Journal of Medical Entomology, 2017, 1–7
doi: 10.1093/jme/tjx059
Research Article

Chemosensory Responses to the Repellent *Nepeta* Essential Oil and Its Major Component Nepetalactone by *Aedes aegypti* (Diptera: Culicidae), a Vector of Zika Virus

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Subject Editor: Patricia Scaraffia

Received 31 August 2016; Editorial decision 28 February 2017

Abstract

Nepeta essential oil (*N*eo; catnip) and its major component, nepetalactone, have long been known to repel insects including mosquitoes. However, the neural mechanisms through which these repellents are detected by mosquitoes, including the yellow fever mosquito *Aedes aegypti* (L.), an important vector of Zika virus, were poorly understood. Here we show that *N*eo volatiles activate olfactory receptor neurons within the basiconic sensilla on the maxillary palps of female *Ae. aegypti*. A gustatory receptor neuron sensitive to the feeding deterrent quinine and housed within sensilla on the labella of females was activated by both *N*eo and nepetalactone. Activity of a second gustatory receptor neuron sensitive to the feeding stimulant sucrose was suppressed by both repellents. Our results provide neural pathways for the reported spatial repellency and feeding deterrence of these repellents. A better understanding of the neural input through which female mosquitoes make decisions to feed will facilitate design of new repellents and management strategies involving their use.

Key words: *Nepeta*, catnip, taste, olfaction, mosquito

The yellowfever mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) is an important vector of human disease agents including yellow fever, chikungunya, dengue, and Zika viruses (Monath 1994, Burt et al. 2012, Bhatt et al. 2013, Gatherer and Kohl 2016). Numerous methods are utilized to control mosquitoes including insect repellents which exert their effects by decreasing contacts between the mosquito vector and its host (Dickens and Bohbot 2013, Debboun et al. 2015). Spatial repellents have their effects at some distance from the host and are detected by olfactory receptor neurons (ORNs) housed in sensilla mainly on the antennae or maxillary palps. Contact repellents or feeding deterrents have their effects at close range and are detected by gustatory receptor neurons (GRNs), most of which are present on various appendages including the legs and mouthparts (Sanford et al. 2013; Sparks and Dickens 2016a,b).

Mosquito repellents may be man-made synthetic compounds, e.g., DEET (*N*,*N*-diethyl-3-methylbenzamide), picaridin [2-(2-hydroxyethyl)-1-piperidine carboxylic acid 1-methylpropyl ester], and IR3535 [3-(*N*-butyl-*N*-acetyl)-aminoproprionic acid ethyl ester] (Debboun et al. 2015), or naturally occurring compounds including *p*-menthane-3,8-diol (PMD) (Trigg 1996), geraniol (Weldon et al. 2011), and 2-undecanone (Barton 2003, Roe 2004). Both synthetic repellents and naturally occurring compounds may act as spatial repellents or feeding deterrents (Debboun et al. 2015).

Nepeta essential oil (catnip, herein *N*eo) and its major component, nepetalactone, have long been known to repel insects (Eisner 1964). The presence of nepetalactone and related compounds in plants was thought to be involved in protecting plants from insect feeding. More recently, *N*eo and nepetalactone were shown to repel mosquitoes including *Ae. aegypti* from a distance and deter feeding (Bernier et al. 2005, Chauhan et al. 2005). Subsequently, an ORN housed within sbtl1 sensilla on the antennae of *Ae. aegypti* females was shown to respond to nepetalactone, thus potentially providing a channel for its

detection (Ghaninia et al. 2008) and a mechanism for its spatial repellency. However, the mechanism by which *N*eo and nepetalactone deter feeding remains unknown and the possibility that other ORNs may be involved in their detection has not been investigated.

Here we investigate the possibility that ORNs housed within the capitate basiconic sensilla on the palps of female *Ae. aegypti* detect *N*eo, and examine detection of *N*eo and nepetalactone by GRNs on the labella as a mechanism for their feeding deterrent effects.

Materials and Methods

Insects

Aedes aegypti were reared from eggs obtained from the Center for Medical and Veterinary Entomology, USDA, ARS, in Gainesville, FL. Larvae were fed ground Tetramin fish food while held in an environmental chamber under a photoperiod of 12:12 (L:D) h at 27 °C. Upon emergence, adults were maintained in an environmental chamber at 27 °C, 70% relative humidity, and fed a 10% sucrose solution. The experimental adults received only water for 20–30 h prior to use.

Gas Chromatography/Mass Spectrometry

Essential oil from *Nepeta rtanjensis* Diklić & Milojević, an endemic and critically endangered plant from Serbia, was used for its high *trans,cis*-nepetalactone (4a-α,7-β,7a-α-nepetalactone synonymous with *E,Z*-nepetalactone and *trans,cis*-nepetalactone) content (Mišić et al. 2015). Qualitative analysis and relative quantification of *N. rtanjensis* essential oil was performed by gas chromatography coupled mass spectrometry (GC/MS). An HP-5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a split–splitless injector and an HP-5 column (25 m by 0.32 mm, 0.53 µm film thickness), was used. Carrier gas flow rate (H_2) was 1 ml min^{-1}, injector temperature 250 °C, and detector temperature 300 °C. Column temperature increased from 40 °C to 260 °C, at rate of 4 °C min^{-1}, and then was held at 260 °C for 10 min. Two microliters of essential oil solution in ethanol (0.2%) was injected in split mode (1:30). Gas chromatography coupled mass spectrometry analysis was performed under the same analytical conditions as GC/FID, using an HP G 1800C Series II GCD system (Hewlett-Packard, Palo Alto, CA) and an HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness). Helium was used as a carrier gas, while the transfer line was heated at 260 °C. Mass spectra were acquired in EI mode (70 eV), in m/z range of 40–450. The constituents were identified by comparison of their mass spectra to those from Wiley275 and NIST/NBS libraries. The experimental values for retention indices were determined using calibrated Automated Mass Spectral Deconvolution and Identification System Software (AMDIS ver. 2.1, National Institute of Standards and Technology [NIST], Standard Reference Data Program, Gaithersburg, MD), compared to those from available literature (Adams 2007), and used as an additional tool to approve MS findings. α-Pinene, β-pinene, and 1,8-cineole identities were also verified using analytical standards (>99%, Symrise GmbH & Co. KG, Holzminden, Germany). For the purpose of relative quantification, area percent reports obtained by FID as a result of standard processing of chromatograms, were used as base for the quantification purposes.

Chemical Stimuli

Nepeta essential oil was isolated from air-dried flowering tops of *N. rtanjensis* by hydrodistillation for 2 h in a Clevenger-type apparatus (Ljaljević-Grbić et al. 2008). *E,Z-* and *Z,E*-nepetalactone, 99%, were derived from *Nepeta cataria* L. (catnip) oil (Chauhan et al. 2005). Racemic 1-octen-3-ol (>98%) was obtained from Fluka Chemical Corp., Milwaukee, WI. Sucrose (>99%) was obtained from Sigma, St. Louis, MO.

Electrophysiology

Olfactory Recordings

Electrical responses from ORNs housed within the capitate basiconic sensilla on the maxillary palps of 5–10-d-old females were recorded using tungsten electrodes made from 125-µm tungsten wire electrolytically sharpened to tip diameters of <1 µm. A female mosquito was immobilized on a glass microscope slide using a small amount stickem and cellophane tape to expose the maxillary palps and allow electrode access to the base of individual sensilla; the indifferent electrode was inserted into the compound eye. Signals were amplified and filtered (bandpass 300 hz to 1,000 hz) with a Grass P15D AC amplifier (Grass Instrument Corp., Quincy, MA). Data were collected, stored and analyzed using a microcomputer equipped with AutoSpike software (Syntech, Kirchzarten, Germany).

Serial dilutions of *N*eo prepared in nanograde hexane were delivered as volatiles emanating from 5-µl aliquots placed on a filter paper strip inserted into a glass odor cartridge. A stream of synthetic air (Ultra Zero Grade; >0.5 ppm CO_2; 665 mls/min) carried molecules over the preparation by switching between a purge stream and a stimulus-laden stream using a Syntech CS-55 Stimulus

Table 1. Chemical composition of *Nepeta* essential oil as revealed by GC/FID/MS analysis

Peak no.	Asignment	RT/MS (min)	RT/FID (min)	% m/m	RRT/FID	CI
1	α-Pinene	6.91	10,769	2,97	0.377	42
2	β-Pinene	8.22	12,342	0,38	0.432	5
3	1,8-Cineole	10.02	14,414	0,25	0.505	4
4	α-Campholenal	13.36	18,144	0,32	0.636	3
5	2-Methoxy-para-cresol	16.47	21,624	1,63	0.757	24
6	4a-α,7-a,7a-α-Nepetalactone	21.32	27,208	15,72	0.953	226
7	a-Copaene	21.66	27,522	0,83	0.964	12
8	4a-α,7-β,7a-α-Nepetalactone	22.49	28,550	69,42	1.000	1000
9	γ-Cadinene	25.81	31,554	0,14	1.105	2
10	δ-Cadinene	26.23	32,480	0,57	1.138	8
11	α-Calacorene	26.82	32,814	0,11	1.149	2
12	α-Cadinol	30.04	36,687	0,14	1.285	2
13	Cis-14-nor-Muurol-5-en-4-one	30.91	37,754	0,29	1.322	3

RT, retention time; % (m/m), percentage of component in EO (mass on mass); RRT, relative retention time; CI, concentration index.

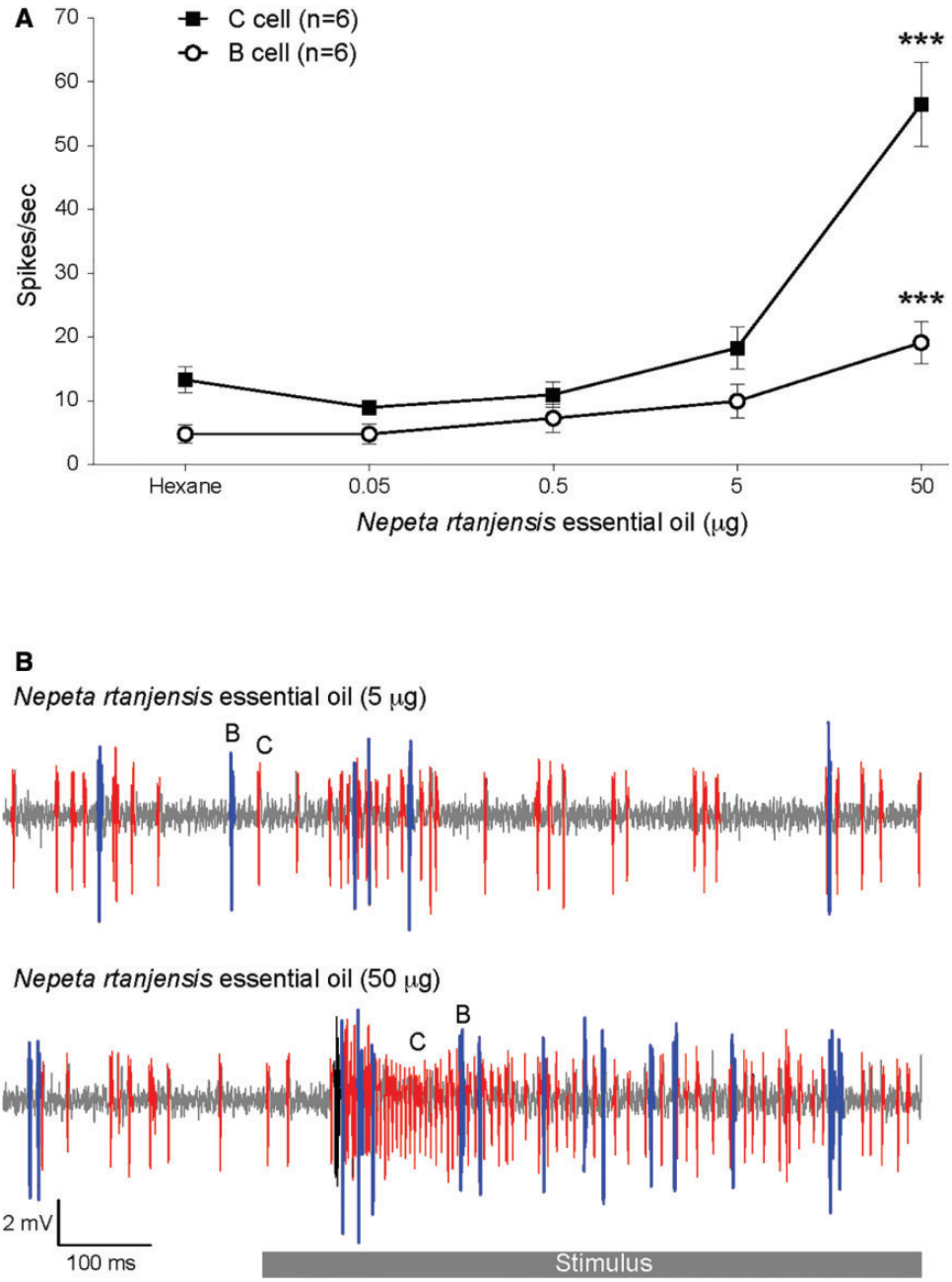

Fig. 1. Recordings from capitate basiconic sensilla on the maxillary palps of *Ae. aegypti* females. (**A**) Dose response curves to serial dilutions of *Nepeta rtanjensis* essential oil (*N*eo) revealed a threshold for significant increase in spikes of both the "C" cell and "B" cell at the 50 μg stimulus load (one-way ANOVA followed by Dunnett's multiple comparison post test, *** $P < 0.001$). Vertical bars represent $\pm$ standard errors. (**B**) Representative recording of electrical activity in response to 5 and 50 μg stimulus loads of *Nepeta rtanjensis* essential oil (*N*eo). Activity of the "B" cell and "C" cell are designated in blue and red, respectively.

Controller (Syntech). Between stimulations the preparation was bathed for 3 min in synthetic air to allow for recovery.

Gustatory Recordings

Electrical activity from GRNs housed within individual sensilla on the labella of 5–7-d-old females was recorded using the "tip recording" technique (Hodgson et al. 1955, Sparks and Dickens 2014). Recordings were from the same long uniporous hairs near the distal end of the labella ($n = 4$ for each stimulus). In brief, narrow strips of cellophane tape were used to immobilize a female on a platform. An indifferent electrode composed of an electrolytically sharpened tungsten wire was inserted into the thorax; the recording electrode was fashioned as a glass capillary containing a silver wire pulled to a tip diameter of 15 μm to allow recording from individual sensilla. The recording electrode contained both an electrolyte (1 mM NaCl) and the experimental chemical being tested. The electrodes were connected to a Taste Probe preamplifier designed for recording from gustatory sensilla in insects (Syntech). Electrical signals acquired and conditioned using an IDAC-4 data acquisition controller were collected and analyzed using a microcomputer equipped with AutoSpike software (Syntech).

Serial dilutions of Neo and nepetalactone were prepared in 1 mM NaCl and 10% ethanol; the organic solvent, herein ethanol,

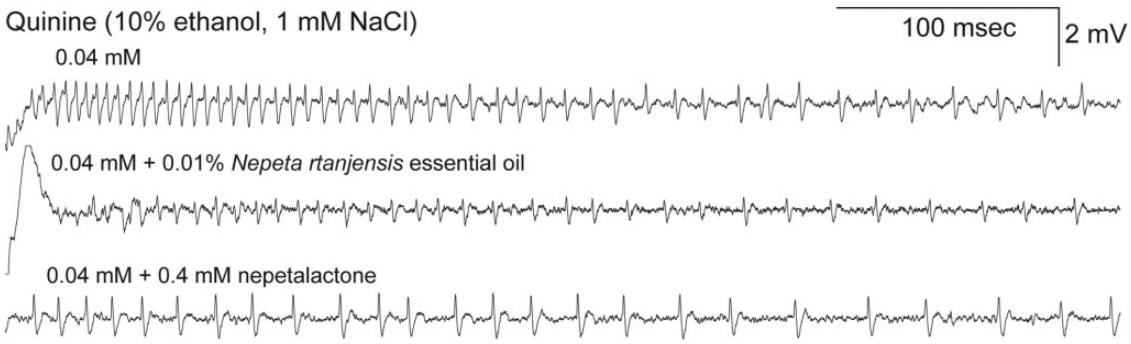

Fig. 2. Recordings of electrical activity from a gustatory sensillum on the labella of *Ae. aegypti* females in response to quinine 0.04 mM alone and quinine 0.04 mM + either 0.001% *Nepeta rtanjensis* essential oil (*N*eo) or 0.4 mM nepetalactone ($n = 4$ for each). Traces start 50 ms following the stimulus artifact and last for 500 ms. Note that a single amplitude spike predominates in responses to quinine alone and quinine + *N*eo or nepetalactone.

facilitated solubilization of the apolar repellents as previously described (Sanford et al. 2013; Sparks and Dickens 2016a,b).

Results

Gas Chromatography/Mass Spectrometry

Gas chromatographic analysis of *N*eo revealed 13 identifiable peaks based on mass spectral data (Table 1). Two major peaks were predominant and comprised 85% of the extract. These two peaks were characterized as isomers of the oxygenated monoterpene nepetalactone: 4a-α,7-β,7a-α-nepetalactone (69.42%) and 4a-α,7-α,7a-α-nepetalactone (15.72%). Minor peaks included monoterpene hydrocarbons—α-pinene (2.97%), β-pinene (0.38%), 1,8-cineole (0.25%); oxygenated monoterpenes—α-campholenal (0.32%), α-copaene (0.83%); sesquiterpene hydrocarbons—δ-cadinene (0.57%), γ-cadinene (0.14%), α-calocorene (0.11%), α-copaene (0.83%); oxygenated sesquiterpenes—α-cadinol (0.14%), *cis*-14-nor-muurol-5-en-4-one (0.29%); and a cresol—2-methoxy-paracresol (1.63%).

Olfactory Receptor Neurons on the Palp Respond to *Neo*, But Only at High Concentrations

Dose response curves constructed from responses to serial dilutions of *N*eo revealed an increase in the number of action potentials elicited from the "C" neuron but only at the highest stimulus load tested (50 µg; Fig. 1). A small increase in the number of spikes also occurred for the "B" neuron.

Neo and Its Major Component, Nepetalactone, Activate the GRN Sensitive to the Feeding Deterrent, Quinine

Recordings from the gustatory sensilla on the labella of females revealed that the feeding deterrent quinine elicited single amplitude spike responses (Fig. 2, $n = 4$). Mixtures of quinine and either *N*eo (0.01%) or nepetalactone (0.04 mM) also elicited single amplitude spike responses ($n = 4$), thus indicating these gustatory stimuli activated the same GRN. Increasing concentrations of either *N*eo alone or nepetalactone alone elicited increasing numbers of spikes of a single amplitude (Fig. 3A, $n = 6$). Dose response curves revealed similar thresholds for both *N*eo and nepetalactone, ca. 0.001% for *N*eo and 40 µM for nepetalactone (Fig. 3B). Moreover, shapes of dose response curves for both were nearly identical within the limits of standard errors.

Neo, Nepetalactone, and Sucrose Are Mutually Antagonistic Gustatory Stimuli

As shown previously (Sanford et al. 2013), the feeding stimulant sucrose at 4 mM stimulates a GRN with a large amplitude spike (Fig. 4, upper trace, $n = 4$). When sucrose at 4 mM was admixed with super threshold concentrations of either 0.01% *N*eo or 0.4 mM nepetalactone (see Fig. 3), spikes of two amplitudes are present: one responsive to the feeding stimulant, the other responding to the feeding deterrents, *N*eo or nepatalactone (Fig. 4, lower traces, $n = 4$). However, the combination of the feeding stimulant and a feeding deterrent resulted in diminished numbers of spikes for both the feeding stimulant (sucrose) and the feeding deterrents (*N*eo and nepetalactone) relative to the numbers elicited by either stimulus alone (e.g., compare to traces in Fig. 3A).

Discussion

Olfactory sensilla on the maxillary palps are named the capitate basiconic pegs and house three ORNs: an ORN ("A") with the largest amplitude spike responds to CO_2, an ORN with an intermediate spike ("B") has an ill-defined specificity, while the ORN with the smallest amplitude spike ("C") responds to racemic 1-octen-3-ol, especially the enantiomer (R)-(-)-1-octen-3-ol (Grant and Dickens 2011). Here we show that the neuron responsive to 1-octen-3-ol is also mildly activated by *N*eo, but only at extremely large stimulus loads, requiring a 1,000–10,000× larger stimulus load than for its natural ligand 1-octen-3-ol. An even smaller number of spikes were elicited from the intermediate spike at the highest stimulus load tested. Thus, it seems unlikely that the spatial repellency observed for *N*eo or nepetalactone is regulated by neural input through these sensilla. The spatial repellency of nepetalactone is likely mediated by an ORN in sbtI1 sensilla on the antennae as demonstrated in a previous study (Ghaninia et al. 2008).

Gustatory sensilla on the labella of *Ae. aegypti* house at least three types of GRNs with different specificities: a GRN with a large spike responds to salt (NaCl), a second GRN with a large spike amplitude responds to the feeding stimulant sucrose, while a third GRN represented by a small amplitude spike responds selectively to the feeding deterrent quinine and certain repellents including DEET (Sanford et al. 2013). Here we show that the neuron responding to the feeding deterrent quinine also responds to the repellents *N*eo and nepetalactone. This correlates well with studies showing contact or topical repellency for both *N*eo and its major component for several mosquito species including *Ae. aegypti* and the common malaria mosquito *Anopheles quadrimaculatus* (Bernier et al. 2005, Chauhan et al. 2005). The GRN responsive to the feeding deterrent

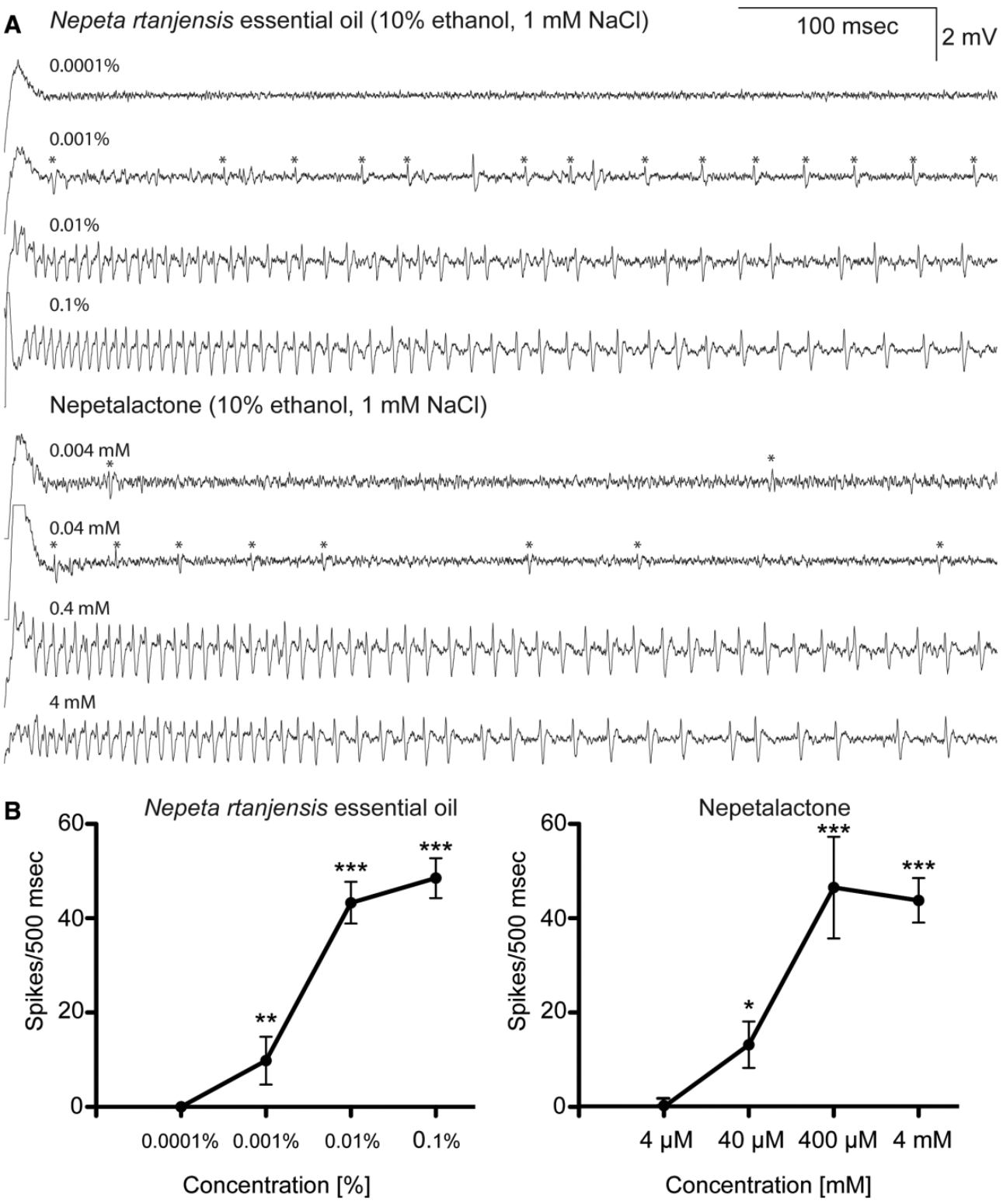

Fig. 3. (**A**) Recordings of electrical activity from gustatory sensilla on the labella of *Ae. aegypti* females in response to serial dilutions of *Nepeta rtanjensis* essential oil (*N*eo) and nepetalactone. Traces start 50 ms following the stimulus artifact and last for 500 ms ($n = 6$). Asterisks indicate spikes corresponding to *N*eo or nepetalactone stimulation. Note that a single amplitude spike predominates in each recording. (**B**) Dose response curves constructed from the mean numbers of spikes elicited by increasing concentrations of *N*eo or nepetalactone. As the *N*eo used in this experiment is ~85% (by mass) nepetalactone, 0.1% *N*eo represents a concentration of nepetalactone on the order of 4 mM. These curves reveal a threshold for significant increases in spikes as compared to control stimuli at 0.001% *N*eo (** $P = 0.003$) and 40 µM nepetalactone (* $P = 0.026$; one-way ANOVA followed by Dunnett's multiple comparison post test, *** $P < 0.001$). Vertical bars represent ± standard errors.

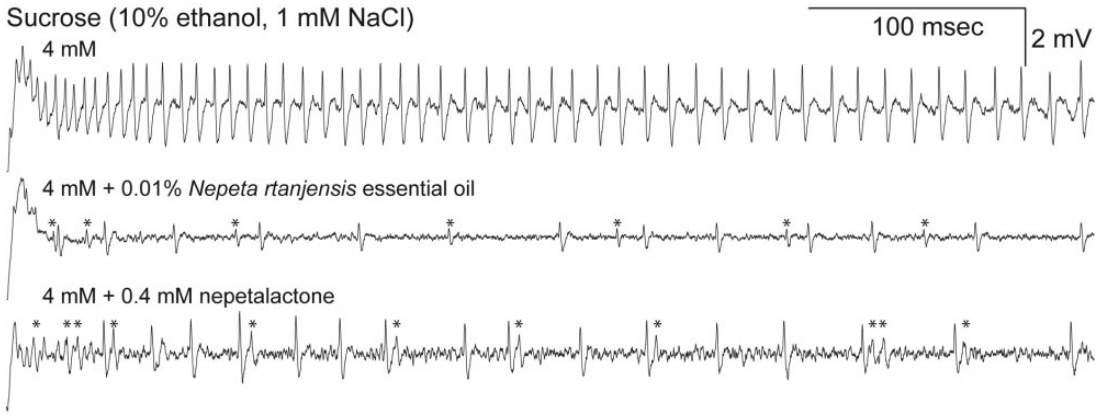

Fig. 4. Recordings of electrical activity from a gustatory sensillum on the labella of *Ae. aegypti* females in response to: sucrose 4 mM alone and sucrose 4 mM + either 0.01% *Nepeta rtanjensis* essential oil (*N*eo) or 0.4 mM nepetalactone ($n = 4$ for each). Traces start 50 ms following the stimulus artifact and last for 500 ms. Asterisks indicate spikes corresponding to *N*eo or nepetalactone stimulation. Note only a single amplitude spike responding to sucrose while two spikes are present in responses to sucrose + *N*eo or nepetalactone.

Table 2. Effects of *Nepeta* sp. essential oil on olfactory and gustatory receptors in *Ae. aegypti* females

Olfactory effects	Antenna	Maxillary palp
	Activates neuron in sbt1 sensillum[a]	Activates "C" neuron and to a lesser extent "B" neuron at high concentrations
Gustatory effects	Labellum	
	Activates quinine-sensitive neuron Suppresses response of the sucrose-sensitive neuron to its cognate stimulus	

[a] Ghaninia et al., 2008.

quinine was also shown to be sensitive to both naturally occurring and synthetic insect repellents in both *Ae. aegypti* (Sanford et al. 2013) and *An. quadrimaculatus* (Sparks and Dickens 2016a,b).

The reciprocal antagonistic effects of the feeding stimulant sucrose and feeding deterrent quinine on activity of their respective GRNs was previously demonstrated in *An. quadrimaculatus* (Sparks and Dickens 2016a, b). Here we show similar antagonistic effects of sucrose and *N*eo or nepetalactone in *Ae. aegypti*, thus providing a peripheral mechanism for modulating feeding decisions by female mosquitoes when confronted with mixed gustatory stimuli. This trait likely appears in many modern mosquito species, as *Ae. aegypti* and *An. quadrimaculatus* represent lineages (Culicinae and Anophelinae) whose common ancestors are perhaps >200 million years old (Reidenbach et al. 2009).

Although catnip oil (*N. cataria* L.) has been formulated as an alternative repellent, here we provide an insight on the effectiveness of essential oil from another *Nepeta* species, *N. rtanjensis*, in targeting the same GRN that responds to deterrent quinine, and its possible mechanism(s) of action in repelling mosquitoes. Nepetalactones are the major constituents of *N. rtanjensis* essential oil, and are most likely responsible for the observed repellent activity. However, the effect of other minor compounds and possible synergistic and antagonistic interactions between all the constituents should not be neglected. Some minor constituents of *N. rtanjensis* essential oil, including 1,8-cineole and α- pinene, are reported to possess repellent activity against various insects (Nerio et al. 2010).

In conclusion,we have shown that *N*eo and its major component nepetalactone affect both the olfactory and gustatory receptor systems in *Ae. aegypti* females, further adding to an earlier report on their effect on the olfactory system (Ghaninia et al. 2008; Table 2.). Olfactory receptor neurons housed within olfactory sensilla on the maxillary palps of female *Ae. aegypti* respond to *N*eo only at very high stimulus loads, and thus are likely not involved in spatial repellency observed in previous studies. We also show that the GRN responding to the feeding deterrent quinine, responds to both *N*eo and nepetalactone, thus providing a neural pathway for the reported feeding deterrence of these repellents. The interaction between the feeding stimulant and feeding deterrent enhances our knowledge of the mechanisms involved in feeding decisions by female *Ae. aegypti*. A better understanding of the neural input through which female mosquitoes make decisions to feed will facilitate design of future investigations and implementation of novel management strategies involving the use of repellents.

Acknowledgments

We are grateful for the gift of nepetalactone from Dr. Kamal Chauhan of the USDA, ARS, NEA, BARC, Invasive Insects Biocontrol and Behavior Laboratory, Beltsville, MD. This work was supported in part by a grant to J.C.D. from the Deployed War Fighter Protection (DWFP) Research Program, funded by the U.S. Department of Defense through the Armed Forces Pest Management Board (AFPMB).

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