intermediate (e.g. reactive oxygen species) and final products (e.g. surrounding of pathogens using melanin) deal with the elimination (the case of reactive oxygen species) or isolation (the case of melanin) of pathogens (González-Santoyo & Córdoba-Aguilar, 2012).

Bacteria

All instars of the triatomine life cycle ingest blood, which is sterile (i.e. free of bacteria). However, by inhaling air before ecdysis and through coprophagia, triatomines acquire bacteria that eventually reach the intestine (Beard *et al.*, 2002; Balczun *et al.*, 2008; Sassera *et al.*, 2013). In fact, the intestinal microbiota in triatomines is composed principally of bacteria, which multiply rapidly (100–10,000-fold) after the ingestion of blood (Azambuja *et al.*, 2004). However, to obtain some missing nutrients, symbiotic bacteria may also participate in triatomine metabolism either by providing particular dietetic elements (e.g. vitamin B) or by being digested (Beard *et al.*, 2002; Sassera *et al.*, 2013).

Included in this microbiota is *Serratia marcescens*, a bacteria whose development correlates with a decrease in *T. cruzi* populations, because it directly attacks the parasite membrane (Azambuja *et al.*, 2004; Gourbière *et al.*, 2012). The mechanism of this interaction is not entirely clear but, in response to the colonization of *T. cruzi* in the gut, *S. marcescens* seems to act as a haemolytic bacteria that lyses erythrocytes via the production of a pigment called prodigiosin, that ultimately impedes the establishment of *T. cruzi*, without killing the insect (Azambuja *et al.*, 2004). We are unaware, however, whether this possible negative effect of *S. marcescens* has been implemented as a tool for biological control (e.g. using *S. marcescens* as a potential dietary component in triatomine blood sources). On the other hand, it has been observed that the insect immune system produces antimicrobial peptides (AMPs) such as defensins and lysozymes (see subsections 'Defensins' and 'Lysozymes ' below) in response to a population increase of pathogens and symbiotic bacteria, an immune response that negatively affects trypanosomes (Gourbiére *et al.*, 2012). Some immune components against bacteria are cited below.

Antimicrobial peptides

It is known that insects use a variety of AMPs, such as defensins, lysozymes, attacins, cecropins and prolixicins to combat Gram-positive or -negative bacteria. Production of AMPs takes place mainly in the fat body, haemocytes and digestive tract (Boulanger *et al.*, 2006; Vieira *et al.*, 2014). The primary

https://doi.org/10.1017/S0007485315000504 Published online by Cambridge University Press

role of AMPs in triatomine, as well as in other insects, is defending them against pathogens (including, but not limited to *T. cruzi*) (e.g. Ursic-Bedoya *et al.*, 2008). For example, *Rhodococcus rhodnii* (a genetically modified bacteria) produces Cecropin A, an AMP that eliminates *T. cruzi* in the insect (Beard *et al.*, 2002; Dotson *et al.*, 2003; Kollien *et al.*, 2003).

That pathogens have the ability to trigger AMPs in triatomines was elucidated by the presence of different types of bacteria such as *Staphylococcus aureus* (Gram-positive) or *Escherichia coli* (Gram-negative) (Vieira *et al.*, 2014). In the first case *S. aureus* was able to induce transcripts of mRNA for Defensin a and b. In relation to transcripts, a recent study in *Rhodnius prolixus* detected that the largest expression of transcripts for AMPs took place in the anterior midgut via the presence of Defensins or lysozymes. In contrast, a novel AMP, called Prolixicin, was detected in the posterior midgut (Vieira *et al.*, 2014).

Defensins

Defensins (with a molecular weight of 4 kDa) are cysteine-rich proteins that were first analysed in the vector *R. prolixus* (Lopez *et al.*, 2003; Araújo *et al.*, 2006; Boulanger *et al.*, 2006). Defensins are highly widespread products in different insect orders that include Diptera, Hymenoptera, Hemiptera, Coleoptera, Lepidoptera, Odonata and Hemiptera. The primary role of defensins is to fight Gram-positive and -negative bacteria as well as fungi. Their mechanism of action is the formation of holes and canals that disrupt the cytoplasmic membrane of bacteria (Yi *et al.*, 2014).

From the haemolymph of *R. prolixus*, three isoforms of genes encoding different defensins a, b and c have been isolated, with 46, 56 and 51 base pairs (bp), respectively. These peptides have sequences that are similar to those of the defensins found in other insects of the Hemipteran order, and are mainly used against Gram-positive bacteria (Lopez *et al.*, 2003). Interestingly, in *R. prolixus*, the peptide transcription did not take place immediately in the haemocoel, but it did in tissues that were not directly stimulated such as the intestine, suggesting a delayed or systemic transcription of defensins (Lopez *et al.*, 2003). Actually, this systemic effect could be seen in the expression of other AMPs (Ursic-Bedoya *et al.*, 2008). It can be inferred that the transcription of peptides was due to a signaling cascade, because such transcription did not occur immediately after inoculation (Lopez *et al.*, 2003). However, the molecules involved in this signalling cascade have not yet been described in detail (Lopez *et al.*, 2003).

Another vector in which defensins have been isolated is *Triatoma brasiliensis* – *def1* and *def2*- (Araújo *et al.*, 2006; Waniek *et al.*, 2011). In the majority of insect taxa, the sequence of defensins begins with alanine–threonine aminoacids, which is similar to the sequence found in *R. prolixus* and *T. brasiliensis* (Lopez *et al.*, 2003; Araújo *et al.*, 2006). This was supported by sequence alignment and identity of *R. prolixus* with other Hemiptera and Coleoptera (Lopez *et al.*, 2003). The fact that all these insect species, including triatomines, share such defensin sequences, imply that defensins have changed very little among insect lineages; thus, these defensins seem highly conserved enzymes. Despite the similarity in the structure of defensins, their expression varies in different host organs. The expression of *def1* was very low in the small intestine (the site where ingested blood is digested), rectum and salivary glands, but high in the stomach (the site where the greatest quantity of symbionts exists) (Araújo *et al.*, 2006). This suggests a role of this defensin in controlling symbionts in the intestinal tract (Araújo *et al.*, 2006). The second defensin encoding gene, *def2*, showed a coding region of 282 bp, and its active form was similar to that of *def1* (identity of 88.3%), suggesting similar functions.

Finally, other defensins, *def3* and *def4*, have been studied in *T. brasiliensis* (Waniek *et al.*, 2009). At days 3 and 5 post-feeding, *def3* was principally expressed in the fat body, salivary glands and small intestine, and was activated mainly in the stomach and fat body (Araújo *et al.*, 2006; Waniek *et al.*, 2009).

Lysozymes

Lysozymes (with a molecular weight of 15 kDa) were the first AMPs isolated and purified from insects, initially in *Galeria mellonella* and *Bombyx mori* (Azambuja *et al.*, 1997) and then corroborated in other insects orders (Fujita, 2004). These enzymes hydrolyse the β−1, 4-glycosidic bond between the *N*-acetylmuramic and *N*-acetylglucosamine acids of peptidoglycans that are present on the cellular wall of Gram-positive bacteria, including *Bacillus megatarium* and *Micrococcus luteus* (Azambuja *et al.*, 1997; Kollien *et al.*, 2003). This hydrolysation causes cellular rupture (Azambuja *et al.*, 1997; Araújo *et al.*, 2006; Balczun *et al.*, 2008; Ursic-Bedoya *et al.*, 2008).

Four groups of lysosymes have been described: chicken type (c), goose-type (g), invertebrate type (i) and viral type (v), with insects belonging to the c group (Araújo *et al.*, 2006; Balczun *et al.*, 2008). Although lysozymes are only active against Gram-positive bacteria, they can create a synergic effect when associated with other AMPs such as cecropins and attacins (Azambuja *et al.*, 1997; Ursic-Bedoya *et al.*, 2008; Vieira *et al.*, 2014). This synergism implies an augmented antibacterial activity such as degradation of the bacterial cell-wall, lytic activity that affects the permeability of the outer membrane and extended action against Gram-negative bacteria such as *E. coli* (Engstrom *et al.*, 1984; Azambuja *et al.*, 1997; Kollien *et al.*, 2003). Another example of this synergism is the combination of different AMPs, extracted previously from other insect orders, which cause a potential toxic effect to *T. cruzi* (Fieck *et al.*, 2010).

Among haematophagous insects, the production of lysozymes has been detected in the salivary glands of Tsetse flies (*Glossina* spp.) and the digestive tract of *R. prolixus* (Azambuja *et al.*, 1997; Fujita, 2004). Furthermore, an increase in the concentration of this enzyme was induced in the haemolymph of *R. prolixus* after a direct injection of *Micrococcus lysodeicticus* bacteria into the haemocoel (Azambuja & Garcia, 1987). Given all these sites where lysozymes have been detected, it is unclear where they are regulated. However, evidence in *T. infestans* suggests that lysozyme1 (*lys1*) is regulated in the digestive tract but only after ecdysis and feeding, and that its role is a digestive one (Kollien *et al.*, 2003). A second lysozyme was characterized in the intestine of *T. infestans* too, lysozyme2 (*lys2*), after a bloodmeal, and its catalytic active residues are valine and tyrosine, although it is unclear whether it is involved in immune functions (Balczun *et al.*, 2008). Further research is needed to clarify whether there are other body regions where these lysozymes are regulated.

The function of lysozymes in triatomines is still unclear. Some evidence suggests that lysozymes seem involved in the digestion of symbiotic bacteria or with the movement of bacteria through the digestive tract. For example, in *T. brasiliensis*, lys1 shows a large expression in the stomach which, as aforementioned, is where bacteria multiply rapidly after a bloodmeal (Araújo *et al.*, 2006). Another example comes

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from *R. prolixus* from which two lysozymes, RpLys-A and RpLys-B (with a molecular weight of 15.8 and 15.1 kDa, respectively), were found after inoculation with *E. coli* and *M. luteus* in the haemocoel (Ursic-Bedoya *et al.*, 2008). These lysozymes showed different expression in spite of their localization in the vector, where RpLys-A was among the enzymes in the digestive tract (anterior midgut), and their function correlated with digestion, whereas RpLys-B participates in the immune response and was located in the fat body (Ursic-Bedoya *et al.*, 2008). One final example is that of lysozyme RpLys-A which has a role in immune response after its activity was induced with blood that contained *S. aureus* or *E. coli* (Vieira *et al.*, 2014).

Cecropins

Cecropins are highly alkaline molecules with activity against Gram-positive and -negative bacteria, as well as fungi (Azambuja *et al.*, 1997; Yi *et al.*, 2014). Hultmark *et al.* (1980) inoculated the pupae of *Hyalophora cecropia* (Lepidoptera: Saturniidae) with genetically modified bacteria that induced the production of peptides with antibacterial activity. These peptides were called cecropins and have a molecular weight of 4 kDa (Azambuja *et al.*, 1997). To date cecropins have not been identified in Hemiptera.

Regarding the use of cecropins in *T. cruzi* control, genetically modified bacteria, such as *R. rhodnii*, induced cecropin A production of *H. cecropia* in the lumen of the small intestine of *R. prolixus*, and these impeded the development of *T. cruzi* (Beard *et al.*, 2001). As expected, *T. cruzi* was able to develop in samples of *R. prolixus* that were not inoculated with genetically modified symbionts (Azambuja *et al.*, 1997; Lopez *et al.*, 2003; Ursic-Bedoya *et al.*, 2008, 2011). The inoculation of insects with transformed symbionts has been called paratransgenesis (Durvasula *et al.*, 1999; Beard *et al.*, 2001, 2002), and has been proposed as an alternative for control of Chagas disease through the expression of toxic cecropins in the insect vector to kill *T. cruzi*. Interestingly, also Cecropin A has been combined with other potential AMPs extracted from different insects: *Apis mellifera* (apidaecin and melittin), *Xenopus laevis* (magainin II), *B. mori* (moricin) and *Penaeus monodon* (penaidin) (Fieck *et al.*, 2010). The results showed a potential effect against *T. cruzi* through the inhibition of parasite growth (Fieck *et al.*, 2010).

Attacins and prolixicins

Attacins with a molecular weight of 20–23 kDa are a family of proteins rich in glycine whose activity is limited to a few Gram-negative bacteria such as *E. coli* (Engstrom *et al.*, 1984; Azambuja *et al.*, 1997). Of all the proteins in the insect humoral system, these possibly have the most specific bactericidal activity. This AMP was purified first from haemolymph of *H. cecropia* that was inoculated with bacteria, and two classes of attacins were described: basic (A–D) and acidic (E–F). The characterization of these attacins was achieved from insects such as lepidopteran and dipteran species (Yi *et al.*, 2014). The fact that these attacins play a role in *Glossina* Tsetse flies against *Trypanosoma brucei* (Hu & Aksoy, 2005), suggests that they can be used against *T. cruzi* in triatomines.

Recently, a glycine AMP called prolixicin was characterized in *R. prolixus*. Its sequence showed a region related to the diptericin/attacin family (Ursic-Bedoya *et al.*, 2011). This peptide showed a strong action against Gram-negative bacteria even when it did not show high activity (Ursic-Bedoya *et al.*, 2011). A possible site of expression for this AMP is the posterior midgut and so its function could be the avoidance of bacterial expansion (Vieira *et al.*, 2014).

Virus

The only virus known to attack triatomines is the Triatoma virus (TrV). The discovery of TrV was triggered by the death of fifth instar nymphs of *T. infestans* after feeding (Muscio *et al.*, 1987). Upon observing the intestinal content of the dead insects, spherical particles of 30 nm in diameter were found (Muscio *et al.*, 1987). The content was processed and used to inoculate fifth instar nymphs, which died within 36 h. The immediate symptom was hind leg paralysis (Muscio *et al.*, 1987). These spherical particles were characterized as uncoated viral particles whose viral genome is composed of a positive-sense single-stranded RNA (+ssRNA) (Muscio *et al.*, 1987). The capsid is composed of three main polypeptides of 33, 37 and 39 kDa and a minor one of 45 kDa (Muscio *et al.*, 1987). TrV replicates within the gut cells of triatomines (Rozas-Dennis *et al.*, 2000).

In relation to the specificity of TrV, Rozas-Dennis *et al.* (2000) reported that it not only infects *T. infestans*, but also *T. platensis*, *T. delpontei*, *T. pallidipennis*, *T. rubrovaria* and *R. prolixus*. However, TrV is not pathogenic for vertebrates (Querido *et al.*, 2013). The ultimate effects of TrV, belonging to the Dicistroviridae family (previously called the Picorna virus), are a high mortality rate, delayed development and reduced fecundity (Muscio *et al.*, 1997; Rozas-Dennis *et al.*, 2000).

TrV can be transmitted among triatomines transovarially and through the faecal–oral route (Muscio *et al.*, 2000). The triatomine immune response to TrV is as yet unknown. It would be expected that triatomines rely on PO, NO and/or endosymbiotic bacteria in response to a TrV infection, as has been reported for other insects (Ourth & Renis, 1993; Johnson, 2015).

Fungi

There is a shortage of information about the fungi–triatomine relationship. *R. prolixus* was the first triatomines species from which fungi were isolated, first finding *Nocardia rhodnii* (Moraes *et al.*, 2000) and later *Aspergillus versicolor* (Moraes *et al.*, 2000, 2004). Moreover, the principal fungi from the digestive tract of triatomine adults and nymphs (including *T. brasiliensis*, *T. infestans*, *T. pseudomaculata* and *T. sordida* under laboratory conditions and *T. vitticeps* in wild specimens) were *Aspergillus flavus*, *A. ochraceus*, *A. parasiticus*, *Fusarium* sp., *Trichoderma harzianum* and *Verticillium* sp. (Moraes *et al.*, 2000). In spite of the presence of these fungi, the affected triatomines showed no signs of infection (Moraes *et al.*, 2000). In the digestive tract of nymphs and adults of *Panstrongylus megistus*, *R. prolixus*, *R. neglectus* and *Dipetalogaster maxima*, the following fungal species were also observed: *Aspergillus niger*, *Penicillium corylophilum* and *Acremonium* sp. (Moraes *et al.*, 2000).

Interestingly, *T. cruzi* was not present when these fungi were found in nymphs, suggesting that natural fungal flora present in the digestive tract may influence *T. cruzi* colonization (Moraes *et al.*, 2004). Actually, it is not clear if fungus could deal with *T. cruzi* directly, as has been shown in other vectors, such as *Anopheles* spp. infected with *Plasmodium* species (Thomas & Read, 2007). Moraes *et al.* (2004) just reported high susceptibility to both fungi and *T. cruzi* in *P. megistus*

https://doi.org/10.1017/S0007485315000504 Published online by Cambridge University Press

and *R. prolixus*, and low susceptibility of these pathogens in *D. maxima* and *R. neglectus*. Perhaps the immune response used by triatomines has the same effect on both the fungus and parasite. Further experimental research is needed to test whether the main function of these fungi is to prevent *T. cruzi* colonization.

In general, the fungus–insect interaction has been well documented. If a fungus can overcome the insect immune response, it proceeds to degrade the cuticle, leading to insect death (Thomas & Read, 2007). Indeed, fungi have two principal strategies when facing the host immune response: cryptic forms of growth (blastospores) that evade host defence mechanisms, and the production of immunomodulatory substances (mycotoxins such as dextruxins) that inhibit the host immune response (Thomas & Read, 2007). Furthermore, the two fungal strategies work complementarily: while the component present in the cell wall of the conidia (β−1, 3-glucans) acts as an immunosuppressor, blastospores avoid recognition by the insect immune system (Boucias & Pendland, 1991). How these functions work in triatomines is as yet unknown.

As aforementioned, extremely little is known about the triatomine immune response to fungi. However, some details can be extrapolated from the study of other insects. Different types of immunomodulatory mycotoxins in insects are specific to each type of entomopathogenic fungus. For example, *Metarhizium anisopliae* produces dextruxins and *Beauveria bassiana* beauvericins (Rohlfs & Churchill, 2011), both being important in pathogenesis (Boucias & Pendland, 1991). The action of dextruxin A was evaluated in *R. prolixus* in the Malpighian tubule system, where it inhibits fluid secretion and leads to a decrease in electrical potential (Ruiz-Sanchez *et al*., 2010). This inhibitory capacity against fungi has been found in the insects of agricultural importance (Gillespie *et al*., 2000), and likely also occurs in triatomines.

Fungi produce a number of enzymes (e.g. proteases, lipases, esterases and chitinases) that facilitate invasion of the haemocoel in a number of insects (Boucias & Pendland, 1991; Thomas & Read, 2007). The entire invasion process occurs within 24 h after the adhesion of the conidia to the cuticle and the secretion of dextruxin by the fungus. This mycotoxin acts as an inhibitor and alters haemocyte morphology (Avulova & Rosengaus, 2011). Negative effects of dextruxins include a reduction of insect PO activity (see 'Parasites' subsection 'The PO system' below), and a decreased rate of phagocytosis and encapsulation (Rohlfs & Churchill, 2011). The hyphal bodies, another form of reproduction in the host, initiate the production of a protective covering of collagen that encapsulates β−1, 3-glucans and in this way avoids the encapsulating immune response of the host (Avulova & Rosengaus, 2011). The interactions at immune level between symbiotic or entomopathogenic fungi need to be assessed in triatomines.

Parasites

To date, the only parasites of triatomines whose biology has been studied are *T. cruzi* and *Trypanosoma rangeli*. It is known that *T. cruzi* is able to cause important physiological changes in triatomines such as a delay in nymphal development in *T. infestans* (Schaub, 1989), and a reduction in adult longevity in *R. prolixus* (Schaub, 1989). However, *T. cruzi* did not have such effects in *Triatoma dimidiata* (Schaub, 1989), although in this species it reduces bloodmeal ingestion in infected nymphs, which leads to an immunosuppression as the triatomine is not obtaining enough metabolites (e.g. iron; Schaub, 1989; Schaub *et al*., 2011). These pathological effects have not been considered by other authors and require further study to determine whether or not *T. cruzi* is capable of stressing its vector.

Antimicrobial peptides

Even when AMPs have been studied in the context of antibacterial function, some research has been done with *T. cruzi* (Ursic-Bedoya *et al*., 2011; Waniek *et al*., 2011; Moreira *et al*., 2014). These AMPs include defensins, lysozymes and prolixicins, which could participate in clearing of ingested blood meal and as signalling molecules (Boulanger *et al*., 2006). For instance, it has been shown that the defensin *def1* of *T. brasiliensis* fifth instar nymphs attenuates *T. cruzi* proliferation but does not eliminate the parasite (Araújo *et al*., 2006). Moreover, the role of *def1* depends on the intestinal region: in the stomach it regulates the quantity of symbiotic organisms while in the small intestine it may control *T. cruzi* populations (Waniek *et al*., 2011).

Another AMP, prolixicin (with a molecular weight of 11 kDa), was recently isolated from *R. prolixus*. Paradoxically, it showed no activity against *T. cruzi* (Ursic-Bedoya *et al*., 2011). This enzyme is produced in the fat body and the small intestine of adult insects as a response to infection by Gram-negative bacteria such as *E. coli* (Ursic-Bedoya *et al*., 2011; Vieira *et al*., 2014). A recent study demonstrated that the quantity of prolixicin in the small intestine was insufficient for eliminating *T. cruzi*. One reason for this action is that proteases present in the digestive tract of the host could affect the production of prolixicin peptide (Ursic-Bedoya *et al*., 2011). The question of why *R. prolixus* still produces prolixicin despite its apparent inefficacy awaits further investigation. One possibility is that this response was useful in the evolutionary past but is no longer effective. The fact, however, that immune responses are energetically costly (Schmid-Hempel, 2005) implies that if a response is not needed, it should be selected against. This does not seem to be the case for *R. prolixus*.

The PO system

PO is an enzyme involved in the oxidation of phenols and quinones. This oxidation consists of a cascade of reactions that hydrolyses monophenols and O-diphenols and oxidizes the latter to form O-quinones (Gregorio & Ratcliffe, 1991; Cerenius *et al*., 2008; González-Santoyo & Córdoba-Aguilar, 2012). PO is highly reactive and covalently binds to proteins to form polymers like melanin that encapsulate pathogens. PO normally exists as an inactive precursor called proPO, found in the plasmatic fraction of the haemolymph or in haemocytes of invertebrates (Gomes *et al*., 2003; Genta *et al*., 2010; González-Santoyo & Córdoba-Aguilar, 2012). PO can be activated by enzymes such as trypsin, chymotrypsin and components on the cellular wall of bacteria and fungi (Laughton & Siva-Jothy, 2011).

PO acts against many invasive agents in insects (González-Santoyo & Córdoba-Aguilar, 2012) but has only been assessed in a few species of triatomines. For example, as a response to the presence of *T. rangeli* in *R. prolixus*, higher levels of PO led to an increase in the number of lysozymes and haemocytes as well as to the formation of melanin nodules (Azambuja *et al*., 1999). Interestingly, *T. rangeli* seems to have mechanisms for evading the negative effects of PO, according to the following evidence: (a) although *T. rangeli* is recognized

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and encapsulated by host defence cells, it is capable of surviving and utilizing haemocytes for proliferation (Azambuja *et al.*, 1999); (b) *T. rangeli* has been shown to inhibit PO activity in *R. prolixus*; (c) in *R. prolixus* adults that were infected orally with *T. rangeli* epimastigotes, the proPO system was inhibited at the haemolymph level, the site where the parasite develops (Gregorio & Ratcliffe, 1991; Gomes *et al.*, 2003). This inhibition may actually be the explanation for the negative effect of invasive agents in spite of PO production that has been found a number of insect studies (reviewed by González-Santoyo & Córdoba-Aguilar, 2012). Interestingly, the inhibitory action by *T. rangeli* may not be direct, but instead via the disruption of juvenile hormone regulation, which in turn would affect PO production (Nakamura *et al.*, 2007).

The assumed inhibition of PO by *T. rangeli*, however, needs to be considered in a broader context. PO production in triatomines is condition-dependent, and one key factor driving condition is diet (González-Santoyo & Córdoba-Aguilar, 2012). Thus, whether a reduced PO production is prompted by *T. rangeli* or diet needs to be clarified. On the other hand, inhibitory action cannot be considered a generalized property in triatomines. For example, infection by *T. cruzi* was followed by a high PO activity in the host haemocoel during the first 24 h, but decreased after this time (Mello *et al.*, 1995).

Nitric oxide

NO is a powerful free radical and a highly toxic gas produced by the oxidation of L-arginine to citrulline mediated by the NO synthase (NOS) enzyme (Rivero, 2006). Although NO has been well described as a powerful defence mechanism against parasites, the information is limited about the role played by NO (and free radicals in general) against bacteria, fungi or virus. In a first exploration of this topic, Whitten *et al.* (2001) studied two strains of *T. rangeli* (H14 and Choachi) in *R. prolixus*. These authors concluded that high levels of free radicals (including superoxide and NO) reduced parasite survival and completion of the life cycle. At least for the defence of *R. prolixus* against *T. rangeli*, this suggests a key role of free radicals.

More recently, and also in relation to *R. prolixus*, it was found that the expression of NO takes place in the fat body, haemocytes, haemolymph, stomach epithelium, small intestine, rectum and salivary glands (Whitten *et al.*, 2007), areas where *T. cruzi* and *T. rangeli* are present. However, both parasites seem to interact differently with respect to NO. Whereas the presence of *T. cruzi* activates NOS expression in haemocytes and the stomach, *T. rangeli* suppresses the expression of this molecule (Whitten *et al.*, 2001). One explanation is that the early stimulation of NO production in haemocytes in response to *T. cruzi* impedes its colonization in the haemocoel (Whitten *et al.*, 2001). On the other hand, the inhibition of NO in haemocytes and the fat body by *T. rangeli* likely represents a defence mechanism of the parasite that allows for its development in the haemocoel and therefore its later passage to the salivary glands (Whitten *et al.*, 2001). Furthermore, a high concentration of NO is induced in the rectum during a *T. rangeli* infection, which could force the parasite to leave the middle intestine and then complete its life cycle in the salivary glands (Whitten *et al.*, 2007). However, whether the migration of *T. rangeli* to these glands is a consequence of escaping the triatomine's immune response or a use of NO by the parasite as a signal to complete its life cycle in the glands is unknown.

There are other much less studied effects of nitrogen-based compounds involved in triatomine immune defence. The high concentration of nitrites in the small intestine could possibly avoid penetration of the intestinal epithelium by *T. cruzi* (Whitten *et al.*, 2007; Castro *et al.*, 2012), which would block its dissemination to the haemocoel. Though it is unclear why nitrite concentrations decrease in the small intestine following infection by *T. rangeli* (Whitten *et al.*, 2007), the reason may lie in an inhibitory effect similar to that that occurs with PO. Further studies are needed on the role of NO and its oxygen intermediates in the capacity of triatomines to combat parasitic infections.

Lectins (agglutinins)

Lectins or agglutinins are glycoproteins that have the capacity to agglutinate cells and precipitate glycoconjugates (Azambuja *et al.*, 1997). Lectins are found in the haemolymph and digestive tract of a number of invertebrates, and their synthesis is carried out principally in haemocytes and the fat body (Azambuja *et al.*, 2004). It has been reported that they can act as opsonins, facilitating phagocytosis by haemocytes (Azambuja *et al.*, 1997).

Very little is known about the action of lectins in triatomines. A study that described the activity of agglutinins in the intestine and haemolymph of *R. prolixus* suggested a role for these glycoproteins in the regulation of parasite-vector interactions (Mello *et al.*, 1995; Ratcliffe *et al.*, 1996). Mello *et al.* (1995) showed that there are differences in the agglutination of three different strains of *T. cruzi* – Dm28c, CI and Y – in the stomach as well as the haemolymph of *R. prolixus*, and that these differences correlate with a variation in the carbohydrates on the surface of the parasite. The ability to agglutinate trypanosomes may depend on the amount of lectins produced by the host. *T. infestans* has more lectins than *R. prolixus*, and the former has greater resistance to infection by *T. rangeli* (Azambuja *et al.*, 1999).

Cellular defence mediated by haemocytes

The mechanisms of cellular defence are mediated mainly by haemocytes in insects. Once again, very little information is available on this subject in relation to triatomines. The types of haemocytes in insects vary among taxa, with four main classes that are based on their morphology, histochemistry and functional characteristics (Lavine & Strand, 2002; Borges *et al.*, 2008; Strand, 2008): (a) granulocytes, which have a function of phagocytosis; (b) plasmatocytes, which adhere to parasites to form cellular capsules; (c) oenocitoids, which contain precursors of PO that are involved in the production of melanin; and (d) prohaemocytes, which are stem cells. It is now known that there are six types of haemocytes in *R. prolixus, R. neglectus, Triatoma infestans, P. megistus* and *D. maxima*: prohaemocytes, plasmocytes, cistocysts, oenocytoids, adipohaemocytes and giant granular cells (Azambuja *et al.*, 1997).

Concerning the morphological and functional characterization of cell types involved in the immune response, five different haemocytes were identified in *R. prolixus*: prohaemocytes, plasmatocytes, oenocytoids, adipocytes and granulocytes (Borges *et al.*, 2008). Plasmatocytes and prohaemocytes are the most abundant cells, perhaps due to their direct action against bacteria via phagocytosis. On the other hand, granulocytes and oenocytoids also changed in response to *S. aureus* bacteria. In response to this bacterium no events concerning melanization

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were observed (Borges *et al*., 2008). Despite the lack of evidence, it is likely that triatomine haemocytes are involved in other typical insect haemocyte functions such as nodule formation and encapsulation. Clearly, studying functions of triatomine haemocytes is an open research line.

Signalling mechanisms in triatomines/insect immune response

Our knowledge of signalling mechanism in triatomines is highly limited but one can gain some insights from other taxa (Hoffman & Reichhart, 2002). Similar to other insects, triatomines are likely to rely on three principal pathways to attack pathogens such as bacteria and fungi: IMD, Toll and Jak-STAT (Kingsolver & Hardy, 2012). To trigger any of these pathways, it is necessary to recognize foreign agents and one mechanism is via the PAMPs present on the pathogen's surface. For this, insects use host PRRs which have been identified such as peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins, thioester containing proteins, scavenger receptors, C-type lectins, galactosidose-binding lectins and fibrinogen-like domain molecules (Michel & Kafatos, 2005; Kingsolver & Hardy, 2012). The anterior PRRs could be circulating in the haemolymph and bounding to cells (Michel & Kafatos, 2005). In *R. prolixus* (Vieira *et al*., 2014) and other insects (Hoffman & Reichhart, 2002; Kingsolver & Hardy, 2012), the IMD pathway could be activated by Lipopolysaccharides present in Gram-negative bacteria, and then the induction of different AMPs like defensins could be achieved. The Toll pathway seems to be induced by the presence of PAMPs like β−1, 3-glucans in fungi, and peptidoglycans present in Gram-positive bacteria, that bind with their respective recognition protein: βGRP or PGRP (Boulanger *et al*., 2006; Ursic-Bedoya & Lowenberger, 2007). These two pathways induce a nuclear factor κB (NF-κB)-like transcription factor (Boulanger *et al*., 2006), that could be involved in proteolytic cascades such as melanisation, coagulation, phagocytosis and encapsulation by haemocytes (Ursic-Bedoya & Lowenberger, 2007).

With respect to *T. cruzi* or *T. rangeli*, there is scarce evidence of pathways activated. Worth mentioning is the study of Ursic-Bedoya & Lowenberger (2007) in *R. prolixus*. These authors found that the expression of genes in response to bacteria (*E. coli* and *M. luteus*) or *T. cruzi*, led to the identification of six molecules involved in the above pathways – Transferrin, Nitrophorins, β−1, 3-glucan recognition protein (GRP), haemolymph protein, Rel/Dorsal and Mucin/Peritrophin like.

Recently, the transcriptome of the digestive tract of *R. prolixus* has been sequenced (Ribeiro *et al*., 2014). This study found a high number of genes expressed belonging to the Toll pathway in contrast with other groups of insects such as Dipterans. On the other hand, a low abundance of transcripts related to IMD and STAT pathways were present in the digestive tract (Ribeiro *et al*., 2014). An explanation for these results is that lysozymes and lectins (Toll-regulated) are used more due to their defensive action in comparison with IMD and STAT pathways whose products are for digestive functions (Ribeiro *et al*., 2014).

Conclusions

Scarce information is available on the immune response of triatomines to pathogens, whether in terms of quality (i.e. the description of the immune machinery or pathogenic agents in triatomines) or quantity (i.e. of the ca. 130 triatomine species, no more than 10 have been studied). This can be contrasted to the abundant research on mosquitoes (Cator *et al*., 2012; Clayton *et al*., 2014), whose interactions with other infective agents are well known. For example, by combining the information available on the antiviral responses of mosquitoes and genomics techniques, it may be possible to disrupt RNA interference and the JAK/STAT pathway, thus leaving mosquitoes immune depressed against several arbovirus types (reviewed by Rückert *et al*., 2014). On the other hand, studies on the interaction between triatomines and invading agents have centred on describing the latter, providing very little information on the effects of invasion or the immune response of triatomines. In the case of fungi, for example, a number of species have been described, but their effects and, consequently, the immune response by triatomines are completely absent.

One does not need to start from scratch to understand triatomine defence mechanisms, as there is already information on other insects (e.g. agricultural insect pests; Shah & Pell, 2003). One case is the study of Ribeiro *et al*. (2014) who identified the transcripts of the intestinal tract of *R. prolixus*. As indicated before, these authors found that Toll pathway-related genes that code for lysozymes and lectins were more highly expressed in comparison with other insect orders. The immune responses dictated by these genes were presumed from studies in other insects (Ribeiro *et al*., 2014). Studies like this can serve to design studies on potential triatomine responses. Some strategies that can be explored are the following: (a) the use of already described powerful infective organisms, such as the bacteria *R. rhodnii* (Dotson *et al*., 2003; Kollien *et al*., 2003) and *S. marcescens* (Azambuja *et al*., 2004; Gourbiére *et al*., 2012), as well as TrV (Muscio *et al*., 1987) and fungi (Moraes *et al*., 2000), to kill triatomines; and (b) enhancement of the triatomine immune response to trypanosomes, which consists of cecropins (Beard *et al*., 2001), defensins, lysozymes and prolixicins (Mello *et al*., 1995; Gomes *et al*., 1999; Ursic-Bedoya *et al*., 2011; Waniek *et al*., 2011; Moreira *et al*., 2014), free radicals (Whitten *et al*., 2001) and lectins (Azambuja *et al*., 1999).

Finally and for biological control purposes, several other strategies can be explored. Now that the genome of *T. cruzi* has been elucidated (Grisard *et al*., 2014), researchers should be able to establish new types of adaptive associations between triatomines and trypanosomes, as have been described between humans and trypanosomes (Sistrom *et al*., 2014; in this case using *T. brucei*). One necessary step here is to elucidate the genome of triatomines, which among other possibilities would allow for the identification of triatomine genes that respond to trypanosome infection, and the consequent use of this information to target the immune response for manipulation and biological control. Another possible strategy is to understand the biochemical intimacy of the *T. cruzi*–triatomine interaction. In this sense, Gutiérrez-Cabrera *et al*. (2014) identified the changes in glycoproteins that take place in the triatomine intestinal membrane, allowing for *T. cruzi* adhesion and development. With this information, such glycoprotein composition could perhaps be altered, leading to new strategies for the control of trypanosome establishment and colonization. By last, a multi-pathogen approach could be conceived. One example is the combined use of fungi and nematodes, as has been described for agricultural pests (e.g. Bedding & Molyneux, 1982). In this extent, results in those pests have shown that fungi can weaken the insect cuticle, which allows nematodes to penetrate (Bedding & Molyneux, 1982). Actually, one variable that can be added to this multi-pathogen approach is toxic elements such as what Fieck *et al*. (2010) when including different Cecropins.

https://doi.org/10.1017/S0007485315000504 Published online by Cambridge University Press

Supplementary material

The supplementary material for this article can be found at http://www.journals.cambridge.org/S0007485315000504

Acknowledgements

A. Laura Flores-Villegas acknowledges the scholarship and financial support provided by the Consejo Nacional de Ciencia y Tecnología (CONACyT-326511). This paper constitutes a partial fulfillment of the doctoral work of A. Laura Flores-Villegas in the Doctorado en Ciencias Biológicas of the Universidad Nacional Autónoma de México (UNAM). M. Cabrera-Bravo, P. M. Salazar-Schettino and M. I. Bucio-Torres had a financial grant from PAPIIT-UNAM (IT 200913 and IN 211613), and so did A. Córdoba-Aguilar (IN 222312).

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