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Reverse chemical ecology-based approach leading to the accidental discovery of repellents for *Rhodnius prolixus,* a vector of Chagas diseases refractory to DEET

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Abstract

Rhodnius prolixus is one of the most important vectors of Chagas disease in Central and South America for which repellents and attractants are sorely needed. Repellents like DEET, picaridin, and IR3535 are widely used as the first line of defense against mosquitoes and other vectors, but they are ineffective against *R. prolixus.* Our initial goal was to identify in *R. prolixus* genome odorant receptors sensitive to putative sex pheromones. We compared gene expression of 21 ORs in the *R. prolixus* genome, identified 4 ORs enriched in male (compared with female) antennae. Attempts to de-orphanize these ORs using the *Xenopus* oocyte recording system showed that none of them responded to putative sex pheromone constituents. One of the them, RproOR80, was sensitive to 4 compounds in our panel of 109 odorants, namely, 2-heptanone, γ-octalactone, acetophenone, and 4- methylcychohexanol. Interestingly, these compounds, particularly 4-methylcyclohexanol, showed strong repellency activity as indicated not only by a significant decrease in residence time close to a host, but also by a remarkable reduction in blood intake. 4-

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Author Contributions

A.C.A.M. and W.S.L. conceived the project. A.C.A.M., W.S.L. and P.X. designed the experiments and performance of the data analysis. T.A.F. designed and performed kissing bug bioassays. X.W. performed mosquito bioassays. T.A.F., N.F.B., D.S.O. and M.F.M. performed qPCR experiments. C.R.U. synthetized putative sex pheromones. A.C.A.M. and W.S.L. wrote the manuscript. All authors provided input, read, and approved the final version of the manuscript.

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

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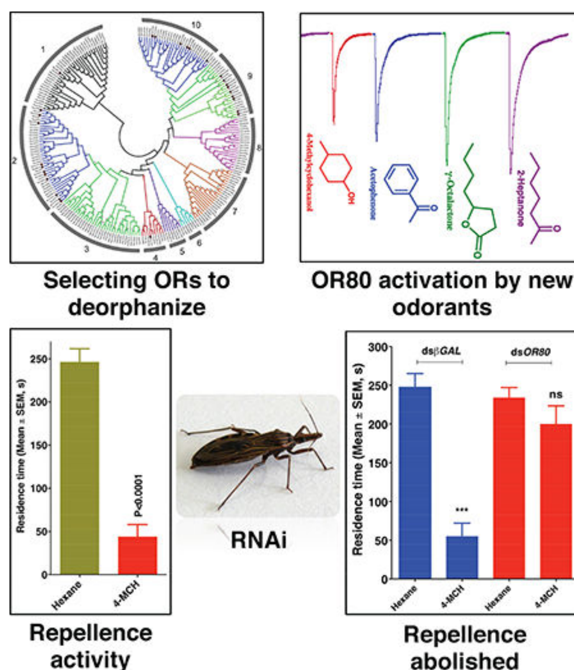
Methylcyclohexanol-elicited repellency activity was abolished in RNAi-treated insects. In summary, our search for pheromone receptors led to the discovery of repellents for *R. prolixus.*

Graphical Abstract



Keywords

odorant receptor; *Rhodnius prolixus*; repellent; RNAi; DEET; acetophenone; 4-methylcyclohexanol; γ-octalactone; 2-heptanone

1. Introduction

Chagas disease, also known as American trypanosomiasis, is a life-threatening infection caused by the protozoan *Trypansosoma cruzi,* which is mainly transmitted by kissing bugs, with *Rhodnius prolixus* (Hemiptera: Reduviidae) being the main vector in Colombia and Venezuela (Moncayo and Silveira, 2009; Monteiro et al., 2003). Discovered in 1909 (Perez-Molina and Molina, 2017), Chagas disease is one of the neglected diseases, which still affects 7.5 million people around the world (WHO, 2013). Socioeconomics plays a crucial role in the dissemination of this disease given that in endemic areas most people are infected in their own homes where they coexist with insect vectors, which found a nonrural favorable environment in cracks and cavities of poorly constructed houses (Perez-Molina and Molina, 2017). With increasing immigration in a globalized world, Chagas diseases has also been reported in nonendemic areas (Antinori et al., 2017). Because vaccines are not available, vector control is the most suitable means for reducing transmission. However, chemical control of Reduviidae using insecticides has failed more than once, primarily due to insecticide resistance (Mougabure-Cueto and Picollo, 2015). Albeit useful for protection against mosquitoes (Leal, 2014; Lupi et al., 2013) and other vectors, repellents, such as

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DEET, picaridin, and IR3535, are ineffective against triatomines (Zermoglio et al., 2015). Therefore, novel repellents that fend off triatomines and/or attractants that may be used for trapping vectors are sorely needed.

Despite being a model for insect physiology studies for a long time (Nunes-da-Fonseca et al., 2017), details about chemical communication in *R. prolixus* are just emerging. Putative sex pheromones have been identified by an in-depth investigation of the constituents of metasternal glands in males and females (Pontes et al., 2008). The most abundant compounds, 2-methyl-3-buten-2-ol, (2*S*)-pentanol, (3*E*)-2-methyl-3-penten-2-ol, and (2R/2*S*)-4-methyl-3-penten-2-ol, were enriched in female glands and released at a time coinciding with the period of sexual activity (Pontes et al., 2008). Additionally, male bugs showed a significant preference for a blend of these compounds as compared with clean air control (Bohman et al., 2018). We surmised that male-enriched or male-specific odorant receptor (OR) genes in *R. prolixus* genome are involved in reception of these putative sex pheromones. Using quantitative PCR, we studied differential expression of 21 selected *OR* genes in male and female antennae. Four ORs, namely RproOR1, 3, 74, and 80, were significantly enriched in male antennae. Attempts to deorphanize these ORs using the *Xenopus* oocyte recording system showed that when coexpressed with the obligatory odorant receptor coreceptor Orco, RproOR1, 3 and 74 did not respond to sex pheromones. Although RproOR80•RproOrco-expressing oocytes were not activated by any of the putative sex pheromones, they elicited robust, dose-dependent currents when challenged with racemic 4-methylcyclohexanol (4-MCH), acetophenone, γ-octalactone, and 2- heptanone. High transcript levels of *RproOR80* were also found in 5th instar male nymphs, particularly in antennae. Behavioral analysis confirmed that *R. prolixus* is indeed insensitive to DEET, but was repelled by OR80 ligands, particularly 4-MCH. These odorants not only caused a reduction in the time a test insect remained closed to a host, but also led to a remarkable decrease in blood ingestion. Repellency behavior against 4-MCH was abolished when *RproO80* was silenced by RNAi. In summary, our failure to identify putative pheromone receptors led to the accidental discovery of repellents effective against *R. prolixus.*

2. Materials and Methods

2.1. *Rhodnius prolixus* Maintenance and Ethics Statement

R. prolixus adults were maintained at 28°C and 80–90% relative humidity in the Insect Biochemistry Laboratory at the Institute of Medical Biochemistry at the Federal University of Rio de Janeiro, Brazil. Adult insects were fed on rabbit at 3-week intervals, except for 5th instar nymphs that were fed at 5-week intervals. All animal care and experimental protocols were conducted following the guidelines of the institutional care and use committee (Committee for Evaluation of Animal Use for Research from Federal University of Rio de Janeiro-CAUAP, under register number CEAU- UFRJ#1200.001568/2013–87, 155/13). Technicians dedicated to the animal facility at Federal University of Rio de Janeiro carried out all aspects related to rabbit husbandry under strict guidelines to ensure careful and consistent handling of the animals.

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2.2 RNA Isolation, cDNA Synthesis and Cloning

Total RNA (t-RNA) was extracted from the antennae, proboscis, and legs of blood-fed adult males and females, and from antennae of male 5th instar nymphs using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was then eluted into nuclease-free water. The concentration of t-RNA was estimated at 260 nm (Lee and Schmittgen, 2006) using a SmartSpect plus spectrophotometer (Bio-Rad, Hercules, CA, USA). We followed previously published protocols (Franco et al., 2016; Oliveira et al., 2018). The following primers were used. The underlined sequences are restriction sites for *XmaI* or *XbaI.* Bold represents Kozak sequences (Kozak, 1987).

RproORl (1230 bp), Forward:

GATCAATTCCCCGGG**ACC**ATGGAAGAATTTGCTGGGATTGATG; Reverse:

CAAGCTTGCTCTAGATTATTTAGTTGATGATTTCACCGAGCC: RproOR3 (1323 bp), Forward:

GATCAATTCCCCGGG**ACC**ATGGATATCTTGCAAAGATTTAAAGATTTCCTCC: Reverse:

CAAGCTTGCTCTAGATTAAGTATTTTTGAAAGCCAACAGTAGACTGAAGTAT; RproOR74 (1122 bp), Forward:

GATCAATTCCCCGGG**ACC**ATGAATTTTAAAAGGCTTTCACCAATACA: Reverse:

CAAGCTTGCTCTAGAAGCTGTTTCCAAGTTGC: RproOR80 (1137 bp), Forward:

GATCAATTCCCCGGG**ACC**ATGTCTCAGGTAACGGAAAATTTATCTCCAGTAAA G; Reverse:

CAAGCTTGCTCT AGAT GAT AAT CAACCT GGAGACAGATTA.

Each purified fragment was cloned into the pGEM-T Easy vector (Promega), and then transformed into One Shot TOP10 (Invitrogen) cells using the heat shock method. Plasmids were extracted and purified from the transformed cells using a QIAprep Spin Miniprep Kit (Qiagen), and then subcloned sequences were validated by sequencing (Davis Sequencing Inc, Davis, CA). RproOR1 was a synthetic gene prepared by GenScript Biotech Corp. (Piscataway, NJ 08854).

2.3 Expression Analysis by Real-Time Quantitative PCR (qPCR)

Quantitative PCR was performed using cDNA extracted from adult antennae using specific primers (Supplementary Table S1). Each qPCR reaction was performed on biological triplicates, which were each run with 3 technical replicates using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) with the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The qPCR experiments were performed according to the Minimum Information Required for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin et al., 2009). *R.*

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prolixus ribosome protein 18 - *RproR18S* (accession#AJ421962.1; RPRC017412-RA) was used as a reference gene to normalize the expression levels among the samples (Majerowicz et al., 2011). Raw C_t normalized against the *RproR18S* standard values were used to calculate relative expression levels in the samples using the $2^{- \quad Ct}$ method (Livak and Schmittgen, 2001). qPCR products were analyzed on 1% agarose gels followed by staining with GelRed™ (Biotium).

2.4 Synthesis of dsRNA

A pGEM-T Easy vector, carrying the full-length *RproOR80* gene together with primers containing the T7 RNA polymerase promoter and terminator sequences, were used to generate a 371-bp PCR product that was used for dsRNA synthesis. *Cquiβ-galactosidase* (CPIJ003337-RA) (Zhu et al., 2013) was used as an unrelated gene for RNAi. The following primers were used: RproOR80 (371 bp), Forward: TAATACGACTCACTATAGGGTGTGCAAGCCCTTTCAGTAA; Reverse: TAATACGACTCACTATAGGGATACGCAGGTGGTGAACTCC; β-Gal (492 bp), Forward: TAATACGACTCACTATAGGGAATGGTTCAGGTCGAAAACG; Reverse: TAATACGACTCACTATAGGGCCGCCTCGTACAAAACAAGT (sequence representing T7 promoter are underlined). PCR products were used as templates for dsRNA synthesis using the MEGAscript RNAi kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. dsRNAs were quantified using a spectrophotometer at 260 nm (Lee and Schmittgen, 2006)[26], and their integrity was assessed by electrophoresis on 1% agarose gels stained with GelRed™ (Biotium). dsRNAs were purified on a large scale using a MEGAclear kit (Ambion) and were then precipitated in 5 M ammonium acetate to yield 10 μg/μL of OR80-dsRNA and β-GAL-dsRNA.

2.5. dsRNA Treatment

One microgram of OR80-dsRNA or β-GAL-dsRNA diluted in 2 pL of RNase-free water was injected into the metathoracic space between the 2nd and 3rd thoracic segments of unfed male 5th instar nymphs as previously described (Franco et al., 2016; Mansur et al., 2014; Oliveira et al., 2018). Likewise, 2 pL of RNase-free water was injected into the H_2O control group. Antennae from different groups were subjected to RNA extraction and cDNA synthesis as described above. Transcription levels of the silenced genes were analyzed by qPCR.

2.6. In Vitro Transcription, Oocytes Microinjection and Electrophysiology

In vitro transcription, oocytes microinjection, and electrophysiology were performed as previously described by (Xu et al., 2014). Briefly, in vitro transcription of cRNAs was performed by using an mMESSAGE mMACHINE T7 kit (Ambion) according to the manufacturer's protocol. Plasmids were linearized with NheI, SphI, or PstI, and capped cRNAs were transcribed using T7 RNA polymerase. cRNA samples were purified with LiCl precipitation solution and resuspended in nuclease-free water at a concentration of 200 μg/mL and stored at −80°C in aliquots. RNA concentrations were determined by UV spectrophotometry. cRNA samples were microinjected (2 ng of RproORx cRNA and 2 ng of RproOrco cRNA) into stage V or VI *Xenopus laevis* oocytes (EcoCyte Bioscience, Austin TX). Oocytes were then incubated at 18°C for 3–7 days in modified Barth's solution [in

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mM: 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.82 MgSO4, 0.33 Ca(NO3)2, 0.41 CaCl2, 10 HEPES, pH 7.4] supplemented with 10 μg/mL of gentamycin, 10 μg/mL of streptomycin, and 1.8 mM sodium pyruvate. A two-electrode voltage clamp (TEVC) was used to detect inward currents. Oocytes were placed in a perfusion chamber and challenged with a panel of more than 100 compounds, including putative sex pheromones (Bohman et al., 2018; Pontes et al., 2008), in a random order (flow rate was 10 mL/min). Odorant-induced currents were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT), voltage held at −70 mV, low-pass filtered at 50 Hz and digitized at 1 kHz. Data acquisition and analysis were carried out with Digidata 1440A and pCLAMP 10 software (Molecular Devices, LLC, Sunnyvale, CA).

2.7. Panel of Odorants

The following compounds were tested: (3*E*)-2-methyl-3-penten-2-ol, (2S/2R)-4-methyl-3-penten-2-ol, (*S*)-(+)-2-pentanol, 2-methyl-3-buten-2-ol, 1-butanol, 1-pentanol, 1- hexanol, 1-heptanol, 1-octanol, 1-nonanol, 2,3-butanediol, 2-butoxyethanol, 3-methyl- 1-butanol, 2-hexen-1-ol, 3-hexen-1-ol, 1-hexen-3-ol, 1-heptene-3-ol, 3-octanol, 1-octen- 3-ol, 2-octanol, 2-butanol, 2-nonen-1-ol, racemic 4-methylcyclohexanol, 1- hexadecanol, menthyl acetate, methyl acetate, ethyl acetate, propyl acetate, butyl acetate, pentyl acetate, hexyl acetate, heptyl acetate, octyl acetate, nonyl acetate, decyl acetate, ethyl 3-hydroxybutanoate, (*E*)-2-hexenyl acetate, (*Z*)-3-hexenyl acetate, α- terpinene, γ-terpinene, ethyl lactate, methyl salicylate, geranyl acetate, octadecyl acetate, acetaldehyde, propanal, butanal, pentanal, hexanal, (*E*)-2-methyl-2-butenal, heptanal, octanal, nonanal, decanal, undecanal, 1-dodecanal, (*E*)-2-hexenal, (*Z*)-8-undecenal, (*E*)-2-heptenal, (*E*)-2-nonenal, 2-butanone, 2-heptanone, geranyl acetone, 6-methyl-5- hepten-2-one, (−)-menthone, fenchone, cyclohexanone, acetophenone, phenol, 2- methylphenol, 3-methylphenol, 4-methylphenol, 4-ethylphenol, 3,5-dimethylphenol, 2,3-dimethylphenol, guaiacol, 2-tridecanone, 2-methoxy-4-propylphenol, 2- phenoxyethanol, (+/−)- limonene, linalyl acetate, α-humulene, linalool oxide, geraniol, nerol, thymol, (+/−)-linalool, eucalyptol, citral, eugenol, α-pinene, ocimene, (±)- citronellal, indole, 3-methylindole, 3-pentanol, 3-methyl-2-butanol, 3-methyl-2-buten-1- ol, γ-valerolactone, γ-hexalactone, γ-octalactone, γ-decalactone, α-phellandrene, nerolidol, γ-dodecalactone, 2,4-dimethylphenol, 2,5-dimethylphenol, 2,6-dimethylphenol, 3,4-dimethylphenol. All compounds were acquired from Sigma-Aldrich, except for 1-dodecanol (Acros), 1-butanol (Fisher Scientific), octadecyl acetate (a gift from Bedoukian Research, Inc.), and (3E)-2-methyl-3-penten-2-ol and (2S/2R)-4-methl-3-penten-2-ol, which were previously synthesized (Pontes et al., 2008).

2.8. Biological Effects

R. prolixus behavior towards a living host was measured using a previously described protocol (Zermoglio et al., 2015), with minor modifications (see Supplementary Video S1 - temporarily uploaded to YouTube to facilitate review; https://youtu.be/T-CHYpS4N4). Briefly, a 50 mL-polystyrene tube was used, with a mesh placed 1.3 cm away from the open end to avoid direct contact and feeding on the human subject. A slit gate was placed 2 cm away from the closed end where the test insect was kept for 5 min before starting measurement. Test compounds (50μL) in the specified concentrations were loaded on a piece of filter paper (1 × 3 cm), which was placed in the area between the extreme and the

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mesh, also referred to as the host zone (HZ) (Zermoglio et al., 2015). By contrast, the opposite area at the closed end of the tube refers to refuge zone (RZ), and the space between HZ and RZ is called the intermediate zone (IZ) (Zermoglio et al., 2015). When ready to start, the open end of the tube was brought in contact with the human subject's arm and then the test insect was released from RZ. The residence time in each zone was recorded for 5 min for each tested insect. For each of the following treatments 10 insects were tested. Host (no filter paper); filter paper with hexane; DEET at 1% in hexane; 4- MCH, acetophenone, γ-octalactone, and 2-heptanone; all at 0.01, 0.1, or 1% in hexane. All test compounds were from Sigma-Aldrich, with >97% purity.

All protocols involving human volunteers were submitted to the Human Research Ethics Committee of the Hospital Clementino Fraga Filho at Federal University of Rio de Janeiro (CEP/HUCFF/UFRJ) and were based on Resolution from National Health Council/Brazil (CNS#466/2012 and ON-CNS # 001/2013). The protocol used in this study was approved by the CEP/HUCFF/UFRJ under registration number CAAE- UFRJ#82075818.1.0000.5257–2/2018. A 30-year-old Latino man served as host for all tests.

2.9. Effects on Blood Ingestion

A previously reported protocol (Franco et al., 2016) was used to test the ability of insects to ingest a blood meal in the presence of semiochemicals. Briefly, insects were placed on the bottom of a cylindrical glass (10 cm x 6 cm). The glass top open end was protected by mesh to prevent insect escape. A filter paper (10 cm x 6 cm) was folded and kept inside the glass to form a ladder and allow access to the vertebrate host whose ear was in contact with the mesh at the top of the glass. Twenty-one-day starved 5th instar insects were individually introduced at the bottom of the glass, and each insect was allowed to acclimate for 5 min. Treatments were hexane (N=10), DEET (N=10), 4-methylcyclohexanol (N=10), acetophenone (N=10), γ-octalactone (N=10) and 2-heptanone (N=10), with all compounds diluted to 1% with hexane. Test compounds (200 μL) were separately loaded in the top part of the filter paper at 2 cm away from the vertebrate ear to make a possible repellent barrier. Each insect was allowed to engorge on blood for 30 min. The insects were weighed 2 h before and soon after a blood meal. The difference in weight was regarded as the amount of blood ingested by the insect. Insect blood-fed protocol was approved by Committee for Evaluation of Animal Use for Research from Federal University of Rio de Janeiro- CAUAP, under register number CEAU-UFRJ#1200.001568/2013–87, 155/13.

2.10. Mosquito Repellency Assay

Repellency behavior was measured using a modified surface landing and feeding assay (Xu et al., 2014). Two hundred female *Culex quinquefasciatus* (Merced/Davis strain, (Choo et al., 2018)) (4 days after emergence) were aspirated and transferred to the arena 2 h before each experiment. All openings were sealed, and the cage was kept near the base of the arena. Thirty minutes after water started circulating, the assay cage was then inserted into the base. Defibrinated sheep blood (100 pl; supplied by the VetMed shop, University of California, Davis) was gently pipetted onto one end of dental cotton plugs (Primo Dental Products, #2 Medium), which were placed in between CO_2 dispensing needles and Dudley tube, one on the control and the other in the test side of the arena. These were surrounded by filter paper

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rings, one loaded with 200μ1 of solvent (hexane) only and the other with 200μ1 of a test sample. Test compounds were DEET, 2-heptanone, γ-octalactone, acetophenone, and 4-MCH; all at 1% m/v concentration in hexane. CO_2 flow was initiated, and the assay was recorded during the scotophase with a camcorder equipped with a Super NightShot Plus infrared system (Sony Digital Handycam, DCR-DVD 910). The number of mosquitoes landing on cotton rolls or in their vicinity were recorded every 5 min. Then, responding mosquitoes were gently removed and treatment and control were rotated. Each assay lasted for 30 min. Then, it was repeated using DEET as a positive control. In short, for each compound tested, the same groups of mosquitoes were tested against DEET.

2.11. Statistical Analysis

All data are expressed as mean ± SEM of 3 independent experiments. Significant differences between qPCR groups were evaluated using the $2^{-\quad Ct}$ values and analyzed using the *t* test or one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons on bioassay. A E-value <0.05 was regarded as statistically significant. All statistical analyses were performed using PRISM 6.0 software (GraphPad Software, San Diego, California, USA).

3. Results and Discussion

3.1. Deorphanization of RproORs

Previously, the *R. prolixus* genome has been sequenced (Mesquita et al., 2015), which coupled with transcriptome analysis (Latorre-Estivalis et al., 2017) led to the identification of genes encoding putative odorant-binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptors (ORs), and ionotropic receptors (IRs) (for definitions, see (Leal, 2012)). In an attempt to identify ORs involved in the reception of the constituents of *R. prolixus* sex pheromone system (Bohman et al., 2018; Pontes et al., 2008), we analyzed previously identified OR vis-á-vis their ligands and then selected 21 *RproOR* genes in clusters with other *OR* genes related to reception of sex and or aggregation pheromones (Fig. S1). Previous differential expression analysis did not show evidence of ORs specific to or enriched in male antennae (Latorre-Estivalis et al., 2017), which might suggest a function specific to males. Therefore, by using qPCR expression of the selected *OR* genes, we compared the presence of *OR* genes in male and female antennae. In agreement with previous work (Latorre-Estivalis et al., 2017), most OR transcripts were equally expressed in male and female antennae or more expressed in female than in male antennae (Fig. S2). Four *OR* genes were enriched or predominantly expressed in male than in female antennae (Fig. S2). Specifically, RproOR1 (VectorBase identity, RPRC000579), RproOR3 (RPRC000059), RproOR74 (RPRC000200), and RproOR80 (RPRC000441) were considered candidate pheromone receptors given the predominance of their transcripts in male antennae. To test this hypothesis, we cloned these receptors and subcloned into pGEMHE. Because we have difficulty cloning the entire ORF for RproOR1, we obtained a synthetic RproOR1 gene and subcloned it in pGEMHE. We then expressed each of the test receptors along with the obligatory odorant receptor coreceptor (RproOrco) (Franco et al., 2016) in *Xenopus* oocytes and recorded electrophysiological responses (Katada et al., 2003) when oocytes were challenged with a panel of 109 odorants. First, we challenged each tested

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RproOR»RproOrco-expressing oocyte with an Orco agonist, 2-{[4-ethyl-5-(4-pyridinyl)-4H-1,2,4-triazol-3yl]sulfanyl}N-(4-isopropylphenyl)acetamide (OCL12) (Chen and Luetje, 2012). When a receptor is properly coexpressed along with Orco, the oocytes tend to generate larger OCL12-induced currents than those recorded from oocytes expressing only Orco (Chen and Luetje, 2012). On the basis of this comparative response, one can infer whether a "silent receptor" might be due to the lack of a key ligand in the test panel or proper expression in tested oocytes.

RproOR1•RproOrco-expressing oocytes gave strong responses to OCL12 but did not respond to any of the constituents of the sex pheromone. These oocytes gave moderate responses to 3-methyl-1-butanol, methyl butyrate, (*Z*)-3-hexenyl acetate, pentanal, 1-pentanol, 2,3-butanedione, 2-undecanone, and menthone, thus suggesting that RproOR1 is a nonspecific receptor. By contrast, both RproOR3•RproOrco- and RproOR74•RproOrco-expressing oocytes were not activated by any of the 109 odorants in our panel. We conclude that these ORs too are not pheromone receptors, and based on their moderate responses to OCL12, we speculate that our panel could be missing key ligands.

Lastly, RproOR80•RproOrco-expressing oocytes did not respond to any of the constituents of the sex pheromones, ie, 2-methyl-3-buten-2-ol, (25*)-pentanol, (3E)-2-methyl-3-penten-2-ol, and (2R/25)-4-methyl-3-penten-2-ol, but gave robust responses to 2-heptanone, γ-octalactone, acetophenone and 4-MHC (Fig. 1) in a dose-dependent manner (Fig. S3). We then compared by using qPCR *RproOR80* transcript levels in adult and immature insects and found that RproOR80 is expressed also in high levels in 5th instar male nymph antennae and to some extent in proboscis (Fig. 2). Previous transcriptome analysis suggested that RproOR80 is equally expressed at low levels in male (3.06 FPKM) and female (3.06 FPKM) antennae of adults, but not in nymph (0.22 FPKM) (Latorre-Estivalis et al., 2017). Such discrepancies between RNA-Seq qPCR data are common. In our own hands, for example, we validated RNA-Seq data from *Culex* mosquitoes with qPCR data for the 5 top OR genes matching nicely with differential expression calculated by RNA-Seq (Leal et al., 2013). Later, we found that the 6th OR in the rank by RNA-Seq was by far the OR gene most expressed in *Cx. quinquefasciatus* antennae (Choo et al., 2018).

3.2. Repellence tests

We surmised that the 4 odorants that elicited robust currents in RproOR80•RproOrco-expressing oocytes might have a role in the chemical ecology of both adults and immature insects. *R. proxilus* takes blood meals throughout its life, with immature insects requiring it for growth and molting (Resh and Cardé, 2003). On the other hand, at least 1 of the 4 odorants, γ-octalactone (Bedoukian, 2013; Xu et al., 2014) is a known mosquito repellent, whereas 4-methylcyclohexanone has been identified as a repellent for human lice (Pelletier et al., 2015) and an oviposition attractant for mosquitoes (Linley, 1989). We then tested whether 2-heptanone, γ-octalactone, acetophenone and/or 4-MHC might repel *R. prolixus.*

With the same protocol used to demonstrate that DEET is not an effective repellent against *R. prolixus* (Zermoglio et al., 2015), we first confirmed that indeed DEET does not repel *R. prolixus* 5th instar male nymph (Fig. 3). No significant difference existed whether attraction was measured in the presence of 1% DEET, with the host alone, or with the solvent (hexane)

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used to deliver DEET (unpaired *t* test, N=10 each, hexane vs. DEET, $P = 0.8858$; hexane vs. host, $P = 0.5695$) (Fig. 3). By contrast, the time of residence in the host zone decreased significantly in the presence of 2-heptanone, γ-octalactone, acetophenone, or 4- MCH (all at 1%, $P < 0.0001$) (Fig. 3). The time of residence in the presence of hexane (control) was 246.4±15.3 s, whereas in the presence of 1% 4-MCH the mean time was reduced to 44.1±13.9 s. At lower doses, the residence time in the host zone and in the presence of 4-MCH was significantly lower than in the control: 0.1% 4-MCH, 39.5±11.1 s, and 0.01% 4-MCH, 67.9±17.3 s. Likewise, 5th instar female nymphs were repelled by 4- MCH. The time of residence in the presence of hexane (control) was 216±13.8 s, whereas in the presence of 1% 4-MCH the mean time was reduced to 33.5±12.8 s (P<0.0001; two-tailed, Mann-Whitney test). At lower doses, the residence time in the host zone and in the presence of 4-MCH was significantly lower than in the control: 0.1% 4-MCH, 42.7±19.4 s (P<0.0001), and 0.01% 4-MCH, 92.4±23.5 s (P<0.0005, two-tailed, Mann-Whitney test).

Next, we measured whether these repellents would affect the insects' ability to ingest a blood meal. Insects used in this bioassay were male 5th instar nymphs starved for 21 days, which can feed up to 10 times their body weight in blood, making it relatively easy to measure the ingested blood amount. This strategy makes it possible to evaluate whether the presence of physiologically active chemicals could also interfere directly in the blood-feeding behavior. Blood ingestion in the presence of DEET or hexane did not differ significantly (unpaired *t*-test, N=10, $P = 0.5484$) (Fig. 4). By contrast, in the presence of any of the 4 repellents, 5th instar nymphs did not consume a blood meal (P < 0.0001 in all 4 cases; N=10 each) (Fig. 4). In summary, insects in control groups ingested an average of 136.6±16.1 mg of blood, whereas insects in treatment groups consumed no more than 1 mg. This result shows a remarkable reduction in the insects' ability to intake a blood meal. Blood intake did not increase significantly when 2-heptanone, acetophenone, γ-octalactone, or 4-MCH were tested at lower doses (0.1 and 0.01%). For example, a slight increase occurred in blood intake when γ-octalactone was tested at lower doses, but was still significantly lower than in the control (P < 0.0001 in all cases): 1%, 0.96±0.45 mg; 0.1%, 6.55±4.14 mg; 0.01%, 17.5±7.7 mg.

3.3. RNAi and phenotype behavior

Next, we tested whether reducing *RproOR80* transcripts by RNAi would affect the insect response to repellents. First, we injected OR80-dsRNA, water, or β-GAL-dsRNA in the thorax of 5th instar males, following previously reported protocols (Franco et al., 2016; Mansur et al., 2014; Oliveira et al., 2018), and compared *RproOR80* transcript levels 2 days postinjection. Transcript levels were significantly reduced (Fig. 5A) compared with those in negative controls. Then, we tested whether the phenotype would respond differently to the most active of all 4 repellents, 4-MCH, and used DEET as a negative control. As previously observed (Fig. 3), water- injected nymphs were repelled by 4-MCH, but not DEET (Fig. 5B). Likewise, β-GAL-dsRNA-injected nymphs behaved normally thus being repelled by 4-MCH, but not by DEET (Fig. 5B). By contrast, responses of nymphs with reduced transcripts of RproOR80 to 4-MCH did not differ significantly from the response to DEET or hexane (control) (Fig. 5B). We, therefore, concluded that 2-heptanone, γ-octalactone, acetophenone, and 4-MCH are *R. prolixus* repellents.

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3.4. Mosquito responses to *R. prolixus* repellents

We then tested whether these compounds would also repel *Cx. quinquefasciatus.* In agreement with the literature (Bedoukian, 2013; Xu et al., 2014), in our surface landing and feeding assays, blood-seeking female mosquitoes were repelled by DEET ($P < 0.0001$) and γ-octalactone ($P < 0.0001$), but no repellence was observed with 2-heptanone, acetophenone, or 4-MCH (Fig. S4).

3.5. Conclusions

Taken together, these findings confirm that, although DEET is a broad-spectrum repellent (Leal, 2014), it is not effective against the bloodsucking *R. prolixus.* More importantly, 2-heptanone, γ-octalactone, acetophenone, and 4-MCH are effective repellents for *R. prolixus* at a low dose (1%), but except for γ-octalactone, these repellents are not effective against *Cx. quinquefasciatus* mosquitoes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Antinori S, Galimberti L, Bianco R, Grande R, Galli M, Corbellino M, 2017 Chagas disease in Europe: A review for the internist in the globalized world. Eur J Intern Med 43, 6–15. [PubMed: 28502864]

Bedoukian RH, 2013 Control and repellency of mosquitoes. Google Patents.Bohman B, Weinstein AM, Unelius CR, Lorenzo MG, 2018 Attraction of Rhodnius prolixus males to a synthetic female-pheromone blend. Parasit Vectors 11, 418. [PubMed: 30012183]

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT, 2009 The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55, 611–622. [PubMed: 19246619]

Chen S, Luetje CW, 2012 Identification of new agonists and antagonists of the insect odorant receptor co-receptor subunit. PLoS One 7, e36784. [PubMed: 22590607]

Choo YM, Xu P, Hwang JK, Zeng F, Tan K, Bhagavathy G, Chauhan KR, Leal WS, 2018 Reverse chemical ecology approach for the identification of an oviposition attractant for *Culex quinquefasciatus*. Proc Natl Acad Sci U S A 115, 714–719. [PubMed: 29311316]

Franco TA, Oliveira DS, Moreira MF, Leal WS, Melo AC, 2016 Silencing the odorant receptor co-receptor RproOrco affects the physiology and behavior of the Chagas disease vector Rhodnius prolixus. Insect Biochem Mol Biol 69, 82–90. [PubMed: 25747010]

Katada S, Nakagawa T, Kataoka H, Touhara K, 2003 Odorant response assays for a heterologously expressed olfactory receptor. Biochem Biophys Res Commun 305, 964–969. [PubMed: 12767924]

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Kozak M, 1987 An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 15, 8125–8148. [PubMed: 3313277]

Latorre-Estivalis JM, Robertson HM, Walden KK, Ruiz J, Goncalves LO, Guarneri AA, Lorenzo MG, 2017 The molecular sensory machinery of a Chagas disease vector: expression changes through imaginal moult Sci Rep 7, 40049. [PubMed: 28059141]

Leal WS, 2012 Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annu Rev Entomol 58, 373–391. [PubMed: 23020622]

Leal WS, 2014 The enigmatic reception of DEET - the gold standard of insect repellents. Curr Opin Insect Sci 6, 93–98. [PubMed: 25530943]

Leal WS, Choo YM, Xu P, da Silva CS, Ueira-Vieira C, 2013 Differential expression of olfactory genes in the southern house mosquito and insights into unique odorant receptor gene isoforms. Proc Natl Acad Sci U S A 110, 18704–18709. [PubMed: 24167245]

Lee EJ, Schmittgen TD, 2006 Comparison of RNA assay methods used to normalize cDNA for quantitative real-time PCR. Anal Biochem 357, 299–301. [PubMed: 16930526]

Linley JR, 1989 Laboratory tests of the effects of p-cresol and 4-methylcyclohexanol on oviposition by three species of Toxorhynchites mosquitoes. Med Vet Entomol 3, 347–352. [PubMed: 2577519]

Livak KJ, Schmittgen TD, 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408. [PubMed: 11846609]

Lupi E, Hatz C, Schlagenhauf P, 2013 The efficacy of repellents against *Aedes, Anopheles, Culex* and *Ixodes* spp. - a literature review. Travel Med Infect Dis 11, 374–411. [PubMed: 24201040]

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. [PubMed: 21929722]

Mansur JF, Alvarenga ES, Figueira-Mansur J, Franco TA, Ramos IB, Masuda H, Melo AC, Moreira MF, 2014 Effects of chitin synthase double-stranded RNA on molting and oogenesis in the Chagas disease vector *Rhodniusprolixus*. Insect Biochem Mol Biol 51, 110–121. [PubMed: 24398146]

Mesquita RD, Vionette-Amaral RJ, Lowenberger C, Rivera-Pomar R, Monteiro FA, Minx P, Spieth J, Carvalho AB, Panzera F, Lawson D, Torres AQ, Ribeiro JM, Sorgine MH, Waterhouse RM, Montague MJ, Abad-Franch F, Alves-Bezerra M, Amaral LR, Araujo HM, Araujo RN, Aravind L, Atella GC, Azambuja P, Berni M, Bittencourt-Cunha PR, Braz GR, Calderon-Fernandez G, Carareto CM, Christensen MB, Costa IR, Costa SG, Dansa M, Daumas-Filho CR, De-Paula IF, Dias FA, Dimopoulos G, Emrich SJ, Esponda-Behrens N, Fampa P, Fernandez- Medina RD, da Fonseca RN, Fontenele M, Fronick C, Fulton LA, Gandara AC, Garcia ES, Genta FA, Giraldo-Calderon GI, Gomes B, Gondim KC, Granzotto A, Guarneri AA, Guigo R, Harry M, Hughes DS, Jablonka W, Jacquin-Joly E, Juarez MP, Koerich LB, Latorre-Estivalis JM, Lavore A, Lawrence GG, Lazoski C, Lazzari CR, Lopes RR, Lorenzo MG, Lugon MD, Majerowicz D, Marcet PL, Mariotti M, Masuda H, Megy K, Melo AC, Missirlis F, Mota T, Noriega FG, Nouzova M, Nunes RD, Oliveira RL, Oliveira-Silveira G, Ons S, Pagola L, Paiva- Silva GO, Pascual A, Pavan MG, Pedrini N, Peixoto AA, Pereira MH, Pike A, Polycarpo C, Prosdocimi F, Ribeiro-Rodrigues R, Robertson HM, Salerno AP, Salmon D, Santesmasses D, Schama R, Seabra-Junior ES, Silva-Cardoso L, Silva- Neto MA, Souza-Gomes M, Sterkel M, Taracena ML, Tojo M, Tu ZJ, Tubio JM, Ursic-Bedoya R, Venancio TM, Walter-Nuno AB, Wilson D, Warren WC, Wilson RK, Huebner E, Dotson EM, Oliveira PL, 2015 Genome of *Rhodnius prolixus,* an insect vector of Chagas disease, reveals unique adaptations to hematophagy and parasite infection. Proc Natl Acad Sci U S A 112, 14936–14941. [PubMed: 26627243]

Moncayo A, Silveira AC, 2009 Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. Mem Inst Oswaldo Cruz 104 Suppl 1, 17–30. [PubMed: 19753454]

Monteiro FA, Barrett TV, Fitzpatrick S, Cordon-Rosales C, Feliciangeli D, Beard CB, 2003 Molecular phylogeography of the Amazonian Chagas disease vectors Rhodnius prolixus and R. robustus. Mol Ecol 12, 997–1006. [PubMed: 12753218]

Mougabure-Cueto G, Picollo MI, 2015 Insecticide resistance in vector Chagas disease: evolution, mechanisms and management. Acta Trop 149, 70–85. [PubMed: 26003952]

Nunes-da-Fonseca R, Berni M, Tobias-Santos V, Pane A, Araujo HM, 2017 Rhodnius prolixus: From classical physiology to modern developmental biology. Genesis 55.

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Oliveira DS, Brito NF, Franco TA, Moreira MF, Leal WS, Melo ACA, 2018 Functional characterization of odorant binding protein 27 (RproOBP27) From *Rhodnius prolixus* antennae. Front Phsysiol 9, 1175.

Pelletier J, Xu P, Yoon KS, Clark JM, Leal WS, 2015 Odorant receptor-based discovery of natural repellents of human lice. Insect Biochem Mol Biol 66, 103–109. [PubMed: 26494014]

Perez-Molina JA, Molina I, 2017 Chagas disease. Lancet.

Pontes GB, Bohman B, Unelius CR, Lorenzo MG, 2008 Metasternal gland volatiles and sexual communication in the triatomine bug, *Rhodnius prolixus*. J Chem Ecol 34, 450–457. [PubMed: 18317844]

Resh VH, Cardé RT, 2003 Encyclopedia of insects. Academic Press, Amsterdam; Boston.

WHO, 2013 Chagas disease (American trypanosomiasis) - fact sheet (revised in March 2013). Wkly Epidemiol Rec 340.

Xu P, Choo YM, De La Rosa A, Leal WS, 2014 Mosquito odorant receptor for DEET and methyl jasmonate. Proc Natl Acad Sci U S A 111, 16592–16597. [PubMed: 25349401]

Zermoglio PF, Martin-Herrou H, Bignon Y, Lazzari CR, 2015 Rhodnius prolixus smells repellents: Behavioural evidence and test of present and potential compounds inducing repellency in Chagas disease vectors. J Insect Physiol 81, 137–144. [PubMed: 26210345]

Zhu F, Xu P, Barbosa RM, Choo YM, Leal WS, 2013 RNAi-based demonstration of direct link between specific odorant receptors and mosquito oviposition behavior. Insect Biochem Mol Biol 43, 916–923. [PubMed: 23911547]

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Highlights

- Quantitative PCR analysis showed that out of 21 candidate pheromone receptors in *Rhodnius prolixus* genome, 4 genes were enriched in male antennae.
- Deorphanization with the *Xenopus* oocyte expression system demonstrated that none of 4 receptors responded to sex pheromones.
- One receptor, RproOR80, was sensitive to 2-heptanone, γ-octalactone, acetophenone, and 4- methylcyclohexanol.
- In the presence of these compounds, nymphs spent significantly less time close to a human host and did not take a blood meal from a vertebrate host.
- Repellency activity was abolished in RproOR80-dsRNA-injected nymphs, but not in control insects injected with water or β-GAL-dsRNA.

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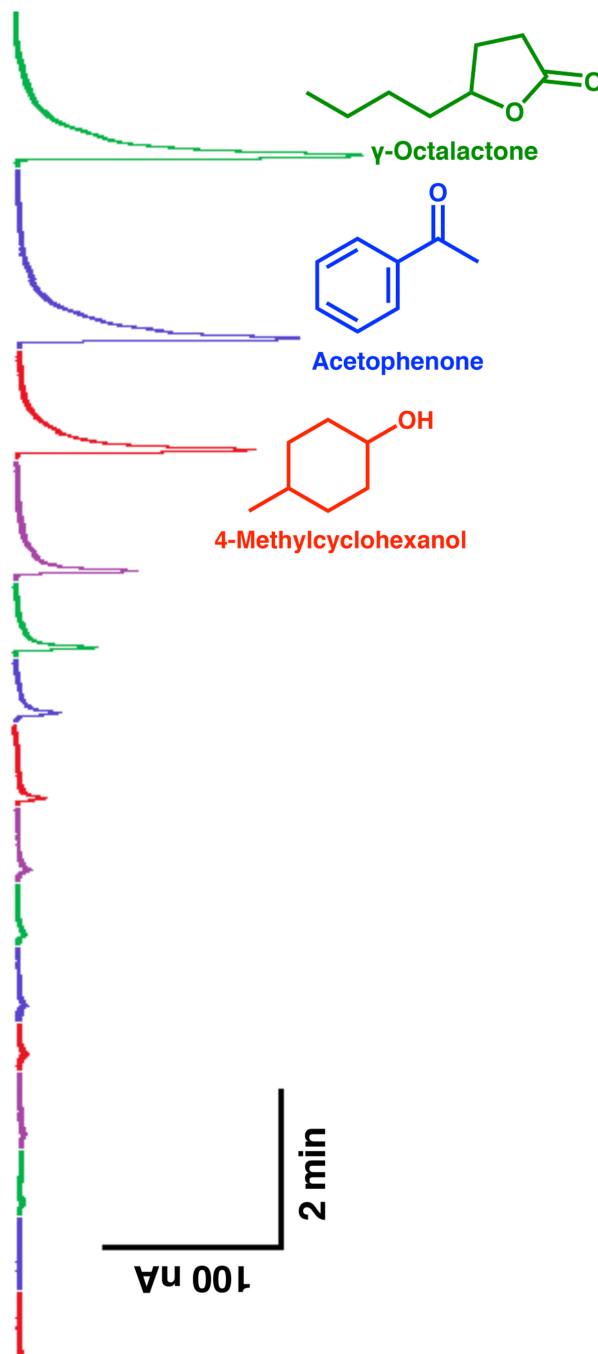

Fig 1. Traces obtained with RproOR80•RproOrco-expressing oocytes when challenged with test compounds in a dose-dependent manner.

Traces are colored to match the structure of the compounds: 4-methylcyclohexanol (maraschino), acetophenone (blueberry), γ-octalactone (clover), and 2-heptanone (plum). In this trace, oocytes were challenged with these compounds in the order above from left to right, with 1 μM, 10 μM, 100 μM, and 1 mM.

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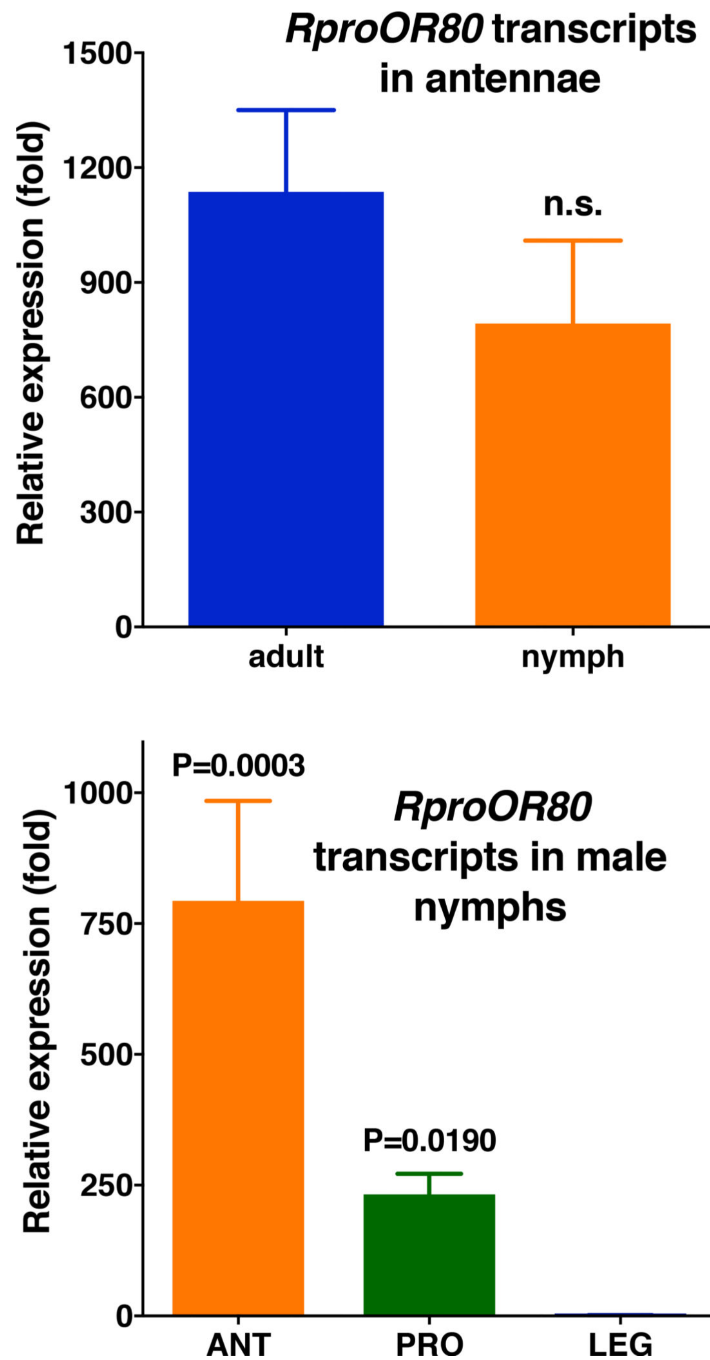

Fig 2. Quantitative analysis of RproOR80 expression.

(A) Comparison of *RproOR80* transcript levels in antennae of male adults and male 5th instar nymphs. n. s. : not significant, unpaired t-test, P=0.3332. (B) Quantitative RproOR80 expression profile in antennae (ANT); proboscis (PRO) and legs (LEG) of male 5th instar nymphs. Friedmann test, with Dunn's multiple comparisons test. P adjusted values on the top of bars are for ANT and PRO compared to LEG.

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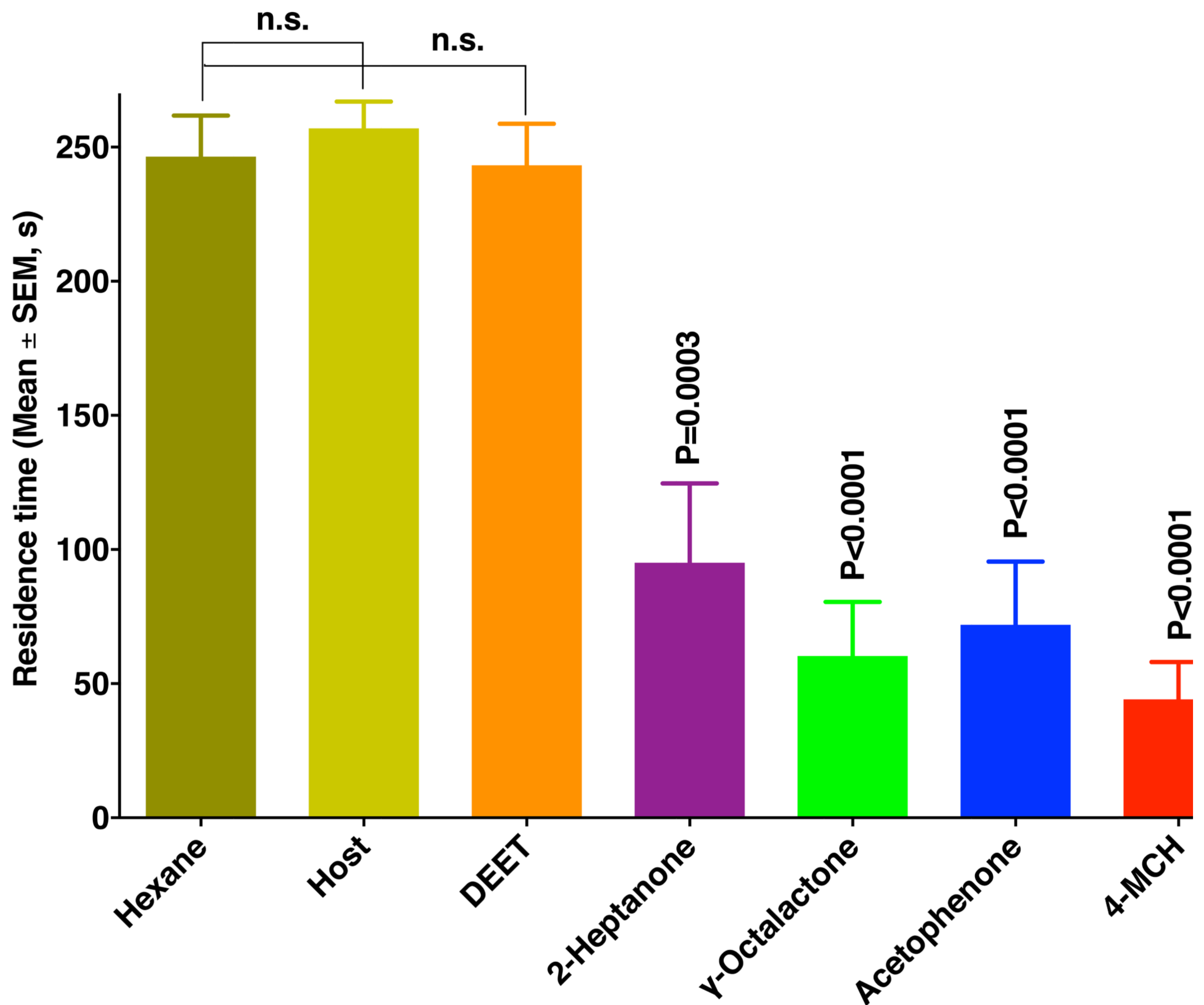

Fig. 3. *R. prolixus* behavioral response to human host in the absence and presence of physiologically relevant compounds.

Male 5th instar nymphs spent considerable time in the host zone (approximately 250 s out of 300 s recorded) in the presence of 1% DEET or hexane control. In the presence of 2-heptanone, γ-octalactone, acetophenone, or 4- methylcyclohexanol (4-MCH) at 1%, insects spent significantly less time close to the host.

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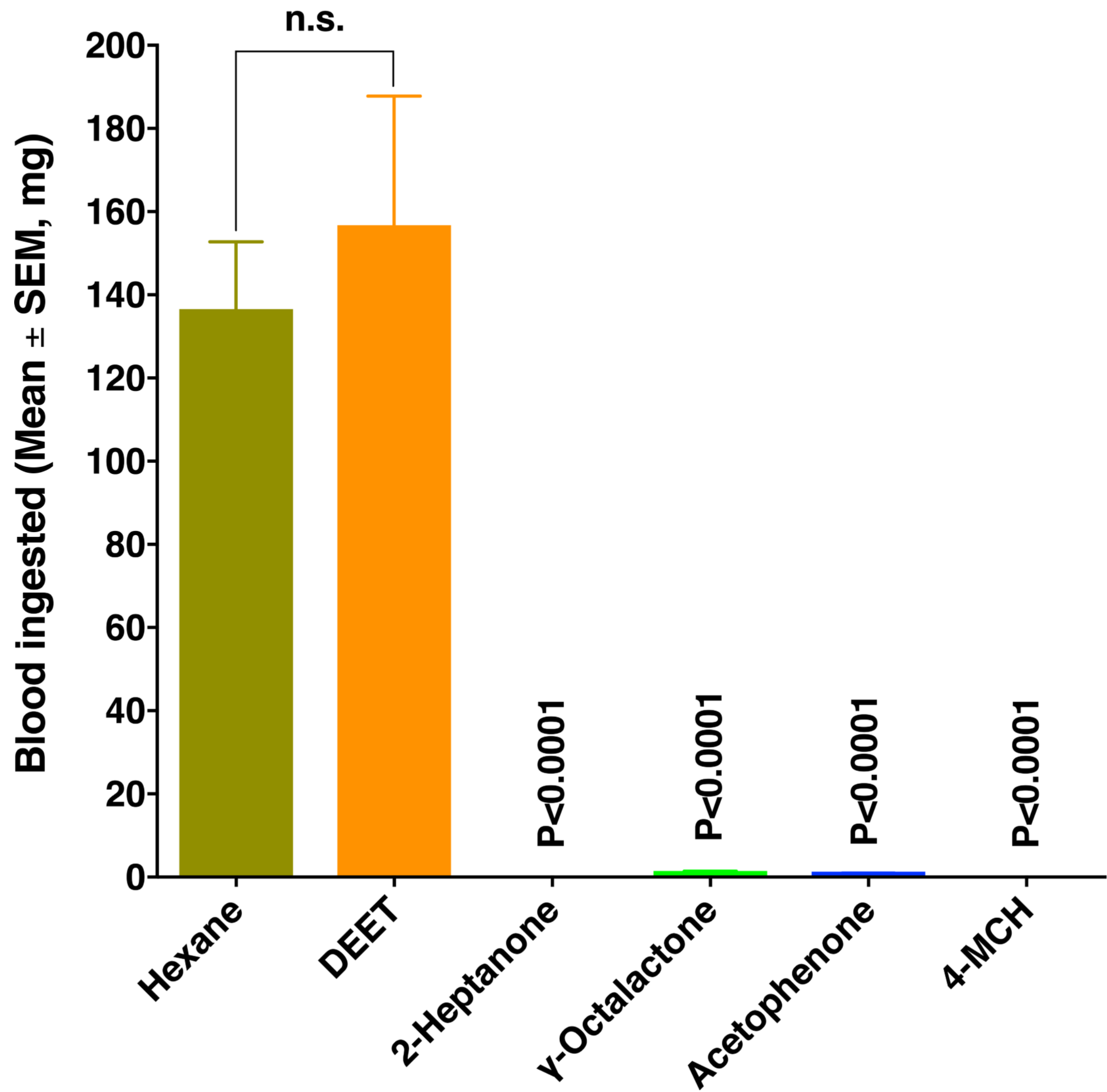

Fig. 4. *R. prolixus* feeding behavior in the presence or absence of physiologically relevant compounds.

Starved 5th instar male engorgement in the presence of DEET and control (hexane) did not differ significantly. In the presence of 2-heptanone, γ-octalactone, acetophenone, or 4-methylcyclohexanol (4-MCH) at 1%, feeding was prevented.

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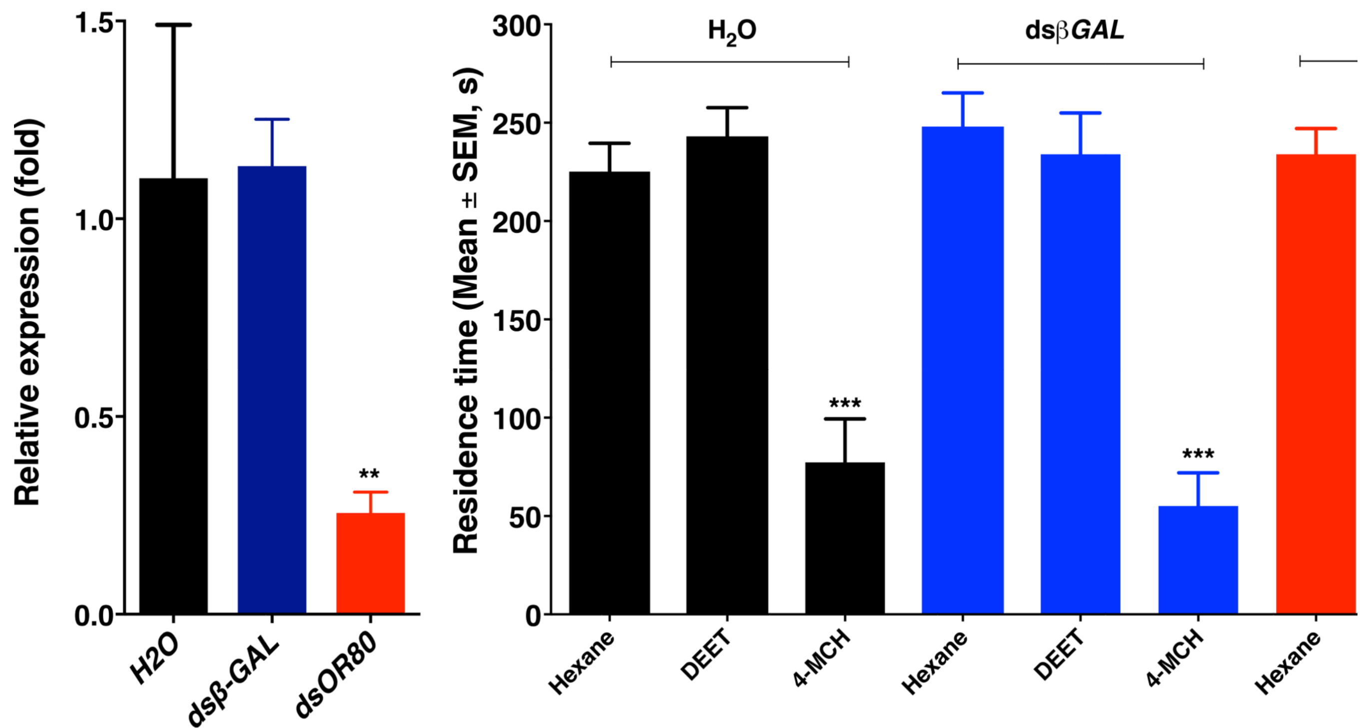

Fig. 5. Effect of reducing *RproOR80* transcript by RNAi on *R. prolixus* response to 4-methylcyclohexanol.

(A) Quantitative analysis of *RproOR80* expression in antennae of insects injected with H_2O, *βGAL-dsRNA,* and *RproOR80-dsRNA.* Ribosomal gene *Rpro18S* was used as an endogenous control. (B) Response of phenotypes to human host in the presence of DEET or 4-methylcyclohexanol (4-MCH). Tested insects were injected with water, *β-GAL-dsRNA,* or *OR80-dsRNA.* 4-MCH-elicited repellence was abolished in *OR80-* dsRNA-injected nymphs.