Preferential Induction of Canonical IMD and Toll Innate Immune Receptors by Bacterial Challenges in Triatoma Pallidipennis Primed with Gram-Negative and Gram-Positive Bacteria, Respectively

Juárez-Palma Lilia, Alejandro Alvarado-Delgado & Mario Henry Rodriguez

Av. Universidad 655

ABSTRACT

Insects lack an adaptive immune defense against invading microorganisms, but they possess humoral and cellular response similar to that of vertebrates. The Immune Deficiency (IMD) and Toll are the major signaling pathways to produce humoral antimicrobial peptides AMPs. Pathogen molecular patterns (PAMs) of Gram-negative bacteria activate Pattern recognition receptors (PRR) of the IMD pathway, while PAMS of Gram-positive activate PRR of the Toll pathway.

Although the IMD pathways is incomplete in Hemipterans, in Triatoma pallidipennis, there is a preferential participation of the IMD pgrp-lc and toll receptors in the responses to Gram- negative and Gram-positive bacteria, respectively. Still, as in other insects, cross induction was observed.

Keywords: priming, *Triatoma pallidipennis*, tppgrp-lc, tptoll, IMD pathway, toll pathway.

Classification: NLM Code: QW 568

Language: English

Great Britain Journals Press

LJP Copyright ID: 392811

London Journal of Medical and Health Research

Volume 24 | Issue 2 | Compilation 1.0

Preferential Induction of Canonical IMD and Toll Innate Immune Receptors by Bacterial Challenges in *Triatoma Pallidipennis* Primed with Gram-Negative and Gram-Positive Bacteria, Respectively

Juárez-Palma Lilia[α], Alejandro Alvarado-Delgado[σ] & Mario Henry Rodriguez[ρ]

ABSTRACT

Insects lack an adaptive immune defense against invading microorganisms, but they possess humoral and cellular response similar to that of vertebrates. The Immune Deficiency (IMD) and Toll are the major signaling pathways to produce humoral antimicrobial peptides AMPs. Pathogen molecular patterns (PAMs) of Gram-negative bacteria activate Pattern recognition receptors (PRR) of the IMD pathway, while PAMS of Gram-positive activate PRR of the Toll pathway.

Although the IMD pathways is incomplete in Hemipterans, in Triatoma pallidipennis, there is a preferential participation of the IMD pgrp-lc and toll receptors in the responses to Gram-negative and Gram-positive bacteria, respectively. Still, as in other insects, cross induction was observed. An enhanced protection after a previous exposure to a pathogen, termed priming, functionally homologous to the adaptive immune memory of vertebrates, has been documented in several insect Orders but not in Hemiptera, and the participation of the components of the immune signaling cascades remains poorly explored. We present evidence for immune priming to Micrococcus luteus (Gram-positive) and Escherichia coli (Gram-negative) bacteria in T. pallidipennis. The preferential participation of receptors of the IMD and Toll pathways in the responses to each bacterial challenge was recorded.

Keywords: priming, *Triatoma pallidipennis*, tp*pgrp-lc*, tp*toll*, IMD pathway, toll pathway.

Author α σ ρ: Centro de Investigación Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Av. Universidad 655, C. P. 62100 Cuernavaca, Morelos, México.

Correspondence Author ρ: Centro de Investigación Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Av. Universidad 655, C. P. 62100 Cuernavaca, Morelos, México

I. INTRODUCTION

Insects lack an adaptive immune defense system against invading microorganisms but respond with humoral and cellular effector mechanisms whose components have counterparts in vertebrates' innate immune responses [1- 4]. These were mostly unraveled from studies in *Drosophila melanogaster* and *Aedes aegypti* [5 - 7]. The principal effectors comprise lectins, melanin, and antimicrobial peptides (AMPs) [8-13] produced mainly in the fat body, hemocytes and the digestive tract [14].

The Immune Deficiency (IMD) [15] and Toll [16] signaling pathways, whose counterparts in vertebrates are the tumor necrosis factor receptor (TNFR) and the interleukin-1 receptor (IL-1R), and the Toll-like receptors (TLRs) [17], respectively, are the major signaling pathways to produce AMPs [18]. Pathogen–associated molecular patterns (PAMPs) of invading microorganisms, that are recognized by pattern-recognition receptors (PRRs) [12, 13, 19, 20], activate these immune pathways. The canonical pathways were described mainly in the Dipterans *Drosophila* flies and *Aedes* mosquitoes.

Accordingly, Gram-negative bacteria are recognized by a transmembrane receptor that recruits and activates the IMD cascade leading to the expression of the AMP genes *cecropin, attacin, diptericin, drosomycin* [21, 22].

Gram-positive bacteria and fungi bound to proSpatzle induce its proteolytic cleavage, Spatzle

binds to the Toll receptor on the cell membrane [23], which in turn triggers the Toll cascade leading to the expression of the AMP genes *drosomycin, defensin B,* and *metchnikowin* [19, 24, 25). Although Gram-negative and Gram-positive bacteria preferentially activate IMD and Toll signaling pathways, respectively, there are cross interactions among these and other immune pathways such as JAK/STAT (26- 28], and they could be synergetic [29].

The Toll and IMD pathways are fully present in most insects [25], but this is not true for several Hemipterans, including reduvids. In this family, ortholog molecules, including the PRRs, *pgrp-lc* and *pgrp-la,* and AMPs have been identified [30-32]. In addition, there is evidence for the presence of key members of the IMD pathway (IMD and Relish) in *R. prolixus*, [34]. But genes coding this pathway appear incomplete or absent in *Triatoma dimidiata, Triatoma infestans and Triatoma pallidipennis* [31, 33]. Nevertheless, it was documented in *T. pallidipennis* the preferential participation of the IMD *pgrp-lc* and *toll* receptor genes and the *Relish* transcription factor in the regulation of responses to Gram-positive and Gram-negative infections, respectively, but as in other insects, cross induction was observed [35].

In contrast to vertebrates, the mechanisms for adaptation and selection of immune effector molecules are lacking in insects. However, enhanced protection after a previous exposure to a pathogen, termed immune priming [36, 37], has been documented in Diptera, Coleoptera, Lepidoptera, Homoptera, Hymenoptera, and Orthoptera (revised by Contreras *et al.* [38]). This immune priming is functionally homologous to the adaptive immune memory of vertebrates [36, 37, 39].

As no adaptive modifications of the protective molecules, such as AMPs occur, the molecular mechanisms participating in the induction of immune priming are mainly unknown. The increased response to a second encounter is associated with an increased production of immune effector molecules [40, 41]. Still, the participation of the components of the immune signaling cascades remains poorly explored [42].

We present herein evidence for immune priming responses to sublethal doses of *Micrococcus luteus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacteria in *T. pallidipennis*, a primary vector of Chagas disease in Mexico [43].

In addition, we documented the preferential participation of the IMD and Toll immune pathways receptors in the responses to each bacterial challenge, respectively.

II. METHODS

2.1 Insects Rearing

Triatoma pallidipennis was obtained from a colony initiated with specimens collected in different locations in the Morelos State, Mexico. The colony was maintained in the insectary of the National Institute of Public Health of Mexico.

Fifth-stage nymphs were maintained at 28°C and 70–80% relative humidity under a photoperiod of 12 h light and 12 h dark. They were fed rabbit blood after molting, using artificial feeders. All experiments were conducted using 10-12 days-post-feeding-fifth-instar nymphs. The protocols were approved by the Biosafety, Ethics and Research Committees of the National Institute of Public Health (file number, CB17-229, CB:1491, CI:1500).

2.2 Cultures of Escherichia Coli and Micrococcus luteus

Gram-negative *Escherichia coli* (DH5α8739 strain atcc.org) was grown overnight at 37°C in Luria-Bertani Broth (Dibico, Mexico) with agitation. 100µL was added to 2mL of fresh medium in a test tube and incubated until reaching an optical density of 0.7 (O.D. 600 nm).

The culture was centrifuged at 8000 rpm at 4°C, and the pellet recovered in PBS-pH 7.4 (137mM NaCl, 2.7mM KCl, 10mM sodium phosphate). Bacteria concentrations were quantified by colony forming units (CFU) in Luria Bertani-Broth medium with 1.5% bacteriological agar (BD Bioxon, Becton Dickinson, Mexico). Lyophilized Gram-positive *Micrococcus luteus* (Sigma-Aldrich, M-0508) was diluted in 5mL of PBS-pH 7.4. The viability of the sample was determined in an aliquot of 10µL using Trypan Blue at 0.4% in

PBS. (Corning USA) and counted in a Neubauer chamber. Translucent white colonies were counted individually in an optical microscope (Olympus Optical Co, LTD, Japan) at 100X. Bacteria concentrations were determined by counting CFU of cultures in Luria Bertani-Broth medium with 1.5% bacteriological agar (BD Bioxon, Becton Dickinson, Mexico).

2.3 Infection of T. Pallidipennis with Gram-Negative and Gram-Positive Bacteria

Groups of ten fifth-stage nymphs, 10 to 12 days post-feeding, cold-anesthetized (4 °C), were injected through the cuticular inter-tegument between the head and the thorax with live 10^3 CFU of *M. luteus*, 10^6 CFU of *E. coli* in 20µL of PBS, control groups were inoculated with 20 µL of sterile PBS using a syringe (31GX8mm needle).

On 1-, 15-, and 21 days post-inoculation (priming), five specimens of each group were dissected, and their fat body tissues were recuperated. Tissue samples were stored in 200 µLTRIzol (Thermo Fisher Scientific, Waltham, MA, USA) at −70 °C until processing for quantitative real-time polymerase chain reaction (qPCR) of transcripts.

After 21 days post-inoculation, five to seven nymphs per group were challenged with the same quantities of *M. luteus*, *E. coli* bacteria or PBS as previously described. At one- and three- day post-challenge, the fat bodies of five cold-anesthetized specimens of each group were individually obtained in PBS. Tissue samples were stored in Eppendorf tubes in 200 µL TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) at −70 °C until processed for total RNA extraction. Each treatment had three replicates per group.

2.4 RNA Extraction and cDNA Synthesis.

Total RNA from fat body tissues was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations. Briefly, about 50 mg of each fat body sample in 200 µL TRIzol in Eppendorf tubes (Thermo Fisher Scientific) were macerated using a biovortex. After adding 40 µL of chloroform (Sigma- Aldrich, St. Louis, MO, USA), the preparations were mixed and centrifuged for 15 min at 10,000g at 4 °C. The aqueous phase was recovered, and 200 µL of cold isopropanol (Sigma-Aldrich) was added, mixed, and incubated at −20 °C for one h. The samples were centrifuged at 10,000g for 10 min, and the pellets were washed with 500 µL 75% ethanol and centrifuged at 10,000g for 15 min (Scilogex, D3024R centrifuge). The supernatants were removed, and the pellets were suspended in 40 µL diethyl pyrocarbonate (DEPC, Sigma-Aldrich)-treated water. RNA was quantified with a NanoDrop 1000 spectrophotometer v. 3.7 (Thermo Fisher Scientific) and visualized using electrophoresis in agarose gels stained with EpiQuik DNA stain (EpiGentek, Farmingdale, NY, USA). Ten micrograms of total RNA were treated with 1 unit of DNAse I (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 37 °C, and subsequently inactivated with 1uL 25mM EDTA (ethylenediaminetetraacetic acid) at 75°C for 15 min.

First-strand cDNA synthesis was performed in 25 µL reactions containing 10 µg total RNA using an oligo dT 18 mero primer (Thermo Fisher Scientific) with 200 units of Reverse Transcriptase enzyme IV (SuperScript ®, Thermo Fisher Scientific). The synthesis reactions were incubated for one h at 42 °C, and then inactivated at 75°C for 15 min. The synthesized cDNA was diluted 1:10 with DEPC water and stored at −70 °C until use.

2.5 PCR of Tppgrp-lc, Tptoll, and AMP Transcripts

The transcription of Tp*pgrp-lc*, Tp*toll*, Tp*defensin B,* and Tp*prolixicin* was confirmed in cDNA templates by RT-PCR, using the *T. pallidipennis* *β*-actin gene as control. Oligonucleotides sequences and PCR reaction conditions are reported in [35].

2.6 Quantitative PCR

We used qPCR to analyze the expression of Tp*pgrp-lc* and Tp*toll* receptors and *prolixicin* and *defensin B* transcripts in individual cDNA samples of fat body tissue after the priming and challenge with *M. luteus* and *E. coli* as previously reported [35]. Briefly, each reaction was performed in a final volume of 10 µL, containing 2 µL of cDNA (1:10), 1.5 pmol of each oligonucleotide, and 5 µL of SYBR Green 2X Mix (NZY qPCR Green Master

Mix, nzytech, Lisbon, Portugal). qPCR was performed in a Rotor-Gene Q 5plex (Qiagen, Hilden, Germany). The qPCR conditions used were as follows:50°C for 2min, 95°C for 5 min, 35 cycles of 95 °C for 20s and 65 °C for 60s. A melt curve analysis was conducted to confirm the specificity of the reaction. Controls without templates were included with each primer set to verify the absence of exogenous DNA and oligonucleotide dimers.

2.7 Statistical Analysis

The relative differences in the expression of transcripts were calculated using the 2−ΔΔCt method [44]. As endogenous control, we used the *β-actin* gene. The values obtained from the ΔCt analysis (Ctvalue of problem transcript – Ctvalue of *β-actin*) were used to compare each transcript between groups (ΔΔCt) in all experiments (e.g., ΔCprolixicin priming group – Δcprolixicin PBS group). ANOVA not RM tests were performed to determine differences in gene expression between each treatment and their controls adjusted by Tukeys analysis. Graphs were made using GraphPad Prism 6. *P*-values of $P < 0.05$ were considered significant.

III. RESULTS

The expression of Tp*pgrp-lc*, Tp*toll*, Tp*defensin B*, and Tp*prolixicin* was estimated by PCR in the fat body of specimens of all experimental groups. Inoculation with *E. coli* and *M. luteus* increased the transcription of immune response genes, and significant differences were observed in some transcripts after the first bacterial infections and after challenges.

Tprolixicin increased significantly in insects infected (priming) with *E. coli* (4.08-fold, SE 2.00–6.15) on day one after priming, and its expression was reduced on days 15 to 21 after priming, but it increased to levels higher than those of the priming response (13.68-fold, SE 7.67-20.82– $P = 0.0146$ and 51.19-fold, SE 19.18-149.08 $P = 0.0064$) on days 1 and 3 respectively after challenge with the same bacteria. (Fig. 1A, Table1).

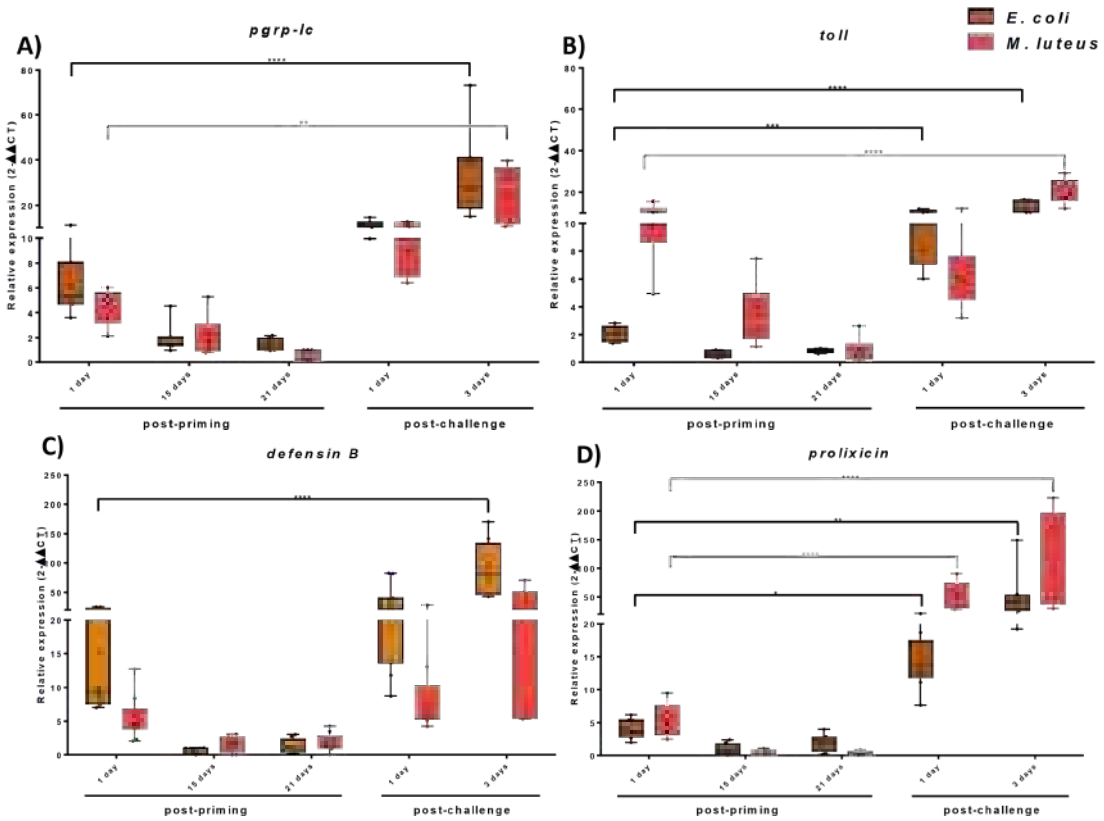

Figure 1: Relative expression of Tp*prolixicin (A),* Tp*defensin B* (B), Tp*pgrp-lc* (C), *and* Tp*toll* (D) transcripts in fat body of priming and challenge *T. pallidipennis* with *E. coli* or *M. luteus*. In priming bugs, Tp*prolixicin* and Tp*toll* transcripts increased against both bacteria after a day post-priming and these increase was greater against *M.*

luteus after one and three-days post-challenge (A and D). Similar results were observed in Tp*defensin B* and *Tppgrp-lc*, but the transcripts expression was greater against *E. coli* (B and C). Relative expression (2−ΔΔCT) is the quantified change of expression between transcripts, asterisks indicate significative *p value* (*$p = 0.0146$, ** $p = 0.0064$, *** $p = 0.0030$ and **** $p < 0.0001$), bars represent the mean transcript levels ± 95% CI. Groups were normalized with unchallenged and pbs group adjusted for *β-actin*.

Table 1: Comparasion of the relative expression of Tp*prolixicin*, Tp*defensin B*, Tp*prgp-lc* and Tp*toll* transcripts during the first bacterial infection (1-day post priming) and the second bacterial infection (1 and 3 days post challenge). Priming values are shown in gray, challenge values with increased expression are shown in green and those without significant increase are shown in red. All values are shown in fold change. Standard errors and stadistical significance are shown in parentheses.

	1 day post-priming	15 days post-priming	21 days post-priming	1 day post-challenge	3 days post-challenge
defensin B vs *E. coli*	13.36 (7.03-25)	0.69 (0.010-1.020)	1.27 (0.15-3.0)	34.0 (8.69-82.70, ns $p > 0.05$)	90.29 (43.10-170, $p < 0.0001$)
defensin B vs *M. luteus*	5.48 (2.07-12.70)	1.49 (0.01-3.02)	1.80 (0.04-4.28)	9.21 (4.25-28.24, ns $p > 0.05$)	26.73 (5.33-70.52, ns $p > 0.05$)
prolixicin vs *E. coli*	4.08 (2.0-6.15)	0.98 (0.040-2.39)	1.89 (0.13-4.0)	13.68 (7.67-20.82, $p = 0.0146$)	51. 19 (19.18-149.08 $p = 0.0064$)
prolixicin vs *M. luteus*	5.27 (2.46-9.45)	0.51 (0.11-1.08)	0.30 (0.06-0.86)	51.40 (27.85-90.50, $p < 0.0001$)	112.81 (29.44-222.86, $p < 0.0001$)
pgrp-lc-E. coli	6.31 (3.60-11.10)	1.87 (0.95-2.06)	1.49 (0.95-2.15)	11.51 (9.95-14.52, ns $p > 0.05$)	33.78 (14.92-73.00, $p < 0.0001$)
pgrp-lc vs *M. luteus*	4.47 (2.14-6.03)	2.07 (0.80-5.27)	0.60 (0.15-1.00)	9.61 (6.40-12.50, ns $p > 0.05$)	25.97 (12.29-39.65, $p = 0.0064$)
toll vs *E. coli*	2. 07 (1.40-2.84)	0.61 (0.35-0.90)	0.85 (0.65-1.00)	9.42 (6.02-12.01, $p = 0.0003$)	12.98 (10.28-16.28, $p < 0.0001$)
toll vs *M. luteus*	12.52 (4.92-15.60)	4.12 (1.14-7.46)	1.04 (0.11-2.63)	7.63 (3.22-12.14, ns $p > 0.05$)	24.02 (17.80-29.12, $p < 0.0001$)

Like the infection with *E. coli*, the infection (priming) with *M. luteus* resulted in significant increases in the expression of *Tprolixin* (5.27-fold, 2.46-9.45), but its expression was more significant after 1 and 3 days of the challenge (51.40-fold, SE 27.85-90.50 $P < 0.0001$ and 112.81-fold, SE 29.44–222.86 $P < 0.0001$), respectively (Fig.1A, Table1). The increase in the levels of Tp*prolixin* transcripts induced by the challenge with *M. luteus* was higher than those by the challenge with *E. coli* (Fig. 2, Table 2).

Priming with *E. coli* produced an increase of Tp*defensin B* transcripts on day one post-priming (13.36-fold, SE 7.03-25), and these decreased at days 15 to 21 post-priming, but the challenge with the same bacterium produced increased levels of the transcript (34-fold, SE 8.69–82.70 $P > 0.05$ and 90.29-fold, SE 43.10–170 $P < 0.0001$) on days 1 and 3 post-challenge with the same bacterium, respectively (Fig. 1B, Table 1).

Priming with *M. luteus* resulted in a moderate increase of Tp*defensin* (5.48-fold, SE 2.07–12.70) on day one post-priming, and its level decreased on day 15 and remained low until day 21 post. priming. Challenge with the same bacterium did not induce statistically significant increases on days 1 and 3 post-challenge (9.21-fold, SE 4.25–28.24 $P > 0.05$ and 26.73-fold, SE 5.33-70.52 $P > 0.05$). However, the increases in Tp*defensin B* transcripts induced by the challenge with *E. coli* were higher than those by the challenge with *M. luteus* (Fig. 2, table 2).

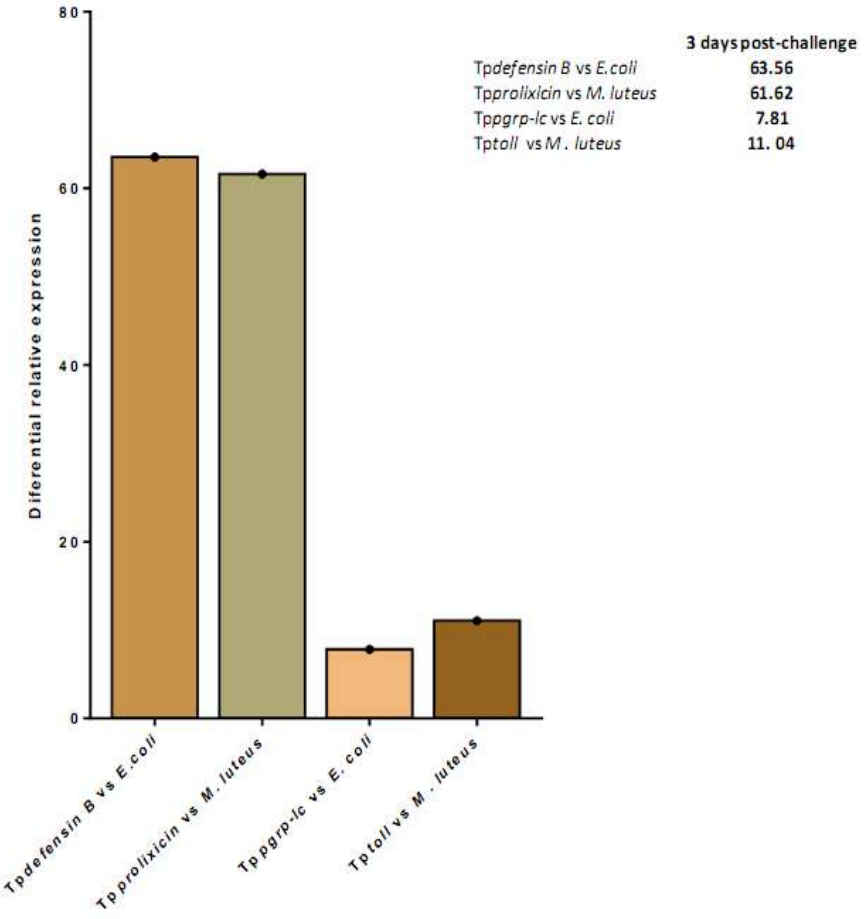

Figure 2: Differential relative expression of AMPs and receptors of *T. pallidipennis* after three days post-challenge with *E. coli* or *M. luteus*. Tp*defensin B* and Tp*pgrp-lc* transcripts were more intensely expressed when inoculated with *E. coli*, while Tp*prolixicin* Tp*toll* were more expressed in response to *M. luteus*.

Table 2: Differential expression of AMPs, and Tp*pgrp-lc* and Tp*toll* receptors during the 1-and 3-days post challenge with *E. coli* or *M. luteus*. *Tpdefensin B* and *Tppgrp-lc* were more expressed against *E. coli*, while Tp*prolixicin* and Tp*toll* against *M. luteus***.** The differential values with increased expression are shown with an asterisk. Differential expression was obtained by comparing the values of a specific transcript in response to infection of each bacterial species. The values with increased expression are shown in green and those with no significant increase are shown in red. All values are shown in fold change.

	1 day post-challenge	3 days post-challenge		1 day post-challenge	3 days post-challenge
Tp*defensin B* vs *E. coli*	34	90.29	Tp*prolixicin* vs *E. coli*	13.68	51. 19
Tp*defensin B* vs *M. luteus*	9.21	26.73	Tp*prolixicin* vs *M. luteus*	51.4	112.81
	24.79	63.56*		37.72*	61.62*
Tp*pgrp-lc* vs *E. coli*	11.51	33.78	Tp*toll* vs *E. coli*	9.42	12.98
Tp*pgrp-lc* vs *M. luteus*	9.61	25.97	Tp*toll* vs *M. luteus*	7.63	24.02
	1.9	7.81*		1.79	11.04*

	3 days post-challenge	1 day post-challenge
Tp*defensin B* vs *E. coli*	63.56*	24.79
Tp*prolixicin* vs *M. luteus*	61.62*	37.72*
Tp*pgrp-lc* vs *E. coli*	7.81*	1.9
Tp*toll* vs *M . luteus*	11. 04*	1.79

Tp*pgrp-lc* increased after infection with *E. coli* (6.31-fold, SE 3.60–11.10) but transcript levels decreased on days 15 and 21. After challenge with the same bacterium, Tp*pgrp-lc* increased significantly on three days post inoculation, (33.78-fold, SE 14.92-73 $P < 0.0001$) (Fig. 1C, Table1).

After priming with *M. luteus,* increases of Tp*pgrp-lc* levels were observed on day one post-inoculation (4.47-fold, SE 2.14-6.03), and the transcript levels remained low up to 21 days. After a challenge with the same bacterium, transcription increased at levels higher than those observed during the first infection. Increased levels were higher after three days post-challenge (25.97-fold, SE 12.29–39.65 $P < 0.0064$). On day one, no statistically significant increases were observed (9.61-fold, SE 6.40–12.50 $P > 0.05$). The increase in Tp*pgrp-lc* transcripts induced by the challenge with *E. coli* was higher than those by the challenge with *M. luteus*.

Tp*toll* transcripts increased after priming with *E. coli* (2.07-fold, SE 1.40-2.84) levels below those of pre-priming were observed on days 15 and 21. After challenge, Tp*toll* transcripts increased (9.42-fold, SE 6.02–12.01 $P = 0.0003$ and 12.98-fold, SE 10.28-16.28 $P < 0.0001$) on the first- and third- day post-injection, respectively. Using *M. luteus,* Tp*toll* transcripts increased during the priming (12.52-fold, SE 4.92-15.60), and the challenge with the same bacterium resulted in a new rise in *TpToll*, reaching higher levels that those observed after the first infection (24.02-fold, SE 17.80–29.12 $P < 0.0001$) on day three post-challenge. The increase in the levels of Tp*toll* transcripts induced by the challenge with *M. luteus* was higher than those by the challenge with *E. coli* (Fig. 2).

IV. DISCUSSION

We documented in *T. pallidipennis* the participation of *Tppgrp-lc* and Tp*toll* receptors in the induction of immune priming, and in the enhanced immune response after a second challenge with the same bacteria. In addition, we confirmed our previous observations on the preferential participation of these receptors in the recognition and induction of the primary immune innate response to bacterial infections.

As we employed sub-lethal doses of *E. coli* and *M. luteus* for the challenge, we could not assess any possible protection effect of the priming induced by first bacterial infections. Nevertheless, the *defensin B* and *prolixin* transcription responses after a second bacterial challenge fulfilled the essential parameters of priming: specificity [36] and long-lasting biphasic response [37, 39]. However, in analyzing the molecular specificity of the reactions, some precisions are required. Although the infection with Gram-positive and Gram-negative bacteria induced the transcription of both AMPs, and transcription levels decreased with time in both infections. The Tp*defensin B* initial response was higher after infection with *E. coli* than with *M. luteus*, confirming the preferential induction of the IMD pathway by the Gram-negative bacterium. The response to *M. luteus* could indicate possible Tp*pgrp-lc* isoforms responding to Gram-positive bacteria, or the direct induction of components of the IMD pathway. Similar primary responses were observed in *D. melanogaster* [21, 26]

A second challenge with *E. coli* resulted in higher levels of Tp*defensin B* transcript, while a second challenge with *M. luteus* did not induce significant changes in its transcription. After priming, the differences between the responses support a preferential response to the Gram-negative bacterium, which is in accord with a preferential IMD pathway induction. Although we did not explore possible changes in the transcription of other components of the IMD pathway, changes in *Tppgrp-lc* transcription after challenge with *E. coli* followed a similar pattern to that of Tp*defensin B*. In contrast, after challenge with *M. luteus,* this was not significant. We speculate that the increased Tp*pgrp-lc* receptor, augmented the recognition of the *E. coli* used for challenge, thus the enhanced Tp*defensin B* response. This confirms a relative degree of the specificity of the priming response within the cross interacting IMD and Toll system, a condition favouring a broad-spectrum innate Immune response [21].

On the other hand, *Tprolixin* and Tp*toll* transcription levels increased after the first

infection with both bacteria, but they were higher after infection with *M. luteus*. The participation of Tp*toll* and other Toll receptors in responses to Gram-positive, indicates higher receptor affinity to the Gram-positive bacterium. Toll pathway induction by Gram-positive and Gram-negative bacteria was observed in other insects [45, 46] and could explain our observations. In the same way, the transcription of *Tprolixin* and Tp*toll* was higher after a second challenge with both bacteria, but the challenge with *M. luteus* resulted in higher transcription levels than with the challenge with *E. coli*. The increase of the Toll receptor after priming could explain the enhanced response to *M. luteus* during the challenge.

Our observations reflect the diversity in the induction of the innate immune pathways among insects. It could be explained by cross-interactions between the members of two central innate immune cascades. The capacity of the receptors to interact with diverse molecules in Gram-positive and Gram-negative bacteria is shown by diverse toll receptors that could mediate the activation of immune responses against Gram-negative bacteria [4]. On the other hand, as bacteria contain a mixture of antigens, it is possible that although Gram-negative bacteria preferentially induce the IMD immune system and Gram-positive the Toll system, some bacterial antigens may induce the other immune cascade than the canonically, up to now accepted. Nevertheless, the higher responses after the challenge are in order with the specificity of the priming inductor.

The mechanisms underlying immune priming remain poorly understood; however, specific receptor induction is expected for specific responses. Specific, or in the case of *T. pallidipennis* preferential, induction began with activating a particular receptor. Our results indicate that transcription of *Tppgrp-lc* and Tp*toll* receptors increased after the first exposition to their respective bacterium, indicating that a mechanism activates the production of more receptors than those already present before exposition. Similar results were observed in *Anopheles gambiae* exposed to bacteria [47].

Increased numbers of receptors could be responsible of the primary response,and having already more receptors could explain an increased response to the second exposure. As stablished in canonical priming (biphasic response), the transcription effectors Tp*prolixin* and Tp*defesin B* subdued after three weeks, so did their receptors, probably because exhaustion of the respective antigens. Interestingly, an even higher receptors' transcription occurred after the challenge. This indicates possible not yet defined mechanisms responsible for induction of the transcription during the first pathogen encounter, specifically (or preferentially) triggered again with the challenge.

We did not explore the participation of other members of the IMD and Toll immune cascades that could play a role in directly modulating the immune response at the transcription level. There is evidence in *Drosophila* that Toll, but not IMD, is necessary, although insufficient for priming induction (48), but its participation still requires revealing. Epigenetic DNA/RNA dynamic modifications by methylation could regulate gene expression [49], possibly explaining "memory", but the underlaying mechanism for enhancement is not responded. Endoreplication (DNA synthesis without cell division) has been documented in *An. Albimanus* infected with *Sacharomyces cerevisiae* [50] and *Plasmodium berghei,* probably increasing the number of immune genes copies during the first encounter with the pathogens. This, along with the transcription of regulatory elements of the cell cycle [51], could indicate the involvement of enhanced transcription in the immune response but does explain further enhancement after challenge.

In conclusion, we have documented in the Hemipteran T. pallidipennis the response to Gram-negative and Gram-positive bacteria with the main features of priming: biphasic and enhanced AMPs transcription response after a second challenge. Both phases were associated to the preferential induction of IMD and Toll receptors, respectively. The mechanisms underlying the induction of their transcription warrant further research.

ACKNOWLEDGEMENTS

This study was supported by the Consejo Nacional de Ciencia y Tecnología de México (CONACyT) (Grant 1500). The authors would like to thank Carlos Isaac Roldan, for helping with maintaining the triatomine colony.

The authors declare that they have no conflict of interest related to the findings or techniques in the present study.

List of abbreviations:

qPCR (quantitative polymerase chain reaction)
pgrp-lc (Peptidoglycan recognition protein-long chain)
AMPs (Antimicrobial peptides)
IMD (Immune deficiency signalling pathway)

REFERENCES

1. Leclerc V., Reichhart J.-M. The immune response of *Drosophila melanogaster*. Immunol. Rev. 2004198, 59–71.
2. Müller U., Vogel P., Alber G., Schaub G.A. The innate immune system of mammals and insects. Contrib. Microbiol. 2008; 15, 21–44.
3. Li G., Wang H., Wang S., Xing G., Zhang C., Zhang, W., Liu J., Su S., Zhou J.,. Insights into the genetic and host adaptability of emerging porcine circovirus 3. Virulence 2018; 9: 1301–1313.
4. Vilmos P. Insect immunity: evolutionary roots of the mammalian innate immune system. Immunol. Lett. 1998. 62, 59–66.
5. Ferrandon D., Imler J. L., Hetru C., Hoffmann J. A. The *Drosophila* systemic immune response: sensing and signaling during bacterial and fungal infections. Nat. Rev Immunol. 2007; 7: 862–74.
6. Ramirez J. L., Muturi E. J., Barletta A. B. F., Rooney A. P. The *Aedes aegypti* IMD pathway is a critical component of the mosquito antifungal immune response. Dev. Comp. Immunol. 2019; 95:1–9.
7. Xi Z., Ramirez J. L., Dimopoulos G. The *Aedes aegypti* toll pathway controls dengue virus infection. PLOS Pathog. 2008;4:e1000098.
8. Vilmos P., Kurucz E. Insect immunity: evolutionary roots of the mammalian innate immune system. Immunol. Lett. 1998; 62: 59–66.
9. Leclerc V, Reichhart J.M. The immune response of *Drosophila melanogaster*. Immunol. Rev. 2004, 198: 59–71.
10. Akira S., Uematsu S., Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006; 124:783–801.
11. Flores-Villegas A. L., Salazar-Schettino P. M., Cordoba-Aguilar A., Gutierrez-Cabrera A. E., Rojas-Wastavino G. E., Bucio-Torres M. I., et al. Immune defense mechanisms of triatomines against bacteria, viruses, fungi and parasites. Bull. Entomol. Res. 2015; 105:523–32.
12. Akira S., Uematsu S., Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006; 124, 783–801.
13. Lu Y., Su F. Li, Q. Zhang J., Li Y., Tang T., Hu Q,, Yu X-Q. , . Pattern recognition receptors in *Drosophila* immune responses. Dev. Comp. Immunol. 2020;102, 103468.
14. Vieira C. S., Waniek P. J, Castro D. P., Mattos D. P., Moreira O.C., Azambuja P. Impact of *Trypanosoma cruzi* on antimicrobial peptide gene expression and activity in the fat body and midgut of *Rhodnius prolixus*. Parasit. Vectors. 2016;9:1–12.
15. Myllymaki H., Valanne S., Ramet M. The *Drosophila* Imd signaling pathway. J Immunol. 2014; 192: 3455–62.
16. Valanne S., Wang J-H, Ramet M. The *Drosophila* Toll signaling pathway. J. Immunol. 2011; 186: 649–56.
17. Belvin M.P., Anderson K.V. A conserved signaling pathway: the *Drosophila* toll-dorsal pathway. Annu. Rev. Cell. Dev. Biol. 1996; 12: 393–416.
18. Xu J., Cherry S. Viruses and antiviral immunity in *Drosophila*. Dev. Comp. Immunol. 2014; 42:67–84.
19. Lemaitre B., Hoffmann J. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 2007;25:697–743.
20. Hultmark D. *Drosophila* immunity: paths and patterns. Curr. Opin. Immunol. 2003; 15: 12–9.
21. Tanji T., Yun E.Y., Ip Y.T. Heterodimers of NF-κB transcription factors DIF and Relish

regulate antimicrobial peptide genes in *Drosophila*. Proc. Natl. Acad. Sci. 2010; 107: 14715–20.
22. Ursic-Bedolla R., Buchhop J. B. J., Durvasala R., Lowenberber C. Prolixin: a nivel antimicrobial peptide isolated from *Rhodnius prolixus* with differential activity against bactaria and *Trypanosoma cruzi*. Insect Mol. Biol. 2011: 20: 775-786
23. Weber A. N. R., Moncrieffe M. C., Gangloff M., Imler J-L., Gay N.J. Ligand receptor and receptor-receptor interactions act in concert to activate signaling in the *Drosophila* toll pathway. J Biol Chem. 2005. https:// doi.org/ 10. 1074/ jbc. M5020 74200.
24. Imler J.L., Hoffmann J. A. Signaling mechanisms in the antimicrobial host defense of *Drosophila*. Curr. Opin. Microbiol. 2000; 3:16–22.
25. Wang Q., Ren M., Liu X., Xia H., Chen K. Peptidoglycan recognition proteins in insect immunity. Mol. Immunol. 2019;106, 69–76.
26. Nishide Y., Kageyama D., Yokoi K., Jourak, A., Tanaka H., Futahashi R., et al., 2019. Functional crosstalk across IMD and Toll pathways: insight into the evolution of incomplete immune cascades. Proc. Biol. Sci. 286, 20182207.
27. Koyama H., Kato D., Minakuchi, C., Tanaka T., Yoko K., Miura, K. Pepti- doglycan recognition protein genes and their roles in the innate immune path- ways of the red flour beetle, *Tribolium castaneum* . J. Invertebr. Pathol. 2015; 132, 86–100.
28. Wan Y., Yang, F., Cao X., Zou Z., Lu Z., Kanost MR., Jiang H. Hemolymph protease- 5 links the melanization and Toll immune pathways in the tobacco hornworm, *Manduca sexta*. Proc. Natl. Acad. Sci. USA 2020; doi: 10.10 73/pnas.2004761117.
29. Tanji T, Hu X, Weber ANR, Ip YT. Toll and IMD pathways synergistically activate an innate immune response in *Drosophila melanogaster*. Mol. Cell. Biol. 2007;27:4578.
30. Lopez L., Morales G., Ursic R., Wolff M, Lowenberger C. Isolation and characterization of a novel insect defensin from *Rhodnius prolixus*, a vector of Chagas disease. Insect Biochem Mol Biol. 2003;33:439–47.
31. Zumaya-Estrada F. A., Martinez-Barnetche J., Lavore A., Rivera-Pomar R., Rodriguez M. H. Comparative genomics analysis of triatomines reveals common first line and inducible immunity-related genes and the absence of Imd canonical components among hemimetabolous arthropods. Parasites Vectors. 2018; 11:1–16.
32. Salcedo-Porras N., Noor S., Cai C, Oliveira P. L., Lowenberger C. *Rhodniusprolixus* uses the peptidoglycan recognition receptor rpPGRP-LC/LA to detect Gram-negative bacteria and activate the IMD pathway. Curr. Res.Insect Sci. 2021;1:100006.
33. Mesquita R. D. et al. 2015 Genome of *Rhodnius prolixus*, an insect vector of Chagas disease reveals unique adaptations to hematophagy and parasite infection. Proc. Natl Acad. Sci. USA 112: 14 936–14 941.
34. Salcedo-Porras N., Guarneri A., Oliveira,P. L., Lowenberger C. *Rhodnius prolixus*: identification of missing components of the IMD immune signaling pathway and functional characterization of its role in eliminating bacteria. PLoS One 2019; 14, e0214794.
35. Alvarado-Delgado A., Juarez-Palma L., Martinez-Bartneche J., Rodriguez M. H. The IMD and Toll canonical immune pathways of *Triatoma pallidipennis* are preferentially activated by Gram-negative and Gram-positive bacteria, respectively, but cross-activation also occurs. Parasit Vectors 2022; 15: 256.
36. Kurtz J. Specific memory within innate immune systems. Trends Immunol. 2005; 26, 186–192.
37. Schmid-Hempel P. *Evolutionary Parasitology*. Oxford University Press, Oxford, U.K. Ecol. Entomol. 2016; 41:351–366.
38. Contreras- Garduño J, Lanz-Mendoza H., Franco B, Nava A, Pedraza-Reyes M, Canales-Lazcano M. Insect immune priming: ecology and experimental Evidences. Ecol. Entomol. 2016; 41: 351-366.
39. Brehélin M., Roch P. Specificity, learning and memory in the innate immune response. Inverter. Surv. J. 2008; 5: 103–109.
40. Rodrigues J., Brayner F. A., Alves L.C., Dixit R., Barillas-Mury. C Hemocyte differentiation

mediates innate immune memory in *Anopheles gambiae* mosquitoes. Science. 2010; 329: 1353–1355.
41. Contreras-Garduño J., Rodríguez M. C., Martínez S. H., Barnetche J. M., Alvarado A., Izquierdo, J. *et al. Plasmodium berghei* induced priming in *Anopheles albimanus* independently of bacterial co-infection. Dev. Comp. Immunol. 2015; 52: 172–181.
42. Schulenburg H., Boehnisch C., Michiels N.K. How do invertebrates generate a highly specific innate immune response? Mol. Immunol. 2007; 44: 3338–3344.
43. Ramsey J. M., Townsend Peterson A., Carmona-Castro O., Moo-Llanes D. A, Nakazawa Y., Butrick M., et al. Atlas of Mexican Triatominae (Reduviidae: *Hemiptera*) and vector transmission of Chagas disease. Mem. Inst. Oswald. Cruz. 2015; 110: 339.
44. Livak K. J., Schmittgen T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402–40845. Kawai T., Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011: 34:637–50.
45. Takeuchi O., Hoshino K., Kawai T., Sanjo H., Takada H., Ogawa T., et al. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. Immunity. 1999;11:443–51.
46. Dimopoulus G., Christophides G. K., Mesister S., Kafatos F. C. Genome expression analysis of *Anopheles gambiae*: Responses to injury, bacterial challenge, and malaria infection PNAS 2002; 99: 8814-8819
47. Pham L.N. Dionne M.S. Shirasu-Hiz M., Scheneider DS. A specific primed immune response in Drosophila is dependent on phagocytes. PLoS Pathog. 2007 3: e26.
48. Ottaviani E. Invertebrate immunological memory: could the epigenetic changes play the part of lymphocytes? Inverteb Surv J. 2015; 12, 1–4.
49. Hernández-Martínez S., Román-Martínez, U, Martínez-Barnetche J., Garrido E., Rodríguez M. H., Lanz-Mendoza H. Induction of DNA synthesis in *Anopheles albimanus* tissue cultures in response to a *Saccharomyces cerevisiae* challenge. Arch. Insect. Biochem. Physiol. 2006; 63: 147–158.
50. Maya-Maldonado K., Cime-CastillJ., Maya-Lucas O., Argotte-Ramos R., Rodríguez M. C., Lanz-Mendoza H. Transcriptome analysis uncover differential regulation in cell cycle, immunity, and metabolism in *Anopheles albimanus* during immune priming with *Plasmodium berghei*. Dev. Comp. Immunol. 2021; 120, 104046.