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TRPA1 modulates noxious odor responses in *Lygus hesperus*

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ABSTRACT

Lygus hesperus is a key pest of many economically important crops across western North America. Central to many aspects of the lives of these insects is chemical signalling, with identified roles in host plant selection, aggregation and passive mate guarding. The development of novel monitoring and control approaches for this insect will rely on a sound understanding of how these cues are perceived and processed, and their impact on behavior. Towards this end, we investigated allyl isothiocyanate, cinnamaldehyde and citronellal, compounds that are noxious repellents to other insects. We found that *L. hesperus* avoided areas containing the three compounds and that exposure induced increases in movement velocity and duration in both nymphs and adults. This suggests these compounds may work as repellents. To better understand the underlying physiology of this response, RNA interference by dsRNA injection was used to inhibit the expression of two chemosensory-associated proteins, the odorant receptor co-receptor (Orco) and the transient receptor potential A (TRPA1) channel. While knockdown of Orco did not change the reaction of adult females to citronellal, TRPA1 silencing effectively eliminated the induced increase to movement, suggesting a chemoperceptory role in citronellal detection.

1. Introduction

The western tarnished plant bug, *Lygus hesperus*, is an abundant and highly destructive polyphagous pest found throughout western North America (Scott, 1977; Jackson et al., 1995; Slosser et al., 2006). Use of broad-spectrum pesticides to control populations is currently effective, but the approach has negative impacts on the environment and populations of beneficial insects. Over-reliance on pesticide application can also lead to a loss of efficacy; the sister species *L. lineolaris*, which is more heavily controlled, has exhibited increased resistance over time to a suite of chemicals (Parys et al., 2017). Ensuring the long-term utility of pesticides will require the adoption of alternative control approaches that complement existing technologies. One potential route for controlling Lygus is through manipulation of their chemosensory environment (Van der Goes and Carlson, 2006; Witzgall et al., 2010). Chemical odorants and tastants play crucial roles for insects, allowing them to distinguish appropriate plants on which to feed and oviposit, locate and select appropriate mates, and avoid threats (Stengl, 2017). Male Lygus have been shown to rely on such cues to distinguish sex, reproductive maturity, and mating status in females (Brent, 2010a; Brent and Byers, 2011; Brent et al., 2017; Byers et al., 2013). Lygus nymphs and adult females have also been shown to discriminate host plant odors (Blackmer et al., 2004; Blackmer and Cañas, 2005). Despite the potential advantages for controlling Lygus populations by disrupting their chemoperception, little is known about which compounds Lygus respond to and even less is known about their olfactory and gustatory physiology.

The perception and transduction of odorant and tastant signals requires a complex interplay of numerous components. Perception starts at chemosensory sensilla, where sensory neurons for olfaction and gustation are located. In insects, these are typically present on antennae, mouth parts, legs, wings and the ovipositor (Joseph and Carlson, 2015). Olfactory receptor neurons (ORNs), used to detect volatile compounds, are principally located on the antennae (Stengl, 2017), while gustatory receptor neurons (GRNs), used to detect low volatility tastants, are found on mouth parts and legs (Scott, 2018). Each sensillum can contain several receptor neurons, each of which is responsive to a limited range of chemistries. ORNs normally express a single type of OR, but the diversity of odorant receptors (ORs) associated with ORNs can be quite wide, depending on the species and developmental stage, which allows insects to discriminate a variety of relevant odors. One extreme is that of *Tribolium castaneum*; the genome predicts as many as 341 distinct odorant receptors (ORs) (Touhara and Vosshall, 2009). In contrast to this diversity, one OR, the odorant receptor co-receptor (Orco), is expressed in most insect ORNs and its structure is strongly conserved across species. A fully functional odorant-sensing unit is dependent on the heterodimerization of Orco with other ORs (Carey and Carlson 2011). ORNs also express members

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of the transient receptor potential (TRP) superfamily of ion channels that have been linked with virtually all sensory modalities. TRPA1 has been implicated in a number of behavioral responses to aversive stimuli, such as temperature (Kang et al., 2011), reactive oxygen species (Du et al. 2016; Guntur et al., 2017), phototoxins (Du et al., 2019), and a diverse array of noxious odorants and tastants (Fowler and Montell, 2013). The mediatory functions of TRPA1 appear to vary by species and isoform, with alternative splicing linked to differences in the sensitivity to varying stimuli. Unlike ORNs, GRNs can express multiple gustatory receptors (GRs). GRNs tuned to bitter compounds also express subsets of the TRP channels, including TRPA1, that can form complexes with the GRs to enhance the signal transduction of typically bitter compounds, such as aristolochic acid (Afroz et al., 2013; Kim et al., 2010) and camphor (Liman et al., 2014; Zhang et al., 2013). Once activated by a chemical compound, ORNs and GRNs convey the information through neuronal complexes where signals are collectively processed, before being sent to the brain, where an appropriate behavioral response will be determined (Scott, 2018; Wilson, 2013).

Although Lygus management strategies that target the mechanisms underlying chemoperception have significant potential to disrupt crucial life functions, the complexity and variability across insects complicates implementation. Given their broad functions, conserved structures, and central roles in chemoperception, Orco and TRPA1 have been proposed as early candidates for targeted disruption and/or modulation. However, our understanding of their functions in Lygus biology remain limited relative to other pests. Here, we examine the behavior of *L. hesperus* to known noxious compounds and then use RNAi-mediated knockdown to determine if Orco and/or TRPA1 play a role in mediating the behavioral responses.

2. Methods and materials

2.1. Insect rearing

Test subjects were the F1 or F2 progeny of 400–500 adult *L. hesperus* collected from alfalfa (*Medicago sativa* L.) near Maricopa, AZ. Field collections were made in October and November 2017. Collected adults were maintained in incubators set at 27.0 ± 1 °C with a photoperiod of 14:10 (L:D) h. They were held in collapsible cages (30.5 × 30.5 × 30.5 cm; BioQuip, Rancho Dominguez, CA) provisioned with shredded paper and a sheet of Hexcel (PN1, Hexcel, Pleasanton, CA) for refuge, and saturated cotton as a water source. The insects were fed raw sunflower seeds (*Helianthus annuus* L.) and fresh green bean pods (*Phaseolus vulgaris* L.) three times weekly. Experimental insects were generated from eggs deposited in Parafilm M agarose packs. Resulting hatches were collected daily and groups of mixed-sex nymphs were reared in 1890-ml waxed chipboard cups (Huhtamaki, De Soto, KS, USA) under the same environmental conditions as their parents. Each container was provisioned with approximately 20 g of green beans and 12 g of artificial diet (Debolt, 1982) packaged in Parafilm M (Pechiney Plastic Packaging, Chicago, IL, USA) (Patana, 1982). Provisions were replaced every 48 h. Containers were covered with nylon mesh to ensure adequate ventilation and light exposure. When needed for assays, 4th and 5th instars were removed within 1 day of molting. Daily monitoring allowed adults to be collected within 24 h of emergence. Cohorts of same-aged adults were separated by gender and reared under conditions matching those for nymphs to prevent any behavioural changes resulting from mating (Brent, 2010a,b; Brent and Byers, 2011). Nymphs and adults were maintained at densities known to minimally affect development (50–100/container; Brent, 2010c).

2.2. Laboratory bioassay and chemical repellents

An enclosed arena assay was used to test the responses of 4th and 5th instars, and adult females and males to noxious odors. Nymphs were tested 1–2 days after molting. Adults were aged 7 d post-eclosion to ensure maturity. Adults were separated by sex from eclosion onward to prevent behavioural shifts induced by mating (Strong et al., 1970; Brent, 2010b). Assays were conducted in plastic plates with six 2.0 × 3.5 cm wells. At the bottom of each well was placed an 18 × 18 mm glass coverslip. Three compounds (Sigma-Aldrich) were selected for their known efficacy in provoking behavioral responses in other insect species: allyl isothiocyanate (Al-Anzi et al., 2006; Blackwell et al., 1997; Freitas et al., 2016), cinnamaldehyde (Chang et al., 2006; Cheng et al., 2006; Kasim et al., 2014) and citronellal (Du et al., 2015; Kwon et al., 2010; Müller et al., 2008; Yang and Ma, 2005). These were diluted in DMSO (Sigma-Aldrich) to create solution concentrations of 0.0 (DMSO control), 0.08, 0.4, 2.0, and 10.0%. For each treatment, a 2 µl aliquot was applied to a 6 mm diameter disk of Whatman #2 filter paper. Treated disks were air dried for 5 min, then centered on the slip covers in the wells. Lygus were immobilized on ice, then placed individually into each well. The plate was covered with a sheet of glass. All six individuals on a plate were exposed to the same treatment, and each plate was used for only one specific type of treatment to avoid contamination effects. Lygus were allowed to recover from cooling for 5 min prior to being observed. A Pro HD C920 camera (Logitech, Lausanne, Switzerland) mounted over the plate recorded movement to a computer using a frame rate of 30 Hz over a 10 min period. Data were collected and summarized by Ethovision XT (v. 10.1.856, Noldus Information Technology, Wageningen, Netherlands). After an observation bout was complete, coverslips and paper disks were discarded, and the plate and glass were thoroughly cleaned with 90% EtOH. For each treatment combination, there were 16 observation bouts, resulting in 96 individuals tracked. Treatment bouts were conducted over many days and in a randomized order to avoid confounding effects of any possible circadian changes to perceptive ability. The null hypothesis that *L. hesperus* showed no difference in the velocity or duration of movement when exposed to the test odorant relative to the DMSO carrier was tested using Kruskal-Wallis ANOVA on ranks and a Tukey test for multiple comparisons when the ANOVA indicated significance. A non-parametric approach was necessary because Shapiro-Wilk tests indicated that the data was non-normally distributed.

A second behavioural assay was used to differentiate whether *L. hesperus* any responses in the movement assay were due to induced changes in activity or to an aversion to the chemicals propelling the insects to move away. A plexiglass arena (10 × 40 cm), with a mesh top to ensure air circulation, was divided into four equally sized zones, A through D. A DMSO-treated disk on a slip cover, as above, was introduced to the center of zone A. A second disk treated with a 10% solution of one of the three odorants was placed in the center of Zone D.

Table 1
Results of Kruskal-Wallis ANOVAs on ranks for movement velocity and duration in *L. hesperus* exposed to volatilizing allyl isothiocyanate, cinnamaldehyde, or citronellal.

	Velocity	Duration
Allyl Isothiocyanate		
4th Instar	$H = 33.93$, $df = 4$, $p \leq 0.001$	$H = 20.77$, $df = 4$, $p \leq 0.001$
5th Instar	$H = 56.09$, $df = 4$, $p \leq 0.001$	$H = 52.14$, $df = 4$, $p \leq 0.001$
Female	$H = 100.33$, $df = 4$, $p \leq 0.001$	$H = 95.00$, $df = 4$, $p \leq 0.001$
Male	$H = 107.21$, $df = 4$, $p \leq 0.001$	$H = 81.20$, $df = 4$, $p \leq 0.001$
Cinnamaldehyde		
4th Instar	$H = 19.31$, $df = 4$, $p \leq 0.001$	$H = 29.93$, $df = 4$, $p \leq 0.001$
5th Instar	$H = 17.64$, $df = 4$, $p \leq 0.001$	$H = 22.06$, $df = 4$, $p \leq 0.001$
Female	$H = 143.35$, $df = 4$, $p \leq 0.001$	$H = 196.98$, $df = 4$, $p \leq 0.001$
Male	$H = 43.73$, $df = 4$, $p \leq 0.001$	$H = 81.20$, $df = 4$, $p \leq 0.001$
Citronellal		
4th Instar	$H = 11.65$, $df = 4$, $p \leq 0.001$	$H = 28.62$, $df = 4$, $p \leq 0.001$
5th Instar	$H = 54.32$, $df = 4$, $p \leq 0.001$	$H = 66.60$, $df = 4$, $p \leq 0.001$
Female	$H = 84.58$, $df = 4$, $p \leq 0.001$	$H = 181.19$, $df = 4$, $p \leq 0.001$
Male	$H = 42.95$, $df = 4$, $p \leq 0.001$	$H = 73.19$, $df = 4$, $p \leq 0.001$

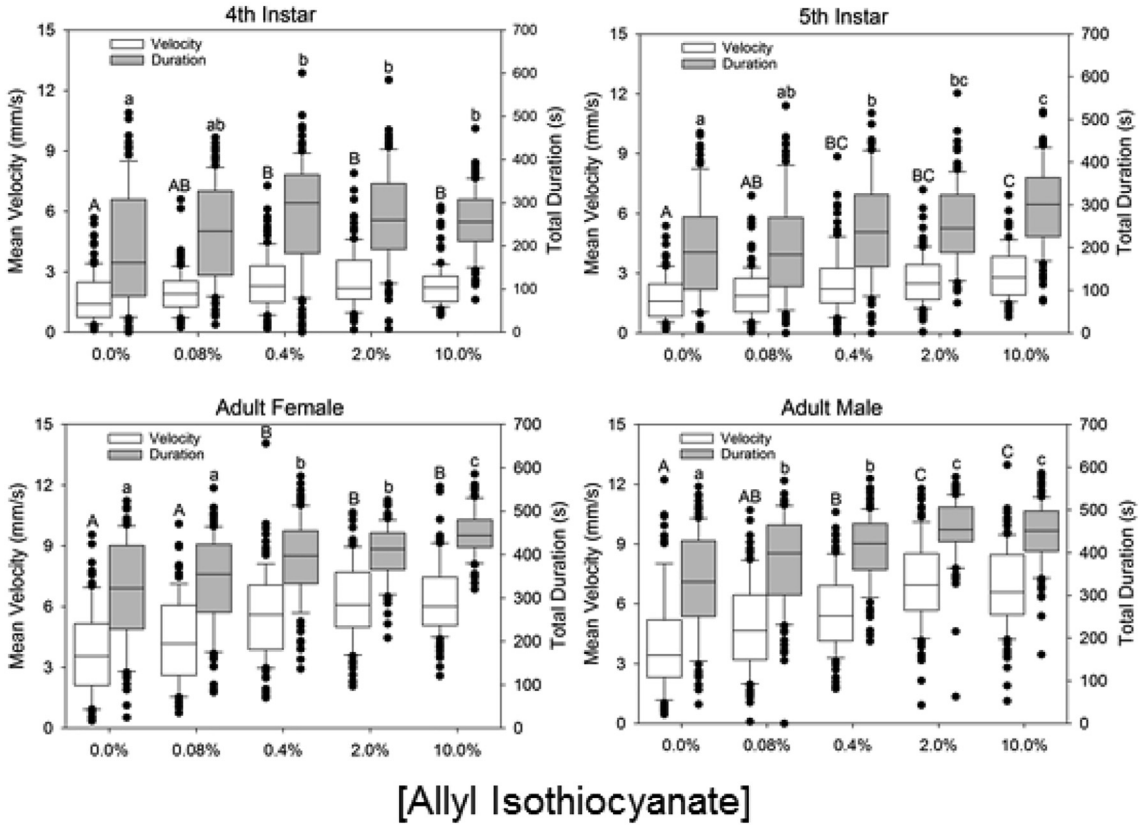

Fig. 1. Effects of differing allyl isothiocyanate concentrations on the movement of *L. hesperus*. The mean velocities and total durations of movement are summarized for 4th and 5th instar nymphs of both sexes, and female and male adults at 7 d post-eclosion (n = 96). Presented are the medians, interquartile ranges, 90th and 10th percentiles (whiskers), and any outlier data points (●) exceeding the outer bounds. Significant differences (Kruskal-Wallis ANOVA on ranks followed by Dunn's post hoc analysis, $\alpha = 0.05$) between groups are indicated by different letters over the boxes, with upper-case used for velocity, and lower-case for duration.

Ten 7 d old adults were introduced to the center of the arena, between zones B and C. After 15 min, the number of insects in each chamber section was recorded. For each of the three odorants, and for each sex, 36 trials were run. After each trial the chamber was cleaned thoroughly, and its orientation was flipped to reduce any positional effects on movement. The null hypothesis that *L. hesperus* would exhibit an equal distribution across every zone regardless of odorant exposure was tested using Kruskal-Wallis ANOVA on ranks and a Tukey test for comparing among zones when significance was indicated.

2.3. Bioinformatics

To identify the *L. hesperus* TRPA1 (*LhesTRPA1*) homolog, previously assembled transcriptomes (Hull et al., 2014; Tassone et al., 2016) were queried using tBLASTn (*e* value $\leq 10^{-5}$) with TRPA1 sequences from *Drosophila melanogaster* (AEU17952), *Apolygus lucorum* (Fu et al., 2016), *Acyrthosiphon pisum* (XP_00194450), and *Tribolium castaneum* (EFA01253). The longest *L. hesperus* variant identified was re-evaluated against the NCBI nr database with BLASTx, and the conceptually translated sequence was scanned for defined protein motifs using ScanProsite (Sigrist et al., 2010) and the HMMscan module on the HMMER webserver (Finn et al., 2011). Transmembrane domain predictions were performed using TOPCONS (Bernsel et al., 2009). To examine phylogenetic relationships, a multiple sequence alignment consisting of the putative *L. hesperus* TRPA1 (LhTRPA1), four *A. lucorum* TRPA1 variants, and TRP-like sequences from *D. melanogaster* and *T. castaneum* (accession numbers listed in Table S1) was generated using default settings for MUSCLE (Edgar, 2004) in Geneious 10.1.3 (Biomatters Ltd., Auckland, New Zealand). Phylogenetic inferences were constructed in MEGA X (Kumar et al., 2018) using the maximum likelihood method with the Le and Gascuel model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (2 categories: +G, parameter = 2.8670) with the analysis incorporating 33 amino acid sequences. All positions with less than 95% site coverage were eliminated, *i.e.*, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option) with the final dataset consisting of 383 positions.

2.4. LhesTRPA1 cloning and sequencing

Total RNAs were isolated from pooled female heads at 7–9 days post-eclosion using TRI Reagent (Life Technologies, Carlsbad, CA) and RNeasy Mini Kit Spin Columns (Qiagen, Germantown, MD). First-strand complementary DNAs (cDNAs) were generated from 500 ng DNase I-treated total RNA using Superscript III Reverse Transcriptase (Life Technologies) and custom made random pentadecamers (IDT, San Diego, CA). The full-length *LhesTRPA1* open reading frame (ORF) was PCR amplified using primers (Table S2) designed to the predicted start and stop sites in conjunction with Sapphire Amp Fast PCR Master Mix (Takara Bio USA Inc., Mountain View, CA). PCR was performed on a Biometra TRIO multiblock thermocycler (Biometra, Göttingen, Germany) in a 30-µl reaction volume containing 0.5 µl cDNA template and 0.13 µM of each primer. The thermocycler conditions consisted of: 95 °C for 2 min followed by 40 cycles at 95 °C for 20 s, 55 °C for 20 s, 72 °C for 2.5 min, and a final extension at 72 °C for 7 min. The resulting product was separated on a 1% agarose gel using a tris/acetate/EDTA

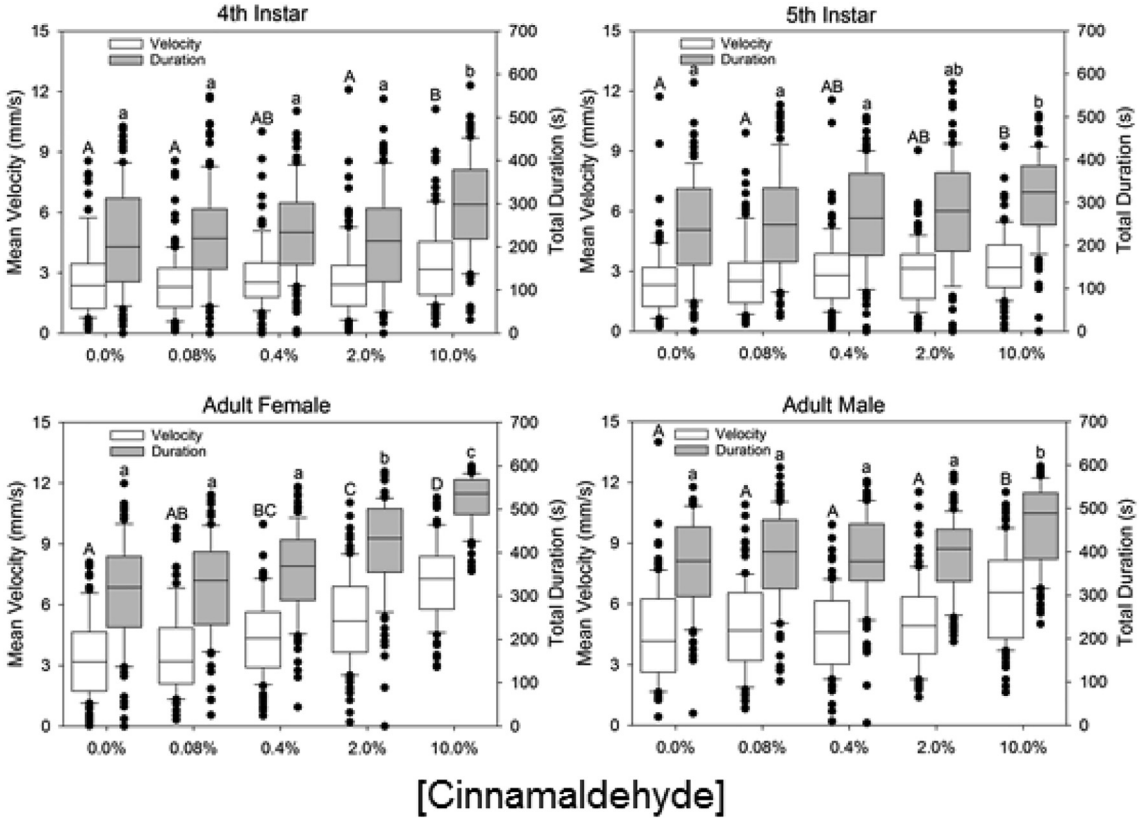

Fig. 2. Effects of differing cinnamaldehyde concentrations on the movement of *L. hesperus*. The mean velocities and total durations of movement are summarized for 4th and 5th instar nymphs of both sexes, and female and male adults at 7 d post-eclosion (n = 96). Presented are the medians, interquartile ranges, 90th and 10th percentiles (whiskers), and any outlier data points (●) exceeding the outer bounds. Significant differences (Kruskal-Wallis ANOVA on ranks followed by Dunn's post hoc analysis, $\alpha = 0.05$) between groups are indicated by different letters over the boxes, with upper-case used for velocity, and lower-case for duration.

buffer system and visualized with SYBR Safe (Life Technologies). The reaction was subcloned into pCR2.1-TOPO TA (Life Technologies) and sequenced at the Arizona State University DNA Core Laboratory (Tempe, AZ). A consensus sequence for the longest variant has been deposited with GenBank under accession number MN230873.

2.5. RT-PCR expression profiling

Total RNAs were isolated as above from two biological replicates of pooled eggs, nymphs (1st-5th instar) and mixed sex adults (0 and 7 d post-eclosion) as well as tissues from 7 to 9 d mixed sex adults including, bodies, heads, and chemosensory-associated tissues (antennae, proboscises, legs, and cuticle). cDNAs were prepared from 500 ng DNase I-treated total RNAs using Superscript III Reverse Transcriptase as above. Each of the cDNAs was PCR-screened for *LhTRPA1* transcript expression using primers (Table S2) designed to amplify a 481-bp fragment (nt 685–1166). A 501-bp fragment of *L. hesperus* actin (GBHO01044314.1) was likewise amplified. PCR was performed in a 20-µl reaction volume using 1 µl cDNA template and 0.2 µM of each primer pair with Sapphire Amp Fast PCR Master Mix and a Biometra TRIO multiblock thermal cycler with cycling conditions consisting of: 95 °C for 2 min followed by 37 cycles at 95 °C for 20 s, 56 °C for 20 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were separated on 1.5% agarose gels and visualized as before. Gel images were obtained using an AlphaImager Gel Documentation System (ProteinSimple, San Jose, CA) and then processed in Photoshop CS6 v13.0 (Adobe Systems Inc., San Jose, CA).

2.6. RNAi-mediated knockdown

The complete 687-bp ORF of Venus (a yellow fluorescent protein variant) and ~500-bp fragments of *LhesOrco* (nt 802–1292) and *LhesTRPA1* (nt 1306–1806) were amplified from validated plasmid DNAs using primers (Table S2) containing a 5′ T7 promoter sequence and Sapphire Amp Fast PCR Master Mix. Thermocycler conditions consisted of 95 °C for 2 min followed by 35 cycles at 95 °C for 20 s, 61°C for 20 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The resulting PCR products were gel purified with an EZNA gel extraction kit (Omega Bio-Tek, Norcross, GA), and then used as templates to generate double-stranded RNA (dsRNA) with a MEGAscript RNAi kit (Life Technologies) according to the manufacturer's instructions. Purified dsRNAs were quantitated on a Synergy H4 hybrid multi-mode microplate reader (BioTek Instruments, Winooski, VT), and then diluted to 1 µg/µl in RNase-free water. Following cold immobilization on ice for 5 min, newly emerged adult females were injected between the 5th and 7th abdominal tergites with 500 nl of 1 µg/µl dsRNAs using a Nanoject III programmable nanoliter injector (Drummond Scientific Company, Broomall, PA) as described previously (Brent and Hull, 2019). It was assumed that male responses to the odorants would be mediated by the same mechanisms as for females, so only the latter sex was tested. Injected females were allowed to recover for 10 min, and then maintained under normal rearing conditions. Those failing to recover were discarded. Treatment groups consisted of 40 per dsRNA set, with three replicates of each. A fourth group consisting of non-injected females from the same cohort was established at the same time. Groups were housed in rearing conditions described above and allowed to mature to seven days post-injection. To examine the potential involvement of *LhesOrco* or *LhesTRPA1* in the noxious compound behavioral response, females were subjected to the arena assay described above and exposed to 2% citronellal, a concentration shown in the earlier assays to promote movement. Neither AITC or CA were tested because TRPA1 is likely to mediate responses to all three compounds

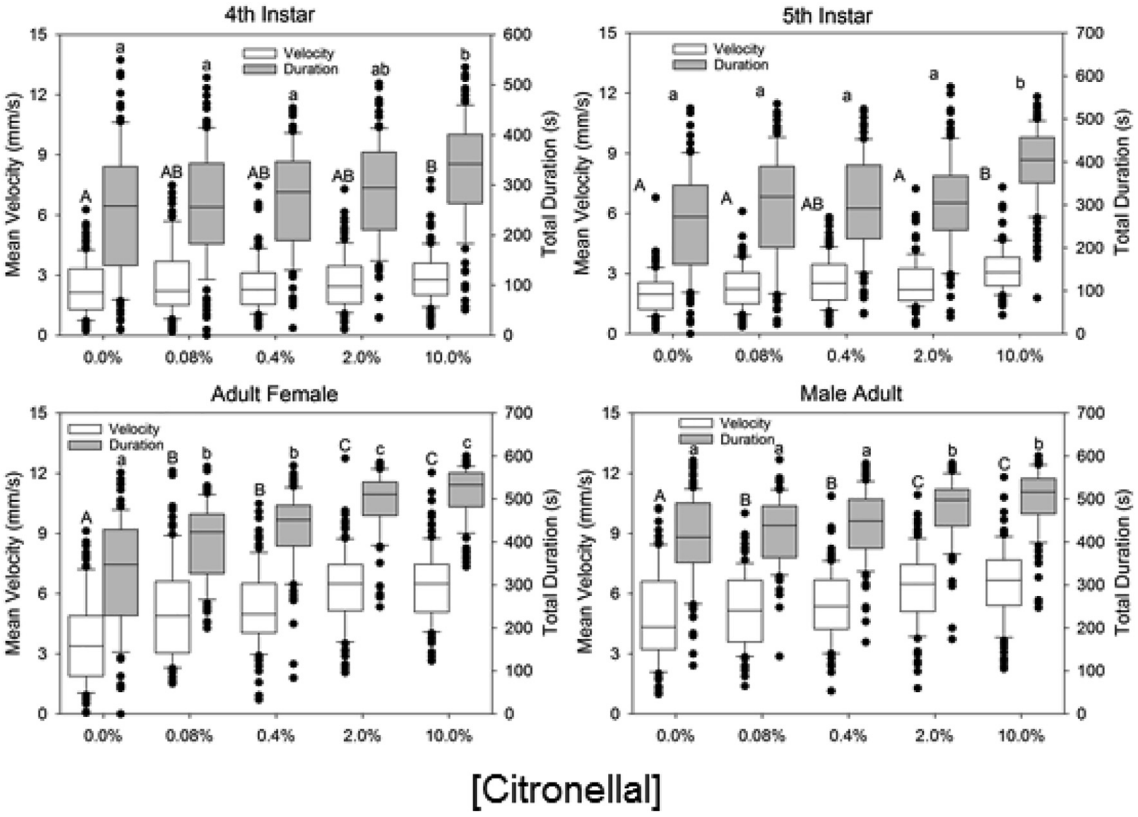

Fig. 3. Effects of differing citronellal concentrations on the movement of *L. hesperus*. The mean velocities and total durations of movement are summarized for 4th and 5th instar nymphs of both sexes, and female and male adults at 7 d post-eclosion (n = 96). Presented are the medians, interquartile ranges, 90th and 10th percentiles (whiskers), and any outlier data points (●) exceeding the outer bounds. Significant differences (Kruskal-Wallis ANOVA on ranks followed by Dunn's post hoc analysis, $\alpha = 0.05$) between groups are indicated by different letters over the boxes, with upper-case used for velocity, and lower-case for duration.

Table 2
Results of Kruskal-Wallis ANOVAs on ranks for distribution of *L. hesperus* in an arena of four tandem zones, with one zone containing volatilizing allyl isothiocyanate (AITC), cinnamaldehyde (CA), or citronellal.

	Female	Male
AITC	H = 67.25, df = 3, p ≤ 0.001	H = 73.56, df = 3, p ≤ 0.001
CA	H = 57.94, df = 3, p ≤ 0.001	H = 61.01, df = 3, p ≤ 0.001
Citronellal	H = 48.76, df = 3, p ≤ 0.001	H = 58.37, df = 3, p ≤ 0.001

(Kang et al., 2011; Kwon et al., 2010; Du et al., 2015). Across replicates, a total of 15 runs were made, resulting in 90 individuals tested per treatment combination. For instances in which the camera and software were unable to track movement properly, or in which the individuals failed to move, data was discarded, resulting in sample sizes of 80–88 per treatment. The null hypothesis that *L. hesperus* injected with dsRNAs targeting *LhesOrco* or *LhesTRPA1* showed no difference in the velocity or duration of movement relative to non-injected or *Venus* dsRNA-injected controls was analyzed using Kruskal-Wallis ANOVA on ranks and a Tukey test for multiple comparisons when the ANOVA indicated significance.

2.7. Quantitative real-time PCR confirmation of transcript knockdown

At two days post-injection, target transcript levels in 10 Lygus from each treatment group were individually assayed by quantitative real-time PCR (qRT-PCR). cDNAs were generated using total RNA isolated from individual whole Lygus bodies. Primers (Table S2) were designed to amplify fragments of *LhesOrco* (nt 356–478), *LhesTRPA1* (nt 2448–2568), and *L. hesperus actin* (nt 683–787). Primer efficiencies were determined using a 5-log dilution series of linearized plasmid DNAs (*LhesOrco* – 94%, $R^2 = 0.999$; *LhesTRPA1* – 87%, $R^2 = 0.999$) or whole body cDNAs (*actin* – 92%, $R^2 = 0.999$). qRT-PCR was performed on a BioRad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using a KAPA SYBR FAST qPCR kit (Kapa Biosystems Inc., Woburn, MA) in 20 µl reaction volumes containing 1 µl cDNA template and 0.125 µM of each primer pair. Samples were assayed in triplicate with thermocycler conditions consisting of: 95 °C for 3 min followed by 40 cycles at 95 °C for 5 s and 63 °C for 10 s with a final melt curve analysis (95 °C for 10 s followed by 65 °C for 5 s which then ramped at 0.5 °C per 5 s to 95 °C) to assess reaction specificity. Each run included three no-template control replicates in which RNase-free water was substituted for the cDNA template. qRT-PCR results were analyzed via the ΔΔCq method (Livak and Schmittgen, 2001) in CFX Maestro v1.1 (Bio-Rad Laboratories) with primer efficiencies incorporated and statistical significance ($p < 0.5$) calculated using ANOVA and Tukey HSD post-hoc analysis.

3. Results

3.1. Response to noxious odorants

All three of the tested odorants produced responses consistent with their being found noxious to Lygus. Compared to the DMSO control exposure, the odorants induced significant concentration-dependent increases in velocity and duration (Table 1), however, sensitivity varied by chemical and test group. For allyl isothiocyanate (AITC), the response threshold was low at 0.4% for nymphs and adult females, and 0.08% for males (Fig. 1). A concentration of 10% cinnamaldehyde (CA) was needed to evoke a significant response in nymphs and adult males, but adult females reacted to concentrations of 0.4% (Fig. 2). Similarly, citronellal enhanced movement in nymphs exposed to 10% solutions,

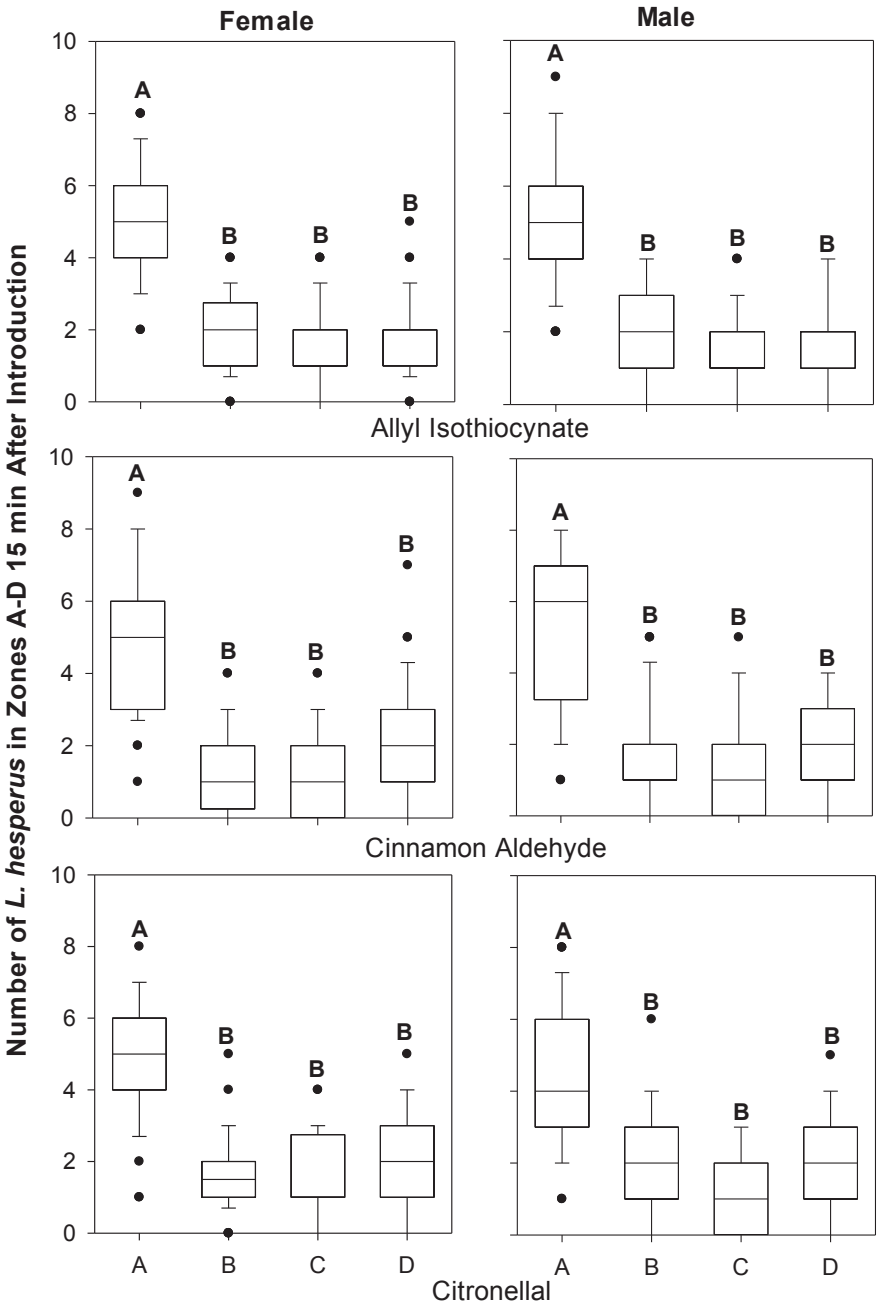

Fig. 4. The effect of three noxious compounds on the positioning of *L. hesperus* female and male adults. Ten insects were introduced into the center of an arena with four tandem zones of equal size (A-D) then exposed to volatilizing DMSO in zone A, and to allyl isothiocyanate, cinnamaldehyde, or citronellal located in zone D. After 15 min introduction, the number in each zone were counter for each of 36 trials conducted for each permutation. Presented are the medians, interquartile ranges, 90th and 10th percentiles (whiskers), and any outlier data points (●) exceeding the outer bounds. Significant differences (Kruskal-Wallis ANOVA on ranks followed by Tukey's post hoc analysis, $\alpha = 0.05$) between groups are indicated by different letters over the boxes.

while adults of both sexes responded to concentrations of just 0.08% (Fig. 3).

The movement responses of females and males to these odorants appear to be driven by aversion, creating significantly uneven distributions in the linear arenas (Table 2). Both sexes consistently gathered in greater numbers in the zone (A) furthest from the tested compounds (Fig. 4), despite zone A containing a disk saturated in DMSO. The numbers in zone D were equivalent to the low counts observed in zones B and C, despite the fact that Lygus typically congregates in arena corners (personal observation).

3.2. *LhesTRPA1 identification*

TRPA1 and/or Orco functionalities mediate the detection of AITC, CA and citronellal in a number of insect species (Kang et al., 2011; Kwon et al., 2010; Zhao et al., 2010; Yi et al., 2014; Du et al., 2015). In *L. hesperus*, the *Orco* homolog has been identified and its expression profile characterized (Hull et al., 2012). In contrast, the Lygus *TRPA1* has yet to be cloned. To identify this homolog, we searched current *L. hesperus* transcriptomic datasets (Hull et al., 2014; Tassone et al., 2016) for sequences exhibiting similarity with known TRPA1 proteins from *D. melanogaster*, *A. lucorum*, *A. pisum*, and *T. castaneum*. The longest sequence identified exhibited significant similarity with transcripts annotated as transient receptor potential cation channel subfamily A members (Table 3). Consistent with most TRP-like proteins, the Lygus sequence (LhesTRPA1) is predicted to contain multiple N-terminal ankyrin repeats and six transmembrane domains (Table 3).

Furthermore, the sequence sorted phylogenetically with the TRPA1 clade rather than with one of the other TRP-like subfamilies (Supplemental Fig. 1). Multiple clones encoding the complete 3747-bp *LhesTRPA1* ORF with > 99% nucleotide identity to the transcriptomic sequence were amplified from adult female *L. hesperus* head cDNAs.

Table 3
Bioinformatics analysis of LhesTRPA1.

TOP5 BLASTx hits Description	Accession No.	E Value	% Identical Sites
transient receptor potential cation channel subfamily A member 1 [*Cimex lectularius*]	XP_024082341.1	0.00E+00	927/1269 (73%)
transient receptor potential cation channel subfamily A member 1 [*Nilaparvata lugens*]	XP_022190720.1	0.00E+00	848/1159 (73%)
TRPA1 [*Nilaparvata lugens*]	AOR81469.1	0.00E+00	837/1128 (74%)
transient receptor potential cation channel subfamily A member 1 isoform X3 [*Cryptotermes secundus*]	XP_023702416.1	0.00E+00	839/1141 (74%)
transient receptor potential cation channel subfamily A member 1 isoform X4 [*Zootermopsis nevadensis*]	XP_021938940.1	0.00E+00	838/1153 (73%)
Motif Analysis			
Motif	Amino Acids	Algorithm	
ankyrin repeat region (PS50297)	120–676	ScanProsite[a]	
ankyrin repeat (PS50088)	154–186	"	
"	187–220	"	
"	256–277	"	
"	331–363	"	
"	402–434	"	
"	509–541	"	
"	542–564	"	
"	578–610	"	
"	610–630	"	
"	644–676	"	
ankyrin 2 (PF12796.7)	125–219	HMMR[b]	
"	361–433	"	
"	536–608	"	
"	602–675	"	
ankyrin (PF00023.3)	256–287	"	
ion transport (PF00520.31)	811–1093	"	
TOPCONS Transmembrane Prediction[c]			
TM residues	TM domains		
791–811, 847–867, 884–904, 913–933, 953–973, 1032–1052	6		

[a] Sigrist et al., 2010.
[b] Finn et al., 2011.
[c] Bernsel et al., 2009.

Although additional *TRPA1* variants with N-terminal deletions similar to those reported in *A. lucorum* (Fu et al., 2016) were identified in the transcriptomic database, those sequences were 5′ fragments that lacked stop codons.

3.3. LhesTRPA1 transcript expression

Because noxious odorant-induced movement was observed in both nymphal and adult stages (Figs. 1–3), *LhesTRPA1* expression was examined across Lygus development. Similar to *LhesOrco* (Hull et al., 2012), the *LhesTRPA1* transcript was amplified from all five nymphal instars as well as newly emerged and reproductively mature adults (Fig. 5A). In addition, the expression of *LhesTRPA1* in tissues and appendages typically associated with chemosensation (*i.e.* antenna, proboscis, leg, and cuticle) supports a role in noxious compound detection (Fig. 5B). *LhesOrco* was likewise amplified from chemosensory tissue cDNAs (Hull et al, 2012).

3.4. RNAi-mediated knockdown of LhesTRPA1 and LhesOrco

To examine the functional role of *LhesTRPA1* and *LhesOrco* in noxious odorant detection, we injected dsRNAs corresponding to ~500-bp fragments of the respective coding sequences into newly emerged female adults. Control females were injected with dsRNA corresponding to the complete coding sequence of the fluorescent protein Venus. Reduced target transcript levels were observed in adults injected with *LhesOrco* (Fig. 6A) and *LhesTRPA1* (Fig. 6B) dsRNAs, but only the latter treatment produced a noticeable behavioural change (Fig. 7). Relative to the non-injected and *Venus* dsRNA injected controls, females injected with *TRPA1* dsRNAs had lower movement velocity and duration when exposed to 2% citronellal, indicating reduced responsiveness.

4. Discussion

Although the role of chemosensory systems in detecting noxious plant-derived compounds that trigger avoidance behaviors (*i.e.* motivated movement) has been elucidated in a number of insect species, our understanding of these behaviors and the odorants that activate them is limited in *L. hesperus*. Using movement-based assays, we found that AITC (an organosulfur compound responsible for the pungency of mustard and wasabi), CA (a phenylpropanoid that is a major component of cinnamon bark oil), and citronellal (a monoterpenoid that is the main component of citronella oil) all induced significant movement in *L. hesperus* regardless of developmental stage or sex (Figs. 1–3), and the movement appeared to be due to aversion (Fig. 4). Similar chemotactic effects have been reported for these compounds in insects of multiple orders; however, responses are frequently species-dependent with considerable variation in the degree of sensitivity shown for each compound and the behavior triggered.

Among hemipteran pests, AITC has been reported to be an attractant for *Nysius raphanus* and *Murgantia histrionica*, but a repellent for *Aphis fabae* (Demirel and Cranshaw, 2006; Nottingham et al., 1991; Thrift et al., 2018). This variation might reflect ecological adaptation of the species as both *N. raphanus* and *M. histrionica* feed on brassicaceous crops in which AITC occurs naturally. The host plant range of mirid plant bugs includes the Brassicaceae (Young 1986), with *L. hesperus* pest activity reported in *Brassica* oilseed rape (Butts and Lamb 1990, 1991). The pronounced effects of AITC exposure on *L. hesperus* movement in nymphs and adults of both sexes (Figs. 1, 4), however, suggests the compound can act as a chemorepellent rather than a chemoattractant, at least at the relatively high concentrations tested here. Surprisingly, AITC had minimal effects on *A. lucorum* movement (Fu et al., 2016).

As with AITC, CA is present in the floral blends (Knudsen et al., 2006) of some Lygus host plants (Young 1986) and has been reported to be attractive to *Lygus rugulipennis* (Koczor et al., 2012). Conversely, CA

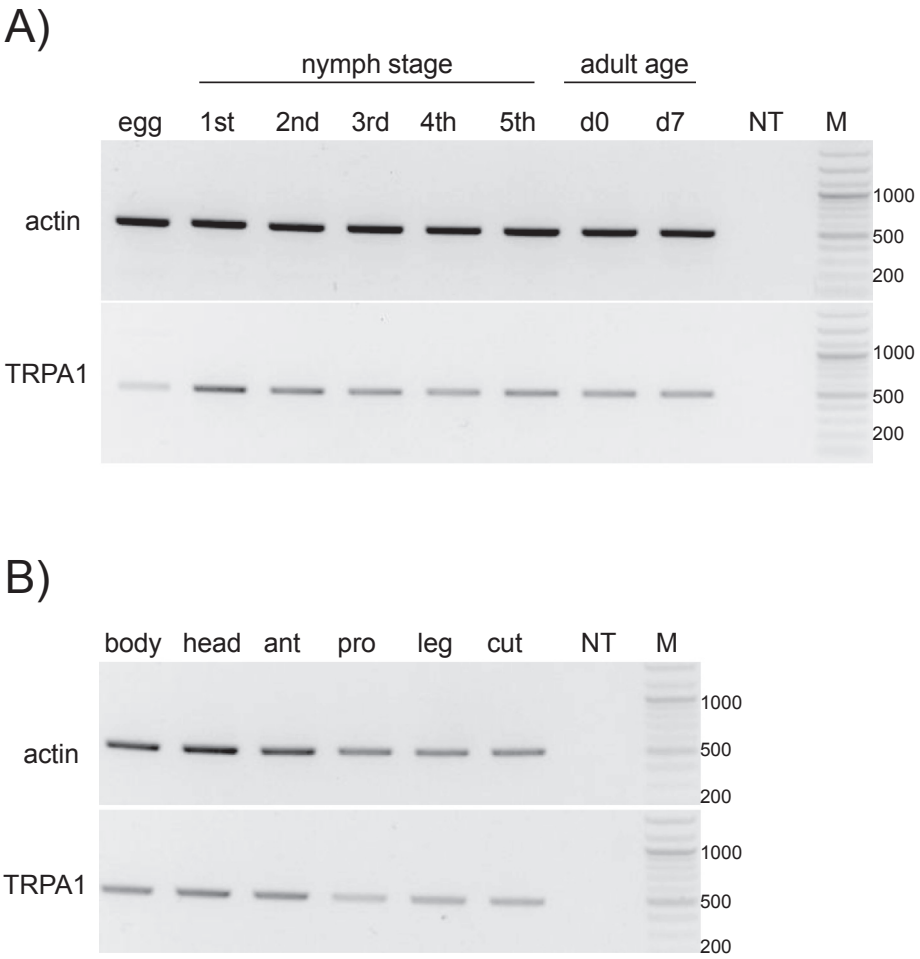

Fig. 5. *LhesTRPA1* transcript expression profile. Endpoint RT-PCR amplification using cDNAs spanning *L. hesperus* development (A) as well as from adult chemosensory-associated tissues/appendages (B). Amplimers correspond to ~500-bp fragments of the transcripts of interest. Abbreviations: *upper panel* - d0, newly emerged adult; d7, 7d old reproductively mature adult; NT, no template; M, DNA ladder; *lower panel* - ant, antennae; pro, proboscis; cut, cuticle; NT, no template; M, DNA ladder.

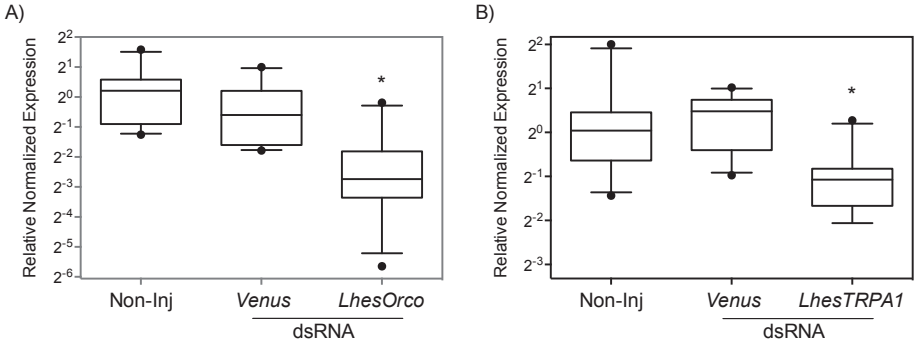

Fig. 6. qPCR confirmation of transcript knockdown. Relative normalized transcript expression of *LhesOrco* (A) and *LhesTRPA1* (B) in *L. hesperus* adults at 2 days post-dsRNA injection. Transcript levels were normalized to *actin*. Data represent the medians, interquartile ranges, 90th and 10th percentiles (whiskers), and any outlier data points (●) exceeding the outer bounds. Values were generated from 10 individual Lygus per injection group assayed in triplicate. Statistical analysis performed by ANOVA followed by Tukey post hoc analysis. Asterisks indicate significant differences at $p < 0.05$.

promoted avoidance behavior in *A. lucorum* (Fu et al., 2016) and induced significant movement in *L. hesperus* regardless of developmental stage or sex (Fig. 2). Despite the literature-based evidence that might suggest a chemoattractant role for CA, Lygus movements in our assay were more reflective of repellence than attraction as contact with and even proximity to the cinnamaldehyde infused filter disc was avoided. The chemorepellency of CA is not unique to our assay as similar effects have been reported for *Bemisia tabaci* (Deletre et al., 2015, 2016), *Megalurothrips sjostedti* (Abtew et al., 2015), and *Sitophilus zeamais* (Zaio et al., 2018). However, it is possible that *L. hesperus* might be attracted by much lower concentrations of the odorant.

The citronellal-induced movements observed for *L. hesperus* (Figs. 3, 4) are consistent with the avoidance response in *A. lucorum* (Fu et al., 2016). This effect differs from that reported for a number of other hemipteran pests including *Bemisia tabaci*, *Bagrada hilaris*, *Triatoma rubida*, *Triatoma protracta*, and *Triatoma recurve*, all of which exhibited little to no response to citronellal (Deletre et al., 2016; Joseph 2017; Zamora et al., 2015).

The detection of diverse noxious compounds has been linked to TRPA1 (Fowler and Montell, 2013) and, in some species, Orco (Kwon et al., 2010; Yi et al., 2014; Zhao et al., 2010). To examine the potential role of these two proteins in odorant-induced *L. hesperus* movement, we

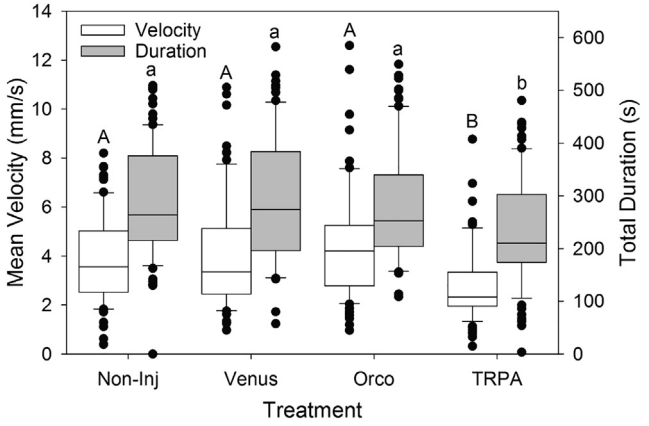

Fig. 7. Effects of RNAi-mediated knockdown on the movement of *L. hesperus* in response to 2% citronellal. Females injected with *LhesTRPA1* dsRNA (n = 79) exhibited diminished movement velocity and duration relative to uninjected females (n = 81) or females injected with either *Venus* dsRNA (n = 88) or *LhesOrco* dsRNA (n = 80). Presented are the medians, interquartile ranges, 90th and 10th percentiles (whiskers), and any outlier data points (●) exceeding the outer bounds. Significant differences (Kruskal-Wallis ANOVA on ranks followed by Dunn's post hoc analysis, α = 0.05) between groups are indicated by different letters over the boxes, with upper-case used for velocity, and lower-case for duration.

used transcriptomic data to facilitate identification and cloning of the putative *LhesTRPA1* homolog and extended previous characterization of the *LhesOrco* homolog (Hull et al., 2012). Consistent with olfactory detection of noxious volatiles, *LhesTRPA1* transcripts are expressed in chemosensory-associated tissues, with highest expression in antennae (Fig. 4B). Similar antennal-dominant expression of TRPA1 has been reported for other insect species including, *A. lucorum* (Fu et al., 2016), *Helicoverpa armigera* (Wei et al., 2015), *Bactrocera dorsalis* (Su et al., 2018), *Solenopsis invicta* (Wang et al., 2018), and *Anopheles gambiae* (Wang et al., 2009). The non-olfactory expression of *LhesTRPA1* likely reflects additional sensory modalities (gustatory discrimination of aversive compounds, detection of UV via H_2O_2 reactivity, temperature and mechanical sensation) associated with different isoforms of TRPA1 (Kang et al., 2011; Fowler and Montell, 2013; Du et al., 2016, 2019; Guntur et al., 2017). The significant reduction in citronellal-induced movement in adult female *L. hesperus* injected with *LhesTRPA1* dsRNA rather than those injected with *LhesOrco* dsRNA suggests that avoidance behavior activated in response to noxious odorants proceeds via an Orco-independent pathway. Although *in vivo* determination of the role TRPA1 plays in mediating odorant responses is limited, our findings contrast with that reported for a number of other insects. In *D. melanogaster*, dual pathways are required for the citronellal response – a primary Orco-dependent pathway that is directly activated by citronellal and a second pathway in which TRPA1 is activated downstream of a citronellal G protein-coupled receptor (Kwon et al., 2010). The Orco pathway likewise predominates in *B. dorsalis* as the oviposition deterrence effects of citronellal were significantly reduced following RNAi-mediated knockdown of the Orco homolog (Yi et al., 2014). Orco knockdown also disrupted the chemotactic responses of *Phyllotreta striolata* to AITC (Zhao et al., 2010). However, in both *B. dorsalis* and *Phyllotreta striolata*, the role of TRPA1 was not assessed, so that like in *D. melanogaster*, TRPA1 may play some secondary role.

The lack of an effect following Orco knockdown in our assay system could reflect an insufficient reduction in the olfactory receptor, however, qPCR analysis confirmed *LhesOrco* transcript levels were lower following dsRNA injection. Regardless, the data clearly support a role for TRPA1 in citronellal-induced movement of *L. hesperus*. Furthermore, the activation of TRPA1 channels by both AITC and CA (Fu et al., 2016; Kang et al., 2011; Kwon et al., 2010; Wang et al., 2018; Wei et al., 2015) suggests that the behaviors induced by these two odorants in our movement assays are likely also mediated by LhesTRPA1. Differential responsiveness of the dipteran TRPA1(A) and TRPA1(B) variants to citronellal has been reported (Kang et al., 2011; Du et al., 2015) with higher sensitivity in the A variant, which is distinguished by an extended N terminus. Sequence alignments of the LhesTRPA1 sequence presented here with potential variant fragments identified in the *L. hesperus* transcriptomes suggests the cloned sequence has an N-terminal extension similar to the citronellal sensitive dipteran TRPA1(A) variants. However, because the dsRNA injected in our assays targeted a portion of the *LhesTRPA1* shared across the variants we are unable to assign sensitivities to the specific variants. As a consequence, knockdown and the associated phenotypic effects would be variant-independent.

Chemical-induced activation of mammalian TRPA1 channels has been linked to covalent modification of key residues in the N terminus (Cys414, Cys421, Cys621, Cys641, Cys665, and Lys710 in human TRPA1) by AITC and CA (Hinman et al., 2006; Macpherson et al., 2007). These residues, with the exception of Cys421 and Cys694, are well-conserved across most species including LhesTRPA1 (Fig. 8 and Kang et al., 2011). Reduced responses of the *D. melanogaster* TRPA1 to AITC following Ser substitution of Cys621/Cys641 (Cys650/Cys670 in *D. melanogaster*) suggests that the mechanism for sensing reactive electrophiles has been evolutionarily conserved between insects and mammals. The role of the non-conserved residues in chemically-induced activation of insect TRPA1 channels remains to be determined. Surprisingly, mutation of *D. melanogaster* Cys650/Cys670 had no effect on citronellal-based activation of TRPA1 suggesting that the citronellal mechanism is independent of that used in sensing reactive electrophiles (Du et al., 2015). More recently, allosteric activation of human TRPA1 by CA has been linked to five ankyrin repeats in the N terminus that contain a Thr/Ser-Pro-Leu-His tetrapeptide motif (Hynkova et al., 2016). Four of the five are conserved in the LhesTRPA1 and DmelTRPA1 sequences, but their functional importance in channel activation outside of mammalian models remains to be determined.

The divergent responses of the TRPA1 channel to chemical activation across insect species (Du et al., 2015; Wang et al., 2018) and in

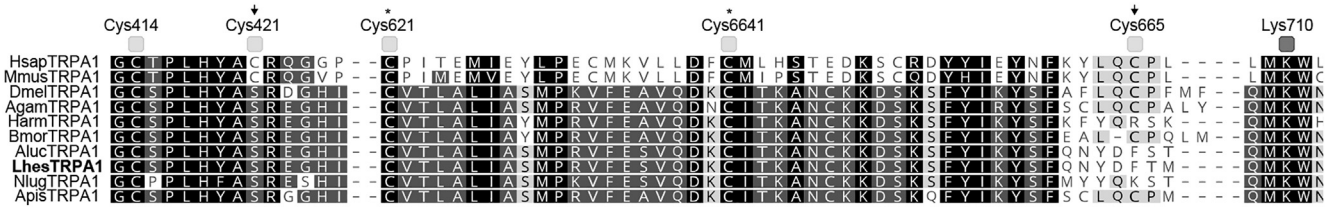

Fig. 8. Evolutionary conservation of residues critical to reactive electrophile-induced activation of TRPA1 channels. Electrophile reactive residues in mammalian TRPA1 channels are indicated. Cys numbering is based on human TRPA1. Poorly conserved Cys residues are marked with an arrowhead. Cys residues critical for AITC activation of *D. melanogaster* TRPA1 are indicated by the asterisks. The *Lygus hesperus* TRPA1 (LhesTRPA1) sequence is indicated in bold font. TRPA1 species abbreviations and accession numbers are: HsapTRPA1 (*Homo sapiens*, XP_011515926.1), MmusTRPA1 (*Mus musculus*, EDL14332.1), DmelTRPA1 (*Drosophila melanogaster*, NP_648263.5), AgamTRPA1 (*Anopheles gambiae*, ACC86138.1), HarmTRPA1 (*Helicoverpa armigera*, AHV83756.1), BmorTRPA1 (*Bombyx mori*, XP_004932880.1), AlucTRPA1 (*Apolygus lucorum*, Fu et al. 2016), NlugTRPA1 (*Nilaparvata lugens*, AOR81469.1), ApisTRPA1 (*Acyrthosiphon pisum*, XP_001944501.2).

relation to mammalian channels (Du et al., 2015) have stirred interest in exploring the channels as potential targets for new chemistries and repellents (Kim 2013; Nagata 2007; Salgado 2017). Our demonstration of the functional role that LhesTRPA1 plays in mediating noxious odorant responses lays the foundation for additional studies to assess the suitability of targeting the protein in Lygus management.

CRediT authorship contribution statement

J. Joe Hull: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Supervision. **Yu-Wen Yang:** Investigation. **Katelyn Miyasaki:** Investigation. **Colin S. Brent:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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