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Jaburetox affects gene expression and enzyme activities in *Rhodnius prolixus*, a Chagas' disease vector

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ABSTRACT

Jaburetox, a recombinant peptide of ~11 kDa derived from one of the *Canavalia ensiformis* (Jack Bean) urease isoforms, is toxic and lethal to insects belonging to different orders when administered orally or via injection. Previous findings indicated that Jaburetox acts on insects in a complex fashion, inhibiting diuresis and the transmembrane potential of Malpighian tubules, interfering with muscle contractility and affecting the immune system. *In vitro*, Jaburetox forms ionic channels and alters permeability of artificial lipid membranes. Moreover, recent data suggested that the central nervous system (CNS) is a target organ for ureases and Jaburetox. In this work, we employed biochemical, molecular and cellular approaches to explore the mode of action of Jaburetox using *Rhodnius prolixus*, one of the main Chagas' disease vectors, as experimental model. *In vitro* incubations with fluorescently labeled Jaburetox indicated a high affinity of the peptide for the CNS but not for salivary glands (SG). The *in vitro* treatment of CNS or SG homogenates with Jaburetox partially inhibited the activity of nitric oxide synthase (NOS), thus disrupting nitrinergic signaling. This inhibitory effect was also observed *in vivo* (by feeding) for CNS but not for SG, implying differential modulation of NOS in these organs. The inhibition of NOS activity did not correlate to a decrease in expression of its mRNA, as assessed by qPCR. UDP-*N*-acetylglucosamine pyrophosphorylase (UAP), a key enzyme in chitin synthesis and glycosylation pathways and a known target of Jaburetox in insect CNS, was also affected in SG, with activation of the enzyme seen after both *in vivo* or *in vitro* treatments with the peptide. Unexpectedly, incubation of Jaburetox with a recombinant *R. prolixus* UAP had no effect on its activity, implying that the enzyme's modulation by the peptide requires the participation of other factor(s) present in CNS or SG homogenates. Feeding Jaburetox to *R. prolixus* decreased the mRNA levels of UAP and chitin synthase, indicating a complex regulation exerted by the peptide on these enzymes. No changes were observed upon Jaburetox treatment *in vivo* and *in vitro* on the activity of the enzyme acid phosphatase, a possible link between UAP and NOS. Here we have demonstrated for the first time that the Jaburetox induces changes in gene expression and that SG are another target for the toxic action of the peptide. Taken together, these findings contribute to a better understanding of the mechanism of action of Jaburetox as well as to the knowledge on basic aspects of the biochemistry and neurophysiology of insects, and might help in the development of optimized strategies for insect control.

Abbreviations: Jbtx, Jaburetox; CNS, central nervous system; NOS, nitric oxide synthase; SG, salivary glands; NO, nitric oxide; UAP, UDP-*N*-acetylglucosamine pyrophosphorylase; UDP-GlcNAc, UDP-*N*-acetylglucosamine; GPI, glycophosphatidylinositol; rUAP, recombinant UAP; AP, acid phosphatase; JBU, Jack Bean Urease; GIPLs, glycoinositolphospholipids.

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1. Introduction

Ureases are nickel-dependent enzymes that catalyze the hydrolysis of urea into carbon dioxide and ammonia. Over the years, several other roles were characterized for these proteins, revealing their moonlighting properties (Carlini and Ligabue-Braun, 2016). Ureases present fungi- and entomotoxic effects independent of their enzymatic function (Follmer et al., 2004; Becker-Ritt et al., 2007; Carlini and Polacco, 2008). Insecticidal activity of *Canavalia ensiformis* ureases resides, in part, in a peptide released upon enzymatic hydrolysis by cathepsin-like peptidases present in the digestive system of some insects groups such as Hemiptera and Coleoptera (Stanisçuaski and Carlini, 2012). Based on those findings, a urease-derived recombinant peptide was generated by heterologous expression in *Escherichia coli* and named Jaburetox (Jbtx) (Mulinari et al., 2007; Postal et al., 2012). Jaburetox turned out to be toxic for insects of different orders, including those not affected by urease, but it was atoxic for mammals such as rats and mice (Mulinari et al., 2007), thus presenting biotechnological potential as an insect control tool (Stanisçuaski and Carlini, 2012; Carlini and Ligabue-Braun, 2016).

Several lines of evidence point out that Jbtx can interfere and act upon the nervous and neurolocomotor systems. Martinelli et al., 2014, reported in the cockroach *Nauphoeta cinerea* that the Jbtx induced a diminution of resting tension of the coxal muscle and reduced the strength amplitude of the muscular contraction, probably due to a direct interaction on N-metil-D-aspartate (NMDAR) and/or gamma-aminobutyric acid (GABA) receptors. Recent findings showed that the central nervous system (CNS) of *Triatoma infestans*, a hematophagous triatomine vector of Chagas' disease, is a target of the Jbtx (Galvani et al., 2015). Insects injected with the peptide showed signs of neurotoxicity such as paralysis of the legs, proboscis extension and abnormal movements of the antennae. The presence of Jbtx was demonstrated in CNS homogenates by means of western blot and the peptide was detected in the antennal lobe and subesophageal ganglion by immunohistochemistry. The specific location of the injected Jbtx in the nervous tissue as well as the induced behavioral effects led the authors to investigate the role of the nitric oxide synthase (NOS), an enzyme involved in nitrinergic signaling. As determined by spectrophotometric measurements, the toxic peptide altered NOS activity and nitric oxide levels *in vivo* and *in vitro* (Galvani et al., 2015).

NOS represents a family of enzymes that form nitric oxide from L-arginine (Davies, 2000). They present different isoforms with various locations, regulations and catalytic properties. In vertebrates, three isoforms are found: nNOS (also known as Type I, NOS-I and NOS 1), distributed mainly in neuronal tissue; iNOS (Type II, NOS-II and NOS-2) which is inducible in a wide range of cells and tissues, and finally eNOS (Type III, NOS-III and NOS-3), described for the first time in endothelial cells (Alderton et al., 2001). NOS activity is well known in insects and it was reported for the first time in the salivary glands (SG) of *R. prolixus* (Ribeiro and Nussenzveig, 1993). Basic studies of comparative physiology and biochemistry in simple organisms have provided strong evidence that nitric oxide (NO) signaling is functional and evolutionary conserved (Davies, 2000). Hematophagous insects use anticoagulants in the saliva during the feeding process, which inhibit platelet aggregation and vasoconstriction in the host. *R. prolixus*' nitrophorin is a protein that acts as a salivary vasodilator, containing a heme group that stores and delivers NO (Nussenzveig et al., 1995). NO is also involved in invertebrate immune response due to its capacity to induce oxidation of heme groups and nitrosylation of amino-acid residues, causing changes in the structure of proteins in pathogens, thus compromising parasite viability (Radi, 2004).

Immunoproteomic approaches applied to *T. infestans* have identified UDP-*N*-acetylglucosamine pyrophosphorylase (UAP) as a Jbtx binding protein in the CNS of treated insects (Galvani et al., 2015). UAPs employ the pyrophosphate high energy bonds of UTP to produce the sugar nucleotide UDP-*N*-acetylglucosamine (UDP-GlcNAc), essential for chitin synthesis, protein glycosylation, production of glycophosphatidylinositol (GPI) anchors and other secondary metabolites (Kramer and Muthukrishnan, 2005; Merzendorfer, 2006; Moussian, 2008). Chitin synthases are large proteins of the plasma membrane that catalyze the polymerization of *N*-acetylglucosamine into chitin from intracellular pools of UDP-GlcNAc (Coutinho and Henrissat, 1999). Chitin in insects has been identified as a component of the cuticle, SG, trachea, tracheiolas and peritrophic matrix (Merzendorfer, 2006; Merzendorfer, 2011) and has been recently identified in the midgut and perimicrovillar membrane of *R. prolixus* (Alvarenga et al., 2016).

Since the mechanism of action of Jbtx is not completely understood, we employed the model organism *R. prolixus* to explore the effects of the toxic peptide on several parameters of the insect physiology, focusing on enzymatic pathways of the CNS and SG. Our findings expanded the previous knowledge on the mode of action of this toxin and shed light on new aspects related to the physiology of triatomines.

2. Materials and methods

2.1. Chemicals

PureLink® RNA Mini Kit, High Fidelity Polymerase Phusion, Buffer Tango 10X (Thermo Fisher Scientific, Waltham, MA, USA); pET-15b plasmid (Novagen Merck Millipore, Billerica, MA, USA); T4 DNA Ligase (Ludwig Biotecnologia Ltda., Brazil); EZ-10 Spin Column PCR Purification and EZ-10 Spin Column DNA Gel Extraction kit (BioBasic, Markham, Canada); High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA); Chelating-Sepharose Fast Flow (GE Healthcare Life Sciences, Little Chalfont, UK); Pure Link Quick Plasmid Miniprep (Invitrogen, Carlsbad, CA, USA) and GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) were purchased from the indicated commercial sources. Fluorescein isothiocyanate (FITC), protease inhibitor cocktail (cat. number P8340) and all the remaining reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Jaburetox production and FITC labelling

The recombinant peptide used in this study was expressed and purified as described by Lopes et al. (2015). After the purification and before its use for incubations or feeding experiments, Jbtx was diluted in 20 mM sodium phosphate buffer (PB, pH 7.4) or *Rhodnius* saline (150 mM NaCl; 8.6 mM KCl; 2 mM $CaCl_2$; 8.5 mM $MgCl_2$; 4 mM $NaHCO_3$; 34 mM glucose; 5 mM HEPES, pH 7) (Lane et al., 1975). FITC labelling of Jbtx was performed in dimethyl sulfoxide (DMSO) as described elsewhere (Hermanson, 1996). The same procedure was done with bovine serum albumin (BSA) as a non-specific interaction control.

2.3. Insects

The experiments were conducted with unfed fifth stage nymphs of *R. prolixus* (3 weeks after ecdysis). The insects were kindly supplied by Dr. José Rodrigues Coura (Institute Oswaldo Cruz, RJ, Brazil) and Dra. Alessandra Aparecida Guarneri (Institute Oswaldo Cruz, MG, Brazil) and housed under controlled conditions of light (L:D = 12:12, lights on at 7.00 a.m.), temperature (27 ± 1 °C) and relative humidity (60%). The colony was fed at regular intervals on human blood poured over Parafilm-covered acrylic plates main-

tained at 37 °C on a warming table as previously described (Fruttero et al., 2016).

2.4. Dissection and tissue collection

The CNS (composed by brain and ganglia) and SG were dissected from treated (saline alone or saline plus Jbtx) or non-treated insects under cold PB plus protease inhibitors (10.4 mM AEBSF, 8 μM aprotinin, 0.4 mM bestatin, 0.14 mM E-64, 0.2 mM leupeptin and 0.15 mM Pepstatin A) and kept at −80 °C until use.

2.5. In vitro *incubation with Jbtx-FITC*

Jbtx-FITC and BSA-FITC (23 μM each) in *Rhodnius* saline were incubated for 1 h at room temperature with freshly dissected SNC or SG from non-treated fifth instar nymphs, followed by three washes with saline of 30 min each. Autofluorescence controls omitting the incubation step with the fluorescent proteins were also carried out (data not shown). The organs were placed in Lab-Tek Chamber Slides (Thermo Fisher Scientific, Waltham, MA, USA) and analysed under a Zeiss Axiovert 200 inverted fluorescence microscope equipped with an Axiocam MRc camera (Carl Zeiss, Jena, Germany) and the images were acquired using AxioVision Rel 4.8 Software.

2.6. Feeding experiments

The insects weighting an average of 32 ± 7 mg, were artificially fed on Parafilm-covered acrylic plates, kept at 37 °C for 30 min on a warming table containing *Rhodnius* saline supplemented with 1 mM ATP (control) or saline plus 1 mM ATP and Jbtx. To estimate the dose incorporated, preliminary experiments were conducted weighting the insects before and after feeding. The average volume ingested was 238 ± 49 μL and the concentration of the feeding solution was adjusted for the insects to receive a dose of ∼0.1 μg Jbtx/mg of body weight. Only the insects that fed a volume of solution within the desired range were selected for the final experiments. SG and SNC were dissected 18 h afterwards.

2.7. Determination of NOS activity

Dissected organs from treated insects (pools of 10 organs for each treatment) were homogenized in 20 mM Tris–HCl, pH 7.4, containing 0.32 M sucrose, 2 mM Na_2EDTA, 2 mM dithiothreitol (DTT) and protease inhibitors. The homogenates were centrifuged at 10,000 xg for 10 min at 4 °C and the protein concentration was measured in the supernatant according to Bradford (1976). NOS activity was determined as described in Galvani et al. (2015) with a reaction mix containing 50 mM PB pH 7.0, 1 mM $CaCl_2$, 1 mM L-arginine, 100 μM NADPH, 10 μM DTT, 0.1 μM catalase, 4 μM superoxide dismutase (SOD), 5 μM oxyhemoglobin and 10 μg of protein, at 37 °C. The formation of methemoglobin was registered at 401 nm in a SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). In order to confirm that the oxidation occurred only by NO production, controls were carried out using the specific NOS inhibitor, NG-methyl-L-arginine (L-NMMA, 1 mM).

The experiments designed to evaluate the *in vitro* effect of Jbtx on NOS activity were conducted preincubating the supernatant of tissues from non-treated insects (pools of 10 organs) with 1 nM Jbtx or with no addition (controls) for 45 min at 4 °C. Afterwards, the NOS activity was determined as above.

2.8. RNA extraction, cDNA synthesis and gene expression analysis

Total RNA was extracted from CNS and SG from saline and Jbtx-treated insects using commercial PureLink® RNA Mini Kit, following manufacturer's instructions. Briefly, organs were washed with PB and homogenized in 1.2 mL lysis buffer at 4 °C. The homogenate was vortexed for 2 min and centrifuged at 12,000 xg for 2 min at 4 °C, and the supernatant was transferred to the column for total RNA separation and elution. Total RNA was eluted twice in 50 μL of RNAse free water, quantified at 260 nm and stored at −20 °C.

cDNA was synthetized using the High-Capacity cDNA Reverse Transcription Kit, employing 100 ng of total RNA (in 28.4 μL), 4 μL of 5 μM primer Oligo dT primer, 4 μL of Buffer 10X, 1.6 μL of 100 mM dNTPs mix and 2 μL reverse transcriptase 50 U/μL per tube, totalizing a 40 μL volume. The reaction was incubated for 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C. The product was diluted 5 times and stored at −20 °C.

The gene expression experiments were conducted using the equipment Eco Real-Time PCR System and software Eco (Illumina Inc., San Diego, CA, USA). Briefly, 8 μL of diluted cDNA were added to each well, along with 1 μL of *forward* primer, 1 μL of *reverse* primer and 10 μL of qPCR Master Mix, containing all the necessary reagents for the reaction. All samples were analyzed in quadruplicate. The reaction parameters were: initial denaturation at 95 °C, for 2 min, followed by 40 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 15 s) and extension (72 °C, 15 s), finishing in a melting curve (55–95 °C, with 0.1 °C increments/second).

To analyze the expression profile of *NOS* gene in saline and Jbtx-fed insects, the primers 5′-GCACGATGTTTGATGTCCTG-3′ and 5′-GCAAAGCGAACCATTTCAAG-3′ were designed based on the available sequence (Yuda et al., 1996). The identification of *UAP* and *chitin synthase* genes from *R. prolixus* was carried out performing a BLAST search using *Aedes aegypti* homologue sequences (AAEL001627 and AAEL002718, respectively) deposited in the VectorBase (https://www.vectorbase.org/, Giraldo-Calderón et al., 2015) as a query. The corresponding genes for *R. prolixus UAP* (RPRC015300) and *chitin synthase* (RPRC008031) were identified and its expression analyzed employing the following primers: 5′-GCGGAGCTAAAGTTGTGGAG-3′ and 5′-CATCGTTGTTACGCATTTCAGC-3′ for *UAP* and 5′-CATGAAAGCAATGGTGGATGTTGTAGCAATTG-3′ and 5′-ACTGAATGTTTAGAGACATAGTTCTCCCACC-3′ for *chitin synthase*. Primers for the normalizers *actin* (5′-CAGAGAAAAGATGACGCAGATAATG-3′ and 5′-CGGCCAAATCCAATCG-3′) and *elongation factor 1* (5′-GATTCCACTGAACCGCCTTA-3′ and 5′-GCCGGGTTATATCCGATTTT-3′) were based on the available literature (Defferrari et al., 2014; Majerowicz et al., 2011). The results were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.9. Determination of UAP activity

Dissected organs from treated insects (pools of 10 organs for each treatment) were homogenized in 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.5) with protease inhibitors and centrifuged (10,000 xg, 10 min, 4 °C). The supernatant was recovered and, after measuring the protein concentration, UAP activity was determined following the methodology described by Mio et al. (1998) with slight modifications (Galvani et al., 2015). The assays were carried out in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 25 μM UTP, 20 μM N-acetyl-α-D-glucosamine 1-phosphate, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.4 units/ml pyrophosphatase and 3–5 μg of supernatant protein as enzyme source. The reaction mix was incubated for 15 min at 37 °C and an equal volume of the color reagent comprising 0.03% (w/v) malachite green, 0.2% (w/v) ammonium molybdate, 0.05% (v/v) Triton X-100 in 0.7 M HCl was added. The

color was developed for 5 min at 37 °C and then absorbance readings were registered at 655 nm. Specific activity was expressed as the absorbance (A_{655}) per mg of protein.

The experiments directed to assess the *in vitro* effect of Jbtx on UAP activity were carried out preincubating the supernatant of non-treated insects (pools of 10 organs) with different concentrations of Jbtx or with no addition for the controls for 1 h at room temperature. Afterwards, the UAP activity was determined as above.

2.10. Determination of acid phosphatase activity

Dissected organs from treated insects (pools of 10 organs for each treatment) were homogenized in 20 mM sodium acetate buffer pH 4.0 containing 10 mM DTT, 10 mM Na_2EDTA and protease inhibitors and centrifuged at 14,000 x*g* for 5 min at 4 °C. Supernatants were recovered, the protein concentration determined and the acid phosphatase (AP) activity was measured following the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) to *p*-nitrophenol (*p*NP) as described previously (Leyria et al., 2015). The assays were conducted incubating 5 mM *p*NPP as substrate and 2–4 μg of protein homogenates as enzyme source for 30 min at 37 °C. The reaction was stopped with 1 M NaOH and the amount of *p*NP released into the medium was registered at 405 nm.

The *in vitro* effect of Jbtx on AP activity was assessed preincubating the supernatant of non-treated insects (pools of 10 organs) with different Jbtx concentrations or with no addition (controls) for 1 h at 4 °C. Afterwards, the AP activity was determined as described above.

2.11. Statistical analyses

For measurements of UAP, NOS and AP activities, groups 10 insects were pooled and processed. Four to nine independent experiments were performed and data was expressed as mean ± Standard Error of the Mean (SEM). Graphs and statistical tests were performed using GraphPad Prism and GraphPad Instat 5.0 (GraphPad Software, San Diego, CA, USA). Results from the *in vitro* effect of Jbtx on UAP and AP activities were analysed by one-way parametric ANOVA for comparisons between means and employing Student–Newman–Keuls as post-test. All other comparisons were made using the Student's *t*-test. A P value < 0.05 was considered statistically significant. The degrees of freedom (*df*) are informed in the legends of the figures.

3. Results

3.1. In vitro *Jbtx-FITC incubations*

Several lines of evidence indicated that ureases and derived peptides, including Jbtx, act on the nervous system as part of their toxic mechanism (Carlini and Ligabue-Braun, 2016). *In vitro* incubation experiments with the toxic peptide labelled with FITC were conducted to evaluate the potential affinity of Jbtx towards the insect CNS and other organs of interest such as the SG, employing BSA-FITC as a non-specific binding control. As shown in Fig. 1, Jbtx binds to the CNS, reinforcing the view that it is a target of the toxic peptide. Only a slight binding of Jbtx was observed for SG.

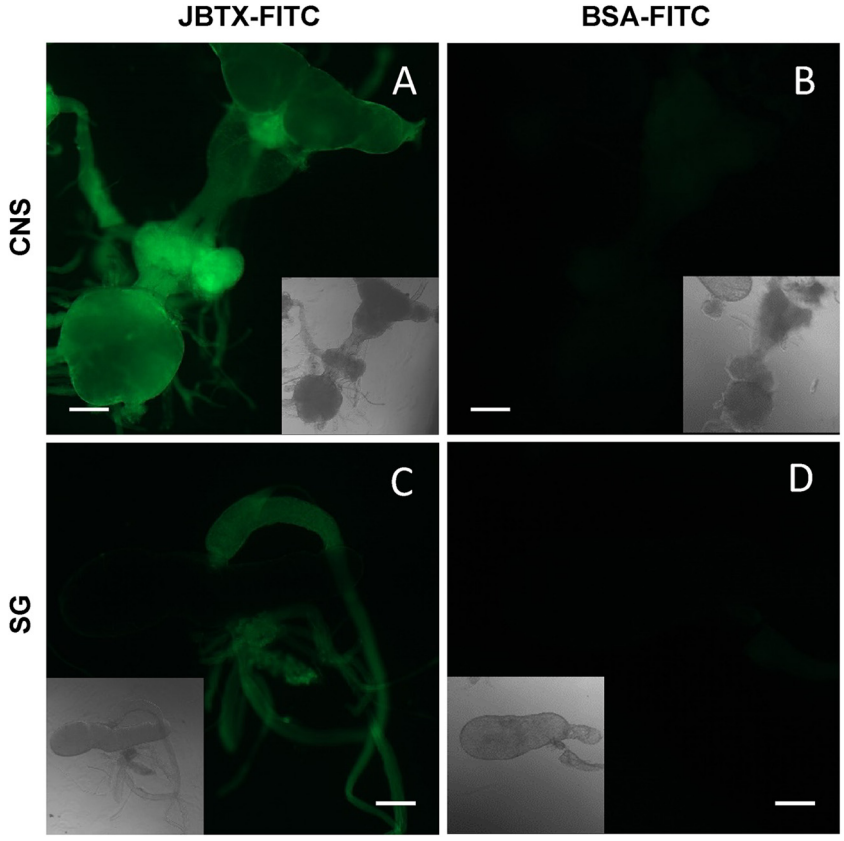

Fig. 1. *in vitro* incubation of organs with Jaburetox-FITC. The central nervous system (CNS) (A-B) and the salivary glands (SG) (C-D) of the insects were dissected and incubated for 1 h at room temperature with 23 μM Jaburetox-FITC (A, C) or with 23 μM BSA-FITC as control (B, D). The insets are the corresponding bright field images and the figure shows representative experiments of three independent assays. Bars = 200 μm.

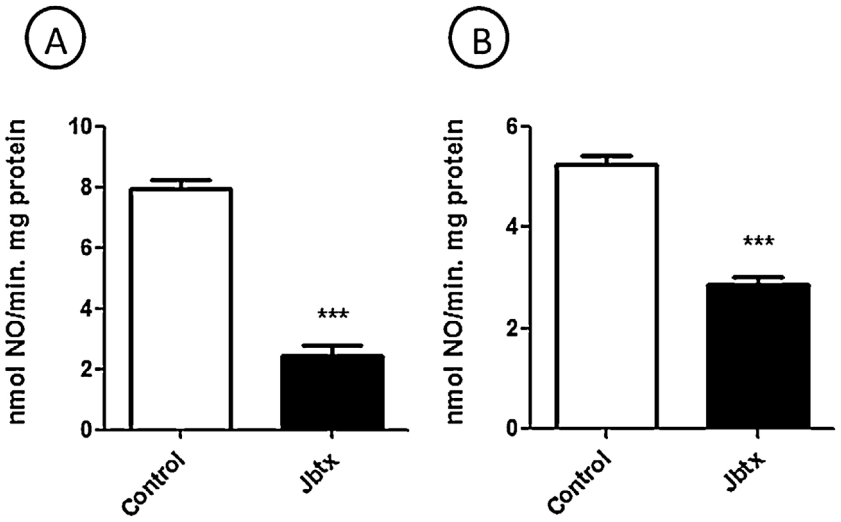

Fig. 2. Effect of Jaburetox on NOS activity after the *in vitro* treatment. NOS activity was determined in CNS **(A)** and SG (B) homogenates. The organs were dissected, pooled, homogenized and incubated with 1 nM Jaburetox or with equivalent volumes of buffer (control) for 45 min at 4 °C. Results are expressed as nmol of NO produced/min.mg protein and are means ± SEM. $^{***}P < 0.0001$, Student *t*-test, (A) (n = 4–6, *t*-test: $t = 11.83$, $df = 8$, $P < 0.0001$), (B) (n = 4–6, *t*-test: $t = 10.33$, $df = 8$, $P < 0.0001$). CNS: central nervous system, SG: salivary glands.

3.2. Jbtx effects on NOS

Galvani and collaborators (2015) demonstrated in *T. infestans* that treatments with Jbtx either *in vivo* (by injection) or *in vitro* reduced NOS activity and NO levels in homogenates of the insect's CNS, without modifying the expression of the protein. In *R. prolixus*, the *in vitro* incubation of homogenates with 1 nM Jbtx induced a statistically significant decrease in NOS activity both in the CNS (Fig. 2A, $P < 0.0001$) and in the SG (Fig. 2B, $P < 0.0001$).

To investigate if the inhibitory effect of Jbtx on NOS could also be induced *in vivo*, fifth instar nymphs were fed a dose of 0.1 μg Jbtx/mg of body weight in *Rhodnius* saline or saline alone (controls), dissected and processed for NOS measurements 18 h afterwards. The *in vivo* treatment with Jbtx caused a considerable decrease in NOS activity in the insect CNS when compared to controls (Fig. 3A, $P < 0.05$). Nevertheless, no differences were detected in NOS activity in SG homogenates of control and Jbtx-fed insects (Fig. 3B). On the other hand, while the *in vivo* treatment with Jbtx did not alter the NOS mRNA expression in the CNS, it led to an increment of its expression in the SG (Fig. 4). The same expression profile was observed with both normalizer genes.

3.3. Jbtx effects on UAP

Earlier reports from our group (Galvani et al., 2015) pointed out that *in vitro* incubations of Jbtx with *Dysdercus peruvianus*' brain homogenates as well as the injections of the toxic peptide into *T. infestans* led to an increased activity of UAP in the CNS. To test this effect in *R. prolixus*, CNS and SG homogenates were preincubated with different concentrations of Jbtx and then the UAP activity was measured. As shown in Fig. 5A, 10 nM and 50 nM Jbtx promoted a significant increase in UAP activity in the CNS ($P < 0.0001$) whereas a concentration of 50 nM Jbtx was necessary to notably boost the activity in SG homogenates ($P < 0.0001$, Fig. 5B). The availability of *R. prolixus*' genome allowed the identification of the *UAP* sequence. To gain insight into this *in vitro* effect of the

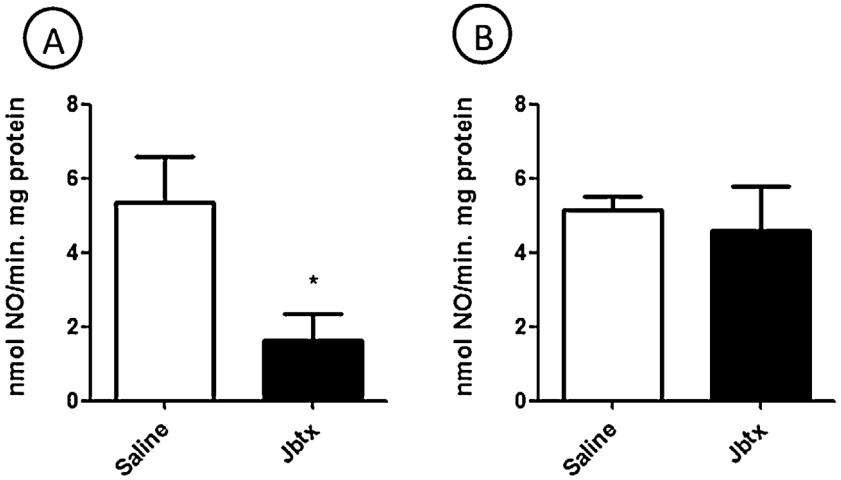

Fig. 3. Effect of Jaburetox on NOS activity after *in vivo* treatment. Insects were fed with Jaburetox (0.1 μg/mg of body weight) in *Rhodnius* saline or with saline alone (control) and dissected 18 h afterwards. CNS and SG were homogenized and assayed for NOS activity using colorimetric techniques. (A) NOS activity in CNS. Results are expressed as specific activity (nmol of NO produced/min.mg protein) and are means ± SEM, Student *t*-test (n = 7-8, *t*-test: $t = 2.477$, $df = 13$, $P = 0.0278$). (B) NOS activity in SG. Results are expressed as specific activity (nmol of NO produced/min.mg protein) and are means ± SEM, Studentt test. (n = 6, *t*-test: $t = 0.4641$, $df = 10$, $P = 0.6525$). CNS: central nervous system, SG: salivary glands.

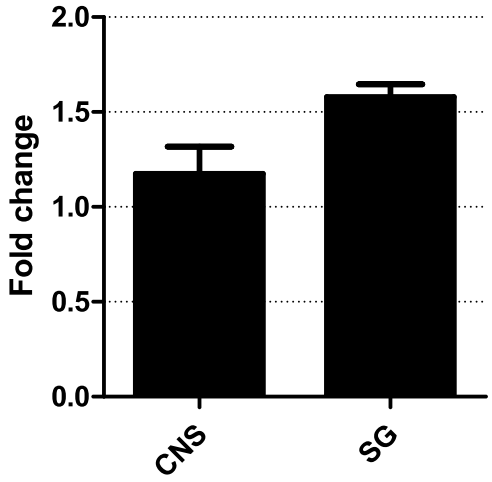

Fig. 4. Effect of Jaburetox *in vivo* treatment on *NOS* gene expression as measured by qPCR. Insects were fed with Jaburetox (0.1 μg/mg of body weight) in *Rhodnius* saline or with saline alone (control) and dissected 18 h afterwards. CNS and SG were homogenized and processed as described in Materials and Methods. Elongation factor 1 and actin (not shown) were employed as normalizers. The results are expressed in relation to insects fed on saline alone. CNS: central nervous system, SG: salivary glands. Results are expressed as Fold Change and are means ± SEM (n = 4) of a typical experiment.

peptide on the enzyme's activity, a recombinant UAP from *R. prolixus* was produced (supplementary data). This rUAP presented an apparent molecular weight of ~59 kDa, compatible with other UAPs from insects (Arakane et al., 2011; Liu et al., 2013). No significant changes were observed when the activity of rUAP (200 nM) alone was compared with rUAP preincubated with 600 nM Jbtx for 1 h at room temperature (100 ± 4.02% for rUAP alone as compared to 104.35 ± 6.20% for rUAP plus Jbtx).

Upon feeding the insects a dose of 0.1 μg Jbtx/mg of body weight, a significant increase in UAP activity was observed in the CNS and the SG ($P < 0.001$, Fig. 6A and $P < 0.05$, Fig. 6B, respectively), similar to the results obtained with tissue homogenates *in vitro* (Fig. 5). Expression of UAP mRNA upon Jbtx feeding was then analyzed. The observed expression profile was markedly different from what could be expected from the effect of Jbtx on the enzyme activity as described above, since the treatment with the toxic peptide caused a decrease in the UAP mRNA expression, both in the CNS and in the SG (Fig. 7). The levels of UAP mRNA were similar in both tissues of saline-fed insects and the same expression profile was observed using either of the two normalizer genes.

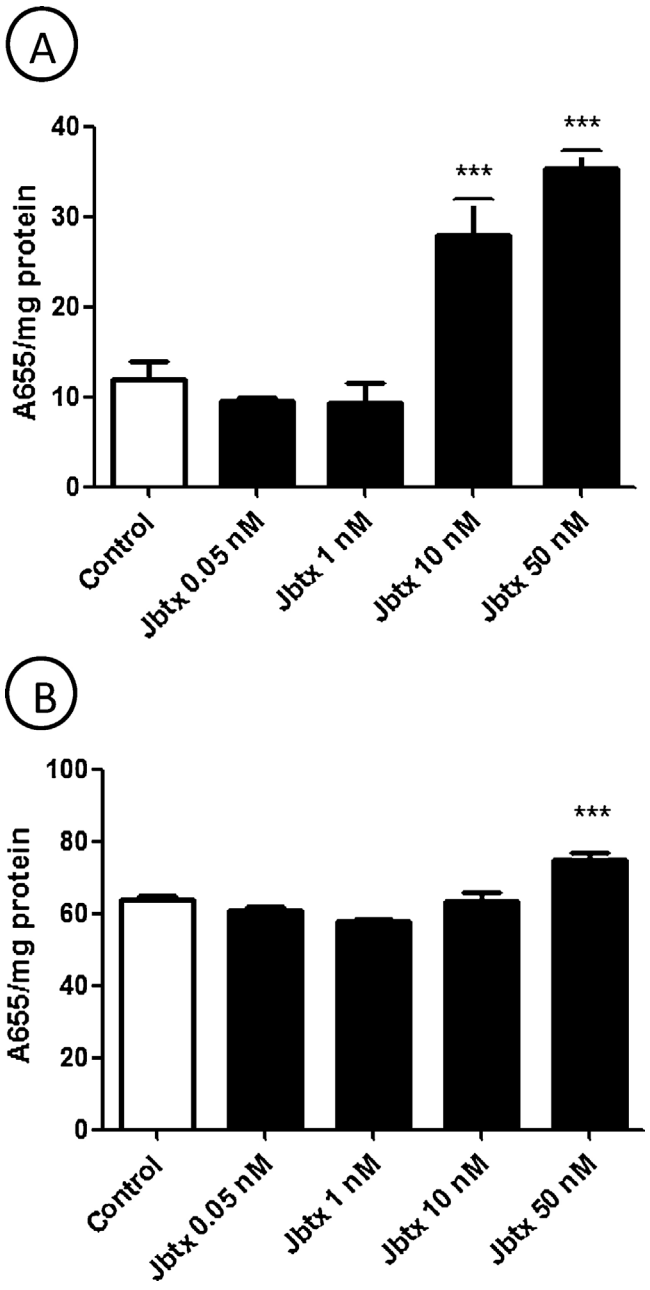

Fig. 5. Effect of Jaburetox on UDP-*N*-acetylglucosamine pyrophosphorylase (UAP) activity after *in vitro* treatment. The organs were dissected, pooled, homogenized and incubated with different concentrations of Jaburetox or with equivalent volumes of buffer (control) for 1 h at room temperature. (A) UAP activity in the CNS. Results are expressed as specific activity (A_{655} nm/mg protein) and are means ± SEM (n = 4-6). $^{***}P < 0.0001$, One-way parametric ANOVA (ANOVA: $F = 29.10$, $df = 23$, $P < 0.0001$). (B) UAP activity in the SG. Results are expressed as specific activity (A_{655} nm/mg protein) and are means ± SEM (n = 4). $^{***}P < 0.0001$, One-way parametric ANOVA (ANOVA: $F = 13.73$, $df = 19$, $P < 0.0001$). CNS: central nervous system, SG: salivary glands.

3.4. Jbtx effects on chitin synthase expression

Once established that the Jbtx affects both, the UAP activity and its mRNA expression, we decided to analyze whether an enzymatic route requiring UAP products, such as that of chitin synthase, could also be altered by the peptide. Insects fed 18 h earlier on a Jbtx-containing diet exhibited a diminution of chitin synthase mRNA expression in the CNS and in the SG (Fig. 8), similarly to what was observed for the UAP mRNA expression profile. The levels of chitin synthase mRNA were comparable in both tissues of saline-fed insects. The same expression profile was observed using either of the two normalizer genes.

3.5. Jbtx effects on AP

Since protein phosphorylation-dephosphorylation is an important mechanism of regulation of NO production (Alderton et al., 2001) and due to the fact that glycoinositolphospholipids (GIPLs), an indirect product of UAP activity, can also influence NOS activity (Gazos-Lopes et al., 2012), we decided to assess if Jbtx would induce a modification in AP activity. However, no significant differences were found on AP activity of CNS and SG homogenates upon incubation with Jbtx (Fig. 9A and B) nor in the tissues of Jbtx-fed insects when compared to controls (Fig. 10A and B).

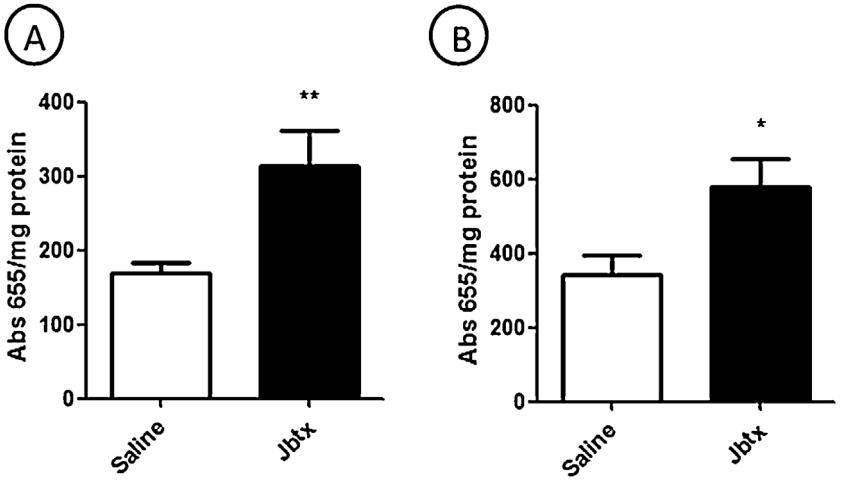

Fig. 6. Effect of Jaburetox on UDP-*N*-acetylglucosamine pyrophosphorylase (UAP) activity after *in vivo* treatment. Insects were fed with Jaburetox (0.1 μg/mg of body weight) in *Rhodnius* saline or with saline alone (control) and dissected 18 h afterwards. CNS and SG were homogenized and assayed for UAP activity using colorimetric techniques. (A) UAP activity in CNS. Results are expressed as specific activity (A_{655} nm/mg protein) and are means ± SEM (n = 8-9). $^{**}P < 0.001$, Student *t*-test (*t*-test: $t = 3.183$, $df = 15$, $P = 0.0062$). (B) UAP activity in SG. Results are expressed as specific activity (A_{655} nm/mg protein) and are means ± SEM (n = 7). $^{*}P < 0.05$, Student *t*-test (*t*-test: $t = 2.557$, $df = 12$, $P = 0.0251$). CNS: central nervous system, SG: salivary glands.

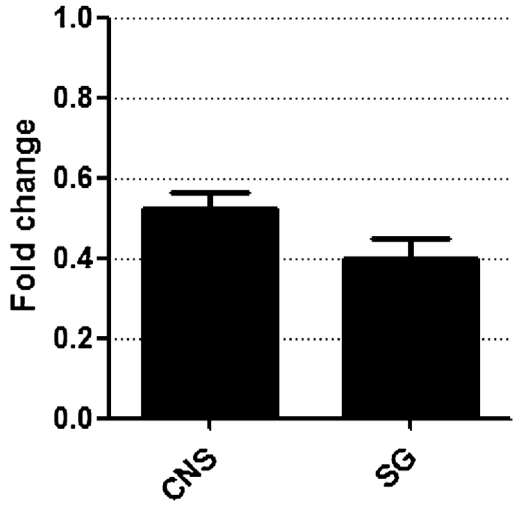

Fig. 7. Effect of the *in vivo* Jaburetox treatment on *UAP* expression as measured by qPCR. Insects were fed with Jaburetox (0.1 μg/mg of body weight) in *Rhodnius* saline or with saline alone (control) and dissected 18 h afterwards. CNS and SG were homogenized and processed as described in Materials and Methods. Elongation factor 1 and actin (not shown) were employed as normalizers. The results are expressed in relation to insects fed on saline alone. CNS: central nervous system, SG: salivary glands. Results are expressed as Fold Change and are means ± SEM (n = 4) of a typical experiment.

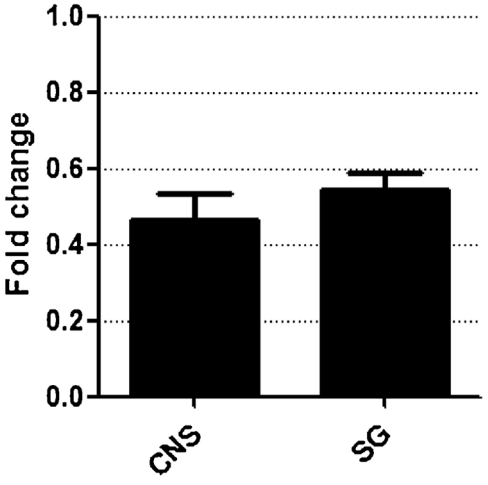

Fig. 8. Effect of the *in vivo* Jaburetox treatment on *chitin synthase* expression as measured by qPCR. Insects were fed with Jaburetox (0.1 μg/mg of body weight) in *Rhodnius* saline or with saline alone (control) and dissected 18 h afterwards. CNS and SG were homogenized and processed as described in Materials and Methods. Elongation factor 1 and actin (not shown) were employed as normalizers. The results are expressed in relation to insects fed on saline alone. CNS: central nervous system, SG: salivary glands. Results are expressed as Fold Change and are means ± SEM (n = 4) of a typical experiment.

4. Discussion

The developing of insect resistance is the most pressing reason to discover and characterize new compounds that can be used as insecticides. Ideally, these substances should be toxic only to harmful species and environment-friendly (Hardy, 2014). Regarding these points, a strategy employing transgenic plants expressing insecticidal peptides or formulations containing entomotoxic peptides can be a safer alternative for pest control, in comparison to chemical pesticides, due to their degradability (Follmer, 2008). The recombinant peptide Jbtx was proven entomotoxic to several insect species and harmless, at least given acutely, for two mammalian species (Mulinari et al., 2007). Moreover, preliminary results with transgenic crops of sugarcane, maize and soybean plants expressing Jbtx showed higher resistance to the attack of insects (reviewed in Carlini and Ligabue-Braun, 2016).

Rhodnius prolixus is one of the main Chagas' disease vectors among the 148 described species of triatomines (Justi et al., 2014) and it has been employed as a model for physiological research since the pioneer works of Wigglesworth (1931). In the context of entomotoxin research, the administration of the *C. ensiformis* urease isoforms Jack Bean Urease (JBU) (Stanisçuaski et al., 2010; Defferrari et al., 2014) and canatoxin (Carlini et al., 1997), as well as Jbtx (Martinelli et al., 2014), proved to be lethal to *R. prolixus* in periods ranging from hours to days, depending on the dose, the toxin and whether the compounds were injected or fed. Jaburetox at very low doses (femtomolar) induced antidiuresis in isolated Malpighian tubules of *R. prolixus*, as part of the lethal mechanism (Stanisçuaski et al., 2009; Paluzzi et al., 2012). Our results here indicated that, *in vitro*, the toxic peptide binds to and presents

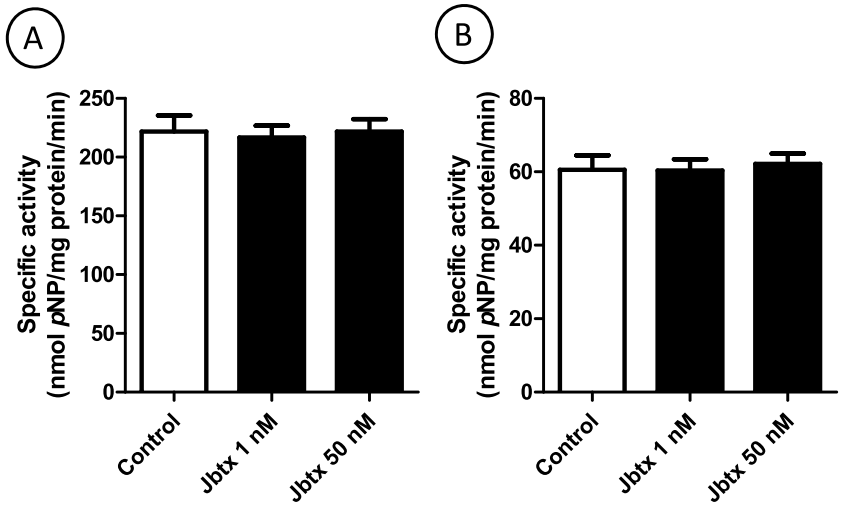

Fig. 9. Effect of Jaburetox *in vitro* treatment on acid phosphatase (AP) activity. The organs were dissected, pooled, homogenized and incubated with 1 and 50 nM Jaburetox or with equivalent volumes of buffer (control) for 1 h at 4 °C. The AP activity was determined in CNS (A) and SG (B) homogenates by colorimetric techniques. Results are expressed as specific activity (nmol *p*NP/mg protein/min) and are means ± SEM (n = 4). (A) (ANOVA: $F = 0.05878$, $df = 11$, $P = 0.9472$). (B) (ANOVA: $F = 0.08807$, $df = 11$, $P = 0.9165$).

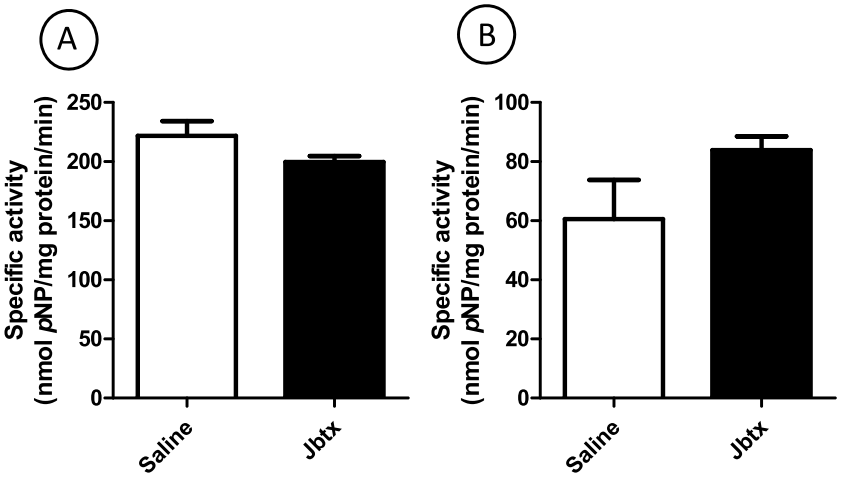

Fig. 10. Effect of Jaburetox *in vivo* treatment on AP activity. Insects were fed with Jaburetox (0.1 μg/mg of body weight) in *Rhodnius* saline or with saline alone (control) and dissected 18 h afterwards. CNS and SG were homogenized and assayed for AP activity using colorimetric techniques. (A) AP activity in CNS. Results are expressed as specific activity (nmol *p*NP/mg protein/min) and are means ± SEM, Student *t*-test (n = 6) (*t*-test: $t = 1.627$, $df = 10$, $P = 0.1349$). (B) AP activity in SG. Results are expressed as specific activity (nmol *p*NP/mg protein/min) and are means ± SEM, Student *t*-test (n = 6) (*t*-test: $t = 1.657$, $df = 10$, $P = 0.1285$).

higher affinity for the CNS than to the SG. Similar findings were observed for the *in vitro* incubations of Jbtx with the nerve cord of the cockroach *N. cinerea* (unpublished results). Jaburetox also interacts with artificial membranes, such as large unilamellar vesicles (LUVs) (Barros et al., 2009), planar lipid bilayers (Piovesan et al., 2014) and multilamellar liposomes (Micheletto et al., 2016), leading to permeabilization of LUVs and formation of cation selective ion channels in the lipid bilayers. Moreover, it was shown by Micheletto et al. (2016) that the toxic peptide is able of inserting itself within the hydrophobic core of the membrane lipid bilayer. The difference in Jbtx's affinity between the CNS and the SG could be explained in terms of the different compositions of the membranes, since it was previously demonstrated that Jbtx binds to acidic phospholipids but not to neutral ones (Barros et al., 2009) and that the insertion of the peptide was easier in negatively charged membranes (Piovesan et al., 2014).

According to our searches in the *R. prolixus*' genome, this species possess only one NOS isoform. In the present work, we demonstrated that Jbtx induced a decrease of NOS activity in *R. prolixus* CNS both *in vivo* and *in vitro*. Galvani et al. (2015) working with *T. infestans* adults, also described an inhibitory effect of Jbtx *in vitro* and *in vivo* (by injection) on NOS activity in the insect brain. Moreover, preliminary assays on *N. cinerea* nerve cords homogenates also pointed out to a Jbtx-provoked inhibition of NOS activity (unpublished data), suggesting that this effect is more general, affecting also tissues from non-hemipteran insects. On the other hand, reduction of NOS activity in the SG was only seen *in vitro*, by adding the peptide to the homogenate, suggesting that Jbtx was somehow "inactivated" or did not reach the SG of insects upon feeding at the necessary levels to cause significant modifications. Worthy to mention, injections of Jbtx into *R. prolixus* nymphs also did not alter NOS activity in the hemolymph, as recently reported by Fruttero et al. (2016), pointing to tissue-specific effects of the peptide upon the enzyme. Gazos-Lopes et al. (2012) using the SG of *R. prolixus* reported that trypanosomatid parasites or their GIPLs caused NOS downregulation, inhibiting the production of NO, the enzyme activity and its protein expression without modifying the mRNA levels. Here our results have shown that Jbtx-induced NOS regulation in *R. prolixus* is a complex one. While no changes in NOS mRNA levels were observed in the CNS, an increase was detected in

the SG mRNA levels when compared to controls. The fact that the NOS mRNA expression profile did not match the observed reduction in the enzyme activity is an indicative that the toxic peptide could be acting on more than one regulation level, i.e., affecting directly the NOS enzymatic machinery as suggested by the *in vitro* assays and/or, indirectly, through modifications on the expression of its gene, or even altering the membrane properties of target cells. Regulation of the NOS enzymes has been described at all levels from gene transcription, specific inhibition, allosteric modulation and covalent modifications. In particular, the processes of phosphorylation and dephosphorylation are key mechanisms of regulation of NO production (Alderton et al., 2001). In the SG of *R. prolixus*, injections of GILPs from *Trypanosoma cruzi* led to a decrease in the activity of phosphotyrosine phosphatase in the glands (Gazos-Lopes et al., 2012). Moreover, sodium orthovanadate (a general AP inhibitor) was capable of diminishing NO production in a way similar to that of *T. cruzi* GIPLs, establishing a link between phosphatase(s) and NOS enzymatic activities. The mechanism of action of Jbtx on NOS seems to work differently from that of tripanosomatid GIPLs because the diminution in enzyme activity induced by the toxic peptide was not accompanied by changes in AP enzyme levels, either in *vivo* or *in vitro*.

Our findings demonstrated that Jbtx increased UAP activity, both after *in vivo* or *in vitro* treatments, not only in the *R. prolixus* CNS but also in their SG. This increase was previously observed by our group (Galvani et al., 2015) in the CNS of *T. infestans* (*in vivo* upon injection) and of *Dysdercus peruvianus* (*in vitro*), indicating that this toxic effect of Jbtx could be a generalized feature among insects, or at least in hemipterans. This previous work also established a physical interaction between Jbtx and UAP. Nevertheless, the experiments performed in the present work with a fully active rUAP and Jbtx did not produce activation of the enzyme. This result precludes a direct allosteric mechanism to explain the activation of UAP by Jbtx, as seen in brain homogenates in the presence of the peptide, and suggests that another factor(s) present in the tissue homogenates could be required for the modulation. *Rhodnius prolixus*' rUAP is, to our knowledge, the first recombinant UAP of an insect to be obtained and presents similar features to those reported in the literature (Mio et al., 1998). A full characterization of *R. prolixus*' rUAP is currently being carried out and will be part of a separate work. In contrast to the upregulation of the enzyme activity, *in vivo* treatment of *R. prolixus* by feeding on Jbtx unexpectedly led to a marked decrease in the UAP (and chitin synthase) mRNA levels. As discussed above for the Jbtx's effect on NOS, it is possible that the peptide affects UAP at more than one level of regulation. In this case, the Jbtx-induced fall in the mRNA levels of UAP could be a compensatory mechanism of the insect to cope with the increase in the enzyme activity. In fact, such a negative feedback mechanism in which an increase in the enzymatic activity is compensated by a decrease of gene expression has been previously described in the literature for different biological systems including 5-aminolevulinate synthase (Hamilton et al., 1991); semicarbazide-sensitive amine oxidase (Nordquist et al., 2002) and acetylcholinesterase (Ferreira et al., 2012).

Arakane et al. (2011) identified two UAP genes in the beetle *Tribolium castaneum* and described their differential expression patterns during the development. Both proteins were critical for development and survival, although one of them was more related to chitin production and the other was involved in the other UAP functions, including glycosylation. Likewise, two UAP genes were also described in the locust *Locusta migratoria*, also presenting variable expression patterns and specialized functions (Liu et al., 2013). In contrast, our search for UAPs in *R. prolixus* retrieved only one gene. This fact indicates that this species' single UAP has to carry out its diverse functions, in contrast to the specialized variants of *T. castaneum* and *L. migratoria*. Several studies in insects focusing on UAP gene profiles report that the expression of this enzyme's mRNA fluctuates in response to changes in the metabolism such as development and molting, among others (Araújo et al., 2005; Schimmelpfeng et al., 2006; Tonning et al., 2006; Arakane et al., 2011; Humphreys et al., 2013; Liu et al., 2013). Besides, UAP's expression responsiveness to external stimuli has been reported, as is the case of cancer cells upregulating UAP when challenged with antitumoral compounds (Itkonen et al., 2015).

5. Conclusions

In this work, we confirmed the effect of Jbtx on insect SNC and showed that the SG are also a target for the peptide's toxic action, widening the span of Jbtx-affected organs *in vivo*. Apart from confirming NOS and UAP as Jbtx's molecular targets in *R. prolixus*, our findings identified chitin synthase as a novel enzymatic pathway altered by the peptide. In general, the CNS was more susceptible to Jbtx effects than SG, exhibiting increased binding of Jbtx-FITC and an *in vivo* inhibitory effect of NOS activity in treated insects that was not observed for SG. On the other hand, SG were the only organ where an increase of NOS mRNA levels was seen in response to the *in vivo* Jbtx treatment. This is the first report to demonstrate that Jbtx modifies the gene expression in *R. prolixus*, adding yet another level of complexity to its entomotoxic mode of action.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2017.01.009.

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