Accepted Manuscript

The *limpet* transcription factors of *Triatoma infestans* regulate the response to fungal infection and modulate the expression pattern of defensin genes

M. Constanza Mannino, Flavia R. Paixão, Nicolás Pedrini

PII: S0965-1748(18)30386-2

DOI: https://doi.org/10.1016/j.ibmb.2019.03.010

Reference: IB 3145

To appear in: *Insect Biochemistry and Molecular Biology*

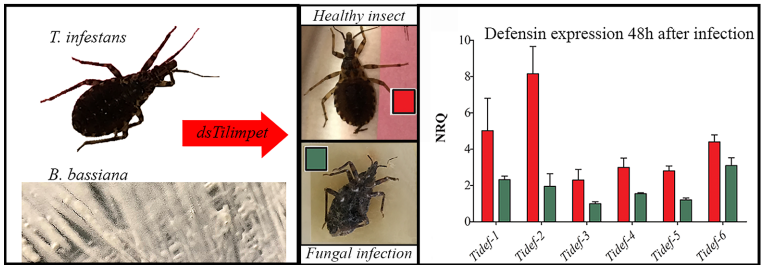
Received Date: 24 October 2018

Revised Date: 19 March 2019

Accepted Date: 22 March 2019

Please cite this article as: Mannino, M.C., Paixão, F.R., Pedrini, Nicolá., The *limpet* transcription factors of *Triatoma infestans* regulate the response to fungal infection and modulate the expression pattern of defensin genes, *Insect Biochemistry and Molecular Biology* (2019), doi: https://doi.org/10.1016/j.ibmb.2019.03.010.

Effect of *Tilimpet* silencing on defensins expression pattern



The *limpet* transcription factors of *Triatoma infestans* regulate the response to fungal infection and modulate the expression pattern of defensin genes

M. Constanza Mannino[a]; Flavia R. Paixão[a]; Nicolás Pedrini[a]*

[a]Instituto de Investigaciones Bioquímicas de La Plata (CONICET CCT La Plata-UNLP), Universidad Nacional de La Plata, Argentina.

* Corresponding author at: INIBIOLP, calles 60 y 120, 1900 La Plata, Argentina

E-mail address: npedrini@med.unlp.edu.ar

Declarations of interest: none

Abstract

As part of the innate humoral response to microbial attack, insects activate the expression of antimicrobial peptides (AMPs). Understanding the regulatory mechanisms of this response in the Chagas disease vector *Triatoma infestans* is important since biological control strategies against pyrethroid-resistant insect populations were recently addressed by using the entomopathogenic fungus *Beauveria bassiana*. By bioinformatics, gene expression, and silencing techniques in *T. infestans* nymphs, we achieved sequence and functional characterization of two variants of the *limpet* transcription factor (*Tilimpet*) and studied their role as regulators of the AMPs expression, particularly defensins, in fungus-infected insects. We found that *Tilimpet* variants may act differentially since they have divergent sequences and different relative expression ratios, suggesting that *Tilimpet-2* could be the main regulator of the higher expressed defensins and *Tilimpet-1* might play a complementary or more general role. Also, the six defensins (*Tidef-1* to *Tidef-6*) exhibited different expression levels in fungus-infected nymphs, consistent with their phylogenetic clustering. This study aims to contribute to a better understanding of *T. infestans* immune response in which *limpet* is involved, after challenge by *B. bassiana* infection.

Key words: *Beauveria bassiana*, Chagas disease vector, Insect immunity, Triatomine bugs.

1. Introduction

~~Insects display complex and sophisticated innate immunity responses since they do not have an adaptive immunity as vertebrates, although seem to contain some characteristics of an adaptive immune system~~ Insects rely almost exclusively on an innate immune system to protect themselves from pathogens as they do not have an advanced adaptive immune system. (Cooper and Eleftherianos, 2017). The defense mechanisms include both humoral and cellular immunity, each consisting of different strategies to fight and overcome the barrage of invasive microbes that either cohabit with or infect them. Cellular responses involve hemocytes and can include processes such as phagocytosis, encapsulation, and nodulation (Lavine and Strand, 2002). On the other hand, humoral immune responses act through melanization (Cerenius et al., 2008), production of oxygen reactive species (Nappi and Ottaviani, 2000) and production of antimicrobial peptides (AMP~~s~~) (Bulet et al., 1999; Hultmark, 2003). The AMP~~s~~ comprise a group of different molecules which are the hallmark of humoral response in insects after immune challenge (Pal and Wu, 2009). An extensively characterized group of AMP~~s~~ are defensins, since a vast amount of information is available. This short peptide~~s~~ family -around 50 amino acids in length- is evolutively conserved and has six characteristic cysteine residues that form three disulfide bonds which confer structural stability (Tonk et al., 2015a). Their amino acidic sequences and biological functions have a considerable level of diversity in ~~the~~ insects ~~they have been characterized~~ (Rajamuthiah et al., 2015; Seufi et al., 2011; Tonk et al., 2015b). Although there are some previous reports about defensins from the triatomine bugs *Rhodnius prolixus* (Lopez et al., 2003; Ursic-Bedoya and Lowenberger, 2007), *Triatoma brasiliensis* (Waniek et al., 2009), and *T. pallidipennis* (Diaz–Garrido et al., 2018), functional and structural characteristics are scarce in *T. infestans*. Only two defensins have been described in this species (de Araújo et al., 2015) and recently four

more sequences were found in an integument transcriptome (Calderón-Fernández et al., 2017).

AMPs expression is regulated mainly by a battery of immunity-related genes through the Toll, IMD, JAK-Stat and RNAi regulation pathways, which are activated by Gram-positive bacteria and fungi (Lemaitre et al., 1997; Leulier et al., 2000; Rutschmann et al., 2002, 2000). Among them, genes participating in AMPs regulation, the transcription factor *limpet* was related to the primary immune response in *Drosophila* (Jin et al., 2008). This protein contains Zinc fingers structures and a typical repetition of LIM domains (InterPro #IPR001781) accompanied by a PET domain (InterPro #IPR010442), therefore they are named LIMPET. Functional characterization was only reported by Jin et al. (2008) in *D. melanogaster*, and its potential function was mentioned by Altincicek et al. (2008) in *Tribolium castaneum.*

Triatoma infestans is the main vector of Chagas disease (American Trypanosomiasis) in the southern cone of South America (WHO, 2000). Chagas disease has a considerable medical and socioeconomic impact since around 7 to 8 million people are estimated to be affected by the parasite *Tripanosoma cruzi*, and causing around 12,000 deaths per year in the world (mostly in the Americas) are related to this affection (Dias et al., 2002; Lee et al., 2013; WHO, 2012). For several years, pyrethroid residual spraying was a successful tool for triatomines control; however, an increasing number of highly resistant *T. infestans* populations in the Gran Chaco region were identified posing a challenge in vector control (Mougabure-Cueto and Picollo, 2016). Biological control is a worldwide strategy used as a part of integrated pest management programs, and in the last decade the ability of the hypocrealean entomopathogenic fungus *Beauveria bassiana* to colonize and kill *T. infestans* has been an active topic of research in our laboratory (Forlani et al., 2015; 2011; Mannino et al., 2018; Pedrini et al., 2009). *Beauveria bassiana* penetrates the host through the cuticle and proliferates inside the hemocoel, triggering the *T. infestans* immune response (Lobo et al.,

2015; Pedrini, 2018). It was proposed and tested in both laboratory and field as a safe and effective biological tool to control not only pyrethroid-susceptible but also pyrethroid-resistant populations of *T. infestans* (Forlani et al., 2015; 2011; Pedrini et al., 2009).

A better understanding of the regulation of *T. infestans* innate immune response in its interaction with the entomopathogenic fungus *B. bassiana* is crucial to the development and improvement of integrated vector control strategies against triatomine bugs. In this study, we identified and characterized two genes encoding for *limpet* transcription factors in *T. infestans* and studied their role as regulators of AMPs expression, particularly defensins.

2. Materials and methods

2.1. Insects

Fourth instar nymphs of *T. infestans* came from a colony regularly maintained and reared at 30 °C, 50–60% relative humidity, under a 12 h photophase, and fed on ketamine-anesthetized rats (Paim et al., 2017), at the INIBIOLP, Facultad de Ciencias Médicas, La Plata, Argentina. All animal care and laboratory experimental protocols were approved by the Directive Board of the INIBIOLP (Animal Welfare Assurance No. A5647–01) and carried out following the AVMA Animal Welfare Policies and AVMA Guidelines on Euthanasia: https:// www.avma.org/kb/policies/pages/default.aspx, https// www.avma.org/KB/Policies/Documents/euthanasia.pdf, accessed October 2, 2018. For all the assays, 4-week-old nymphs were used, two weeks after a blood meal. For the different treatments, each sample consisted of an individual insect.

2.2. Identification of *limpet* and *defensin* transcripts

~~Two and six nucleic acid sequences of interest for *limpet* and *defensin*, respectively, were identified~~ The nucleic acid sequences for two *limpet* and six *defensins* were identified and

retrieved from *T. infestans* expressed sequence tag (EST) libraries from the integument (GenBank, BioProject PRJNA314811) (Calderón-Fernández et al., 2017) and salivary glands (GenBank, BioProject PRJNA238208) (Schwarz et al., 2014). The sequences putatively encoding for either *limpet* or *defensin* were further searched using BLASTN (Basic Local Alignment Search tool-N)(Altschul et al., 1990) against the non-redundant database at the National Centre for Biotechnology Information (NCBI, http://www. ncbi.nlm.nih.gov/) to confirm its identity with other known insect *limpet* or *defensin*. The GenBank codes of the sequences used for BLASTN search of the related sequences were JAS01664 (*limpet*) and JAS02103 (*defensin*). Also, alignments to identify homology with the related triatomine bug *R. prolixus* (whole genome sequenced) (Mesquita et al., 2015) were performed trough VectorBase BLASTN (https://www.vectorbase.org/blast).

2.3. Nucleic acid ~~manipulation~~

Total RNA was extracted from whole insects by using the Tri Reagent® (Molecular Reagent Center, USA) technique, according to manufacturer instructions. Quantity and quality of RNA were assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and 1% (w/v) agarose gel electrophoresis, respectively. For cDNA synthesis, iScript™ cDNA Synthesis Kit (BioRad, USA) was used according to manufacturer's instructions. One microgram of each sample of total RNA was used for cDNA synthesis. The resultant cDNA was diluted 1/10 for further use in PCR as well as in qPCR. Gene Runner 3.1 (generunner.net) was used for all primer design, PCR, qPCR and silencing primers. Primers are listed in Table S1. To confirm and ~~complete~~ obtain the full length sequence of *limpet*, including its 5´end, the primers ~~used to amplify and obtain a larger *limpet* sequence are~~ listed in Table S1 were used. PCR was performed with an initial denaturation at 94°C for 1 min, followed by 35 cycles each consisting of 15s at 94°C, 30s at 58°C, and 30s at 72°C, and a final extension step of 4 min at 72°C. The PCR products were cleaned up using 3M sodium

acetate and chilled absolute ethanol precipitation. Products were sequenced in both directions (Macrogen Inc., South Korea).

2.4. Phylogenetic analysis of *limpet* and *defensin* transcripts

The MEGA 7.0.26 program (www.megasoftware.net) (Tamura et al., 2007) was used to perform multiple sequence alignments using the ClustalW 2 algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and to construct the phylogenetic trees. Consensus phylogenetic trees were constructed using the unweighted pair group method with arithmetic means (UPGMA). To evaluate the branch strength of the phylogenetic tree, bootstrap analysis of 5,000 replications was performed.

2.5. Fungal cultures

Beauveria bassiana strain GHA (Laverlam International, USA) was grown on Potato Dextrose Agar (PDA) (Merk, Germany) plates. Plates were incubated at 26 °C for 12 days. Suspensions of conidia were prepared by rinsing fungal cultures with sterile distilled water and rubbing the sporulating surface with a bent needle. After filtering debris, the liquid was diluted in sterile distilled water containing 0.01% Tween 80. Fungal blastospores were produced in Sabouraud dextrose + 1% yeast extract liquid broth cultures (SDY), using conidia harvested from PDA plates to final concentration of 5×10^5 conidia ml^{-1} as the inoculum. Cultures were grown for 3 days at 26 °C under shaking (200 rpm) and filtered (twice) through sterile folded gauze to remove mycelia. Blastospores were obtained by centrifugation and the pellet resuspended in sterile distilled water. Final blastospore concentrations were determined by direct counts using a Neubauer chamber.

2.6. Infection assay

2.6.1. RNAi construction, insect inoculation and infection assay sampling

149 The dsRNAi construction was obtained through PCR using the primers listed in Table S1
150 and the MEGAscript™ RNAi Kit (Ambion, USA), according to the manufacturer's
151 instructions. In order to avoid potential off-target effects, silencing primers were designed in
152 two non-overlapping regions of the *Tilimpet* variants to obtain two double-strand RNA,
153 named ds*Tilimpet* A and ds*Tilimpet* B. After verifying that both fragments exerted a similar
154 effect both in the *limpet* silencing and in the expression of *defensin* genes at 48 h post
155 injection (see results), all the assays were done with ds*Tilimpet* A. Four sets of insects
156 (control and *limpet* dsRNA, with or without fungal blastospores) were inoculated with 1µl of
157 different solutions. All injections were performed with 10 µl Hamilton syringes as we
158 previously described (Dulbecco et al., 2018). Both control and *limpet* dsRNA to achieve
159 RNA interference were injected in a final concentration of 1µg µl^{-1}. The control dsRNA
160 consists in a fragment of *Xenopus* elongation factor 1α gene, which is provided by the kit
161 used. From now on, these controls will be referred as "healthy insects". Also, a dose 120
162 blastospores/nymph (Lobo et al., 2015) was co-injected mixed with either the control or
163 *limpet* dsRNA using the same final concentration of interference RNA as previously used.
164 For each of the four set mentioned, five biological replicates (with 5 insects each) were
165 assayed. After injection, samples consisting of one entire insect each were taken every 12 h
166 for a period of 48 h. An additional group of no injected naïve insects were also sampled at 48
167 h. Sampling time points were chosen based on Lobo et al. (2015) and previous infection
168 experiments (data not shown). Then, RNA extraction and cDNA preparation were done as
169 described in section 2.3.

170 The same bioassay, including the four sets of insects (control and *limpet* dsRNA, with or
171 without fungal blastospores, each consisting in five biological replicates) were repeated in
172 order to check the insect mortalities each 12 h. Cadavers were placed in individual humid
173 chambers at 26 ºC to confirm fungal infection as is described by Lobo et el. (2015). A colony

control without injection was also monitored for insect survival; no dead insects were detected in this group during the trial period.

2.6.2. Gene expression analysis

qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) to assess both expression and silencing of the *limpet* variants and to measure all defensins expression levels. The expression of both *limpet* and *defensin* genes were also assayed in no injected naïve insects. The cycling parameters were 95°C for 5 min followed by 40 cycles of 95°C for 10s, and 60°C for 45s ending with melting curve product amplification. Relative gene expression was analyzed by the multiple reference gene method (Hellemans et al., 2007). Elongation factor 1-alpha (*ef1-α*) and *RP ribosomal protein 18S* of *T. infestans* were used as the internal reference genes, as they has been used in other insects (Lourenço et al., 2008; Rong et al., 2013). To analyze the expression profiles, we applied the NRQ model, consisting of the conversion of quantification cycle values (Cq) into normalized relative quantities (NRQs), the adjustment for differences in PCR efficiency between the amplicons (Pfaffl, 2001), and the normalization of the data using multiple reference genes (Hellemans et al. 2007). We calculated the relative quantities and normalized the data following the formulas detailed in Hellemans et al. (2007). The comparative Ct (ΔΔCt) method was employed to calculate the relative expression ratios (RER). Three technical replicates were performed for each of the four independent biological replicates assayed. Standard curves were obtained to evaluate the PCR efficiency of each primer pair used. Oligonucleotide sequences, amplicon lengths, and PCR efficiencies are shown in Table S1. Statistical analysis was performed using ANOVA, Bonferroni's post test, and *t*-test when it corresponded. All graphs were constructed with Prism GraphPad 5 (GraphPad Sofware, USA).

198

199 ## 3. Results

200 ### 3.1. Sequence analysis and characterization

201 #### 3.1.1. *Limpet*

202 Two variants of the *limpet* transcription factor were identified by searching in
203 previously sequenced *T. infestans* transcriptomes (Calderón-Fernández et al., 2017; Schwarz
204 et al., 2014). Nucleotide alignments of each full-length *limpet* sequences showed two highly
205 homologous regions corresponding to PET and LIM domains, being the LIM region the most
206 conserved and the PET more variable (Fig. S1). The sequences were named as *Tilimpet-1* and
207 *Tilimpet-2* and annotated in GenBank. The former transcript (accession no. MH998010)
208 exhibited a series of LIM domains and a PET domain, showing high homology with a gene
209 (accession no. MH998013) of the related triatomine bug *R. prolixus*. *Tilimpet-2* (accession
210 no. MH998011) presented the characteristic set of LIM domains typically associated with
211 these proteins but lacked a PET domain; it also showed high homology to the *R. prolixus*
212 gene (MH998012). Comparisons of both gene structures described for *R. prolixus* (Fig. 1A)
213 and their respective transcript variants (Fig. 1B) are shown as a reference along with *T.*
214 *infestans* mRNA variants (Fig. 1C). When compared to *R. prolixus* sequence MH998011, a 5´
215 fragment was missing. The obtained and sequenced fragment was identical to that of *R.*
216 *prolixus.* For a further characterization, phylogenetic trees were constructed with model and
217 related insect species (Fig. 2A). It is interesting to note that the two variants observed for *T.*
218 *infestans* clustered in two different clades (75% cutoff was considered). *T. infestans*
219 sequences in both cases clustered together with *R. prolixus* as the closest species. Similarly,
220 other species that were analyzed, such as *Drosophila willistoni*, *Cimex lectularius* and

Halymorpha halys, showed *limpet* variants that group in each of the different major clusters (Fig. 2A).

3.1.2. *Defensins*

Six putative defensin sequences were identified by searching in previously sequenced *T. infestans* transcriptomes (Calderón-Fernández et al., 2017; Schwarz et al., 2014). Nucleotide alignments of each full-length sequence showed high homologous regions corresponding to defensins in other insects. These sequences were annotated in GenBank (accession no. MH998014, MH998009, MH341003, MH341004, MH341005, MH341006 for *Tidef-1* to *Tidef-6*, respectively), and compared to sequences belonging to representative species of the major insect orders (*R. prolixus, D. melanogaster, Apis mellifera, Spodoptera frugiperda* and *T. castaneum*). As shown in Fig. 2B, ten clades were clustered considering a 75% of similitude cutoff. *T. infestans* defensins were distributed in four sub-clusters, the first one containing *Tidef-1* and *Tidef-2* together with *R. prolixus* defensins. The second cluster is entirely composed by *T. infestans* defensins, *Tidef-4 and Tidef-5*. Finally, *Tidef-3* and *Tidef-6* appear as separated branches, being the most divergent sequences of the group. When analyzing defensins expression profiles, clustering and expression levels can be linked (see below).

3.2. Gene expression in healthy insects

3.2.1. *Limpet*

The natural variation of *Tilimpet-1* and *Tilimpet-2* expression in healthy insects (i.e., not injected with *B. bassiana* blastospores) were quite different in the time period assayed, *Tilimpet-2* displayed always higher expression levels than *Tilimpet-1*. The expression of *Tilimpet-2* increased significantly at 36 h after the beginning of the experiment (injection with control dsRNA), whereas for *Tilimpet-1* the expression remained at low levels showing

only a small increase at 48 h (Fig. 3A). The expression level at 48 h of both *limpet* genes in naïve insects (not injected) were the same than those found in healthy insects (injected with dsRNA but not with *B. bassiana* blastospores) (Fig. S2).

3.2.2. *Defensins*

The basal expression pattern of the six T. *infestans* defensins was also measured. *Tidef-1* was the highest expressed peaking at 12 h after ds-RNA injection and subsequently lowering to an expression level comparable to the other defensin genes (Fig. 3B). The rest of the genes exhibited similar expression levels trough time and among themselves, all remained under half the expression of *Tidef-1* peak. *Tidef-2* slightly lowered its expression at 24 h but then recovered the expression level at 36 and 48 h (Fig. 3B). *Tidef-3* showed the same expression pattern at every time point, being among the lowest expressed defensins (Fig. 3B). Finally, *Tidef-4*, *Tidef-5* and *Tidef-6* displayed small changes but always at very low expression levels. Both naïve and healthy insects showed similar expression level for the six *defensin* genes at 48 h (Fig. S2).

3.3. *Tilimpet* silencing, immune challenge and insect mortality

Mortality bioassays were conducted in *T. infestans* 4th instar nymphs in order to assess the effect of silencing both *limpet* variants (ds*Tilimpet*) on *B. bassiana* infection. Cumulative mortality is shown in Figure 4. In insect not subjected to immune challenge, ds*Tilimpet* displayed higher mortality rate than controls (injected with dsRNA) from 36 h to the end of the trial, reaching around 20% mortality increase compared with the control at 72 h. This result shows that insect viability is somewhat affected after *limpet* silencing. When analyzing the fungus-infected insects, ds*Tilimpet* exhibited significantly higher mortality rates than controls (Fig. 4) at almost all time points except at 72 h, when cumulative mortality

reached 100% in ds*Tilimpet* and around 80% in non silenced insects but infected with fungal blastospores.

3.4. Gene expression in fungus-infected insects

3.4.1. Time course expression of *T. infestans limpet* genes

The relative expression ratios (RER) for *Tilimpet-1* in *B. bassiana*-infected *T. infestans* were higher than in healthy insects at early time points ($F=23.15$; $dF=17$; $P < 0.0001$) (Fig. 5A); however, after 24 h the differences disappeared ($P > 0.05$). A different pattern was observed when analyzing *Tilimpet-2*, RER levels were always significantly higher in fungus-infected insect compared with healthy bugs after 12h ($F=37.83$; $dF=15$; $P < 0.0001$). A noteworthy peak of induction of *Tilimpet-2* was observed at 24-36 h ($0.01 < P < 0.001$), indicating that *Tilimpet-2* displayed the highest induction when the fungal pathogen was present (Fig. 5A).

3.4.2. Time course expression of *T. infestans defensin* genes

Four of all six analyzed defensins showed significant interaction between the time and treatment factors ($F=16.39$; $dF=17$; $P < 0.0001$); therefore, analyses and comparisons were carried out point by point. *Tidef-1* and *Tidef-2*, both grouped in the first cluster of the phylogenetic tree (Fig. 2B), had the highest induction ($0.0001 < P < 0.01$) (Fig. 5B). *Tidef-4* and *Tidef-5*, which clustered together as shown in Fig. 2B, did not show differences through time and expression ratios were around those shown by healthy insects. The same was observed for *Tidef-3*, except at 48 h ($P < 0.003$) when it shows a small induction. *Tidef-6* showed high expression ratios later in time, at 36 ($P < 0.0002$) and 48 h ($P < 0.0004$) (Fig. 5B), reaching the induction levels that *Tidef-1* displayed at the entire time period assayed.

3.5. Functional analysis of *T. infestans limpet* variants by RNAi

Sequence-specific *limpet* dsRNA (ds*Tilimpet* A) was synthesized *in vitro* and injected into the fourth instar nymphs of *T. infestans*, which were then sampled every 12 hours at least for two days and at 72 h when possible. Statistically significant differences in expression of both *Tilimpet-1* ($F=16.39$; $dF=14$; $P < 0.0001$) and *Tilimpet-2* ($F=4.77$; $dF=14$; $P < 0.0187$) were observed between silenced and control groups (Table 1), showing that the silencing construct worked well for both variants in healthy and infected insects, ranging from 78.2 to 99.8 % (P values ranged between $P < 0.00001$ and $P < 0.05$). A second silencing fragment (ds*Tilimpet* B) was used to assess potential off-target effects, the silencing efficiency at 48 h for *Tilimpet-1* and *Tilimpet-2* resulted in 78.0 ($P < 0.00001$) and 99.9% ($P < 0.00001$), respectively.

3.5.1. The effect of *Tilimpet* silencing on defensin expression

To assess the effect of *limpet* silencing on the expression of defensins, we measured the expression pattern of the six defensins genes on fungus-infected insects, normalized with healthy nymphs, for both controls and *limpet*-silenced insects through time. As shown in Figure 6 (A and B), the highest differences in RER corresponded to *Tidef-1* and *Tidef-2* from 12 to 48 h ($F=16.39$; $dF=17$; $P < 0.0001$ and $F=4.77$; $dF=16$; $P < 0.0187$, respectively). RERs for *Tidef-3*, *Tidef-4*, and *Tidef-5* showed lower to no difference at all ($F=1.770$; $dF=14$; $P < 0.210$; $F=3.11$; $dF=14$; $P < 0.0706$ and $F=2.81$; $dF=14$; $P < 0.0889$, respectively) (Fig. 6C-E). *Tidef-6* had lower RER differences at early time points but at 36 and 48 h, RER differences between healthy and fungus infected insects was similar to *Tidef-1* and *Tidef-2* ($F=50.49$; $dF=14$ and $P < 0.0001$) (Fig. 6F). Similar values were obtained for the six defensins 48 h after injection with ds*Tilimpet* B (Fig. S3).

4. Discussion

Limpet transcription factors typically display two characteristic domains: a single PET domain followed by a repetition of LIM domains (Zn finger motif). In most insect species two genes are linked to this function, one of them is longer and has 13 to 14 exons and several splice variants, and the other is much shorter, displaying only two exons and only one transcript variant (www.vectorbase.org; http://ibeetle-base.uni-goettingen.de; www.flybase.org). After we identified two variants of the *limpet* transcription factor in *T. infestans* and completed their sequences, the phylogenetic analysis clustered the variants into two different tree branches, grouping each variant in different clusters (Fig. 2A). In the analysis, species of the more abundant insect orders were considered, and a similar separation of *limpet* variants was observed. The cluster which grouped *Tilimpet-2* showed a higher level of homology than the second cluster, where *Tilimpet-1* grouped, that in turn could be divided into two subgroups under more stringent cut-off values. The restriction of a higher cut-off value would generate a new sub-cluster where *R. prolixus* and *T. infestans* are separated from the rest of the compared insects (Fig. 2A). These findings suggested that, to date, the two variants which were identified in many insect species were also present in *T. infestans* and the related kissing bug *R. prolixus*. The expression pattern of both *limpet* variants observed in ~~both~~ naïve, healthy and fungus-infected insects suggest that the main regulation was carried out by *Tilimpet-2*; whereas *Tilimpet-1* could be linked to either a more general response in healthy insects or only at early stages after the fungus enters the hemolymph. Thus, *Tilimpet-1* and *Tilimpet-2* may act concomitantly to aid each other in a fungal infection immune response. It is possible that some transcription factors have evolved to take part in different metabolic processes and to present multiple or divergent functions even having a similar nucleotide sequence (Chen and Rajewsky, 2007). Interestingly, *Tilimpet-2* is the shorter sequence, which did not include a PET domain but had two more LIM domains than *Tilimpet-1*. It would be possible that LIM domains play a fundamental role in this case as

gene expression regulation is listed among the variety of biological functions associated with this family of proteins (www.rcsb.org).

The number of defensin genes present in different species varies, although most of them typically present three different sequences. In some species, it was described that they act differentially depending on the injury suffered by the insect (Altincicek et al., 2008; Mingyue et al., 2016; Yokoi et al., 2012). The observation of the phylogenetic analysis performed on the six identified defensins in *T. infestans* showed that they cluster in four different branches, four of them among or closely to *R. prolixus* defensins and the other two completely separated. Even though conserved, it should be noted that *Tidef*-3 and *Tidef-6* seem both to be more divergent than in other species compared, since only *A. mellifera* had a similar clustering while in the rest of the considered insects, including examples from the major Insecta orders, the identified defensins clustered together in the same branch (Fig. 2B). This higher variability in *T. infestans* defensins could be linked to their function. The discussed results were in agreement with a series of different defensin sequence analysis in arthropods and even mammals and plants (Altincicek et al., 2008; Crovella et al., 2005; Gruber and Muttenthaler, 2012; Mingyue et al., 2016; Tonk et al., 2015a); therefore, this AMP~~s~~ family shows transphyletic conservation, keeping in mind that a certain degree of variability also exists. It is interesting to note that defensin general expression level correlated with the phylogenetic cluster where they were grouped, especially for *Tidef-1* and to a lesser extent for *Tidef-2*, which had the highest expression ratios in ~~both~~ naïve, healthy and fungus-infected insects. These two defensins acting throughout the time interval considered, together with *Tidef-6* gaining importance in the later time period post infection, could be the main responsible for the antifungal immune response. A slight induction of *Tidef-3* at 48 h was also remarkable and could indicate a small contribution in the infection fighting process. The rest of the evaluated defensins -which also clustered together- did not seem to be affected by the

presence of *B. bassiana´s* blastospores. The relatively small induction observed might be due to the fact that all measurements were made from whole insects, the induction values would have been probably higher (as commonly found for immune related genes after microbial challenge) in isolated tissues. Also, it is known that different pathogens elicit different signalization pathways and have differential responses, the fact that only a group of defensins show differences of expression can be related to the fact that fungal pathogens activate a specific group of defensins and the rest may respond to other pathogens or immune challenges. An overlapped observation of both *limpet* and *defensins* RER patterns indicates that *Tilimpet-2* might regulate the induction of *Tidef-1* and *Tidef-2* at early stages of infection and also *Tidef-6* later in time, since it peaks after *Tilimpet-2* peaked. In this case, the result is consistent what was expected for effectors expression which is lagged to transcription factors action. *Tilimpet-1* could be of aid to *Tilimpet-2* especially at early time points where its expression is induced.

After the attempt of silencing both *Tilimpet* variants with only one primer pair, we achieved silencing levels that were in every case in the range of 78-99% when assayed from 12 to 48 hours post injection. The designed primer system in the most conserved region of the transcripts for silencing both variants accomplished the goal. Similar results were obtained after injection of a different non-overlapping dsRNA fragment, thus discarding the possibility of an unwanted off-target effect. Then, we tested the effect of *limpet* silencing on fungal infection as well as on regulation of the immune response of the six defensins previously mentioned. We found that *limpet* silencing had an impact on fungus-free insect survival, which agree with existing data reporting these transcription factors as part of innate immune response in other insects (Altincicek et al., 2008; Jin et al., 2008), and also suggest that they have a direct role in protecting *T. infestans* from opportunistic pathogens. After *B. bassiana* infection, ds*Tilimpet* exhibited significantly higher mortality, meaning that the absence of the

limpet variants made the insects more susceptible to the fungal blastospores, and allowed *B. bassiana* to kill them faster than to the controls.

The defensin expression pattern was reduced by the effect of *Tilimpet* silencing (Fig. 6). Both *Tidef-1* and *Tidef-2* displayed the higher differences between ds*Tilimpet* and control samples, and later in time *Tidef-6* showed the same behavior. For the three remaining defensins, RER values were close between both samples, which might indicate that this group is not directly involved in the defense against fungal infection. In *limpet*-silenced insects, all defensins exhibited RERs < 1 (Fig. 6), perhaps due to the (low) expression levels observed; which might prevent obtaining accurate values after normalization with *limpet*-silenced insects, since both groups are not expected to significantly express defensins. The lower values on defensin expression found in fungus- infected ds*Tilimpet* compared with those observed in healthy *ds*Tilimpet might be also related to a metabolic cost inherent to the fungal exposure: the immune system of *T. infestans* is not capable of fighting the infection when lacking *Tilimpet* transcription factor, while the fungus is activating other immune pathways. The participation of more than one regulation factor is very likely to happen especially when the faith of the immune challenge outcome is compromised. Tight regulation of immunity involving more than one factor would imply that the defense mechanism system evolved to not be overcome easily. This might also explain the existence of variants of the *limpet* factor, as well as the many factors that play a role in immunity whose function remain unknown (Altincicek et al., 2008; Jin et al., 2008). The peak of *Tilimpet* expression is in agreement with the orchestrated functioning of different regulation factors that act earlier or later in the infection timeline, being *Tilimpet* an early involved factor. Further research would lead to the identification of the later acting factors in this immune network.

These results agreed with the described functional differences that defensins present in different organisms, suggesting that the fungal infection triggers the expression of three

defensins in *T. infestans* over the rest. In a previous study in *T. infestans* carried out by Lobo et al. (2015), the action of AMPs was analyzed in a general approach during *B. bassiana* infection correlating the course of fungal infective genes and insect immunity genes at different stages of the infective process. The particular defensin analyzed had a high induction after 24-48 h; this defensin is the same as *Tidef-1* analyzed in this work and the obtained results were consistent. *Tidef-1* not only was one of the most inducted genes but this induction was also sustained throughout the infection process. In summary, *Tilimpet* regulates the expression of the defensins at all stages of infection, although not only defensins are regulated by *Tilimpet*. Their expression levels are related to the cluster they belong to and they have different roles related to the type of immune challenge the insects were subjected to.

Conclusion

In this work we identified and characterized two variants of *limpet* transcription factor and linked their function with the humoral innate immune response in *T. infestans*. *Tilimpet* variants may act differentially, since they have divergent sequences and different expression patterns, suggesting that *Tilimpet-2* could be the main regulator and *Tilimpet-1* might play a complementary or more general role in defensins regulation. The six analyzed defensins exhibited different behavior and expression levels consistent with their sequence clustering; suggesting that two clusters were responsible for most of the defensive response. The fact that some defensins are either tissue-specific expressed or induced only by the presence of Gram-positive bacteria (Ursic-Bedoya and Lowenberger, 2007) might be the reason to explain the low expression or no induction observed for some of defensin genes in the whole body of fungus-infected *T. infestans*. Further research in the many unidentified sequences which are involved in humoral immunity response is necessary to disentangle the pathways

involving the two versions of *Tilimpet* and affecting the regulation of defensins expression patterns after the insects' immune system had been challenged by fungal infections.

Acknowledgments

The authors would like to give special thanks to Cecilia Fusé and Facundo Bozzolo for insect rearing and to Rosana Del Cid for language revision. This research was supported by grants from the National Agency for Scientific and Technological Promotion (PICT 2015-2763) and the National Scientific and Technical Research Council (CONICET) (PUE 2016). NP is a member of the CONICET Researcher's Career, Argentina.

References

Altincicek, B., Knorr, E., Vilcinskas, A., 2008. Beetle immunity: Identification of immune-inducible genes from the model insect *Tribolium castaneum*. Dev. Comp. Immunol. 32, 585–595.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic Local Alignment Search Tool. J. Mol. Biol. 215, 403–410.

Araújo, C.A.C., Lima, A.C., Jansen, A.M., Galvão, C., Jurberg, J., Costa, J., Azambuja, P., Waniek, P.J., 2015. Genes encoding defensins of important Chagas disease vectors used for phylogenetic studies. Parasitol. Res. 114, 4503–4511.

Bulet, P., Hetru, C., Dimarcq, J., Hoffman, D., 1999. Antimicrobial peptides in insects; structure and function. Dev. Comp. Immunol. 23, 329–344.

Calderón-Fernández, G.M., Moriconi, D.E., Dulbecco, A.B., Juárez, M.P., 2017.

461 Transcriptome Analysis of the *Triatoma infestans* (Hemiptera: Reduviidae) Integument.
462 J. Med. Entomol. 54, 1531–1542.

463 Cerenius, L., Lee, B.L., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in
464 invertebrate immunity. Trends Immunol. 29, 263–271.

465 Chen, K., Rajewsky, N., 2007. The evolution of gene regulation by transcription factors and
466 microRNAs. Nature, 93-103.

467 Cooper, D., Eleftherianos, I., 2017. Memory and specificity in the insect immune system:
468 Current perspectives and future challenges. Front. Immunol. 8, 539.

469 Crovella, S., Antcheva, N., Zelezetsky, I., Boniotto, M., Pacor, S., Verga Falzacappa, M.V.,
470 Tossi, A., 2005. Primate beta-defensins--structure, function and evolution. Curr. Protein
471 Pept. Sci. 6, 7–21.

472 Dias, J., Silveira, A., Schofield, C., 2002. The impact of Chagas disease control in Latin
473 America—A review. MemInst Oswaldo Cruz 97, 603–612.

474 Díaz-Garrido, P., Sepúlveda-Robles O., Martínez-Martínez , I., Espinoza, B., 2018.
475 Variability of defensin genes from a Mexican endemic Triatominae: *Triatoma (Meccus)*
476 *pallidipennis* (Hemiptera: Reduviidae). Biosci. Rep. 28, 38(5).

477 Dulbecco, A.B., Moriconi, D.E., Calderón-Fernández, G.M., Lynn, S., McCarthy, A., Roca-
478 Acevedo, G., Salamanca-Moreno, J.A., Juárez, M.P., Pedrini, N., 2018. Integument CYP
479 genes of the largest genome-wide cytochrome P450 expansions in triatomines
480 participate in detoxification in deltamethrin-resistant *Triatoma infestans*. Sci. Rep. 8,
481 10177.

482 Forlani, L., Pedrini, N., Girotti, J.R., Mijailovsky, S.J., Cardozo, R.M., Gentile, A.G.,
483 Hernández-Suárez, C.M., Rabinovich, J.E., Juárez, M.P., 2015. 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. 9(5), e0003778.

Forlani, L., Pedrini, N., Juárez, M.P., Juárez, P., 2011. Contribution of the horizontal transmission of the entomopathogenic fungus *Beauveria bassiana* to the overall performance of a fungal powder formulation against *Triatoma infestans*. Res. Rep. Trop. Med. 2, 135–140.

Gruber, C.W., Muttenthaler, M., 2012. Discovery of defense- and neuropeptides in social ants by genome-mining. PLoS ONE 7(3), e32559.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.

Hultmark, D., 2003. Drosophila immunity: Paths and patterns. Curr. Opin. Immunol. 15, 12–19.

Jin, L.H., Shim, J., Yoon, J.S., Kim, B., Kim, J., Kim-ha, J., 2008. Identification and functional analysis of antifungal immune response genes in *Drosophila*. PLoS Pathog. 4(10), e1000168.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Lavine, M., Strand, M., 2002. Insect hemocytes and their role in immunity. Insect Immunol. 32, 25–47.

Lee, B.Y., Bacon, K.M., Bottazzi, M.E., Hotez, P.J., 2013. Global economic burden of Chagas disease: a computational simulation model. Lancet Infect. Dis. 13, 342–348.

507 Lemaitre, B., Reichhart, J.-M., Hoffmann, J.A., 1997. *Drosophila* host defense: Differential
508 induction of antimicrobial peptide genes after infection by various classes of
509 microorganisms. Proc. Natl. Acad. Sci. 94, 14614–14619.

510 Leulier, F., Rodriguez, A., Khush, R.S., Abrams, J.M., Lemaitre, B., 2000. The *Drosophila*
511 caspase Dredd is required to resist Gram-negative bacterial infection. EMBO Rep. 1,
512 353–358.

513 Lobo, L.S., Luz, C., Fernandes, É.K.K.K., Juárez, M.P., Pedrini, N., 2015. Assessing gene
514 expression during pathogenesis: Use of qRT-PCR to follow toxin production in the
515 entomopathogenic fungus *Beauveria bassiana* during infection and immune response of
516 the insect host *Triatoma infestans*. J. Invertebr. Pathol. 128, 14–21.

517 Lopez, L., Morales, G., Ursic, R., Wolff, M., Lowenberger, C. (2003) Isolation and
518 characterization of a novel insect defensin from *Rhodnius prolixus*, a vector of Chagas
519 disease. Insect Biochem. Mol. Biol. 33, 439–447.

520 Lourenço, A., Mackert, A., Cristino, A., Simões, Z., 2008. Validation of reference genes for
521 gene expression studies in the honey bee , Apis mellifera , by quantitative real-time RT-
522 PCR. Apidologie 39, 372–385.

523 Mannino, M.C., Juárez, M.P., Pedrini, N., 2018. Tracing the coevolution between T*riatoma*
524 *infestans* and its fungal pathogen *Beauveria bassiana*. Infect. Genet. Evol. 66, 319–324.

525 Mesquita, R.D. et al., 2015. Genome of *Rhodnius prolixus*, an insect vector of Chagas
526 disease, reveals unique adaptations to hematophagy and parasite infection. Proc. Natl.
527 Acad. Sci. 112, 14936–14941.

528 Mingyue, L., Mohamed, A.A., Zhang, L., Zhang, P., Zhang, L., 2016. A family of CS αβ
529 defensins and defensin-like peptides from the migratory locust, *Locusta migratoria*, and

their expression dynamics during mycosis and nosemosis. PLoS ONE 11(8): e0161585.

Nappi, A.J., Ottaviani, E., 2000. Cytotoxicity and cytotoxic molecules in invertebrates. BioEssays 22, 469–480.

Paim, R.M.M., Nascimento, B.W.L., Nascimento, A.M.D., Pacheco, D.E., Soares, A.C., Araujo, R.N., Sant'Anna, M.R.V., Pessoa, G.C.D., Gontijo, N.F., Pereira, M.H., 2017. Functional aspects of salivary nitric oxide synthase of *Rhodnius prolixus* (Hemiptera, Reduviidae) and nitric oxide trafficking at the vector-host interface. Sci. Rep. 22, 7(1):16036.

Pal, S., Wu, L.P., 2009. Lessons from the fly: pattern recognition in *Drosophila melanogaster*. In: Kishore, U. (Ed.), Target pattern recognition in innate immunity. Springer, New York, pp. 162–174.

Pedrini, N., 2018. Molecular interactions between entomopathogenic fungi (Hypocreales) and their insect host: Perspectives from stressful cuticle and hemolymph battlefields and the potential of dual RNA sequencing for future studies. Fungal Biol. 122(6), 538-545.

Pedrini, N., Mijailovsky, S.S.J., Girotti, J.J.R., Stariolo, R.R., Cardozo, A., Juárez, M.P., Cardozo, R.M., Gentile, A., Juárez, M.P., 2009. Control of pyrethroid-resistant Chagas disease vectors with entomopathogenic fungi. PLoS Negl. Trop. Dis. 3, e434.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT – PCR. Nucleic Acids Res. 29(9):e45.

Rajamuthiah, R., Jayamani, E., Conery, A.L., 2015. A defensin from the model beetle *Tribolium castaneum* acts synergistically with Telavancin and Daptomycin against multidrug resistant *Staphylococcus aureus*. PLoS ONE 10(6): e0128576.

Rong, S., Li, D.-Q., Zhang, X.-Y., Li, S., Zhu, K.Y., Guo, Y.-P., Ma, E.-B., Zhang, J.-Z.,

2013. RNA interference to reveal roles of β-N -acetylglucosaminidase gene during molting process in *Locusta migratoria*. Insect Sci. 20, 109–119.

Rutschmann, S., Jung, A.C., Hetru, C., Reichhart, J.M., Hoffmann, J.A., Ferrandon, D., 2000. The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*. Immunity 12, 569–580.

Rutschmann, S., Kilinc, A., Ferrandon, D., 2002. Cutting Edge: The Toll pathway is required for resistance to Gram-positive bacterial infections in *Drosophila*. J. Immunol. 168, 1542–1546.

Schwarz, A., Medrano-Mercado, N., Schaub, G.A., Struchiner, C.J., Bargues, M.D., Levy, M.Z., Ribeiro, J.M.C., 2014. An updated insight into the sialotranscriptome of *Triatoma infestans*: developmental stage and geographic variations. PLoS Negl. Trop. Dis. 8(12): e3372.

Seufi, A.E.M., Hafez, E.E., Galal, F.H., 2011. Identification, phylogenetic analysis and expression profile of an anionic insect defensin gene, with antibacterial activity, from bacterial-challenged cotton leafworm, *Spodoptera littoralis*. BMC Mol. Biol. 12, 47.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. 101, 11030–11035.

Tonk, M., Cabezas-Cruz, A., Valdés, J., Rego, R., Grubhoffer, L., A, E.-P., Vilcinskas, A., Kotsyfakis, M., Rahnamaeian, M., 2015a. *Ixodes ricinus* defensins attack distantly-related pathogens. Dev. Comp. Immunol. 53, 358–365.

Tonk, M., Knorr, E., Cabezas-cruz, A., Valdés, J., Kollewe, C., Vilcinskas, A., 2015b.

576 *Tribolium castaneum* defensins are primarily active against Gram-positive bacteria. J.
577 Invertebr. Pathol. 132, 208–215.

578 Ursic-Bedoya, R. J., Lowenberger, C. A., 2007. *Rhodnius prolixus*: Identification of
579 immune-related genes up-regulated in response to pathogens and parasites using
580 suppressive subtractive hybridization. Dev. Comp. Immunol. 31, 109–120.

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

585 WHO, 2012. Research priorities for Chagas disease, human African trypanosomiasis and
586 leishmaniasis. Geneva, Switzerland.

587 WHO, 2000. Control of Chagas disease, second report of the WHO expert committee.
588 Brasilia, Brazil.

589 Yokoi, K., Koyama, H., Minakuchi, C., Tanaka, T., Miura, K., 2012. Results in immunology
590 antimicrobial peptide gene induction , involvement of Toll and IMD pathways and
591 defense against bacteria in the red flour beetle, *Tribolium castaneum*. Results Immunol.
592 2, 72–82.

593

594 **Figure legends**

595 **Figure 1. The structure of *limpet* genes from triatomines.** Genes (A) and transcripts (B) of
596 *Rhodnius prolixus*, and transcript variants of *Triatoma infestans* (C). Bars indicate 100bp
597 length distance.

598 **Figure 2. Phylogenetic analyses of *limpet* (A) and *defensin* (B) sequences.** The
599 evolutionary history was inferred using the UPGMA method. The optimal tree with the sum
600 of branch length = 3.45570653 is shown. The tree is drawn to scale, with branch lengths in
601 the same units as those of the evolutionary distances used to infer the phylogenetic tree. All
602 positions containing gaps and missing data were eliminated (Tamura et al., 2004). A 75%
603 similarity cutoff was used to define clusters. Ti: *Triatoma infestans*, Rp: *Rhodnius prolixus*,
604 Cl: *Cimex lectularius*, Hh: *Halymorpha halys*, Am: *Apis mellifera*, Aa: *Aedes aegypti*, Dw:
605 *Drosophila willistoni*, Dm: *Drosophila melanogaster*, Nl: *Nasonia longicornis*, Ag:
606 *Anopheles gambiae*, At: *Arabidopsis thaliana*, Sf: *Spodoptera frugiperda*, Tc: *Tribolium*
607 *castaneum*. Sequences from *T. infestans* and *R. prolixus* are boxed in red.

608 **Figure 3. Basal expression of *limpet* (A) and *defensin* (B) genes in non-infected *Triatoma***
609 ***infestans*.** One-way ANOVA followed by Bonferroni post-test was performed for each gene.
610 Four independent biological replicates assayed. Different letters indicate significant
611 differences for a single gene through time. Asterisks indicate significant differences in gene
612 expression at each time point. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

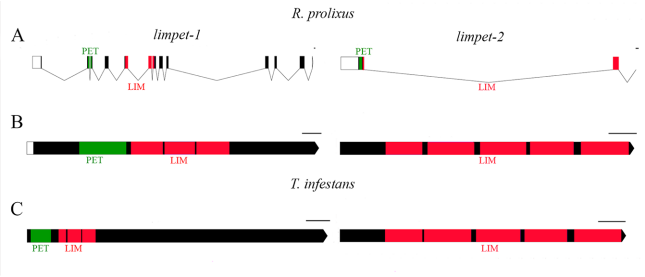
613 **Figure 4. Mortality bioassays of *Beauveria bassiana* (*Bb*)-infected *Triatoma infestans* on**
614 **either control or *limpet* dsRNA- injected nymphs (ds*Tilimpet*).** Data represent mean
615 cumulative mortality percentage ± SD from five biological replicates. Asterisks indicate
616 significant differences ($P < 0.05$).

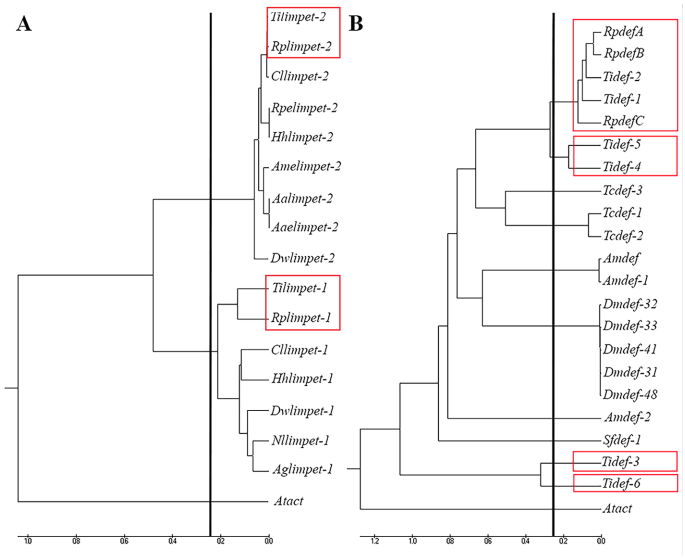
617 **Figure 5. Expression pattern of *limpet* (A) and *defensin* (B) genes in *Beauveria bassiana*-**
618 **infected *Triatoma infestans*.** Relative expression ratio (RER) is shown at different time
619 periods after 4th-instar nymphs' injection with blastospores, normalized to expression in
620 healthy insects. Four independent biological replicates were assayed. Statistically different
621 values are marked with different letters.

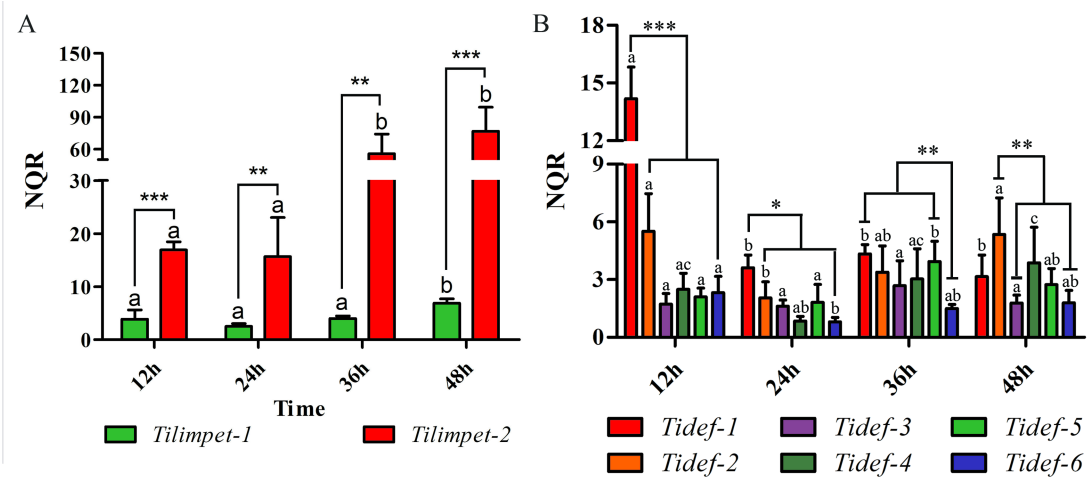
622 **Figure 6. Effect of *Tilimpet* silencing on defensins expression.** Relative expression ratio
623 (RER) of *T. infestans* defensin genes (*Tidef-1* to *Tidef-6*) is shown at different time periods in
624 *Beauveria bassiana*-infected insects, normalized to expression in healthy insects, in both
625 *limpet*-silenced and control *Triatoma infestans*. Four independent biological replicates were
626 assayed.

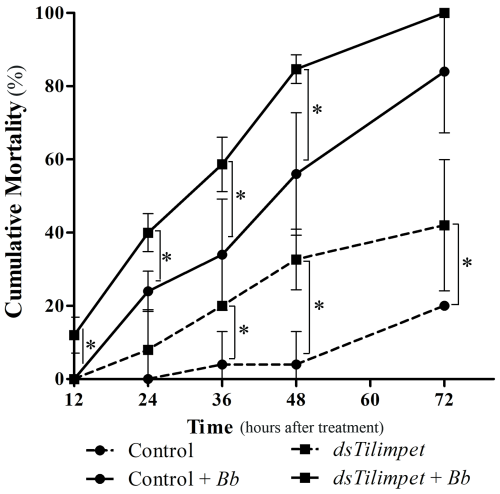
Table 1. The silencing efficiency of ds*Tilimpet* (RNAi). Relative expression ratios (RER) of *Tilimpet-1* and *Tilimpet-2* genes at different time periods in 4th-instar *T. infestans* nymphs injected with ds*Tilimpet*, normalized with nymphs injected with control double-stranded RNA. Values are means ± standard deviation, *P* value is shown in brackets.

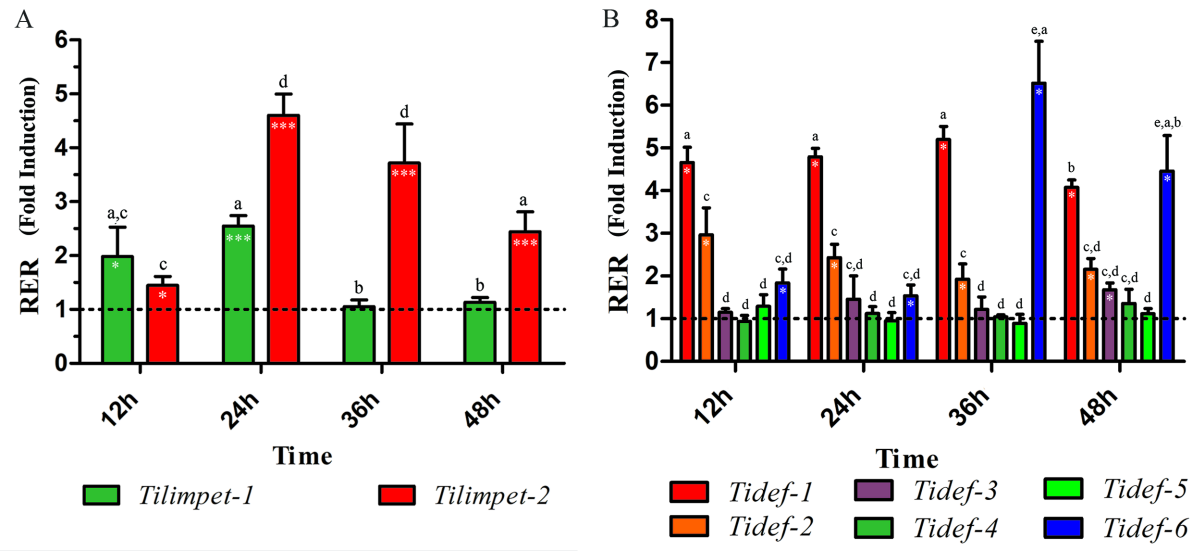
Time	*Tilimpet-1*	*Tilimpet-2*
12 h	0.06 ± 0.02 (5.3E-11)	0.009 ± 0.007 (3.3E-09)
24 h	0.04 ± 0.03 (7.8E-06)	0.005 ± 0.007 (4.7E-03)
36 h	0.04 ± 0.03 (3.9E-07)	0.011 ± 0.06 (1.3E-05)
48 h	0.02 ± 0.04 (3.6E-08)	0.01 ± 0.02 (2.38E-12)

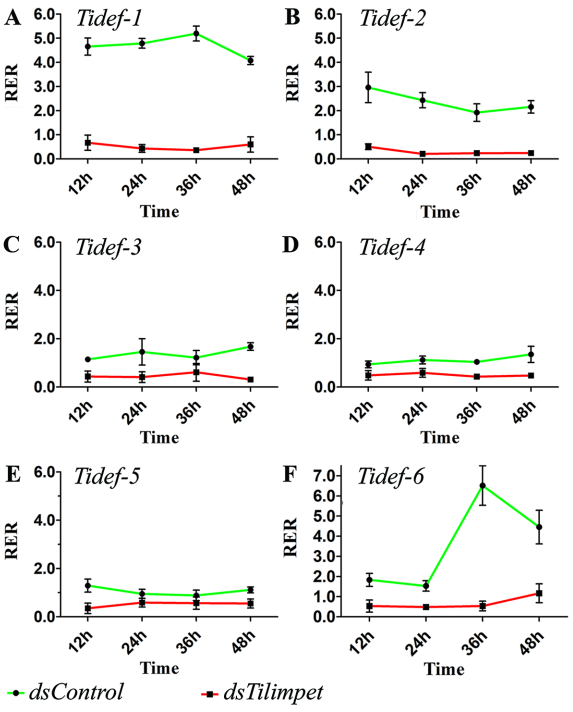












- Two divergent *limpet* transcription factors (*Tilimpet*) were found in *T. infestans*
- Both variants were linked to *T. infestans* humoral immune response
- *Tilimpet-2* could be the main regulator in fungal infections
- Defensins (*Tidef)* expression pattern was linked to their phylogenetic clustering
- Both *Tidef-1* and *Tidef-2* were the more affected defensins by *limpet* silencing