Transcriptomics supports local sensory regulation in the antennae of the kissing bug *Rhodnius prolixus*

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ABSTRACT

Rhodnius prolixus is becoming a model for revealing the molecular bases of insect sensory biology due to the publication of its genome, its well characterized behavioural repertoire and the advent of NGS technologies. Gene expression modulates behaviour-triggering processes at peripheral and central levels. Still, the regulation of sensory-related gene transcription in sensory organs is poorly understood. This study characterizes the genetic bases of antennal plasticity in sensory function, using *R. prolixus* as an insect model. Antennal expression of neuromodulatory genes such as neuropeptides, neurohormones and their receptors was characterized by means of RNA-Seq. Nuclear receptor and takeout gene sequences were identified, as well as those of enzymes involved in the biosynthesis and processing of neuropeptides and biogenic amines. We report a broad repertoire of neuromodulatory and endocrine genes expressed in antennae and suggest that they modulate sensory neuron function locally. Most neuropeptide-coding genes showed consistent expression in the antennae of all stages studied. Future studies should characterize the contribution of modulatory components acting over antennal sensory processes to assess the relative contribution of peripheral and central regulatory systems on the plastic expression of insect behaviour.

INTRODUCTION

Rhodnius prolixus has been an important insect model for neuroethological studies for many decades [1,2]. Relevant aspects of its neuroethology, such as host odour-mediated behaviour [3,4], circadian modulation [5], the action of biogenic amines and neuropeptides [6] or the expression of behavioural plasticity [7,8] have been thoroughly studied. Recently, molecular processes related to sensory function have been characterized for *R. prolixus*, such as the tissue-specific expression profiles of odorant receptor genes [9] and related changes associated to development and nutrition [10]. Additionally, neuropeptide precursor genes were described for *R. prolixus* [11] and the dynamics of neuropeptide expression or release at diverse physiological conditions were characterized for processes such as feeding or ecdysis [12,13]. Based on the current knowledge on its behaviour and physiology, and the recent publication of its genome sequence, it is reasonable to suggest that *R. prolixus* has become an appropriate model for revealing the molecular bases of neuroethological processes in insects. Furthermore, neuroethological research in kissing-bug insects is of medical interest given their role as vectors of *Trypanosoma cruzi*, the causative agent of Chagas' disease, which is considered a neglected disease affecting over 8 million people worldwide (http://www.who.int/chagas/disease/en/).

Kissing-bug antennae are multimodal sensory organs dedicated to detect diverse stimuli associated to hosts [4], microenvironmental features and intraspecific communication [2]. The physiological bases of sensory processes sit on receptor neurons that express specific membrane proteins that confer them an ability to react to specific stimuli present in the environment. These neurons are mostly found in tiny hair-like structures called sensilla, which can house from one to several dozen sensory cells [14]. In a recent study, the expression of sensory-receptor coding genes was characterized in *R. prolixus* antennae by means of RNA-Seq [15]. Therefore, the antennal expression of a large set of genes related to diverse stimulus transduction processes was reported (chemoreceptors, odorant binding proteins - OBPs, chemosensory proteins - CSPs, transient receptor potential - TRP channels and pick pockets - PPKs receptors) [15].

The triggering of behaviors as a response to relevant external stimuli can be modulated at peripheral and central levels [16]. Insect behavior shows plasticity depending on age, physiological status (i.e., phase of daily cycle, nutritional or reproductive status) and experience [16]. For instance, mature kissing-bugs

seek host cues promptly, but they do not express proper host-seeking behaviour during the first week after ecdysis [17] or after engorgement [8]. Electroantennography and single sensillum recordings performed on different insect species have reported a high degree of physiological plasticity at sensory levels [18,19], at least partially explaining behavioral changes triggered by feeding or development. Similar changes have been documented at the molecular level, with altered gene expression associated to feeding [20] or age [21]. In fact, variations in gene expression depending on nutritional status or development have been described for olfactory correceptors in the antennae of *R. prolixus* [10]. Nevertheless, information about elements regulating sensory gene transcription and the abundance of the corresponding proteins in insect peripheral organs is very limited [22-25]. Physiological mechanisms modulating peripheral responses to sensory stimuli involve signaling controlled by biogenic amines, hormones, and neuropeptides, as well as their target G-protein coupled receptors (GPCRs) and nuclear receptors, overall controlling the functional status of sensory processes [16]. The main objective of this study is to characterize modulatory components potentially involved in the local regulation of antennal sensory function using *R. prolixus* as a model insect. For this purpose, we characterized the expression of a diverse set of genes known for their neuromodulatory and endocrine roles in the antennae of 5th instar larvae and adults of *R. prolixus* by means of RNA-Seq.

RESULTS

Manual gene curation

Neuropeptide and neurohormone precursor genes - A total of 17 neuropeptide precursor gene models that were absent from the RproC3 version of the *R. prolixus* genome were included in the genome GFF file (Supplementary Table S1). The long neuropeptide F (LNPF) and orcokinin (OK) predictions were corrected according to Sedra and Lange [26] and Sterkel, et al. [27], respectively. The RYamide gene model was fixed based on our antennal transcriptomes. Besides, IDLSRF-like peptide, glycoprotein hormones alpha-2 (GPA2) and beta-5 (GPB5), and bursicon-beta (also known as partner of bursicon) genes were identified in the *R. prolixus* genome. A new isoform of the *R. prolixus* adipokinetic hormone (AKH) gene, originated through alternative splicing, was identified in the antennal assemblies (Supplementary Table S1). Both AKH isoforms share the signal peptide and the active conserved peptide, but differ in the C-terminal region. Whereas the previously reported isoform encodes the core peptide and a single spacer peptide, the

isoform presented here encodes the core peptide and two non-conserved spacer peptides. The gene models of eclosion hormone (EH); ion transport peptide (ITP) isoform A; NVP-like; orcokinin-B; and orcokinin-C remained incomplete because it was impossible to fix them due to problems in the genome assembly, e.g. some fragments were located in the opposite strand or were absent from the genome assembly (Supplementary Table S1).

G-protein coupled receptors - Most of the biogenic amine-related GPCR gene models were edited (Supplementary Table S2). However, many of these genes models are still incomplete. In the case of Family A neuropeptide receptor genes, a total of 15 gene models based on Ons [40] were included in the GFF file of the *R. prolixus* genome (Supplementary Table S2). Besides, 11 gene models of this receptor family were edited in the existing GFF file of the genome. Two isoforms (alfa and beta) of the Corazonin (CZ) receptor gene were described Hamoudi, et al. [28]. Nevertheless, our antennal transcriptome only presented the alfa isoform (GenBank Acc. N° AND99324). A second kinin receptor (previously described as an orphan receptor by Ons, et al. [29]) and a Tachykinin 86C-like receptor were identified. Most of the Family B neuropeptide receptor gene models were also fixed (Supplementary Table S2). A phylogenetic tree was built to annotate both the calcitonin-like (CT) and the corticotropin-releasing factor-related like (CRF) diuretic hormone (DH) receptors (Supplementary Fig. 1S). Two CT/DH-like receptors were previously described in *R. prolixus* by Zandawala, et al. [30]: receptor 1 and receptor 2, the ortholog of *D. melanogaster hector* gene (FlyBase Acc. Number CG4395). The resulting phylogenetic tree suggested that a third CT/DH like-receptor previously described by Ons, et al. [29] seems to be exclusive of heteropteran insects (Supplementary Fig. S1). The CRF/DH-like receptors 1 and 2 (including isoforms 2A and 2B) were grouped in a different clade as shown in Zandawala, et al. [30].

Biogenic amine biosynthesis enzymes - All enzymes known to mediate biogenic amine biosynthesis in other insects were annotated in the last version of the *R. prolixus* genome [31]; however, minor changes would be needed to fix some of them (Supplementary Table S3). These models include: 1) Tyrosine 3-monooxigenase (TH), which synthesizes dopamine from L-tyrosine; 2) Aromatic L-amino acid decarboxylases (AADCs) 2 and 4 (involved in the synthesis of dopamine from L-DOPA and in the synthesis of

tyramine from L-tyrosine, respectively) and; 3) Tryptophan 5-hydroxylase-1 (TPH1), which synthesizes serotonin from L-tryptophan.

Neuropeptide processing enzymes - The neuropeptide processing enzymes were not previously annotated in the *R. prolixus* genome [31]. Using sequences from *Drosophila* as queries, we were able to identify a total of 9 enzyme genes that seem to correspond to *R. prolixus* orthologues (Supplementary Table S4). The processing of neuropeptides involves the following enzymes: 1) signal peptidase (SP), which cleaves the signal peptides from their N-terminals; 2) three members of the furin subfamily (dFUR1, dFUR2a and dFUR2b), which are Subtilisin-like endoproteases that cleave the propeptide at monobasic (Arg) and dibasic (Arg-Arg/Lys-Arg) sites; 3) prohormone convertase 2 (*amontillado* or PC2), which cleaves mono (Arg) and dibasic (Arg-Arg; Lys-Arg; Arg-Lys; Lys-Lys) sites; 4) the carboxypeptidase M (two new isoforms were identified in the antennal assemblies with differences in the 3´ region) and D (known as *silver*, which trims C-terminal Arg and Lys after Furins/PC2 cleavage reaction); 5) the PHM (Peptidylglycine alfa-hydroxylating mono-oxygenase) amidating enzyme, which is responsible for the alpha-amidation of the peptide C-terminal; 6) a prolyl endoprotease belonging to the Peptidase 9 protein family, for which no functional information is available for insects (Supplementary Table S4); 7) the amidating enzymes, the peptidyl alfa-hydroxyglycine alfa-amidating lyase (PAL) 1 and 2.

Nuclear receptors - The ecdysone receptor (*Eip75B*) gene was the only annotated nuclear receptor in the *R. prolixus* genome so far [31]; however, no information about isoforms was included in the annotation. In the antennal assemblies, the sequence of the *RproEip75B* was identified using *DmelEip75B* and posteriorly compared to the VectorBase prediction. This comparison allowed correcting the VectorBase prediction and identifying it as isoform A (by means of the two distinctive exons in the N-terminal-region) and the antennal sequence as isoform B (with the first exon located in the second intron of the A isoform)[32]. Besides *RproEi75B*, a total of 20 nuclear receptor genes were identified (Supplementary Table S5) and annotated based on their phylogenetic relations to those of *Cimex lectularius*; *Pediculus humanus*; and *D. melanogaster* nuclear receptor sequences (Supplementary Fig. S2). The orthologues of *D. melanogaster eagle* and hormone receptor like-83 genes were not identified either in the *R. prolixus, C. lectularius* or *P. humanus* genomes (Supplementary Fig. S2).

***Takeout* genes** - Three takeout (*to*) genes had been previously identified in the *R. prolixus* genome: *to1* (RPRC010098); *to2* (RPRC002313); and *to3* (RPRC01009) [31]. A total of 12 new *to* gene sequences were identified in our assemblies (Supplementary Table S6) and annotated based on their phylogenetic relations (Figure 4). Considering this analysis, RPRC002313 and RPRC010096 were annotated as *to6* and *to2*, respectively. *R. prolixus to* genes were separated into two different clades: *to1-to9* and *to10-to15*. All the structural characteristics of *to* genes were identified in *R. prolixus to* sequences: presence of signal peptide; two conserved cysteine residues in the N-terminal region and two conserved motifs [33]. As expected, the length of all *to* sequences was close to 250 amino acids (Supplementary Fig. S3). Finally, it was observed that 11 out of 15 *to* genes clustered in KQ034137 and KQO34102 supercontigs, with 8 and 3 genes each (Supplementary Fig. S4).

Antennal expression profiles

Neuropeptide and neurohormone precursor genes - A total of 32 neuropeptide precursor genes were found to be expressed in *R. prolixus* antennae, considering a value of >1 Fragments Per Kilobase Million (FPKM) in at least one library as an exclusion threshold (see Supplementary Database 3). Fifteen out of 46 *R. prolixus* neuropeptide genes showed FPKM values higher than 10 in at least one library. Allatostatin-CC (AstCC), allatostatin-CCC (AstCCC), ITG-like, IDLSRF-like peptide and OK were the most highly expressed neuropeptide genes in the antennae of *R. prolixus* (Fig. 1a and Supplementary Database 3). The gene encoding for AstCC was the one showing highest expression in our database, especially in larval antennae (larvae FPKM value = 888; female FPKM value = 98.5 and male FPKM value = 55). Indeed, the lower expression of this gene in male antennae was statistically significant (FDR<0.05) when compared to that observed in larval antennae (Table S7). For AstA and myoinhibitory peptide (MIP), a significant lower expression (FDR<0.05) was also observed in the antennae of both adult stages when compared to larvae (Table S7). The antennal expression of allatotropin (AT); OK and IDLSRF-like peptide seems to increase after imaginal moult (Fig. 1a). The expression reported for OK; Dh31; CAPA; AKH and ITP is the sum of their different isoforms or splicing variants.

GPCRs - Data suggest that more than half of Family A neuropeptide receptor genes (23 out of 43 genes) were expressed in the antennae (FPKM values >1 in at least one library; Supplementary Database 3).

Crustacean cardioactive peptide (CCAP) receptor 1; NPF receptor 1; ITP; GPA2/GPB5 receptor and RFamide peptide receptor were the most highly expressed Family A receptor-coding genes (Fig. 1b and Supplementary Database 3). The expression of the AKH receptor was significantly lower (FDR=0.06) in females, as compared to larval, antennae (Table S7). Interestingly, the expression of kinin receptor 2 increased significantly in the antennae of adults (FDR=0.058 and FDR=0.014 for female and male, respectively; Fig.1b and Table S7). The antennal expression reported for ACP/CZ related peptide, Capability (CAPA) and CZ receptors, as well as for Pyrokinin receptor 2 was the sum of their different isoforms.
In the case of Family B neuropeptide receptor genes, only calcitonin-like diuretic hormone (CT-DH) receptor 2 showed FPKM values lower than 1 (Supplementary Database 3). Five out of seven receptor genes belonging to this family presented FPKM values higher than 10 in at least one library (Supplementary Database 3). CT/DH receptor 3, which according to our phylogenetic analysis seems to be exclusive of heteropterans, showed the highest expression for this family. In fact, its expression showed a significant increase in the antennae of adults (FDR=0.0978 and FDR=0.041 for female and male, respectively) when compared to those from larvae (Fig. 1b and Table S7). A similar expression pattern was observed for CT/DH receptor 1 gene (isoforms B and C included) and for the corticotropin releasing factor like diuretic hormone (CRF/DH) receptor 2 (isoforms A and B included) (Fig. 1b). Regarding other GPCRs, transcripts of UV opsin receptor, free fatty acid receptor-4 like and NinaE were detected in all three libraries (Fig. 1b).

Tyrosine kinase and guanylyl cyclase type receptors - The neuropeptide-like precursor 1 (NPLP1) putative receptor (tyrosine kinase-type) and the potential neuroparsin (guanylyl cyclase receptor) seem to be expressed in the antennae of *R. prolixus* (Fig. 1b).

Neuropeptide processing enzymes - All enzymes involved in neuropeptide processing, except prohormone convertase 1, seem to be expressed in the antennae of *R. prolixus*, presenting values higher than 10 FPKM in at least one library (Supplementary Table S7). The peptidyl-amidating monooxigenase, signal peptidase and furin-like protease 1 genes showed the highest expression (Fig. 1c).

Biogenic amine related genes - Expression of at least 14 out of 18 biogenic amine receptor genes was detected in the antennae of *R. prolixus* (FPKM value >1 in at least one library). Muscarinic acetylcholine (M-AC) receptor DM1-like; orphan receptor 1; serotonin 2b receptor and serotonin-like receptor 1 and 2b

genes presented the highest antennal transcription within this group (Fig. 2a and Supplementary Database 3). The expression of the octopamine (Oct) receptor 3R showed a significant increase (FDR=0.071) in male antennae compared to larvae (Fig. 2a), while octopamine receptors beta 1R and 2R showed a similar trend. All genes encoding for enzymes involved in the biosynthetic pathway of biogenic amines were detected in the antennae of *R. prolixus* (Fig. 2b). The gene that encodes for Tyrosine-3-monoxigenase, which synthesizes DOPA from L-tyrosine, was the most highly expressed of this enzyme group (Fig. 2b).

Nuclear receptor genes - Ecdysone-induced protein 75, hepatocyte nuclear factor 4, hormone receptor-like in 96 and *ultraspiracle* were the genes with the highest expression, with FPKM values >10 in the three libraries (Fig. 3; Supplementary Database 3). The expression of hormone receptor-like in 46 increased significantly after imaginal moult in male antennae (FDR= 0.017; Table S7). Six nuclear receptor genes had no expression (FPKM value < 1 in the three libraries) in the *R. prolixus* antennal transcriptomes, these were: *Dissatisfaction*; Ecdysone-induced protein (EIP) 78C; Hormone receptor (HR) like in 51; *Knirps*; *Tailless* and *Seven up* (Fig. 3; Supplementary Database 3).

***Takeout* genes** - These genes were highly expressed in *R. prolixus* antennae (Fig. 4), 6 out 15 presenting FPKM values higher than 1000 in at least one library (Supplementary Database 3). While most *to* genes tended to present an increased expression in adult antennae, a few seemed to follow the opposite pattern. For example, *to11* gen showed a significant decrease after imaginal molt (FDR <0.05 in both sexes; Table S7), while *to2*, decreased its expression significantly only for male adults (FDR=0.012; Table S7). Nevertheless, the expression of *to3* showed a significant increase in both adult stages after molting (FDR<0.05; Table S7), and those of *to*4, *to*7, *to*8, *to*10, *to*12, *to*14 and *to*15 followed a similar profile. The genes included in the clade of *to1-to9* tended to present higher expression level in antennae.

DISCUSSION

The molecular bases of sensory plasticity at the local antennal level have been sparsely analysed (revised by Gadenne, et al. [16]). Our study has characterized the expression profile of a diverse set of genes encoding different modulatory elements (neuropeptides, GPCRs, nuclear receptors and *takeout* genes) in the antenna of *R. prolixus*. The antennal transcription of a broad repertoire of these genes suggests that

diverse local systems may be dedicated to the modulation of antennal functions, such as the detection of host cues and communication signals [2].

The presence of neurosecretory cells in insect antennae has only been previuosly described for mosquitoes [34], but no other study has reported neuropeptide synthesis in these organs. The existence of similar cells could be the basis for the detection of a broad set of neuropeptide transcripts in the antennae of kissing-bugs (a total of 36 neuropeptides seems to be expressed) and other insects [23,35,36]. The expression of neuropeptide processing enzyme genes in the antennae was also detected. As far as we know, this is the first report on the expression of this type of enzyme genes in insect antennae (Figure 1c). The results presented herein add evidence supporting the antennal production of neuropeptides. However, immunohistochemistry and microscopy experiments would be necessary to confirm the existence of neurosecretory cells in the antennae of kissing bugs and other insects.

Antennal neurosecretory cells have been shown to project collaterals that form synaptoid sites on the dendrites of sensory neurons in mosquito antennae [34,37]. In recent years, modulatory actions of different neuropeptides have been shown on both antennal and labral laberal chemosensory neurons [22-25]. Nevertheless, no evidence supporting a local origin for these neuromodulatory signals has been found. The current study shows that *R. prolixus* antennae produce a diversity of neuropeptide coding transcripts, among them high levels of AstCC and ITG-like peptide in all three libraries (Fig. 1a). Functional RNAi or CRISPR/CAS9 studies should be performed in order to elucidate their role.

Orcokinin and IDLSRF-like peptide presented increased antennal expression after the imaginal moult, suggesting that these peptides may be related to adult-specific sensory processes underlying dispersion by flight and mating in kissing-bugs. On the other hand, the decreased antennal expression of AstA and MIP in adults when compared to larvae suggests a role in immature instars. Instar-specific functional studies with both allatostatins will be necessary in order to understand their antennal function. The significantly lower expression of AstCC in male antennae may suggest a sex-specific antennal role.

The expression of 30 out of 46 neuropeptide and neurohormone receptor genes (FPKM value >1, Supplementary Database 3), the other fundamental component of the neuropeptidergic system, suggests

that diverse local regulatory processes can react to a similarly complex set of modulatory signals. Indeed, 14 neuropeptides and their corresponding receptors presented expression higher than 1 FPKM value in at least two conditions (Table 1), reinforcing the hypothesis of a local regulatory system. The expression of neuropeptide receptor genes in antennae has been already described in other insects [35,36]. The high expression shown in all conditions by LNPF receptor 1, GPA2/GPB5 receptor (also known as leucine-rich repeat-containing G protein-coupled receptor 1 - LGR1) and CT/DH receptor 1 (Fig. 1b), suggests an important regulatory role on antennal function. Interestingly, a LNPF-based system modulates responsiveness to food odours of a specific class of OSN in *D. melanogaster* [24]. Whether this could also be the case for OSNs in *R. prolixus* antennae deserves consideration. The significantly augmented expression of CT/DH receptor 3 and Kinin receptor 2 observed in the antennae of adults (Fig. 1b and Supplementary Table S7) suggests a regulatory function of adult-specific sensory processes. A similar increased adult expression profile was previously observed in the antennae of *R. prolixus* for several chemoreceptors [15]. Therefore, it would be interesting to study whether these are functionally connected in the adult phase. The significant decrease observed on the expression of the AKH receptor gene in female antennae may suggest a relation to the modulation of pheromone perception and production as observed for *D. melanogaster* in a sex-specific and starvation dependent manner [38]. Again, it would be interesting to analyse its functional role in kissing-bugs.

Peripheral effects of biogenic amines and their antennal production in insects have been reviewed by Zhukovskaya and Polyanovsky [39]. As observed for neuropeptides [22,24], the modulation of chemosensation and other sensory modalities by biogenic amines [40] depends on their antennal levels [39], as well as the abundance of their receptors [41]. Actually, *in situ* hybridization allowed detecting octopamine and tyramine receptor gene transcripts by or close to the cell soma of olfactory receptor neurons of different insects [23,42]. This supports the existence of direct modulatory effects of biogenic amines on peripheral sensory processes. Biogenic amines such as octopamine have been proposed to directly affect transduction and spike generation on ORNs [43]. Consistent with these findings, a diverse set of transcripts of biogenic amine receptors was identified in the antennal transcriptome of *R. prolixus* (a total of 14 biogenic amine receptor genes seem to be expressed in them) and in those from other insects [25,35,36]. As observed for neuropeptides,

most of the genes coding for enzymes involved in the biosynthesis of biogenic amines seem to be expressed in *R. prolixus* antennae (Fig. 2b). Serotonergic nerve fibres innervate the antennae of mosquitoes [44] which could relate to the high antennal expression observed for 5-HT receptors in *R. prolixus*, since the DM1-like muscarinic acetylcholine (mAch) receptor and three serotonin (5-HT) receptors (Fig. 2a) showed highest expression in all libraries. Octopamine receptors may have a modulatory role on male sensory processes, as they showed increased expression, this being significant in the case of receptor 3, in the antennae of male adults (Fig. 2a; Supplementary Table S7). A role of octopamine receptors in the modulation of male sensory physiology was observed in male moths in which this molecule enhances ORN sensitivity to specific sexual pheromone components [43].

Hormonal regulation on insect sensory systems has been poorly studied at the peripheral organs [45]. Here we show that most nuclear receptors present in the genome are expressed in the antennae of an insect (Fig. 3 and Supplementary Database S3), suggesting that these organs have broad capacity to respond to endocrine signals. It is worth mentioning that *Eip75B* and hepatocyte nuclear factor 4 (*Hnf4*) genes are the most expressed nuclear receptor in *R. prolixus* antennae (Fig. 3). Considering ecdysteroid signalling, the detection of *Eip75B* transcripts indicates a potential capacity of kissing-bug antennae to respond to the EcR-*USP* complex (Ecdysone receptor + *Ultraspiracle*), as observed for *Spodoptera litoralis* [45]. Besides, *Eip75B* and Hormone receptor-like 51 (also known as *unfulfilled*) have been identified in central clock cells of *D. melanogaster* and control the expression of clock genes, playing an important role in the maintenance of locomotor rhythms [46,47]. Therefore, we suggest that these nuclear receptors may have a similar regulatory role at the periphery, considering that the presence of a peripheral circadian clock has been reported for insect antennae [48]. The *Hnf4* gene, which induces the expression of enzymes that drive lipid mobilization and β-oxidation as a response to starvation in *D. melanogaster*[49], also showed high expression in antennae. The relatively low nutritional status of the insects used in our studies could relate to its high expression in *R. prolixus* antennae. Functional studies would need to be performed in order to evaluate the potential role of this gene as a nutritional sensor in insect antennae. An increased expression of the Hormone receptor-like in 46 (also known as Hormone receptor 3), which is the heterodimer partner of Eip75B, in male specimens suggest a sex-specific role in antennae.

Fifteen *takeout* genes were identified in the *R. prolixus* genome, while Ribeiro, et al. [50] identified 18 potential takeout transcripts in a midgut transcriptome of this species and Marchant, et al. [51] identified 25 takeout transcripts in the transcriptome of the kissing-bug *Triatoma brasiliensis*. Consistently, these numbers match the scale of those found in *Anopheles gambiae* (10); *Acyrthosiphon pisum* (17); and *Bombyx mori* (14) genomes [52]. *R. prolixus to* genes present a cluster organization (Supplementary Fig.4S), probably due to gene duplication events, as it was previously observed in other insects [52]. The antennal expression of *takeout* genes has already been reported in Dipterans [53,54]. Furthermore, it has been shown that starvation induces the expression of these genes [54] that have also been related to foraging activity [55]. This putative function could explain the high expression observed in the three antennal libraries (Fig. 4), however, functional studies need to be performed to be able to confirm these roles in the antennae of kissing-bugs. Two *to* genes presented significant differences between larval and adult antennal transcriptomes (*to11* and *to3,* with an up and downregulation, respectively) and *to2* is significantly down-regulated when male antennae are compared to those of larvae (Supplementary Table S7). If these *to* genes are related to sex, as it was observed in *D. melanogaster* [56], or are related to other adult specific behaviour, deserves to be studied, however, the putative ligands and intracellular receptors of *to* genes are still unknown.

Given that antennal cells are bathed by haemolymph but not so the dendrites of sensory neurons, it is certain that there is modulation by central signals like circulating hormones, biogenic amines and neuropeptides in insect antennae [16]. However, the antennal detection of neuropeptide transcripts and of those of enzymes involved in their biosynthesis (and that of biogenic amines) suggests the existence of a local regulatory system, which could also modulate the sensitivity of peripheral neurons. Future RNA-seq, peptidomics, in *situ* hybridisation and other functional genetic experiments should confirm that these regulatory components are present in the antennae of insects and unveil the interaction between central and peripheral regulatory systems to understand their relative roles in the control of antennal sensory physiology.

METHODS

Transcriptomic data analysis - Read sequences and *de novo* assemblies were obtained from Latorre-Estivalis, et al. [15]. In this study, three antennal transcriptomes of unfed 21 day-old 5th instar larvae and female and male adults from *R. prolixus* (colony originated from Honduras and held at the Centro de Pesquisas René Rachou – FIOCRUZ) were obtained. A total of sixty antennae were collected per sample and used for RNA extraction for subsequent RNA-Seq library preparation and sequencing as described in Latorre-Estivalis, et al. [15]. Briefly, sequencing was performed at the W. M. Keck Centre for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign, IL, USA) on an Illumina HiSeq2000 from both ends to a total read length of 100 nucleotides. Read sequences were obtained from PRJNA281760/SRP057515 project at NCBI, which contains data from the three conditions analysed: SRS923612/SRX1011796/SRR2001242 (antennal library from larvae); SRS923595/SRX1011769/SRR2001240 (antennal library from female adults); and SRS923599/SRX1011778/SRR2001241 (antennal library from male adults). Reads were mapped to the *R. prolixus* genome assembly (version RproC3) by means of STAR v.2.6.0 [57] and an edited genome GFF file. Raw read counts were used for differential expression analyses among stages and between sexes using the edgeR (v3.6.8). The FDR adjusted p-value (False Discovery Rate) <0.1 was set as threshold to define the significance level. Heat maps showing gene expression (expressed as Fragments Per Kilobase Million - FPKM value +1 following by Log10 transformation) of the different protein families in the conditions tested were prepared using the gplot package in R.

Manual gene curation - Manual curation of genome project databases by means of the inclusion and correction of gene models, using transcriptomic data and published studies, is fundamental for increasing database quality. The use of reliable genome databases, which need to be as complete and validated as possible, is especially relevant for performing adequate quantitative transcriptomic and functional genetic studies. Most of the target sequences curated herein were obtained from Ons, et al. [58]; Ons, et al. [29]; Ons [59]; Mesquita, et al. [31]; and Yeoh, et al. [60] (details in Supplementary Tables S1 and S2). Therefore, all sequences were compared to the SOAPdenovo and Trinity generated antennal assemblies from Latorre-Estivalis, et al. [15]. The discrepancies observed between target gene models from the *R. prolixus* genome (Gene set: RproC3.3, available on 24 Oct 2017) and the transcripts from the *de novo* antennal assemblies are reported

in Supplementary Tables S1-S6. In the case of neuropeptide precursor and GPCRs genes that were manually corrected/extended, new Generic Feature Format (GFF) files were created and included in the RproC3.3 version of the *R. prolixus* genome GFF file. In case of the other gene families, new gene models were created only for those genes that were absent from the VectorBase gene prediction database or those whose gene models were partially constructed. The modified GFF file of the genome was used for read mapping. The protein sequences of all genes analysed and the edited GFF file are included in the Supplementary Material (Database S1 and Database S2, respectively).

Identification of new genes - Orthologous sequences from *D. melanogaster* [61,62] were used in tBLASTn searches in the *R. prolixus* genomic database (www.vectorbase.org) to identify nuclear receptor genes and enzymes related to prepropeptide/preproprotein processing. Sequences of *takeout* (*to*) genes previously annotated for *R. prolixus* [31] were used as query to search for new sequences in the genome. Subsequently, all sequences were manually corrected/extended according to our *de novo* antennal transcriptomes and annotated based on their phylogenetic relations to other insect sequences. In addition, the structural characteristics of *to* genes, such as the presence of a signal peptide (detected by means of SignalP 4.0 [63]); of two conserved cysteine residues in the amino terminal region implicated in disulfide bond formation and ligand binding [64]; and of two conserved motifs [33] were confirmed in *R. prolixus to* sequences

Phylogenetic analysis - For building the phylogenetic trees, protein sequences of *R. prolixus* and other insect species were aligned using G-INS-I strategy in MAFFT v.7 (mafft.cbrc.jp/alignment/server), and manually edited in Jalview v2.6.1. Finally, maximum likelihood trees were built in PhyML v.3.0. Branch support was determined using the approximate Likelihood Ratio Test (aLRT). Non-parametric branch support based on the Shimodaira-Hasegawa-like (SH) procedure.

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AUTHORS' CONTRIBUTIONS

M.G.L conceived the project. M.G.L and J.M.L.E designed the experiments and performed data analyses. J.M.L.E generated insects for RNA-Seq. J.M.L.E carried out the bioinformatic analyses and provided RNA-Seq data. J.M.L.E, M.S, S.O, and M.G.L wrote the manuscript and provided comments on versions. All authors read and approved the final manuscript.

ADDITIONAL INFORMATION

Competing financial interests- The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FIGURES AND TABLES

Figure 1 - Heat map comparing the expression levels of (a) neuropeptide precursor genes, (b) G protein-coupled receptor genes, and (c) neuropeptide processing enzymes in the antennae of *R. prolixus* larvae (L), female (F) and male (M) adults.

Expression levels (displayed as Log10 FPKM +1) represented by means of a colour scale, in which blue/red represent lowest/highest expression. Abbreviations: R, receptor; H, hormone. The complete names of neuropeptide precursor genes, their receptors and enzymes are detailed in Supplementary Table S1-3.

Figure 2 - Heat map comparing antennal expression levels of *R. prolixus* genes coding for putative (a) BA-detecting GPCRs and for (b) enzymes involved in BA synthesis in the antennae of larvae (L), female (F) and male (M) adults.

Expression levels (displayed as Log10 FPKM +1) represented by means of a colour scale, in which red/red represent lowest/highest expression. Abbreviations: AC, acetylcholine; R, receptor; Dop, Dopamine, M-Ach, Muscarinic Acetylcholine; Oct, Octopamine; Tyr, Tyramine; Ser, Serotonine; AADC, Amino acid decarboxylase. Complete names of biogenic amine receptors and enzymes are detailed in Supplementary Table S4-5.

Figure 3 - Heat map comparing the expression levels of *R. prolixus* nuclear receptors in the antennae of larvae (L), female (F) and male (M) adults.

Expression levels (displayed as Log10 FPKM +1) represented by means of a colour scale, in which blue/red represent lowest/highest expression. Abbreviations: R, receptor; Eip, Ecdysone-induced protein; TF, transcription factor; NF, nuclear factor; HR, hormone receptor; PNR, photoreceptor-specific nuclear receptor. Complete names of these genes are detailed in Supplementary Table S6.

Figure 4 - Heat map comparing the expression levels of *R. prolixus takeout* (*to*) genes in the antennae of larvae (L), female (F) and male (M) adults.

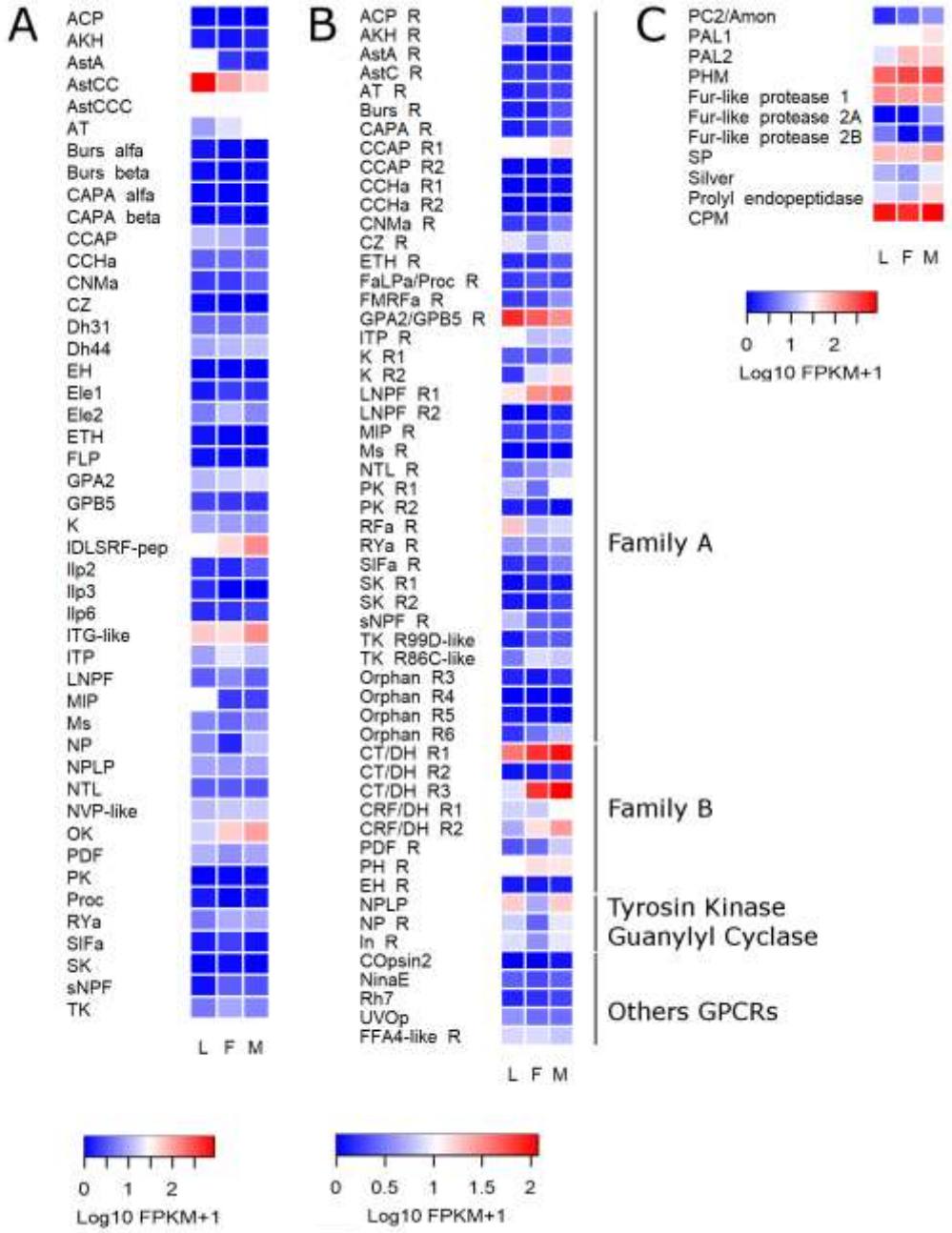
Expression levels (displayed as Log10 FPKM +1) represented by means of a colour scale, in which blue/red represent lowest/highest expression. The evolutionary history of R. *prolixus takeouts* was inferred by using the Maximum Likelihood method in PhyML v3.0. The support values on the bipartitions correspond to SH-

like P values, which were calculated by means of aLRT SH-like test. The LG substitution amino-acid model was used.

Table 1 – Antennal expression (represented as Fragments Per Kilobase Million - FPKM - values) of neuropeptides and their corresponding receptors with FPKM values higher than 1 in at least two of the analysed conditions. Complete names are detailed in Supplementary Table S1 and S2.

Neuropeptide	Larvae	Female	Male	Receptor	Larvae	Female	Male
CCAP	11.3	9.8	4.5	CCAP r	12.1	9.8	14.1
Dh31	3.4	3.3	4.8	CT/DH r1	41.8	81.1	116.7
				CT/DH r3	7.5	79.3	131.6
Dh44	7.7	10.7	12.4	CRF/DH r1	6.5	6.1	10.9
				CRF/DH r2	4.1	14.8	29.2
GPA2	10.5	14	17.5	GPA2/GPB5 r	87.9	54.1	32.5
GPB5	1.37	1.06	1.02				
LK	8.7	7.2	6	Kinin r 1	1.3	1.5	2.2
				Kinin r 2	0.7	7.4	14.1
ITP	7.5	20.1	12	ITP r	11.3	5.2	5.9
LNPF	2.5	5.1	2.6	LNPF r1	13.5	31.8	39.8
Ntl	2.4	2.3	2.1	Ntl r	1.7	2.9	5.4
NP	5.1	0.7	11.5	NP r	6.2	1.6	8.2
NPLP1	7.7	7.1	7.9	NPLP r	1.3	0.7	1.3
PDF	9.6	5.8	8.1	PDF r	1.2	1.7	6.1
RYa	3.9	8.9	8	RYa r	3.25	3.2	3.87
sNPF	0.2	2.5	1.9	sNPF r	5.3	1.6	1.5
TK	3.8	8.1	4.8	TK 86C-like r	2.1	7.2	5.8
				TK 99D-like r	0.2	1.3	1.4

FIGURE 1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ACP
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K
IDLSRF-pep
Ilp2
Ilp3
Ilp6
ITG-like
ITP
LNPF
MIP
Ms
NP
NPLP
NTL
NVP-like
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RYa
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SK
sNPF
TK
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0 1 2
Log10 FPKM+1
B
ACP R
AKH R
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AT R
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CNMa R
CZ R
ETH R
FaLPa/Proc R
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ITP R
K R1
K R2
LNPF R1
LNPF R2
MIP R
Ms R
NTL R
PK R1
PK R2
RFa R
RYa R
SIFa R
SK R1
SK R2
sNPF R
TK R99D-like
TK R86C-like
Orphan R3
Orphan R4
Orphan R5
Orphan R6
CT/DH R1
CT/DH R2
CT/DH R3
CRF/DH R1
CRF/DH R2
PDF R
PH R
EH R
NPLP
NP R
In R
COpsin2
NinaE
Rh7
UVOp
FFA4-like R
L F M
0 0.5 1 1.5 2
Log10 FPKM+1
Family A
Family B
Tyrosin Kinase
Guanylyl Cyclase
Others GPCRs
C
PC2/Amon
PAL1
PAL2
PHM
Fur-like protease 1
Fur-like protease 2A
Fur-like protease 2B
SP
Silver
Prolyl endopeptidase
CPM
L F M
0 1 2
Log10 FPKM+1

FIGURE 2

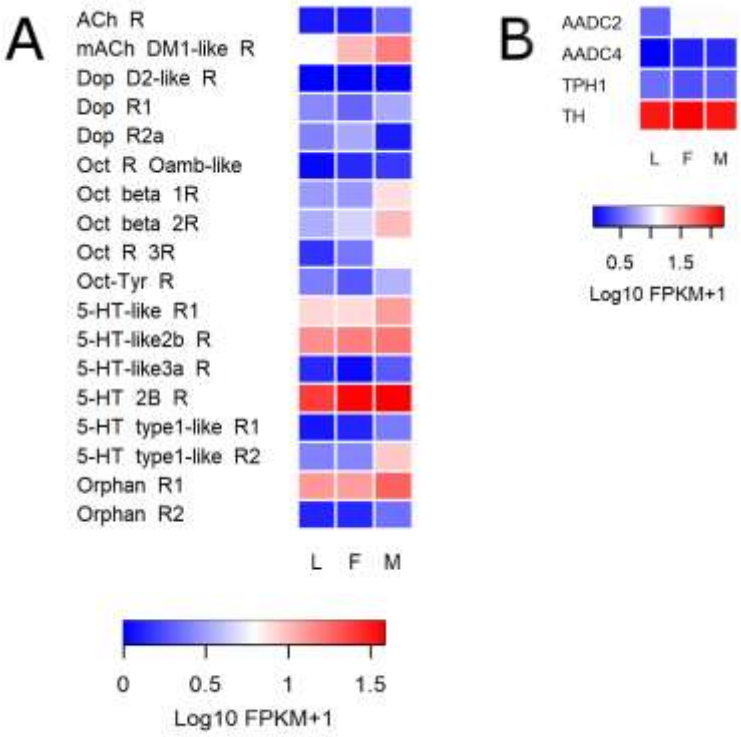


FIGURE 3

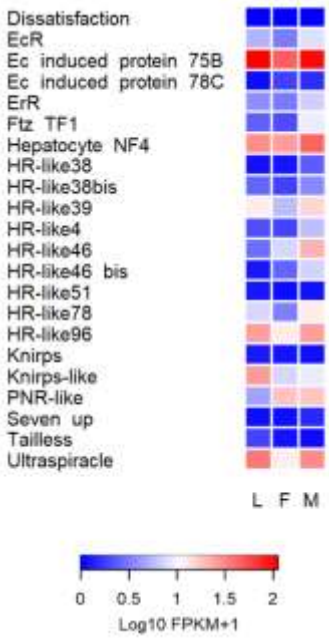
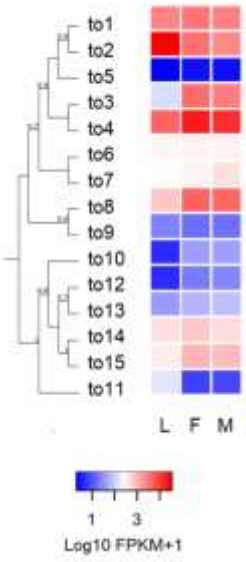


FIGURE 4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L F M
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Log10 FPKM+1