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Thermosensation and the TRPV channel in *Rhodnius prolixus*

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ABSTRACT

The thermal sense of triatomine bugs, vectors of Chagas disease, is unique among insects. Not only do these bugs exhibit the highest sensitivity to heat known in any animal up to date, but they can also perceive the infrared radiation emitted by the body of their warm-blooded hosts. The sensory basis of this capacity has just started to be unravelled. To shed additional light on our understanding of thermosensation, we initiated an analysis of the genetic basis of the thermal sense in *Rhodnius prolixus*. We tested the hypothesis that a TRPV (transient receptor potential vanilloid) channel receptor is involved in the evaluation of heat in this species. Two different approaches were adopted. Initially, we analysed the expression of a TRPV candidate for this function, i.e., *RproIav*, in different tissues. Subsequently, we tested the effects of capsaicin and capsazepine, two molecules known to interact with mammal TRPV1, using three different behavioural protocols for evaluating thermal responses: (1) proboscis extension response (PER), (2) thermopreference in a temperature gradient and (3) spatial learning in an operant conditioning context. Bioinformatic analyses confirmed that the characteristic features typical of the TRPV channel subfamily are found in the RproIav protein sequence. Molecular analysis showed that *RproIav* is expressed in *R. prolixus*, not only in the antennae, but also in other body structures bearing sensory organs. Behavioural experiments consistently revealed that capsaicin treated insects are less responsive to heat stimuli and prefer lower temperatures than non-treated insects, and that they fail to orient in space. Conversely, capsazepine induces the opposite behaviours. The latter data suggest that triatomine thermoreception is based on the activation of a TRP channel, with a similar mechanism to that described for mammal TRPV1. The expression of *RproIav* in diverse sensory structures suggests that this receptor channel is potentially involved in bug thermoreception. This constitutes solid evidence that thermosensation could be based on the activation of TRP receptors that are expressed in different tissues in *R. prolixus*. Whether *RproIav* channel is a potential target for the compounds tested and whether it mediates the observed effects on behaviour still deserves to be confirmed by further research.

1. Introduction

Transient receptor potential (TRP) proteins are a superfamily of selective cation channels involved in a wide range of sensory processes, including thermal, tactile, gustatory, osmolar and fluid flow sensing, in both vertebrates and invertebrates (Damann et al., 2008; Huang, 2004; Olszewska, 2010; Pedersen et al., 2005; Ramsey et al., 2006; Venkatachalam and Montell, 2007). TRPs are divided into seven subfamilies due to their structure and amino acidic sequences, but this division does not reflect functional categories (Huang, 2004; Fowler and Montell, 2013). One of the main functions in which TRPs are involved is thermosensation. Different TRPs belonging to three subfamilies are considered to mediate thermosensation: melastatine (TRPMs), ankyrin (TRPAs), and vanilloid receptors (TRPVs). In mammals, thermo-related channels from TRPM and TRPA subfamilies are involved in sensing low temperatures, while TRPVs are related with ambient or high temperature sensing (Damann et al., 2008; Dhaka et al., 2007; Knowlton et al., 2010; Jordt et al., 2003; Ramsey et al., 2006; Palkar et al., 2015). Conversely, in insects, TRPAs are involved in sensing warm and high temperatures, while TRPV receptors have been shown to only mediate responses to low temperatures (Dhaka et al., 2006; Hamada et al., 2008; Hwang et al., 2012;

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Kwon et al., 2010; Neely et al., 2011; Rosenzweig et al., 2008; Tracey et al., 2003; Zhong et al., 2012).

Among invertebrates, the TRPA subfamily is present in *Daphnia pulex* and *Caenorhabditis elegans* but no function related to thermosensation was described in these cases (Khan-Kirby and Bargmann, 2006; Matsuura et al., 2009). In insects, TRPA channels have been identified in different species of Lepidoptera, Coleoptera, Hymenoptera, Phthiraptera and Diptera (Matsuura et al., 2009). In *Drosophila melanogaster*, three channels belonging to the TRPA subfamily are related to thermosensation (dTRPA1, *painless* and *pyrexia*) and mediate responses to moderate and high temperatures (Dhaka et al., 2006; Hamada et al., 2008; Barbagallo and Garrity, 2015), fact that has also been recently observed in other insect species (Kim et al., 2015). *painless* and *pyrexia* channels are present in other insect species (like *Bombyx mori*, *Tribolium castaneum*, *Apis mellifera*, *Nasonia vitripennis* and *Pediculus humanus*), suggesting that channels for the detection of noxious temperatures are conserved among different insect taxa (Matsuura et al., 2009). In *Anopheles gambiae*, AgamTRPA1 in antennal structures is activated by temperature increases from 25 °C to 37 °C, and this would potentially allow mosquitoes to detect increasing temperature along gradients when approaching hosts (Wang et al., 2009).

The vanilloid receptor (TRPV) subfamily is one of the best known among TRPs. There are several TRPVs, and, among other functions, some respond to high temperatures (Benham et al., 2003; Caterina, 2007; Nilius and Voets, 2005; Szallasi and Blumberg, 1999; Vennekens et al., 2008). Six vanilloid receptors have been discovered in mammals, TRPV1 to 6, of which the first four are thermoTRPs (Ramsey et al., 2006). TRPV channels are also present in invertebrates. Particularly in insects, two TRPVs were found in the fruit fly, *D. melanogaster*: *nanchung* (*DmelNan*) and *inactive (DmelIav)*. Both have a structure similar to that of mammal vanilloid receptors, but do not seem to respond to substances that activate other TRPV channels, such as capsaicin (Huang, 2004). It has been found that *nanchung* is not related with thermosensation, while *inactive* is required for sensing cool temperatures (17.5–18 °C) but it appears not to be activated by them in vitro (Fowler and Montell, 2013; Kwon et al., 2010; Rosenzweig et al., 2008). The mammal TRPV1 channel, also known as capsaicin receptor, is directly involved in responses to high temperature and noxious stimuli (nociception). TRPV1 is widely expressed in the peripheral and central nervous system (Vennekens et al., 2008). It has been reported that these channels are directly activated in response to capsaicin, a substance found in chilli peppers that produces the known "hot" sensation, by binding the receptor (O'Neil and Brown, 2003; Tominaga et al., 1998). While heat opens TRPV1, capsaicin and protons lower the activation threshold of the receptor, resulting in activation at lower temperatures (Tominaga et al., 1998). Conversely, capsazepine is known to be a competitive antagonist of mammalian TRPV1 that inhibits the response to vanilloid compounds (Bevan et al., 1992; Walpole et al., 1994). Although capsazepine has been shown to counteract the effect of capsaicin in a number of bioassays, it can also inhibit other thermo-sensitive channels (TRPM8), confirming its non-selective mode of action (Szallasi and Blumberg, 1999; Vennekens et al., 2008). Administration of capsaicin can alter the thermopreference of insects in thermal gradients, as well as the amount of carbon dioxide released, but these responses are highly species-specific (Maliszewska and Tegowska, 2012; Olszewska et al., 2010; Tegowska et al., 2005). Effects of capsazepine on *Tenebrio molitor* larvae were also tested by Olszewska and Tegowska (2011), who found opposite effects to those elicited by capsaicin. Although no TRPV1 homologues have yet been identified in insects, the fact that capsaicin and capsazepine affect thermally-mediated responses indicates that a receptor that is functionally similar to the one found in mammals is still to be described. After searching for orthologues belonging to the TRPV subfamily (Kwon et al., 2010) in the *Rhodnius prolixus* genome sequence, a single candidate sequence potentially related to thermoreception was found. This putative *R. prolixus inactive* channel gene (*RproIav*) was reported as part of the *R. prolixus* genome (Mesquita et al., in preparation).

For haematophagous insects, thermosensation is of paramount importance since heat plays a central role in their orientation towards hosts (Lazzari, 2009). In this work, we studied thermosensation in blood-sucking bugs, since they exhibit the highest thermal sensitivity reported in animals (Lazzari, 2009; Lazzari and Núñez, 1989). Triatomines can detect infrared radiation (Lazzari and Núñez, 1989; Schmitz et al., 2000; Zopf et al., 2014a,b) and their responses are triggered when there is contrast between the source of heat and ambient temperatures (i.e., bugs only respond to stimuli from objects at host's temperature, 30–35 °C, provided that the environment is colder than the object; Ferreira et al., 2007; Fresquet and Lazzari, 2011). Heat receptors in triatomines are concentrated on the antennae, but are also present in different regions of the body (Insausti et al., 1999; Lazzari and Wicklein, 1994; Zopf et al., 2014a,b). Several studies have analysed the effect of changes in temperature on triatomine thermopreference and cognitive abilities (Schilman and Lazzari, 2004; Vinauger et al., 2013). However, in spite of all the knowledge accumulated on the behavioural responses of triatomines to heat, the underlying molecular pathways remain unknown and associated channels have yet to be recognised (Latorre-Estivalis et al., 2013). In the present report, we aim to initiate the study of the genetic bases of thermoreception in blood-sucking bugs by identifying and characterising the expression of *RproIav*, a putative thermosensitive channel gene. Furthermore, we test the effects of capsaicin and capsazepine on thermally-mediated behaviours in these insects and evince that they modulate triatomine behavioural responses to heat.

2. Materials and methods

2.1. Genetic analysis

2.1.1. Structural characterisation of R. prolixus inactive TRP channel

Diverse functional and structural features characteristic of the TRP gene family were evaluated using different software and protein databases, as follows. The presence of a signal peptide was evaluated using SignalIP v4.1 (Petersen et al., 2011). Subsequently, the presence of functional domains such as the ion channel and ankyrin repeats characteristic of these receptors were identified using InterProScan v5 (Jones et al., 2014). Finally, TOPCONS and TMHMM v2.0 were used to assess the location and number of predicted transmembrane domains existing in the sequence (Bernsel et al., 2009; Krogh et al., 2001). CLUSTAL X v2.0 allowed aligning the RproIav sequence with those of orthologues from other insects (*A. mellifera*, *B. mori*, *D. melanogaster*, *T. castaneum* and *P. humanus*) for confirming the degree of conservation of the diverse domains along the sequence and their correct location (Matsuura et al., 2009; Thompson et al., 1997). Comparison of the RproIav protein sequence was performed using a BLASTp v2.2.30 search of potential orthologues in the UniProtKB/TrEMBL database (Bairoch et al., 2005). A total of fourteen Iav protein sequences from diverse insect orders, two protein sequences from mammal TRPV1 and four protein sequences from insect and mammal TRPA1 were obtained. Subsequently, sequences were aligned with CLUSTAL X v2.0 (Thompson et al., 1997) and manually edited in Jalview v2.6.1 (Waterhouse et al., 2009). For the phylogenetic reconstruction, a total of 12 different evolutionary models (JTT, LG, DCMut, MtREV, MtMam, MtArt, Dayhoff, WAG, RtREV, CpREV, Blosum62, and VT) were tested using

the ProtTest v2.4 webserver (Abascal et al., 2005). Finally, a maximum likelihood tree was built in MEGA6.0 (Tamura et al., 2013) with 1000 pseudo-replicates and using TRPA1 sequences as an outgroup.

2.1.2. Insects used for molecular biology experiments

For molecular biology assays adult females were obtained from a colony originated from *R. prolixus* captured at Honduran intradomiciles two decades ago and reared ever since at CPqRR. This colony is permanently kept at 26 ± 1 °C, under natural illumination and 65 ± 10% RH regimes.

2.1.3. Primer design

Primers were designed for reverse transcription polymerase chain reaction (RT-PCR) experiments using the Primer3 4.0.0 software [http://primer3.wi.mit.edu] (Rozen and Skaletsky, 2000). Primer specificity was tested *in silico* using BLASTn in the *R. prolixus* genome database (Altschul et al., 1990). The characteristics of the primers are described on Table 1.

2.1.4. RNA extraction and cDNA synthesis

Total RNA was extracted from pools of 40 antennae, 20 rostri, 120 tarsi, 80 tibial pads and 10 ovipositors from a batch of 20 individuals. Specifically, ovipositors were excised from half of the females from this batch to avoid excess material during sample processing. RNA extraction was performed using 500 μL of TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted RNA was resuspended in 25 μL of DEPC-treated water (Life Technologies, Carlsbad, CA, USA), and its concentration determined using a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Genomic DNA was eliminated using RQ1 RNase-Free DNase kit (Promega, Fitchburg, WI, USA). Finally, cDNA was synthesized in a MasterCycler® Gradient Thermal Cycler (Hauppauge, NY, USA) using the SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA), a 1:1 mix of Random Hexamers and 10 μM Oligo (dT)20 primers in a final volume of 20 μL. The amount of treated RNA used for RT reactions was 890 ng. All the cDNAs produced were stored at −20 °C until use.

2.1.5. PCR

Reactions were made using 1 μL of pure cDNA, 1.1 μL of a 1 mM dNTPs solution, 0.6 μL of a 10 μM primer solution and 1 U of Taq polymerase (Promega, Fitchburg, WI, USA) in a final volume of 12 μL. Reactions were performed during 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The size of the resulting PCR products was observed by means of electrophoresis in 2% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA). The Glucose-6-phosphate dehydrogenase gene (*G6PDH*) was used as a positive control to evaluate cDNA integrity. Template free negative controls were also included to confirm absence of reagent contamination.

2.2. Behavioural experiments

2.2.1. Insects used for behavioural experiments

Fifth instar *R. prolixus* were used throughout these experiments. Bugs were reared in the laboratory at 25 ± 2 °C under a 12L:12D illumination and 65 ± 5% RH regime. Unfed 2–6 week old fifth instar nymphs were used.

2.2.2. Drug administration

We performed several behavioural experiments in order to establish whether thermal receptor agonists and antagonists affected bug thermosensation. In order to test the effect of a thermal receptor agonist on triatomine thermally-mediated behaviour, we used a capsaicin analogue, the N-vanyllylnonanamide ($C_{17}H_{27}NO_3$, also known as pseudocapsaicin, or synthetic capsaicin, from now on pCap) and its antagonist, capsazepine ($C_{19}H_{21}ClN_2O_2S$, from now on Cpz). pCap and Cpz were diluted in Ringer solution at different concentrations and 10 μl of one of these solutions were injected through the membrane of the second leg coxa in each bug. As a control, a group of bugs was injected 10 μl of Ringer solution. Different concentrations of the drugs were tested in the first protocol (PER) and, based on the results of these experiments, we determined the optimal concentration to be used in the following experiments.

2.2.3. PER

The proboscis extension reflex (PER) has been extensively used as a bioassay to study cognitive abilities in honey bees and it has recently been used as an indicator of heat detection in appetitive and aversive contexts with *R. prolixus* (Bodin et al., 2009a, 2009b; Vinauger et al., 2013).

Insects were randomly assigned to one of seven treatments: Ringer solution (control group, $N = 67$), pCap 3.41 μM ($N = 20$), pCap 34.1 μM ($N = 20$), pCap 341 μM ($N = 21$), Cpz 2.65 μM ($N = 20$), Cpz 26.5 μM ($N = 20$) or Cpz 265 μM ($N = 20$). Higher concentrations (mM) of the drugs were evaluated in preliminary tests. Although some bugs survived drug administration long enough to allow performing the experiments, these solutions were found to be lethal in a longer term. Therefore, the corresponding results were not included in our analyses.

Bugs were kept in tubes for 60 min after injection and then dorsally attached to a steel rod with double-sided adhesive tape in order to allow performing experiments in an open-loop design (device was designed after Fresquet and Lazzari, 2011). Once mounted in the setup, bugs were allowed to secure a Styrofoam ball in order to provide tarsal contact to record their behaviour in open-loop locomotion. The temperature of a water cooled Peltier element (4 × 4 cm, 12 V, 72 W, QuickCool, Wuppertal, Germany) was defined by an accurate controller (Peltron GmbH Peltier-Technik, Germany) which allowed precise presentation of thermal stimuli. Insects were placed at a distance in relation to the Peltier element so that they could barely contact its surface by extending their proboscises.

Before each trial, bugs were familiarised with the device and the temperature of the Peltier element was fixed at 25 °C. Trials begun after 1 min of familiarisation. Each trial consisted of three

Table 1
Specific primers used for RT-PCR experiments with *RprolaV* TRP channel and *G6PDH* genes.

Gene	Vector base code	Primer sequences	Amplicon length (bp)	Intron length (bp)
RprolaV	RPRC002111	For-TAAACCAAACAGAGTCGCCC/Rev-TAATGGGTCTGGTGGTGAGT	106	Intron –Exon junction
RproG6PDH	R4G5X8 (UniProtKB/TrEMBL access. number)	For-AGCCTGGAGAAGCGGTTTACGTTA/Rev-GTGAGCCACAGAATACGTCGAGT	162	923

consecutive stimulation cycles in which the bugs were exposed to the Peltier element for 10 s at 35 °C followed by 50 s at 25 °C. Every time the proboscis of an insect was observed to be fully extended a PER event was recorded. The number of PERs elicited by bugs was recorded for the different experimental series and the proportion of bugs that elicited 0–3 PERs was calculated for each treatment.

2.2.4. Thermal preference in a temperature gradient

To investigate whether the thermal preference of triatomines is affected by capsaicin and capsazepine, bug thermopreference was studied in a thermal gradient. The gradient was generated by using a graded aluminium surface (12 × 50 cm) attached to a resistor (230 V/65 W) in one extreme and connected to a voltage regulator that allowed changing the maximum temperature. The gradient was set to keep a maximum temperature of 35 °C on one thermal extreme and a minimum of 25 °C on the cold side. Room temperature remained constant (20.5 ± 1 °C) throughout the experiments.

Individuals were injected as above with pCap solution (341 μM, $N = 29$) or with Cpz solution (26.5 μM, $N = 22$) and their position in the thermal gradient was recorded over time. Controls for each treatment were performed using insects injected with Ringer solution (pCap $N = 29$, Cpz $N = 22$). Bugs were injected and immediately released individually at the warmest extreme of the gradient and their position was recorded every 30 min during two hours. Insect locations were converted to temperature values applying a third-grade polynomial function that was constructed measuring the temperature of the gradient every 10 cm with a type k thermocouple. Since bug thermopreference varies along the daily cycle, all assays were performed during the photophase and under constant low intensity illumination (Lazzari, 1992).

2.2.5. Spatial learning in the hot-box

In order to test whether pCap and Cpz modify the learning capabilities of *R. prolixus*, an operant conditioning protocol was applied in a hot-box experimental design (Wustmann et al., 1996). Insects were randomly assigned to one of four treatments: 10 μl injection 341 μM capsaicin, injection of 26.5 μM capsazepine, injection of Ringer solution and no injection.

The experimental device consisted of a twin-chamber Plexiglas box (5 × 2 × 3.5 cm) in which two individual bugs were simultaneously tested (no visual contact). The floor of the chambers was made of a Peltier element (4 × 4 cm, 12 V, 72 W, QuickCool, Wuppertal, Germany) connected to a controller (Peltron Gmbh Peltier-Technik, Germany) that allowed presenting fast temperature changes. Additionally, a manual switch allowed setting up two different temperatures. The temperature of the Peltier surface was monitored by means of a PT-1000 thermocouple covered by conductive material to promote thermal conductivity. The opposite side of the Peltier element was placed over a cooling system that allowed heat dissipation (20 °C/4–5 s). The experiments were performed in darkness and monitored with the aid of a small CCD camera (lambda = 900 nm, invisible to the insects, Reisenman et al., 1998) to track insect movement. At the beginning of each experiment, a thin sheet of filter paper was placed on the hot surface of the Peltier element to prevent chemical contamination by bugs. A line perpendicular to the major axis of each chamber was drawn on the filter paper, virtually defining two zones: "punishment" and "no-punishment" sides. This device allows testing insect cognitive abilities. In brief, the position of an insect was monitored and whenever it crossed the line separating both sides and entered the zone previously defined as punished, the temperature of the chamber was raised (punishment), while whenever the insect returned to the no-punishment side, the temperature was lowered.

Experiments consisted of a one minute pre-training phase (at 25 °C) during which the position of each bug was recorded, followed by an 8 min training phase. Individuals were released on the middle zone of each chamber and their position was monitored. For standardization purposes, a zone-change event was defined when the whole head of an individual crossed the line from one side of the chamber to the other, as the antennae of these insects are expected to hold most surface thermoreceptors. Whenever an insect crossed to the punishment side, the temperature of the chamber was raised to 45 °C, and every time it returned to the no punishment side, the temperature was decreased to 25 °C. For every trial, two insects were released simultaneously, one in each chamber. In one chamber, a conditioned insect received thermal shocks (45 °C) whenever its head crossed to the punishment side. In the other chamber, a pseudoconditioned insect was exposed to punishment every time the first insect entered the punishment side. In this way, the pseudoconditioned insect was punished irrespective of its own position. For each trial, the time spent on the punishment and no punishment sides was recorded by means of an event-recording software (Event recorder 1.2.4).

2.3. Data analyses

2.3.1. PER

For each treatment, the proportion of insects performing three consecutive PERs was analysed with tests of homogeneity of proportions which are multiple Tukey type comparison tests (Zar, 2010). When significant differences were found, *a posteriori* comparisons of the proportion of insects performing 3 PERs were performed between insects injected with different concentrations of pCap or Cpz and those injected with Ringer solution, i.e., the control series. This procedure is analogous to the Dunett's test but applied when proportions are used (Zar, 2010).

2.3.2. Thermal preference in a temperature gradient

In order to determine the preferred temperature of bugs injected with pCap and Cpz, compared to the control, *t*-tests (Zar, 2010) were performed for each time recorded (30, 60, 90 and 120 min).

2.3.3. Spatial learning in the hot-box

The learned spatial preference of insects was estimated using a Performance Index (PI) that was calculated as the subtraction between the time insects stayed in the no-punishment (T(25), chamber temperature 25 °C) and punishment sides (T(45), chamber temperature 45 °C), relative to the total assay time, as indicated by the following equation:

$$PI = T(25) - T(45)/T(25) + T(45)$$

PI ranges from −1 (avoidance of the no-punishment side) to 1 (preference of the no-punishment side). A value near 0 indicates that the insect spent the same amount of time in each zone, therefore evincing a lack of spatial preference.

In order to assess whether a significant conditioning had occurred, the mean PI (of the Conditioned Group) calculated for the last minute of training was compared against that of the first minute of training, the last minute of training of the pseudoconditioned group and zero. Mean differences in the PI of the experiments were compared by means of non-parametric statistics because tests for normality yielded mixed results. Pairs of independent groups were compared by Mann–Whitney tests. The test of Wilcoxon was applied to compare the mean PI against zero and to compare pairs of dependent groups.

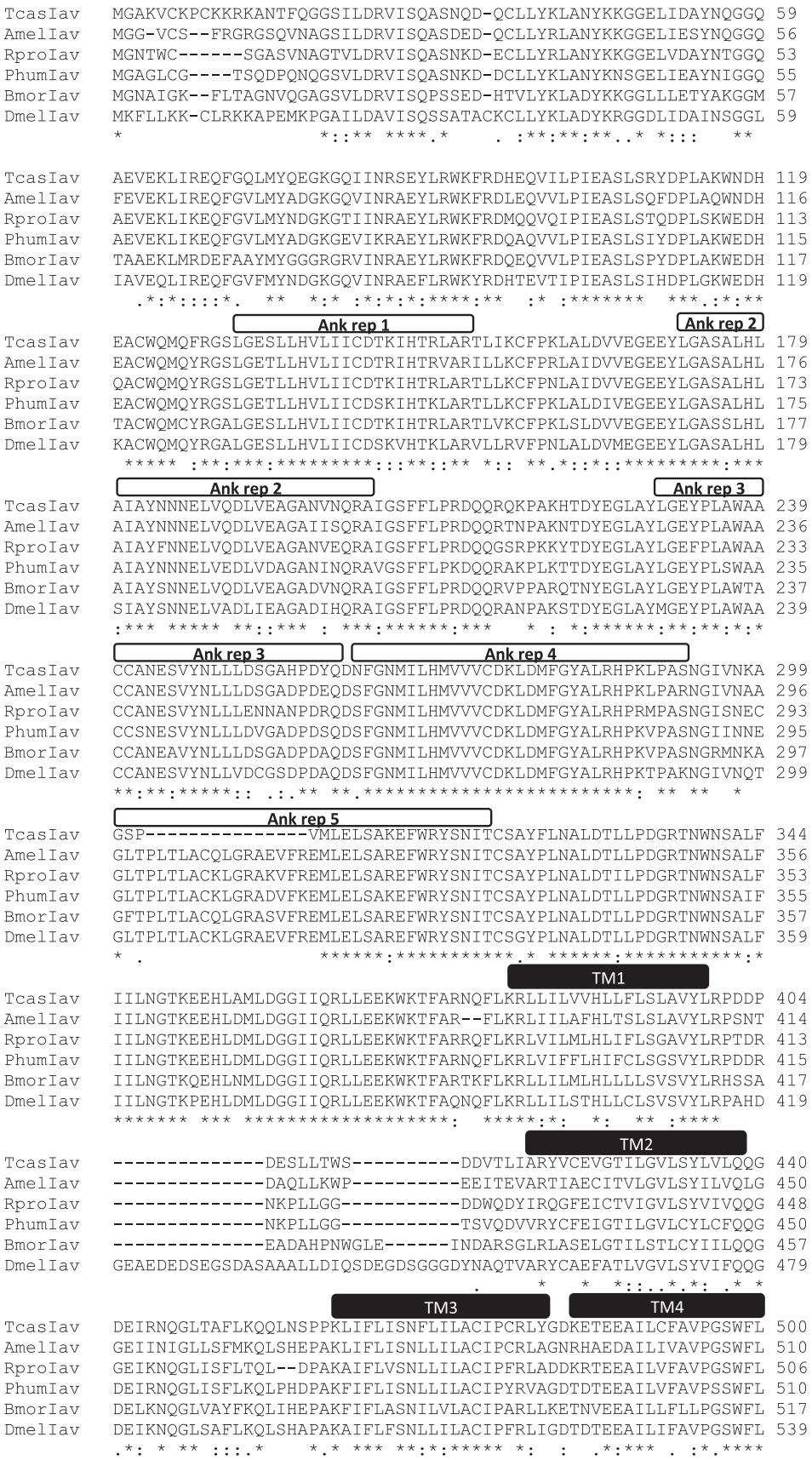

Fig. 1. Alignment of the protein sequence of Rprolav with orthologous sequences from other insects. Asterisks indicate identical amino-acids, double points show conserved exchanges and single points show homologous amino acids. Light grey boxes indicate the location of the ion transport domain. Black and white boxes indicate the locations of transmembrane domains (TM) and ankyrin repeats (Ank rep). The location of the pore region was established based on Kim et al. (2003) and is indicated by with a black dotted line All sequences features were proposed using their location in *Dmellav* as reference. Species abbreviations: Tcas = *Tribolium castaneum*; Amel = *Apis mellifera*; Rpro = *Rhodnius prolixus*; Phum = *Pediculus humanus*; Bmor = *Bombyx mori* and Dmel = *Drosophila melanogaster*.

Fig. 1 (*continued*)

```
TcasIav   QSSSTDT---VNNEIN-EKNIENSDLDYAEERIKLVKESLKQ-------VVDVAQIRP-- 1021
AmelIav   SSTSMDRNPRYQSLLNGHENSIDRPIESRECSIEAINSQIKQNGPCETMKAKVQKKRPKT 1083
RproIav   NGSTIDTKINLLSWMYKSGNSSASSADNDPPPYTPLPTAPP------IIKRKSMKRPKT 944
PhumIav   SSSTNSEDFSCLTENYINKNIKVN-ANFSENSLRSNRSSANL------VVKKFGRRSGLK 995
BmorIav   VVPEIQQSNENQKPEQVHQDYLRELIVLAEKPATTNLELKQL-------AEKAADLRDVP 944
DmelIav   SSVWAPPGLDVDTGFHFDEAVAEEVLTIEQEAEVETEDGNGGQDSEDIPTAEEVHATMKQ 1047
                                .

TcasIav   ---INIDVALEEQVSVTISETMRVQGSGDGAEVQSSNVK--PKRKKRSKTAKNNK----- 1071
AmelIav   ARNRCINLQHADLDDKDIETILHWHNTYRNTVASGKEIRGNPGPQRPAKFMMEVMWDDEL 1143
RproIav   AKPNRVAPEPEVPP-RPGSSAVPASEIKRQNSPPDPLEPWSTREITNMNAILAWQPSDQD 1003
PhumIav   SSTNRIAPADLSPVGNVSKYSETLCNSYCITSSSDVLYQWSIKGITNMNTLLGLE--NED 1053
BmorIav   EIDININMAAKSARKMVAGAVSGLFGVAADTPAPDAGWRRDRHDNSDSDPISGTI----- 999
DmelIav   FHLRKCQPAQDEAARRAKSARVRRRNKVSPEQSDDPDERSQRGRSAYTRRTQSPPDPLEP 1107
                                                  .

TcasIav   ----------------------
AmelIav   ALIARRWVVQCNLLEKDQCRDVGK 1167
RproIav   SM-------------------- 1005
PhumIav   SM-------------------- 1055
BmorIav   ----------------------
DmelIav   WSTRELQDINKILARK-------- 1123
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Fig. 1 (*continued*)

3. Results

3.1. Genetic analysis

3.1.1. Structural and phylogenetic characterisation of the R. prolixus inactive TRP channel sequence

The protein sequence of *RproIav* presented ion transport (InterPro Database code: IPR005821) and ankyrin repeat-containing domains (InterPro Database code: IPR020683). Specifically, *RproIav* presented a total of five ankyrin repeats. Both, TOPCONS and TMHMM, indicated that a total of six transmembrane domains exist in the sequence. As expected, no signal peptide was found for *RproIav*.

BLASTP analyses of protein sequence alignments showed that RproIav has a 74% sequence identity with the Iav sequences of *D. melanogaster* and *A. mellifera*; 71% with that of *B. mori*; 70% with *T. castaneum* Iav and 63% with that from *P. humanus*. The alignment of RproIav with orthologous sequences showed the low conservation degree of this gene at the intracellular C-terminus region (Fig. 1) with only 25% of its residues conserved. On the other hand, the intracellular N-terminus (including the ankyrin repeats) and the six transmembrane domains showed 82% of its residues conserved (Fig. 1).

The LG model amino-acid replacement matrix (Le and Gascuel, 2008) was the best fit model for protein evolution. The phylogenetic tree is composed of two clades of TRPV receptor sequences, one including insect Iav sequences and other with mammal TRPV1 sequences, with an outgroup composed of insect and mammal TRPA1 sequences (Fig. 2). RproIav grouped together with those of the other hemimetabolous insects, suggesting a proper characterisation of the predicted protein sequence of these bugs.

3.1.2. Expression pattern in putative adult sensory tissues

The detection of expression of the *RproG6PDH* gene by RT-PCR in adult tissues confirmed the integrity of all cDNAs produced (Fig. 3). Fig. 3 shows that all sensory structures tested (antennae, rostri, tarsi, tibial pads and genitalia) presented clear evidence of expression of the *RproIav* channel gene.

3.2. Behavioural responses

3.2.1. PER

In this experiment, we expect bugs to be less responsive to heat stimuli when injected with pCap, as the drug lowers the activation threshold of its receptor but the insect's PER response depends on the perception of a difference between the ambient and the stimulus temperatures. Given that capsazepine has an opposite mode of action, we expect that bugs injected with Cpz will show a higher responsiveness.

The proportion of insects eliciting three consecutive PERs depended on the concentration of the drug injected. The proportion of insects that elicited three consecutive PERs was lower in bugs injected with pCap (34.1 μM, 0.308, $Q = 3.833$, $P < 0.01$; 341 μM, 0.467, $Q = 2.469$, $P < 0.05$) compared to controls (0.714). This reduction in the response was not significant with the lowest concentration of the drug (3.41 μM, 0.786, $Q = 0.837$, $P > 0.05$). In the case of injections with Cpz, three different results were found. When insects were injected the highest concentration of this compound (265 μM), the proportion of bugs presenting three consecutive PERs was lower than that of control insects (0.200, $Q = 5.845$, $P < 0.01$). However, when insects were injected 26.5 μM Cpz of, the proportion of bugs that elicited three consecutive PERs was higher than that of controls (0.875, $Q = 2.019$, $P < 0.05$). Finally, these differences in responses compared to control bugs were not significant with 2.65 μM Cpz (0.786, $Q = 0.580$, $P > 0.05$). In light of these results, the following experiments were performed injecting 341 μM pCap of and 26.5 μM Cpz.

3.2.2. Thermal preference in a temperature gradient

Given the action mode of the drugs injected (i.e., pCap lowers the channel activation threshold, while Cpz does the opposite), we expect bugs injected with pCap to choose higher temperatures in the thermal gradient and bugs injected with Cpz to choose lower temperatures.

Bugs injected either pCap or Cpz had different thermopreference than those in the Ringer control group. The effect of both, pCap and Cpz, was evident 90 min after injection and lasted for at least 120 min.

After 90 min, insects in the pCap injected group preferred higher temperatures than those in the Ringer control group (>2.6 °C difference, $t_{0.01(1),28} = 3.08$, $P < 0.01$, Fig. 4). Conversely, 90 min after injection Cpz injected insects preferred lower temperatures than those in the Ringer control (>1.8 °C difference, $t_{0.01(1),21} = 3.67$, $P < 0.01$, Fig. 4).

3.2.3. Spatial learning in the hot-box

In spatial learning experiments, we expect that bugs injected with pCap will not be able to learn under a heat

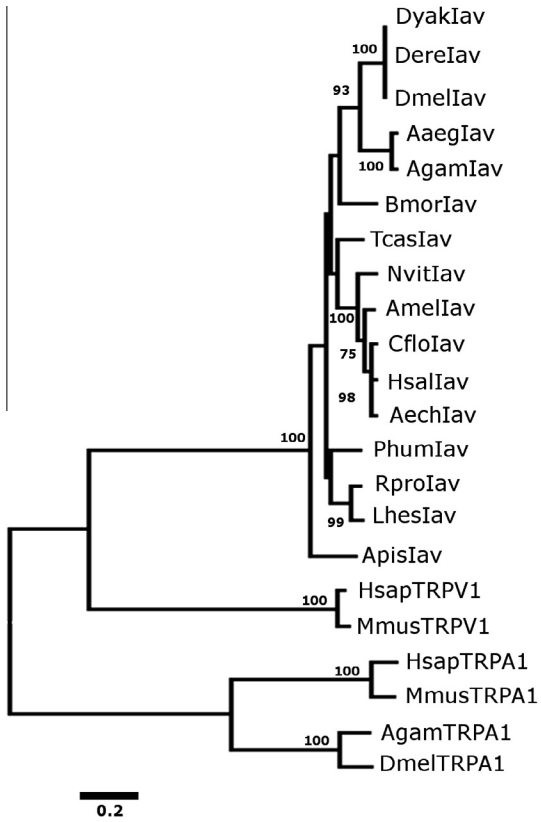

Fig. 2. Molecular phylogenetic analysis of Iav, TRPV1 and TRPA1 protein sequences by the maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method based on the Le and Gascuel (2008) model. The tree with the highest log likelihood (−11048251) is shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Percentages higher than 70 are indicated by numbers. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a LG model. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was rooted using TRPA sequences as the outgroup. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Species abbreviations (accession number to UniProtKB database): AaegIav = *Aedes aegytpi* (Q16WC0); AechIav = *Acromyrmex echinatior* (F4WYD6); AgamIav: *Anopheles gambiae* (Q7QFD0); AgamTRPA1 = *Anopheles gambiae* (B3G3I7); AmelIav = *Apis mellifera* (A0A087ZPY6); ApisIav = *Acyrthosiphon pisum* (J9JUJ3); BmorIav = *Bombyx mori* (H9J469); CfloIav = *Camponotus floridanus* (E2AXW0); DereIav = *Drosophila erecta* (B3NXW2); DmelIav = *Drosophila melanogaster* (Q9W3W0); DmelTRPA1 = *Drosophila melanogaster* (Q7Z020); DyakIav = *Drosophila yakuba* (B4PZ84); HsalIav = *Harpegnathos saltator* (E2BHT3); HsapTRPA1 = *Homo sapiens* (O75762); HsapTRPV1 = *Homo sapiens* (Q8NER1); LhesIav = *Lygus hesperus* (A0A0A9Y583); MmusTRPA1 = *Mus musculus* (Q8BLA8); MmusTRPV1 = *Mus musculus* (Q704Y3); NvitIav = *Nasonia vitripennis* (K7IWA0); PhumIav = *Pediculus humanus* (E0W1R2); RproIav = *Rhodnius prolixus* and TcasIav = *Tribolium castaneum* (D6WNB8).

stimuli/punishment protocol, since they would not be able to sense high temperatures as different from room temperatures. In contrast, we expect insects injected with Cpz to show a similar or higher learning performance as compared to control animals.

Insects belonging to both conditioned control groups, intact and Ringer injected, gradually improved their performance in the hot-box with time, i.e., learning to avoid the punishment. Their Performance Index (PI) was significantly increased from t1 (first minute in the hot-box) to t8 and was different from a PI of 0 (Table 2 and Fig. 5). The same was observed in conditioned bugs injected with Cpz (Table 2 and Fig. 5). Conversely, bugs previously injected with pCap did not show any sign of spatial learning, their performance not differing along the training interval and from a PI of 0 (Table 2 and Fig. 5). Moreover, the last minute training PI of pCap injected insects was lower than that of Ringer injected insects (Table 2 and Fig. 5). On the contrary, the last minute training PI of Cpz injected insects was similar to that scored for Ringer injected insects, thus reinforcing the results previously obtained (Table 2 and Fig. 5). The PI calculated for all pseudoconditioned insects was similar to that of control group bugs, independent of treatment.

4. Discussion

In this work we aimed to initiate the study of the genetic basis of the thermal sense of *R. prolixus*, one of the main vectors of Chagas disease. We tested the hypothesis that a TRPV (transient receptor potential vanilloid) channel is involved in the evaluation of heat in this species by adopting two different approaches: using basic bioinformatics and molecular biology we characterised the gene structure of *RproIav* and analysed its expression on diverse sensory structures and, from a second approach, we tested thermally-triggered behavioural responses in bugs injected with either of two molecules known to interact with mammal TRPV1 (capsaicin and capsazepine).

Initially, the characteristic structural and functional features known for the TRPV gene subfamily (Venkatachalam and Montell, 2007) were found in RproIav, confirming its proper functional annotation (Fig. 1). The N-terminal region and the six transmembrane domains which contain the channel domain were the most conserved parts of the RproIav sequence. Interestingly, the N-terminal intracellular domain of TRPV1 is apparently necessary for the recognition of capsaicin and noxious temperatures (Schumacher et al., 2000). Whether this gene region is related to the formation of receptors constituted by several subunits or not deserves to be studied in the future for the *inactive* gene (Erler et al., 2004). The pore region of TRPV1 has also been indicated to be relevant for capsaicin action (Welch et al., 2000) and shows a relevant degree of sequence identity with that of DmelIav (Kim et al., 2003), which also showed a high sequence identity with RproIav (Fig. 1). The phylogenetic tree presented in this report suggests its proper characterisation, as RproIav grouped properly with those of other hemimetabolous insects (Fig. 2).

Our behavioural experiments were performed in order to test the effects of capsaicin and capsazepine on bug thermosensation. We used three different independent behavioural protocols that, together, allowed us to test general heat sensing, rather than particular context-dependent responses. All three protocols showed

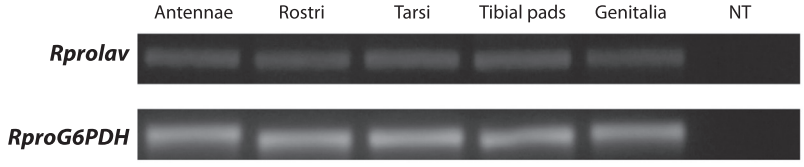

Fig. 3. *RproIav* expression profiles in different *Rhodnius prolixus* tissues. Reverse transcription PCR (RT-PCR) was performed using specific primer pairs and cDNAs from different adult tissues: antennae, rostri, tarsi, tibial pads and genitalia. PCR products were analysed on agarose gel. *RproG6PDH* was used as a reference of quality and quantity for all cDNAs.

Table 2
Results (statistic and *p*) of the performance of *Rhodnius prolixus* under the conditioning protocol for conditioned insects. Comparisons between independent groups were performed with the Mann–Whitney *U* test, while the Wilcoxon test was used for comparisons between non-independent groups. C and PS stand for the conditioned and the pseudoconditioned groups, pCap: capsaicin, Cpz: capsazepine.

	No injection	With injection		
		Ringer	pCap	Cpz
	Statistic; *p*	Statistic; *p*	Statistic; *p*	Statistic; *p*
Last minute training vs. 0	U = 20; <0.01	U = 40; <0.01	U = 180; n.s	U = 0; <0.01
Last minute training C vs. last minute training PS	U = 100; <0.01	U = 113; <0.01	U = 188; n.s	U = 45;<0.01
1° min training vs. last minute training	W = 0; <0.01	W = 0; <0.01	W = 101; n.s	W = 5; <0.01
Last minute training vs. last minute training ringer	–	–	U = 76; <0.01	U = 162; n.s

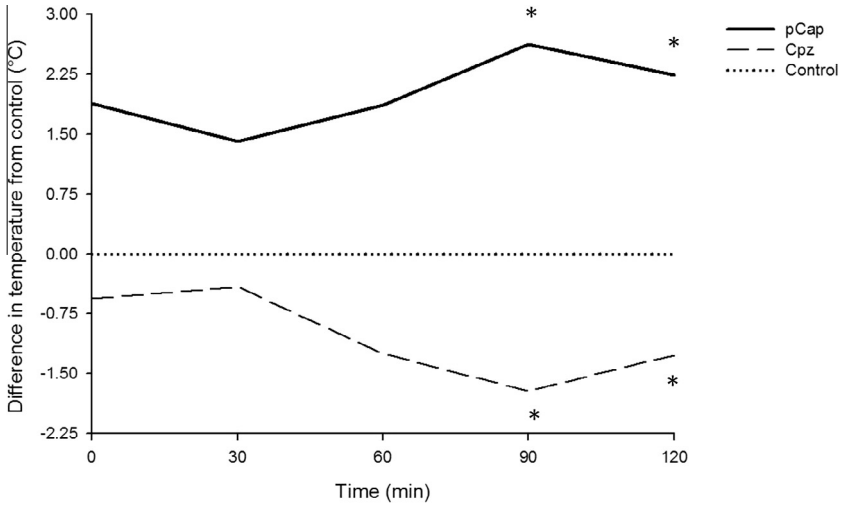

Fig. 4. Changes in preferred temperature induced by injection of 10 μl of 341 μM capsaicin (pCap) or 26.5 μM capsazepine (Cpz), in relation to the choice of control insects (injected with Ringer solution). Choices were represented at 30 min intervals during 120 min experiments. *Significant difference.

that insects treated with capsaicin are less responsive to heat, while insects treated with capsazepine show the opposite effects. We found that responses are dose-dependent and that high concentrations of any of the drugs (data not shown) are lethal, which, at least for capsaicin, is consistent with previous observations of this drug more broadly affecting organisms when applied in high doses (i.e., it alters the consistency of biological membranes, Lundbaek et al., 2005).

One of the behavioural tests we used was the proboscis extension reflex (PER), in which the insects were presented with a source of heat in an appetitive context (Vinauger et al., 2013). When injected with capsaicin, insects showed a decrease in their responsiveness (i.e., extend their proboscis to bite the warm object). In triatomines, PER is driven by stimulation of antennal thermoreceptors (Ferreira et al., 2007), and hence our results suggest that these were blocked by *pCap*. When injected with the highest concentration of capsazepine insects were less responsive than those in the control group, and we attribute this fact to secondary effects of the drug, as the majority of these insects died within 24 h after the experiment. Insects injected with the intermediate concentration of the drug were more responsive to heat, which suggests that capsazepine is also interacting with antennal receptors, but in the opposite fashion. Our results are consistent with previous findings of the antagonistic effect of capsaicin and capsazepine on mammalian TRPV1 and on insects (i.e., *T. molitor* larvae, Bevan et al., 1992; Gonzalez-Reyes et al., 2013; Olszewska and Tegowska, 2011; Walpole et al., 1994).

Our second behavioural protocol tested thermopreference in a gradient. We found that injection of capsaicin made insects prefer higher temperatures while injection of capsazepine induced the opposite effect. It has been shown that temperature preference in triatomines is species-specific and varies along the daily period and with nutritional status (Guarneri et al., 2003; Lazzari, 1991; Pires et al., 2002; Schilman and Lazzari, 2004). Although, for *R. prolixus* in particular, Schilman and Lazzari (2004) showed that differences in preferred temperatures in different sexes, day times and degrees of starvation are small (i.e., less than 1 °C). The alterations of the preferred temperatures observed under the effects of these drugs were greater than 2.6 °C and 1.8 °C (with capsaicin and capsazepine, respectively). Hence, our results are independent of those factors normally affecting thermal preference in this species and the effects evinced seem to be produced by the action of the drugs. The thermal preference of triatomines is thought to be driven by antennal receptors, as well as by receptors in other parts of the body (Lorenzo Figueiras et al., 2013). Therefore, we suggest that, given that our candidate channel gene is expressed in several body parts bearing sensory structures, the drugs could be generally affecting the response of these receptors.

Finally, our third behavioural approach consisted of inducing operant conditioning in a spatial learning paradigm. Here again, we found that capsaicin and capsazepine promoted opposite effects on the thermally-mediated behaviour of the insects. Capsaicin injected individuals were not capable of learning to avoid thermal punishment. Although we cannot discard the possibility of the drug affecting other neuronal pathways involved in the learning process, our results, together with those of the two previous behavioural protocols, suggest that insects were not able to learn to avoid the thermal punishment because their thermal sensing was impaired by the drug.

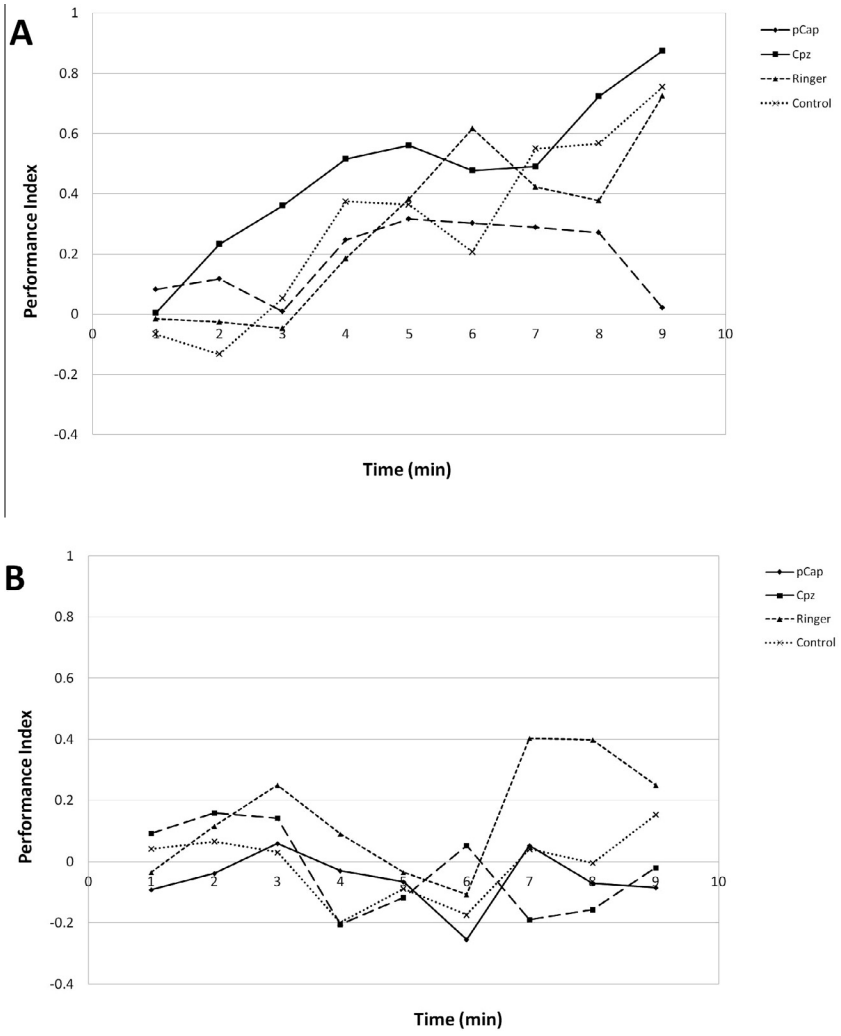

Fig. 5. Performance Index (PI) over time in *R. prolixus* individuals subjected to (A) conditioning and (B) pseudoconditioning thermal learning protocols after injection of 10 μl of 341 μM capsaicin, 26.5 μM capsazepine or Ringer solution. "Control" refers to performances of bugs that were not injected. PI is the difference between time spent in the unpunished zone and that spent in the punished zone. Training phase extends between minutes 2 and 9, while minute 1 corresponds to the pre-training phase.

Our study began to unravel the molecular bases of thermally-mediated behaviours in blood-sucking bugs. Triatomine bugs have one of the most sensitive temperature detection systems, which is directly related to their blood-sucking lifestyle by mediating host-location, but little is known about the molecular pathways controlling heat-related behaviours (Lazzari, 2009). We found that *R. prolixus inactive* gene, which is relatively conserved in comparison to orthologous genes of other insects, is expressed in diverse bug tissues bearing sensory structures (Matsuura et al., 2009). Subsequently, we found that compounds that are known to interact with TRPV1 channels (namely capsaicin and capsazepine) induced altered responses in different contexts in which thermal stimuli are crucial for the insects: biting, environmental thermal choices and aversive learning. Given the similarities in the structure of mammal TRPV1 and *RproIav*, and that capsaicin acts on TRPV1 by binding directly to an intracellular binding site on the channel that is apparently similar in *RproIav*, this gene is a good candidate for being the target of the drugs tested (O'Neil and Brown, 2003). Furthermore, we suggest that TRPV participates, alone or together with other TRP channels still uncharacterised in this species, in mediating triatomine thermosensation, being its action modulated by these compounds in a similar fashion as with mammal TRPV1.

Although the structural homologies among TRPs do not necessarily correlate with their functions, certain groups are known to be involved in particular pathways (Huang, 2004). Temperature sensing, for instance, is related to channels belonging to three different TRP subfamilies: TRPM, TRPA and TRPV (Fowler and Montell, 2013). While TRPVs mediate mammal responses to high temperature, in insects this function has only been associated with TRPAs (Dhaka et al., 2006; Fowler and Montell, 2013; Hamada et al., 2008; Lee et al., 2005; Neely et al., 2011; Ramsey et al., 2006; Tracey et al., 2003; Wang et al., 2009). Although further research is needed to understand the molecular mechanisms underlying the interaction between receptor channels and the compounds used in our study, this work constitutes the first evidence of a TRPV channel, *RproIav*, mediating thermosensation and temperature responses in an haematophagus insect.

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