http://dx.doi.org/10.1590/1678-4162-13027

Arq. Bras. Med. Vet. Zootec., v.76, n.1, p.77-83, 2024

Repellence and insecticidal activity mediated by necrosis in *Aedes aegypti* mosquitoes exposed to thymol

[*Repelência e atividade inseticida mediada por necrose em mosquitos* Aedes aegypti *expostos ao timol*]

G.J. *Nascimento*, L.H.G. *Oliveira*[2], H.L. *Rique*[2], R.T. *Leite*[3], F.C. *Nunes*[4]*

[1]Graduate, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, RJ, Brasil
[2]Graduate, Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, *Campus* UFPE, Recife, PE, Brasil
[3]Centro de Biotecnologia, Universidade Federal da Paraíba, João Pessoa, PB, Brasil
[4]Universidade Federal da Paraíba, João Pessoa, PB, Brasil

ABSTRACT

The mosquito *Aedes aegypti* is a major threat to public health because it spreads several arboviruses. Since this insect is an anthropophilic mosquito that has evolved to thrive in urban settings and is common in Brazil and other tropical nations, controlling its population growth is difficult. The two basic methods of control are eradicating its breeding grounds and applying pesticides. There have been reports of pesticide resistance emerging as well as DEET's potential danger for people, particularly youngsters. Therefore, research has been done to find novel insecticides and repellents. In this way, the goal of this research was to assess thymol's insecticidal and repellent activities in *Ae. aegypti*. Bioassays were performed with eggs, larvae and adults exposed to different concentrations of thymol. The findings demonstrated that thymol has insecticidal effect in both the immature and adult phases, eliminating the larvae in 24 hours. In addition, necrosis was observed in hemocytes, without alteration in the production of nitric oxide by the insects. When evaluating repellency, thymol showed 99.5% of landing inhibition. This leads to the conclusion that thymol can be employed as an active component in the formulation of insecticides and repellents for the *Ae. aegypti* mosquito.

Keywords: culicidae, monoterpene, essential oils, arboviruses

RESUMO

O Aedes aegypti *é um mosquito que causa grande impacto na saúde pública por ser o vetor de diversas arboviroses. Controlar a proliferação desse inseto é desafiador, já que é um mosquito antropofílico, altamente adaptado a ambientes urbanos e disseminado no Brasil e em outros países de clima tropical. A principal forma de combatê-lo é destruindo seus criadouros ou utilizando inseticidas. Há relatos do desenvolvimento de resistência aos inseticidas e da potencial toxicidade do DEET para os seres humanos, sobretudo crianças. Dessa forma, estudos vêm sendo realizados, a fim de buscar novas substâncias inseticidas e repelentes. Nesse sentido, o objetivo deste trabalho foi avaliar a atividade inseticida e repelente do timol em mosquitos* Ae. aegypti. *Foram realizados bioensaios com ovos, larvas e adultos expostos a diferentes concentrações de timol. Os resultados demonstraram que o timol possui ação inseticida tanto nas fases imaturas quanto no adulto, matando 100% das larvas em 24h. Além disso, observou-se necrose em hemócitos, sem alteração na produção de óxido nítrico pelos insetos. Ao avaliar a repelência, o timol apresentou 99,5% de inibição do pouso. Dessa forma, conclui-se que o timol pode ser utilizado como princípio ativo na composição de inseticidas e repelentes contra o mosquito* Ae. aegypti.

Palavras-chave: culicídeos, monoterpeno, óleos essenciais, arboviroses

*Corresponding author: fabiola@cbiotec.ufpb.br
Submitted: March 27, 2023. Accepted: August 14, 2023.

INTRODUCTION

The *Aedes* (*Stegomyia*) *aegypti* mosquito (Linnaeus, 1762) represents a major public health problem since it is considered the main vector of dengue, chikungunya, and Zika viruses (Terra *et al.*, 2017). Controlling the population of *Ae. aegypti* has been a challenge, particularly in developing countries, where resources are often scarce. The efficiency of conventional ways of managing *Aedes sp.* is jeopardized by issues with city infrastructure, such as inadequate rubbish collection coverage and inconsistent water supply. (Zara *et al*., 2016; Coelho, 2008). The selection of resistant insect populations has been facilitated by the indiscriminate use of insecticides and environmental repellents. Consequently, it is essential to search for alternate vector control strategies that are more secure, efficient, and biodegradable (Silva *et al*., 2014; Brasil, 2016).

Monoterpenes and sesquiterpenes are the most prevalent chemicals in the largest class of terpenoids found in natural plant products (Dubey *et al*., 2003). Thymol (5-methyl-2-(1-methylethylphenol), a monoterpene that is present in thyme and oregano essential oils (Budavari, 1989), is notable for its antibacterial, antifungal, anti-inflammatory, anticancer, and insecticide properties (Guimares *et al*., 2012; Yin *et al*., 2012) (Figure 1). Thus, the purpose of this study was to investigate the insecticidal and repellent activity of thymol on *Ae. aegypti*.

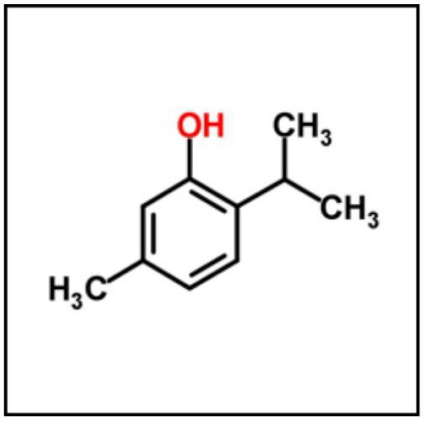

Figure 1. Chemical structure of thymol (2-Isopropyl-5-methylphenol). Monoterpene with an aromatic structure, a meta-cresol, with formula C10H14O, molar mass 150.22 g. mol-1.

MATERIAL AND METHODS

Thymol (2-Isopropyl-5-methylphenol) was obtained from Sigma Aldrich. The test solution was prepared by solubilizing Thymol in distilled water and DMSO (2%).

*Ae. aegypt*i mosquitoes of the Rockefeller strain from a cyclical colony kept at the Laboratory of Biotechnology Applied to Parasites and Vectors Lapavet/Cbiotec/UFPB were utilized in the biological experiments. The mosquitoes were housed in a biological oxygen demand (BOD) chamber with a constant temperature of 27±2 °C, relative humidity of 75.5%, and a 12-hour photoperiod (WHO, 2013; Nunes *et al*. 2015). In the trials, mosquito eggs, larvae in the fourth stage of development (L4), and adults were used. The study was approved by the Committee on Ethics in the Use of Animals of the Federal University of Paraíba (CEUA), protocol nº 131/2017.

Filter paper strips containing 25 eggs of *Ae. aegypti* were immersed in plastic tubes containing 25mL of thymol solution (0.1mg/mL). The hatching rate of the eggs was evaluated after 24 hours. In control groups, eggs were exposed to water and DMSO 2%. Tests were performed in triplicate.

In the larvicidal activity tests, groups with 15 larvae in the fourth stage (L4) were transferred to falcon tubes containing 30mL of thymol solution in different concentrations (0.01-0.1mg/mL). Larvae mortality was verified at 24h. In control groups, larvae were exposed to water and DMSO 2%. In the adulticidal activity tests, 10

mosquitoes, anesthetized by cold, received 10µL of the test substance (0.1mg/mL), which was the lowest concentration capable of killing 100% of the larvae in the larvicidal activity test. After contact with the test substance, the mosquitoes were transferred to an insectarium and observed after 24 hours to verify mortality. The experiments were performed in triplicate.

Primary culture of hemocytes from the hemolymph of 200 larvae (L4) was used for the cytotoxicity experiments. Briefly, 20mL of Leibovitz medium, 0.10g of Fluconazole, 5g of ampicillin, and 10% fetal bovine serum were combined with the collected hemolymph. The culture was then incubated at 28°C for 5 days. After the incubation period, the cells were resuspended in a fresh Leibovitz medium. Concentrations of 1 x 10^5 cells from the primary culture were plated in 12-well plates, being exposed to a concentration of 0.25mg/mL of the test substance for 24 hours. The cells were then exposed to 15µL of propidium iodide and incubated for 15 minutes in the dark. Fluorescence microscopy was utilized to separate healthy cells from red-stained necrotic cells.

To evaluate the production of nitric oxide by hemocytes exposed to thymol, the supernatant of the cytotoxicity assay was used. NO dosage was performed using the Griess method (Green *et al*. 1981). Griess reagent and 50 microliters of cell supernatant were blended and incubated at 37°C for 15 minutes in the dark. The control group was only exposed to PBS and the absorbance was read at 562nm. Assays were performed in triplicate and NO was quantified using a NaNO2 standard curve as a reference.

The repellency tests were carried out in a plastic chamber measuring 30 x 30 x 30 cm, containing 10 *Ae. aegypti* female. For each test, 1 mouse (Mus musculus) previously anesthetized received 10µL of thymol solution on the shaved skin of the back. In the negative controls, distilled water was applied and in the positive controls, repellent based on DEET 10% was applied. In each test, the number of landings or bites for 60 minutes was recorded. Tests were performed in triplicate.

GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) was used to calculate LC10, LC50, and LC90. Analysis of variance (ANOVA) and Tukey's test ($p<0.05$) were applied to determine significant differences between groups.

RESULTS AND DISCUSSION

Several bioassays were conducted to assess the insecticidal and repellent activities of thymol on *Ae. aegypti* in the hatchability assay, it was observed that thymol (0.1 mg/mL) prevented *Ae. aegypti* eggs from hatching in 24 hours as compared to the control (0 vs. 22%) (Fig. 2). In their investigation, Pineda-Cortel *et al*. (2019) found that the crude ethanolic extract of *Artocarpus blancai* inhibited 100% of hatchability, but the necessary concentration was 1000 ppm, whereas it was 100 ppm in our study.

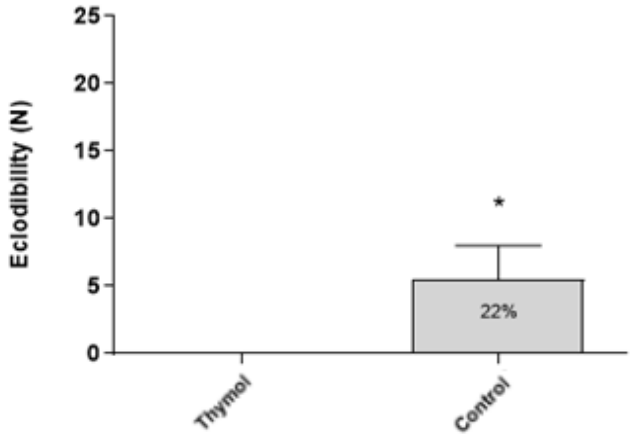

Figure 2. Hatchability rate of *Ae. aegypti* eggs exposed to thymol (0.1 mg/mL) after 24 hours. Control group eggs were exposed only to water and 2% DMSO. * Significant statistical differences were considered when $P<0.05$ compared to its control.

It is crucial to note that the various components of crude plant extracts can interact with one another and enhance or even decrease the insecticidal effects of the separate compounds. Regarding larvicidal activity, it was observed that thymol at concentrations of 0.025, 0.05, and 0.1 mg/mL killed respectively 30±2.6, 55±3.2, and 100% of *Ae. aegypti* after 24 h (Fig. 3). Thymol was reported to be the most toxic substance to *Ae. aegypti* among the different chemicals tested in the study by Lee and Ahn (2013), presenting a CL50 of 11.72mg/L, corroborating our results. Regarding adulticidal activity, it was observed that thymol (0.1mg/mL) resulted in the death of 26±1.1% of mosquitoes (Fig. 3).

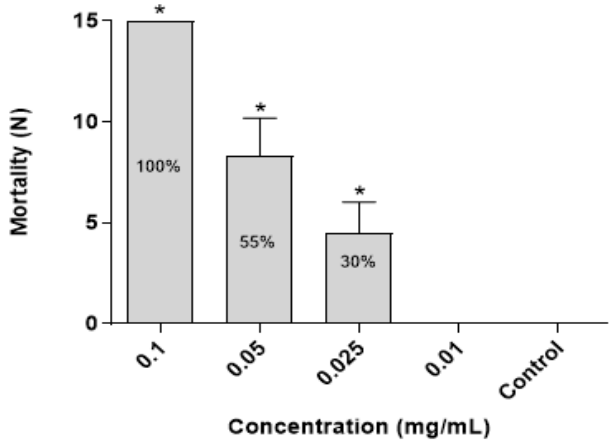

Figure 3. Mortality of *Ae. aegypti* larvae exposed to different thymol concentrations (0.01 - 0.1mg/mL). The control group was exposed only to water and 2% DMSO. * Significant statistical differences were considered when P<0.05 compared to its control.

Cell viability assays and measurement of NO production were also carried out to better understand the mechanisms of action involved in the insecticidal activity. In this sense, cell death can follow the path of necrosis or apoptosis, the first being always pathological, while the second can also occur in normal physiological processes of the organism. These processes may be initiated by several external causes, which may then cause several intracellular events, such as membrane damage that affects the mitochondria and, in turn, reduces ATP generation and cell death (Robbins & Cotran, 2016). In our study, it was shown that the experimental group had 4 times more necrotic cells than the control group when analyzing the effects of thymol on hemocytes (43.0 ± 6.8 Vs. 10.0±1.1) (Figure 4). The difference between groups was considered statistically significant at a significance level of 95%.

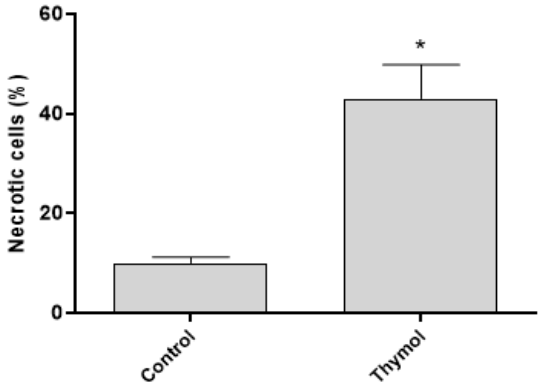

Figure 4. Mean rate of necrotic hemocytes after exposure to thymol (0.25 mg/mL) for 24 h. * Significant statistical differences were considered when P<0.05 compared to its control.

In their research, Nunes *et al*. (2015) showed that the crude *Agave sisalana* extract led to hemocyte necrosis. In their investigation of *H. velutina*, Fernandes *et al*. (2020) noted a related phenomenon. In this way, necrosis could be at least partially responsible for the insecticidal effect we saw in our investigation.

Regarding NO production, there was no statistical difference between the experimental and control groups (0,058 ± 0,010 Vs. 0,049 ± 0,058 µM). NO plays an important role in the immune response of insects, modulating the immune system through cell signaling. Therefore, its production is increased in situations of chemical or biological stress (Castro *et al*., 2012). Its synthesis occurs during the transformation of the semi-essential amino acid L-arginine into L-citrulline, in a reaction mediated by the enzyme nitric oxide synthase (NOS), in the presence of oxygen, NADPH, and calcium, among other substances (Chatkin, 2000). Studies have shown that insecticidal substances can alter NO production in the hemocytes of *Ae. aegypti*. Nunes *et al*. (2015) showed that the crude extract of *A. sisalana* caused a decrease in NO levels in the hemocytes of *Ae. aegypti* exposed to the test substance. Fernandes *et al*. (2020) observed that different components of the *Helicteres velutina* plant caused an increase in NO production in *Ae. aegypti*. These results differ from the findings in our study, which leads us to believe that the insecticidal action of thymol is not associated with the NO pathway.

Some studies have shown that thymol has inhibitory properties of the enzyme acetylcholinesterase (AChE) (Lee and Ahn, 2013).

External sensory stimuli generate a nerve impulse, which will be converted into motor activity after traveling through the nervous system and activating muscle cells. This transmission can be electrical or chemical. Chemical transmission takes place at synapses with the help of neurotransmitters, mainly acetylcholine. After fulfilling its role, acetylcholine needs to be degraded by acetylcholinesterase, so that the adjacent cell does not have Na+ channels constantly open. With the inhibition of acetylcholinesterase, there is an accumulation of acetylcholine in the synapses, thus causing nervous hyperactivity and, consequently, a collapse of the insect's nervous system. Although further studies are still needed to fully understand the mechanisms involved in Thymol's insecticidal activity, it appears to be a combination of cytotoxic action and AChE inhibition.

When evaluating repellency, it was found that thymol was capable of repelling 99.5% of mosquitoes, with a better result than DEET (98%) (Figure 5).

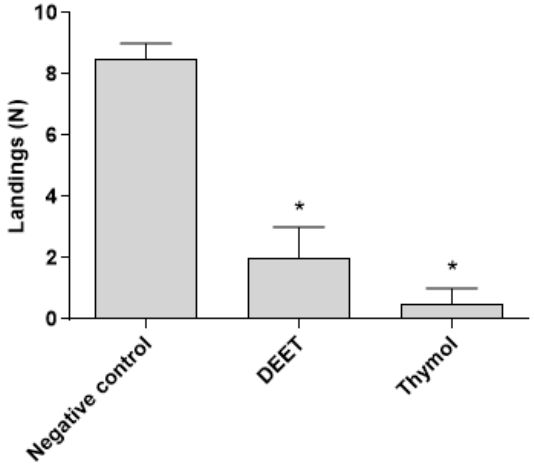

Figure 5. Evaluation of repellency by landing or bites of *Ae. aegypti* mosquitoes exposed to Thymol (test group), DEET (positive control group) and water (negative control group). *Significant statistical differences were considered when P<0.05 compared to its control.

The olfactory system of insects is composed of several mechanisms involving a range of receptors and proteins. Odors are detected by olfactory receptor neurons that work in conjunction with other molecules (Clark and Ray, 2016). The human body produces several attractive odors, such as lactic acid and CO2 released in breathing, which is the key to the link between anthropophilic insects and humans (Zwiebel and Takken, 2004). Studies have shown that different insect repellents use a similar mode of action, binding and interacting with specific odorant and taste receptors, altering their activity, and exerting their deterrent effects (Dickens and Bohbot, 2013; Xu *et al*., 2014). Thymol is a substance with a strong odor, which is present in several essential oils, such as Thyme oil for example (Kowalczyc *et al*., 2020). In the study by Luker *et al.* (2023) the authors draw attention to the diversity of methods used to assess repellency. Different methodologies may present variations in results for the same substance. In methodologies like the one used in our study, contact between the mosquito and the host activates sensory organs such as the labella and tarsus. These organs have receptors that allow the mosquito "to taste".

CONCLUSIONS

Thymol demonstrated insecticidal activity, preventing *Ae. aegypti* eggs from hatching and killing larvae and adults. Our investigation revealed the necrotizing action of Thymol in *Ae. aegypti* hemocytes in addition to the inhibitory effect of AChE activity that has already been shown in the literature, helping to understand the processes underlying insecticidal activity. In addition to the insecticidal activity, there was an important repellent activity against the *Aedes aegypti* mosquito, an insect with a great impact on public health. To help suppress the arboviruses spread by *Ae. aegypti*, thymol can be employed as an active ingredient in the formulation of novel insecticides and repellents.

REFERENCES

BRASIL Ministério da Saúde. Secretaria de Vigilância em Saúde. Recomendações técnicas ao Sistema Nacional de Vigilância Sanitária para colaborar no combate ao Aedes aegypti e prevenção e controle da Dengue, Chikungunya e infecção pelo vírus Zika. Brasília-DF, 2016.

BUDAVARI, S. The Merck index - an encyclopedia of chemicals, drugs and biological. 17ed. Rahway: Merck & Co., Inc., 1989. 1606p.

CASTRO, D.P.; MORAES, C.S.; GONZALES, M.S. *et al.* Trypanosoma cruzi immune response modulation decreases microbiota in Rhodnius prolixus gut and is crucial for parasite survival and development. PLoS One. 2012;7(5):e36591.

CHATKIN, J.M. Óxido nítrico exalado no diagnóstico e acompanhamento das doenças respiratórias. *J. Pneumol*., v.26, p.36-43, 2000.

CLARK, J.T.; RAY, A. Olfactory mechanisms for discovery of odorants to reduce insect-host contact. *J. Chem. Ecol*., v.42, p.919-930, 2016.

COELHO, G.E. Dengue: desafios atuais. *Epidemiol. Serv. Saúde*, v.17, p.231-233, 2008.

DICKENS, J.C.; BOHBOT, J.D. Mini review: mode of action of mosquito repellents. *Pestic. Biochem. Physiol*., v.106, p.149-155, 2013.

DUBEY, V.S.; BHALLA, R.; LUTHRA, R.; DONALÍSIO M.R. O dengue no espaço habitado. *J. Biosci*., v.28, p.637-646, 2003.

FERNANDES, D.A.; OLIVEIRA, L.H.G.; RIQUE, H.L.; SOUZA, M.D.F.V.D.; NUNES, F.D.C. Insights on the larvicidal mechanism of action of fractions and compounds from aerial parts of helicteres velutina K. Schum against Aedes aegypti L. *Molecules*, v.25, p.3015, 2020.

GREEN, L.C.; RUIZ DE LUZURIAGA, K.; WAGNER, D.A. *et al*. Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci.,* v.78, p.7764-7768, 1981.

GUIDELINES for efficacy testing of spatial repellents. Control of neglected tropical diseases Who pesticide evaluation scheme. WHO/CDS/CPC/ MAL/13.12., Geneva: WHO, 2013. 58p.

GUIMARÃES, A.G.; XAVIER, M.A.; SANTANA, M.T. *et al*. Carvacrol attenuates mechanical hypernociception and inflammatory response. *Naunyn-Schmiedeberg's Arch. Pharmacol*., v.385, p.253-263, 2012.

KOWALCZYK, A.; PRZYCHODNA, M.; SOPATA, S.; BODALSKA, A.; FECKA, I. Thymol and thyme essential oil-new insights into selected therapeutic applications. *Molecules*, v.25, p.4125, 2020.

LEE, D.C.; AHN, Y.J. Laboratory and simulated field bioassays to evaluate larvicidal activity of pinus densiflora hydrodistillate, its constituents and structurally related compounds against aedes albopictus, aedes aegypti and culex pipiens pallens in relation to their inhibitory effects on acetylcholinesterase activity. *Insects*, v.4, p.217-229, 2013.

LUKER, H.A.; SALAS, K.R.; ESMAEILI, D. *et al*. Repellent efficacy of 20 essential oils on Aedes aegypti mosquitoes and Ixodes scapularis ticks in contact-repellency assays. *Sci. Rep*., v.13, p.1705, 2023.

NUNES, F.C.; LEITE, J.A.; OLIVEIRA, L.H. *et al*. The larvicidal activity of Agave sisalana against L4 larvae of Aedes aegypti is mediated by internal necrosis and inhibition of nitric oxide production. *Parasitol. Res*., v.114, p.543-549, 2015.

PINEDA-CORTEL, M.R.B.; CABANTOG, R.J.R.; CAASI, P.M. *et al*. Larvicidal and ovicidal activities of Artocarpus blancoi extracts against Aedes aegypti. *Pharm. Biol.*,v.57, p.120-124, 2019.

SILVA, S.L.C.; GUALBERTO, S.A.; CARVALHO, K.S.; FRIES, D.D. Avaliação da atividade larvicida de extratos obtidos do caule de Croton linearifolius Mull. Arg. (Euphorbiaceae) sobre larvas de Aedes aegypti (Linnaeus, 1762) (Diptera: Culicidae). *Biotemas*, v.27, p.79-85, 2014.

TERRA, M.G.; SILVA, R.S.; PEREIRA, M.G.N.; LIMA, A.F. Aedes aegypti e as arbovíroses emergentes no Brasil. *Rev. Uningá*, v.30, p.52-60, 2017.

XU, P.Y.M.; CHOO, A.R.; LEAL, W.S. Mosquito odorant receptor for DEET and methyl jasmonate. *Proc. Natl. Acad. Sci*., v.111, p.16592-16597, 2014.

YIN, Q.H.; YAN, F.X.; ZU, X.Y. *et al*. Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. *Cytotechnology*, v.64, p.43-51, 2012.

ZARA, A.L.D.S.A..; SANTOS, S.M.D; FERNANDES-OLIVEIRA, E.S.; CARVALHO, R.G.; COELHO, G.E. Estratégias de controle do Aedes aegypti: uma revisão. *Epidemiol. Serv. Saúde*, v.25, p.391-404, 2016.

ZWIEBEL, L.J.; TAKKEN, W. Olfactory regulation of mosquito-host interactions. *Insect. Biochem. Mol. Biol*., v.34, p.645-652, 2004.