“The neuropeptide CCHamide 2 regulates diuresis in the Chagas’ disease vector *Rhodnius prolixus*”

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Summary statement: The neuropeptide RhoprCCHamide2 has a dual diuretic effect, enhancing the serotonin-induced secretion by Malpighian tubules, and inhibiting serotonin-induced absorption across the anterior midgut in *Rhodnius prolixus*.

List of abbreviations:

calcitonin-like diuretic hormone

CAP2b peptide-A: CAPA

central nervous system: CNS

CRF-like diuretic hormone: CRFDH

double strand RNA: dsRNA

G-protein coupled receptors: GPCRs

Serotonin: 5-hydroxytryptamine. 5HT

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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 CCHamide 2 is involved in the regulation of the post-prandial diuresis in the kissing bug *R. prolixus*. Our results suggest a dual effect of *Rhopr*CCHamide2 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.

1. Introduction

Hematophagous insects, including triatomines, take a large amount of blood in every meal, which can be equivalent to several fold 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 following 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, 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 is a severe neglected tropical disease which affects up to 6-7 million people worldwide (Rassi et al., 2010).

Given the relevance of triatomines excretion for Chagas' disease transmission and the convenience of *R. prolixus* as a model for physiological studies in insects (Ons, 2017), diuresis

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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 to 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. Around 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 following 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 the 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, while 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 different physiological function since it has opposite effects on Malpighian tubules and anterior midgut.

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CCHamide is a brain-gut neuropeptide precursor family conserved in insect genomes, where is usually represented by two paralogues named CCHamide1 and CCHamide2 (Hansen et al., 2011; Reiher et al., 2011). The physiological role of CCHamides has been studied in *Drosophila melanogaster*. DromeCCHamide1 has been involved in alimentary behavior and sensorial perception of food (Farhan et al., 2013; Ida et al., 2012), whereas *Drome*CCHamide2 seems to regulate the appetite (Ida et al 2012; Ren et al., 2015) and the coordination of growth with nutrition (Sano et al., 2015). *R. prolixus* genome (Mesquita et al., 2015) encodes both CCHamide 1 and CCHamide 2 paralogues. CCHamide2 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 *Rhopr*CCHamide 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 *Rhopr*CCHamide2 in the post-prandial diuresis. Our results suggest a dual effect of *Rhopr*CCHamide2 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 strand RNA (dsRNA) encoding a fragment of *Rhopr*CCHamide2 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.

2. Material and Methods

2.1 Insects

A colony of *R. prolixus* was maintained in our laboratory in a 12:12 hour light/dark period at 28 ± 2 ºC. 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 WHO Biosecurity Handbook (ISBN 92 4 354 6503).

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2.2 RT-PCR:

The procedures performed for RT-PCR has 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) under 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 µg of RNA from each sample was treated with DNAseI (Promega, Wisconsin, USA) and used to synthesize cDNA with the M-MLV Reverse Transcriptase kit (Promega). PCR amplifications of the specified transcripts were performed on triplicate in a 20 µL final volume (primers detailed in Table 1). *Rhopr*β-Actin 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 sec; 50°C for 30 sec and 72°C for 30 sec. Three biological replicates were performed.

2.3 dsRNA synthesis:

For a detailed description on dsRNA synthesis see Wulff et al 2017. Briefly, a 270 bp fragment encoded in both isoforms of *Rhopr*CCHamide2 was PCR-amplified using Pegasus Taq Polymerase (Productos Bio-Lógicos, Argentina) and primers containing a fragment of T7 promoter sequence at 5' end (CCHaRNAiFw and CCHaRNAiRv, see primers sequences in Table 1). One µl of the PCR product was used for a secondary PCR using T7-promoter primer (T7 full, see primer sequence in Table 1). PCR product were used to generate *Rhopr*CCHa-dsRNAs by *in vitro* transcription using T7-RNA polymerase (Promega, USA) or the MEGAscript™ T7 kit (Ambion, São Paulo, Brazil), according to the manufacturer instructions. Reactions were performed at 37°C, overnight. The products of transcription were treated with DNAse and RNAseA (Fermentas, USA). Subsequently a precipitation with AcNa 3M was performed. The precipitate was resuspended in saline solution. A 274 bp fragment from 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 *R. prolixus* genome, and was used as a control dsRNA. PCR products were sequenced at Macrogen facility (Seoul; Korea) to corroborate the specificity of the fragment obtained. The formation of dsRNAs was confirmed by running a 2% agarose gel

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and quantified from an image of the gel using the software ImageJ 1.32 (NIH, USA). The dsRNA was stored at -20°C until use.

2.4 RNAi mediated gene silencing of Rhopr*CCHamide* 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 , either dsCCHamide or control) diluted in *R. prolixus* saline solution (in mM: 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) .

2.5 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 with the formula "final weight - initial weight". After this, the animals were individually placed in 1.5 ml microtubes previously weighed in a precision balance. Fifteen, 30, 45, 60, 90, 120, 150, 180 and 240 minutes after feeding each insect was changed to a new weighed microtube and the volume of excreted urine was estimated by subtracting the weight of the empty tube to the weight of the tube after the visit of the insect.

2.6 qRT-PCR

qRT-PCR was performed as described previously (Wulff et al 2018). Briefly, twenty-four hours after finished the *in vivo* diuresis assay, insects were dissected to obtain CNS, anterior midgut and Malpighian tubules separately. Five tissues/sample were pooled in a microtube containing Trizol reagent (Ambion, São Paulo, Brazil). cDNA was prepared as described above.

cDNA amplifications were performed for each sample in triplicate, in a 20 µl final volume (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 sec; (iii)50, 58 or 60°C (depending on the primers melting temperature) for 30 sec; (iv) steps (i) and (ii) were repeated for 40 cycles. A control without

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a template was included in all batches. GAPDH and β - Actin 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).

2.7 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 control group and the remaining two were assigned to the CCHamide2 group. Each tubule was placed into a 90 μl droplet containing either saline solution (control group) or synthetic *Rhopr*CCHa-2 1 μM (GGCSAFGHSCFGGH-NH_2) (Genscript Corporation; Piscataway, New Jersey, 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 period, 1 μM serotonin was added to the medium in both groups to stimulate secretion (this is the 5HT concentration that generate 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 serotonin. The secreted droplets were photographed using a microscope digital camera (MiniVid, LW Scientific, Lawrenceville, GA, USA) and the diameter measured offline with the software Image J 1.32 (NIH, USA). 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.

2.8 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 hour with either saline solution, 5HT 0.1 μM 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), synthetic *Rhopr*CCHa2 0.1 μM in saline solution, or 5HT 0.1 μM plus *Rhopr*CCHa2 0.1 μM

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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 weight - final weight"/ 60 min was evaluated as the rate of absorption of the anterior midgut.

2.9 Anterior midgut contraction assay

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 part of the paraffin with a lighter and placing gently adjusting the animal until it solidifies. The dorsal cuticle was removed under saline solution, 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 minutes. Anterior midgut contraction rate was measured by counting the number of contractions in 30 s. This procedure was repeated 6 times for each preparation, and the results of the 6 measurements were averaged. After this, the saline was removed and replaced with an equal volume of solution containing *Rhopr*CCHa2 at different concentrations (0.001, 0.01, 0.1, 1 and 10 µM). The anterior midgut rate was measured and expressed as % of the control (saline). Ten animals were analyzed for control and for each *Rhopr*CCHa-2 concentration.

3. Results

3.1 Expression pattern of Rhopr*CCHamide2 and CCHamide GPCRs in tissues:*

The expression pattern of *Rhopr*CCHamide2 and the two putative RhoprCCHamide GPCRs, encoded in the predicted transcripts RPRC00776 and RPRC000608 of the *R. prolixus* genomic sequence (www.vectorbase.org; RproC3 dataset), were determined using RT-PCR (Figure 1). The transcript encoding neuropeptide RhoprCCHamide2 precursor was detected in the CNS, anterior midgut and Malpighian tubules, but not in posterior midgut nor in hindgut (Figure 1). Besides, a transcript encoding RPRC007766 was identified in PM and RPRC000608 transcript was PCR-amplified in AM, MT and PM (Figure 1).

3.2 Rhopr*CCHamide2 regulates post-prandial diuresis:*

The tissue-specific transcription of *Rhopr*CCHamide2 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 *Rhopr*CCHamide2. Unfed fourth instar *R. prolixus* nymphs were injected with control dsRNA (n=25) or dsRNA encoding a fragment of *Rhopr*CCHamide2 (dsCCHa group, n=44).

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Seven days after the injections, control and dsCCHa insects were fed for 15 min. No significant differences in the weight of blood ingested were detected between control and dsCCHa groups (0.098 +/- 0.070 mg and 0.104 mg +/- 0.150 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 ($p<0.001$) minutes of post-prandial diuresis (Figure 2A). In the 60 min time point, no significant difference was detected between groups in the volume of urine excreted. Conversely, in the lasts 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; Figure 2A).

In order to evaluate the net effect of *Rhopr*CCHamide2 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; Figure 2B).

Twenty-four hours after finished the *in vivo* diuresis assay, *Rhopr*CCHamide2 transcription was evaluated by qRT-PCR in tissues from control and dsCCHa treated insects. When evaluated dsCCHa treated vs. control animals, we detected an 84 % decrement in the levels of transcript for *Rhopr*CCHamide in the anterior midgut (n=5, $p<0.01$, Student's t-test), a 40 % of decrement in the CNS (n= 5, p=0.0951) and a 30 % of decrement in the Malpighian tubules (n= 5, p=0.153) (Figure 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.

3.3 Rhopr*CCHamide2 stimulates fluid secretion by Malpighian tubules:*

We evaluated the activity of the neuropeptide *Rhopr*CCHamide2 in the secretory function of the Malpighian tubules from fifth instar *R. prolixu*s nymphs using a secretion assay. Stimulation was achieved by the addition of 1 μM 5HT into the bathing droplet containing either saline solution (control group) or synthetic 1 μM *Rhopr*CCHa (CCHa group).

As expected, the stimulation of the tubules with 5HT activated fluid secretion; the secretion rate with 1 μM serotonin in the bath was 12.05 nl/min (Figure 3). Our results showed that synthetic *Rhopr*CCHamide stimulated secretion in 5HT-activated tubules, generating a secretion rate of 18.29 nl/min (Figure 3). This represents a significant increase

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of 1.52 fold in the secretion rate (p<0.001; Figure 3). Furthermore, the accumulated secreted volume was significant higher for *Rhopr*CCHamide2 incubated tubules at 30 min (p<0.05) and 40 min (p<0.01) after stimulation with 5HT (Two-ways ANOVA; n= 37; 47). Synthetic *Rhopr*CCHa was not able to induce fluid secretion in non-stimulated tubules during 40 min incubations (data not shown).

3.4 Rhopr*CCHamide2 regulates fluid transport by* R. prolixus *anterior midgut:*

Treatment with serotonin (0.1 μM) 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) (Figure 4). The transport rate was 76.0 +/- 9.6 nl/min, in agreement with a previous report (Ianowski et al., 2010). Exposing the tissue to synthetic *Rhopr*CCHa2 (1 μM in bathing saline solution) had no effect on the fluid transport rate (n=11) (Figure 4). However, *Rhopr*CCHamide2 significantly reduced the stimulatory effect of serotonin on anterior midgut (n=11; p<0.05, One-way ANOVA and Tukey-Kramer contrasts) (Figure 4). Even though the fluid transport is higher in the group treated with 5HT+synthetic RhoprCCHamide2 respect to saline and RhoprCCHamide2 groups, the differences were not statistically significant in our analysis (Figure 4). Finally, *Rhopr*CCHamide had no effect on the contractility of the anterior midgut at concentrations of 0.001 to 10 μM (data not shown).

4. Discussion

In this work we demonstrate that CCHamide2, a scarcely studied neuroendocrine peptide in insects, possess a role in the regulation of 5HT-stimulated excretory organs in *R. prolixus*. Furthermore, RNAi-mediated gene silencing of *Rhopr*CCHamide2 alters the time course and intensity of the post-prandial diuresis, suggesting that it may play a role on osmoregulation after feeding.

Like most insect neuropeptides, the transcript of *Rhopr*CCHamide2 was detected in the CNS. We also found this transcript in the anterior midgut, suggesting that it is a brain-gut neuropeptide such as *Drome*CCHamide and *Bommo*CCHamide (Reiher et al., 2011; Roller et al., 2008). *Rhopr*CCHamide2 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 *Rhopr*CCHamide could regulate Malpighian tubules function in an autocrine or paracrine way. Furthermore, the transcripts of the putative GPCRs for *Rhopr*CCHamide were

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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 *Rhopr*CCHamide2 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 to controls, followed by a reduction respect to controls towards the end of the diuretic process. Despite this temporal modulation, dsCCHamide-treated insects excreted a higher accumulated volume after completion of diuresis (Figure 2 B), indicating a net anti-diuretic role for this neuropeptide. However, given that the reduction in gene expression was more robust in anterior midgut than in CNS and Malpighian tubules (Figure 2C), we cannot rule out that the net antidiuretic effect and/or the temporal modulation are mainly related to the role of *Rhopr*CCHamide2 in the anterior midgut than in other structures. Animals that were treated with dsCCHamide2 were able to feed and molt normally. Thus, in the experimental conditions the effects of dsCCHamide2 on diuresis do not deleteriously compromise homeostasis. However, this regulation could be important for the animal to survive in particular stressful situations, such as for example starvation for long periods.

In vitro tests demonstrated that *Rhopr*CCHamide has an effect on both the 5HT-stimulated Malpighian tubules and in the 5HT-stimulated anterior midgut, albeit with opposite effects. Treatment with *Rhopr*CCHamide 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 Malpighian tubules and 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 *Rhopr*CCHamide (1µM) on 5HT-treated Malpighian tubules, with a significant increase in the secretion rate. This stimulatory effect is lower than the observed for *Rhopr*CRF-DH, which has a strong secretory-enhancing effect in non-stimulated tubules (Te Brugge et al., 2011). Moreover, *Rhopr*CTDH caused a small stimulatory effect in the rate of secretion of Malpighian tubules, and an additive effect when acting in conjunct with 5HT (Te Brugge et al., 2005). In the case of *Rhopr*CCHamide, the enhancing effect in the secretory activity of the Malpighian tubules was observed only when they were previously stimulated with 5HT. On the other hand, we observed that *Rhopr*CCHamide has an inhibitory effect on

the anterior midgut, by reducing fluid transport rate across the anterior midgut previously stimulated with 5HT, in a similar way to the effect reported for *Rhopr*CAPA (Ianowski et al., 2010).

The physiological function of *Rhopr*CCHamide is still to be determined. However, we can hypothesize from its mode of action that 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, *Rhopr*CCHamide 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 *Rhopr*CCHamide2 is near double of the effect of *Rhopr*CCHamide observed in the stimulated Malpighian tubules (see Figures 3 and 4). Thus, it would uncouple the function of those organs by simultaneously slowing midgut fluid absorption and stimulating Malpighian tubules secretion. This would necessarily result in a decrease in the hemolymph volume. Moreover, *Rhopr*CCHamide2 does not have an effect on the excretory organs when applied alone, but rather has an effect only on stimulated organs. We propose that *Rhopr*CCHamide2 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 amphibian hosts. In contrast, the Malpighian tubules seem to have a more constant secretion rate that is not dependent on Na^+ content of the hemolymph. Tubules exposed to Na^+ concentrations ranging from 98 to 137 mM 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 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 neuroendocrine regulation of diuresis could provide target candidates for new-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

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the control of Chagas' disease transmission. Pseudopeptides and non-peptidic mimetics could be modeled in order to interfere with *Rhopr*CCHamide2 function. This kind of molecules has been already assayed in with success in *R. prolixus* for other neuropeptide family (Lange et al., 2016). Hence, our finding of a new member of the hormonal system that regulate diuresis in this species is relevant for basic and applied entomology.

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8. References

Audsley, N., Down, R.E., 2015. G protein coupled receptors as targets for next generation pesticides. Insect biochemistry and molecular biology 67, 27-37.

Cabello, D.R., 2001. Resistance to starvation of Rhodnius neivai Lent, 1953 (Hemiptera: Reduviidae: Triatominae) under experimental conditions. Memorias Instituto Oswaldo Cruz 96, 587-91.

Capriotti, N., Mougabure-Cueto, G., Rivera-Pomar, R., 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 neglected tropical diseases 8, e2659.

Coast, G.M., 2009. Neuroendocrine control of ionic homeostasis in blood-sucking insects. The Journal of experimental biology 212, 378-386.

Fabro, J., Sterkel, M., Capriotti, N., Mougabure-Cueto, G., Germano, M., Rivera-Pomar, R., 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. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 12, 487-491.

Farhan, A., Gulati, J., Grobetae-Wilde, E., Vogel, H., Hansson, B.S., Knaden, M., 2013. The CCHamide 1 receptor modulates sensory perception and olfactory behavior in starved Drosophila. Scientific reports 3, 2765.

Farmer, J., Maddrell, S.H., Spring, J.H., 1981. Absorption of Fluid by the Midgut of Rhodnius. Journal of Experimental Biology 94, 301-316.

Hansen, K., Hauser, F., Williamson, M., Weber, S., Grimmelikhuijzen, J.P., 2011. The Drosophila genes CG14593 and CG30106 code for G-protein-coupled receptors

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specifically activated by the neuropeptides CCHamide-1 and CCHamide-2. Biochemical and Biophysical Research Communications 404, 184-189.
Ianowski JP1, Christensen RJ, O'Donnell MJ., 2004. Na+ competes with K+bumetanide-sensitive transport by Malpighian tubules of Rhodnius prolixus. Journal of Experimental Biology. 207: 3707-3716.
Ianowski, J.P., Paluzzi, J.P., Te Brugge, V.A., Orchard, I., 2010. The antidiuretic neurohormone RhoprCAPA-2 downregulates fluid transport across the anterior midgut in the blood-feeding insect Rhodnius prolixus. American journal of physiology. Regulatory, integrative and comparative physiology 298, R548-557.
Ida, T., Takahashi, T., Tominaga, H., Sato, T., Sano, H., Kume, K., Ozaki, M., Hiraguchi, T., Shiotani, H., Terajima, S., Nakamura, Y., Mori, K., Yoshida, M., Kato, J., Murakami, N., Miyazato, M., Kangawa, K., Kojima, M., 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. Frontiers in endocrinology 3, 177.
Lange, A.B., Nachman, R.J., Kaczmarek, K., 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.
Maddrell, S.H., 1964. Excretion in the Blood-Sucking Bug, Rhodnius Prolixus Stal. Ii. The Normal Course of Diuresis and the Effect of Temperature. The Journal of experimental biology 41, 163-176.
Maddrell, S.H., Gardiner, B.O., 1975. Induction of transport of organic anions in Malpighian tubules of Rhodnius. The Journal of experimental biology 63, 755-761.
Maddrell, S.H., 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. The Journal of experimental biology 61, 155-171.
Maddrell, S.H., O'Donnell, M.J., Caffrey, R., 1993. The regulation of haemolymph potassium activity during initiation and maintenance of diuresis in fed Rhodnius prolixus. The Journal of experimental biology 177, 273-285.
Maddrell, S.H., Pilcher, D.E., 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. The Journal of experimental biology 54, 779-804.
Majerowicz D, Alves-Bezerra M, Logullo R, Fonseca-de-Souza AL, Meyer-Fernandes JR, Braz GR, Gondim KC., 2011. Looking for reference genes for real-time quantitative PCR experiments in Rhodnius prolixus (Hemiptera: Reduviidae). Insect Mol Biol 20: 713-722.
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., Torres, A.Q., Ribeiro, J.M., Sorgine, M.H., Waterhouse, R.M., Montague, M.J., Abad-Franch, F., Alves-Bezerra, M., Amaral, L.R., Araujo, H.M., Araujo, R.N., Aravind, L., Atella, G.C., Azambuja, P., Berni, M., Bittencourt-Cunha, P.R., Braz, G.R., Calderon-Fernandez, G., Carareto, C.M., Christensen, M.B., Costa, I.R., Costa, S.G., Dansa, M., Daumas-Filho, C.R., De-Paula, I.F., Dias, F.A., Dimopoulos, G., Emrich, S.J., Esponda-Behrens, N., Fampa, P., Fernandez-Medina, R.D., da Fonseca, R.N., Fontenele, M., Fronick, C., Fulton, L.A., Gandara, A.C., Garcia, E.S., Genta, F.A., Giraldo-Calderon, G.I., Gomes, B., Gondim, K.C., Granzotto, A., Guarneri, A.A., Guigo, R., Harry, M., Hughes, D.S., Jablonka, W., Jacquin-Joly, E., Juarez, M.P., Koerich, L.B., Lange, A.B., Latorre-Estivalis, J.M., Lavore, A., Lawrence, G.G., Lazoski, C., Lazzari, C.R., Lopes, R.R., Lorenzo, M.G., Lugon, M.D., Majerowicz, D., Marcet, P.L., Mariotti, M., Masuda, H., Megy, K., Melo, A.C., Missirlis, F., Mota, T.,

Noriega, F.G., Nouzova, M., Nunes, R.D., Oliveira, R.L., Oliveira-Silveira, G., Ons, S., Orchard, I., Pagola, L., Paiva-Silva, G.O., Pascual, A., Pavan, M.G., Pedrini, N., Peixoto, A.A., Pereira, M.H., Pike, A., Polycarpo, C., Prosdocimi, F., Ribeiro-Rodrigues, R., Robertson, H.M., Salerno, A.P., Salmon, D., Santesmasses, D., Schama, R., Seabra-Junior, E.S., Silva-Cardoso, L., Silva-Neto, M.A., Souza-Gomes, M., Sterkel, M., Taracena, M.L., Tojo, M., Tu, Z.J., Tubio, J.M., Ursic-Bedoya, R., Venancio, T.M., Walter-Nuno, A.B., Wilson, D., Warren, W.C., Wilson, R.K., Huebner, E., Dotson, E.M., Oliveira, P.L., 2015. Genome of Rhodnius prolixus, an insect vector of Chagas disease, reveals unique adaptations to hematophagy and parasite infection. Proceedings of the National Academy of Sciences of the United States of America 112, 14936-14941.

Omondi, B. A., J. M. Latorre-Estivalis, I. H. Rocha Oliveira, R. Ignell and M. G. Lorenzo., 2015. Evaluation of reference genes for insect olfaction studies. Parasites and Vectors 8: 243.

Ons, S., 2017. Neuropeptides in the regulation of Rhodnius prolixus physiology. Journal of insect physiology 97, 77-92.

Ons, S., Lavore, A., Sterkel, M., Wulff, J.P., Sierra, I., Martinez-Barnetche, J., Rodriguez, M.H., Rivera-Pomar, R., 2016. Identification of G protein coupled receptors for opsines and neurohormones in Rhodnius prolixus. Genomic and transcriptomic analysis. Insect biochemistry and molecular biology 69, 34-50.

Ons, S., Sterkel, M., Diambra, L., Urlaub, H., Rivera-Pomar, R., 2011. Neuropeptide precursor gene discovery in the Chagas disease vector Rhodnius prolixus. Insect molecular biology 20, 29-44.

Orchard, I., 2006. Serotonin: a coordinator of feeding-related physiological events in the blood-gorging bug, Rhodnius prolixus. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology 144, 316-324.

Paluzzi, J.P., Orchard, I., 2006. Distribution, activity and evidence for the release of an anti-diuretic peptide in the kissing bug Rhodnius prolixus. The Journal of experimental biology 209, 907-915.

Paluzzi, J.P., Orchard, I., 2010. A second gene encodes the anti-diuretic hormone in the insect, Rhodnius prolixus. Molecular and cellular endocrinology 317, 53-63.

Paluzzi, J.P., Russell, W.K., Nachman, R.J., 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.

Picollo, M.I., Vassena, C., Santo Orihuela, P., Barrios, S., Zaidemberg, M., Zerba, E., 2005. High resistance to pyrethroid insecticides associated with ineffective field treatments in Triatoma infestans (Hemiptera: Reduviidae) from Northern Argentina. Journal of medical entomology 42, 637-642.

Quinlan, M.C., Tublitz, N.J., 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. Journal of experimental biology 200, 2363-7.

Rassi, A., Jr., Rassi, A., Marin-Neto, J.A., 2010. Chagas disease. Lancet 375, 1388-1402.

Reiher, W., Shirras, C., Kahnt, J., Baumeister, S., Isaac, R.E., Wegener, C., 2011. Peptidomics and peptide hormone processing in the Drosophila midgut. Journal of proteome research 10, 1881-1892.

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., Sondergaard, L., Grimmelikhuijzen, C.J., 2015. CCHamide-2 Is an Orexigenic Brain-Gut Peptide in Drosophila. PloS one 10, e0133017.

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Roller, L., Yamanaka, N., Watanabe, K., Daubnerova, I., Zitnan, D., Kataoka, H., Tanaka, Y., 2008. The unique evolution of neuropeptide genes in the silkworm Bombyx mori. Insect biochemistry and molecular biology 38, 1147-1157.
Sano, H., Nakamura, A., Texada, M.J., Truman, J.W., Ishimoto, H., Kamikouchi, A., Nibu, Y., Kume, K., Ida, T., Kojima, M., 2015. The Nutrient-Responsive Hormone CCHamide-2 Controls Growth by Regulating Insulin-like Peptides in the Brain of Drosophila melanogaster. PLoS genetics 11, e1005209.
Sierra, I., Capriotti, N., Fronza, G., Mougabure-Cueto, G., Ons, S., 2016. Kdr mutations in Triatoma infestans from the Gran Chaco are distributed in two differentiated foci: Implications for pyrethroid resistance management. Acta tropica 158, 208-213.
Sterkel, M., Oliveira, PL, Urlaub, H., Hernandez-Martinez, S., Rivera-Pomar, R., Ons, S, 2012. OKB, a novel family of brain-gut neuropeptides from insects.Insect Biochem Mol Biol 42, 466-473.
Te Brugge, V., Ianowski, J.P., Orchard, I., 2009. Biological activity of diuretic factors on the anterior midgut of the blood-feeding bug, Rhodnius prolixus. General and comparative endocrinology 162, 105-112.
Te Brugge, V., Paluzzi, J.P., Schooley, D.A., Orchard, I., 2011. Identification of the elusive peptidergic diuretic hormone in the blood-feeding bug Rhodnius prolixus: a CRF-related peptide. The Journal of experimental biology 214, 371-381.
Te Brugge, V.A., Lombardi, V.C., Schooley, D.A., Orchard, I., 2005. Presence and activity of a Dippu-DH31-like peptide in the blood-feeding bug, Rhodnius prolixus. Peptides 26, 29-42.
Verlinden, H., Vleugels, R., Zels, S., Dillen, S., Lenaerts, C., Crabbe, K., Spit, J., Vanden Broeck, J., 2014. Receptors for Neuronal or Endocrine Signalling Molecules as Potential Targets for the Control of Insect Pests, in: Elsevier (Ed.), Advances in Insects Physiology. Elsevier.
Villalobos-Sambucaro, M.J., Lorenzo-Figueiras, A.N., Riccillo, F.L., Diambra, L.A., Noriega, F.G., Ronderos, J.R., 2015. Allatotropin modulates myostimulatory and cardioacceleratory activities in Rhodnius prolixus (Stal). PloS one 10, e0124131.
Wulff, J. P., Capriotti, N., Ons, S., 2018. Orcokinins regulate the expression of neuropeptide precursor genes related to ecdysis in the hemimetabolous insect Rhodnius prolixus. Journal of insect physiology 108, 31-39.
Wulff, J.P., Sierra, I., Sterkel, M., Holtof, M., Van Wielendaele, P., Francini, F., Broeck, J.V., Ons, S., 2017. Orcokinin neuropeptides regulate ecdysis in the hemimetabolous insect Rhodnius prolixus. Insect biochemistry and molecular biology 81, 91-102.
Zandawala, M., Lytvyn, Y., Taiakina, D., Orchard, I., 2012. Cloning of the cDNA, localization, and physiological effects of FGLamide-related allatostatins in the blood-gorging bug, Rhodnius prolixus. Insect biochemistry and molecular biology 42, 10-21.
Zandawala, M., Paluzzi, J.P., Orchard, I., 2011. Isolation and characterization of the cDNA encoding DH(31) in the kissing bug, Rhodnius prolixus. Molecular and cellular endocrinology 331, 79-88.
Zandawala, M., Poulos, C., Orchard, I., 2015. Structure-activity relationships of two Rhodnius prolixus calcitonin-like diuretic hormone analogs. Peptides 68, 211-213.

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Tables

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			
*Rhopr*β-ActinFw	ACACCCAGTTTTGCTTACGG	58	300	ND	RT-PCR
*Rhopr*β-ActinRv	GTTCGGCTGTGGTGATGA	57			
*Rhopr*GAPDHFw	GACTGGCATGGCATTCAGAGTT	60	182	102.5	qPCR
*Rhopr*GAPDHRv	CCCCATTAAAGTCCGATGACACC	60			
*Rhopr*ActinFw	ATCTGTTGGAAGGTGGACAG	58	125	102.3	qPCR
*Rhopr*ActinRv	CCATGTACCCAGGTATTGCT	58			
T7 full	ATAGAATTCTCTCTAGAAGCTTAATACGACTCACTATAGGG	61	---	ND	dsRNA synthesis

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Figures

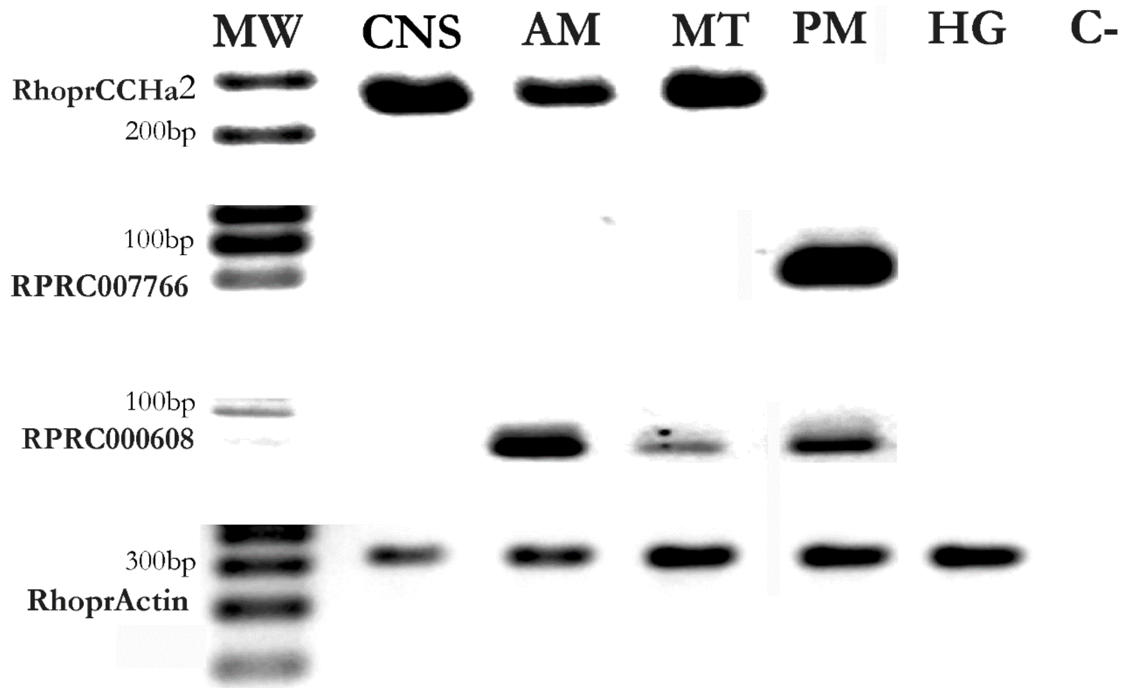

Figure 1: Transcripts encoding *Rhopr*CCHamide and its GPCRs (RPRC00776 and RPRC000608) were PCR-amplified in different tissues of *R. prolixus*. MW: molecular weight marker, CNS: central nervous system, AM: anterior midgut, MT: Malpighian tubules, PM: posterior midgut, HG: hindgut and (C-): negative control without template was assayed for each primer pair. Rhopr*Actin* transcript was PCR-amplified from every tissue as a positive control to test the quality of the cDNAs. An agarose gel image representative of the three biological replicates performed is showed.

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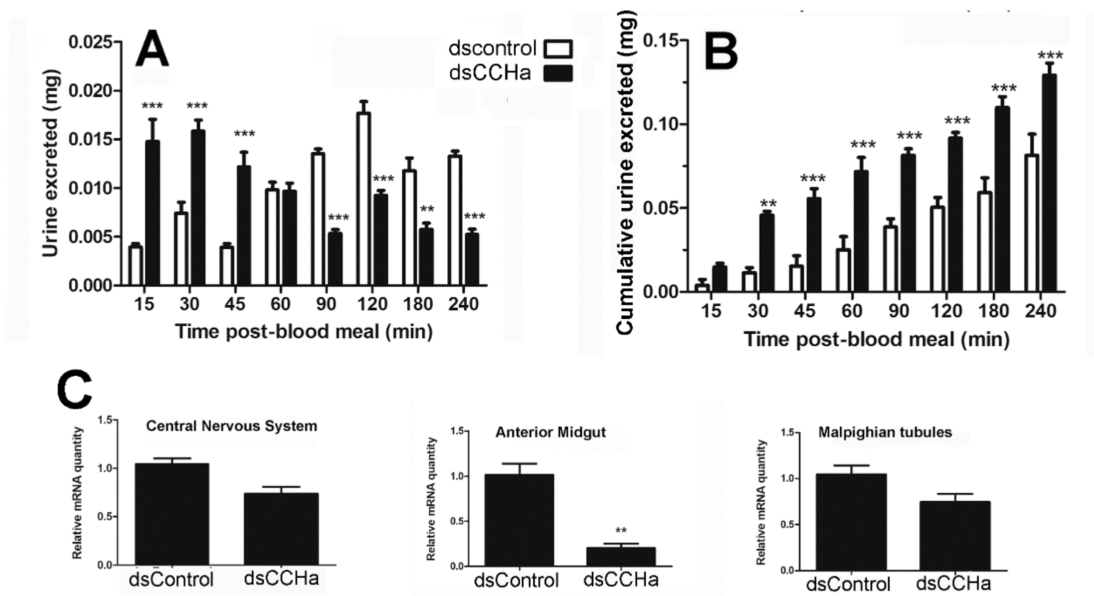

Figure 2: dsCCHa injection stimulates urine excretion by *R. prolixus*. A) Milligrams of urine excreted at different time points post-blood intake in dsControl (n=25) and dsCCHa (n=44) injected insects. Each data represents the mean ± standard error. Two-way ANOVA with repeated measures, Tukey test was used for post-hoc comparisons. *=p<0.05; **=p<0.01; ***=p<0.001. B) Cumulative volume excreted for the dsControl (n=25) and dsCCHa (n=44) groups. Each data represents the mean ± standard error. Two-way ANOVA with repeated measures, Tukey test was used for post-hoc comparisons. **=p<0.01, ***=p<0.001. C)Effect of dsCCHa injection on the mRNA levels of *Rhopr*CCHamide2 in the Anterior Midgut, Central Nervous System and Malpighian tubules (n=5). **=p <0.01 Student's t-test.

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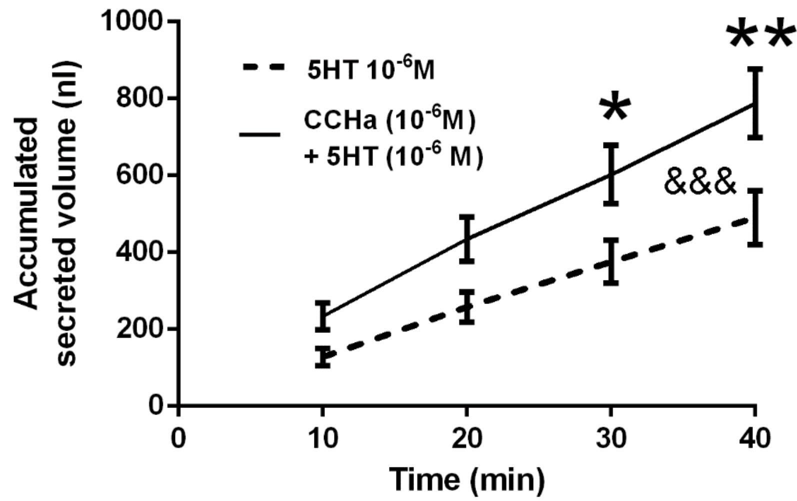

Figure 3: Malpighian tubules secretion volume at different times after stimulation with serotonin (1 μM) in tubules incubated in saline solution (n=47) or synthetic *Rhopr*CCHa (1 μM) (n=37). *=p <0.05; **=p <0.01. Two-ways ANOVA with repeated measures followed by Bonferroni's contrasts. Secretion rate slopes in both experimental groups are significant different (&&&=p<0.001, linear regression analysis).

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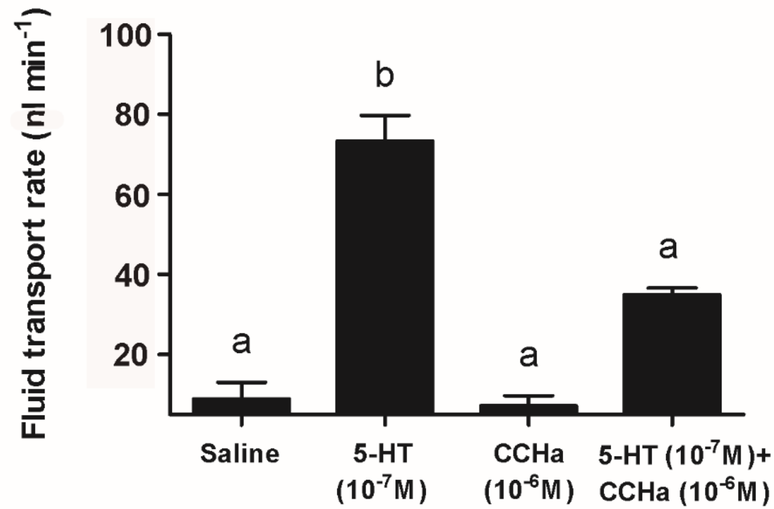

Figure 4: Effect of *Rhopr*CCHamide on the fluid transport rate in the anterior midgut of 5th instar *R. prolixus*. The tissue was incubated with either saline (n=8), serotonin 0.1 µM (n=10), *Rhopr*CCHa2 1 µM (n=11) and serotonin 0.1 µM + *Rhopr*CCHa2 0.1 µM (n=11). Different letters indicate significant differences (One-way ANOVA, Tukey-Kramer contrasts, $p<0.05$).

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