Insect Biochemistry and Molecular Biology 69 (2016) 71–81

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Patterns of expression of odorant receptor genes in a Chagas disease vector

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A R T I C L E I N F O

Article history:
Received 6 October 2014
Received in revised form
29 April 2015
Accepted 1 May 2015
Available online 21 May 2015

Keywords:
Olfaction
Receptors
Triatomines
Physiology and Gene Expression

A B S T R A C T

Rhodnius prolixus is a triatomine bug acting as a relevant vector of Chagas disease for which the genome sequence has been recently made available. Based on this information, a set of olfactory (ORs) and ionotropic receptor (IRs) genes potentially related to olfactory processes was characterized, and the expression patterns along bug development and in different structures potentially involved in promoting chemosensory-mediated behaviors were studied. For this, diverse bioinformatic procedures were used to validate gene models analyzing their structural and functional features and designing specific primers. Evolutionary relationships among *R. prolixus* olfactory coreceptors (*RproOrco*, *RproIR25a*, *RproIR8a* and *RproIR76b*) and their orthologues from other insects were shown to have mostly good bootstrap support values in phylogenetic trees. Moreover, antennal expression was confirmed for most genes included in the study. Both ORs and IRs showed antennal expression along the whole development of bugs of this species, with few exceptional receptors showing gradually increasing expression or expression restricted to the antennae of adult bugs. Finally, the expression of most of the selected genes was confirmed in other structures, such as rostri, tarsi, tibial pads and genitalia, which are potentially involved in promoting chemosensory-mediated behaviors. These results are discussed in terms of their relevance to advance in the understanding of the molecular bases of triatomine behavior.

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1. Introduction

Rhodnius prolixus is the second most important insect vector of Chagas disease (Lent and Wygodzinsky, 1979; Guhl et al., 2009; Hashimoto and Schofield, 2012). In addition, its friendly rearing conditions and bulky size, allowing surgical procedures (Garcia et al., 1990), make this species an excellent model organism for the study of different aspects of insect physiology (Wigglesworth, 1934), biochemistry (Champagne et al., 1995) immunology (Feder et al., 1997), and neuroethology (Guerenstein and Lazzari, 2009; Ons et al., 2011; Manrique and Lorenzo, 2012). For example, neuroethological studies have allowed the understanding of several aspects of triatomine behavior, such as mechanisms underlying host search (Guerenstein and Lazzari, 2009) and pheromone communication (Ward, 1981; Lorenzo and Lazzari, 1996; Manrique et al., 2006; Manrique and Lorenzo, 2012). Diverse chemically-triggered behaviors make up an elaborated repertoire allowing these insects to interact with their environment and react to relevant signals (Guerenstein and Lazzari, 2009). These behaviors are known to be expressed in the presence of both volatile and contact clues emitted by their hosts or conespecifics. Nevertheless, the molecular bases of triatomine detection processes need to be uncovered to allow a deeper comprehension of the underlying mechanisms. Furthermore, the development of these sensory capabilities should also be studied, as immature instars are known to be able to detect host odors (Núñez, 1982; Barrozo and Lazzari, 2004; Manrique and Lorenzo, 2012) and react to alarm pheromones (Manrique et al., 2006), but it is not known whether they are capable of detecting sexual pheromones. The publication of the *R. prolixus* genome sequence gives a new perspective to these studies (Latorre-Estivalis et al., 2013), offering genetic information that will transform *R. prolixus* into a more powerful, appealing and complete model.

Olfaction is critical for insect survival, since this sense is fundamental to detect chemical stimuli related with the presence

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http://dx.doi.org/10.1016/j.ibmb.2015.05.002
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of food, sexual partners, danger and pathogens. Insects have developed a large and diverse repertoire of receptors to detect these signals (Kaupp, 2010; Carey and Carlson, 2011). The main groups of insect proteins involved in the reception of odors are olfactory receptors (ORs), ionotropic receptors (IRs), odorant-binding proteins (OBPs), chemosensory proteins (CSPs), odorant-degrading enzymes (ODEs), and sensory neuron membrane proteins (SNMPs) (Leal, 2013). The genes coding for receptors mediating odor recognition (ORs and IRs) are expressed by olfactory sensory neurons (OSNs), which are housed in olfactory sensilla mostly located on insect antennae.

Insect ORs, first identified in *Drosophila melanogaster*, are characterized by the presence of seven transmembrane domains and a reverse membrane topology when compared with mammalian olfactory receptors (Gao and Chess, 1999; Vosshall et al., 1999; Benton et al., 2006; Lundin et al., 2007). These receptors are expressed in OSNs mostly housed in trichoid and basiconic sensilla (Couto et al., 2005). Each OR is produced within a subpopulation of OSNs (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000). Nevertheless, all of them are co-expressed with a chaperoning coreceptor protein known as Orco, which is involved in their localization to the ciliated dendrites of the OSN (Krieger et al., 2003; Larsson et al., 2004; Pitts et al., 2004) and in the signal transduction process (Benton et al., 2006; Sato et al., 2008; Wicher et al., 2008). Olfactory receptors represent a large and extremely divergent gene family, with no apparent subfamilies or close orthologies across insects, except from *Orco* (Hansson and Stensmyr, 2011).

A newly described chemosensory receptor family was recently characterized in the OSNs of coeloconic sensilla: the ionotropic receptors (Benton et al., 2009). These receptors are a divergent subfamily proposed to be derived from ionotropic glutamate receptors (iGluRs) with which they share a similar molecular structure, including an extracellular ligand-binding domain with two lobes separated by an ion channel domain (Benton et al., 2009). Nevertheless, at least one of the three characteristic residues that interact with glutamate in the iGluR ligand-binding domain is expected to be altered in IRs (Benton et al., 2009; Olivier et al., 2011). Ionotropic receptors have been proposed to act as dimers or trimers of subunits co-expressed in the same neuron (Benton et al., 2009). Accordingly, these complexes are composed by an odor-specific receptor and one or two coreceptors (Abuin et al., 2011). The latter include the proteins coded by the genes named *IR25a*, *IR8a* and *IR76b* (Abuin et al., 2011). Two different subfamilies are distinguished within these receptors: the conserved "antennal IRs" and the more species-specific "divergent IRs". The former subfamily derives from animal iGluR ancestors and is probably the first olfactory receptor family of insects (Croset et al., 2010). The divergent IR subfamily is involved in taste and food assessment and evolved from "antennal IRs" (Croset et al., 2010).

Information about the molecular bases of sensory processes carried out in tissues other than insect antennae is very scarce. The best understood example of olfactory processes carried out by these structures is CO_2 detection by capitate peg sensila in the mosquito maxillary palp (Jones et al., 2007; Lu et al., 2007). Therefore, characterizing whether other insect tissues potentially involved in sensory processes show expression of putative chemosensory genes would be relevant.

The present study characterized the sequences of all olfactory coreceptors (*RproOrco*, *RproIR8a*, *RproIR25a* and *RproIR76b*) and a subset of ORs and IRs from *R. prolixus*, describing their structural and functional features. All sequences included in this study were previously annotated in VectorBase and their identity will be reported in a manuscript presenting the fundamental characteristics of the genome of this species (Mesquita et al., in preparation). In the case of the coreceptors, we analyzed the amino acid sequence diversity focusing on the identification of protein signatures in orthologues from other insects and establishing evolutionary relationships among them. Furthermore, the expression patterns shown by the four coreceptors and the subset of ORs and IRs have been characterized for embryos, antennae of all instars and other sensory tissues of *R. prolixus* adults.

2. Material and methods

2.1. Sequence and structure analysis of R. prolixus olfactory and ionotropic receptors

A total of 116 OR and 33 IR sequences have been annotated in VectorBase for *R. prolixus* (https://www.vectorbase.org/organisms/rhodnius-prolixus), as part of the genome sequence characterization (Mesquita et al., in preparation). A functional and structural characterization of all coreceptors and a subset of olfactory and ionotropic receptors was performed using different programs and protein databases. Firstly, the presence of a signal peptide was assessed by means of SignalP v4.1 (Petersen et al., 2011). Subsequently, functional domains were identified using InterProScan v5 (Jones et al., 2014), and the Conserved Domains (CDD) (Marchler-Bauer et al., 2011) and Pfam v27.0 databases (Finn et al., 2013). Additionally, the number and location of predicted transmembrane domains were identified using TOPCONS (Bernsel et al., 2009), TMHMM Server v2.0 (Krogh et al., 2001) and TMpred softwares (Hofmann, 1993).

Comparison of the *R. prolixus* coreceptor protein sequences was performed using BLASTp v2.2.30 searches of potential orthologues from other insects in the UniProtKB/TrEMBL Database (Bairoch et al., 2005). In order to generate the alignments presented in the results section, the protein sequences encoded by the olfactory (*RproOrco*) and ionotropic (*RproIR8a*, *RproIR25* and *RproIR76b*) coreceptor genes (Mesquita et al., in preparation) were aligned with CLUSTAL X v2.0 (Thompson et al., 1997) with a group of orthologues from other insect species (Tables s1 and s2 – Supplementary Material).

To build phylogenetic trees, alignments of sequences (detailed in Tables s1 and s2 – Supplementary Material) were manually edited in Jalview v2.6.1 (Waterhouse et al., 2009) and subsequently aligned using G-INS-I strategy in MAFFT (Katoh et al., 2009). For the phylogenetic reconstruction we tested 12 different evolutionary models (JTT, LG, DCMut, MtREV, MtMam, MtArt, Dayhoff, WAG, RtREV, CpREV, Blosum62, and VT) using the ProtTest v2.4 webserver (Abascal et al., 2005). Finally, maximum likelihood trees with 1000 pseudo-replicates of the original data were built in PhyML v3.0 (Guindon et al., 2009) using the Phylemon v2.0 webserver (Sánchez et al., 2011). Graphical representations were generated using FigTree v1.4.0.

2.2. Insects and tissues

Insects were obtained from a colony of *R. prolixus* housed at the Centro de Pesquisas René Rachou (generated from intradomiciliary insects donated by Dr. Carlos Ponce, Ministerio de Salud Pública, Honduras). Experimental insects were reared under a natural light regime, controlled temperature of 26 ± 1 °C and relative humidity of $65 \pm 10\%$. Two different experiments were performed to characterize the expression of odorant receptors. In a first study, antennae from the five larval instars and male and female adult insects were obtained by dissection. In this experiment, whole eggs were also used to analyze odorant receptor expression in embryos. In a second study, diverse tissues potentially involved in

chemoreception were dissected separately from male and female adults: rostri, tarsi, tibial pads and genitalia.

2.3. Primer design

A subset of 10 ORs, including the coreceptor *Orco*, and 20 ionotropic receptors, including the three IR coreceptors, were selected from the *R. prolixus* genome (Mesquita et al., in preparation) for primer design. Specific primers for these receptor candidates were designed using the Primer3 4.0.0 (http://primer3.wi.mit.edu) (Rozen and Skaletsky, 2000) and Oligoanalyser (Integrated DNA Technologies, Inc. IA, USA) softwares. The specificity for each primer was tested *in silico* using BLASTn (Altschul et al., 1990) in the *R. prolixus* genome database. The primer sequences and their corresponding amplicon lengths are detailed on Table s3 and s4 in the Supplementary Material.

2.4. RNA extraction and cDNA synthesis

Total RNAs were extracted from pools of 40 antennae from each of the different larval stages and male and female adults. In the case of eggs, RNA was obtained from a pool of 20 whole eggs. For the tissue expression study, RNA was isolated from different sex pools of 20 rostri, 120 tarsi, 80 tibial pads from fore and middle legs and 10 genitalia. RNA extractions were made using 500 μL of TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) for each larval and adult tissue sample. This experiment was replicated in batches that always included samples from all developmental stages obtained simultaneously. RNAs were resuspended in 30 μL of DEPC-treated water (Life Technologies) and subsequently quantified using a Qubit® 2.0 Fluorometer (Life Technologies). After DNase treatment with RQ1 RNase-Free DNase (Promega, Fitchburg, WI, USA), cDNAs were synthetized using SuperScript III Reverse Transcriptase (Life Technologies) and a 1:1 mix of Random Hexamers and 10 μM Oligo(dT)$_{20}$ primers in a final volume of 20 μL. All reverse transcription reactions were performed in a MasterCycler® Gradient Thermal Cycler (Hauppauge, NY, USA) using the same amount of treated RNA per sample for all batch samples to allow comparison between them. The amount of treated RNA used for RT reactions on embryo and antennal samples was 200 ng. In the case of the sensory tissue expression study, 890 ng of treated RNA were used for all RT reactions. All the cDNAs produced were stored at −20 °C.

2.5. Tissue and developmental expression profiles

PCR reactions were performed on cDNAs from whole eggs, antennae of all developmental stages and several tissues obtained from adult bugs. The reactions were performed for 40 cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) with 1 μL of pure cDNA, 1.1 μL of a 1 mM dNTP solution, 0.6 μL of a 10 μM primer solution and 1 U of Taq polymerase (Promega) in a final volume of 12 μL. Amplification product sizes were confirmed by running 5 μL of sample on a 2% agarose gel stained with GelRed™ (Biotium) and subsequently exposing it to ultraviolet light for visualization. Negative controls (free of template) were also included. The *R. prolixus* Glucose-6-phosphate dehydrogenase gene or *RproG6PDH* (R4G5X8 in UniProtKB/TrEMBL) has been used to check the integrity of all cDNAs. Reproducibility was confirmed by amplification of cDNAs from at least two different sets of biological samples. In the case of the antennal development experiment, expression was evaluated for a total of 10 OR and 20 IR genes. Besides, a subset of 7 ORs and 14 IRs was used to evaluate expression in the different sensory tissues studied. Amplicons for all the genes studied were sequenced to confirm primer specificity and sequence identity.

3. Results

3.1. Sequence and structure analysis of R. prolixus olfactory and ionotropic receptors

All ORs studied in the present report lacked a signal peptide in their protein sequences and had a number of amino acids ranging between 372 and 473 (Table 1). In all cases, ORs presented functional domains characteristic for this protein family (InterPro Database code IPR004117-odorant receptor family; Pfam code PF02949-seven transmembrane receptor family; CDD code CL20237-seven transmembrane receptor family; Table 1). The number of predicted transmembrane domains varied between 5 and 7, depending on the predictive method applied, i.e., TOPCONS, TMHMM and TMpred (Table 1).

In the case of IRs, most sequences presented a signal peptide and their length varied between 352 and 921 amino acids (Table 2). Functional domains typical of this protein family had a variable presence, but those predicted by the InterPro (code IPR00132-ionotropic glutamate receptor protein family) and Pfam (code PF00060) were more consistently present (Table 2). Depending on the predictive method applied, i.e., TOPCONS, TMHMM and TMpred, the number of predicted transmembrane domains varied mostly between 3 and 5, with few cases presenting predictions of up to 7 domains (Table 2). The absence of at least one of the three characteristic iGluR glutamate binding residues is a criterion for IR identification (Benton et al., 2009) and was confirmed for all IRs studied (Table 2).

BLASTp analyses of protein sequence alignments showed that RproOrco shares 84% amino acid identity with those of mirid sequences (*Apolygus lucorum*, *Lygus lineolaris* and *Lygus hesperus*), 59% with those of *Pediculus humanus* and *Sitobion avenae*, and 57% with that of *D. melanogaster*. The alignment of RproOrco amino acid sequence with those from other insects showed a high degree of conservation, except for the intracellular loop located between the 4th and 5th predicted transmembrane domains (Fig. s1 – Supplementary Material). As expected, the region comprised between the 6th and 7th transmembrane domains was highly conserved (Benton et al., 2006; Wicher et al., 2008), presenting identical amino acid positions in 72.6% of residues (Fig. s1 – Supplementary Material). Additionally, several residues considered to be important for Orco protein function were identified in the alignment: four phosphorylation sites (Pro/Ser159; Asp248; Ser/Val289 and Thr314, described by Sargsyan et al., 2011), one motif related with ion permeability found between positions 379 and 386 (Wicher et al., 2008), a residue (Asp453) considered to affect spontaneous and induced action potentials in the receptor complex (Nakagawa et al., 2012); and a residue (Tyr465) described by Kumar et al. (2013) considered to be fundamental for channel activity (Fig. s1 – Supplementary Material). The sequence of RproIR25a shared 72% amino acid identity with that of *Acyrthosiphon pisum*, 69% with that of *P. humanus*, 66% with that of *Tribolium castaneum*, 64% with that of *Schistocerca gregaria* and 61% with that of *D. melanogaster*. The RproIR8a coreceptor shared 49% amino acid identity with *S. gregaria*, 48% with *P. humanus*, *A. pisum* and *T. castaneum* and 45% with *D. melanogaster*. For the RproIR76b coreceptor, *R. prolixus* shared 44% amino acid identity with *A. pisum*, 43% with *T. castaneum*, 41% with *P. humanus* and 40% with *D. melanogaster*. The three predicted transmembrane domains and the pore region were the most conserved segments for the three ionotropic coreceptor sequences (Figs. s2–s4 – Supplementary Material). Moreover, the amino terminal domain and S1 lobe of IR25a showed a higher degree of conservation when compared to those of the other two coreceptors (Fig. s1 – Supplementary Material). None of the *R. prolixus* IR coreceptor sequences

Table 1
Functional and structural characterization of *Rhodnius prolixus* olfactory receptors. The presence of a signal peptide was assessed using SignalP 4.1 Server; functional domains were identified using InterProScan5, Pfam and the CDD and the number and location of predicted transmembrane domains was assessed using TOPCONS, TMHMM and TMpred softwares. Numbers in parentheses indicate domain location in the protein sequence.

Gene	Amino acids[d]	Signal peptide	Functional domains			Number of predicted transmembrane domains		
			IPR004117[a]	PF02949[b]	cl20237[c]	TOPCONS	TMHMM	TMpred
RproOrco	473	No	Yes (1-473)	Yes (77-459)	Yes (77-459)	7	7	7
RproOR13	441	No	Yes (7-441)	Yes (187-430)	Yes (187-430)	7	6	7
RproOR18	432	No	Yes (1-432)	Yes (78-420)	Yes (78-420)	7	6	7
RproOR19	408	No	Yes (1-406)	Yes (179-407)	Yes (179-407)	7	6	6
RproOR20	437	No	Yes (1-44)	Yes (282-422)	Yes (282-422)	5	5	7
RproOR32	432	No	Yes (1-431)	Yes (139-419)	Yes (139-419)	5	6	7
RproOR53	372	No	Yes (36-372)	Yes (62-361)	Yes (62-361)	7	6	6
RproOR82	376	No	Yes (26-375)	Yes (60-363)	Yes (60-363)	7	5	6
RproOR98	406	No	Yes (5-406)	Yes (115-396)	Yes (115-396)	7	6	6
RproOR104	394	No	Yes (11-391)	Yes (124-371)	Yes (124-371)	7	6	6

[a] InterPro code corresponding to odorant receptor family.
[b] Pfam code corresponding to seven transmembrane receptor family, probably *Drosophila* odorant receptor.
[c] CDD code corresponding to seven transmembrane receptor family, probably *Drosophila* odorant receptor.
[d] Number of amino acids for each receptor was obtained from Mesquita et al. in preparation.

Table 2
Functional and structural characterization of *Rhodnius prolixus* ionotropic receptors. The presence of a signal peptide was assessed using SignalP 4.1 Server; functional domains were identified using InterProScan5, Pfam and the CDD and the number and location of predicted transmembrane domains was assessed using TOPCONS, TMHMM and TMpred softwares. Numbers in parentheses indicate domain location in the protein sequence.

Gene	Amino acids[g]	Signal peptide	Functional domains					Number of predicted transmembrane domains			iGluR residues[f]		
			IPR001320[a]	PF00060[b]	cl10011[c]	cl19076[d]	cl18192[e]	TOPCONS	TMHMM	TMpred	R	T	D/E
RproIR8a	858	Yes	Yes (363-782)	Yes (484-782)	No	Yes (379-467)	Yes (586-749)	3	3	4	R	A	E
RproIR25a	921	Yes	Yes (422-854)	Yes (547-854)	Yes (422-854)	Yes (432-530)	Yes (651-820)	3	3	4	R	S	D
RproIR76b	506	Yes	Yes (169-370)	Yes (171-426)	No	No	No	3	4	5	S	I	T
RproIR21a	784	Yes	Yes (383-665)	Yes (383-665)	No	Yes (257-354)	No	3	4	7	R	W	L
RproIR40a	633	No	Yes (314-592)	Yes (316-592)	No	No	Yes (409-541)	3	2	3	R	S	T
RproIR41a	657	Yes	Yes (354-627)	Yes (355-626)	No	No	No	5	6	5	P	V	S
RproIR41b	654	Yes	Yes (353-624)	Yes (353-616)	No	No	No	5	4	6	P	V	S
RproIR41c	647	Yes	Yes (343-620)	Yes (344-616)	No	No	No	3	2	5	V	T	T
RproIR75a	631	Yes	Yes (331-564)	Yes (331-593)	No	Yes (263-309)	No	3	3	4	R	D	S
RproIR75d	617	Yes	Yes (328-585)	Yes (328-585)	No	No	No	3	3	4	A	L	L
RproIR75e	625	No	Yes (330-586)	Yes (331-588)	No	No	No	5	4	5	R	L	P
RproIR75k	614	Yes	No	Yes (321-588)	No	No	No	3	4	4	R	A	D
RproIR75m	598	Yes	Yes (317-494)	Yes (317-568)	No	No	Yes (429-440)	3	6	4	R	L	N
RproIR75n	604	Yes	Yes (317-408)	Yes (317-514)	No	No	No	3	3	4	R	L	N
RproIr93a	854	No	Yes (554-823)	Yes (555-823)	Yes (80-260)	Yes (418-524)	Yes (655-796)	3	4	4	R	T	M
RproIR103	595	No	Yes (310-582)	Yes (311-582)	No	No	No	5	4	4	L	T	D
RproIR104	576	Yes	No	No	No	No	No	5	1	4	N	S	G
RproIR105	616	Yes	Yes (323-593)	Yes (324-593)	No	No	No	5	3	5	K	K	D
RproIR106	615	Yes	Yes (324-566)	Yes (324-566)	No	No	No	5	4	7	R	K	Q
RproIR107	352	No	No	Yes (98-317)	No	No	No	3	3	3	N	N	–

[a] InterPro code corresponding to ionotropic glutamate receptor protein family.
[b] Pfam code corresponding to ligand-gated ion channel domain.
[c] CDD code corresponding to the Type 1 periplasmic binding fold superfamily.
[d] CDD code corresponding to periplasmic binding protein superfamily.
[e] CDD code corresponding to eukaryotic homologues of bacterial periplasmic substrate binding protein superfamily.
[f] Functional characteristics of iGluR residues. R (arginine) binds the glutamate α-carboxyl group; T (threonine) binds the glutamate γ-carboxyl group and D (aspartate) or E (glutamate) that bind the α-amino group of glutamate (Benton et al., 2009).
[g] Number of amino acids for each receptor was obtained from Mesquita et al. in preparation.

presented the three residues (Arg; Thr and Glu/Asp) located in the S1 and S2 binding domain lobes that characterize iGluRs (Figs. s2–s4 Supplementary Material). Overall, the conserved IR coreceptor features were restricted to functionally relevant regions described for these genes (Benton et al., 2009).

The LG amino-acid replacement matrix (Le and Gascuel, 2008) was the best fit model of protein evolution for the four coreceptors. The *Orco* phylogeny showed a division of holometabolous and hemimetabolous insects (Fig. 1), with *RproOrco* grouping with those of other bugs and aphids. Trees obtained for the three ionotropic coreceptors presented similar topologies with bootstrap values supporting the clades (Figs. 2–4), with the exception of the *RproIR8a* tree in which two species of hymenopterans appeared intermingled within the hemimetabolous representatives.

3.2. Expression profile through developmental stages

The expression profiles of olfactory and ionotropic receptors were characterized in embryos, antennae of the five larval instars and of male and female adults (Figs. 5 and 6). Expression of the housekeeping gene *G6PDH* was confirmed in all cDNAs used for this part of the study (Figs. 5 and 6). The transcription of *RproOrco* was detected in antennae from all developmental stages analyzed (Fig. 5). Similar expression patterns were observed for *RproOR18*,

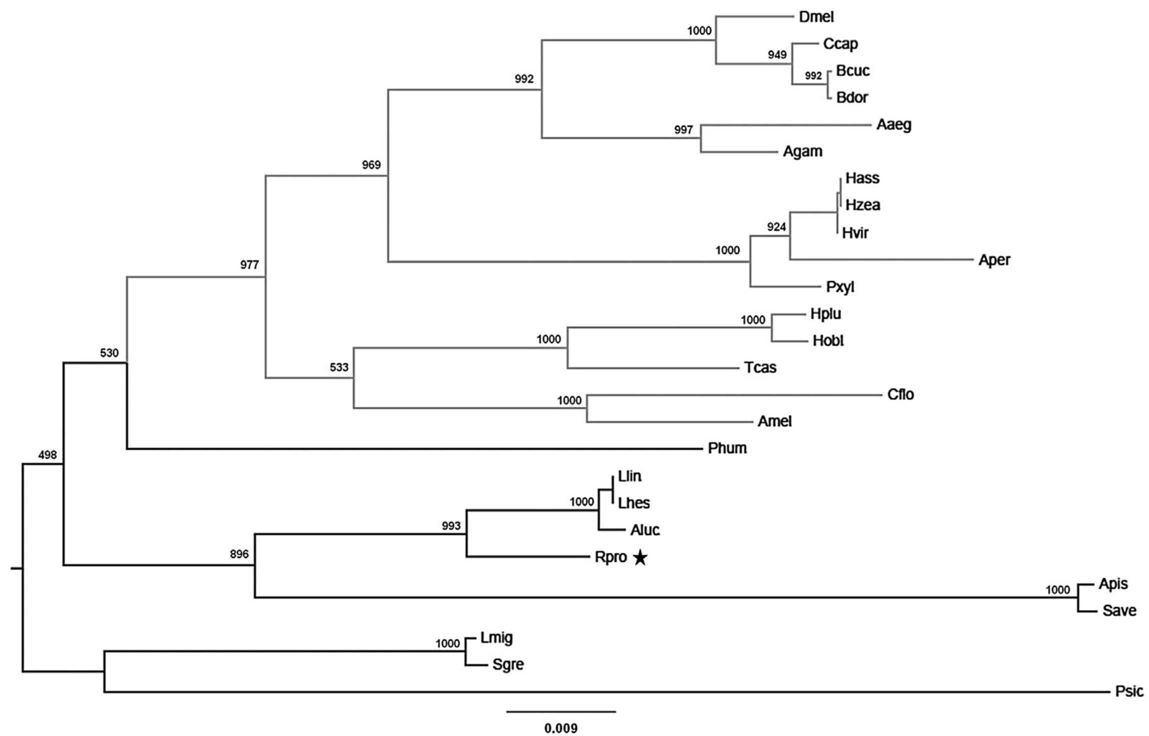

Fig. 1. Phylogenetic relationships of the RproOrco sequence and those of other insect species. The maximum likelihood tree was constructed using PhyML v3.0 based on a MAFFT alignment of Orco sequences from *R. prolixus* and other insects listed in Table s1 – Supplementary Material. The section of the tree comprising holometabolous insects is shown in gray, while the position of RproOrco is denoted by a black star. Bootstrap support values are based on 1000 pseudo-replicates. The scale bar represents the number of amino acid substitutions per site. The tree was rooted using *Phyllium siccifolium* (Psic) *Locusta migratoria* (Lmig) and *Schistocerca gregaria* (Sgreg) as out-groups. Abbreviations: Apis = *Acyrthosiphon pisum*; Aaeg = *Aedes aegypti*; Agam = *Anopheles gambiae*; Aper = *Antheraea pernyi*; Amel = *Apis mellifera*; Aluc = *Apolygus lucorum*; Bcur = *Bactrocera cucurbitae*; Bdor = *Bactrocera dorsalis*; Cflo = *Camponotus floridanus*; Ccap = *Ceratitis capitata*; Dmel = *Drosophila melanogaster*; Hass = *Helicoverpa assulta*; Hzea = *Helicoverpa zea*; Hvir = *Heliothis virescens*; Hobl = *Holotrichia oblita*; Hplu = *Holotrichia plumbea*; Lhes = *Lygus hesperus*; Llin = *Lygus lineolaris*; Phum = *Pediculus humanus*; Pxyl = *Plutella xylostella*; Save = *Sitobion avenae*; and Tcas = *Tribolium castaneum*.

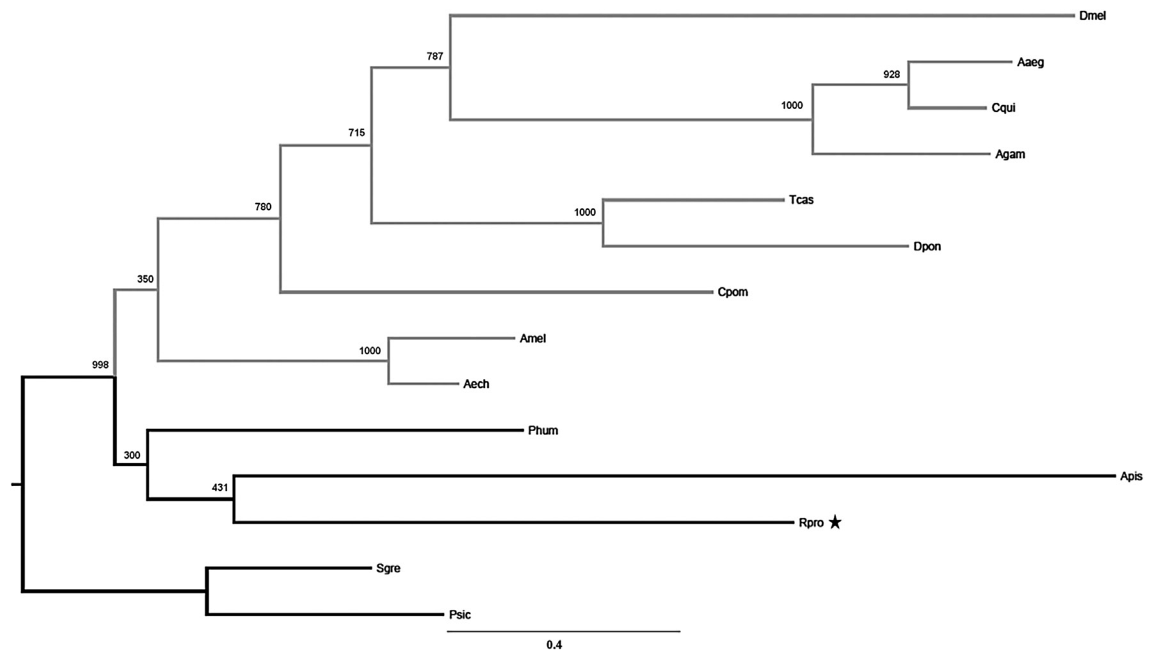

Fig. 2. Phylogenetic relationships of the RproIR8a coreceptor sequence and those of other insect species. The maximum likelihood tree was constructed using PhyML v3.0 based on a MAFFT alignment of IR8a sequences from *R. prolixus* and other insects listed in Table s2 – Supplementary Material. The section of the tree comprising holometabolous insects is shown in gray, while the position of RproIR8a is denoted by a black star. Bootstrap support values are based on 1000 pseudo-replicates. The scale bar represents the number of amino acid substitutions per site. The tree was rooted using *Phyllium siccifolium* (Psic) and *Schistocerca gregaria* (Sgreg) as out-groups. Abbreviations: Aech = *Acromyrmex echinatior*; Amel = *Apis mellifera*; Apis = *Acyrthosiphon pisum*; Aaeg = *Aedes aegypti*; Agam = *Anopheles gambiae*; Cqui = *Culex quinquefasciatus*; Cpom = *Cydia pomonella*; Dpon = *Dendroctonus ponderosae*; Dmel = *Drosophila melanogaster*; Phum = *Pediculus humanus*; and Tcas = *Tribolium castaneum*.

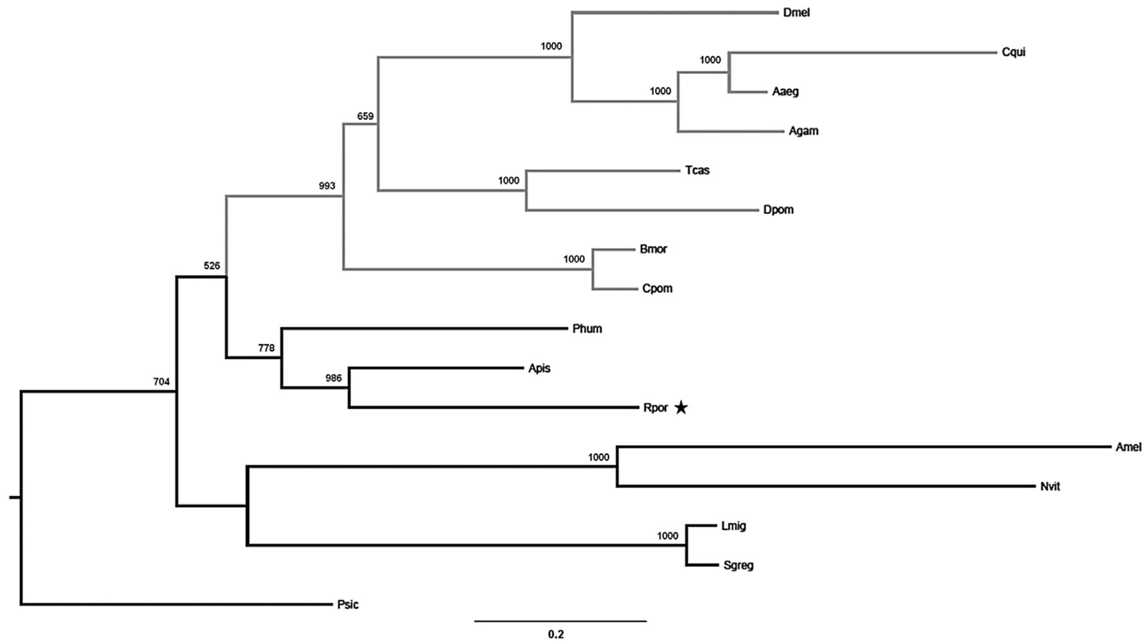

Fig. 3. Phylogenetic relationships of the RproIR25a coreceptor sequence and those of other insect species. The maximum likelihood tree was constructed using PhyML v3.0 based on a MAFFT alignment of IR25a sequences from *R. prolixus* and other insects listed in Table s2 – Supplementary Material. The section of the tree comprising holometabolous insects is shown in gray, while the position of RproIR25a is denoted by a black star. Bootstrap support values are based on 1000 pseudo-replicates. The scale bar represents the number of amino acid substitutions per site. The tree was rooted using *Phyllium siccifolium* (Psic) as out-group. Abbreviations: Apis = *Acyrthosiphon pisum*; Aaeg = *Aedes aegypti*; Agam = *Anopheles gambiae*; Amel = *Apis mellifera*; Bmor = *Bombyx mori*; Cqui = *Culex quinquefasciatus;* Cpom = *Cydia pomonella*; Dpon = *Dendroctonus ponderosae*; Dmel = *Drosophila melanogaster*; Lmig = *Locusta migratoria*; Nvit = *Nasonia vitripennis*; Phum = *Pediculus humanus*; Sgreg = *Schistocerca gregaria*; and Tcas = *Tribolium castaneum*.

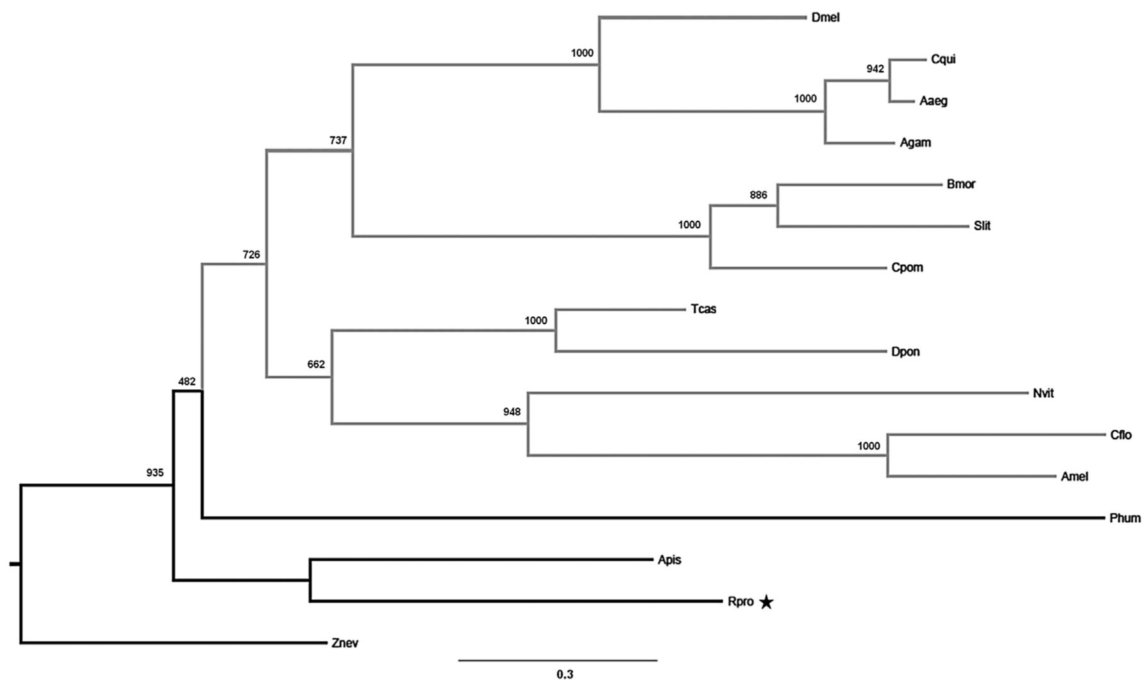

Fig. 4. Phylogenetic relationships of the RproIR76b coreceptor sequence and those of other insect species. The maximum likelihood tree was constructed using PhyML v3.0 based on a MAFFT alignment of IR76b sequences from *R. prolixus* and other insects listed in Table s2 – Supplementary Material. The section of the tree comprising holometabolous insects is shown in gray, while the position of RproIR76b is denoted by a black star. Bootstrap support values are based on 1000 pseudo-replicates. The scale bar represents the number of amino acid substitutions per site. The tree was rooted using *Zootermopsis nevadensis* (Znev) as out-group. Abreviations: Apis = *Acyrthosiphon pisum*; Aaeg = *Aedes aegypti*; Agam = *Anopheles gambiae*; Amel = *Apis mellifera*; Bmor = *Bombyx mori*; Cflo = *Camponotus floridanus*; Cqui = *Culex quinquefasciatus*; Cpom = *Cydia pomonella*; Dpon = *Dendroctonus ponderosae*; Dmel = *Drosophila melanogaster*; Nvit = *Nasonia vitripennis*; Phum = *Pediculus humanus*; Slit = *Spodoptera littoralis*; and Tcas = *Tribolium castaneum*.

Fig. 5. Expression profiles of odorant receptors in embryo and in antennae of *R. prolixus* from different developmental stages obtained by RT-PCR. Abbreviations: I: first instar larvae; II: second instar larvae; III: third instar larvae; IV: fourth instar larvae; V: fifth instar larvae; M: male adult; F: female adult and NT: no-template.

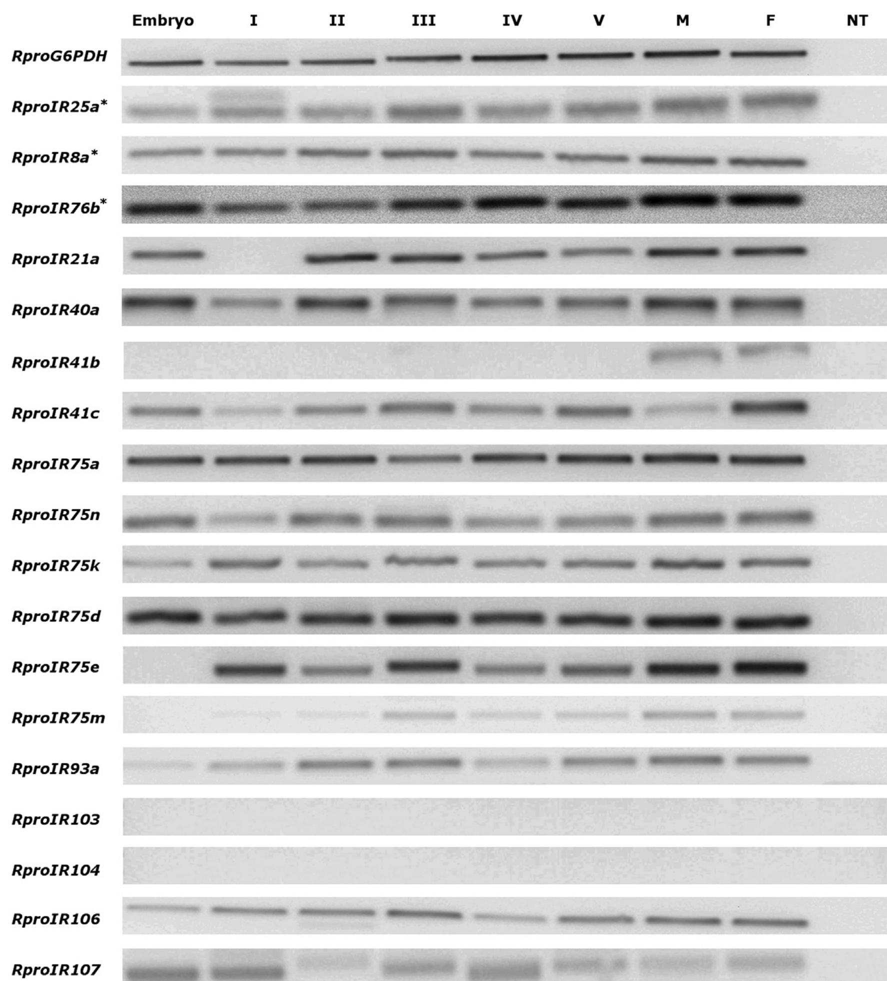

Fig. 6. Expression profiles of ionotropic receptors in embryo and in antennae of *R. prolixus* from different developmental stages obtained by RT-PCR. Abbreviations: I: first instar larvae; II: second instar larvae; III: third instar larvae; IV: fourth instar larvae; V: fifth instar larvae; M: male adult; F: female adult; NT: no-template; and (*) indicates the ionotropic correceptors.

RproOR20, *RproOR53* and *RproOR104*. Transcription was not detected in embryos in the case of *RproOR13* and *RproOR98*. An increase in expression along development was apparent for *RproOR13*, *RproOR19* and *RproOR32*, for which weak or no expression could be detected in embryos. Weak expression of *RproOR82* was detected in embryos, 3rd instar larvae and adult antennae.

As observed with *RproOrco*, the three IR coreceptors (*RproIR8a*, *RproIR25a* and *RproIR76b*) were transcribed in antennae of all developmental instars (Fig. 6). Similarly, expression of a subset of eleven IRs was observed in the antennae of all developmental instars analyzed (Fig. 6). The expression of *RproIR75e* was not observed in embryos and the expression of *RproIR21a* was not observed in first instar larvae. Antennal expression of *RproIR75m* showed an apparent increase after the second instar, while antennal expression of *RproIR41b* was restricted to adult insects (Fig. 6). Two divergent IRs, *RproIR103* and *RproIR104* did not show antennal expression at any instar (Fig. 6).

The amplicons of all genes studied were sequenced, confirming gene identity in 26 out of 28 cases. Nevertheless, sample quality impeded the correct confirmation of *RproIR41a* and *RproIR105* amplicon sequences.

3.3. Expression profile in different male and female adult tissues

The chosen subsets of olfactory and ionotropic receptors presented expression in most tissues tested (Fig. 7a and b). The expression of *RproG6PDH* gene was confirmed for all tissues (Fig. 7a and b) and all tissues analyzed showed expression of *RproOrco*, *RproOR13*, *RproOR20* and *RproOR104* (with a weak band for male rostri) (Fig. 7a). Transcripts of *RproOR19* were detected in all studied tissues of both sexes, except for female rostri. The expression of *RproOR53* was detected exclusively in tibial pads and genitalia of males, while the expression of *RproOR32* was restricted to antennae.

Regarding IRs, expression of *RproIR8a* was absent from female rostri, male tarsi and male genitalia (Fig. 7b). The other IR coreceptors presented expression in all male and female tissues analyzed (Fig. 7b). The expression of *RproIR41b* and *RproIR75m* was restricted to antennae (Fig. 7b). None of the tissues showed expression of *RproIR103* and *RproIR104* (Fig. 7b). The remaining IRs showed expression in all tissues (Fig. 7b).

4. Discussion

The characterization of a subset of olfactory receptor gene sequences in *R. prolixus* is reported by the present study. Moreover, the antennal expression of most receptor genes studied along bug development was confirmed. While many of these receptors presented consistent antennal expression through bug development, several appeared to increase expression in older stages or their expression was restricted to adult bugs. These expression patterns may suggest different roles related to olfactory processes performed in all stages or exclusively in adults. Furthermore, several ORs and IRs studied showed expression in other sensory structures of these insects, suggesting that they are involved in chemosensory driven behaviors.

Diverse functional and structural features characteristic of all coreceptor protein sequences have been described, as well as the presence of critical conserved residues necessary for proper function (Figs s1–s4 – Supplementary Material). The high consistency shown by phylogenetic trees (Figs. 1–4) suggests that the quality of these sequences is adequate for use in functional genetics studies, e.g., primer design or RNAi. All members of the subset of selected ORs and IRs showed proper structural and functional domains as expected for their protein families (Tables 1 and 2). Most receptors studied in the present report, i.e., 28 out of 30, presented consistent expression in *R. prolixus* antennae, confirming their potential roles in bug chemoreception. Interestingly, the expression of many of these receptors was also observed in other sensory structures, suggesting the involvement of these structures in triatomine chemoreception.

Alignments performed allowed us to confirm that the region between the transmembrane domains 6 and 7 of RproOrco is the most conserved, as expected due to the relevant functions of several residues located there (Benton et al., 2006; Wicher et al., 2008; Nakagawa et al., 2012; Kumar et al., 2013). Specifically, RproOrco presented a motif (GYL) described to be relevant for ion permeability in residues 383–385 (Wicher et al., 2008). Moreover, other relevant residues considered to affect spontaneous and evoked action potentials (Tyr465) in the receptor complex (Nakagawa et al., 2012) or important for channel activity (Asp453) have also been found in RproOrco (Kumar et al., 2013). Importantly, Orco has been described to present an intracellular loop between transmembrane domains 6 and 7 which has been said to be relevant for complex formation with specific ORs (Benton et al., 2006). As expected, this region is highly conserved in RproOrco, as shown by aligning its sequence with those of other insects (Fig. s1 – Supplementary Material). As seen for other Orco sequences compared to date, the presence of several putative protein kinase C target sites (Pro/Ser159; Asp248; 289 Ser/Val and Thr314) has been confirmed in the extracellular loop 2 and the intracellular loop 2 of RproOrco (Wicher et al., 2008; Sargsyan et al., 2011). Moreover, the region between TM4 and TM5 showed a degree of conservation, as reported elsewhere (Hull et al., 2012).

RproIR25a appeared as the most conserved IR coreceptor sequence, suggesting its ancestrality in relation to other IRs, as could be anticipated from phylogenetic studies (Croset et al., 2010). On the other hand, RproIR76b appeared as the shortest and most variable IR coreceptor in this species. Besides, the three transmembrane domains and the predicted ion channel appeared as the most conserved regions of all IR coreceptor protein sequences. The amino terminal region of RproIR25a showed to be another conserved part of this sequence, as seen in other reports (Olivier et al., 2011) and it has been proposed that in iGluRs, this region is involved in the assembly of subunits within the receptor complex (Ayalon and Stern-Bach, 2001).

Expression experiments reinforced the predicted chemosensory role of most genes studied, as their transcripts were found in antennae and other putative sensory structures. *R. prolixus* coreceptors had consistent expression through all developmental instars suggesting that triatomine hatchlings have functional chemosensory receptors since early life. This seems consistent with reported results obtained with other insects (Olivier et al., 2011; Wang et al., 2012; Yang et al., 2012; Dong et al., 2013; Xu et al., 2013; Guo et al., 2014). Indeed, Franco et al. (2015) obtained similar *RproOrco* expression profiles along developmental instars and in different chemosensory tissues of adult insects by means of qPCR. Most other ORs and IRs seem to present a similar antennal expression profile through triatomine development, reinforcing the proposition of a competent chemosensory function for the antennae of these insects since early life. First instar triatomine larvae are expected to have the weakest tolerance to starvation compared to all other instars and would consequently benefit from being able to find hosts efficiently after hatching from eggs (Cabello, 2001). Interestingly, three ORs and one IR seemed to increase the abundance of their antennal transcripts along development, suggesting that these receptors may not present a role for the initial instars. Nevertheless, as the expression of *RproG6PDH* also showed an apparent increase with development, further experiments will be necessary to confirm the biological relevance of this

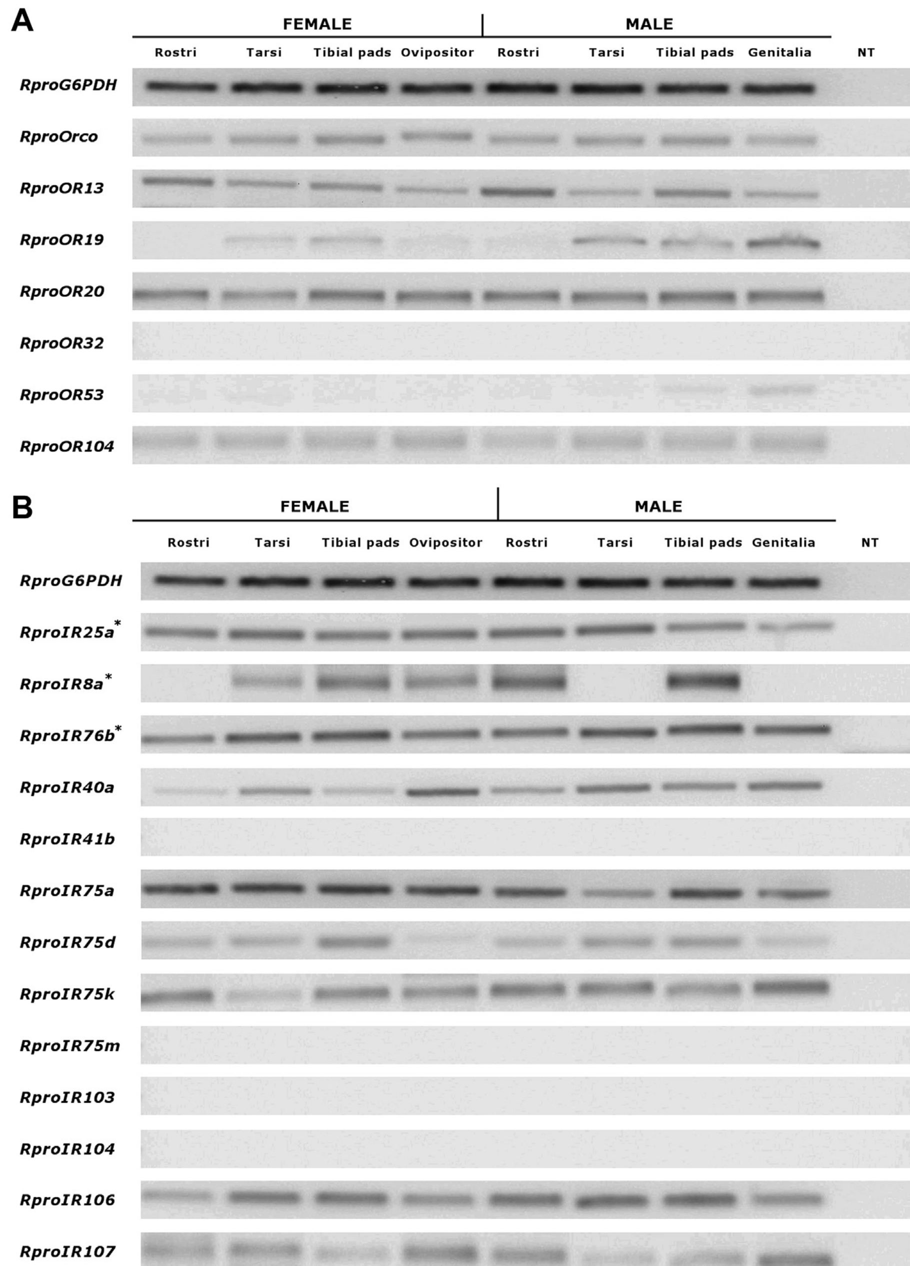

Fig. 7. Expression profiles of olfactory (A) and ionotropic (B) receptors in different male and female adult tissues of *R. prolixus* obtained by RT-PCR. NT: no-template and (*) indicates the ionotropic correceptors.

pattern. Finally, the expression of two receptors, *RproOR82* and *RproIR41b*, was more intensely detected in the antennae of adult insects. This might suggest a role related to the detection of chemical signals whose function is restricted to adult life. In fact, it has been shown that triatomines use sexual pheromones to mediate encounters between males and females (Pontes et al., 2008; Vitta et al., 2009; Zacharias et al., 2010; Pontes and Lorenzo, 2012; May-Concha et al., 2013; Pontes et al., 2014). Most coreceptors presented a widespread expression pattern in the sensory structures studied here, the exception being *RproIR8a*. Furthermore, several other ORs and IRs presented a similar expression pattern. Therefore, it is suggested that these structures present chemosensory function abilities. Several reports on triatomine behavior indicate that these insects use volatile and contact signals to exchange information by means of pheromones. For example, it has been shown that female *R. prolixus* increase their oviposition rate in the presence of chicken feathers, on which they glue their eggs (Schilman et al., 1996). Whether the recognition of these substrates is mediated by chemosensory hairs located in the ovipositor of females would need to be determined. Our results suggest this possibility deserves to be studied. Additionally, the recognition of substrate borne aggregation pheromones (Lorenzo Figueiras and Lazzari, 1998) could also be mediated by chemoreceptors that contact the floor of triatomine shelters, such as those probably located in their tibial pads and tarsi (Insausti et al., 1999). Chemosensory capabilities on all these structures could also play a role in the recognition of sexual partners (Cocchiararo-Bastias et al., 2011). Finally, the recognition of host surfaces by means of chemical properties could also be mediated by OR and IR expressing sensory structures such as rostri and tarsi. Widespread expression of

chemosensory receptors has already been reported for other insects (Xia and Zwiebel, 2006; Olivier et al., 2011; Hull et al., 2012; Wang et al., 2012; Yang et al., 2012; Xu et al., 2013), suggesting the observed expression patterns may be characteristic of insect chemosensory capabilities over diverse appendages and structures.

This is the first report on the characterization of protein features and gene expression of a subset of two of the main chemosensory protein families in a triatomine bug. The confirmation of expression of the genes studied here opens the possibility of developing functional experiments to characterize their roles. As expected, this represents a new area of vector insect research allowing the identification of potential targets to interfere with specific aspects of their biology (Turner et al., 2011; Boyle et al., 2013).

Sponsorships

INCTEM (Project number: 573959/2008-0), FAPEMIG (Project number: APQ-01359-11), PROEP-FIOCRUZ (Project number: 401973/2012-3), CNPq (Project number: 483805/2013-0) and FIOCRUZ Visiting Researcher fellowship program (fellowship JMLE 550017/2012-7).

Acknowledgments

Authors would like to acknowledge the assistance of Prof. Hugh Roberston for editing our manuscript. The assistance of Prof. Hernán Dopazo on the elaboration of phylogenetic trees is also acknowledged. Mr. Yesid Cuesta and Mr. Luis Villegas have critically commented our text, tables and figures and their support is greatly recognized. Dr. Maria Victoria Periago has edited the English style and we feel grateful for her support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.05.002.

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