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RESEARCH ARTICLE

The neuropeptide CCHamide2 regulates diuresis in the Chagas disease vector *Rhodnius prolixus*

Natalia Capriotti[1], Juan P. Ianowski[2], Paula Gioino[2] and Sheila Ons[1,*]

ABSTRACT

Given that hematophagous insects ingest large quantities of blood in a single meal, they must undergo a rapid post-prandial diuresis in order to maintain homeostasis. In the kissing bug *Rhodnius prolixus* (Hemiptera: Reduviidae), the coordinated activity of the Malpighian tubules and anterior midgut maintains water and ion balance during the post-prandial diuresis. Three to four hours after the meal, the diuretic process finishes, and the animal enters an antidiuretic state to ensure water conservation until the next blood intake. The diuretic and antidiuretic processes are tightly regulated by serotonin and neuropeptides in this insect. In the present work, we report that the neuropeptide precursor CCHamide2 is involved in the regulation of the post-prandial diuresis in *R. prolixus*. Our results suggest a dual effect of RhoprCCHamide2 peptide, enhancing the serotonin-induced secretion by Malpighian tubules, and inhibiting serotonin-induced absorption across the anterior midgut. To our knowledge, this is the first report of a hormone presenting opposite effects in the two osmoregulatory organs (i.e. midgut and Malpighian tubules) in insects, probably reflecting the importance of a well-tuned diuretic process in hematophagous insects during different moments after the blood meal.

KEY WORDS: Insect, Anterior midgut, Malpighian tubules, Kissing bug, Hematophagy

INTRODUCTION

Hematophagous insects, including triatomines, take a large amount of blood in every meal, which can be equivalent to several times their own body weight. A rapid post-prandial diuresis takes place in order to maintain the osmotic and ionic balance, and to recover mobility to escape from predators (Coast, 2009). *Trypanosoma cruzi*, the protozoan parasite that is the causative agent of Chagas disease, is ingested by triatomines with the blood of an infected host. The parasite multiplies and differentiates to its infective form in the gut of the insect; when the infected triatomine takes a subsequent blood meal, it releases *T. cruzi* with its feces. The parasite enters the host through the wound of the bite or through intact mucosal membranes. Despite the efforts to prevent vectorial transmission of Chagas disease, it is still occurring in endemic regions of Latin America. Non-vectorial ways of transmission can also take place by blood transfusions, organ transplantation or transplacentally. Chagas disease is a neglected severe tropical disease which affects up to 6–7 million people worldwide (Rassi et al., 2010).

Given the relevance of triatomine excretion for Chagas disease transmission and the convenience of *Rhodnius prolixus* as a model for physiological studies in insects (Ons, 2017), diuresis in this species has been intensely explored since the pioneering experiments of Simon Maddrell (Maddrell, 1964; Maddrell and Gardiner, 1975; Maddrell and Gee, 1974). Water and ions from the blood meal are absorbed into the hemolymph through the anterior midgut. From the hemolymph, the fluid is transferred to the lumen of the Malpighian tubules (reviewed in Coast, 2009). The upper (distal) segment of the tubule secretes a primary urine that is modified in the lower (proximal) segment to form urine, which is expelled by the anus. Approximately 50% of the blood volume ingested is excreted during the first 3 h post-feeding. Then, the insect enters an antidiuretic state that allows it to survive long periods without ingesting water or nutrients for up to several months (Cabello, 2001), until the subsequent blood intake event (Quinlan et al., 1997). The transport processes must be precisely coordinated to ensure that the animal excretes the excess plasma fraction of the blood, without nutritional value, but at the same time conserves sufficient water and ions to survive long periods of water stress. Thus, the function of the excretory system, i.e. Malpighian tubules and the anterior midgut, is tightly regulated by a system of signals (e.g. hormones) that is not fully understood (Ons, 2017).

In *R. prolixus*, serotonin (5-hydroxytryptamine; 5HT) is a diuretic factor released into the hemolymph by the abdominal nerves (Orchard, 2006). 5HT triggers fluid uptake in the anterior midgut (Farmer et al., 1981), ion (Na^+, K^+ and Cl^-) and water secretion in the distal part of the Malpighian tubules (Maddrell et al., 1971) and the reuptake of K^+ in the proximal part of the tubule (Maddrell et al., 1993). The neuropeptide corticotropin releasing factor-like diuretic hormone (CRF-DH) is a potent diuretic peptide (Te Brugge et al., 2009; Te Brugge et al., 2011), and CAP2b peptide-A (CAPA) presents strong anti-diuretic activity (Ianowski et al., 2010; Paluzzi and Orchard, 2006, 2010). Both neuropeptides act by regulating absorption from midgut and excretion from the Malpighian tubules. Other neuropeptides that have been associated with diuresis in *R. prolixus* are calcitonin-like diuretic hormone (CT-DH) (Te Brugge et al., 2009; Te Brugge et al., 2005; Zandawala et al., 2011; Zandawala et al., 2015) and allatotropin (Villalobos-Sambucaro et al., 2015).

The diuretic (5HT, CRF-like and CT-like) and antidiuretic (CAPA) factors described so far have concordant actions on both components of the diuretic system (i.e. Malpighian tubules and anterior midgut). The diuretic factors stimulate ion transport in both organs, whereas the antidiuretic factors inhibit ion transport (Te Brugge et al., 2009; Te Brugge et al., 2005; Zandawala et al., 2011; Ianowski et al., 2010; Paluzzi and Orchard, 2006, 2010). Here, we describe a neuropeptide, RhoprCCHamide2, with a fundamentally

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List of abbreviations

5HT	serotonin: 5-hydroxytryptamine
CAPA	CAP2b peptide-A
CCHa	CCHamide
CNS	central nervous system
CRF-DH	corticotropin releasing factor-like diuretic hormone
CT-DH	calcitonin-like diuretic hormone
dsRNA	double-stranded RNA
GPCR	G-protein coupled receptor

different physiological function as it has opposite effects on Malpighian tubules and the anterior midgut.

CCHamide (CCHa) is a brain–gut neuropeptide precursor family conserved in insect genomes, where it is usually represented by two paralogs, CCHa1 and CCHa2 (Hansen et al., 2011; Reiher et al., 2011). The physiological role of CCHa has been studied in *Drosophila melanogaster*. DromeCCHa1 has been involved in alimentary behavior and sensorial perception of food (Farhan et al., 2013; Ida et al., 2012), whereas DromeCCHa2 seems to regulate the appetite (Ida et al., 2012; Ren et al., 2015) and the coordination of growth with nutrition (Sano et al., 2015). The *R. prolixus* genome (Mesquita et al., 2015) encodes both *CCHa1* and *CCHa2* paralogs. *CCHa2* is transcribed in two isoforms that give rise to identical mature peptides (Ons, 2017; Ons et al., 2011). Two Family A G-protein coupled receptors (GPCRs) for RhoprCCHa were identified by database searches in the genomic sequence followed by phylogenetic analysis (Ons et al., 2016). However, no functional studies have been reported for this neuroendocrine system in *R. prolixus* to date.

Here, we report a role of RhoprCCHa2 in post-prandial diuresis in *R. prolixus*. Our results suggest a dual effect of RhoprCCHa2 on 5HT-stimulated diuresis, increasing the secretory effect of 5HT on Malpighian tubules, while blocking the effect of 5HT on the anterior midgut. Injections of double-stranded RNA (dsRNA) encoding a fragment of RhoprCCHa2 transcript resulted in a reduction in the amount of transcript detected an in an increment in the urine volume produced during post-prandial diuresis in fourth instar *R. prolixus* nymphs. To our knowledge, this is the first report of a hormone presenting opposite effects in different osmoregulatory organs in insects.

MATERIALS AND METHODS

Insects

A colony of *Rhodnius prolixus* Stål 1859 was maintained in our laboratory in a 12 h:12 h light:dark period at 28±2°C. Insects were weekly fed on chickens, which were housed, cared, fed and handled in accordance with resolution 1047/2005 (Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET) regarding the national reference ethical framework for biomedical research with laboratory, farm and nature collected/wild animals. This framework is in accordance with international standard procedures. Biosecurity considerations agree with CONICET resolution 1619/2008, which is in accordance with the World Health Organization Biosecurity Handbook (https://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2006_6.pdf).

RT-PCR

The procedures performed for reverse transcription PCR (RT-PCR) have been previously described (Sterkel et al., 2012). Briefly, the complete central nervous system (CNS), anterior and posterior midgut, rectum and Malpighian tubules were microdissected from starved fifth instar *R. prolixus* nymphs (3 weeks after molt) in saline solution using a binocular microscope. The structures were separated and placed in a microtube containing Trizol® (Ambion, Sao Paulo, Brazil). Pools of five organs were used for RNA extractions, performed with Trizol according to the manufacturer's instructions. One microgram of RNA from each sample was treated with DNAseI (Promega, Madison, WI, USA) and used to synthesize cDNA with the M-MLV Reverse Transcriptase kit (Promega). PCR amplifications of the specified transcripts were performed in triplicate in a final volume of 20 µl (primers detailed in Table 1). *Rhoprβactin* was used as a positive control of the reaction. The program used in the amplifying reaction was 95°C for 5 min, and 30 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 30 s. Three biological replicates were performed.

dsRNA synthesis

For a detailed description on dsRNA synthesis, see Wulff et al. (2017). Briefly, a 270 bp fragment encoded in both isoforms of *RhoprCCHa2* was PCR-amplified using Pegasus Taq Polymerase (Productos Bio-Lógicos, Argentina) and primers containing a fragment of the T7 promoter sequence at the 5′ end (CCHaRNAiFw and CCHaRNAiRv, see primer sequences in Table 1). One microliter of the PCR product was used for a secondary PCR using the T7 promoter primer (T7 full, see primer sequence in Table 1). PCR products were used to generate RhoprCCHa-dsRNAs by *in vitro* transcription using T7-RNA polymerase (Promega) or the MEGAscript™ T7 kit (Ambion), according to the manufacturer's instructions. Reactions were performed at 37°C, overnight. The products of transcription were treated with DNAse and RNAseA (Fermentas, Waltham, MA, USA). Subsequently, a precipitation with AcNa 3 mol l^{-1} was performed. The precipitate was resuspended in saline solution. A 274 bp fragment from the β-lactamase bacterial gene was PCR-amplified from the pGEM®-T vector with specific primers (ARNiAMPrFw and ARNiAMPrRv). This is an unspecific sequence that is absent from the *R. prolixus* genome, and was used as a control dsRNA. PCR products were sequenced at the Macrogen facility (Seoul, Korea) to corroborate the specificity of the fragment obtained. The formation of dsRNA was confirmed by running a 2% agarose gel and quantified from an image of the gel using the software ImageJ 1.32 (National Institutes of Health, Bethesda, MD, USA). The dsRNA was stored at −20°C until use.

RNAi-mediated gene silencing of RhoprCCHa *in vivo*

Fourth instar *R. prolixus* nymphs (2 weeks after molt and starved since the last eclosion) were injected into the abdomen with 2 µl of dsRNA (1 µg $µl^{-1}$, either dsCCHa or control) diluted in *R. prolixus* saline solution (in mmol l^{-1}: 129 NaCl, 8.6 KCl, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 glucose, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4 and 8.6 Hepes; pH 7).

In vivo diuresis assay

Insects were weighed and individually differentiated by color marks with non-toxic acrylic paint in the posterior legs. All the animals from dsCCHa and control groups were mixed together in a single jar for feeding, to avoid potential differences on the blood meal conditions. The insects were allowed to feed for 15 min on an immobilized chicken. Those insects that did not feed to repletion (homogeneously distributed in both experimental groups) were discarded for the analysis. Once the feeding period was finished, the insects were weighed. The volume of blood ingested was estimated as final mass–initial mass. After this, the animals were individually placed in 1.5 ml microtubes previously weighed in a precision

Table 1. Sequences of primers

Primer name	Sequence 5′→3′	Melting (°C)	Amplicon length (bp)	PCR efficiency (%)	Use
CCHaRNAiFw	TAATACGACTCACTATAGGGCGGCTCTGTAGATCGTTCTCT	58	270	ND	dsRNA synthesis
CCHaRNAiRv	TAATACGACTCACTATAGGGCCTTTGAAAGCGGCTCCAT	58			
CCHaRpFwd	TACGATCCAGGGACACCAT	58	99	80	qPCR
CCHaRpRv	CATGGACGGTGAGCAGTAAG	58			
rCCHa7766RpFw	CAGAGAATGCTATCACAGTCATACC	57	90	ND	RT-PCR
rCCHa7766RpRv	GACGAGGAATATCACAGCGAATAC	57			
rCCHa608RpFw	TAAGCAACGTAGAAGAATACACAC	54	90	ND	RT-PCR
rCCHa608RpRv	ACACCGACTAGGAACATCAC	56			
ARNiAMPrFw	TAATACGACTCACTATAGGGCCAGTGCTGCAATGATAC	53	274	ND	Control dsRNA synthesis
ARNiAMPrRv	TAATACGACTCACTATAGGGGAGCTGAATGAAGCCATAC	53			
Rhoprβ-ActinFw	ACACCCAGTTTTGCTTACGG	58	300	ND	RT-PCR
Rhoprβ-ActinRv	GTTCGGCTGTGGTGATGA	57			
RhoprGAPDHFw	GACTGGCATGGCATTCAGAGTT	60	182	102.5	qPCR
RhoprGAPDHRv	CCCCATTAAAGTCCGATGACACC	60			
RhoprActinFw	ATCTGTTGGAAGGTGGACAG	58	125	102.3	qPCR
RhoprActinRv	CCATGTACCCAGGTATTGCT	58			
T7 full	ATAGAATTCTCTCTAGAAGCTTAATACGACTCACTATAGGG	61	–	ND	dsRNA synthesis

ND, not determined.

balance. At time points of 15, 30, 45, 60, 90, 120, 150, 180 and 240 min after feeding, each insect was changed to a new weighed microtube and the volume of excreted urine was estimated by subtracting the mass of the empty tube from the mass of the tube after the visit of the insect.

qRT-PCR

Quantitative reverse transcription PCR (qRT-PCR) was performed as described previously (Wulff et al., 2018). Briefly, 24 h after finished the *in vivo* diuresis assay, insects were dissected to obtain CNS, anterior midgut and Malpighian tubules separately. Five tissues per sample were pooled in a microtube containing Trizol reagent (Ambion). cDNA was prepared as described above.

cDNA amplifications were performed for each sample in triplicate, in a final volume of 20 µl (see primer sequences in Table 1). Gene expression levels were quantified using FastStart SYBR Green Master (Roche) in iQ single color in an Arial Mx Real-time PCR instrument (Applied Biosystems). The schedule used for the amplification reaction was: (i) 95°C for 5 min; (ii) 95°C for 30 s; (iii) 50, 58 or 60°C (depending on the primers melting temperature) for 30 s; and (iv) steps (i) and (ii) were repeated for 40 cycles. A control without a template was included in all batches. *GAPDH* and *βactin* were used as a reference genes; these genes were previously validated as stables in *R. prolixus* under different conditions (Majerowicz et al., 2011; Omondi et al., 2015).

Malpighian tubule secretion assay

Malpighian tubules were isolated from fifth instar *R. prolixus* nymphs under saline solution using a binocular microscope. From each insect, two tubules were assigned to the control group and the remaining two were assigned to the CCHa2 group. Each tubule was placed into a 90 µl droplet containing either saline solution (control group) or synthetic RhoprCCHa2 1 µmol l^{-1} (GGCSAFGHSCFGGH-NH_2) (Genscript Corporation, Piscataway, NJ, USA) surrounded with mineral oil. The segment of the tubule proximal to the hindgut was wrapped to a pin held 2 mm away from the drop. The more distal portion of the tubule was exposed to the solution. A nick was carefully made with a fine forceps in the region of the tubule exposed to the mineral oil, in order to allow fluid secretion. After this procedure, 1 µmol l^{-1} 5HT was added to the medium in both groups to stimulate secretion (this is the 5HT concentration that generates the maximal rate of secretion by the Malpighian tubules; Te Brugge et al., 2011). Secreted droplets were collected from the nicked segment of the tubule using a glass probe at 10 min intervals for 40 min after stimulation with 5HT. The secreted droplets were photographed using a microscope digital camera (MiniVid, LW Scientific, Lawrenceville, GA, USA) and the diameter was measured offline with the software ImageJ 1.32. The volume of each drop of urine was calculated with the sphere equation ($V=4/3\pi r^3$). The cumulative volume secreted over 40 min was calculated.

Anterior midgut fluid transport assay

The anterior midgut was microdissected from fifth instar nymphs 2 weeks after ecdysis and starved since eclosion under saline solution using a binocular microscope. The tissue was washed once for 5 min with gentle agitation to remove the remnants of blood. Subsequently, the posterior end was ligated with a silk thread and filled with 50 µl of saline solution colored with Bromophenol Blue, in order to detect possible leaks. The anterior end was ligated with another strand of silk thread. The tissue was gently dried with an absorbent paper and weighed in a precision balance (model 1207 MP2, Sartorius, Göttingen, Germany). Finally, the preparation was incubated for 1 h with either saline solution, 0.1 µmol l^{-1} 5HT in saline solution (this is the concentration of 5HT that more strongly stimulate the rate of absorption across the epithelium of the anterior midgut; Te Brugge et al., 2009), 0.1 µmol l^{-1} synthetic RhoprCCHa2 in saline solution, or 0.1 µmol l^{-1} 5HT plus 0.1 µmol l^{-1} RhoprCCHa2 in saline solution (Ianowski et al., 2010). The tissue was removed from the incubation medium, gently dried with absorbent paper and weighed. The difference (initial mass–final mass)/60 min was evaluated as the rate of absorption of the anterior midgut.

Anterior midgut contraction assay

The anterior midgut contraction assay has been previously described (Wulff et al., 2018). Briefly, fifth instar *R. prolixus* nymphs (2–3 weeks after eclosion) were placed ventral surface down on a Petri dish covered with paraffin. The insects were fixed by melting a small amount of the paraffin with a lighter, placing the animal in the paraffin and gently adjusting it until the paraffin solidified. The dorsal cuticle was removed under saline solution,

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and the internal organs were exposed. The semi-intact preparation was equilibrated in 90 µl saline solution for 20 min at room temperature (25±2°C), replacing the fluid with fresh saline every 5 min. The anterior midgut contraction rate was measured by counting the number of contractions in 30 s, and is expressed as a percentage of the control (saline). This procedure was repeated six times for each preparation, and the results of the six measurements were averaged. After this, the saline was removed and replaced with an equal volume of solution containing RhoprCCHa2 at different concentrations (0.001, 0.01, 0.1, 1 and 10 µmol l^{-1}). Ten animals were analyzed for control and for each RhoprCCHa2 concentration.

RESULTS

Expression pattern of RhoprCCHa2 and CCHamide GPCRs in tissues

The expression pattern of RhoprCCHa2 and the two putative RhoprCCHa GPCRs, encoded in the predicted transcripts RPRC00776 and RPRC000608 of the *R. prolixus* genomic sequence (www.vectorbase.org; RproC3 dataset), was determined using RT-PCR (Fig. 1). The transcript encoding neuropeptide RhoprCCHa2 precursor was detected in the CNS, anterior midgut and Malpighian tubules, but not in the posterior midgut or the hindgut (Fig. 1). Besides, a transcript encoding RPRC007766 was identified in the posterior midgut and RPRC000608 transcript was PCR-amplified in the anterior midgut, Malpighian tubules and posterior midgut (Fig. 1).

RhoprCCHa2 regulates post-prandial diuresis

The tissue-specific transcription of RhoprCCHa2 and its putative receptors suggested an involvement in the regulation of diuresis. In order to test this hypothesis, we performed an *in vivo* experiment using RNAi-mediated gene silencing for *RhoprCCHa2*. Unfed fourth instar *R. prolixus* nymphs were injected with control dsRNA (n=25) or dsRNA encoding a fragment of *RhoprCCHa2* (dsCCHa group, n=44).

Seven days after the injections, control and dsCCHa insects were fed for 15 min. No significant differences in the mass of blood ingested were detected between control and dsCCHa groups (0.098±0.070 and 0.104±0.150 mg, respectively).

dsCCHa-treated insects excreted significantly higher volumes of urine than the control group during the first 15 (P<0.001), 30 (P<0.001) and 45 min (P<0.001) of post-prandial diuresis (Fig. 2A). At the 60 min time point, no significant difference was detected between groups in the volume of urine excreted. Conversely, in the last time points evaluated, the volume excreted was significantly lower for the treated group (P<0.001 for 90, 120 and 240 min; P<0.01 for 180 min; two-way ANOVA with repeated measures, n=25–44; Fig. 2A).

In order to evaluate the net effect of *RhoprCCHa2* silencing in the total volume excreted, we calculated the cumulative volume expelled by adding the volume excreted during every time point by each animal. We found that the cumulative volume was significant higher for treated insects (P<0.01 at 30 min; P<0.001 at 45, 60, 90, 120, 180 and 240 min post-feeding; two-way ANOVA with repeated measures, n=25–44; Fig. 2B).

Twenty-four hours after finishing the *in vivo* diuresis assay, *RhoprCCHa2* transcription was evaluated by qRT-PCR in tissues from control and dsCCHa-treated insects. When evaluating dsCCHa-treated versus control animals, we detected an 84% decrement in the levels of transcript for *RhoprCCHa* in the anterior midgut (n=5, P<0.01, Student's t-test), a 40% decrement in the CNS (n=5, P=0.0951) and a 30% decrement in the Malpighian tubules (n= 5, P=0.153; Fig. 2C). Treated and control insects that were not dissected for qRT-PCR successfully molted to fifth instar, undergoing ecdysis around day 13 post-blood meal.

RhoprCCHa2 stimulates fluid secretion by Malpighian tubules

We evaluated the activity of the neuropeptide RhoprCCHa2 in the secretory function of the Malpighian tubules from fifth instar *R. prolixus* nymphs using a secretion assay. Stimulation was achieved by the addition of 1 µmol l^{-1} 5HT into the bathing droplet containing either saline solution (control group) or synthetic 1 µmol l^{-1} *RhoprCCHa* (CCHa group).

As expected, the stimulation of the tubules with 5HT activated fluid secretion; the secretion rate with 1 µmol l^{-1} 5HT in the bath was 12.05 nl min^{-1} (Fig. 3). Our results showed that synthetic RhoprCCHa stimulated secretion in 5HT-activated tubules, generating a secretion rate of 18.29 nl min^{-1} (Fig. 3). This represents a significant increase of 1.52-fold in the secretion rate (P<0.001; Fig. 3). Furthermore, the accumulated secreted volume was significant higher for RhoprCCHa2 incubated tubules at 30 min (P<0.05) and 40 min (P<0.01) after stimulation with 5HT (two-way ANOVA; n=37, 47). Synthetic RhoprCCHa2 was not able to induce fluid secretion in non-stimulated tubules during 40 min incubations (data not shown).

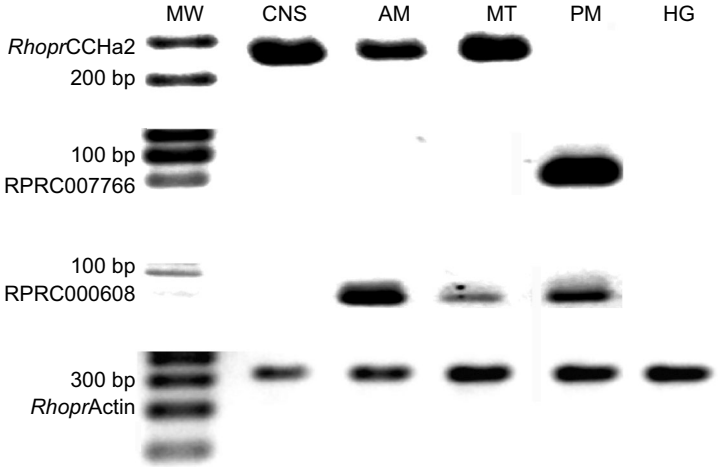

Fig. 1. Transcripts encoding RhoprCCHa2 and its G-protein coupled receptors (RPRC00776 and RPRC000608) were PCR-amplified in different tissues of *Rhodnius prolixus*. Molecular weight marker (MW), central nervous system (CNS), anterior midgut (AM), Malpighian tubules (MT), posterior midgut (PM), hindgut (HG) and negative control without template (C–) were assayed for each primer pair. RhoprActin transcript was PCR-amplified from every tissue as a positive control to test the quality of the cDNA. An agarose gel image representative of the three biological replicates performed is shown.

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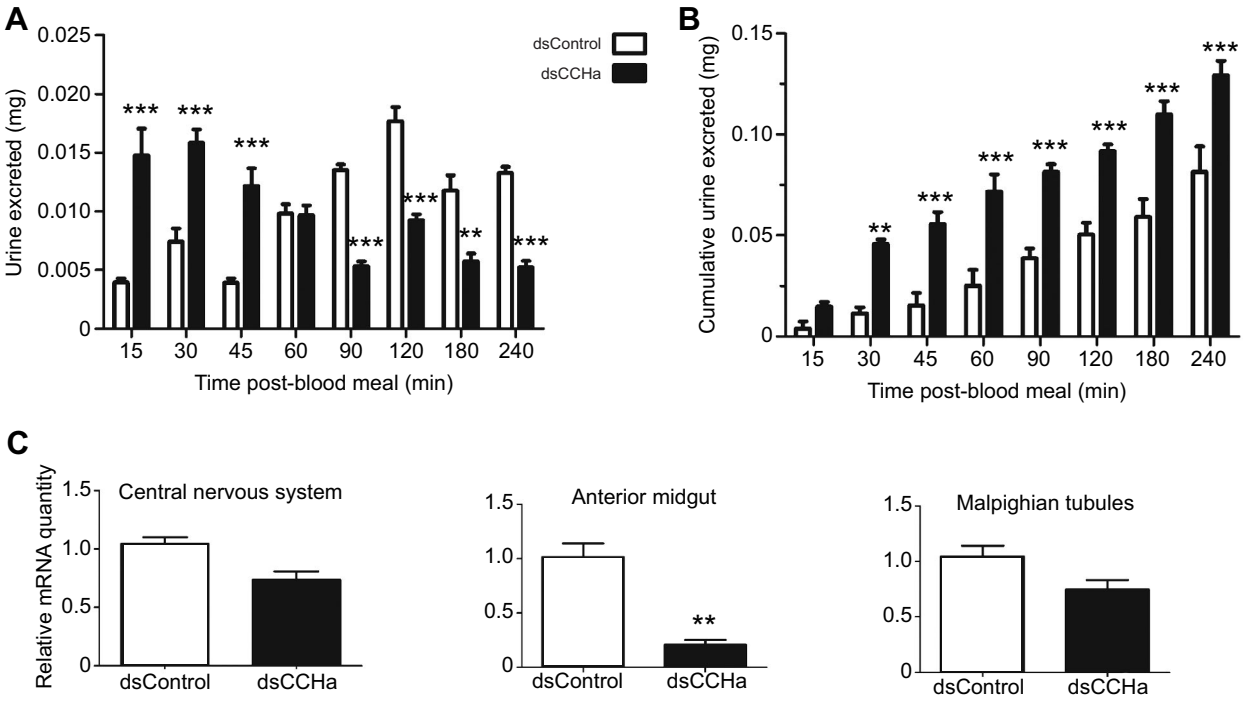

Fig. 2. Injection of dsRNA encoding a fragment of RhoprCCHa2 (dsCCHa) stimulates urine excretion by *R. prolixus*. (A) Milligrams of urine excreted at different time points post-blood intake in dsControl (n=25) and dsCCHa (n=44) injected insects. Two-way ANOVA with repeated measures; Tukey's test was used for *post hoc* comparisons. **P<0.01; ***P<0.001. (B) Cumulative volume excreted for the dsControl (n=25) and dsCCHa (n=44) groups. Two-way ANOVA with repeated measures; Tukey's test was used for *post hoc* comparisons. **P<0.01, ***P<0.001. (C) Effect of dsCCHa injection on the mRNA levels of RhoprCCHa2 in the anterior midgut, central nervous system and Malpighian tubules (n=5). **P<0.01 Student's t-test. Data are means±s.e.m.

RhoprCCHa2 regulates fluid transport by *R. prolixus* anterior midgut

Treatment with 5HT (0.1 µmol l^{-1}) stimulated fluid transport by the anterior midgut of fifth instar *R. prolixus* nymphs from the lumen into the bath (n=8–10; P<0.05, one-way ANOVA and Tukey–Kramer contrasts; Fig. 4). The transport rate was 76.0±9.6 nl min^{-1}, in agreement with a previous report (Ianowski et al., 2010). Exposing the tissue to synthetic RhoprCCHa2 (1 µmol l^{-1} in bathing saline solution) had no effect on the fluid transport rate (n=11) (Fig. 4). However, RhoprCCHa2 significantly reduced the stimulatory effect of serotonin on anterior midgut (n=11; P<0.05, one-way ANOVA and Tukey–Kramer contrasts) (Fig. 4). Even though the fluid transport is higher in the group treated with 5HT+synthetic RhoprCCHa2 with respect to the saline and RhoprCCHa2 groups, the differences were not statistically significant in our analysis (Fig. 4). Finally, RhoprCCHa2 had no effect on the contractility of the anterior midgut at concentrations of 0.001 to 10 µmol l^{-1} (data not shown).

DISCUSSION

In this work we demonstrate that CCHa2, a rarely studied neuroendocrine peptide in insects, possesses a role in the regulation of 5HT-stimulated excretory organs in *R. prolixus*.

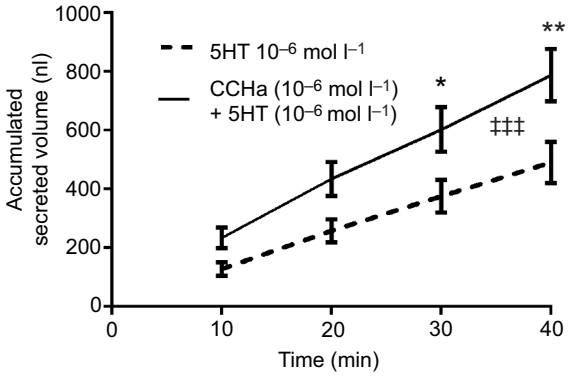

Fig. 3. Malpighian tubule secretion volume at different times after stimulation with serotonin (5HT; 1 µmol l^{-1}) in tubules incubated in saline solution (n=47) or synthetic RhoprCCHa2 (1 µmol l^{-1}; n=37). Two-way ANOVA with repeated measures followed by Bonferroni contrasts (*P<0.05; **P<0.01). Secretion rate slopes in both experimental groups are significantly different (‡‡‡P<0.001, linear regression analysis).

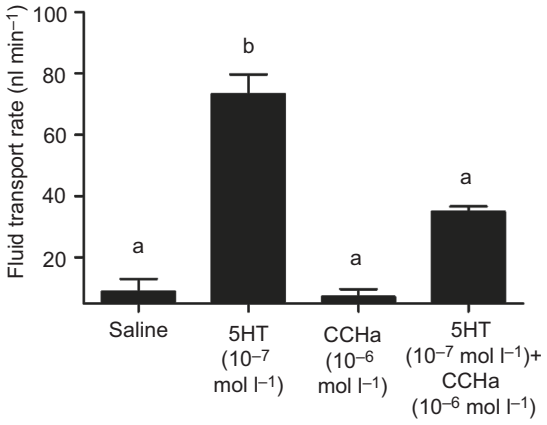

Fig. 4. Effect of RhoprCCHa2 on the fluid transport rate in the anterior midgut of fifth instar *R. prolixus*. The tissue was incubated with either saline (n=8), 0.1 µmol l^{-1} 5HT (n=10), 1 µmol l^{-1} RhoprCCHa2 (n=11) or 0.1 µmol l^{-1} 5HT+0.1 µmol l^{-1} RhoprCCHa2 (n=11). Different letters indicate significant differences (one-way ANOVA, Tukey–Kramer contrasts, P<0.05).

Furthermore, RNAi-mediated gene silencing of *RhoprCCHa2* alters the time course and intensity of the post-prandial diuresis, suggesting that it may play a role in osmoregulation after feeding.

Like most insect neuropeptides, the transcript of RhoprCCHa2 was detected in the CNS. We also found this transcript in the anterior midgut, suggesting that it is a brain–gut neuropeptide such as DromeCCHa and BommoCCHa (Reiher et al., 2011; Roller et al., 2008). RhoprCCHa2 transcript was also detected in Malpighian tubules. Even though the Malpighian tubules are not a common site of neuropeptide precursor expression, *Rhopr*FGLamide was also found in this structure (Zandawala et al., 2012). Our finding may suggest that RhoprCCHa2 could regulate Malpighian tubules function in an autocrine or paracrine way. Furthermore, the transcripts of the putative GPCRs for RhoprCCHa2 were found in the posterior midgut (RPRC007766) and in the anterior midgut, posterior midgut and Malpighian tubules (RPRC000608), also pointing to a possible role in feeding-related events, diuresis and/or excretion.

Through the silencing of RhoprCCHa2 expression by means of RNAi, we determined a biphasic modulation of diuresis over time. An initial increase in the diuresis during the first 45 min compared with controls was followed by a reduction with respect to controls towards the end of the diuretic process. Despite this temporal modulation, dsCCHa-treated insects excreted a higher accumulated volume after completion of diuresis (Fig. 2B), indicating a net anti-diuretic role for this neuropeptide. However, given that the reduction in gene expression was more robust in the anterior midgut than in the CNS and Malpighian tubules (Fig. 2C), we cannot rule out that the net antidiuretic effect and/or the temporal modulation are mainly related to the role of RhoprCCHa2 in the anterior midgut than in other structures. Animals that were treated with dsCCHa2 were able to feed and molt normally. Thus, under the experimental conditions, the effects of dsCCHa2 on diuresis do not deleteriously compromise homeostasis. However, this regulation could be important for the animal to survive in particular stressful situations, such as starvation for long periods.

In vitro tests demonstrated that RhoprCCHa2 has an effect on both the 5HT-stimulated Malpighian tubules and the 5HT-stimulated anterior midgut, albeit opposite effects. Treatment with RhoprCCHa2 triggered an increase in fluid secretion across the distal segment of the Malpighian tubules when they were stimulated with 5HT, but inhibited 5HT-stimulated fluid transport from the midgut lumen into the hemolymph. Although other reports of peptides with diuretic or antidiuretic activity have been described in *R. prolixus*, the effects were always concordant (i.e. stimulation or inhibition) in both the Malpighian tubules and the anterior midgut (Ianowski et al., 2010; Paluzzi and Orchard, 2006; Paluzzi et al., 2008; Te Brugge et al., 2009; Te Brugge et al., 2011). Here, we report a stimulatory effect of RhoprCCHa2 (1 µmol l^{-1}) on 5HT-treated Malpighian tubules, with a significant increase in the secretion rate. This stimulatory effect is lower than that observed for RhoprCRFDH, which has a strong secretory-enhancing effect in non-stimulated tubules (Te Brugge et al., 2011). Moreover, RhoprCTDH caused a small stimulatory effect in the rate of secretion of Malpighian tubules, and an additive effect when acting in conjunction with 5HT (Te Brugge et al., 2005). In the case of RhoprCCHa2, the enhancing effect in the secretory activity of the Malpighian tubules was observed only when they were previously stimulated with 5HT. In contrast, we observed that RhoprCCHa2 has an inhibitory effect on the anterior midgut, by reducing the fluid transport rate across the anterior midgut previously stimulated with 5HT, in a similar way to the effect reported for RhoprCAPA (Ianowski et al., 2010).

The physiological function of RhoprCCHa2 is still to be determined. However, we can hypothesize from its mode of action that it could be involved in hemolymph homeostasis during post-prandial diuresis. In contrast with diuretic and antidiuretic signals described to date, which stimulate or block, respectively, both the Malpighian tubules and the anterior midgut, RhoprCCHa2 has opposite effects on the tubules and anterior midgut. Also, it is interesting to note that the decrease in fluid transport rate in the anterior midgut stimulated with 5HT and treated with RhoprCCHa2 is near double the effect of RhoprCCHa2 observed in the stimulated Malpighian tubules (see Figs 3 and 4). Thus, it would uncouple the function of those organs by simultaneously slowing midgut fluid absorption and stimulating Malpighian tubule secretion. This would necessarily result in a decrease in the hemolymph volume. Moreover, RhoprCCHa2 does not have an effect on the excretory organs when applied alone, but rather has an effect only on stimulated organs. We propose that RhoprCCHa2 may function during post-prandial diuresis to modulate the Malpighian tubule and anterior midgut fluid transport rate to maintain hemolymph volume and composition homeostasis. Such a regulatory mechanism is necessary because the rate of fluid transport across the midgut is dependent on the Na^+ concentration in the blood meal plasma (Farmer et al., 1981). As a result, blood meals with high Na^+ content, such as avian blood, would trigger faster fluid transport across the midgut than blood meals with low Na^+, such as those from amphibian hosts. In contrast, the Malpighian tubules seem to have a more constant secretion rate that is not dependent on the Na^+ content of the hemolymph. Tubules exposed to Na^+ concentrations ranging from 98 to 137 mmol l^{-1} displayed the same secretion rate after 5HT stimulation (Ianowski et al., 2004). Thus, the fluid transport rate of the midgut does not match that of the Malpighian tubules by default, but rather must be modulated to ensure that the amount of fluid absorbed matches that secreted to avoid large fluctuations in hemolymph volume and composition.

Given that *T. cruzi* is transmitted to humans during the post-prandial diuresis of triatomine insects, a detailed understanding of the neuroendocrine regulation of diuresis could provide target candidates for next-generation management strategies (Audsley and Down, 2015; Verlinden et al., 2014). This is specially urgent for triatomines, given the high level of pyrethroid resistance registered in domiciliary populations of *Triatoma infestans* (Capriotti et al., 2014; Fabro et al., 2012; Picollo et al., 2005; Sierra et al., 2016), which is a challenge for the control of Chagas disease transmission. Pseudopeptides and non-peptidic mimetics could be modeled in order to interfere with RhoprCCHa2 function. This kind of molecule has been already assayed in with success in *R. prolixus* for other neuropeptide families (Lange et al., 2016). Hence, our finding of a new member of the hormonal system that regulates diuresis in this species is relevant for basic and applied entomology.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.C., J.P.I., S.O.; Methodology: N.C., J.P.I., P.G., S.O.; Validation: N.C., S.O.; Formal analysis: N.C., J.P.I., S.O.; Investigation: N.C., J.P.I., P.G.,

S.O.; Resources: J.P.I., S.O.; Data curation: N.C., J.P.I., S.O.; Writing - original draft: N.C.; Writing - review & editing: N.C., J.P.I., S.O.; Visualization: N.C., J.P.I.; Supervision: J.P.I., S.O.; Project administration: S.O.; Funding acquisition: J.P.I., S.O.

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References

Audsley, N. and Down, R. E. (2015). G protein coupled receptors as targets for next generation pesticides. *Insect Biochem. Mol. Biol.* **67**, 27-37. doi:10.1016/j.ibmb.2015.07.014

Cabello, D. R. (2001). Resistance to starvation of *Rhodnius neivai* Lent, 1953 (Hemiptera: Reduviidae: Triatominae) under experimental conditions. *Mem. Inst. Oswaldo Cruz* **96**, 587-591. doi:10.1590/s0074-02762001000400023

Capriotti, N., Mougabure-Cueto, G., Rivera-Pomar, R. and Ons, S. (2014). L925I mutation in the Para-type sodium channel is associated with pyrethroid resistance in *Triatoma infestans* from the Gran Chaco region. *PLoS Negl. Trop. Dis.* **8**, e2659. doi:10.1371/journal.pntd.0002659

Coast, G. M. (2009). Neuroendocrine control of ionic homeostasis in blood-sucking insects. *J. Exp. Biol.* **212**, 378-386. doi:10.1242/jeb.024109

Fabro, J., Sterkel, M., Capriotti, N., Mougabure-Cueto, G., Germano, M., Rivera-Pomar, R. and Ons, S. (2012). Identification of a point mutation associated with pyrethroid resistance in the para-type sodium channel of *Triatoma infestans*, a vector of Chagas' disease. *Infect. Genet. Evol.* **12**, 487-491. doi:10.1016/j.meegid.2011.12.006

Farhan, A., Gulati, J., Grobetae-Wilde, E., Vogel, H., Hansson, B. S. and Knaden, M. (2013). The CCHamide 1 receptor modulates sensory perception and olfactory behavior in starved *Drosophila*. *Sci. Rep.* **3**, 2765. doi:10.1038/srep02765

Farmer, J., Maddrell, S. H. and Spring, J. H. (1981). Absorption of fluid by the midgut of *Rhodnius*. *J. Exp. Biol.* **94**, 301-316.

Hansen, K. K., Hauser, F., Williamson, M., Weber, S. B. and Grimmelikhuijzen, C. J. P. (2011). The *Drosophila* genes CG14593 and CG30106 code for G-protein-coupled receptors specifically activated by the neuropeptides CCHamide-1 and CCHamide-2. *Biochem. Biophys. Res. Commun.* **404**, 184-189. doi:10.1016/j.bbrc.2010.11.089

Ianowski, J. P., Christensen, R. J. and O'Donnell, M. J. (2004). Na^+ competes with K^+bumetanide-sensitive transport by Malpighian tubules of *Rhodnius prolixus*. *J. Exp. Biol.* **207**, 3707-3716. doi:10.1242/jeb.01203

Ianowski, J. P., Paluzzi, J.-P., Te Brugge, V. A. and Orchard, I. (2010). The antidiuretic neurohormone RhoprCAPA-2 downregulates fluid transport across the anterior midgut in the blood-feeding insect *Rhodnius prolixus*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, R548-R557. doi:10.1152/ajpregu.00208.2009

Ida, T., Takahashi, T., Tominaga, H., Sato, T., Sano, H., Kume, K., Ozaki, M., Hiraguchi, T., Shiotani, H., Terajima, S. et al. (2012). Isolation of the bioactive peptides CCHamide-1 and CCHamide-2 from *Drosophila* and their putative role in appetite regulation as ligands for G protein-coupled receptors. *Front. Endocrinol.* **3**, 177. doi:10.3389/fendo.2012.00177. eCollection 2012

Lange, A. B., Nachman, R. J., Kaczmarek, K. and Zabrocki, J. (2016). Biostable insect kinin analogs reduce blood meal and disrupt ecdysis in the blood-gorging Chagas' disease vector, *Rhodnius prolixus*. *Peptides* **80**, 108-113. doi:10.1016/j.peptides.2016.01.012

Maddrell, S. H. (1964). Excretion in the blood-sucking bug, *Rhodnius prolixus* Stal. II. The normal course of diuresis and the effect of temperature. *J. Exp. Biol.* **41**, 163-176.

Maddrell, S. H. and Gardiner, B. O. (1975). Induction of transport of organic anions in Malpighian tubules of *Rhodnius*. *J. Exp. Biol.* **63**, 755-761.

Maddrell, S. H. and Gee, J. D. (1974). Potassium-induced release of the diuretic hormones of *Rhodnius prolixus* and *Glossina austeni*: Ca dependence, time course and localization of neurohaemal areas. *J. Exp. Biol.* **61**, 155-171.

Maddrell, S. H., Pilcher, D. E. and Gardiner, B. O. (1971). Pharmacology of the Malpighian tubules of *Rhodnius* and *Carausius*: the structure-activity relationship of tryptamine analogues and the role of cyclic AMP. *J. Exp. Biol.* **54**, 779-804.

Maddrell, S. H., O'Donnell, M. J. and Caffrey, R. (1993). The regulation of haemolymph potassium activity during initiation and maintenance of diuresis in fed *Rhodnius prolixus*. *J. Exp. Biol.* **177**, 273-285.

Majerowicz, D., Alves-Bezerra, M., Logullo, R., Fonseca-de-Souza, A. L., Meyer-Fernandes, J. R., Braz, G. R. and Gondim, K. C. (2011). Looking for reference genes for real-time quantitative PCR experiments in *Rhodnius prolixus* (Hemiptera: Reduviidae). *Insect Mol. Biol.* **20**: 713-722. doi:10.1111/j.1365-2583.2011.01101.x

Mesquita, R. D., Vionette-Amaral, R. J., Lowenberger, C., Rivera-Pomar, R., Monteiro, F. A., Minx, P., Spieth, J., Carvalho, A. B., Panzera, F., Lawson, D. et al. (2015). Genome of *Rhodnius prolixus*, an insect vector of Chagas disease, reveals unique adaptations to hematophagy and parasite infection. *Proc. Natl. Acad. Sci. USA* **112**, 14936-14941. doi:10.1073/pnas.1506226112

Omondi, B. A., Latorre-Estivalis, J. M., Rocha Oliveira, I. H., Ignell, R. and Lorenzo, M. G. (2015). Evaluation of reference genes for insect olfaction studies. *Parasit. Vectors* **8**, 243. doi:10.1186/s13071-015-0862-x

Ons, S. (2017). Neuropeptides in the regulation of *Rhodnius prolixus* physiology. *J. Insect Physiol.* **97**, 77-92. doi:10.1016/j.jinsphys.2016.05.003

Ons, S., Sterkel, M., Diambra, L., Urlaub, H. and Rivera-Pomar, R. (2011). Neuropeptide precursor gene discovery in the Chagas disease vector *Rhodnius prolixus*. *Insect Mol. Biol.* **20**, 29-44. doi:10.1111/j.1365-2583.2010.01050.x

Ons, S., Lavore, A., Sterkel, M., Wulff, J. P., Sierra, I., Martínez-Barnetche, J., Rodriguez, M. H. and Rivera-Pomar, R. (2016). Identification of G protein coupled receptors for opsines and neurohormones in *Rhodnius prolixus*. Genomic and transcriptomic analysis. *Insect Biochem. Mol. Biol.* **69**, 34-50. doi:10.1016/j.ibmb.2015.05.003

Orchard, I. (2006). Serotonin: a coordinator of feeding-related physiological events in the blood-gorging bug, *Rhodnius prolixus*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **144**, 316-324. doi:10.1016/j.cbpa.2005.11.010

Paluzzi, J.-P. and Orchard, I. (2006). Distribution, activity and evidence for the release of an anti-diuretic peptide in the kissing bug *Rhodnius prolixus*. *J. Exp. Biol.* **209**, 907-915. doi:10.1242/jeb.02083

Paluzzi, J.-P. and Orchard, I. (2010). A second gene encodes the anti-diuretic hormone in the insect, *Rhodnius prolixus*. *Mol. Cell. Endocrinol.* **317**, 53-63. doi:10.1016/j.mce.2009.11.004

Paluzzi, J.-P., Russell, W. K., Nachman, R. J. and Orchard, I. (2008). Isolation, cloning, and expression mapping of a gene encoding an antidiuretic hormone and other CAPA-related peptides in the disease vector, *Rhodnius prolixus*. *Endocrinology* **149**, 4638-4646. doi:10.1210/en.2008-0353

Picollo, M. I., Vassena, C., Santo Orihuela, P., Barrios, S., Zaidemberg, M. and Zerba, E. (2005). High resistance to pyrethroid insecticides associated with ineffective field treatments in *Triatoma infestans* (Hemiptera: Reduviidae) from Northern Argentina. *J. Med. Entomol.* **42**, 637-642. doi:10.1603/0022-2585(2005)042[0637:HRTPIA]2.0.CO;2

Quinlan, M. C., Tublitz, N. J. and O'Donnell, M. J. (1997). Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stål: the peptide CAP2b and cyclic GMP inhibit Malpighian tubule fluid secretion. *J. Exp. Biol.* **200**, 2363-2367.

Rassi, A., Jr., Rassi, A. and Marin-Neto, J. A. (2010). Chagas disease. *Lancet* **375**, 1388-1402. doi:10.1016/S0140-6736(10)60061-X

Reiher, W., Shirras, C., Kahnt, J., Baumeister, S., Isaac, R. E. and Wegener, C. (2011). Peptidomics and peptide hormone processing in the *Drosophila* midgut. *J. Proteome Res.* **10**, 1881-1892. doi:10.1021/pr101116g

Ren, G. R., Hauser, F., Rewitz, K. F., Kondo, S., Engelbrecht, A. F., Didriksen, A. K., Schjott, S. R., Sembach, F. E., Li, S., Sogaard, K. C. et al. (2015). CCHamide-2 is an orexigenic brain-gut peptide in *Drosophila*. *PLoS ONE* **10**, e0133017. doi:10.1371/journal.pone.0133017

Roller, L., Yamanaka, N., Watanabe, K., Daubnerová, I., Žitňan, D., Kataoka, H. and Tanaka, Y. (2008). The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. *Insect Biochem. Mol. Biol.* **38**, 1147-1157. doi:10.1016/j.ibmb.2008.04.009

Sano, H., Nakamura, A., Texada, M. J., Truman, J. W., Ishimoto, H., Kamikouchi, A., Nibu, Y., Kume, K., Ida, T. and Kojima, M. (2015). The nutrient-responsive hormone CCHamide-2 controls growth by regulating insulin-like peptides in the brain of *Drosophila melanogaster*. *PLoS Genet.* **11**, e1005209. doi:10.1371/journal.pgen.1005209

Sierra, I., Capriotti, N., Fronza, G., Mougabure-Cueto, G. and Ons, S. (2016). Kdr mutations in *Triatoma infestans* from the Gran Chaco are distributed in two differentiated foci: implications for pyrethroid resistance management. *Acta Trop.* **158**, 208-213. doi:10.1016/j.actatropica.2016.03.014

Sterkel, M., Oliveira, P. L., Urlaub, H., Hernandez-Martinez, S., Rivera-Pomar, R. and Ons, S. (2012). OKB, a novel family of brain-gut neuropeptides from insects. *Insect. Biochem. Mol. Biol.* **42**, 466-473. doi:10.1016/j.ibmb.2012.03.003

Te Brugge, V. A., Lombardi, V. C., Schooley, D. A. and Orchard, I. (2005). Presence and activity of a Dippu-DH31-like peptide in the blood-feeding bug, *Rhodnius prolixus*. *Peptides* **26**, 29-42. doi:10.1016/j.peptides.2004.08.025

Te Brugge, V., Ianowski, J. P. and Orchard, I. (2009). Biological activity of diuretic factors on the anterior midgut of the blood-feeding bug, *Rhodnius prolixus*. *Gen. Comp. Endocrinol.* **162**, 105-112. doi:10.1016/j.ygcen.2009.01.025

Te Brugge, V., Paluzzi, J. P., Schooley, D. A. and Orchard, I. (2011). Identification of the elusive peptidergic diuretic hormone in the blood-feeding bug *Rhodnius prolixus*: a CRF-related peptide. *J. Exp. Biol.* **214**, 371-381. doi:10.1242/jeb.046292

Verlinden, H., Vleugels, R., Zels, S., Dillen, S., Lenaerts, C., Crabbe, K., Spit, J. and Vanden Broeck, J. (2014). Receptors for neuronal or endocrine signalling molecules as potential targets for the control of insect pests. In *Advances in Insect Physiology*, pp. 167-303. Elsevier.

Villalobos-Sambucaro, M. J., Lorenzo-Figueiras, A. N., Riccillo, F. L., Diambra, L. A., Noriega, F. G. and Ronderos, J. R. (2015). Allatotropin modulates

myostimulatory and cardioacceleratory activities in *Rhodnius prolixus* (Stal). *PLoS ONE* **10**, e0124131. doi:10.1371/journal.pone.0124131

Wulff, J. P., Sierra, I., Sterkel, M., Holtof, M., Van Wielendaele, P., Francini, F., Broeck, J. V. and Ons, S. (2017). Orcokinin neuropeptides regulate ecdysis in the hemimetabolous insect *Rhodnius prolixus*. *Insect Biochem. Mol. Biol.* **81**, 91-102. doi:10.1016/j.ibmb.2017.01.003

Wulff, J. P., Capriotti, N. and Ons, S. (2018). Orcokinins regulate the expression of neuropeptide precursor genes related to ecdysis in the hemimetabolous insect *Rhodnius prolixus*. *J. Insect Physiol.* **108**, 31-39. doi:10.1016/j.jinsphys.2018.05.006

Zandawala, M., Paluzzi, J.-P. and Orchard, I. (2011). Isolation and characterization of the cDNA encoding DH(31) in the kissing bug, *Rhodnius prolixus*. *Mol. Cell. Endocrinol.* **331**, 79-88. doi:10.1016/j.mce.2010.08.012

Zandawala, M., Lytvyn, Y., Taiakina, D. and Orchard, I. (2012). Cloning of the cDNA, localization, and physiological effects of FGLamide-related allatostatins in the blood-gorging bug, *Rhodnius prolixus*. *Insect Biochem. Mol. Biol.* **42**, 10-21. doi:10.1016/j.ibmb.2011.10.002

Zandawala, M., Poulos, C. and Orchard, I. (2015). Structure-activity relationships of two *Rhodnius prolixus* calcitonin-like diuretic hormone analogs. *Peptides* **68**, 211-213. doi:10.1016/j.peptides.2014.03.019