Insect Biochemistry and Molecular Biology 69 (2016) 82–90

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Silencing the odorant receptor co-receptor *RproOrco* affects the physiology and behavior of the Chagas disease vector *Rhodnius prolixus*

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ARTICLE INFO

Article history:
Received 30 November 2014
Received in revised form
9 February 2015
Accepted 21 February 2015
Available online 4 March 2015

Keywords:
Rhodnius prolixus
Odorant co-receptor
Orco
Interfering RNA

ABSTRACT

Olfaction is one of the main sensory modalities that allow insects to interpret their environment. Several proteins, including odorant-binding proteins (OBPs) and odorant receptors (ORs), are involved in this process. Odorant receptors are ion channels formed by a binding unit OR and an odorant receptor co-receptor (Orco). The main goal of this study was to characterize the *Orco* gene of *Rhodnius prolixus* (*RproOrco*) and to infer its biological functions using gene silencing. The full-length *RproOrco* gene sequence was downloaded from VectorBase. This gene has 7 introns and is located in the genome SuperContig GL563069: 1,017,713−1,023,165. *RproOrco* encodes a protein of 473 amino acids, with predicted 7 transmembrane domains, and is highly expressed in the antennae during all *R. prolixus* developmental stages. The RNAi technique effectively silenced *RproOrco*, reducing the gene's expression by approximately 73%. Interestingly, the effect of gene silencing persisted for more than 100 days, indicating a prolonged effect of dsRNA that was maintained even after molting. The phenotypic effects of silencing involved the following: (1) loss of the ability to find a vertebrate host in a timely manner, (2) decreased ingested blood volume, (3) delayed and decreased molt rate, (4) increased mortality rate, and (5) decreased egg laying. Our data strongly suggest that dsOrco disrupts *R. prolixus* host-finding behavior, which is further reflected in the blood ingestion, molting, mortality, and egg laying data. This study clearly demonstrates that *Orco* is an excellent target for controlling triatomine populations. Thus, the data presented here open new possibilities for the control of vector-borne diseases.

1. Introduction

Olfaction is one of the most important sensory modalities in insects. Behaviors such as looking for food, mating, choosing oviposition sites, escaping from predators and others depend almost exclusively on the correct interpretation of chemical signals from the environment. The olfactory systems of insects, completely differs from those of vertebrates (Benton, 2006). In insects, odorants are captured by antennae pores, and once inside the antennae, odorants bind to odorant-binding proteins (OBPs) and are then transported to odorant receptors (ORs) that are expressed on the membrane of olfactory receptor neurons (ORNs) (Leal, 2012; Suh et al., 2015). A functional OR consists of a heterodimer formed by a specific OR and a ubiquitous co-receptor named odorant receptor co-receptor or Orco (Larsson et al., 2004; Vosshall and Hansson, 2011). *Orco* has been described in different insect species as a unique gene that is characterized by an extremely highly conserved seven transmembrane domain (7TM) amino acid sequence; specifically, the co-receptor shares up to 94% sequence identity with orthologs in other insects (Abdel-Latief, 2007; Krieger et al., 2003; Melo et al., 2004; Pitts et al., 2004; Xia and Zwiebel, 2006; Yang

Abbreviations: Orco, odorant receptor co-receptor; OR, odorant receptor; OBP, odorant-binding protein.

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http://dx.doi.org/10.1016/j.ibmb.2015.02.012

et al., 2012). This high identity suggests that Orco exhibits similar functions across different insect taxa. In fact, Orco is suggested to be responsible for the OR adopting the correct structure, and it also works as a selective ion channel during olfactory signal transduction (Jones et al., 2012; Martin and Alcorta, 2011; Stengl and Funk, 2013). Recent studies have demonstrated that any disturbance in *Orco* expression induces a complete disruption in the insect olfactory system (DeGennaro et al., 2013; Liu et al., 2010; Zhao et al., 2010; Zhou et al., 2014; Zhu et al., 2013).

RNAi is a powerful tool that is used to understand various aspects of insect physiology (Sanchez-Vargas et al., 2004). By silencing different genes, the roles of diverse proteins in the olfactory signaling pathways have been unraveled (Maleszka et al., 2007; Pelletier et al., 2010; Swarup et al., 2011; Zhao et al., 2010; Zhou et al., 2014).

Rhodnius prolixus is an important vector of the protozoan *Trypanosoma cruzi*, the etiological agent of Chagas disease, which annually affects 18 million people in Latin America (WHO, 2013). The control of vector borne-disease populations faces various challenges, including but not limited to resistance to several insecticides (Srinivas et al., 2004; Vassena et al., 2000; Vontas et al., 2012) and environmental concerns regarding use of pesticides. Thus, it is important to identify a safe alternative for controlling harmful insect species be them agricultural pests or insect of medical importance (Leal, 2014). Based on the importance of *R. prolixus* as a vector of Chagas disease, the *R. prolixus* genome project began in 2005, and *R. prolixus* sequences are now available for download through VectorBase website (Lawson et al., 2007, 2009).

Various studies have demonstrated that *Rhodnius* uses numerous compounds as chemical signals (Ferreira et al., 2011; Otalora-Luna and Guerin, 2014; Pontes et al., 2008); however, the mechanism by which this chemical signaling works in this species remains unknown. Due to the high degree of conservation of the *Orco* gene sequence throughout the insect order, we were able to identify a unique putative *RproOrco* gene within the *R. prolixus* genome by using orthologous sequences as a query. Furthermore, the *Orco* expression profile was evaluated in different tissues of *R. prolixus* and was further analyzed using qPCR. The role of Orco in some aspects of *R. prolixus* behavior, including host finding, blood ingestion, ecdysis, mortality, and egg laying, was investigated using RNAi-mediated gene silencing.

2. Materials and methods

2.1. Rhodnius prolixus maintenance and ethics statement

R. prolixus adults and nymphs were maintained at 28 °C and 80–90 % relative humidity in the Insect Biochemistry Laboratory at the Institute of Medical Biochemistry at the Federal University of Rio de Janeiro, Brazil. Adult insects were fed on rabbit blood at 3-week intervals; 1st, 2nd, 3rd, and 4th instar nymphs were fed on rabbit blood at 4-week intervals; and 5th instar nymphs were fed on blood at 5-week intervals. All animal care and experimental protocols were conducted according to the guidelines of the institutional care and use committee (the Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro, CAUAP-UFRJ, Brazil). The protocols were approved by CAUAP-UFRJ under registry # IBqM001.

2.2. Phylogenetic analysis

Orco amino acid sequences from different insect species were used to create an entry file for phylogenetic analysis in MEGA 6 (Tamura et al., 2013). An unrooted consensus neighbor-joining tree (Saitou and Nei, 1987) was calculated using the default settings with pairwise gap deletions. Branch support was evaluated by bootstrap analysis based on 1000 replicates.

2.3. Alignments

Primary amino acid sequences from different insects were aligned using the MultAlin software package (Corpet, 1988). The GenBank accession number of each of the sequences that was used is indicated in parentheses: *R. prolixus* (RPRC000476-PA), *Tribolium castaneum* (gi|226334904), *Holotrichia parallela* (gi|334700348), *Phyllotreta striolata* (gi|187942541), *Culex quinquefasciatus* (gi|78172236), *Aedes aegypti* (gi|157111190), *Anopheles gambiae* (gi|118782595), *Stomoxys calcitrans* (gi|193795139), *Haematobia irritans irritans* (gi|193795141), *Bactrocera cucurbitae* (gi|302171921), *Drosophila melanogaster* (gi|24644231), *Microplitis mediator* (gi|119888030), *Ceratosolen solmsi marchali* (gi|164370696), *Nasonia vitripennis* (gi|283436213), *Apocrypta bakeri* (gi|164370698), *Philotrypesis pilosa* (gi|164370700), *Bombus terrestris* (gi|340728952), *Apis mellifera* (gi|201023349), *Solenopsis invicta* (gi|322790267), *Harpegnathos saltator* (gi|307206100), *Camponotus floridanus* (gi|307183325), *Acromyrmex echinatior* (gi|332023407), *Heliothis virescens* (gi|22293485), *Mythimna separata* (gi|205361592), *Spodoptera litura* (gi|111434281), *Ostrinia scapulalis* (gi|229365451), *Diaphania indica* (gi|205361598), *Plutella xylostella* (gi|226001153), *Epiphyas postvittana* (gi|211909313), *Argyresthia conjugella* (gi|327420424), *Bombyx mori* (gi|112983084), *Mamestra brassicae* (gi|44977310), *Antheraea pernyi* (gi|32399809), *Sitobion avenae* (gi|253946856), and *Acyrthosiphon pisum* (gi|328723528).

2.4. RNA isolation, cDNA synthesis, semi-quantitative PCR, and cloning

Total RNA (t-RNA) was extracted from the antennae, proboscides, legs, and fat body of 5th instar nymphs (male and female) using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was then eluted into nuclease-free water. The concentration of the t-RNA was estimated at 260 nm (Lee and Schmittgen, 2006) using a SmartSpect plus spectrophotometer (Bio-Rad, Hercules, CA, USA). One microgram of t-RNA was treated with 1 U of RNase-free DNase I (Fermentas, Burling Canada) for 30 min at 37 °C. Then, the reaction was stopped with 50 nmol ethylenediaminetetraacetic acid at 65 °C for 10 min. First-strand cDNA synthesis was then performed using 1000 ng of RNA, the RevertAid H Minus First-Strand cDNA kit and random primers (Fermentas). PCR was performed using the AccuPower PyroHot-Start Taq PCR Pre-Mix (Bioneer, Alameda, CA, USA) with approximately 25 ng/μL cDNA. Specific primers (0.4 μM) were used to amplify a 1420-bp fragment corresponding to the full-length *Orco* cDNA and a 115-bp fragment corresponding to *R. prolixus* ribosome protein 18 (*Rp18S*; accession#AJ421962.1) (Majerowicz et al., 2011) as a reference gene. All primer sequences used in this paper are listed in Table 1. Semi-quantitative PCR was performed for *RproOrco* using the following thermocycler parameters: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 90 s; followed by 72 °C for 10 min. Twenty-five cycles of PCR amplification were used for the reference gene. The PCR products were analyzed on a 1% agarose gel stained with ethidium bromide and visualized using a DNR MiniBis Pro Bio-imaging Systems (BioAmerica Inc., Miami, FL, USA). For *RproOrco* cloning, the PCR products were purified using a QIAquick PCR purification kit (Qiagen, Germantown, MD, USA). The purified 1420-bp fragment was then cloned into the pGEM-T Easy vector (Promega) using the manufacturer's protocol and then transformed into One Shot TOP10 (Invitrogen) cells using the heat shock method. Transformed cells were plated in selective Lysogeny Broth medium (LB) with 100 μg/mL ampicillin/IPTG/XGal and

Table 1
List of primers utilized.

Name	Primer sequence (5′-3′)	Aa sequences	PCR product size (bp)
RproOrco (F)	ATGCAGAAAGTGAAGATGCAT	MQKVKMH	1420
RproOrco (R)	ATTTCAATTGTACCAGCACCA	VLVQLK	
RproOrco RNAi (F)	TAATACGACTCACTATAGGGGGAGTTGTCAGCCGCTTTAG	ELSAAL	543
RproOrco RNAi (R)	TAATACGACTCACTATAGGGCTCTTCAGAGCCGTCGTACC	YDGSEE	
RproOrco qPCR (F)	ATGGTAATGAGTTCGACGTA	GNEFDV	135
RproOrco qPCR (R)	ACCATAAGTTCCTGCTTCTT	KKQELM	
Rp18S qPCR (F)	TGTCGGTGTAACTGGCATGT	VGVTGM	115
Rp18S qPCR (R)	TCGGCCAACAAAAGTACACA	VCTFVGR	

The underline shows the minimum promoter sequence of T7 primer. (F) Forward primer; (R) Reverse primer.

screened by colony PCR using T7 and SP6 primers. Positive colonies were grown in liquid LB medium containing ampicillin (μg/mL) at 37 °C overnight. Plasmids were extracted and purified from the transformed cells using a QIAprep Spin Miniprep Kit (Qiagen) and the sub-cloned sequences were validated by sequencing (Davis Sequencing Inc, Davis, CA).

2.5. Expression analysis by real-time quantitative PCR (qPCR)

Quantitative PCR was performed using cDNA extracted from the antennae, proboscides, legs and fat body using specific primers for the amplification of a 135-bp fragment of *Orco* and a 115-bp fragment of *Rp18S* (Table 1). To test the influence of blood meals on *Orco* expression, the insects (males and females) were divided into 2 groups, one of which received blood meals two days after emergence to the adult phase and the other of which was starved for 21 days. t-RNAs from the antennae of each group were purified, and *RproOrco* expression was analyzed by qPCR. Each PCR reaction was performed on biological triplicates, which were each run with three technical replicates using the Step One Real-Time PCR kit (Applied Biosystems, Foster City, California, USA) with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA). The qPCR experiments were performed according to the Minimum Information Required for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin et al., 2009). The *Rp18S* gene was used as a reference gene to normalize the expression levels among the samples (Majerowicz et al., 2011). Raw C_t normalized against the *Rp18S* standard values were used to calculate the relative expression levels in the samples using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The qPCR products were analyzed on 1% agarose gels followed by staining with ethidium bromide (0.5 mg/mL).

2.6. Synthesis of dsRNA

A pGEM−T Easy vector, carrying the 1420-bp fragment of the full-length *RproOrco* gene together with primers (Table 1) containing the T7 RNA polymerase promoter and terminator sequences, were used to generate a 543-bp PCR product that was used for dsRNA synthesis. The *Escherichia coli maltose-binding* gene (*MalE*) from the Litmus 28iMal plasmid (New England Biolabs, Beverly, MA, USA) was used as an unrelated gene for RNAi. The PCR products were used as templates for dsRNA synthesis using the MEGAscript RNAi kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. The dsRNAs were quantified using a spectrophotometer at 260 nm (Lee and Schmittgen, 2006), and their integrity was assessed by electrophoresis on 1% agarose gels stained with ethidium bromide. The dsRNAs were purified on a large scale using a MEGAclear kit (Ambion) and were then precipitated in 5 M ammonium acetate to yield 10 μg/μL of dsOrco and dsMalE.

2.7. dsRNA treatment

One microgram of dsOrco or dsMalE diluted in 2 μL of RNase-free water was injected into the metathoracic space between the 2nd and 3rd thoracic segments of nymphs as previously described (Mansur et al., 2014). In addition, 2 μL of RNase-free water was injected into the H_2O control group. Antennae from different groups were subjected to RNA extraction and cDNA synthesis as described previously. The transcription level of the *Orco* gene was analyzed by qPCR, with the H_2O control group considered the basal level (or 1).

2.8. Bioassay

A simple bioassay was utilized to test the ability of dsOrco and control insects to find a vertebrate host. A 20 cm cylindrical glass with a diameter of 6 cm and a filter paper (20 cm) inside was used to assess the velocity of insects running in the direction of a vertebrate, whose ear was in contact with the mesh on the top of the glass. Seven days after the injection, 5th instar male insects (dsOrco, N = 20; dsMalE, N = 20; H_2O injected, N = 20) were individually introduced to the bottom of the glass and each insect was allowed to acclimate in the glass for 1 min. The time required for the insect to approach the vertebrate was then measured for a maximum of 3 min. The ground speed (distance/time) was calculated for each insect, and insects that demonstrate no movement toward the host within 3 min were considered unable to identify the vertebrate.

2.9. DsOrco effects on blood ingestion

To test the influence of dsOrco on blood ingestion, male and female 5th instar nymphs were injected with dsRNA (dsOrco, dsMalE or H_2O) as described above. The insects were separated into ten groups for the dsOrco and H_2O control (4 insects/group, N = 20 for each condition) and into five groups (4 insects/group, N = 20) for dsMalE. Seven days after injection, the ability of the groups to engorge on blood for 1 h was assessed. The insects were weighed 2 h before the blood meal and again after the blood meals. The difference in weight (after minus before blood meals) was regarded as the amount of blood ingested by the insect. Fourteen days after ecdysis, the dsOrco and H_2O control adult insects (N = 15) were reassessed.

2.10. DsOrco effects on ecdysis, mortality, and oviposition

The development of the treated insect groups described above (dsOrco, dsMalE, and H_2O control) was observed for 25 days. The insects were monitored, noting the number that were able to undergo ecdysis and the number that died during this period. Additionally, the amount of eggs laid by the females treated with dsOrco was compared with the amount laid by the untreated females.

2.11. Statistical analysis

All data are expressed as the means ± SDs of three independent experiments. Significant differences between qPCR groups were evaluated using the ΔCt values and analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. The bioassay and phenotypic effects of the dsRNA were evaluated by ANOVA followed by Tukey's post-hoc test, and egg laying was evaluated by Student's t test. A p-value<0.05 was regarded as statistically significant. All statistical analyses were performed using the PRISM 4.0 software (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Orthologs of RproOrco and phylogenetic analysis

The *RproOrco* sequence RPRC000476-PA was downloaded directly from the VectorBase website (www.vectorbase.org). The 1420-bp fragment obtained from antennae was cloned and sequenced to confirm the full-length *RproOrco* sequence. The seven transmembrane (7-TM) domains were identified by sequence analysis in Transmembrane Helices Hidden Markov Models (THHMM) server ver. 2.2 (http://www.cbs.dtu.dk/services/TMHMM/), revealing the following information: TM-I = 47-69 amino acids (aa); TM-II = 74-96 aa; TM-III = 135-154 aa; TM-IV = 204-226 aa; TM-V = 346-368 aa; TM-VI = 378-400 aa; and TM-VII = 447-469 aa (Fig. 1A). The Orco protein exhibits extremely high homology across different insect species, including some very evolutionarily distant species, such as dipterans and hemipterans. The 7-TM domains of Orco from different insect species exhibited up to 94% homology (Hill et al., 2002; Krieger et al., 2003; Melo et al., 2004; Pitts et al., 2004), suggesting that this protein exhibits similar function in olfactory signal transduction in each of the different insect species.

A phylogenetic tree was constructed using 35 taxa. The protein accession numbers are given in the experimental procedures and Fig. 1B presents the analysis of the phylogenetic distances between the 35 insect species. As expected, *R. prolixus Orco* was rooted in the hemipteran group with *Acyrthosiphon* and *Sitobion*, both of which are phytophagous insects in the Aphididae family. Reduviidae and Aphididae diverged approximately 180 MYA (Hwang and Weirauch, 2012). *R. prolixus* is a member of the Reduviidae family of hematophagous insects, while both *Acyrthosiphon* and *Sitobion* are phytophagous insects that feed exclusively on plant sap. However, despite the extended time since the divergence that separated these species, they still exhibit high identity in the Orco sequence, suggesting that Orco has maintained its function in olfactory signaling throughout evolution.

3.2. RproOrco is highly expressed in the antennae

Initially, the expression profiles of *RproOrco* in the antennae, proboscides, legs, and fat body were investigated at both the adult and nymphs stages. As shown in Fig. 2, *RproOrco* is only highly expressed in the antennae of both males and females at levels that significantly differ those in other tissues ($p < 0.0001$). This result was not surprising, as all previous studies of *Orco* using quantitative PCR indicated that the gene is expressed almost exclusively in the antennae (Wu et al., 2013; Yang et al., 2012; Zhou et al., 2014). An exception is *Orco* (formerly known as *Or7*) of Culicidae, which is expressed in gustatory tissues in addition to the antennae (Melo et al., 2004; Pitts et al., 2004, 2014; Xia and Zwiebel, 2006). As observed in different insects (Melo et al., 2004; Yang et al., 2012; Zheng et al., 2012), *RproOrco* is also expressed during all developmental stages, from the first nymph stage to adult stages, suggesting that this receptor is important during all stages of *Rhodnius* life (data not shown).

Contrary to what has been reported for other insects (Zheng et al., 2012), *RproOrco* expression is not regulated. An interesting aspect of triatomine life is that all life stages require a vertebrate blood meal to complete the biological cycle because blood contents permit the progression from one stage to another. Therefore, finding a vertebrate to acquire food is of paramount importance to the *R. prolixus* biological cycle. Based on this characteristic of *Rhodnius* physiology, we tested the hypothesis that *RproOrco* expression is regulated by blood meals. Males and females were divided into two groups: i) a group given blood meals two days after emergence to the adult phase, and ii) a group starved for 21 days. In these experiments, t-RNAs from the antennae of each group were purified, and *RproOrco* expression was analyzed by qPCR. Our results indicated that *RproOrco* is not regulated by feeding, as both the starved and fed insects exhibited similar expression profiles (Fig. 3). Interestingly, up-regulation of *Orco* expression by the pheromone methyl eugenol (ME) has been observed in the fruit fly *Bactrocera dorsalis* (Zheng et al., 2012). Mature *B. dorsalis* males exhibited significant taxis toward ME within 0.5 h, and *Orco* was expressed significantly in the attracted adults within the same period (Zheng et al., 2012). Zheng et al. (2012) first demonstrated *Orco* regulation in insects. The regulation of specific ORs was first observed in *An. gambiae* females, where odorant receptor 1 (*AgamOR1*) was shown to be regulated by blood meals. Specifically, *AgamOR1* was down-regulated 12 h after blood feeding, a period during in which substantial reduction in olfactory responses to human odorants was also observed (Fox et al., 2001), suggesting that *AgamOR1* was involved in host seeking.

3.3. The role of RproOrco

To investigate the role of *RproOrco* in *R. prolixus* physiology, the *Orco* gene was silenced using RNAi assays. Notably, in triatomines, dsRNA injection is more effective at inducing RNAi than dsRNA ingestion (Araujo et al., 2006); therefore, different studies on *R. prolixus* have used dsRNA injection to infer the function of specific genes (Araujo et al., 2009; Lee et al., 2013; Mansur et al., 2014; Paim et al., 2013; Souza-Ferreira et al., 2014). Males in the 5th instar nymphs stage were injected with dsOrco, dsMalE (a control gene) and H_2O. dsMalE- and H_2O-injected insects served as control groups. Seven days after injection, the insects were fed blood to promote ecdysis, and t-RNAs were purified after ecdysis to the adult stage (approximately 21 days). Fig. 4A presents the profile of *Orco* silencing insects. A reduction in *RproOrco* expression of approximately 73% was observed in insects treated with *Orco* dsRNA. This gene silencing persisted for a long time, approximately 120 days after the dsRNA injection (Fig. 4B). RNA silencing is a reverse genetic approach that has served as a useful tool in understanding insect physiology; however, this technique is limited by variable sensitivities of insect species to RNAi. For example, *Blattella germanica* is highly susceptible to RNAi, but *Manduca sexta* is refractory to treatment (Garbutt et al., 2013). Meanwhile, *R. prolixus* has proven to be an excellent model for RNAi studies. Persistence of the dsRNA effects in *R. prolixus* is intriguing and has been observed in both the *Orco* (present study) and *chitin synthase* genes (Souza-Ferreira et al., 2014). The exact mechanism by which RNAi functions in *R. prolixus* remains unknown.

3.4. The role of RproOrco in the host-seeking behavior of R. prolixus

Next, the effects of dsOrco silencing on insect movement toward a vertebrate were assessed using a bioassay (see the experimental

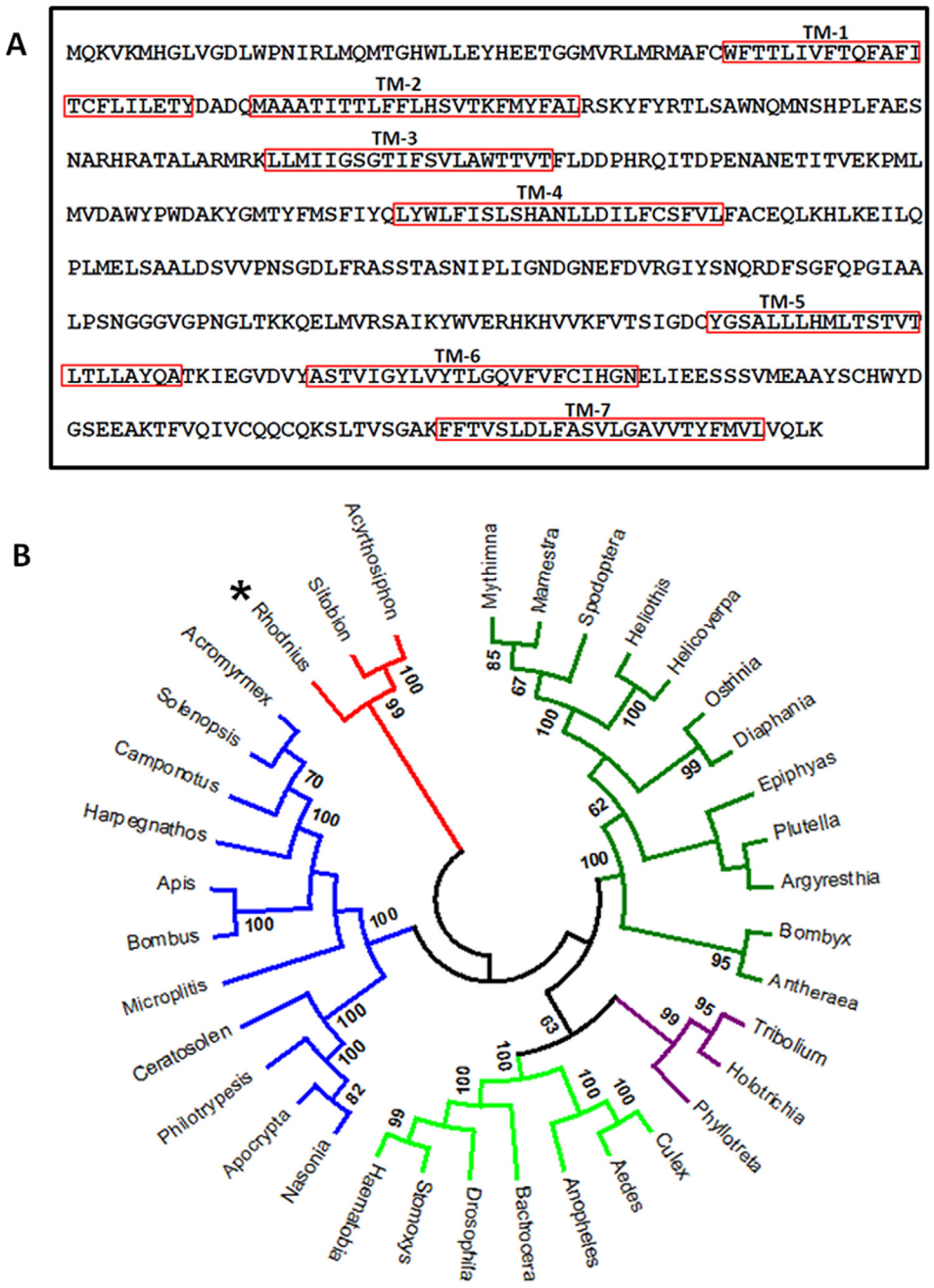

Fig. 1. (**A**) The *R. prolixus* Orco sequence. The full-length RproOrco sequence was obtained by amplification of cDNA from antennae using PCR and a pair of primers followed by cloning and sequencing. (**B**) A phylogenetic tree based on the Orco ortholog sequences was generated using the Mega 6 program with Orco sequence alignment.

procedure). As shown in Fig. 5, insects with silenced *RproOrco* exhibited a statistically significant ($p < 0.0001$) delay in their time required to find a host compared with control insects. In contrast, all of the control insects exhibited the ability to move faster toward the vertebrate (0.66 cm/s for insects injected with H_2O and 1.02 cm/s for insects injected with dsMalE). However, only 60% of the dsRproOrco-treated insects moved toward the host, and the movement occurred at a considerably reduced speed (0.12 cm/s, 5.5-fold slower than control insects). Orco is important for the correct functionality and structure of the odorant receptor in the dendrite membranes of neurons (Stengl and Funk, 2013). Thus, the observation of modified behavior in the *RproOrco*-silenced insects was not surprising and likely occurred as a consequence of a partially incapacitated olfactory system.

3.5. The effects of dsOrco on blood ingestion and R. prolixus physiology

Interestingly, we also observed a loss of the ability to engorge in Orco-deficient insects. Fifth instar nymphs were injected with dsRNA and were fed blood seven days later. All insects were weighed 2 h before and 2 h after taking blood meals. Insects with silenced *RproOrco* ingested approximately 42.5% less blood compared to control insects. Specifically, H_2O- and dsMalE-treated control insects ingested 137 mg and 116 mg of blood, respectively, whereas dsOrco-treated insects ingested only 69 mg of blood (Fig. 6). This effect persisted even after the dsOrco insects became adults. The adults lost approximately 88.2% of their capacity to engorge on a blood meal (data not shown), due to a sustained

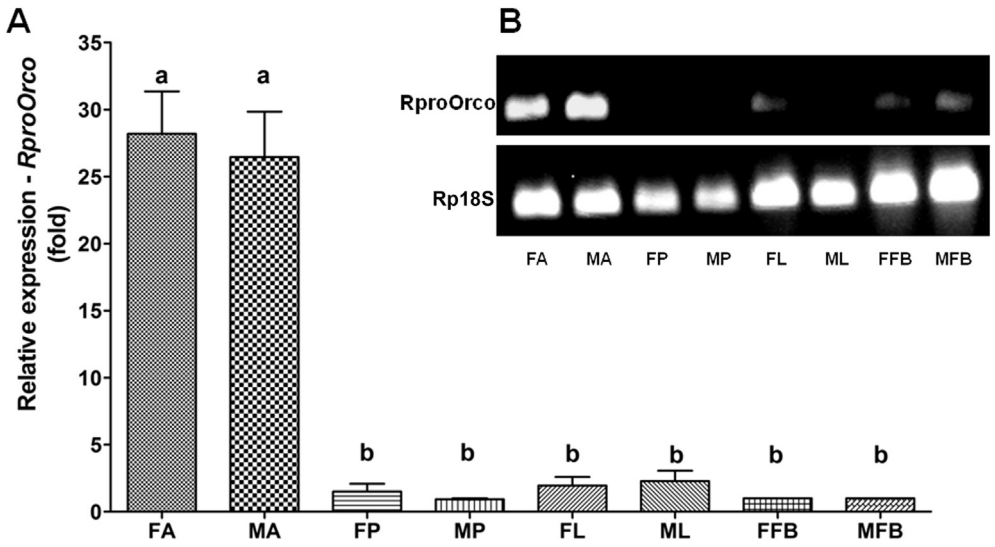

Fig. 2. Analysis of the *RproOrco* expression profile. (**A**) Tissue distribution of *RproOrco* in adults. Error bars represent standard deviation (SD) of the means of three biological replicates. Different letters within the same figure indicate that the values differed significantly (ANOVA followed by Tukey's test; $p < 0.0001$). (**B**) A 1% agarose gel stained with ethidium bromide revealed the profile of *Orco* expression in different tissues: FA = female antennae; MA = male antennae; FP = female proboscides; MP = male proboscides; FL = female legs; ML = male legs; FFB = female fat body; MFB = male fat body.

dsRNA effect after ecdysis. This effect on blood ingestion is intriguing because the region used to induce RNAi was specific to *RproOrco* (BLAST result: *e-value* = 0, identity = 100%). The silenced 543bp-region exhibited no homology to any other odorant receptors or any other gene that could explain a possible off-target effect. Although it remains unclear how decrease in *RproOrco* transcript levels promotes this phenomenon, our RNAi-based approach could lead to both decrease contact of this Chagas vector with humans and the vector chances of successful reproduction (see below). *RproOrco*-deficient insects lost their ability to ingest an entire blood meal, and this fact has further implications regarding the general physiology of *R. prolixus*.

Insects with silenced *Orco* exhibited compromised developmental features. A blood meal is essential for ecdysis in both males and females. Furthermore, such a meal is essential for ovary development and egg production in females because proteins and

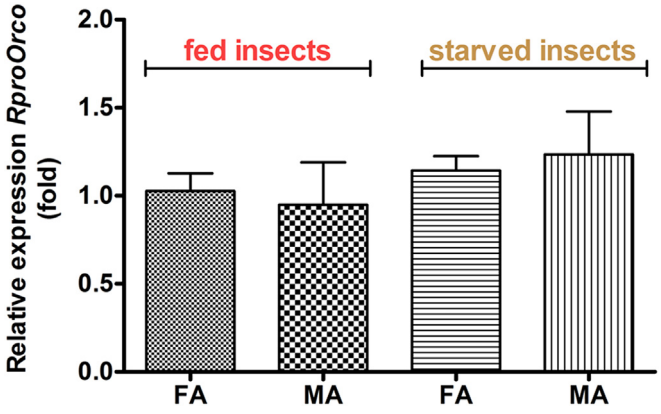

Fig. 3. *RproOrco* expression profile in antennae of blood fed and starved insects. Males and females were divided into two groups: (i) a group given blood meals two days after emergence to the adult phase, and (ii) a group starved for 21 days. t-RNAs from the antennae of each group were purified, and *RproOrco* expression was analyzed by qPCR. The ribosomal gene *Rp18S* was used as an endogenous control. Error bars represent standard deviation (SD) of the means of three biological replicates. No significant difference was observed between the groups (ANOVA followed by Tukey's test; $p > 0.05$). FA = female antennae; MA = male antennae.

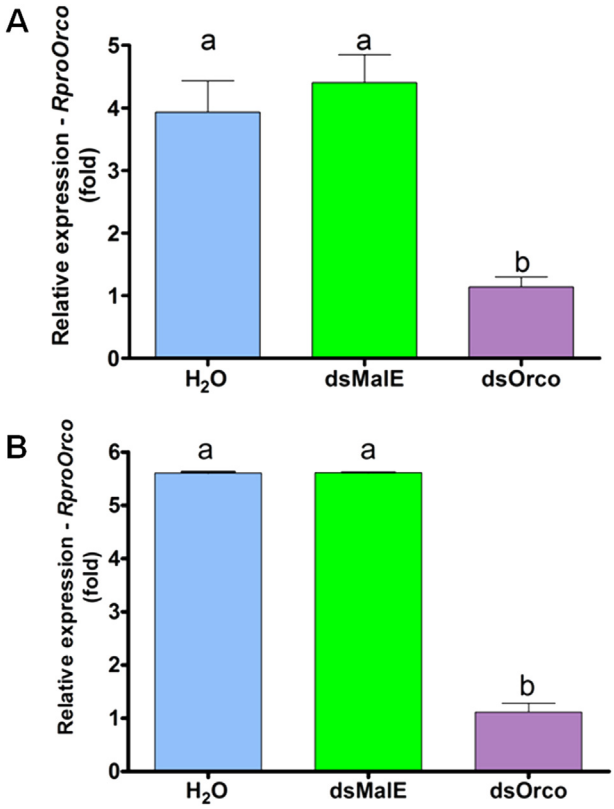

Fig. 4. Quantitative analysis of *RproOrco* gene expression in the antennae of dsRNA-treated insects. Males in the 5th instar nymph stage were injected with dsOrco, dsMalE, or H_2O (controls), and seven days after the dsRNA injection, the insects were fed blood to promote ecdysis. (**A**) Antennae t-RNAs were purified after ecdysis to the adult stage (approximately 21 days later). The cDNA expression levels in the samples were determined using the Step One Real-Time PCR kit and primers specific for the amplification of *RproOrco*; the ribosomal gene *Rp18S* was used as an endogenous control, and dsMalE was used as an unrelated control gene. (**B**) The profile of dsRNA-treated insect adults 120 days after dsRNA injection. Error bars represent standard deviation (SD) of the means of three biological replicates. Different letters within the same figure indicate that the values differed significantly (ANOVA followed by Tukey's test; $p < 0.0001$).

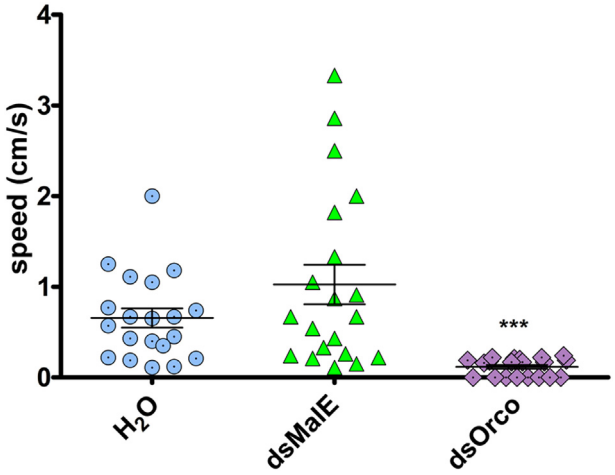

Fig. 5. The ability of dsOrco insects to find a host. The bioassay was based on the time (up to 3 min) that each insect (N = 20 for each of the dsOrco-, dsMalE-, and H_2O-injected groups) took to find a vertebrate. The ground speed (distance/time) was calculated for each insect. Error bars represent standard deviation (SD) of the means of three biological replicates ***Indicates a significant difference in the ground speed between the dsOrco-treated insects and H_2O- and dsMalE-treated insects (ANOVA following Tukey's test; $p < 0.0001$). No significant difference was noted between the control groups (H_2O and dsMalE).

lipids present in the blood are used to produce eggs (Atella et al., 2005; Davey, 2007). Insects with silenced *Orco* lost interest in feeding; consequently, those insects were unable to undergo ecdysis. Only 35% of dsOrco-treated insects progressed from the 5th instar to the adult stage; those insects that developed exhibited a 3-day delay compared with controls (Fig. 7). Whereas control insects started molting on day 14, dsOrco-treated insects began molting on day 17. Thus, 65% of the dsOrco insects remained in the juvenile stage and were consequently incapable of reproduction. Additionally, of the 35% of dsOrco insects that successfully underwent ecdysis, approximately 50% died within 25 days (Fig. 8).

Egg production, another important aspect of *R. prolixus* life, was also affected by dsOrco (Fig. 9). Under normal conditions, *R. prolixus* females produce approximately 42 eggs within 15 days after a

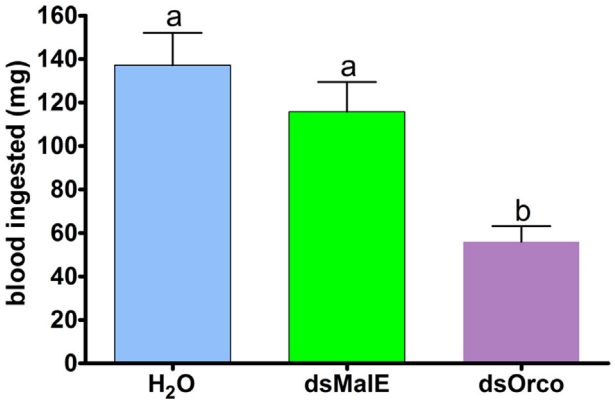

Fig. 6. Blood ingestion by *Orco*-deficient insects. The insects were injected with dsRNA (dsOrco, dsMalE) or H_2O and were separated into ten groups for dsOrco and H_2O control (4 insects/groups, N = 20 for each condition) and into five groups (4 insects/groups, N = 20) for dsMalE. Seven days after the injection, the groups were assessed for the ability to engorge on blood for 1 h. The insects were weighed 2 h before and after the blood meals, and the difference in the weight (after minus before blood meals) is reported as the amount of blood ingested by the insects. Error bars represent standard deviation (SD) of the means of three biological replicates. Different letters within the same figure indicate that the values are significantly different (ANOVA following by Tukey's test; $p < 0.0005$).

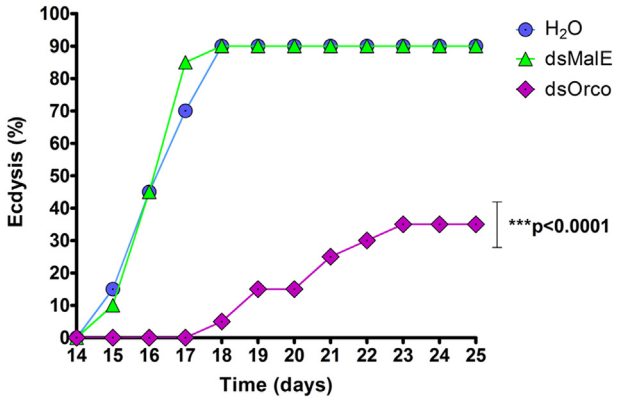

Fig. 7. The effects of dsOrco on ecdysis. The treated insect groups (dsOrco, dsMalE, and H_2O control) were observed for 25 days to assess insect development. Then, the number of insects that underwent ecdysis was determined. ***Indicates a significant difference in ecdysis rates between the dsOrco-treated insects and H_2O- and dsMalE-treated insects (ANOVA following Tukey's test; $p < 0.0001$). No significant difference was noted between the control groups (H_2O and dsMalE).

single blood meal (Atella et al., 2005). In fact, our non-silenced insects produced approximately 48 eggs in 25 days, whereas the *Orco*-silenced females produced roughly half as many eggs (an average of 22.7 eggs/dsOrco female).

Taken together, these results indicate that *Orco* is an excellent target for disrupting the normal behavior of triatomines and directly reducing the insect population (via increased mortality and decreased ecdysis and egg laying). Furthermore, *Orco*-silenced insects lost their capacity to identify a host and were associated with a decreased capacity to ingest blood. These effects reduce the contact between the vertebrate and the insect, which reduces the possibility for the transmission of Chagas disease. In the beetle *T. castaneum*, *Orco* (dsTcOr1) knockdown decreased the response to *Tribolium* aggregation pheromone, supporting the hypothesis that *TcOr1* (*Orco*) plays a similar crucial role in olfaction (Engsontia et al., 2008). *Orco*-knockdown mosquitoes also demonstrated compromised behaviors; they exhibited severely reduced attraction to sugar and did not respond to human scent in the absence of CO_2 (DeGennaro et al., 2013). Disrupted behaviors were also observed in the coleopteran *Phyllotreta striolata*, where *Orco* dsRNA-treated insects altered their host-plant preference and were attracted to other cruciferous vegetables (Zhao et al., 2010). *Orco* silencing was also effective in *Microplitis mediator*. Electroantennogram (EAG) responses of this hymenopteran to two chemical attractants,

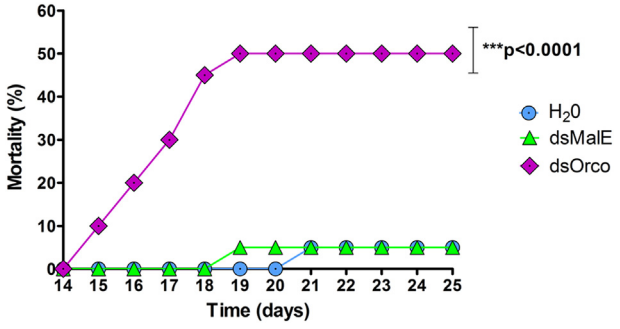

Fig. 8. Mortality rate of dsOrco insects. The treated insect groups (dsOrco, dsMalE, and H_2O control) were observed for 25 days to assess the mortality rate. Then, the number of insects that died was quantified. ***Indicates a significant difference in the mortality rate between the dsOrco-injected insects compared with the H_2O- and dsMalE-treated insects (ANOVA following Tukey's test; $p < 0.0001$). No significant difference was noted between the control groups (H_2O and dsMalE).

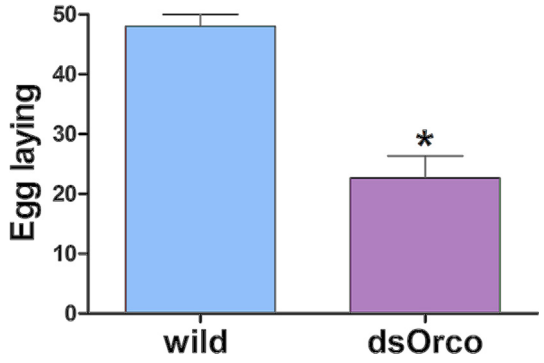

Fig. 9. Oviposition rate in dsOrco insects. The graphic presents the cumulative amount of eggs laid by dsOrco-treated females and untreated females after feeding. The data represent three independent experiments (N = 6).*Indicates significant differences in the egg laying rates between the dsOrco-treated and untreated females (F test followed by Student's *t* test; $p < 0.0279$).

nonanal and farnesene, were significantly reduced (~70%) in RNAi-treated insects compared with controls. RNAi-treated *M. mediator* also responded with reduced rates of walking or flying toward both chemicals compared with controls in a Y-tube olfactometer bioassay. These data provide direct evidence that *MmedOrco* plays an important role in nonanal and farnesene perception in *M. mediator* (Li et al., 2012).

Our data are the first to suggest that the capacity for blood ingestion can be affected by *Orco* knockdown in one of the most important vectors of Chagas disease. These results provide new insight into alternative means of insect population control other than insecticides.

4. Conclusions

- *RproOrco* encodes a 476-amino acid protein with seven transmembrane domains;
- *RproOrco* is highly expressed in the antennae of all stages of *R. prolixus* development (nymphs and adults);
- *RproOrco* expression is not regulated by blood ingestion;
- The RNAi technique effectively silenced *Orco* gene in both males and females;
- *RproOrco* silencing is persistent, even after ecdysis and throughout the adult phase;
- Insects with silenced *RproOrco* are unable to find a vertebrate host in a timely manner;
- *Orco* silencing affects the insects' ability to engorge on blood, and *Orco*-deficient insects ingest significantly less blood than controls;
- dsOrco decreases ecdysis and oviposition rates;
- dsOrco increases the mortality rate;
- The RNAi technique is a powerful tool that can be applied to the development of a control strategy for blood-sucking, disease-carrying insects.

Funding

This work was supported by the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT-EM/CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Work at the University of California–Davis was supported by gifts from Bedoukian Research Inc. (USA) and Fuji Flavor Co. (Japan).

Acknowledgments

The authors are grateful to José S. Lima Júnior and Gustavo T. Ali for their excellent technical assistance.

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