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FLORES VILLEGAS ANY LAURA

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Resumen

La enfermedad de Chagas es catalogada actualmente dentro de las "enfermedades olvidadas" y se considera endémica en muchas áreas de Latino América. El agente etiológico de esta enfermedad es el parásito *Trypanosoma cruzi*, transmitido por las heces de insectos hematófagos de la familia Reduviidae.

El control de los transmisores de la enfermedad de Chagas se ha basado principalmente en el uso de insecticidas químicos. Sin embargo, el uso de estos ha generado problemas de resistencia, contaminación ambiental y riesgos para la salud humana, aunando a esto, la conducta peridoméstica de los triatominos hacen difícil su control. En México más del 74% de la transmisión es vectorial y uno de los principales transmisores es la especie *Meccus pallidipennis* y como alternativa de control se ha propuesto el uso de hongos entomopatógenos: *Isaria fumosorosea* (EH-511/3) *y Metarhizium anisopliae* (EH-473/4) que han sido utilizados en el control biológico de insectos que son plagas agrícolas. Dos características principales en estas especies de hongos es su capacidad para afectar la sobrevivencia de insectos y la inhibición de la respuesta inmune. El objetivo de este estudio fue investigar el efecto de dos hongos entomopatógenos en ninfas de *M. pallidipennis*, en términos de sobrevivencia y respuesta inmune.

Se mantuvieron un grupo infectado y un grupo control para cada especie fúngica estudiada: *M. anisopliae* (EH-473/4) e *I. fumosorosea* EH-511/3, y se obtuvieron datos sobre la sobrevivencia durante 30 días y medidas sobre la fenoloxidasa (PO) y profenoloxidasa (proPO) a las 24, 48, 96 y 144 horas. Para la evaluación de la sobrevivencia se utilizó un análisis de Kaplan-Meier, para la respuesta inmune se utilizó un ANOVA factorial de medidas repetidas.

Los insectos tratados con *M. anisopliae*, murieron antes que los insectos tratados *con I. fumosorosea*. Los insectos infectados con cualquiera de los dos hongos mostraron valores bajos de PO y proPO que en insectos control, con un claro decremento en ambos parámetros a las 24 h y no se observaron cambios posteriores a este tiempo.

Nuestro estudio demuestra la posibilidad de utilizar hongos entomopatógenos para el control de éste tritatomino y el efecto negativo en la PO y proPO parece estar mediado por una baja en la regulación de la respuesta inmune del triatomino.

Abstract

Chagas disease is currently listed within the "neglected diseases" and is considered endemic in many areas of Latin America. An estimated of 7-8 million people are infected worldwide. The etiological agent of this disease is the parasite *Trypanosoma cruzi*, transmitted by the feces of blood-sucking bugs fronm the family Reduviidae.

Chagas disease vector control has been based primarily on the use of chemical insecticides. However, there have been problems of resistance, environmental contamination and risks to human health, furthermore, vectors with peridomestic habits are difficult to control. In Mexico more than 74% of transmission is vectorial and one of the main vectrs is the species *Meccus pallidipennis*, as an alternative control has been proposed the use of entomopathogenic fungi: *Isaria fumosorosea* (EH-511/3) and *Metarhizium anisopliae* (EH-473/4) used in the biological control of insects that are agricultural pests. Two main features in these fungi is their ability to affect the survival of insects and inhibition of their immune response. The aim of this study was to investigate the effect of two entomopathogenic fungi in *M. pallidipennis* nymphs, in terms of survival and immune response.

We had an infected and a control group for each fungal species and assessed: a) insect survival during 30 days; and, b) phenoloxidase (PO) and prophenoloxidase (proPO; two key traits in insect immune response) at 24, 48, 96 and 144 h. For survival we used Kaplan-Meier survival analysis while for immune response we used factorial, repeated measures ANOVA for each fungal species.

Animals treated with *M. anisopliae* died sooner than animals treated with *I. fumosorosea.* Infected animals showed lower PO and proPO values tha sham individuals, with a clear decrease in these parameters at 24 h with no further changes after this time.

Our study widens the possibility of entomopathogenic fungi being used for control this triatomine specie.

The negative effect on PO and proPO seems mediated by a down-regulation of the triatomine immune response.

Capítulo 1.
Introducción general

La enfermedad de Chagas se considera un problema de salud pública en América Latina, causada por el parásito *Trypanosoma cruzi* y transmitida por chinches hematófagas (Hemiptera: Reduviidae) (*Steverding, 2014*). El control de estos insectos transmisores, se ha basado principalmente en el uso de piretroides, los cuáles han demostrado ser inefectivos o no ser efectivos a largo plazo y causando problemas de re-infestación (*Pinchin et al., 1980; Schofield & Dias, 1998; Cortico-Correa et al., 2002; Pedrini et al., 2009*). Alternativamente el uso de hongos entomopatógenos se ha implementado para el control de insectos plaga (*Zimmerman 2007; 2008*), transmisores de enfermedades (*Blandford et al., 2005; Scholte et al., 2005; Thomas & Read, 2007*) y recientemente en triatominos (*Luz et al., 2004; Rocha & Luz, 2011; Rocha et al., 2011; Vázquez-Martínez et al,. 2014; Rodrigues et al., 2014*). Los hongos entomopatogenos han demostrado ser altamente eficientes, ya que inducen una reducción en la respuesta inmune de insectos (Wang & St. Leger, 2006). *Meccus pallidipennis* es uno de los principales vectores de la enfermedad de Chagas en México, dada su amplia distribución (*Carcavallo et al., 1997; Salazar-Schettino et al., 2010*), sus hábitos de predominio peridoméstico (*Martínez-Ibarra et al., 2008*) y altas tasas de infección con *T. cruzi* (*Benítez-Alva et al., 2012*).

1.1 Enfermedad de Chagas y *Trypanosoma cruzi*

En los ciclos de vida de algunas especies de patógenos y parásitos, los insectos son de relevancia fundamental ya que funcionan como vectores a humanos y animales, de esta forma causan enfermedades importantes tales como la malaria, tripanosomiasis, leishmaniasis y oncocercosis *(Boulanger et al., 2006)*.

La enfermedad de Chagas o tripanosomiasis americana es catalogada actualmente dentro de las "enfermedades olvidadas" (Neglected Tropical Disease-NTD) endémica en muchas áreas de Latino América. Se estima que entre 7-8 millones de personas están infectadas alrededor del mundo, de las cuales la mayoría vive en Centro y Sur América (*Nouvellet et al., 2015*). La incidencia de esta enfermedad se ha reducido en lugares donde las campañas de control de vectores y el tamizaje en bancos de transfusión sanguínea han sido llevado a cabo, demostrando el potencial eficaz de políticas en salud pública. Sin embargo, *Trypanosoma cruzi* aún causa 10, 000 muertes y la pérdida de 430, 000 DALYs (Disability Adjusted Life Years) cada año, i. e. la suma de años potenciales de vida perdidos debido a la mortalidad prematura y años de vida productiva perdidos por discapacidad) (*Nouvellet et al., 2015*).

El periodo de incubación en humanos después de la transmisión es de 1 a 2 semanas y es durante este período cuando la parasitemia es detectable. Los síntomas son inespecíficos, incluyen fiebre, hepato-esplenomegalia y linfocitosis atípica. En ocasiones, en el 5% de los casos se hace presente un nódulo en la piel (Chagoma de inoculación) o un edema bipalpebral unilateral indoloro (signo de Romaña), los cuáles indican el sitio de la inoculación. Durante la fase aguda las infecciones no son detectables y regularmente las personas que sobreviven a la fase aguda es gracias a la respuesta inmune celular que es capaz de controlar la replicación de la parasitemia, los síntomas se resuelven espontáneamente y, la parasitemia desaparece de 4 a 8 semanas (*Bern, 2015*). Es entonces cuando los pacientes pueden iniciar con la fase crónica de la enfermedad. La mayoría de las personas cursan de manera asintomática la enfermedad de Chagas pero están infectadas por el resto de sus vidas (70-80%-Forma Indeterminada o sin patología demostrada). Del 20-30% restante (Forma determinada o con patología demostrada) un 30% pueden tener una progresión en el curso de los años hacia una cardiomiopatía chagásica crónica o alteraciones digestivas (*Schofield & Kabayo, 2008; Bern, 2015*).

La enfermedad se considera endémica en América del sur y centro, además de México. Sin embargo, la mitad sur de los Estados Unidos contiene ciclos enzoóticos de *T. cruzi* y casos autóctonos de infecciones humanas por vector se han reportado en Texas, California, Tennessee, Louisiana y Mississippi (*Steverding et al., 2014*). Se estima que residen 300, 000 inmigrantes infectados en los Estados Unidos (*Andrade et al., 2014; Bern, 2015*).

Dicha enfermedad es causada por el protozoario flagelado *Trypanosoma cruzi*, el cuál se multiplica dentro de las células de una gran variedad de tejidos (*Steverding et al., 2014*). Aunque *T. cruzi* es capaz de penetrar la piel intacta de los hospederos vertebrados (*Tay et al., 1980*), en la mayoría de los casos entra al humano a través de microlesiones contaminadas con heces, una vez que las personas se rascan (*Steverding et al., 2014*). El parásito es transmitido por insectos hematófagos de la familia Reduviidae, subfamilia Triatominae. Estos insectos adquieren el parásito tras alimentarse de mamíferos infectados, siendo el intestino medio del vector donde se lleva acabo la reproducción del parásito y en el intestino medio posterior donde se diferencian en la fase infectiva: tripomastigotes metacíclicos *(Garcia y Azambuja, 1991).*

En Latino América se llevaron a cabo programas de control de la enfermedad de Chagas, iniciando en 1960. Por ejemplo, la iniciativa del Cono Sur (SCII-Southern Cone Initiative), formalizada en Noviembre de 1991 por los gobiernos de Argentina, Bolivia, Brasil, Chile, Paraguay y Uruguay, con el objetivo de evitar la transmisión de la enfermedad, eliminando el principal vector domiciliado (*Triatoma infestans*). Sin embargo, no existieron acciones exhaustivas de vigilancia y como resultado, áreas que previamente se consideraron libres del transmisor, ahora están pobladas con insectos infectados con *T. cruzi*, conduciendo a casos de enfermedad de Chagas agudo en la región del Amazonas (*Andrade et al., 2014*).

La transmisión a mamíferos se da cuando el triatomino se alimenta y excreta heces infectadas con el parásito *T. cruzi* sobre el hospedero, el cuál penetra a través de las heridas o mucosas *(Kollien et al., 2000)*.

Otros mecanismos de transmisión incluyen la transfusión sanguínea, transplante de órganos, congénita vía placentaria, o incluso por ingestión de comidas o bebidas contaminadas (*Steverding et al., 2014*). La infección oral con *T. cruzi* actualmente representa la ruta de transmisión más frecuentemente documentada en Brasil. Microepidemias de Chagas agudo han sido reportados en la región del Amazonas y han sido asociados con el consumo de acaí contaminado (*Euterpe plearacea*) y además de jugo de caña (*Andrade et al., 2014*).

Los viajes y migraciones de individuos de áreas endémicas, hacia áreas no endémicas también representa un escenario preocupante para la transmisión congénita, vía transfusión sanguínea y transplante de órganos. Datos epidemiológicos de Europa, estiman entre 59,000-108,000 casos de la enfermedad de Chagas, con prevalencias altas en España e Italia. La mayoría de los casos documentados se refieren a individuos infectados crónicamente, sin embargo, se han identificado casos agudos en estas regiones. Es por ello que los gobiernos de los Estados Unidos, Francia, España y Reino Unido han instituido el tamizaje de órganos y en bancos de sangre (*Andrade et al., 2014)*.

1.2 *Meccus pallidipennis*

En México, se han reportado 32 especies de triatominos, distribuidas en todo el territorio nacional, de las cuales 19 se han encontrado infectadas naturalmente con *T. cruzi* y 13 de ellas están reportadas en la transmisión al humano (*Salazar-Schettino et al., 2010; Rodríguez-Bataz et al., 2011)*; siendo los géneros *Triatoma* y *Meccus* son los más importantes. En el año 2003 se reportó un total de 34 especies colectadas en México (*Galvao et al., 2003*).

La especie *Meccus pallidipennis* (=*Triatoma pallidipennis*) (Hemiptera: Reduviidae) es endémica de México y considerada como uno de los vectores epidemiológicos más importantes de *T. cruzi* en humano y hospederos reservorios animales (*Martínez-Ibarra et al., 2014*) y responsable del 74% de la transmisión vectorial en México (*Ibarra-Cerdeña et al., 2009; Martínez-Ibarra et al., 2012*).

Mazzotti, en 1936, reportó a *Meccus pallidipennis* en Apatzingán, Michoacán y Tetela, Guerrero, como uno de los primeros triatóminos infectados con *T. cruzi* en México (*Mazzotti, 1936*).

Ésta especie pertenece al género *Meccus* (Stal 1859), el cuál engloba otras especies como *M. longipennis*, *M. mazzottii*, *M. picturata* y *M. bassolsae*. Su aspecto general y tamaño de estas especies fue sometido a revalidación del complejo *Triatoma phyllosoma* al género *Meccus* (*Carcavallo et al., 2000*; *Martínez-Ibarrra et al., 2008*). *M. pallidipennis* ha sido reportado en 13 estados: Colima, Estado de México, Guanajuato, Guerrero, Jalisco, Michoacán, Morelos, Nayarit,

Oaxaca, Puebla, Querétaro, Veracruz y Zacatecas *(Carcavallo et al., 1997; Salazar-Schettino et al., 2010; Martínez-Ibarra et al., 2012; Martínez-Ibarra et al., 2014)*, se ha localizado en altitudes de 200 a 1580 m sobre el nivel de mar y es una de las especies más importantes en la transmisión de *T. cruzi (Bautista et al., 2001; Salazar-Schettino et al., 2010),* respecto a su hábitat se conocen como "visitantes" o peridomiciliados. En el estudio realizado por Bautista et al., 1999 se denomina peridomicilio a aquellos sitios localizados en un perímetro de 50 m alrededor de la casa (jardines, bardas de piedra y corrales de animales). Sin embargo, áreas silvestres en el estado de Morelos como nidos de roedores y *tecorrales* (bardas de piedra que alguna vez rodearon las haciendas) (*Bautista et al., 1999*) también se consideran lugares donde se encuentra esta especie. Otra definición refiere que los triatominos peridomiciliados completan algunos estadios de desarrollo en el intradomicilio y son de difícil control entomológico La migración hacia ambientes peridomésticos es el primer paso para la invasión y colonización de la vivienda humana, ya que estos ecotopos sirven como ambientes transicionales entre el área silvestre y la vivienda (*Martínez-Ibarra et al., 2008*).

En cuanto a su ciclo de vida, se ha reportado que el tiempo requerido de huevo a adulto es de 168.7 ± 11.71 días, con una tasa de eclosión del 60% (tiempo de incubación de 16-27 días), y el número de huevos ovipuestos por una hembra es de 498.67 (rango de 408-552) (*Martínez-Ibarra & Katthain-Duchateau, 1999*). Las características biológicas y comportamiento en ésta especie varían con las condiciones de cada localidad. Por ejemplo, el ciclo de vida en poblaciones de *M. pallidipennis* de Taretan, Michoacán fue de 162.37 ± 21.51, Amilcingo, Morelos 143.73 ± 15.97, Mariscala de Juárez, Oaxaca 145.16 ± 16.35, Lluvianos, Estado de México 169.7 ± 19.6, Izúcar de Matamoros, Puebla 141.7 ± 13,8 y en Chilpancingo, Guerrero 144.4 ± 14.5 días (*Martínez-Ibarra et al., 2012*; *Martínez-Ibarra et al., 2014*).

Otros parámetros para considerar a *M. pallidipennis* como un transmisor importante de *T. cruzi* es el tiempo de defecación posterior a la alimentación. Se calcula que la mayoría de los estadios de ninfa en esta especie defecan a los 4 minutos después de alimentarse, ya que se considera que las especies de triatominos que defecan en menos de 10 minutos son vectores efectivos del parásito, de igual forma, sus tiempos de alimentación son cortos, menos de 21 minutos para cada estadio (*Martínez-Ibarra & Novelo-López, 2004*).

Es una especie de tamaño considerable, existe dimorfismo sexual entre machos y hembras, las hembras miden de 3.2-3.5 cm y los machos de 3.1-3.4 cm *(Lent y Wygodzinsky, 1979)*; su cuerpo es ancho, sobre todo en la región abdominal (♂ 12-13mm y ♀ 13-16mm) (Fig. 1).Tienen un color que va del pardo obscuro al negro, con marcas características de color rojo-naranja en el conexivo; posee un par de alas de color blanco-amarillento relativamente angostas, que no cubren la totalidad del conexivo. Su cabeza y sus patas son de color negro. La cabeza es ligeramente más larga que el

pronoto. La región anteocular es tres veces más larga que las posocular. Los tubérculos anteníferos son muy cortos *(Lent y Wygodzinsky, 1979)*.

Figura 1. Macho y Hembra de *Meccus pallidipennis*.

En el control químico de *M. pallidipennis* se han utilizado piretroides sintéticos (bifentrin, cyflutrin y deltametrin (clase II - productos moderadamente tóxicos), aunque después del rociado se ha reportado re-infestación en las casas *(Ramsey et al., 2003)*. Lo anterior, puede estar relacionado con la falta de acción ovicida por piretroides, lo cuál favorece la recuperación de las colonias, por la pérdida de poder residual de estos insecticidas en el peridomicilio e incluso por la falta de ordenamiento y limpieza en esta área que propician refugios naturales para los triatominos *(Cecere et al., 1996; Rojas-Wastavino et al., 2004)*. Por lo anterior resulta importante la búsqueda de estrategias de control ambientalmente seguras contra insectos transmisores de enfermedades y una buena opción es la utilización de agentes microbianos.

1.3 Hongos entomopatógenos

Como alternativa al uso de insecticidas se ha propuesto el uso de hongos entomopatógenos. Ejemplos probados exitosamente en el campo para el control de insectos plaga son: *Isaria fumosorosea* (Hypocreales: Cordycipitaceae) en mosquita blanca (Hemiptera: Aleyrodidae) y *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) en el salivazo de los pastos (Hemiptera: Cercopidae) *(García-Valente et al., 2008 y Hernández-Velázquez et al., 2008)*.

Recientemente diversos hongos entomopatógenos también se han utilizado para el control biológico de insectos transmisores de enfermedades como *Phlebotomus duboscqui* (Diptera: Pshycodidae), *Anopheles arabiensis* y *A. gambiae* (Diptera: Culicidae) *(Ngumbi et al., 2011; Lwetoijera et al., 2010; Scholte et al., 2005)*, *Lutzomya longipalpis* (Diptera: Psychodidae) *(Albano-Amóra et al., 2010)*, *Culex quinquefasciatus* (Diptera: Culicidae) *(Scholte et al., 2003)*, *Xenopsylla brasiliensis* (Siphonaptera: Pulicidae) *(Mnyone et al., 2012)* y *Aedes aegypti* (Diptera Culicidae) *(Reyes-Villanueva et al., 2011)*.

En triatominos, también se han probado su eficacia in vitro, en múltiples especies como *Triatoma infestans*, *Rhodnius prolixus*, *Panstrongylus megistus*, *T. sordida*, *T. brasiliensis*, *T. picturata*, *Dipetalogaster maxima (Enríquez, 2004; Lara Da costa et al., 2003; Lecuona et al., 2001; Luz et al., 1998a; 1998b; 1999; 2003; 2004; Rocha & Luz, 2011; Rocha et al., 2011)* y sobre todo para aquellas especies que son resistentes a la acción de piretroides como *T. dimidiata (Pedrini et al., 2009)*.

Los hongos entomopatógenos infectan por contacto externo y tienen la habilidad de penetrar directamente la cutícula del insecto, tal interacción resulta importante para establecer la infección fúngica, además son excelentes inductores de la respuesta inmune en insectos *(Gillespie et al., 2000a)*.

Al infectar por contacto externo, la cutícula del hospedero es la primera línea de defensa contra la infección fúngica es decir, son ***Barreras fisicoquímicas*** **en el insecto**. La cutícula es una estructura rígida que recubre la parte externa del insecto conformada por dos capas:

1) La **e*picutícula*** cuya composición son grasas, ceras y lipoproteínas, siendo su principal función es evitar la pérdida de agua por transpiración.

2) La ***procutícula*** la cuál actúa como barrera física ante la penetración de patógenos, es la capa más abundante, constituye el 95%, está compuesta de quitina y diversas proteínas estructurales que proporcionan rigidez y la hace difícil de degradar por enzimas líticas de patógenos.

En la cutícula se da la producción de proteasas, peptidasas e inhibidores de proteasas fúngicas que podrían tener un papel importante durante la infección por hongos, además de la presencia de ácidos grasos de cadena corta y lípidos de cutícula que inhiben la germinación de los conidios de hongos *(Téllez-Jurado et al., 2009).*

El mecanismo de infección de los hongos entomopatógenos consiste en la adhesión, penetración, diseminación y salida del hongo del insecto *(Charnley y St. Leger, 1991)*. Los conidios se adhieren a la cutícula del insecto, germinan desarrollando un apresorio (estructura de adhesión y penetración). La cutícula es penetrada por una combinación de presión mecánica y la acción de enzimas que degradan la cutícula, como son las proteasas (Pr1 y Pr2) y quitinasas. El hongo prolifera por crecimiento vegetativo en el hemocele del hospedero y se producen conidios externos hasta la muerte del hospedero.

Una infección exitosa ocurre cuando el hongo penetra la cutícula y vence la respuesta inmune innata del insecto. Los insectos responden celular y humoralmente ante una infección fúngica, esta se presenta desde que ocurre la degradación de la cutícula, durante la penetración. Los hongos tienen dos estrategias principales para vencer la respuesta de defensa: el desarrollo de formas de crecimiento crípticas y la producción de sustancias inmunomodulantes que inhiben el sistema de defensa del hospedero *(Thomas y Read, 2007).*

1.4 Respuesta inmune en insectos

El éxito de las interacciones patógeno-vector depende de la respuesta inmune de los insectos. Los insectos no cuentan con el complejo antígeno-anticuerpo característico de la inmunidad adaptativa en vertebrados, en su lugar, tienen mecanismos de defensa que dependen de ***componentes humorales y celulares de la inmunidad innata*****,** los cuales les permite a los insectos poder sobrevivir en ambientes hostiles y defenderse de patógenos *(Boulanger et al., 2006)*.

Dos enzimas están involucradas principalmente en la respuesta humoral del insecto, la enzima ***fenoloxidasa (PO) y profenoloxidasa (proPO).***

La enzima fenoloxidasa se produce durante la esclerotización de la cutícula, cicatrización de heridas e inicia la biosíntesis de la melanina para generar respuestas de defensa como formación de nódulos, encapsulación, fagocitosis (*Laughton et al., 2011; Smilanich et al., 2009).*

Se ha propuesto que esta enzima está siempre presente como parte de la respuesta inmune del insecto aunque sin mantener los niveles constantes máximos, además de no ser específica contra patógenos o parásitos. Es responsable de eliminar rápidamente la mayor proporción de éstos después de una infección, a diferencia de los péptidos antimicrobianos los cuáles son activados únicamente después de que el patógeno ha sido reconocido y sus efectos son de larga duración *(Laughton y Siva-Jothy, 2010).*

La principal función de la PO en la formación de la melanina (melanogénesis); es decir convertir fenoles a quinonas (*Soderhall & Cerenius, 1998*). La melanina se forma a partir del aminoácido fenilalanina, la cuál es hidrolizada a tirosina, posteriormente la tirosina es hidrolizada para producir DOPA. La DOPA es oxidada a dopaquinona y es convertida inmediatamente a dopacromo por reacciones no enzimáticas y espontáneas. La melanina cumple muchas funciones en los invertebrados además de la pigmentación, varios estudios han mencionado que lesiones mecánicas o la presencia de parásitos (nematodos o parasitoides), resulta en la deposición de melanina alrededor del tejido dañado o del patógeno. Este proceso es llamado melanización (*González-Santoyo & Córdoba-Aguilar, 2012)*.

En artrópodos, la melanización ocurre cuando un objeto extraño es localizado por los hemocitos, a través de cascadas de señalización que involucran lipopolisacáridos, peptidoglicanos, glucanos y sus proteínas de reconocimiento –las β-1,3-Glucanos proteínas de reconocimiento (β GRP). Los hemocitos rodean el objeto extraño y liberan proteínas quimioatractantes (PSP por sus siglás en inglés: plasmatocyte spreading peptide). Estas proteínas atraen a los plasmatocitos, los cuáles forman paredes multicelulares. Las capas internas de la pared de plasmatocitos comienzan a incrementar en tamaño, engrosarse y obscurecerse debido a la producción de melanina. La melanina es eventualmente depositada sobre el patógeno extraño. Las cápsulas de melanina evitan el

crecimiento y reproducción del patógeno y eventualmente conduce a su muerte *(Gillespie et al., 1997; González-Santoyo & Córdoba-Aguilar, 2012)*.

Debido a su naturaleza citotóxica la PO es almacenada comúnmente como un precursor inactivo (zimógeno): ***profenoloxidasa (proPO)***. El procesamiento de proPO a PO está regulado por una compleja cascada proteolítica, la cuál puede ser activada por reconocimiento de los componentes de la pared celular de hongos u otros microorganismos *(González-Santoyo & Córdoba- Aguilar, 2012; Laughton & Siva-Jothy, 2010; Jiménez-Cortés et al., 2012).*

Las proPO son polipéptidos que contienen dos átomos de cobre por molécula de proteína, con un peso total de 50-60 y de 70-80 kDa en su forma activa e inactiva, respectivamente. En artrópodos, las secuencias de aminoácidos de la proPO son homólogas con las hemocianinas (una proteína de transporte de oxígeno en invertebrados) y hexamerinas (proteínas de almacenamiento). Estudios recientes sostienen la idea de que la PO es la enzima ancestral de la cuál las hemocianinas evolucionaron secundriamente y que las hexamerinas divergieron de las hemocianinas *(González-Santoyo & Córdoba-Aguilar, 2012).*

Como activadores artificiales de la proPO, se han utilizado la laminarina, lipopolisacáridos (LPS) y la α-Quimiotripsina, mostrando diferentes grados de activación en insectos. La α-Quimiotripsina es una proteasa que hidroliza la unión de péptidos en la proPO para producir PO en muchos insectos *(Laughton & Siva-Jothy, 2010).*

La actividad de la profenoloxidasa ha sido medida en otro transmisor de la enfermedad de Chagas: *Rhodnius prolixus*, infectado oralmente con *Trypanosoma rangeli*, donde se demostró que el sistema proPO es importante para el establecimiento del parásito en el vector, además se señala que de alguna forma el parásito modula la activación de tal sistema y drásticamente reduce la subsequente activación de la proPO en la hemolinfa *(Gomes et al., 2003).*

También se ha estudiado la respuesta inmune de *Rhodnius prolixus* ante *Trypanosoma cruzi*, y la interacción que existe con los componentes de la hemolinfa. En este estudio se encontró que en la hemolinfa *T. cruzi* no tiene división, no induce moléculas tripanolíticas o antibacteriales, pero induce altos niveles de lisozimas y formación de nódulos *(Mello et al., 1995).* De igual forma correlacionan el número de tripanosomas con la actividad de la PO y señalan que esta actividad decrece inmediatamente que el parásito desaparece, es decir que el parásito no tiene la capacidad de escapar de la respuesta inmune en la hemolinfa *(Azambuja et al., 1999).*

La enzima **óxido nítrico sintasa (NOS)** ha sido asociada a respuestas antimicrobianas, antivirales y antiparasitarias en vectores invertebrados, como los mosquitos y caracoles. Se ha utilizado en el control de parásitos y la capacidad vectorial, juega un papel importante en las vías de señalización del sistema inmune, en donde actúa como un activador de la producción de péptidos antimicrobianos y otros componentes del sistema inmune *(Herrera-Ortíz et al., 2011; Whitten et al., 2007).*

El **óxido nítrico (ON)** es un gas producido de forma intra y extracelular principalmente en el intestino de los insectos y los hemocitos, el cual traspasa la membrana de los patógenos, dañando tanto algunas proteínas como su ADN. Por ejemplo: el mosquito *Aedes aegypti*, vector del virus del dengue, llega a producir altos niveles de ON, y con ello limita la replicación del virus que lo ataca (*Castillo et al., 2006*).

Otro componente importante de la respuesta inmunitaria en insectos es la ***respuesta celular***, que está mediada por hemocitos. Los tipos de hemocitos descritos en insectos y sus nombres pueden varíar entre taxa, aunque se reconocen cuatro tipos principales con base en su morfología, histoquímica y características funcionales (*Lavine & Strand, 2002; Strand, 2008*).

- Los Granulocitos son células adherentes, cuya función primaria es la fagocitosis
- Los Plasmatocitos también son adherentes y forman cápsulas celulares
- Los Enocitoides contienen precursores de la fenoloxidasa involucrados en la producción de melanina
- Los Prohemocitos probables células madre

Se han realizado estudios comparativos en cuanto al número de hemocitos en insectos como *Anopheles gambiae* y *Aedes aegypti* y se plantea si todos los insectos transmisores de enfermedades son capaces de producir tipos de hemocitos similares o diferentes (*Castillo et al., 2006*).

Los hemocitos reconocen patógenos induciendo respuestas como la fagocitosis, encapsulación celular y melanización (*Gillespie et al., 1997*; *Lavine & Strand, 2002*).

- La fagocitosis es un proceso de endocitosis por el cual los hemocitos reconocen, se unen e ingieren patógenos de pequeño tamaño como las bacterias (*Gillespie et al., 1997*).
- La encapsulación celular es una respuesta de defensa contra agentes microbianos de gran tamaño, tales como parásitos, hongos, nematodos, cestodos y huevos de parasitoides que no pueden ser fagocitados individualmente por hemocitos. Durante una respuesta de encapsulación celular, múltiples hemocitos se unen al microorganismo extraño para formar una capa de células superpuestas, que algunas veces producen melanina (*Gillespie et al., 1997; Lavine y Strand, 2002)*.
- La melanización se presenta en algunos insectos que no producen cápsulas celulares y que en su lugar producen acúmulos de melanina alrededor del microorganismo patógeno para formar una cápsula (*Lavine y Strand, 2002; Laughton et al., 2011; Strand, 2008*), este proceso se puede presentar en los parásitos cuando la encapsulación es ineficiente.

En este proceso participan los receptores de reconocimiento de patrones **(PRRs)** para identificar componentes presentes en la pared celular de los patógenos como: peptidoglicanos en bacterias Gram (+); lipopolisacáridos en bacterias Gram (-) y β-1,3-Glucanos en hongos *(Téllez-Jurado et al., 2009).*

Por lo anteriormente citado, resulta interesante conocer los componentes involucrados en la defensa del insecto ante diferentes patógenos, si bien múltiples estudios han remarcado la importancia de tal interacción no sólo en insectos plaga si no además en insectos transmisores de enfermedades.

El objetivo de este estudio fue medir la actividad de dos componentes humorales durante la infección con dos especies de hongos entomopatógenos. Previamente esta actividad ha sido reportada en otros insectos (*Adamo, 2004a; Enríquez-Vara et al., 2012; Gillespie et al., 2000a; 2000b; Mullen & Goldsworthy, 2006;)*, sin embargo no se conoce la respuesta que genera *M. pallidipennis* al ser infectado con *M. anisopliae* e *I. fumosorosea* como agentes de control biológico. La alta activación de componentes como PO y proPO puede reflejar una respuesta inmune eficiente, como se ha señalado en estudios previos *(Adamo, 2004b)*, los hongos entomopatógenos poseen mecanismos importantes para evadir la respuesta inmune del insecto como β-1,3-Glucanos presentes en su pared celular (*Vilcinskas & Gotz, 1999*), causando efectos negativos en el insecto hospedero, por lo tanto, la activación de componentes humorales puede ser uno de los mecanismos importantes en insectos para evitar la infección por hongos entomopatógenos (*Vilcinskas & Matha, 1997*).

Capítulo 2.

Survival and immune response of the Chagas vector *Meccus pallidipennis* (Hemiptera: Reduviidae) against two entomopathogenic fungi, *Metarhizium anisopliae* and *Isaria fumosorosea*

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Survival and immune response of the Chagas vector *Meccus pallidipennis* (Hemiptera: Reduviidae) against two entomopathogenic fungi, *Metarhizium anisopliae* and *Isaria fumosorosea*

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Abstract

Background: Chagas disease is a key health problem in Latin America and is caused and transmitted by *Trypanosoma cruzi* and triatomine bugs, respectively. Control of triatomines has largely relied on the use pyrethroids, which has proved to be ineffective in the long term. Alternatively, the use of entomopathogenic fungi has been implemented to control triatomine bugs. These fungi are highly efficient as they induce a reduction in immune response on insects. *Meccus pallidipennis* is the main triatomine vector of Chagas disease in Mexico. In this work we investigated the effects of two entomopathogenic fungi, *Metarhizium anisopliae* and *Isaria fumosorosea*, on *M. pallidipennis* nymphs in terms of insect survival and immune response.

Methods: We had an infected and a control group for each fungal species and assessed: a) insect survival during 30 days; and, b) phenoloxidase (PO) and prophenoloxidase (proPO; two key traits in insect immune response) at 24, 48, 96 and 144 h. For survival we used Kaplan-Meier survival analysis while for immune response we used factorial, repeated-measures ANOVA for each fungal species.

Results: Animals treated with *M. anisopliae* died sooner than animals treated with *I. fumosorosea*. Infected animals showed lower PO and proPO values than sham individuals, with a clear decrease in these parameters at 24 h with no further changes after this time.

Conclusions: Our study widens the possibility of entomopathogenic fungi being used for triatomine control. The negative effect on PO and proPO seems mediated by a down-regulation of the triatomine immune response.

Keywords: Chagas disease, *Meccus pallidipennis*, Entomopathogenic fungi, Survival, Phenoloxidase, Prophenoloxidase

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Background

Chagas disease is caused by the protozoan *Trypanosoma cruzi* and is considered endemic from Mexico to Argentina [1]. The main transmission source is vectorial, through insect bugs of the subfamily Triatominae (Hemiptera: Reduviidae) [2]. For the case of Mexico, *Meccus pallidipennis* is responsible of approximately 74 % of vectorial transmission (Martínez-Ibarra et al. [3]). The biology of *M. pallidipennis* explains such high transmission rate: a) a wide distribution in Mexico [4, 5] b) its relatively high abundance compared to other triatomines [6] c) its peridomestic nature, meaning close contact with humans [6] d) it is one of the triatomines with the highest *T. cruzi* infection rates [7]; and e) its capacity as highly efficient vector in terms of egg incubation and hatching time, egg-to-adult mortality, oviposition rate and post-feeding defecation times [8].

Given the risk that *M. pallidipennis* implies for *T. cruzi* transmission, there have been several efforts to control it. One of these is the use of synthetic pyrethroids such as bifenthrin, cyfluthrin and deltamethrin insecticides. However, these pyrethroids are not reliable given the high bug re-infestation in dwellings after pyrethroid spraying [9, 10], which is associated with insecticide resistance [11, 12]. Although some other control methodologies have been proposed and/or explored (e.g. using genetically-modified endosymbiont bacteria that impede *T. cruzi* development [13], using natural predators [14]), possibly more than one control action is needed [15]. Here we explored the use of two entomopathogenic fungi, *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) and *Isaria fumosorosea* (Hypocreales: Cordycipitaceae), against *M. pallidipennis* nymphs. Use of these fungi has been highly successful for controlling different insect pests and vectors such as Asian tiger mosquitoes *Aedes albopictus* [16], cattle ticks *Rhipicephalus microplus* [17], Asian citrus psyllids *Diaphorina citri* [18] and diamondback moth *Plutella xylostella* [19]. As a matter of fact, several strains of *M. anisopliae* and *I. fumosorosea*, that are highly efficient to kill triatomines, have been identified [20–23]. Thus, entomopathogenic fungi seem a viable route for triatomine biological control [12, 23–25].

The mechanism that makes entomopathogenic fungi so successful during insect attack, starts with the conidium contact with the insect cuticle [26]. During this, the fungus adheres, penetrates, disseminates and exits the insect body [27]. Once inside, the fungus evades the insect's immune system by: 1) enzyme (e.g. proteases and chitinases) development that degrades the insect cuticle; 2) development of blastospores and hyphal bodies in the hemolymph which inhibit insect immune response; and 3) production of secondary toxic metabolites such as destruxins and beauvericins [28]. However, perhaps the largest attribute of entomopathogenic fungi as a vector and pest control relies on their immunosuppressive action. In support of these, several studies have found that two key insect immune players, phenoloxidase (PO) and, its precursor, prophenoloxidase (proPO), become down-regulated during fungal infection in some, but not all, insect species [29–31]. It is known that during the course of infection, insects make use of PO and proPO against a plethora of pathogens [32, 33]. The enzymatic process from proPO to PO is regulated by a complex proteolytic cascade, which is activated by the recognition of cell wall components of fungi and other pathogens [32]. Furthermore, PO gives rise to cuticle sclerotization and wound repair but also promotes melanine biosynthesis during the formation of nodules and encapsulation of pathogens [34, 35]. Although it is unclear how PO and proPO activity is inhibited, one hypothesis is that fungal destruxins destroy those proteins present in insect cells responsible for proPO production [36, 37].

The main aim of this study was to test the efficiency of two entomopathogenic fungi, *Metarhizium anisopliae* and *Isaria fumosorosea*, to control *M. pallidipennis* nymphs. For this, first we infected bugs using each fungal species individually and assessed survival. Then, to understand the physiological mechanism underlying fungal infection, we recorded the activity of PO and proPO using repeated time measures of the same individual.

Methods

Insects

We used 5th stage nymphs of *M. pallidipennis*, from a colony maintained in the insectary of the Biology of Parasites, Microbiology and Parasitology Department, Faculty of Medicine, Universidad Nacional Autónoma de México. This colony was established in 1998 from insect individuals collected from Oaxtepec village (18°54′ 23″N, 98°58′13″W), state of Morelos, Mexico. Insects were maintained under controlled conditions of 60 % relative humidity, 28 °C, and 12/12 h light/dark cycles at the laboratory.

Nymph infection

Fungi

We used monosporic cultures of *M. anisopliae* EH-473/4 and *I. fumosorosea* EH-511/3 strains whose insect virulence, pheno-and genotypic characterization and safety for mammals are well known [38–40]. These fungi are part of the culture Collection of the Basic Mycology Laboratory, Microbiology and Parasitology Department, Faculty of Medicine, Universidad Nacional Autónoma de México, registered in the "World Federation of Culture Collections" (WFCC) as BMFM-UNAM 834. The original fungal strains were obtained from the "Colección de Hongos Entomopatógenos (CHE)" from the Centro Nacional de Referencia de Control Biológico (CNRCB), Colima City, Mexico. *M. anisopliae* was isolated in 1994, from

Aeneolamia sp. (Hemiptera: Cercopidae), from a sugarcane crop in San Luis Potosi city, Mexico. The label of *M. anisopliae* at CNRCB is CHE-CNRCB 227. The other fungus, *I. fumosorosea* was isolated in 1994 from *Bemisia* sp. (Hemiptera: Aleyrodidae) from a watermelon crop in Colima, Mexico. The label of *I. fumosorosea* at CNRCB is CHE-CNRCB 304. Fungi were previously cultivated in potato dextrose agar (PDA, g/l: 300 g of white potato, 20 g of dextrose, 15 g of agar (BIOXON®, México) and then incubated at 28 °C for eight days [41].

Conidial suspension. The conidia were produced in PDA medium cultures and incubated at 28 °C for 7 days. After incubation, conidia were obtained using 3 ml of 0.5 % Tween 80. This suspension was kept on ice throughout the bioassay, homogenized and two dilutions were performed: 1:10 and 1:100. The number of conidia was counted in a Neubauer chamber and the suspension was adjusted to obtain a final concentration of 1×10^7 conidia/ml for the infection procedure. The whole procedure was performed in a laminar flow hood [42].

Survival assessment

Infection procedure and infected group

We applied 30 μl from a suspension of 1×10^7 conidia/ml of *M. anisopliae* or *I. fumosorosea* on each nymph's pronotum. Each nymph was placed individually in a sterile plastic Petri dish with sterile filter paper and was incubated at 28 °C with a 12/12 h light/dark cycle. After 24 h, all infected nymphs were transferred to 1 % agar-water (to provide appropriate humidity conditions for fungal growth) in plastic Petri dishes (100 × 15 mm) and were again incubated using a Precision 818® incubator at 28 °C and 80 % relative humidity for one month. We corroborated that the infection took place by assessing presence of hyphae and/or mycelium on the insect, and we recorded the number of dead insects daily [40, 42, 43]. We had three criteria to assess that an insect was dead due to fungal infection: a) signs of mycelium presence on the cuticle (by direct observation under a stereoscopic microscope, Olympus SZ40); b) presence of fungal structures inside the insect using imprints on the day when dead insects were recorded; and c) motionless insects. Furthermore, we took fungal samples from nine infected insects of each fungus. This was done by taking some sporulating fungi emerging from the insect cuticle, using an inoculating loop under a flow hood. Fungal samples were cultivated in PDA cultures, to corroborate the micro- and macroscopic characteristics of each infecting fungal species. For imprints, the insect cuticle surface was cleaned using 70 % ethanol and 40 % sodium hypochlorite to remove potential contaminants [42]. Subsequently, a longitudinal (from head to posterior end) cut was gently made using scissors and the cuticle was removed. A drop of blue cotton was then placed on the internal area of the cuticle, and this structure was placed on a slide, covered with a coverslip and sealed with nail varnish to be observed under a microscope at 40×. Each evaluated group had 10 insects with 5 replicates for each fungus, i.e. 50 insects per fungal strain infection.

Sham group

We applied 30 μl of 0.5 % Tween 80 on each nymph's pronotum. Each animal was then moved to a Plastic Petri dish with sterile filter paper and was incubated at 28 °C with a 12/12 h light/dark cycle. Except for the fungal infections, this sham group was treated under the same conditions as the infected groups: 24 h after applying 30 μl of 0.5 % Tween 80, all insects were transferred to agar-water (1 %; again to provide humidity conditions for fungal growth) in plastic petri dishes (100 × 15 mm) (see [43]) and were incubated using a Precision 818® equipment at 28 °C and 80 % relative humidity for one month. Similar to the infected group, mortality of sham insects was recorded daily for one month. To assess whether an insect was dead, we also applied the three criteria expressed above. Similar to the infected groups, we used 10 insects with 5 replicates for each fungus, i. e. 50 insects for each fungal strain.

Negative effects of fungal infection on PO and proPO

We used the same rationale of infection and incubation for those animals described above for the survival experiment. However, rather than assessing survival, we collected hemolymph from both groups every 24, 48, 96 and 144 h after the infection for both fungal species.

Hemolymph extraction

Using a 1 ml micro syringe, we punctured the insect membrane that separates the coxa and trochanter of one of the posterior legs [44], and gently pushed the abdomen. The emerging hemolymph was then collected with a 10 μl micropipette. Hemolymph was mixed with PBS pH 7.2 1 × 2.9 g of $Na_2HPO_4.12H_2O$, 0.2 g of KH_2PO_4, 0.2 g of KCl, 8.0 g of NaCl and deionized water, in a proportion of 1:2.

Protein concentration

For protein quantification and standardization in our samples, we used the Pierce method with the BCA commercial kit (Thermo Fisher Scientific, Rockford, Illinois). For this, in each of 96 microwells of a plate (Costar 96; Corning, New York, New York) we placed 10 μl of hemolymph, 40 μl of PBS pH 7.2 1 X and 150 μl of the Pierce re-agent. We used 2 mg/ml albumin to obtain a standard curve. The plate was covered with foil and was incubated at 37 °C for 30 min. Absorbance was measured in an ELISA plate reader (ELX 800, Biotek) at 562 nm. Protein content was adjusted to 10 μg of protein to record PO and proPO activity [45].

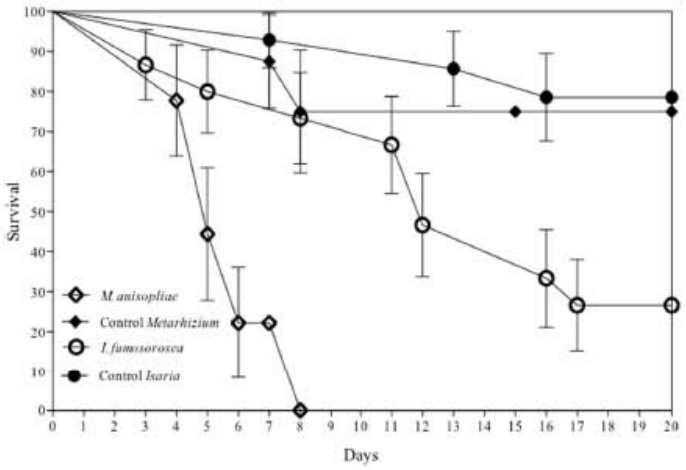

Fig. 1 Survival of fifth instar nymphs of *Mccus pallidipennis* infected with *M. anisopliae* (EH- 473/4), *I. fumosorosea* (EH- 511/3) and their control groups

PO activity

PO activity levels were quantified spectrophotometrically through catalytic conversion of L-dopa 3, 4-dihydroxi-L-phenylalanine (colorless) to dopachrome (brown-red) [46]. Again using a 96 microwell plate, we added 10 μg of protein of each sample contained in 100 μl of PBS. To start PO activation, we added 100 μl of the L-DOPA substrate to a 4 mg/ml concentration. The plate was incubated at 37 °C for 20 min in the dark. After this 20-min period, readings were taken with an ELISA plate reader (ELX 800, Biotek) at 490 nm each 5 min for one hour (giving a total of 12 readings). As blanks we used 100 μl of PBS with 100 μl of L-Dopa. PO readings were obtained in different time periods: after 24, 48, 96 and 144 h. PO activity was expressed as enzyme units (U), where 1 U is the enzyme amount which produces 1 μmol of dopachrome (product) per minute [47].

proPO activity

proPO activity was recorded *via* an artificial activation with α-chymotrypsin [46]. We used 1 μg/ml of α-chymotrypsin (Sigma®). Using the same 96 microwell plate described

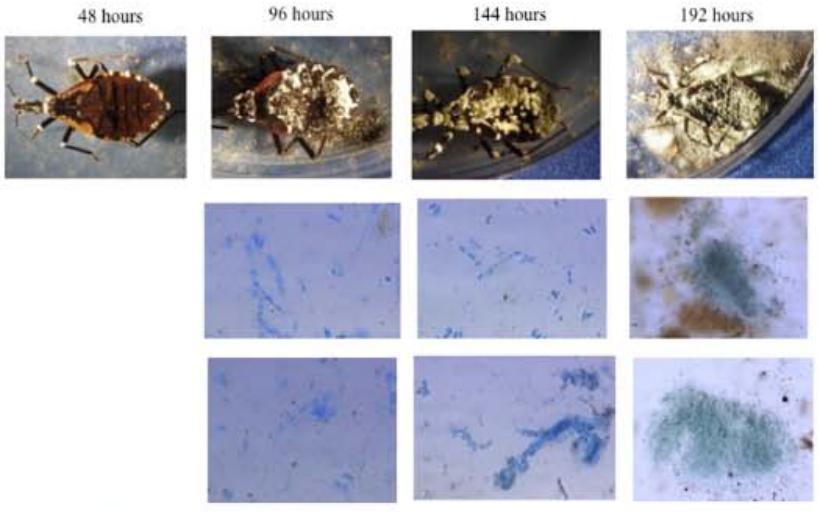

Fig. 2 Fifth instar nymphs of *M. pallidipennis* infected with *M. anisopliae* (EH- 473/4; first row of pictures) and presence of fungal structures (second and third row) on the internal area of the cuticle along time. These fungal structures are shown as hyphal bodies, conidia and conidia columns (40×)

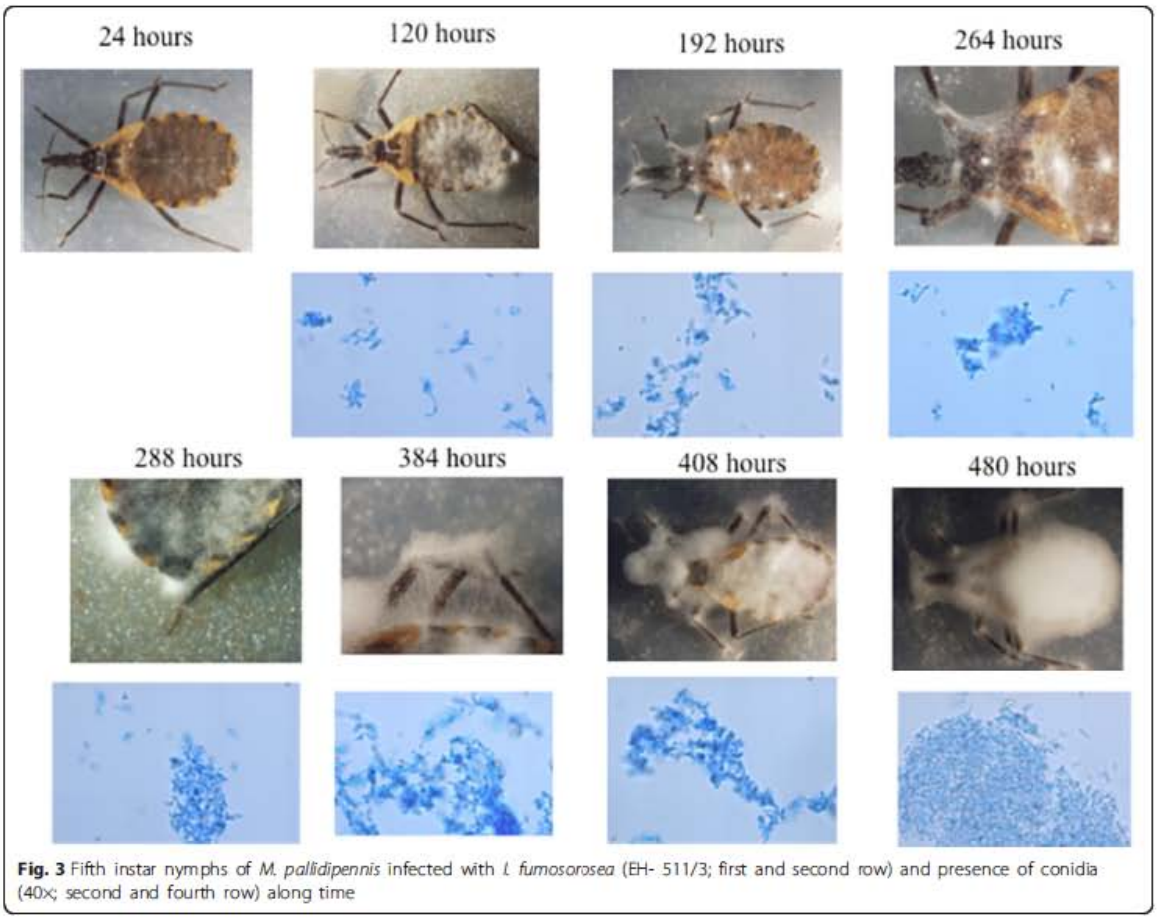

Fig. 3 Fifth instar nymphs of *M. pallidipennis* infected with *I. fumosorosea* (EH- 511/3; first and second row) and presence of conidia (40x; second and fourth row) along time

above, we added 45 µl of PBS, 20 µl of hemolymph sample and 5 µl of α- chymotrypsin. This mixture was incubated at 37 °C for 20 min in the dark. After this 20-min period, 130 µl of L-Dopa was added, and the resulting readings were recorded at 490 nm, each 5 min for one hour (giving a total of 12 readings). As blanks, we used 65 µl of PBS with 130 µl of L-Dopa. proPO readings were obtained in different time periods: after 24, 48, 96 and 144 h. To record specific proPO activity, we did the same as for PO described above.

Statistical analyses

We used Kaplan-Meier analysis for survival. Average survival time as well as risk ratio index were analyzed using a Log-rank (Mantel-Cox) test. For analyzing the effect of treatment on PO and proPO, we used four factorial, repeated-measures ANOVA for each fungal species, with time of PO and proPO assessment as the repeated measure and treatment (experimental, sham) as the between-group variable. Previously, and to fulfill normal distribution assumptions of PO and proPO, we log transformed our data. Analyses were carried out with the repeated measures module of SPSS version 21.

Results

Survival

There were differences in survival distribution for the four treatments ($X^2_{(3)} = 29.12$, $P < 0.0001$; Fig. 1). A comparison of both fungal treatments indicated that individuals treated with *M. anisopliae* died sooner than those treated with *I. fumosorosea* ($X^2_{(1)} = 11.49$, $P = 0.0007$). For those treated with *I. fumosorosea* as well as the control groups of both fungi, no further mortality was detected after 20 days.

Imprints indicate a large invasion of both fungal species within triatomines (Figs. 2 and 3). In relation to assessment of fungal infections, cultures from the nine infected nymphs with *M. anisopliae* showed the following microscopic features: branched conidiophores with basipetal catenulate cylindrical conidia (Fig. 4a). In regards to macroscopical characteristics, initially the colony had a white color that,

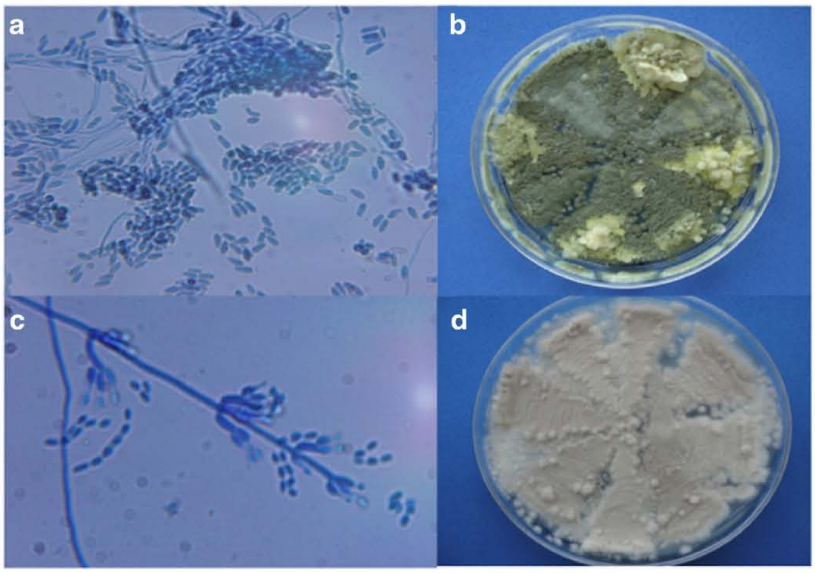

Fig. 4 Microscopic and macroscopic features after fungal growth of samples recovered from nine nymphs infected with *M. anisopliae* (EH- 473/4; **a**, **b**) and *Isaria fumosorosea* (EH- 511/3; **c**, **d**)

after several days, turned into an olive green color (Fig. 4b). Cultures from the nine infected nymphs with *I. fumosorosea* showed microscopic fungal features such as simple conidiophores, with a globose basal portion with emerging fusiform conidia (Fig. 4c). As for macroscopic characteristics, a typical white cotton-like colony was observed with gray-pinkish color when sporulated (Fig. 4d).

PO and proPO activity according to fungal treatment

Metarhizium anisopliae infection

The general model for predicting PO changes indicated that the interaction time*treatment was significant ($F_{3,37}$ = 11.721, $P < 0.0001$). Changes in PO were not significant along time ($F_{3,37} = 0.692$, $P = 0.563$) but treatment was ($F_{1,39} = 69.035$, $P < 0.0001$). According to the latter, PO showed lower values in the infected group (Fig. 5a).

The general model for proPO changes was not significant ($F_{3,32} = 2.192$, $P = 0.108$) with non-significant differences along time ($F_{3,32} = 0.144$, $P = 0.932$) but there was a clear negative effect according to treatment ($F_{1,34} = 31.737$, $P < 0.0001$) where the infected insects ended up with lower values than sham insects (Fig. 5b).

Isaria fumosorosea infection

There was a significant change in PO when the general model for the interaction time*treatment was examined ($F_{3,31} = 4.812$, $P = 0.007$). Time did not predict PO changes ($F_{3,31} = 0.522$, $P = 0.671$) but treatment did, resulting in lower values for infected insects ($F_{1,33} = 61.072$, $P < 0.0001$; Fig. 6a).

ProPO did not show significant changes according to the interaction time*treatment ($F_{3,21} = 2.337$, $P = 0.103$). Time did not affect proPO ($F_{3,21} = 0.662$, $P = 0.584$) but treatment did with lower values for infected insects ($F_{1,23} = 28.543$, $P < 0.0001$; Fig 6b).

Discussion

Our survival experiment indicated differences in killing *M. pallidipennis*, with *M. anisopliae* with a higher virulence than *I. fumosorosea*. As a matter of fact, the former fungus killed all animals at day 8 while the latter took longer than 30 days. These differences in the action of both fungi are likely due to a stronger effect of *M. anisopliae* when compared to *I. fumosorosea*. For example, *M. anisopliae* produces immunosuppressor toxins like destruxin [48] that may lead to damage hemocytes [49] and cause insect paralysis (*via* an increased calcium level in Malpighian tubules) [50]. These effects take place possibly due to the ability of *M. anisopliae* to produce a collagenous coat of hyphal bodies that mask the recognition of β-1,3-glucans by the insect immune system [51]. In contrast, little is known in regards to the effect of *I. fumosorosea*. It is clear that this fungus takes longer to sporulate than *M. anisopliae*, possibly due to a more paused beauvericine production [52] and hyphal growth [43]. How such effects take place *via* inhibition of the insect immune response, is unclear. Our results partly echo findings in other triatomines in which *M. anisopliae* was used [23, 53, 54]. To our knowledge, however, *I. fumosorosea* has been less extensively used against triatomines and one response

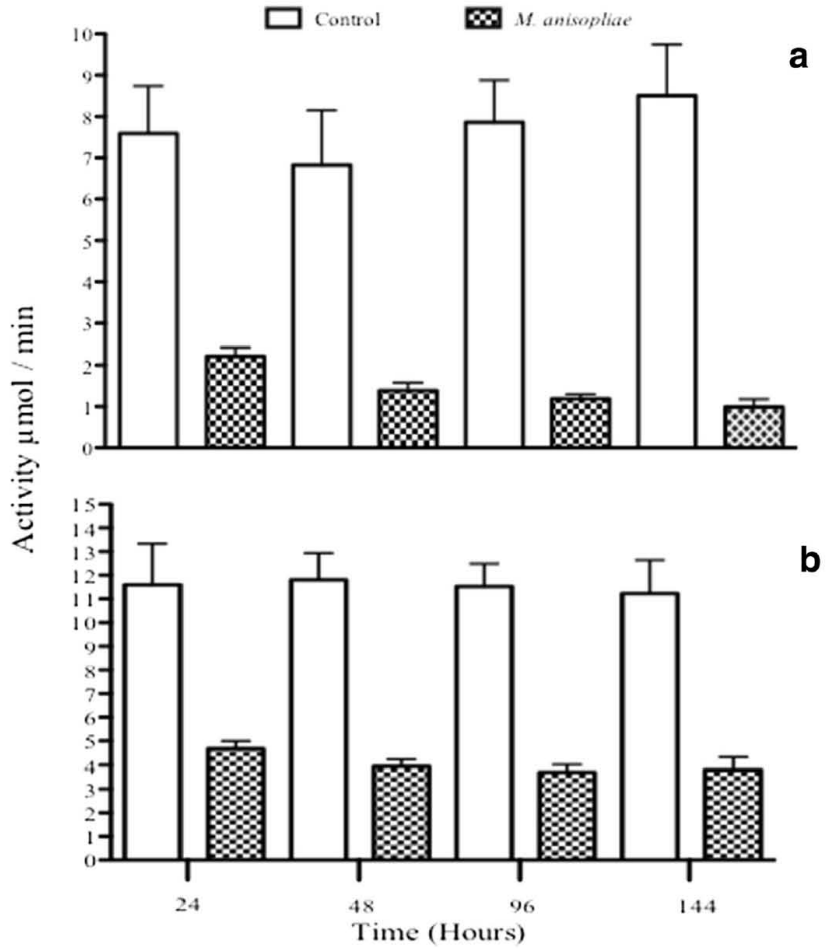

Fig. 5 PO (**a**) and proPO (**b**) activity responses in fifth instar nymphs of *M. pallidipennis* infected with *M. anisopliae* (EH- 473/4) along time

may be that, as we have documented, *M. anisopliae* seems more effective. In fact, other studies in non-triatomines have corroborated that *M. anisopliae* is more effective than *I. fumosorosea* [55–57]. It would be interesting to compare our results with those occurring at other triatomine ages. Unfortunately, such studies have not been carried out yet.

Overall, we found that after fungal infection, both proPO and PO activity decreased. A first defense line in insects is that of the cuticle, where fungistatic fatty acids, phenoloxidases and melanins impede fungal penetration [58]. In case such barrier is overcome, *via* degradation of insect cuticle hydrocarbons [59], then the insect immune system makes use of several humoral and cellular components that include hemocytes that encapsulate fungal parts, PO, reactive oxygen species and antimicrobial peptides [60]. For the case of PO, this synthesizes melanin whose antifungal activity acts directly on the fungal surface which stops fungal development [36, 61]. Despite this role for PO, and its precursor, proPO, these immune components have been shown to decrease during fungal infection [29–31] although the underlying mechanism for such decrease is unclear. In relation to this, fungal infection using *Beauveria bassiana* in the migratory grasshopper *Melanoplus sanguinipes* [62] and of *M. anisopliae* in the locust *Schistocerca gregaria* [29, 63], led to a reduction in hemocyte number. One way this negative action can occur is *via* the use of fungal mycotoxins such as destruxin [48]. This compound reduces PO activity, phagocytosis and encapsulation [52]. It is unclear, however, whether such reduction accompanies or is also a function of a reduction in hemocyte number. One reason why we should expect a relation among these negative actions is that hemocytes carry out the PO cascade so that if these

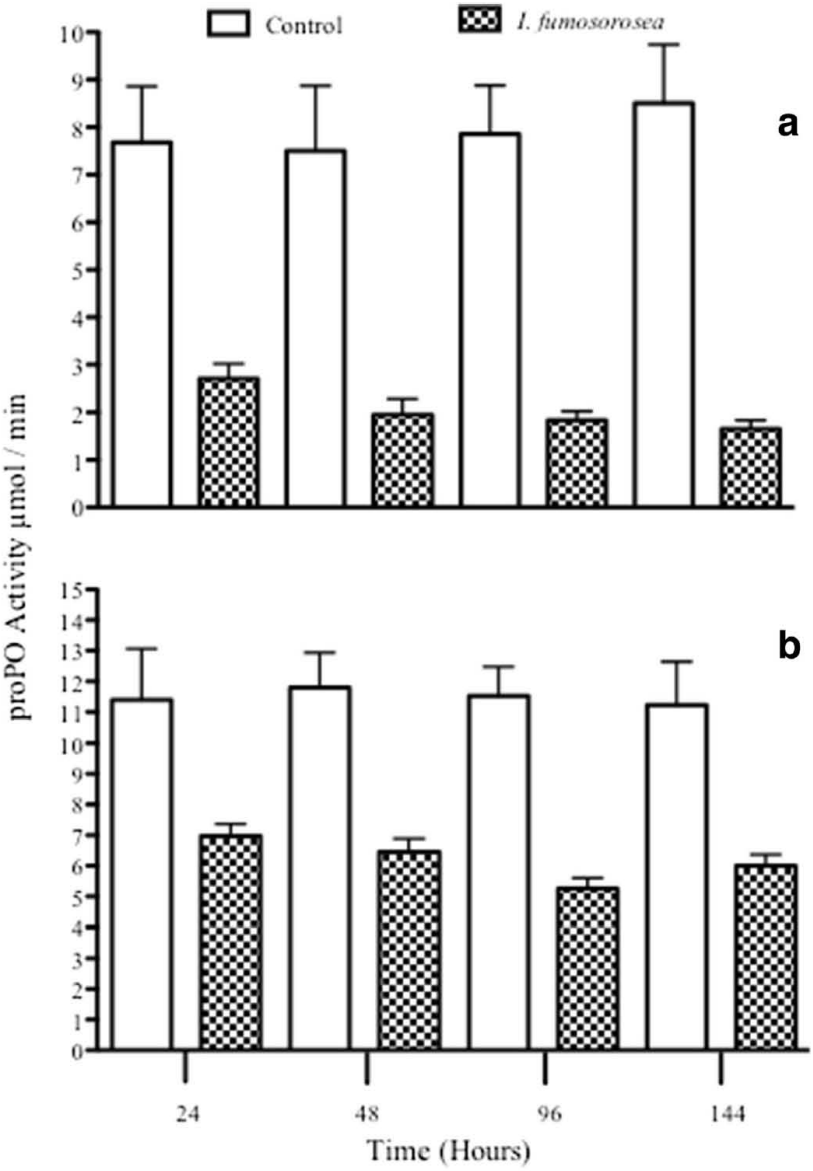

Fig. 6 PO (**a**) and proPO (**b**) activity responses in fifth instar nymphs of *M. pallidipennis* infected with *I. fumosorosea* (EH-511/3) along time

cells are affected by fungus, possibly in the form of lysis [64], proPO and PO are affected too. Furthermore, looking more closely at proPO values after infection by both fungi, this immune response showed higher activity after *I. fumosorosea* than after *M. anisopliae* treatment (Figs. 5 and 6). One has to remember that proPO is the resource tool for PO production [32] so that if both fungi had the same inhibitory PO response, proPO values should remain the same after the infection by either pathogen. Given the hypothesis of an inhibitory response by our fungi, the insect still makes use of some proPO for PO production after *M. anisopliae* treatment (or at least more so than after *I. fumosorosea* infection). This would imply that the insect shows some PO-based immune response against *M. anisopliae* so that the inhibitory response by this fungus is not as complete (or less complete) than that elicited by *I. fumosorosea* infection. This hypothesis would need further testing.

Given a first decrease in PO and proPO at 24 h, we did not detect further changes in these immune components.

Other studies in insects have found an initial increase in PO after 10 min of infection [64], followed by a general decrease at 24 h and no clear changes after this time [29, 64]. This implied an activation of an immune response followed by a negative effect of fungi on the entire PO cascade. Although we did not check what occurred soon after fungal infection, our results confirm previous claims that fungal infection affects PO and proPO [29–31].

Given that there are no vaccines available for controlling Chagas disease, its control relies on local preventive measures which, historically, has been based on the use of insecticides. One alternative to this chemical control is the use of entomopathogenic fungi which has been implemented in some countries [12] but not in Mexico. Our findings indeed seem promising although our conditions still prevent further implementation. For these conditions we refer, for example, to the fact that we used triatomine nymphs. It is known that effects of entomopathogenic fungi on insects may vary according to the ontogenetic insect stage used [65]. This means, that the effect of other stages in *M. pallidipennis* needs to be evaluated. On the other hand, perhaps the use of more than a single strategy is needed to control triatomines [15, 33]. One way to do this is using different pathogens to which insects, or more specifically triatomines, are susceptible. Such pathogens are, for example, the bacteria *Serratia marcescens* [66, 67] Triatoma virus [68, 69] or, even, other fungi [33].

Conclusions

As opposed to the use of pyrethroids, entomopathogenic fungi can be used to control triatomine bugs, the vectors of Chagas disease. We showed that *M. anisopliae* fungus is actually a better control tool than *I. fumosorosea* for killing 5th stage nymphs of *M. pallidipennis*. Our results provide some light to such killing mechanism as, along with fungal infection, we detected a reduction in PO and proPO enzymes, two key components in insect immune defense. Possibly, fungi compromise immune ability *via* mycotoxins such as dextrusin as has been shown in other studies. Thus, our results imply an alternative tool for Chagas disease control.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ALF-V carried out experiments, data collection and statistical analyses, laboratory analyses of immune response, drafted and revised the manuscript; MC-B provided biological material, analyses of experimental data, revised the manuscript; CT provided biological material, analyses of experimental data, revised the manuscript, MIB-T analyses of data, revised the manuscript; PMS-S participated in study design, manuscript drafting and revision; AC-A participated in study design, statistical analyses, manuscript drafting and revision. All authors read and approved the final version of the manuscript.

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References

1. Steverding D. The history of Chagas. Parasit Vectors. 2014;7:317.
2. Schmunis GA, Yadon ZE. Chagas disease: a Latin American health problem becoming a world health problem. Acta Trop. 2010;115:14–21.
3. Martínez-Ibarra JA, Nogueda-Torres B, García-Benavídes G, Vargas-Llamas V, Bustos-Saldaña R, Montañez-Valdez OD. Bionomics of populations of *Meccus pallidipennis* (Stal), (Hemiptera: Reduviidae) from Mexico. J Vect Ecol. 2012; 37:474–7.
4. Carcavallo RU, Galíndez-Girón I, Jurberg J, Galvao C, Lent H. Bibiographic checklist of the American triatominae (Hemiptera: Reduviidae). In: Carcavallo R, Galíndez-Girón I, Jurberg J, Lent H, editors. Atlas of Chagas Disease Vectors in the Americas, Vol. I. Rio de Janeiro: Editora Fiocruz; 1997. p. 15–52.
5. Salazar-Schettino PM, Rojas-Wastavino E, Cabrera–Bravo M, Bucio-Torres M, Martínez-Ibarra JA, Monroy-Escobar MC, Rodas-Retana A, Guevara-Gómez Y, Vences-Blanco MO, Ruíz-Hernández AL, Torres-Gutiérrez E. A revision of thirteen species of Triatominae (Hemiptera: Reduviidae) vectors of Chagas disease in Mexico. J Selva Andina Res Society. 2010;1:57–80.
6. Martínez-Ibarra JA, Grant-Guillén Y, Morales-Corona ZY, Haro-Rodriguez S, Ventura-Rodríguez LV, Nogueda-Torres B, Bustos-Saldaña R: Importance of species of Triatominae (Heteroptera: Reduviidae) in risk of transmission of *Trypanosoma cruzi* in western Mexico. J Med Entomol. 2008;45:476–82.
7. Benítez-Alva JI, Huerta H, Téllez-Rendón JL. Distribución de triatominos (Heteroptera: Reduviidae) asociados a la vivienda humana y posibles zonas de riesgo en seis estados de la República Mexicana. BIOCYT: Biología Ciencia y Tecnología. 2012;5:327–40.
8. Martínez-Ibarra JA, López MN. Blood meals to molt, feeding time and postfeeding defecation delay of *Meccus pallidipennis* (StÅl, 1872) (Hemiptera: Reduviidae) under laboratory conditions. Folia Entomol Mex. 1872;2004(43):313–9.
9. Ramsey JM, Cruz-Celis A, Salgado A, Espinosa L, Ordoñez R, Lopez R, Schofield CJ: Efficacy of pyrethroid insecticides against domestic and peridomestic populations of *Triatoma pallidipennis* and *Triatoma barberi* (Reduviidae: Triatominae) vector of Chagas disease in Mexico. J Med Entomol. 2003;40:912–20.
10. Gorla DE, Ortiz RV, Catalá SS. Control of rural house infestation by *Triatoma infestans* in the Bolivian Chaco using a microencapsulated insecticide formulation. Parasit Vectors. 2015;8:255.
11. Mougabure-Cueto G, Picollo MI. Insecticide resistance in vector Chagas disease: evolution, mechanisms and management. Acta Tropi. 2015;149:70–85.
12. Pedrini N, Mijailovsky SJ, Girotti JR, Stariolo R, Cardozo RM, Gentile A, Juárez MP: Control of pyrethroid-resistant Chagas disease vectors with entomopathogenic fungi. PLoS Negl Trop Dis. 2009;3:e434.
13. Beard CB, Cordon-Rosales C, Durvasula RV. Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. Annu Rev Entomol. 2002;47:123–41.
14. Linares G, Caicedo R. *Pimeliaphilus triatomae* (Acari: Pteregosomidae) utilizado como control biológico de *Meccus pallidipennis* (Hemiptera: Reduviidae) en condiciones de laboratorio. Rev Colomb Entomol. 2004;30:131–5.
15. Gürtler RE, Yadon ZE. Eco-biosocial research on community-based approaches for Chagas disease vector control in Latin America. Trans R Soc Trop Med Hyg. 2015;109:91–8.
16. Lee SJ, Kim S, Yu JS, Kim JC, Nai YS, Kim JS. Biological control of Asian tiger mosquito, *Aedes albopictus* (Diptera: Culicidae) using *Metarhizium anisopliae*JEF-003 millet grain. J Asia Pacific Entomol. 2015;18:217–21.
17. Webster A, Reck J, Santi L, Souza UA, Dall´Agnol B, Klafke GM, Beys-da-Silva WO, Martins JR, Schrank A: Integrated control of an acaricide-resistant strain of the cattle tick *Rhipicephalus microplus* by applying *Metarhizium anisopliae* associated with cypermethrin and chlorpyriphos under field conditions. Vet Parasitol. 2015;207:302–8.

18. Patt JM, Chow A, Meikle WG, Garcia C, Jackson MA, Flores D, Sétamou M, Dunlap CA, Avery PB, Hunter WB, Adamczyk JJ: Efficacy of an autodisseminator of an entomopathogenic fungus, *Isaria fumosorosea*, to suppress Asian citrus psyllid, *Diaphorina citri*, under greenhouse conditions. Biol Control. 2015;88:37–45.
19. Nian XG, He YR, Lu LH, Zhao R. Evaluation of alternative *Plutella xylostella* control by two *Isaria fumosorosea* conidial formulations-oil-based formulation and wettable powder-combined with *Bacillus thuringiensis*. Pest Manag Sci. 2015;71:1675–84.
20. Romaña CA, Fargues J. Relative susceptibility of different stages of *Rhodnius prolixus* to the entomopathogenichyphomycete *Beauveria bassiana*. Mem Inst Oswaldo Cruz. 1992;87:363–8.
21. Juárez MP, Crespo R, Calderón-Fernández G, Lecuona RE, Cafferata LFR. Characterization and carbon metabolism in fungi pathogenic to *Triatoma infestans*, a Chagas disease vector. J Invertebr Pathol. 2000;76:198–207.
22. Lecuona RE, Edelstein JD, Berretta MF, La Rossa FR, Arcas JA. Evaluation of *Beauveria bassiana* (hyphomycetes) strains as potential agents for control of *Triatoma infestans* (Hemiptera: Reduviidae). J Med Entomol. 2001;38:72–179.
23. Rodrigues J, Lobo LS, Fernandes É, Luz C. Effect of formulated *Metarhizium anisopliae* on eggs and eclosing nymphs of *Triatoma infestans*. J Appl Entomol. 2015;139:146–53.
24. Blandford S, Chan BH, Jenkins N, Sim D, Turner RJ, Read AF, Thomas MB: Fungal pathogen reduces potential for malaria transmission. Science. 2005; 308:1638–41.
25. Forlani N, Pedrini N, Girotti JR, Mijailovsky SJ, Cardozo RM, Gentile AG, Hernández-Suárez CM, Rabinovich JE, Juárez MP: Biological control of the Chagas disease vector *Triatoma infestans* with the entomopathogenic fungus *Beauveria bassiana* combined with an aggregation cue: field, laboratory and mathematical modeling assessment. PLoS Negl Trop Dis. 2015;9:e0003778.
26. Boucias DG, Pendland JC. The fungal cell wall and its involvement in the pathogenic process in insects hosts. In: Latgé JP, Boucias D, editors. Fungal Cell Wall and Immune Response. Berlin: Springer; 1991. p. 303–16.
27. Charnley AK, St. Leger RJ. The role of cuticle degrading enzymes in fungal pathogenesis in insects. In: Cole TG, Hoch CH, editors. The fungal Spore and Disease Initiation in Plants and Animals. New York: Plenum Press; 1991. p. 267–86.
28. Thomas MB, Read AF. Can fungal biopesticides control malaria? Nat Rev Microbiol. 2007;5:377–83.
29. Gillespie JP, Burnett C, Charnley AK. The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var *acridum*. J Insect Physiol. 2000;46:429–37.
30. Bogus´MI, Kendra E, Bania J, Szczepanik M, Czygier M, Jablonsky P, Pasztaleniec A, Samborski J, Mazgajska J, Polanowski A. Different defense strategies of *Dendrolimus pini*, *Galleria mellonella*, and *Calliphora vicina* against fungal infection. J Insect Physiol. 2007;53:909–22.
31. Khosravi R, Sendi JJ, Zibaee A, Shokrgozar MA. Immune reactions of the lesser mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) to the entomopathogenic fungus, *Beauveria bassiana* (Bals.-Criv.) Vuill and two developmental hormones. Invert Surviv J. 2014;11:11–21.
32. González-Santoyo IG, Córdoba-Aguilar. Phenoloxidase: a key component of the insect immune system. Entom Exp Appl. 2012;142:1–16.
33. Flores-Villegas AL, Salazar-Schettino PM, Córdoba-Aguilar A, Gutiérrez-Cabrera AE, Rojas-Wastavino GE, Bucio-Torres MI, Cabrera-Bravo M: Immune defence mechanisms of triatomines against bacteria, viruses, fungi and parasites. Bull Entomol Res. 2015;105:523–32.
34. Laughton AM, García JR, Altincieck B, Strand MR, Gerardo NM. Characterisation of immune responses in the pea aphid, *Acyrthosiphon pisum*. J Insect Physiol. 2011;57:830–9.
35. Smilanich AM, Dyer LA, Gentry GL. The insect immune response and other putative defenses as effective predictors of parasitism. Ecology. 2009;90:1434–40.
36. Cerenius L, Thörnqvist PO, Vey A, Johansson MW, Söderhall K. The effect of the fungal toxin destruxin E on isolated crayfish haemocytes. J Insect Physiol. 1990;36:785–9.
37. Park SY, Kim CH, Jeong WH, Lee Jh, Seo SJ, Han YS, Lee IH: Effects of two hemolymph proteins on humoral defense reactions in the wax moth, *Galleria mellonella*. Dev Comp Immunol. 2005;29:43–51.
38. Toriello C. Bioseguridad de *Metharhizium anisopliae* (Metschnikoff) Sorokin (Hyphomycete). Vedalia. 2003;10:107–13.
39. Toriello C, Pérez-Torres A, Burciaga-Díaz A,Navarro-Barranco H, Pérez-Mejía A, Lorenzana Jiménez M, Mier T: Lack of acute pathogenicity and toxicity in mice of an isolate of *Metarhizium anisopliae* var. *anisopliae* from spittlebugs. Ecotoxicol Environ Saf. 2006;65:278–87.
40. Toriello C, Montoya-Sansón E, Zavala-Ramírez M, Navarro-Barranco H, Basilio-Hernández D, Hernández-Velázquez V, Mier T: Virulencia y termotolerancia de cultivos monospóricos de *Metarhizium anisopliae* var. *anisopliae* de la mosca pinta (Hemiptera: Cercopidae). Rev Mex Micol. 2008;28:57–66.
41. Mier T, Toriello C, Ulloa M. Hongos microscópicos saprobios y parásitos: métodos de laboratorio. México: UAM Xochimilco, Instituto de Biología; 2002.
42. Goettel MS, Inglis SD. Fungi: Hyphomycetes. In: Lacey LA, editor. Manual of techniques in insect pathology. Londres: Academic; 1997. p. 213–48.
43. Castellanos-Moguel J, Mier, T, Reyes-Montes MR, Navarro-Barranco H, Zepeda-Rodriguez A, Pérez-Torres A, Toriello, C: Fungal growth development index and ultrastructural study of whiteflies infected by three *Isaria fumosorosea* isolates of different pathogenicity. Rev Mex Micol. 2013;38:23–33.
44. Espinosa de Aquino W. Estudio de moléculas del sistema inmune de *Triatoma pallidipennis* infectado con *Trypanosoma cruzi*. Mexico City, Mexico: Tesis de Maestría en Ciencias, UNAM, 2012.
45. Córdoba-Aguilar A, Ruiz-Silva D, Munguía-Steyer R, Lanz-Mendoza H. Do reproductive activities compromise immunological competence as measured by phenoloxidase activity? Field and experimental manipulation in females of two damselfly species. Physiol Entomol. 2011;36:335–42.
46. Laughton AM, Siva-Jothy MT. A standarised protocol for measuring phenoloxidase and prophenoloxodase in the honey bee, *Apis mellifera*. Apidologie. 2010;42:140–9.
47. Jiménez-Cortés JG, Serrano-Meneses MA, Córdoba-Aguilar A. The effects of food shortage during larval development on adult body size, body mass, physiology and developmental time in a tropical damselfly. J Insect Physiol. 2012;58:318–26.
48. Avulova S, Rosengaus RB. Losing the battle against fungal infection: suppression of termite immune defenses during mycosis. J Insect Physiol. 2011;57:966–71.
49. Kershaw MJ, Moorhouse ER, Bateman R, Reynolds SE, Charnley AK. The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J Invertebr Pathol. 1999;74:213–23.
50. Ruiz-Sanchez E, Orchard I, Lange AB. Effect of the cyclopeptide mycotoxin destruxin A on the Malpighian tubules of *Rhodnius prolixus* (Stal). Toxicon. 2010;55:1162–70.
51. Wang C, St. Leger RJ. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc Nat Acad Sci USA. 2006; 103:6647–52.
52. Rohlf M, Churchill AC. Fungal secondary metabolites as modulators of interactions with insects and other arthropods. Fungal Genet Biol. 2011; 48:23–34.
53. Luz C, Rodrigues J, Rocha LF. Diatomaceous earth and oil enhance effectiveness of *Metarhizium anisopliae* against *Triatoma infestans*. Acta Trop. 2012;122:29–35.
54. Murerwa P, Arama PF, Kamau AW, Maniania NK. Effect of infection by *Metarhizium anisopliae* isolate ICIPE 51 on developmental stage, fecundity and intrinsic rate of increase of *Rhopalosiphum padi* and *Metopolophium dirhodum*. J Entomol Nematol. 2014;6:154–60.
55. Flores A, Pucheta M, Rodriguez S. Mycoinsecticide effects of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosorosea* on the whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae) in different strata of bean. Afr J Microbiol Res. 2012;6:7246–52.
56. Kavallieratos NG, Athanassiou CG, Aountala MM, Kontodimas DC. Evaluation of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosorosea* for control of *Sitophilus oryzae*. J Food Prot. 2014;77: 87–93.
57. Sajap AS, Rozihawati Z, Omar D, Lau WH. *Isaria fumosorosea* and *Metarhizium anisopliae* for controlling *Atteva sciodoxa* (Lepidoptera: Yponomeutidae), a pest of *Eurycoma longifolia*. J Trop For Sci. 2014;26:84–91.
58. Wraight SP, Butt TM, Galaini-Wraight S, Allee LL, Soper RS, Roberts DW. Germination and infection processes of the entomophthoralean fungus *Erynia radicans* on the potato leafhopper, *Empoasca fabae*. J Invertebr Pathol. 1990;56:157–74.
59. Napolitano R, Juárez MP. Entomopathogenous fungi degrade epicuticular hydrocarbons of *Triatoma infestans*. Arch Biochem Biophys. 1997;344:208–14.
60. Yan XD, Scherphof GL, Kamps J. Liposomeopsonization. J Liposome Res. 2005;15:109–39. doi:10.1081/lpr-200064971.
61. Butt TM, Wraight SP, Galaini-Wraight S, Humber RA, Roberts DW, Soper RS. Humoral encapsulation of the fungus *Erynia radicans* (Entomophthorales) by the potato leafhopper, *Empoasca fabae* (Homoptera: Cicadellidae). J Invertebr Pathol. 1988;52:49–56.

62. Bidochka MJ, Khachatourians GC. Hemocytic defense response to the entomopathogenic fungus *Beauveria bassiana* in the migratory grasshopper *Melanoplus sanguinipes*. Entomol Exp Appl. 1987;45:151–6.
63. Milat-Bissaad FZ, Bounaceur F, Halouane F, Behidj N, Chebouti N, Doumandji Mitiche B. Effect of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* var. *acridum* on the haemolymph of the desert locust *Schistocerca gregaria*. Tunis J Plant Prot. 2011;6:127–32.
64. Gillespie JP, Khachatourians GG. Characterization of the *Melanoplus sanguinipes* hemolymph afer infection with *Beauveria bassiana* or wounding. Comp Biochem Physiol. 1992;103:445–63.
65. Mullen LM, Goldsworthy GJ. Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin. J Insect Physiol. 2006;52:389–98.
66. Azambuja P, Feder D, Garcia ES. Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. Exp Parasitol. 2004;107:89–96.
67. Gourbière S, Dorn P, Tripet F, Dumonteil F. Genetics and evolution of triatomines: from phylogeny to vector control. Heredity. 2012;108:190–202.
68. Muscio OA, La Torre JL, Scodeller EA. Small nonoccluded viruses from triatominae bug *Triatoma infestans* (Hemiptera: Reduviidae). J Invertebr Pathol. 1987;49:218–20.
69. Muscio OA, La Torre JL, Bonder MA, Scodeller EA. Triatoma virus pathogenicity in laboratory colonies of *Triatoma infestans* (Hemiptera: Reduviidae). J Med Entomol. 1997;34:253–6.

Capítulo 3.

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

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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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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

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 megaterium* 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

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*

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

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

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.

Supplementary material

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

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References

Araújo, C.A.C., Waniek, P.J., Stock, P., Mayer, C., Jansen, A.M. & Schaub, G.A. (2006) Sequence characterization and expression patterns of defensin and lysozyme encoding genes from the gut of the reduviid bug *Triatoma brasiliensis*. *Insect Biochemistry and Molecular Biology* **36**, 547–560.

Avulova, S. & Rosengaus, R.B. (2011) Losing the battle against fungal infection: Supression of termite immune defenses during mycosis. *Journal of Insect Physiology* **57**, 966–971.

Azambuja, P. & Garcia, E.S. (1987) Characterization of inducible lysozyme activity in the hemolymph of *Rhodnius prolixus*. *Brazilian Journal of medical and Biological Research* **20**, 539–548.

Azambuja, P., Mello, C.B. & Feder, D. (1997) Influence of the triatominae cellular and humoral defense system on the development of trypanosomatids. pp. 709–733 *in* Carcavallo, R., Galíndez-Girón, I. & Jurberg, J. (Eds) *Atlas of Chagas Disease Vectors in the Americas*. Rio de Janeiro, Editora Fiocruz.

Azambuja, P., Feder, D., Mello, C.B., Gomes, S.A.O. & Garcia, E. S. (1999) Immunity in *Rhodnius prolixus*: Trypanosomatid–vector interactions. *Memorias do Instituto Oswaldo Cruz* **94**, 219–222.

Azambuja, P., Feder, D. & Garcia, E.S. (2004) Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. *Experimental Parasitology* **107**, 89–96.

Balczun, C., Knorr, E., Topal, H., Meiser, C.K., Kollien, A.H. & Schaub, G.A. (2008) Sequence characterization of an unusual lysozyme gene expressed in the intestinal tract of the reduviid bug *Triatoma infestans* (Insecta). *Parasitology Research* **102**, 229–232.

Beard, C.B., Dotson, E.M., Pennington, P.M., Eichler, S., Cordon-Rosales, C. & Durvasula, R.V. (2001) Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. *International Journal of Parasitology* **31**, 621–627.

Beard, C.B., Cordon-Rosales, C. & Durvasula, R.V. (2002) Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. *Annual Review of Entomology* **47**, 123–141.

Beckage, N.E. (2011) *Insect Immunology*. San Diego, California, USA, Academic Press.

Bedding, R.A. & Molyneux, A.S. (1982) Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp. (Heterorhabditidae: Nematoda). *Nematologica* **28**, 354–359.

Borges, A.R., Santos, P.N., Furtado, A.F. & Figueiredo, R.C.B.Q. (2008) Phagocytosis of latex beads and bacteria by hemocytes of the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduvidae). *Micron* **39**, 486–494.

Boucias, D.G. & Pendland, J.C. (1991) The fungal cell wall and its involvement in the pathogenic process in insects hosts. pp. 303–316 *in* Boucias, D. & Latgé, J.P. (*Eds*) *Fungal Cell Wall and Immune Response*. Berlin, Springer-Verlag.

Boulanger, N., Bulet, P. & Lowenberger, C. (2006) Antimicrobial peptides in the interactions between insects and flagellates parasites. *Trends in Parasitology* **6**, 262–268.

Casteels, P. (1998) Immune responses in Hymenoptera. pp. 92–110 *in* Brey, P.T. & Hultmark, D. (*Eds*) *Molecular Mechanisms of Immune Responses in Insects*. New York, USA, Chapman & Hall.

Castro, D.P., Moraes, C.S., Gonzalez, M.S., Ratcliffe, N.A., Azambuja, P. & Garcia, E.S. (2012) *Trypanosoma cruzi* immune response modulation decreases microbiota in *Rhodnius prolixus* gut and is crucial for parasite survival and development. *PLoS ONE* **7**, e36591.

Cator, L.J., Lynch, P.A., Read, A.F. & Thomas, M.B. (2012) Do malaria parasites manipulate mosquitoes? *Trends in Parasitology* **28**, 466–470.

Cerenius, L., Lee, B.L. & Soderhall, K. (2008) The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology* **29**, 263–271.

Cisarovsky, G., Schmid-Hempel, P. & Sadd, B.M. (2012) Robustness of the outcome of adult bumblebee infection with a trypanosome parasite after varied parasite exposures during larval development. *Journal of Evolutionary Biology* **25**, 1053–1059.

Clayton, A.M., Dong, Y. & Dimopoulos, G. (2014) The *Anopheles* innate immune system in the defense against malaria infection. *Journal of Innate Immunity* **6**, 169–181.

Dong, Y., Taylor, H.E. & Dimopoulos, G. (2006) AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system. *PLoS Biology* **4**, e229.

Dotson, E.M., Plikaytis, B., Shinnick, T.M., Durvasula, R.V. & Beard, C.B. (2003) Transformation of *Rhodococcus rhodnii*, a symbiont of the Chagas disease vector *Rhodnius prolixus*, with integrative elements of the L1 mycobacteriophage. *Infection, Genetics and Evolution* **3**, 103–109.

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, A., Taneja, J., Kang, A.S., Cordon-Rosales, C., Richards, F.F., Whitham, R.G. & Beard, C.B. (1999) Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via transgenic bacterial symbiont *Rhodococcus rhodnii*. *Medical and Veterinary Entomology* **13**, 115–119.

Engstrom, P., Carlsson, A., Engstrom, Z.J.T. & Bennich, H. (1984) The antibacterial effect of attacins from the silk moth *Hyalophora cecropia* is directed against the outer membrane of *Escherichia coli*. *The EMBO Journal* **3**, 3347–3351.

Fieck, A., Hurwitz, I., Kang, A.S. & Durvasula, R. (2010) *Trypansoma cruzi*: synergistic cytotoxicity of multiple amphipathic anti-microbial peptides to *T. cruzi* and potential bacterial hosts. *Experimental Parasitology* **125**, 342–347.

Fujita, A. (2004) Lysozymes in insects: what role do they play in nitrogen metabolism? *Physiological Entomology* **29**, 305–310.

Genta, F.A., Souza, R.S., García, E.S. & Azambuja, P. (2010) Phenol oxidases from *Rhodnius prolixus*: temporal and tissue expression pattern and regulation by ecdysone. *Journal of Insect Physiology* **56**, 1253–1259.

Gillespie, J.P., Kanost, M.R. & Trenczek, T. (1997) Biological mediators of insect immunity. *Annual Review of Entomology* **42**, 611–643.

Gillespie, J.P., Burnett, C. & Charnley, A.K. (2000) The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var *acridum*. *Journal of Insect Physiology* **46**, 429–437.

Gomes, S.A.O., Feder, D., Thomas, N.E.S., Garcia, E.S. & Azambuja, P. (1999) *Rhodnius prolixus* infected with *Trypanosoma rangeli: in vivo* and *in vitro* experiments. *Journal of Invertebrate Pathology* **73**, 289–293.

Gomes, S.A.O., Feder, D., Garcia, E.S. & Azambuja, P. (2003) Supression of the prophenoloxidase system in *Rhodnius prolixus* orally infected with *Trypanosoma rangeli*. *Journal of Insect Physiology* **49**, 829–837.

González-Santoyo, I. & Córdoba-Aguilar, A. (2012) Phenoloxidase: a key component of the insect immune system. *Entomologia Experimentalis et Applicata* **142**, 1–16.

Gourbière, S., Dorn, P., Tripet, F. & Dumonteil, F. (2012) Genetics and evolution of triatomines: from phylogeny to vector control. *Heredity* **108**, 190–202.

Gregorio, E.A. & Ratcliffe, N.A. (1991) The prophenoloxidase system and *in vitro* interaction of *Trypanosoma rangeli* with *Rhodnius prolixus* and *Triatoma infestans*. *Parasite Immunology* **13**, 551–564.

Grisard, E.C., Teixeira, S.M.R., De Almeida, L.G.P., Stoco, P.H., Gerber, A.L., Talavera-López, C., Lima, O.C., Andersson, B. & De Vasconcelos, A.T.R. (2014) *Trypanosoma cruzi* clone Dm28c draft genome sequence. *Genome Announcements* **2**, 1–2.

Gutiérrez-Cabrera, A.E., Alejandre-Aguilar, R., Hernández-Martínez, S. & Espinoza, B. (2014) Development and glycoprotein composition of the perimicrovillar membrane in *Triatoma* (*Meccus*) *pallidipennis* (Hemiptera: Reduviidae). *Arthropod Structure and Development* **43**, 571–578.

Hoffman, J.A. & Reichhart, J.M. (2002) *Drosophila* innate immunity: an evolutionary perspective. *Nature Immunology* **3**, 121–126.

Hu, Y. & Aksoy, S. (2005) An antimicrobial peptide with trypanocidal activity characterized from *Glossina morsitans morsitans*. *Insect Biochemistry and Molecular Biology* **35**, 105–115.

Hultmark, D. Steiner, H., Rasmuson, T. & Boman, H.G. (1980) Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. *European Journal of Biochemistry* **106**, 7–16.

Johnson, K.N. (2015) Bacterial and antiviral immunity in insects. *Current Opinion in Insect Science* **8**, 1–7.

Kingsolver, B. & Hardy, R.W. (2012) Making connections in insect innate immunity. *Proceedings of the National Academy of Sciences of the United States of America* **46**, 18639–18640.

Kollien, A.H., Fechner, S., Waniek, P.J. & Schaub, G. (2003) Isolation and characterization of a cDNA encoding for a lysozyme from the gut of the Reduviid bug *Triatoma infestans*. *Archives of Insect Biochemistry and Physiology* **53**, 134–145.

Laughton, A.M. & Siva-Jothy, M.T. (2011) A standarised protocol for measuring phenoloxidase and prophenoloxidase in the honey bee, *Apis mellifera*. *Apidologie* **42**, 140–149.

Lavine, M.D. & Strand, M.R. (2002) Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology* **32**, 1259–1309.

Lopez, L., Morales, G., Ursic, R., Wolff, M. & Lowenberger, C. (2003) Isolation and characterization of a novel insect defensin from *Rhodnius prolixus*, a vector of Chagas disease. *Insect Biochemistry and Molecular Biology* **33**, 439–447.

Mello, C.B., Garcia, E.S., Ratcliffe, N.A. & Azambuja, P. (1995) *Trypanosoma cruzi* and *Trypanosoma rangeli* interplay with hemolymph components of *Rhodnius prolixus*. *Journal of Invertebrate Pathology* **65**, 261–268.

Michel, K. & Kafatos, F.C. (2005) Mosquito immunity against *Plasmodium*. *Insect Biochemistry and Molecular Biology* **35**, 677–689.

Moraes, A.M.L., Junqueira, A.C.V., Costa, G.L., Celano, V. & Coura, J.R. (2000) Fungal flora of the digestive tract of 5 species of triatomines vectors of *Trypanosoma cruzi*, Chagas 1909. *Mycopathologia* **151**, 41–48.

Moraes, A.M.L., Junqueira, A.C.V., Celano, V., Lara da Costa, G. & Rodrigues Coura, J. (2004) Fungal Flora of the digestive tract of *Rhodnius prolixus, Rhodnius neglectus, Dipetalogaster maximus* and *Panstrongylus megistus* vectors of *Trypanosoma cruzi*, Chagas, 1909. *Brazilian Journal of Microbiology* **35**, 288–291.

Moreira, C.J.C., Waniek, P.J., Valente, R.H., Carvalho, P.C., Perales, J., Feder, D., Geraldo, R.B., Castro, H.C., Azambuja, P., Ratcliffe, N.A. & Mello, C.B. (2014) Isolation and molecular characterization of a major hemolymph serpin from the triatomine, *Panstrongylus megistus*. *Parasites and Vectors* **7**, 1–16.

Muscio, O.A., La Torre, J.L. & Scodeller, E.A. (1987) Small non-occluded viruses from triatominae Bug *Triatoma infestans* (Hemiptera: Reduviidae). *Journal of Invertebrate Pathology* **49**, 218–220.

Muscio, O.A., La Torre, J.L., Bonder, M.A. & Scodeller, E.A. (1997) Triatoma virus pathogenicity in laboratory colonies of *Triatoma infestans* (Hemiptera: Reduviidae). *Journal of Medical Entomology* **34**, 253–256.

Muscio, O., Bonder, M.A., La Torre, J.L. & Scodeller, E.A. (2000) Horizontal transmission of Triatoma virus through the fecal–oral route in *Triatoma infestans* (Hemiptera: Triatomidae). *Journal of Medical Entomology* **37**, 271–275.

Nakamura, A., Stiebler, R., Fantappié, M.R., Fialho, E., Masuda, H. & Oliveira, M.F. (2007) Effects of retinoids and juvenoids on moult and on phenoloxidase activity in the blood-sucking insect *Rhodnius prolixus*. *Acta Tropica* **103**, 222–230.

Nava-Sánchez, A., Munguía-Steyer, R., González-Tokman, D. & Córdoba-Aguilar, A. (2015) Does mating activity impair phagocytosis-mediated priming immune response? A test using the house cricket, *Acheta domesticus*. *Acta Ethologica*. Doi: 10.1007/s10211-015-0215-y.

Ourth, D.D. & Renis, H.E. (1993) Antiviral melanization reaction of *Heliothis virescens* hemolymph against DNA and RNA viruses *in vitro*. *Comparative Biochemistry and Physiology* **105**, 719–723.

Querido, J.F.B., Agirre, J., Marti, G.A., Guérin, D.M. & Silva, S.M. (2013) Inoculation of Triatoma virus (Dicistroviridae: Cripavirus) elicits a non-infective immune response in mice. *Parasites and Vectors* **6**, 1–6.

Rassi, A. Jr, Rassi, A. & Neto, M. (2010) Chagas disease. *The Lancet* **375**, 1388–1402.

Ratcliffe, N.A., Nigam, Y., Mello, C.B., Garcia, E.S. & Azambuja, P. (1996) *Trypanosoma cruzi* and erythrocyte agglutinins: a comparative study of occurrence and properties in the gut and hemolymph of *Rhodnius prolixus*. *Experimental Parasitology* **83**, 83–93.

Ribeiro, J.C.M., Genta, F.A., Sorgine, M.H.F., Logullo, R., Mesquita, R.D., Paiva-Silva, G.O., Majerowicz, D., Medeiros, M., Koerich, L., Terra, W.R., Ferreira, C., Pimentel, A.C., Bisch, P.M., Leite, D.C., Diniz, M.M.P, Lídio da S.G.V. Junior, J., Da Silva, M.L., Araujo, R.N., Caroline, A., Gandara, P., Brosson, S., Salmon, D., Bousbata, S., González-Caballero, N. Silber, A.M., Alves-Bezerra, M., Gondim, K.C., Silva-Neto, M.A.C., Atella G.C., Araujo, H. Dias, F.A., Polycarpo, C., Vionette-Amaral, R.J., Fampa, P.,

Melo, A.C.A., Tanaka, A.S., Balczun, C., Oliveira, J.H.M., Gonçalves, R.L.S., Lazoski, C., Rivera-Pomar, R., Diambra, L., Schaub, G.A., Garcia, E.S., Azambuja, P., Braz, G.R.C. & Oliveira, P.L. (2014) An insight into the transcriptome of the digestive tract of the bloodsucking Bug, *Rhodnius prolixus*. *PloS Neglected Tropical Diseases* **8**, e2594.

Rivero, A. (2006) Nitric oxide: an antiparasitic molecule of invertebrates. *Trends in Parasitology* **22**, 219–225.

Rohlfs, M. & Churchill, A.C.L. (2011) Fungal secondary metabolites as modulators of interactions with insects and other arthropods. *Fungal Genetics and Biology* **48**, 23–34.

Rozas-Dennis, G.S., La Torre, J.L., Muscio, O.A. & Guérin, M.A. (2000) Direct methods for detecting Picorna-like virus from dead and alive Triatomines insects. *Memorias del Instituto Oswaldo Cruz* **95**, 323–327.

Rückert, C., Bell-Sakyi, L., Fazakerley, J.K. & Fragkoudis, R. (2014) Antiviral responses of arthropod vectors: an update of recent advances. *Virus Disease* **25**, 249–260.

Ruiz-Sanchez, E., Orchard, I. & Lange, A.B. (2010) Effects of the cyclopeptide mycotoxin destruxin A on the Malpighian tubules of *Rhodnius prolixus* (Stal). *Toxicon* **55**, 1162–1170.

Sassera, D., Epis, S., Pajoro, M. & Bandi, C. (2013) Microbial symbiosis and the control of vector-borne pathogens in tsetse flies, human lice and triatomine bugs. *Pathogens and Global Health* **6**, 285–292.

Schaub, G.A. (1989) Does *Trypanosoma cruzi* stress its vectors? *Parasitology Today* **5**, 185–188.

Schaub, G.A., Meiser, C.K. & Balczun, C. (2011) Interactions of Trypanosoma cruzi and Triatomines. pp. 155–178 *in* Mehlhorn, H. (*Ed.*) *Progress in Parasitology*. Berlin, Springer-Verlag.

Schmid-Hempel, P. (2005) Evolutionary ecology of insect immune defenses. *Annual Review of Entomology* **50**, 529–551.

Schofield, C.J. & Galvao, C. (2009) Classification, evolution and species groups within the Triatominae. *Acta Tropica* **11**, 88–100.

Shah, P.A. & Pell, J.K. (2003) Entomopathogenic fungi as biological control agents. *Applied Microbiology and Biotechnology* **61**, 413–423.

Sistrom, M., Evans, B., Bjornson, R., Gibson, W., Balmer, O., Máser, P., Aksoy, S. & Caccone, A. (2014) Comparative genomics reveals multiple genetic backgrounds of human pathogenicity in the *Trypanosoma brucei* complex. *Genome Biology and Evolution* **6**, 2811–2819.

Strand, M.R. (2008) Insect hemocytes and their role in immunity. pp. 25–47 *in* Beckage, N.E. (*Ed.*) *Insect Immunity*. San Diego, California, USA, Academic Press.

Thomas, M.B. & Read, A.F. (2007) Can fungal biopesticides control malaria? *Nature Reviews Microbiology* **5**, 377–383.

Ursic-Bedoya, R.J. & Lowenberger, C.A. (2007) *Rhodnius prolixus*: identification of immune-related genes up-regulated in response to pathogens and parasites using suppressive subtractive hybridization. *Developmental and Comparative Immunology* **31**, 109–120.

Ursic-Bedoya, R.J., Nazzari, H., Cooper, D., Triana, O., Wolff, M. & Lowenberger, C. (2008) Identification and characterization of two novel lysozymes from *Rhodnius prolixus*, a vector of Chagas disease. *Journal of Insect Physiology* **54**, 593–603.

Ursic-Bedoya, R.J., Buchhop, J., Joy, J.B., Durvasula, R. & Lowenberger, C. (2011) Prolixicin: a novel antimicrobial peptide isolated from *Rhodnius prolixus* with differential activity against bacteria and *Trypanosoma cruzi*. *Insect Molecular Biology* **20**, 775–786.

Vieira, C.S., Waniek, P.J., Mattos, D.P., Castro, D.P., Mello, C.B., Ratcliffe, N.A., Garcia, E.S. & Azambuja, P. (2014) Humoral responses in *Rhodnius prolixus*: bacterial feeding induces differential patterns of antibacterial activity and enhances mRNA levels of antimicrobial peptides in the midgut. *Parasites and Vectors* **7**, 1–13.

Waniek, P.J., Castro, H.C., Sathler, P.C., Miceli, L., Jansen, A.M. & Araújo, C.A.C. (2009) Two novel defensin-encoding genes of the Chagas disease vector *Triatoma brasiliensis* (Reduviidae, Triatominae): gene expression and peptide-structure modeling. *Journal Insect Physiology* **55**, 840–848.

Waniek, P.J., Jansen, A.M. & Araújo, C.A.C. (2011) *Trypanosoma cruzi* infection modulates the expression of *Triatoma brasiliensis* def 1 in the midgut. *Vector Borne Zoonotic Diseases* **11**, 845–847.

Whitten, M., Sun, F., Tew, I., Schaub, G., Soukou, Ch., Nappi, A. & Ratcliffe, N. (2007) Differential modulation of *Rhodnius prolixus* nitric oxide activities following challenge with *Trypanosoma rangeli*, *T. cruzi* and bacterial cell wall components. *Insect Biochemistry and Molecular Biology* **37**, 440–452.

Whitten, M.M.A., Mello, C.B., Gomes, S.A.O., Nigam, Y., Azambuja, P., Garcia, E.S. & Ratcliffe, N.A. (2001) Role of superoxide and reactive nitrogen intermediates in *Rhodnius prolixus* (Reduviidae)/*Trypanosoma rangeli* interactions. *Experimental Parasitology* **98**, 44–57.

Yi, H.Y., Chowdhury, M., Huang, Y.D. & Yu, X.Q. (2014) Insect antimicrobial peptides and their applications. *Applied Microbiology Biotechnology* **98**, 5807–5822.

Capítulo 4. Discusión general

4.1 Efecto de los hongos entomopatógenos en la sobrevivencia de triatominos

Se ha señalado que una de las posibilidades para el futuro del control biológico en insectos, es el uso de agentes de control microbiano (microbial control agents MCAs), y el posible desarrollo de pesticidas microbianos y su implementación en campo (*Lacey et al., 2015*). Cerca de 50 especies de virus, bacterias, hongos y nematodos entomopatógenos, son producidos comercialmente y son utilizados como pesticidas microbianos, en una escala global y que aproximadamente de 1-2% de todos los plaguicidas vendidos, son de éste origen y han mostrado un crecimiento constante en comparación con los pesticidas químicos (*Lacey et al., 2015*).

El efecto de los hongos entomopatógenos en insectos ha sido ampliamente estudiado, muchos estudios reflexionan en la capacidad que tienen para disminuir la capacidad de sobrevivencia en su hospedero (*Gillespie et al., 1997; Blandford et al., 2005; Castellanos-Moguel et al., 2008; Baverstock et al., 2010*).

Las especies de hongos entomopatógenos que se comecializan están basados en 3 especies de hongos: *Beauveria spp*, *Metarhizium spp*., *Isaria fumosorosea* (*=Paecilomyces fumosoroseus*) y *Lecanicillium spp*. (*Lacey et al., 2015*).

Un factor importante cuando se elige una especie entomopatógena, incluidos los hongos es la virulencia; es decir, la capacidad que tienen para afectar la sobrevivencia en el insecto. Se sabe que la virulencia en hongos entomopatógenos, generalmente decrece con el sub-cultivo en medios como agar papa dextrosa, y puede ser otra vez activada al pasar por el hospedero insecto. Un aislado de hongos entomopatógenos virulento es aquel que expresa una gran cantidad de proteasas, exoenzimas durante la penetración de la cutícula y la generación de toxinas cuando el hongo coloniza al hospedero (*Lacey et al., 2015*). En este estudio se seleccionaron dos aislados de hongos entomopatógenos previamente caracterizad'os en cuanto su virulencia y patogenicidad: *I. fumosorosea* EH-511/3 y *M. anisopliae* EH-473/4 (*Castellanos-Moguel et al., 2007; Toriello et al,. 2008*). Además han sido caracterizados fenotípicamente y genotípicamente (*Ayala-Zermeño et al., 2011; Castellanos-Moguel et al., 2007; Castellanos-Moguel et al., 2008; Mier et al., 2002; 2005; Toriello, 2003; Toriello et al., 2006; Toriello et al. 2008*). Se ha demostrado que la virulencia en hongos puede ser aumentada a través de la manipulación genética, insertando genes en el genoma fúngico, y con ello promover la expresión de toxinas que incrementan la virulencia del hongo, i. e. inserción de los genes de la toxina del escorpión dentro de *M. anisopliae* y *B. bassiana* (*Wang & St. Leger, 2007*).

El mecanismo de infección en hongos es único; ya que los conidios se adhieren a la cutícula (*Charnley & St. Leger, 1991; Lacey et al., 2015*). En el presente artículo se utilizó una suspensión de $1x10^7$ conidios /ml, aplicados en el pronoto (parte rígida en el tórax de triatominos), lo cuál

permitió observar el efecto de los hongos entomopatógenos por la vía de entrada natural en su hospedero. Dependiendo del patógeno y de la vía de infección, es posible esperar diferentes tipos de respuesta inmune por parte del hospedero. Varios estudios han mostrado altos niveles de PO en respuesta a la inyección de componentes fúngicos tales como blastosporas o conidios, sin embargo como lo menciona *Gillespie et al., 2000b* esta situación no refleja el mecanismo de patogenicidad natural.

Descubrimientos actuales sobre los efectos de los hongos entomopatógenos en mosquitos adultos como *Anopheles stephensi* y *A. gambiae*, mencionan que los insectos infectados con hongos, a su vez evitan el desarrollo del parásito (*Plasmodium spp.*) en el mosquito, lo cuál se ha traducido en un aumento en el desarrollo de investigación enfocada al control de enfermedades transmitidas por mosquitos como malaria (*Blanford et al., 2005; Scholte et al., 2003; 2005*). Sin embargo, la investigación en triatominos infectados con *Trypanosoma cruzi* utilizando hongos entomopatógenos, no ha sido considerada aún.

Aunque los hongos entomopatógenos han sido mencionados principalmente como patógenos de artrópodos, recientemente también se ha demostrado su papel como endófitos de plantas, antagonistas de enfermedades en plantas, colonizadores de rizosferas y promotores del crecimiento en plantas (*Lacey et al., 2015*).

Los hongos entomopatógenos tienen muchas características que son favorables cuando se trata de elegir un buen agente de control biológico. Poseen un riesgo mínimo para organismos no-blanco benéficos tales como abejas, lombrices de tierra y Colémbolos, los cuales son proveedores de servicios ecosistémicos, de igual forma, su efecto es nulo en enemigos naturales de artrópodos como avispas parasitoides y escarabajos depredadores (*Lacey et al., 2015*). Esto fortalece su papel potencial en el manejo integrado de plagas (IPM-integrated pest management), ya que la preservación de enemigos naturales les permite realizar una mejor contribución en la regulación de plagas.

La evasión de la respuesta inmune en insectos infectados por hongos entomopatógenos, es un elemento importante en el proceso de infección, permitiendo al hongo satisfacer sus demandas de nutrientes durante el crecimiento en el hospedero *(Gillespie et al., 2000a)*.

En mi primer artículo hablo de la efectividad de *M. anisopliae* e *I. fumosorosea* para afectar la sobrevivencia de las ninfas de 5to. estadio de *M. pallidipennis*. Se encontró que ambos hongos presentaron la misma capacidad para infectar, sin embargo, *M. anisopliae*, demostró ser más virulento matando a su hospedero a los 8 días posteriores a la infección. En cuanto al tiempo de muerte en el hospedero, en control biológico se enfatiza en la capacidad que tienen para influenciar el comportamiento del insecto, en lugar de matar rápidamente al vector. Esta especie de hongo entomopatógeno, ha sido utilizada en otros triatominos y en diferentes estadios como: huevos, ninfas de 1er. estadio y 3er. estadio de *Triatoma infestans* (*Rodrigues et al., 2014; Luz et al., 2012;*

Flores-Villegas, 2011). *I. fumosorosea* ha sido menos utilizada contra triatominos y en el presente estudio se encontró que su prolongado tiempo afecta la sobrevivencia de *M. pallidipennis* (más de 20 días). Otros estudios en insectos que no son triatominos, han demostrado la eficiencia de *M. anisopliae* (*Flores et al., 2012; Kavallieratos et al., 2014*).

Para comprobar la presencia de ambos hongos entomopatógenos en el trabajo de *Flores-Villegas* se realizaron improntas teñidas con azul de metileno y se pudo observar que el hongo se encontraba en el hemocele del insecto, al inicio de la infección, observándose principalmente estructuras parecidas a hifas y posteriormente conidios, esto es importante, ya que resulta indicativo del proceso de infección y colonización del hongo entomopatógeno (*Flores-Villegas et al., 2016*). En el caso de *M. anisopliae* fue evidente la presencia de "columnas de conidios", lo cuál concuerda con la esporulación del hongo en la superficie del insecto, entre los días cinco ó seis, posteriores a la infección. En el caso de *I. fumosorosea* fueron característicos los conidios en forma de "arroz", lo cuál concuerda con procesos de infección observados en otros insectos (*Charnley & St. Leger, 1991; Humber, 2010; Tulloch, 1976*).

Dada la importancia que ha cobrado el uso de hongos entomopatógenos para el control de insectos transmisores de enfermedades, es importante explorar los mecanismos de respuesta inmune que evaden durante el proceso de infección.

4.2 Efecto de los hongos entomopatógenos en la respuesta inmune de triatominos

La primera línea de defensa en los insectos es la cutícula, la cuál está compuesta de ácidos grasos y componentes como la melanina que impiden la penetración de patógenos y parásitos, incluyendo los hongos (*Charnley & St. Leger, 1991*; *Thomas & Read, 2007*). Sin embargo, algunos microorganismos como los hongos entomopatógenos, son capaces de degradar los hidrocarbonos presentes en la cutícula (*Napolitano & Juárez, 1997*), esto sucede cuando es activada la respuesta inmune humoral y celular. La respuesta celular está compuesta por hemocitos cuya función es la encapsulación, en ellos se lleva a cabo la síntesis de melanina y junto con la fenoloxidasa, péptidos antimicrobianos y especies reactivas de oxígeno, logran contrarrestar una infección. La enzima PO, es sumamente importante en este proceso, como lo han demostrado trabajos anteriores, y está involucrada en la síntesis de melanina, cuya actividad antifúngica, actúa directamente en la superficie celular (*Butt et al., 1988; Cerenius et al., 1990*).

No obstante su papel durante la defensa contra patógenos, la PO y su precursor proPO, han mostrado un decremento durante la infección por hongos entomopatógenos (*Bogus´et al., 2007; Gillespie et al., 2000b; Khosravi et al., 2014*). Resultados similares se obtuvieron en este estudio, donde se observó un decremento en la PO y proPO a las 24 horas posteriores a la infección, sin ningún cambio aparente en estos componentes en el resto de los días.

El uso de *Beauveria bassiana* en el control de el saltamontes *Melanoplus sanguinipes* (*Bidochka & Khachatourians*, 1987) y en *Schistocerca gregaria* (*Gillespie et al., 2000b; Milat-Bissaad et al., 2011*) condujeron a una reducción en el número de hemocitos. Una posible explicación para los resultados obtenidos en éste studio es que las micotoxinas como destruxinas, como en el caso de *M. anisopliae,* pudieron conducir a la reducción en el número de hemocitos y por consecuente a una disminución en la actividad de la PO y su precursor proPO.

Las micotoxinas afectan negativamente la actividad de la fenoloxidasa (*Avulova et al., 2011*), además de influir en los procesos de fagocitosis y encapsulación por parte de los hemocitos (*Rohlf & Churchill, 2011*). Tales acciones explican la posibilidad de lisis por hongos entomopatógenos en hemocitos, los cuáles llevan a acabo la cascada de la PO (*Gillespie & Khachatourians, 1992*), y que por lo tanto estas dos enzimas se vean afectadas.

En el biocontrol de transmisores de enfermedades, no es necesario matar rápidamente al vector con su agente etiológico, más que eso, se enfatiza en el potencial de los entomopatógenos para influenciar en el comportamiento del insecto y de una forma más sutil poder afectar negativamente la transmisión del agente etiológico sin necesidad de reducir la densidad de transmisores *(Scholte et al., 2005)*. Por ejemplo, numerosos estudios en insectos plaga han resaltado el potencial de los hongos para producir efectos pre-letales. Las langostas que se infectan con hongos entomopatógenos desarrollan alteraciones en comportamiento, alimentación, fecundidad, movilidad y respuesta tardía de escape a depredadores antes de que mueran *(Scholte et al., 2005)*. Adicionalmente, se ha visto que los mosquitos transmisores de *Plasmodium sp.* e infectados con entomopatógenos tienden a reducir su alimentación antes de su muerte, por lo tanto se altera la tasa de sobrevivencia del parásito de la malaria dentro del mosquito *(Blandford et al., 2005)*.

En mi estudio fué claro el decremento en la PO y proPO a las 24 horas, no detectándose cambios posteriores en estos mismos componentes. Otros estudios en insectos han reportado un incremento en la actividad de la PO después de 10 minutos de infección con *Beauveria bassiana* en *Melanoplus sanguinipes* (*Gillespie & Khachatourians, 1992*), seguido por un decremento a las 24 horas y sin haber cambios aparentes después de éste tiempo (*Gillespie & Khachatourians, 1992; Gillespie et al., 2000b*). Esto implica una activación de la respuesta inmmune seguida por un efecto negativo de los hongos en la cascada de la fenoloxidasa, por lo tanto, los resultados de este estudio confirman las afirmaciones previas de que las infecciones fúngicas afectan la fenoloxidasa y su precursor profenoloxidasa (*Gillespie et al., 2000b; Bogus´ et al., 2007; Khosravi et al., 2014*).

La fenoloxidasa no sólo ha cobrado importancia como mecanismo de respuesta inmune en insectos, sino que ahora se enfatiza en su papel en aquellos insectos resistentes a insecticidas. Esto se ha analizado recientemente en mosquitos. La resistencia que algunos insectos han generado puede interferir en la inmunidad, ya que la resistencia a insecticidas y los mecanismo inmunes son

energéticamente costosos *(Cornet et al., 2013)*. Es decir, entran en un trade-off o compromiso fisiológico la inmunidad y la resistencia a insecticidas.

Se ha propuesto que las larvas de *Culex pipiens*, poseen un sistema inmune más fuerte que los adultos, y que las hembras son aún más fuertes inmunológicamente hablando que los machos, ya que las hembras al ser las únicas que se alimentan de sangre, están expuestas a un diverso número de parásitos. El término "inmunosenescencia", se refiere a que la inmunidad de hembras y machos declina con la edad, esto es porque la mayor parte de sus recursos están encaminados a maximizar el éxito reproductivo, antes de que mueran *(Cornet et al., 2013)*.

Para apoyar o refutar la idea anterior, se podría medir la respuesta inmune en el estadio adulto del vector, en este estudio se utilizaron ninfas de quinto estadio, en larvas de *Culex pipiens*, se ha reportado que tienen más del doble de cantidad de PO activa y total, esto debido a que las larvas viven en estancamientos de agua donde están expuestos a una gran gama de parásitos *(Cornet et al., 2013)*. En investigaciones donde se ha trabajado con triatominos, las ninfas de 5to. estadio se han encontrado tanto en el intradomicilio como el peridomicilio, lo cual las hace sujeto de igual forma a diversos patógenos.

Estudios previos reportan la virulencia de la cepa EH-473/4 y EH-511/3 en el estadio de huevo de *M. pallidipennis (Flores-Villegas, 2011)* y nuestros resultados apoyan la idea del uso de estos hongos como inhibidores de la respuesta inmune en el vector y como posibles controladores biológicos y por lo tanto una alternativa al uso de insecticidas químicos.

Se ha señalado que el proceso de infección de hongos entomopatógenos en insectos ocurre dentro de las primeras 24 horas y posteriormente el hongo genera metabolitos secundarios como Destruxinas en *M. anisopliae* y Beauvericinas en *I. fumosorosea*. Dcihas toxinas actúan como inmunosupresores, alterando la respuesta inmune humoral, celular y abriendo canales de calcio que pueden causar efectos citotóxicos en hemocitos, túbulos de Malpighi y células epiteliales mesentéricas, en tanto que los niveles de calcio pueden causar parálisis o letargia en el insecto (*Avulova y Rosengaus, 2011)*.

Dentro del insecto, los hongos se reproducen como blastosporas o cuerpos hifales, en *M. anisopliae*, esta última forma de replicación produce una capa protectora de colágeno que evita el reconocimiento de β-1,3-Glucanos, por componentes de la respuesta inmune humoral y celular del insecto (Wang & St. Leger, 2006). Quizá esta es un mecanismo que desarrolló *M. anisopliae* a diferencia de *I. fumosorosea*, para ser más eficiente en evadir la respuesta inmune en insectos, como lo mostraron nuestros resultados.

La respuesta inmune de triatominos ante infecciones con hongos entomopatógenos no ha sido estudiada a detalle, a diferencia de la información generada contra *T. cruzi* y *T. rangeli*. El incremento en la cantidad de proteínas, sugieren un recurso importante en triatominos en su intento

para lidiar contra hongos entomopatógenos, activando la producción de proteínas durante la infección fúngica.

El efecto de inhibición de la PO por *M. anisopliae* corresponde principalmente con el tiempo en que las destruxinas, desarrollo de formas como blastosporas y cuerpos hifales se hacen presentes en el insecto. Con respecto a *I. fumosorosea*, el día 4 muestra un incremento en los niveles de esta enzima, quizá porque el sistema inmune del insecto es capaz de reconocer las blastoesporas. Con base en las curvas de sobrevivencia, se observó que *I. fumosorosea* tarda más tiempo en matar al hospedero y no mata al 100% de los insectos.

Los resultados obtenidos con *M. anisopliae* en mi tesis son similares a lo señalado por *Gillespie et al., 2000b*, al infectar tópicamente *Schistocerca gregaria* con *Metarhizium anisopliae* var *acridum.* En dicho trabajo se menciona que en el día dos posterior a la infección disminuyen los niveles de PO en insectos infectados y permanecen en un nivel por debajo del grupo control a lo largo de los días del experimento, lo cuál posiblemente se explica por inhibición directa del hongo en la activación de la PO, cómo ha sido reportado previamente en otros insectos *(Elbanna et al., 2012).*

La capacidad de inhibición del sistema inmune por *M. anisopliae* es un elemento fundamental para causar patogénesis en el insecto, la cuál ocurre desde la adhesión del conidio a la cutícula, en la hemolinfa a través de la producción de metabolitos secundarios como (Destruxinas) y la capacidad de dimorfismo a través de estructuras como blastosporas y cuerpo hifales. En el caso de este hongo entomopatógeno se ha demostrado la formación de una cubierta de colágeno en cuerpos hifales que evitan el reconocimiento de los β-1,3-Glucanos por el sistema humoral y celular del insecto *(Wang & St. Leger, 2006)*.

Sin embargo, *I. fumosorosea* parece no seguir el mismo comportamiento, ya que en el día 4 se observó un incremento en la actividad de la enzima PO y en el día 6 declinó. Una posible hipótesis es que el sistema inmune del insecto detecta al hongo entomopatógeno y éste no es percibido como una amenaza y por lo tanto el insecto no exhibe una respuesta inmune clara. Tal idea es apoyada en revisiones previas con el hongo entomopatógeno *Beauveria bassiana (Baverstock et al., 2010)*. El tiempo de sobrevivencia con este hongo es mayor con respecto a *M. anisopliae* y no mata al 100% de los insectos prueba, tales resultados se discutirán posteriormente.

En triatominos el reconocimiento y defensa contra tripanosomátidos induce una alta actividad de PO en la hemolinfa y cuerpo graso; siendo el intestino medio anterior el tejido con mayor cantidad de PO (*Genta et al., 2010*).En este sitio también ocurre la multiplicación de bacterias simbiontes como *Rhodococcus rhodnii* y *Serratia marcescens*, aunque este tejido también podría albergar hongos simbiontes o entomopatógenos. Posiblemente al predominar simbiontes en el intestino medio la PO puede actuar como regulador en el control de esta microbiota *(Genta et al., 2010).*

En un estudio realizado en *Rhodnius prolixus* (Hemiptera: Reduviidae), se menciona que la actividad de la PO en ninfas de 5to. estadio es variable, después de la ingesta de sangre, la actividad

de la PO se mantiene constante y después decrece en el día 7 posterior a la infección *(Genta et al., 2010)*. Otro factor que influye en la disminución de la actividad de la PO en insectos no infectados es la inanición *(Adamo, 2004b)*. Al respecto se conoce que la fenilalanina es el aminoácido precursor en la cascada de la PO, la cuál es obtenida de la comida (*Nappi & Christensen, 2005*). Durante el curso del presente experimento los triatominos solo realizaron una ingesta de sangre dos días antes de iniciar el bioensayo, y esto probablemente afectó la actividad de la PO en insectos control.

Bajos niveles en la actividad de la PO se han demostrado al utilizar azadiractina, un potente inhibidor en la síntesis y secreción de la ecdisona, una enzima necesaria para mudar entre estadios ninfales, proponiéndose que la ecdisona regula la respuesta inmune en *Rhodnius prolixus (Genta et al., 2010).*

Medir la actividad de la PO en insectos es un buen indicador de resistencia a patógenos y parásitos porque: (i) es un componente importante del sistema inmune en insectos; (ii) los niveles de la actividad de la fenoloxidasa parecen ser heredables; (iii) la actividad de la fenoloxidasa ha sido correlacionada con la resistencia a algunos patógenos y parásitos.

En nuestro estudio se pudo observar que la actividad de la PO en *M. pallidipennis* es inhibida principalmente por *M. anisopliae*. Los días donde decrece la actividad de la PO, concuerdan con el tiempo en que se encuentran blastosporas y cuerpos hifales en el interior del insecto, es decir los días 2 y 4. Las infecciones por *I. fumosorosea* parecen ser reconocidas por el insecto, pero no exhibe una respuesta inmune y esto se ve reflejado en la sobrevivencia del insecto *(Adamo, 2004a)*.

La PO cuyo objetivo principal es la formación de la melanina, está presente no solo en la hemolinfa, sino también en la cutícula del insecto hospedero, siendo sus funciones la esclerotización de la misma y la cicatrización de heridas al ser activada la profenoloxidasa, mediante mecanismos de señalización que involucran la cascada de las serin-proteasas *(Córdoba-Aguilar et al., 2011; Gillespie et al., 2000a).*

Para activar la PO que no se utilizó durante el proceso de infección se agregó α-Quimiotripsina, en este estudio se pudo observar que la actividad del grupo testigo fue más alta que el grupo infectado y la actividad del grupo infectado disminuyó durante la infección por *M. anisopliae*.

Los resultados indicaron que la actividad de la profenoloxidasa fue mayor que la fenoloxidasa durante el curso de la infección, esto podría indicar que la proenzima fue producida a un nivel mayor del que fue activada. Tal inhibición, como se mencionó previamente, puede estar dada por metabolitos secundarios denominados destruxinas (DTX), y que en *M. anisopliae* han sido identificadas al menos 23 variantes en cultivos, todas conformadas por cinco aminoácidos y un hidroxiácido (*Kershaw et al., 1999)*. La producción de éstas toxinas por el hongo *M. anisopliae* var *acridum* inhiben la PO en los hemocitos de la langosta *Locusta migratoria* (*Gillespie et al., 2000b).*

Existen al menos dos posibles estrategias de virulencia en hongos entomopatógenos: la estrategia de toxinas dada por las DTX y la estrategia de crecimiento por cuerpos hifales y blastosporas *(Kershaw et al., 1999)*.

Durante el proceso de infección la enzima profenoloxidasa se activa para dar lugar a la fenoloxidasa *(Soderhall & Cerenius, 1998)*. La activación de la profenoloxidasa inicia con proteínas de reconocimiento (βGRP, LGRP y PGRP por sus siglas en inglés) que se unen a componentes presentes en la pared celular de los patógenos (β-1,3-Glucanos, Lipopolisacáridos y Peptidoglicanos). Esta interacción activa mecanismos de señalización que involucran serin-proteasas, éstas son hidrolasas con un aminoácido de serina en su centro activo. Las hidrolasas incluyen la tripsina, quimiotripsina y subtilisina. Generalmente se usa quimiotripsina como activador de la proPO, éste activador es útil para cuantificar la PO en la hemolinfa y la PO total en todo el insecto *(Córdoba-Aguilar et al., 2011; Gillespie et al., 2000a; González-Santoyo y Córdoba-Aguilar, 2012)*.

Algunos autores refieren a la proPO como "PO total", es decir la PO activa más proPO inactiva, medir la proPO es un indicador de cuanta proenzima estaba produciendo el insecto hospedero al momento de la infección *(Cornet et al., 2013)*, y al igual que las observaciones con la PO se encontró una disminución importante, a partir del día dos posterior a la infección, en comparación con el grupo testigo.

Desde el punto de vista biológico, la PO mide la cantidad de enzima activada naturalmente, mientras que la "PO total" o proPO mide la inversión global en la defensa inmune del insecto*(Cornet et al., 2013)*.

La melanización es un mecanismo de encapsulación en insectos contra patógenos altamente eficiente, para lo cual se necesita de la activación de la profenoloxidasa (proPO) para convertirse en fenoloxidasa (PO). Por lo tanto la modulación de la respuesta inmune por parte de los hongos entomopatógenos, puede involucrar la unión de moléculas presentes en la pared celular como β-1,3-Glucanos y así evitar la activación de la cascada fenoloxidasa *(Soderhall y Cerenius, 1998)*.

4.3 Respuesta inmune en triatominos ante patógenos

Los insectos como los triatominos están expuestos a ataques por diferentes patógenos y parásitos, estos en respuesta han desarrollado y evolucionado diferentes sistemas de defensa, anteriormente se ha mencionado como primer barrera la cutícula exoesquelética, el epitelio intestinal y glándulas accesorias reproductivas; todos estos componentes pueden secretar lisozimas y compuestos citotóxicos para ayudar a eliminar al patógeno (*Azambuja et al., 1999*).

La entrada de los diferentes patógenos en el triatomino, genera diferentes mecanismos de respuesta inmune, incluso ante la entrada de microorganismos sub-patógenos como *Trypanosoma cruzi* y *T. rangeli*.

Previamente se ha señalado el efecto de hongos entomopatógenos y su capacidad para afectar la sobrevivencia y actividad de dos enzimas importantes en la respuesta inmune: PO y proPO.

Sin embargo, pocos estudios han señalado la importancia de el efecto de diferentes patógenos en la respuesta inmune de triatominos (*Flores-Villegas et al., 2015*), alrededor de 130 especies de triatominos han sido descritas, sin embargo, solamente 10 especies de estas se han estudiado, en contraste con otros vectores como los mosquitos (*Blandford et al., 2005*), cuyas interacciones con diferentes especies de patógenos son mejor conocidas.

Por otra parte los estudios de la interacción entre triatominos y *T. cruzi*, generalmente se han centrado en la descripción del ciclo de vida de *Trypanosoma cruzi* en estos (*Kollien & Schaub, 2000*). Existe muy poca información sobre los efectos de la respuesta del sistema inmune de triatominos (*Mello et al., 1995*; *Azambuja et al., 1999*; *Gomes et al., 2003*; *Whitten et al., 2007*; *Espinosa de Aquino, 2012*), mecanismos de modulación de *T. cruzi* en el triatomino (*Castro et al., 2012; Favila-Ruiz, 2016*) y respuesta inmune asociada a factores bióticos como la altitud (*De Fuentes-Vicente et al., 2016*). En el caso de hongos entomopatógenos, muchas especies han sido descritas como patógenos en triatominos (*Luz et al., 1998a; Lara Da costa et al., 2003; Luz et al., 2004*), sin embargo, su papel a nivel de respuesta inmune es desconocida, a excepción del presente trabajo por *Flores-Villegas et al., 2016*.

Para el entendimiento de la respuesta inmune en triatominos se puede tomar como base estudios previamente realizados en otros insectos (como aquellos de importancia agrícola *Shah & Pell, 2003*).

Un estudio importante fue realizado por *Ribeiro et al., 2014*, el cuál identificó el transcriptoma del tracto intestinal de *R. prolixus*, encontrando datos la expresión de genes relacionados a la vía de señalización Toll que codifican para lisozimas y lectinas. Dichos resultados contrastan a lo registrado en otros órdenes de insectos como Dipteros, lo cuál habla de que esta vía de señalización es más usada en *R. prolixus*, en comparación con vías de señalización como IMD y STAT, al respecto, hace falta indagar más sobre estas vías y su papel en la respuesta inmune.

El principal objetivo de explorar la respuesta inmune en triatominos ante diferentes patógenos, es proponer nuevas estrategias de control utilizando bacterias como: *Rhodococcus rhodnii* (*Dotson et al., 2003; Kollien et al., 2003*) y *Serratia marcescens (Azambuja et al., 2004*; *Gourbiére et al., 2012*), el triatoma virus TrV (*Muscio et al., 1987*) y otras especies de hongos simbiontes (*Moraes et al., 2000*), que regulen el establecimiento de *T. cruzi* y reforzamiento de la respuesta inmune en triatominos, la cuál consiste en péptidos antimicrobianos como cecropinas (*Beard et al., 2001*), defensinas, lisozimas y las recientemente descritas prolixicinas (*Mello et al., 1995; Gomes et al.,*

1999; Ursic-Bedoya et al., 2011; Waniek et al., 2011), además de la generación de radicales libres (*Whitten et al., 2001*) y lectinas (*Azambuja et al., 1999*), las cuáles se encuentran principalmente en el tracto digestivo, sitio de desarrollo del parásito *T. cruzi*.

Finalmente y para propósito de control biológico, se podrían explorar otras estrategias utilizando nuevos patógenos, y gracias a que se conoce actualmente el genoma de *T. cruzi*, establecer asociaciones entre triatominos y tripanosomas, la identificación de genes en triatominos que sean activados durante la infección por *T. cruzi* y la identificación de cambios en las glicoproteínas en el tracto intestinal de triatominos (*Gutiérrez-Cabrera et al., 2014; Rivas-Medina, 2014)*, sitio de adhesión y desarrollo del parásito, y que contribuyan a crear estrategias dirigidas a evitar la colonización por el parásito.

Como se mencionó anteriormente quizá sea necesario la integración de estrategias de control basadas en multipatógenos como las utilizadas por *Bedding & Molyneux, 1982,* utilizando nematodos y hongos, o el uso de elementos tóxicos, como lo cita *Fieck et al., 2010*, cuando combina diferentes péptidos antimicrobianos como las cecropinas.

Capítulo 5. Conclusiones generales

Dado que el principal método de control en triatominos es el uso de insecticidas, se presentó el uso de hongos entomopatógenos como una alternativa de control biológico. Nuestros resultados *in vitro* parecen ser promisorios, sin embargo, faltaría su implementación en campo.

El efecto de los hongos entomopatógenos en *M. pallidipennis* varió de acuerdo al estado ontogenético utilizado, por lo cuál sería apropiado probar otros estadios.

Por otra parte, quizá sea necesario el uso de más de una estrategia para el control de triatominos, es decir, utilizando otros patógenos como bacterias, por ejemplo, *Serratia marcescens,* Triatoma virus o incluso otros hongos entomopatógenos.

Finalmente nuestros resultados, muestran que *M. anisopliae* (EH-473/4):

1) es capaz de penetrar la cutícula del insecto y llegar a la hemolinfa del vector

2) modular la infección en su hospedero, a través de una baja en la activación de la enzima PO y su precursor, afectando de esta forma la sobrevivencia del hospedero.

Adamo SA. 2004a. **Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*.** J Insect Physiol 50: 209-216.

Adamo SA. 2004b. **How should behavioural ecologists interpret measurements of immunity?.** Anim Behav 68: 1443-1449.

Albano-Amóra SS, Leal-Bevilaqua CM, Carneiro-Feijó FM, De Macedo Assuncao-Pereira RH, Dutra- Alves N, De Morais-Freire FA, Toth-Kamura M, Magalhaes-De Oliveira D, Luna-Alves Lima EA, Gadelha-Rocha MF. 2010. **The effects of the fungus *Metarhizium anisopliae* var. *acridum* on different stages of *Lutzomya longipalpis* (Diptera: Psychodidae).** Acta Trop 113: 214-220.

Andrade DV, Gollob KJ, Dutra WO. 2014. **Acute Chagas Disease: New Global Challenges for and Old Neglected Disease.** PLoS Negl Trop Dis 8: e3010.

Avulova S y Rosengaus RB, 2011. **Losing the battle against fungal infection: Supression of the termite immune defenses during mycosis.** J Insect Physiol 57: 966-971.

Ayala-Zermeño MM, Reyes-Montes MR, Arroyo-Vázquez E, Calderón-Esquerro MC, Mier T, Robledo-Retana T, Toriello C. 2011. **An *Isaria fumosorosea* SCAR marker for evaluation of soil, insect, and airborne samples.** Biocontrol Sci Techn 21: 1091-1102.

Azambuja P, Feder D, Mello CB, Gomes SAO, Garcia ES. 1999. **Immunity in *Rhodnius prolixus*: Trypanosomatid-vector interactions.** Mem Inst Oswaldo Cruz, Rio de Janeiro 94: 219-222.

Azambuja P, Feder D, Garcia ES. 2004. **Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector.** Exp Parasitol 107: 89-96.

Bautista NL, García-De la Torre G, De Haro-Arteaga I, Salazar-Schettino PM. 1999. **Importance of *Triatoma pallidipennis* (Hemiptera: Reduviidae) as a vector of *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae) in the state of Morelos, Mexico, and Possible Ecotopes.** J Med Entomol 36: 233-235.

Bautista NL, Rojas G, De Haro I, Bucio M, Salazar-Schettino PM. 2001. **Comportamiento biológico de *Triatoma pallidipennis* (Hemiptera: Reduviidae) en el estado de Morelos México.** Bol chil parasitol 57: 22-27.

Baverstock J, Roy HL, Pell JK. 2010. **Entomopathogenic fungi and insect behaviour: from unsuspecting hosts to targeted vectors.** BioControl 55:89-102.

Beard CB, Dotson EM, Pennigton PM, Eichler S, Cordon-Rosales C, Durvasula RV. 2001. **Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease.** Int J of Parasitol 31: 621-627.

Bedding RA, Molyneux AS. 1982. **Penetration of insect cuticle by infective juveniles of *Heterorhabditis spp.* (Heterorhabditidae: Nematoda).** Nematologica 28: 354–359.

Bern, C. 2015. **Chagas´Disease.** N England J Med. 373: 456-466.

Benítez-Alva JI, Huerta H, Téllez-Rendón JL. 2012. **Distribución de triatominos (Heteroptera: Reduviidae) asociados a la vivienda humana y posibles zonas de riesgo en seis estados de la República Mexicana**. BIOCYT: Biología Ciencia y Tecnología 5: 327-340.

Bidochka MJ, Khachatourians GC. 1987. **Hemocytic defense response to the entomopathogenic fungus *Beauveria bassiana* in the migratory grasshopper *Melanoplus sanguinipes*.** Entomol Exp Appl 1987, 45: 151-156.

Blandford S, Chan BH, Jenkins N, Sim D, Turner RJ, Read AF, Thomas MB. 2005. **Fungal pathogen reduces potential for malaria transmission.** Science 308: 1638-1641.

Bogus´MI, Kendra E, Bania J, Szczepanik M, Czygier M, Jablonsky P, Pasztaleniec A, Samborski J, Mazgajska J, Polanowski A. 2007. **Different defense strategies of *Dendrolimus pini*, *Galleria mellonella*, and *Calliphora vicina* against fungal infection.** J Insect Physiol 53: 909-922.

Boulanger N, Bulet P, Lowenberger C. 2006. **Antimicrobial peptides in the interactions between insects and flagellates parasites.** Trends Parasitol 6: 262-268.

Butt TM, Wraight SP, Galaini-Wraight S, Humber RA, Roberts DW, Soper RS. 1988. **Humoral encapsulation of the fungus *Erynia radicans* (Entomophthorales) by the potato leafhopper, *Empoasca fabae* (Homoptera: Cicadellidae).** J Invertebr Pathol 52: 49–56.

Castellanos-Moguel MJ, González-Barajas M, Mier T, Reyes-Montes MR, Aranda E, Toriello C. 2007. **Virulence testing and extracelular subtilisin-like (Pr1) and trypsin-like (Pr2) activity during propagule production of *Paecilomyces fumosoroseus* isolates from whiteflies (Homoptera: Aleyrodidae).** Rev Iberoam Micol 24: 62-68

Castellanos-Moguel MJ, Cruz-Camarillo R, Aranda E, Mier T, Toriello C. 2008. **Relationship between protease activity and the virulence of *Paecilomyces fumosoroseus* in *Trialeurodes vaporarium* (Hemiptera: Aleyrodidae).** Rev Mex Mic 28: 71-80

Castillo JC, Robertson AE, Strand MR. 2006. **Characterization of hemocytes from the mosquitos *Anopheles gambiae* and *Aedes aegipty*.** Insect Biochem Molec Biol 36: 891-903.

Castro DP, Moraes CS, Gonzalez MS, Ratcliffe NA, Azambuja P, Garcia ES. 2012. ***Trypanosoma cruzi* Immune Response Modulation Decreases Microbiota in *Rhodnius prolixus* Gut and Is Crucial for Parasite Survival and Development.** PloS ONE 7: e36591.

Carcavallo RU, Galíndez-Girón I, Jurberg J, Lent H. 1997. **Bibliographic checklist of the American triatominae (Hemiptera Reduviidae).** In: Carcavallo R, Galíndez-Girón I., Jurberg J, Lent H. (Eds). Atlas of Chagas Disease Vectors in the Americas, Vol. I. Editora Fiocruz, Rio de Janeiro, pp. 15-52.

Carcavallo RU, Jurberg J, Lent H, Noireau F, Galvao C. 2000. **Phylogeny of the triatominae (Hemiptera: Reduviidae). Proposal for taxonomic arrangements.** Entomol Vect 7 (Suppl. 1): 79-81.

Cecere MC, Gurtler RE, Canale D, Chuit R, Cohen JE. 1996. **El papel del peridomicilio en la eliminación de *Triatoma infestans* de comunidades rurales argentinas**. Bol Ofic Sanit Panam 121: 1-10.

Cerenius L, Thörnqvist PO, Vey A, Johansson MW, Söderhall K. 1990. **The effect of the fungal toxin Destruxin E on isolated Crayfish haemocytes**. J Insect Physiol 36: 785-789.

Córdoba-Aguilar A, Ruiz-Silva D, Munguía-Steyer R y Lanz-Mendoza H. 2011. **Do reproductive activities compromise immunological competence as measured by phenoloxidase activity? Field and experimental manipulation in females of two damselfly species.** Physiol Entomol 36: 335-342.

Cornet S, Gandon S, Rivero A. 2013. **Patterns of phenoloxidase activity in insecticide resistant and suceptible mosquitoes differ between laboratory-selected and wild-caught individuals.** Parasit Vectors 6: 2-11.

Cortico-Correa VL, Ferra-Filho AN, Olavo da Rocha-Silva E, Kenge-Ishinata G. 2002. **Triatomine: ovicida action of some pyretroids.** Rev Soc Bras Med Trop 35: 237-241.

Charnley AK, St. Leger RJ. 1991. **The role of cuticle degrading enzymes in fungal pathogenesis in insects.** In: Cole TG, Hoch CH(Eds.). The fungal Spore and Disease Initiation in Plants and Animals. Plenum Press, New York, pp. 267-286.

De Fuentes-Vicente JA, Cabrera-Bravo MA, Enríquez-Vara JN, Bucio-Torres MI, Gutiérrez-Cabrera AE, Vidal-López DG, Martínez-Ibarra JA, Salazar-Schettino PM, Córdoba-Aguilar A. 2016. **Relationships between altitude, triatomine (*Triatoma dimidiata*) immune response and virulence of *Trypanosoma cruzi*, the causal agent of Chagas' disease.** Med Vet Entomol doi: 10.1111/mve.12198.

Dotson EM, Plikaytis B, Shinnick TM, Durvasula RV, Beard CB. 2003. **Chagas disease vector *Rhodnius prolixus*, with integrative elements of the L1 mycobacteriophage.** Infect Genet Evol 3: 103-109.

Elbanna SM, Elhadidy NM, Semida FM, Abdel-Rasool T. 2012. **Physiological and biochemical effect of entomopathogenic fungus *Metarhizium anisopliae* on the 5th instar of *Schistocerca gregaria* (Orthiptera: Acrididae).** J Res Environ Science and Toxicology 1: 7-18.

Enríquez ME. 2004. **Evaluación de la virulencia de diferentes cepas de *Beauveria bassiana* y *Metarhizium anisopliae* en el control biológico de *Triatoma dimidiata*, principal vector del mal de Chagas en Guatemala.** Tesis de Licenciatura. Universidad de San Carlos, Guatemala.

Enríquez-Vara JN, Córdoba-Aguilar A, Guzmán-Franco AW, Alatorre-Rosas R, Contreras-Garduño J. 2012. **Is survival after pathogen exposure explained by host´s immune strength? A test with two species of white Grubs (Coleoptera: Scarabaeidae) Exposed to fungal infections.** Env Entomol 41: 959-965.

Espinosa de Aquino W. 2012. **Estudio de moléculas del sistema inmune de *Triatoma pallidipennis* infectado con *Trypanosoma cruzi.*** Tesis de Maestría en Ciencias, UNAM.

Favila-Ruiz G. 2016. **Actividad de PO en *Meccus pallidipennis* y *Triatoma dimidiata* infectados con *Trypanosoma cruzi.*** Tesis de licenciatura en la carrera de Química Farmacéutica Bióloga, UNAM, México.

Fieck A, Hurwitz I, Kang AS, Durvasula R. 2010. ***Trypansoma cruzi*: synergistic cytotoxicity of multiple amphipathic anti-microbial peptides to *T. cruzi* and potential bacterial hosts.** Exp Parasitol 125: 342–347.

Flores-Villegas AL. 2011. **Evaluación de *Isaria fumosorosea* y *Metarhizium anisopliae* para el control biológico de *Meccus palidipennis* en condiciones de laboratorio.** Tesis de Maestría en Ciencias Biológicas (Biología Experimental), UNAM, México.

Flores-Villegas AL, Cabrera-Bravo M, Toriello C, Bucio-Torres MI, Salazar-Schettino PM; Córdoba-Aguilar A. 2016. **Survival and immunre response of the Chagas vector *Meccus pallidipennis* (Hemiptera: Reduviidae) against two entomopathogenic fungi, *Metarhizium anisopliae* and *Isaria fumosorosea***. Parasit Vectors 9: 176.

Flores A, Pucheta M, Rodriguez S. 2012. **Mycoinsecticide effects of *Beauveria bassiana, Metarhizium anisopliae*, and *Isaria fumosorosea* on the whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae) in different strata of bean.** Afr J Microbiol Res 6: 7246-7252.

Galvao C, Carcavallo R, Rocha Da Silva D, Jurberg J. 2003. **A checklist of the current valid species of the subfamily Triatiominae Jeannel, 1919 (Hemiptera: Reduviidae) and their geographical distribution, with nomenclatural and taxonomic notes.** Zootaxa 202: 1-36.

García ES y Azambuja P. 1991. **Developmental and Interactions of *Trypanosoma cruzi* within the insect vector.** Parasitol Today 7: 240-244.

García-Valente F, Ortega-Arenas LD. 2008. **Mosquita blanca, *Trialeurodes vaporarium* (Hemiptera: Aleyrodidae).** En: Arredondo-Bernal HC, Rodríguez-Del Bosque LA (Eds.). Casos de Control Biológico en México. Grupo Mundi-Prensa, México, pp. 167-176.

Genta FA, Souza RS, García ES, Azambuja P. 2010. **Phenol oxidases from *Rhodnius prolixus*: Temporal and tissue expression pattern and regulation by ecdisone.** J Insect Physiol 56: 1253-1259.

Gillespie JP, Khachatourians GG. 1992. **Characterization of the *Melanoplus sanguinipes* hemolymph afer infection with *Beauveria bassiana* or wounding.** Comp Biochem Physiol 103: 445-463.

Gillespie JP, Kanost MR, Trenczeck T. 1997. **Biological mediators of insect immunity.** Annu Rev Entomol 42: 611-643.

Gillespie JP, Bailey AM, Cobb B, Vilcinskas A. 2000a. **Fungi as elicitors of insect immune response.** Arch Insect Biochem Physiol 44: 49-68

Gillespie JP, Burnett C, Charnley AK. 2000b. **The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var *acridum*.** J Insect Physiol 46: 429-437.

Gomes SA, Feder D, Thomas NES, Garcia ES, Azambuja, P. 1999. ***Rhodnius prolixus* infected with *Trypanosoma rangeli*: in vivo and in vitro experiments.** J Invertebr Pathol 73: 289-293.

Gomes SAO, Feder D, Garcia ES, Azambuja P. 2003**. Supression of the prophenoloxidase system in *Rhodnius prolixus* orally infected with *Trypanosoma rangeli.*** J Insect Physiol 49: 829:837.

González-Santoyo IG, Córdoba-Aguilar A. 2012. **Phenoloxidase: a key component of the insect immune system.** Entomol Exp Appl 142: 1-16.

Gourbière S, Dorn P, Tripet F, Dumonteil F. 2012. **Genetics and evolution of triatomines: from phylogeny to vector control.** Heredity 108: 190-202.

Gutiérrez-Cabrera AE, Alejandre-Aguilar R, Hernández-Martínez S, Espinoza B. 2014. **Development and glycoprotein composition of the perimicrovillar membrane in *Triatoma* (*Meccus*) *pallidipennis* (Hemiptera: Reduviidae).** Arthropod Struct Dev 43: 571–578.

Hernández-Velázquez VM, Toriello C. 2008. **Langosta voladora, *Schistocerca piceifrons piceifrons* (Orthoptera: Acrididae).** In: Arredondo-Bernal HC, Rodríguez-Del Bosque LA (Eds.). Casos de Control Biológico en México. Grupo Mundi-Prensa, México, pp. 47-55.

Herrera-Ortíz A, Martínez-Barnetche J, Smit N, Rodríguez MH, Lanz-Mendoza H. 2011. **The effect of nitric oxide and hydrogen peroxide in the activation of the systemic immune response of *Anopheles albimanus* infected with *Plasmodium berghei*.** Dev Comp Immunol 35: 44-50.

Humber RA, Hansen KS, Wheeler MM. 2010. **ARS Collection of Entomopathogenic Fungal Cultures: Catalog of Species.** p. 462.

Hung SH, Boucias DG. 1992. **Influence of *Beauveria bassiana* on the cellular defence response of the beet army worm, *Spodoptera exigua*.** J Invertebr Pathol 61:179-187.

Ibarra-Cerdeña CN, Sánchez-Cordero V, Towsend Peterson A, Ramsey JM. 2009. **Ecology of North American Triatominae.** Acta Trop 110: 178-186.

Jiménez-Cortés JG, Serrano-Meneses MA, Córdoba-Aguilar A. 2012. **The efects of food shortage during larval development on adult body size, body mass, physiology and developmental time in a tropical damselfly.** J Insect Physiol 58: 318-326.

Kavallieratos NG, Athanassiou CG, Aountala MM, Kontodimas DC. 2014. **Evaluation of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosorosea* for control of *Sitophilus oryzae*.** J Food Prot 77: 87-93.

Kershaw MJ, Moorhouse ER, Bateman R, Reynolds SE, Charnley AK. 1999. **The role of Destruxins in the Pathogenicity of *Metarhizium anisopliae* for Three Species of Insect.** J Invertebr Pathol 74: 213-223.

Khosravi R, Sendi JJ, Zibaee A, Shokrgozar MA. 2014. **Immune reactions of the lesser mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) to the entomopathogenic fungus, *Beauveria bassiana* (Bals.-Criv.) Vuill and two developmental hormones.** Invert Surviv J 11: 11-21.

Kollien AH, Schaub GA. 2000. **The development of *Trypanosoma cruzi* in Triatominae.** Parasitol Today 16: 381:387.

Kollien AH, Fechner S, Waniek PJ, Schaub G. 2003. **Isolation and characterization of a cDNA encoding for a Lysozyme from the gut of the Reduviid bug *Triatoma infestans*.** Arch Insect Biochem Physiol 53: 134-145.

Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M, Goettel MS. 2015**. Insect pathogens as biological control agents: Back to future.** J Invertebr Pathol 132: 1-41.

Lara Da costa G, Lage De Morales AM, Galvao C. 2003. **Entomopathogenic effect of *Aspergillus giganteus* and *Penicillium corylophilum* on two triatomine vectors of Chagas disease.** J Basic Microbiol. 43: 3-7.

Laughton AM, García JR, Altincieck B, Strand MR, Gerardo NM. 2011. **Characterisation of immune responses in the pea aphid, *Acyrthosiphon pisum*.** J Insect Physiol 57:830-839.

Laughton AM, Siva-Jothy MT. 2010. **A standarised protocol for measuring phenoloxidase and prophenoloxodase in the honey bee, *Apis mellifera*.** Apidologie 42: 140-149.

Lavine MD y Strand MR. 2002**. Insect hemocytes and their role in immunity.** Insect Biochem Molec Biol 32: 1259-1309.

Lecuona RE, Edelstein JD, Berretta MF, La Rossa FR, Arcas JA. 2001. **Evaluation of *Beauveria bassiana* (Hyphomycetes) strains as potential agents for control of *Triatoma infestans* (Hemiptera: Reduviidae).** J Med Entomol 38: 172-179.

Lent H, Wygodzinsky P. 1979. **Revision of the Triatominae (Hemiptera, Reduviidae and their significance as vectors of Chagas disease.** Bull Am Mus Nat Hist 163: 125-520.

Luz C, Tigano MS, Silva IG, Cordeiro CM, Aljanabi SM. 1998a. **Selection of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to control *Triatoma infestans*.** Mem Inst Oswaldo Cruz 93: 839-846.

Luz C, Silva IG, Cordeiro CM, Tigano MS. 1998b. ***Beauveria bassiana* (Hyphomycetes) as a posible agent for biological control of Chagas disease vectors.** J Med Entomol 35: 977-979.

Luz C, Silva IG, Magalhaes BP, Cordeiro CM, Tigano MS. 1999. **Control of *Triatoma infestans* (Klug) (Reduviidae: Triatominae) with *Beauveria bassiana* (Bals.) Vuill.: Prelimiary assays on formulation and application in the field.** An Soc Entomol Brasil 28: 101-110.

Luz C, Rocha LF, Humber RA. 2003. **Record of *Evlachovaea* sp. (Hyphomycetes) on *Triatoma sordida* in the state of Goias, Brazil, and Its activity against *Triatoma infestans* (Reduviidae, Triatominae.** J Med Entomol 40: 451-454.

Luz C, Rocha LF, Nery GV, Magalhaes BP, Tigano MS. 2004. **Activity of oil formulated *Beauveria bassiana* against *Triatoma sordida* in peridomestic areas in central Brazil.** Mem Inst Oswaldo Cruz 99: 211-218.

Luz C, Rodrigues J, Rocha LF. 2012. **Diatomaceous earth and oil enhance effectiveness of *Metarhizium anisopliae* against *Triatoma infestans*.** Acta Trop 122: 29-35.

Lwetoijera DW, Sumaye RD, Madmula EP, Kavishe DR, Mnyone LL, Russell TL, Okumu FO. 2010. **An extra domiciliary method of delivering entomopathogenic fungus, *Metarhizium anisopliae* IP 46 for controlling adult populations of the malaria vector, *Anopheles arabiensis.*** Parasit Vectors 3: 1-6.

Martínez-Ibarra JA, Katthain-Duchateau G. 1999. **Biology of *Triatoma pallidipennis* Stal 1945 (Hemiptera: Reduviidae: Triatominae) under laboratory conditions.** Mem Inst Oswaldo Cruz 94: 837-839.

Martínez-Ibarra JA, Novelo-López. 2004. **Blood meals to molt, feeding time and postfeeding defecation delay of *Meccus pallidipennis* (Stal, 1872) (Hemiptera: Reduviidae) under laboratory conditions.** Folia Entomol Mex 43: 313-319.

Martínez-Ibarra JA, Grant-Guillén Y, Morales-Corona ZY, Haro-Rodriguez S, Ventura-Rodríguez LV, Nogueda-Torres B, Bustos-Saldaña R. 2008. **Importance of species of Triatominae (Heteroptera: Reduviidae) in risk of transmission of *Trypanosoma cruzi* in western Mexico.** J Med Entomol 45: 476–82.

Martínez-Ibarra JA, García-Benavidez G, Vargas-Llamas V, Bustos-Saldaña R, Montañez-Valdez OD. 2012. **Bionomics of populations on *Meccus pallidipennis* (Stal), 1872 (Hemiptera: Reduviidae) from Mexico**. J Vect Ecol 37: 474-477.

Martínez-Ibarra JA, Nogueda-Torres B, Salazar-Schettino PM, Vences-Blanco MO, De la Torre-Álvarez FJ, Montañez-Valdez OD. 2014. **Differences on biological attributes of three populations of *Meccus pallidipennis* Stal (Hemiptera: Reduviidae).** J Vector Borne Dis 51: 22-26.

Mazzotti L. 1937. **Infección natural del *Tripanosoma cruzii* de Chagas, en *Triatoma phyllosoma*, Burmeister y *Triatoma pallidipennis*, de la Costa del Pacífico de México.** Med Rev Mex 289: 161-166-.

Mello CB, Garcia ES, Ratcliffe NA, Azambuja P. 1995. ***Trypanosoma cruzi* and *Trypanosoma rangeli:* Interplay with hemolymph components of *Rhodnius prolixus.*** J Inver Pathol 65: 261-268.

Mier T, Toriello C, Ulloa M. 2002. **Hongos microscópicos saprobios y parásitos: métodos de laboratorio.** División de Ciencias Biológicas y de la Salud, primera edición. UAM Xochimilco, Instituto de Biología, UNAM. México D.F., p. 90.

Mier T, Olivares-Redonda G, Navarro-Barranco H, Pérez-Mejía A, Lorenzana M, Pérez-Torres A, Toriello C. 2005. **Acute oral intragastric pathogenicity and toxicity in mice of *Paecilomyces fumosoroseus* isolated from whiteflies.** Antonie Van Leeuwenhoek 88: 103-111.

Milat-Bissaad FZ, Bounaceur F, Halouane F, Behidj N, Chebouti N, Doumandji Mitiche, B. 2011. **Effect of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* var.**

***acridum* on the haemolymph of the desert locust *Schistocerca gregaria*.** Tunis J of Plant Prot 6**:** 127-132.

Mnyone LL, Ng´habi KJ, D Mazigo H, Katakweba AA, N Lyimo I. 2012. **Entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana* reduce the survival of *Xenopsylla brasiliensis* larvae (Siphonaptera: Pulicidae).** Parasit Vectors 5: 204.

Moraes AML, Junqueira ACV, Costa GL, Celano V, Coura J.R. 2000. **Fungal flora of the digestive tract of 5 species of triatomines vectors of *Trypanosoma cruzi*, Chagas 1909.** Mycopathologia 151: 41-48.

Mullen LM, Goldsworthy G. 2006. **Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin.** J Insect Physiol 52: 389-398.

Muscio OA, La Torre JL, Scodeller EA. 1987. **Small Nonoccluded Viruses from Triatominae Bug *Triatoma infestans* (Hemiptera: Reduviidae).** J Invertebr Pathol 49: 218-220.

Napolitano R, Juárez MP. 1997. **Entomopathogenous fungi degrade epicuticular hydrocarbons of *Triatoma infestans*.** Arch Biochem Biophys 344: 208-214.

Nappi AJ, Christensen BM. 2005. **Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity.** Insect Biochem Mol Biol 35: 443-459.

Ngumbi PM, Irungi LW, Ndegwa PN, Maniania NK. 2011. **Pathogenicity of *Metarhizium anisopliae* (Metch.) Sorok and *Beauveria bassiana* (Bals.) Vuill to adult *Phlebotomus duboscqui* (Neveu-Lemaire) in the laboratory.** J Vector Borne Dis 48: 37-40.

Nouvellet PN, Cucunubá ZM, Gourbiere S. 2015. **Ecology, Evolution and Control of Chagas Disease: A Century of Neglected Modelling and a Promising future.** Adv Parasitol 87: 135-191.

Pedrini N, Mijailovsky SJ, Girotti JR, Stariolo R, Cardozo RM, Gentile A, Juárez MP. 2009. **Control of pyrethroid-resistant Chagas disease vectors with entomopathogenic fungi.** PLos Negl Trop Dis 3: 1-11.

Pinchin R, De Oliveira-Filho AM, Pereira AC. 1980. **The flushing-out activity of pyrethrum and synthetic pyrethroids on *Panstrongylus megistus*, a vector of Chagas disease.** Trans Royal Soc Trop Med Hyg 74: 801-803.

Ramsey JM, Cruz-Celis A, Salgado L, Espinosa L, Ordoñez R, Lopez R, Schofield CJ. 2003. **Efficacy of pyrethroid insecticides against domestic and peridomestic populations of *Triatoma pallidipennis* and *Triatoma barberi* (Reduviidae:Triatominae) vectors of Chagas' disease in Mexico.** J Med Entomol 40 (6): 912-920.

Reyes-Villanueva F, Garza-Hernandez JA; Garcia-Mungía AM, Tamez-Guerra P, Howard AFV, Rodriguez-Perez MA. 2011. **Dissemination of *Metarhizium anisopliae* of low and high virulence by mating behavior in *Aedes aegypti*.** Parasit Vectors 4: 171.

Ribeiro JCM, Genta FA, Sorgine MHF, Logullo R, Mesquita, RD, Paiva-Silva GO, Majerowicz D, Medeiros M, Koerich L, Terra WR, Ferreira C, Pimentel AC, Bisch PM, Leite DC, Diniz MMP, Lídio da SGV Junior J, Da Silva ML, Araujo RN, Caroline A, Gandara P, Brosson S, Salmon D, Bousbata S, González-Caballero N, Silber AM, Alves-Bezerra M, Gondim KC, Silva-Neto MAC, Atella GC, Araujo H, Dias FA, Polycarpo C, Vionette-Amaral RJ, Fampa P, Melo ACA, Tanaka AS, Balczun C, Oliveira JHM, Gonçalves RLS, Lazoski C, Rivera-Pomar R, Diambra L, Schaub GA, Garcia ES, Azambuja P, Braz GRC, Oliveira PL. 2014 **An insight into the transcriptome of the digestive tract of the bloodsucking Bug, *Rhodnius prolixus*.** PloS Negl Trop Dis 8: e2594.

Rivas-Medina, EJ. 2014. **Comparación del perfil de glicoproteínas presentes en el intestino medio y posterior de *Meccus pallidipennis* y *Triatoma barberi***. Tesis de licenciatura en Química Farmacéutica Bióloga, UNAM, México.

Rocha LF, Luz C. 2011. **Activity of *Metarhizium* spp. and *Isaria* spp. from the Central Brazilian Cerrado against *Triatoma infestans* nymphs.** Tran R Soc Trop Med Hyg 105: 417-419.

Rocha LF, Silva IG, Luz C. 2011. **Activity of some hypocrealean fungi collected in a Cerrado ecosystem against *Rhodnius* spp. (Hemiptera: Reduviidae) under laboratory conditions.** Acta Trop 18: 63-66.

Rodrigues J, Lobo LS, Fernandes ÉKK, Luz C. 2014. **Effect of formulated *Metarhizium anisopliae* on eggs and eclosing nymphs of *Triatoma infestans*.** J Appl Entomol 139: 146-153.

Rodríguez-Bataz E, Nogueda-Torres B, Rosario-Cruz R, Martínez-Ibarra JA, Rosas-Acevedo JL. 2011. **Triatominos (Hemiptera: Reduviidae) vectores de *Trypanosoma cruzi* Chagas 1909, en el estado de Guerrero, México**. Rev Biomed 22:31-40.

Rohlf M, Churchill AC. 2011. **Fungal secondary metabolites as modulators of interactions with insects and other arthropods.** Fungal Genet Biol 48: 23–34.

Rojas-Wastavino GE, Cabrera M, García G, Vences M, Ruíz A, Bucio M, Guevara Y, Escobar A, Salazar PM. 2004. **Insecticide and community interventions to control *Triatoma dimidiata* in localities of the State of Veracruz, Mexico**. Mem Inst Osw Cruz 99:433-437.

Salazar-Schettino PM, Rojas-Wastavino E, Cabrera–Bravo M, Bucio-Torres M, Martínez-Ibarra JA, Monroy-Escobar MC, Rodas-Retana A, Guevara-Gómez Y, Vences-Blanco MO, Ruiz-Hernández AL, Torres-Gutiérrez E. 2010. **Revisión de 13 especies de la familia**

Triatominae (Hemiptera: Reduviidae) vectores de la enfermedad de Chagas en México. J Selva Andina Res Soc 1: 57-80.

Schofield CJ, Dias JC.1998. **The southern cone initiative against Chagas disease.** Adv Parasitol 42: 1-27.

Schofield CH, Kabayo JP. 2008. **Trypanosomiasis vector control in Africa and Latin America.** Parasit Vectors 1-24.

Scholte EJ, Nijru BN, Smallegange RC, Takken W, Knols BG. 2003. **Infection of malaria *Anopheles gambiae* (s.s.) and filariasis (*Culex quinquefasciatus*) vectors with the entomopathogenic fungus *Metarhizium anisopliae.*** Malaria J 2: 29-31.

Scholte EJ, Ng´habi K, Kihonda J, Takken W, Paaijmans K, Abdulla S, Killeen GF y Knols BG. 2005. **An entomopathogenic fungus for control of adult African malaria mosquitoes.** Science 308: 1641-1642.

Shah PA, Pell JK. 2003. **Entomopathogenic fungi as biological control agents.** Appl Microbiol Biotechnol 61: 413–423.

Smilanich AM, Dyer LA, Gentry GL. 2009. **The insect immune response and other putative defenses as effective predictors of parasitism.** Ecology 90: 1434-1440.

Soderhall K, Cerenius L. 1998. **Role of the prophenoloxidase activating system in invertebrate immunity.** Curr Opin in Immunol 10: 23-28.

Strand MR. 2008. **Insect hemocytes and their role in immunity.** In: Beckage NE (Ed.). Insect Immunity. Academic Press, San Diego, California (USA), pp. 25-47.

Steverding D. 2014. **The history of Chagas disease.** Parasit Vectors 7: 317.

Tay J. y cols. **Evolución del *Trypanosoma cruzi* cepa Mexicana en el huésped vertebrado, invertebrado e in vitro.** Sal Pub Méx 22: 513-520.

Téllez-Jurado A, Cruz Ramírez MG, Mercado Flores Y, Asaff Torres A, Arana-Cuenca A. 2009. **Mecanismos de acción y respuesta en la relación de hongos entomopatógenos e insectos.** Revi Mex Micol 30:73-80.

Thomas MB, Read AF. 2007. **Can fungal bipesticides control malaria?.** Nature 5: 377-383.

Toriello C. 2003. **Bioseguridad de *Metharhizium anisopliae* (Metschnikoff) Sorokin (Hyphomycete).** Vedalia 10: 107-113.

Toriello C, Pérez-Torres A, Burciaga-Díaz A. Navarro-Barranco H, Pérez-Mejía A, Lorenzana Jiménez M, Mier T. 2006. **Lack of acute pathogenicity and toxicity in mice of an isolate of *Metarhizium anisopliae* var. *anisopliae* from spittlebugs.** Ecotoxicol Environ Saf 65: 278-287.

Toriello C, Montoya-Sansón E, Zavala-Ramírez M, Navarro-Barranco H, Basilio-Hernández D, Hernández-Velázquez V, Mier T. 2008. **Virulencia y termotolerancia de cultivos**

monospóricos de *Metarhizium anisopliae* var. *anisopliae* de la mosca pinta (Hemiptera: Cercopidae). Rev Mex Micol 28: 57-66.

Tulloch, M. 1976. **The genus *Metarhizium.*** Mycol Soc 66: 407-411.

Vázquez-Martínez MG, Cirerol-Cruz BE, Torres-Estrada JL, Rodríguez-López MH. 2014. **Potential for entomopathogenic fungi to control *Triatoma dimidiata* (Hemiptera: Reduviidae), a vector of Chagas disease in Mexico**. Rev Soc Bras Med Trop 47: 716-722

Ursic-Bedoya RJ, Buchhop J, Joy JB, Durvasula R, Lowenberger C. 2011. **Prolixicin: a novel antimicrobial peptide isolated from *Rhodnius prolixus* with differential activity against bacteria and *Trypanosoma cruzi*.** Insect Molecul Biol 20: 775-786

Vilcinskas A, Gotz P. 1999. **Parasitic fungi and their interactions with the insect immune system.** Adv Parasitol 43: 267-213.

Vilcinskas A, Matha V. 1997. **Effect of the entomopathogenic fungus *Beauveria bassiana* on the humoral immune response of *Galleria mellonella* larvae (Lepidoptera: Pyralidae).** Eur J Entomol 94: 461-472.

Wang C y St. Leger RJ. 2006. **A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses.** PNAS 103: 6647-6652.

Wang CH, St Leger RJ. 2007. **A scorpion neurotoxin increases the potency of a fungal insecticide.** Nat Biotechno 25: 1455-1456.

Waniek PJ, Jansen AM, Araújo CAC. 2011. ***Trypanosoma cruzi* infection modulates the expression of *Triatoma brasiliensis* def 1 in the midgut.** Vector Borne Zoonotic Dis 11: 845-847.

Whitten MMA, Mello CB, Gomes SAO, Nigam Y, Azambuja P, Garcia E, Ratcliffe NA. 2001. **Role of superoxide and reactive nitrogen intermediates in *Rhodnius prolixus* (Reduviidae)/*Trypanosoma rangeli* interactions.** Exp Parasitol 98: 44-57.

Whitten M, Sun F, Tew I, Schaub G, Soukou Ch, Nappi A, Ratcliffe N. 2007. **Differential modulation of *Rhodnius prolixus* nitric oxide activities following challenge with *Tryapnosoma rangeli*, *T. cruzi* and bacterial cell wall components.** Insect Biochem Mol Biol 37:440-452.

Zimmerman G. 2007. **Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*.** Biocontrol Sci Technol 17: 879-920

Zimmermann G. 2008. **The entomopathogenic fungi *Isaria farinosa* (formerly *Paecilomyces farinosus*) and the *Isaria fumosorosea* species complex (formerly *Paecilomyces fumosoroseus*): biology, ecology and use in biologicalcontrol**. Biocontrol Sci Technol.