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Original article

Molecular evidence for the inhibition of cytochrome p450s and cholinesterases in ticks by the repellent DEET

Cody W. Koloski, Christophe M.R. LeMoine, Adam R. Klonowski, Charlotte M. Smith, Bryan J. Cassone*

Department of Biology, Brandon University, Brandon, MB R78 6A9, Canada

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ABSTRACT

For more than 50 years DEET (*N,N*-Diethyl-m-toluamide) has been considered the gold standard of repellents. It is applied to the skin or clothing to deter mosquitoes and other blood-sucking invertebrate pests from approaching and/or settling, and ultimately it provides temporary protection from bites. Despite rampant global use, surprisingly little is understood about DEET's mode of action and the molecular targets of the active ingredient. Furthermore, the theories into its mechanism for repellency are largely based off fruit fly and mosquito research. Since ticks possess a unique sensory structure, the Haller's organ, the specific genes and pathways associated with DEET avoidance may differ from insects. In these studies, we collected American dog ticks (*Dermacentor variabilis*) from four natural populations within Manitoba, Canada. We first carried out behavior assays, which showed DEET effectively repelled the ticks. RNA sequencing revealed that DEET caused a rapid and substantial reduction in the abundance of transcripts encoding cytochrome P450 and acetylcholinesterase genes, which gradually recovered over the 24 h time course. Finally, enzymatic kinetics provided functional support for DEET's role as an effective inhibitor of P450 s. While many facets of its mode of action remain to be worked out, our study provides valuable insights into the molecular underpinnings of DEET's repellence in ticks.

1. Introduction

Today – just as for the past 50 years – the most commonly used repellent in the world is DEET (Fradin, 1998; Pickett et al., 2008). The active ingredient, *N,N*-Diethyl-m-toluamide, is an oily synthetic substance applied topically to the skin in order to alter the host feeding behaviors of diverse blood sucking arthropods, including fleas, black flies, mosquitoes and ticks. Initially developed for use by United States military in 1946 and registered for commercial use about a decade later (Fradin, 1998; Katz et al., 2008), DEET's formulation has undergone many changes over the years to improve its smell, texture, strength and duration of activity. Almost paradoxically, extensive research has been carried out to determine the effectiveness of the repellent and safe levels/duration of exposure, but its mode of action and molecular targets of the active ingredient remain poorly understood. Given DEET's integral role in the prevention of vector-borne infectious diseases and concerns regarding the evolution of resistance (Stanczyk et al., 2010; Stanczyk et al., 2013), it is imperative that inroads are made towards elucidating the underlying basis of DEET repellence.

Repellents do not kill arthropod pests; rather, they generally work by blocking/modulating the host-seeking (i.e., chemosensory) machinery of the arthropod, preventing it from coming into contact and feeding on humans. While many theories exist, the two predominant hypotheses for the mode of action of DEET are 1) it operates as a "confusant" by acting in conjunction with host odors to drive repellency; or 2) it is perceived as a harmful/adverse odor by the arthropod and is therefore avoided (reviewed in DeGennaro, 2015). Fundamentally based on these theories, some progress has been made towards deciphering the molecular targets (olfactory and gustatory) involved in DEET avoidance in flies, mosquitoes and nematodes (Ditzen et al., 2008; Lee et al., 2010; Pellegrino et al., 2011; Kain et al., 2013; DeGennaro et al., 2013; Dennis et al., 2017). Evidence is also accumulating for novel mechanisms of repellence involving the inhibition of detoxification (cytochrome P450) enzymes (Ramirez et al., 2012; Abd-Ella et al., 2015) or cholinesterases (Corbel et al., 2009). However, documentation of the specific genes and pathways associated with DEET avoidance in ticks is still in its infancy.

Lacking conventional eyesight, antennae and a proboscis, ticks possess sensory innovations used to locate and attach to their vertebrate hosts. The main sensory organ, which is unique to ticks, is the Haller's

* Corresponding author.
E-mail address: cassoneb@brandonu.ca (B.J. Cassone).

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organ. Located on the first tarsus on their foremost pair of legs, the Haller's organ specializes in behavior related to host seeking, infrared detection, body position and mating (Carr et al., 2017; Josek et al., 2018; Mitchell et al., 2017). Their other central sensory organ are the palps, which are found on the mouthparts (chelicerae) and mainly associated with anchoring and chemosensation (Renthal et al., 2017). While studies are slowly accruing, it is not currently understood how ticks detect repellents, or the relative importance of olfaction versus tactile chemoreception in repellency. There is evidence that olfaction is at least partially involved (Dautel et al., 1999; Mcmahon et al., 2003; Sonenshine and Roe, 2013), and the Haller's organ has been implicated in spatial repellency of DEET (Carr et al., 2017). Moreover, the palps are also thought to be involved in repellency through direct contact with repellent via gustatory sensilla (Bissinger and Roe, 2014).

The American dog tick, *Dermacentor variabilis*, is principally found in the United States, east of the Rocky Mountains. Since the 1940s, its range distribution has expanded into Canada, east of Saskatchewan (Matheson, 1950). In Manitoba, it is far and away the most abundant species of human-biting tick, ubiquitously found in the southern part of the province from April to June (Bishopp and Trembley, 1945; Dergousoff et al., 2013). *D. variabilis* feeds on the blood of a variety of mammalian hosts, including dogs, rabbits, raccoons, rats, mice, porcupines, squirrels, voles and humans (Carroll and Nichols, 1986; Koehler et al., 2018). In addition to its pest status, *D. variabilis* is of particular medical importance in North America since it is the primary vector of the pathogens causing Rocky Mountain spotted fever (RMSF) (*Rickettsia rickettsii*) (Burgdorfer, 1975) and tularemia (*Francisella tularensis*) (Reese et al., 2010). As there are no effective vaccines licensed and currently available against these infectious diseases, prevention is predominately achieved through the widespread use of repellents, such as DEET.

To date, little is known about mode of action or the underlying genetic basis of DEET repellency for human-biting ticks. To begin to unveil these mechanisms, we carried out behavioral assays to confirm the effectiveness of DEET as a deterrent for *D. variabilis*. RNA sequencing was then used to build the *D. variabilis* transcriptome and to characterize global changes in gene expression in response to DEET in ticks at 0.25 h, 4 h and 24 h post exposure. Two major patterns emerged from these novel datasets. First, we identified an immediate and substantial reduction in transcript abundance of cytochrome P450 genes, which recovered over time. This finding was corroborated by enzymatic assays, and provides support for the hypothesis that P450 inhibition plays an important part in repellence. Second, we found molecular support for DEET as an acetylcholinesterase inhibitor in ticks. While this work does not fully unravel the repellent's mode of action per say, it provides novel and valuable insights into the responses of ticks to DEET at the molecular level.

2. Materials and methods

2.1. Tick collections and maintenance

Adult *Dermacentor variabilis* were sampled in southwestern Manitoba in May and June 2017 and 2018 on days when the conditions were optimal (e.g., 23 to 27 °C; RH > 65%). Collections were made from four localities in the city of Brandon and surrounding areas (Fig. S1). Sampling was carried out using the dragging method, where 1-m^2 flannel sheets were dragged along the ground in 5 m intervals to gather questing ticks (Rochon et al., 2012). Precautions were taken during collections to ensure the experimental design was not compromised (e.g., tick draggers did not wear repellent or any fragrance). Ticks were stored in 50 mL falcon tubes containing wet paper towel until transport to the laboratory. *D. variabilis* was held in growth chambers under controlled conditions of 20 ± 3 °C, 95% RH with a 12:12 h light-dark cycle included dawn and dusk transitions until commencement of experiments.

2.2. Tick behavioral assays

Our behavioral experiments followed a repeated measures design, as briefly described. A total of 100 color-coded *D. variabilis* were placed into 15 mL conical tubes (10 per tube) and allowed to acclimate for 30 min. An identical 15 mL tube containing two adhesive bands (3/4 inch in width) spanning its diameter was then stacked directly on top of the tube (openings positioned adjacent) and sealed (Fig. S2). Adhesive bands were treated with either 100% ethyl alcohol or DEET:ethyl alcohol (3:1 ratio). Tubes were positioned upright and exposed to an overhead light source in order to simulate questing conditions and sunlight, respectively. To establish baseline data (i.e., control), ticks were first placed at random into ten tubes containing two bands treated with ethyl alcohol. Once the trial commenced, ticks were allowed to move freely within the tube for 30 min, and the capacity of individual *D. variabilis* to contact and breach each adhesive band was recorded. After 24 h, the same ticks were randomly placed into 10 tubes containing one ethyl alcohol band and one DEET/ethyl alcohol band (5 DEET-top and 5 DEET-bottom tubes) and trials followed the same protocol as described. During and post assay, ticks were monitored for changes in behavior. To determine whether ticks were significantly repelled by DEET, each tick was assigned a score (0–2) based on the number of bands it successfully bypassed, and these values were averaged for all the ticks in each tube. Therefore, a tube containing 10 ticks was considered one replicate (i.e., n = 1). A repeated measures one-way Analysis of Variance (ANOVA) was then carried out on the replicates (10 tubes each) of the three different treatments. Consequently, the relative positioning of each treated band was integral to the interpretation of the results; for instance, if a tick were effectively repelled by DEET but not ethyl alcohol as hypothesized, the DEET-bottom treatment would have a lower score (0 for not passing any bands) than the DEET-top treatment (1 for passing the alcohol band).

2.3. Transcriptional analysis of D. variabilis post DEET exposure

2.3.1. Experimental design

Ticks were subjected to a 0.25 h DEET treatment, followed by sample collection at three time points: 0.25, 4 and 24 h post-exposure. Each sample consisted of 20 adult *D. variabilis* (roughly equal numbers of males and females) and each time series was replicated four times using ticks sampled from different localities (12 samples in total). At the onset of each time series, ticks from the maintenance chamber were sorted into pools of 20 and placed into 50 mL falcon tubes containing paper towel soaked in > 98% *N,N*-Diethyl-m-toluamide (TCI America, Portland, OR, USA) at one end. Ticks were initially placed in direct contact with the treated towel but could then move freely within the tube (and out of direct contact) for the duration of the 0.25 h exposure. At each time point, ticks were flash frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Collection of the corresponding 12 control samples was done concurrently and followed an identical design, with the only difference being the replacement of DEET with ddH_20. As the DEET used in these assays was pure, ddH_20 was selected as the control since it is considered a neutral stimulus and is unlikely to elicit a chemosensory response in ticks.

2.3.2. RNA extraction and sequencing

For each pooled replicate, total RNA was extracted from whole-body *D. variabilis* ticks using the Norgen Animal Tissue RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada). RNA was quantified using the Nanophotometer NP80 (Implen Inc., Westlake Village, CA, USA) and run on a 1.5% agarose gel to assess quality. RNA (1 μg/sample) was then sent to the Génome Québec Innovation Centre (McGill University, Montreal, QC, Canada) for mRNA stranded library preparation (New England Biolabs, Ipswich, MA, USA) and sequencing in 100 bp paired-end fashion on a flow cell lane using the Illumina HiSeq4000 platform. The raw sequence reads can be retrieved from the NCBI short sequence

read archive under the accession number SRP150145

2.3.3. Assembly of the D. variabilis transcriptome

Demultiplexed raw reads were imported into FASTQC (Andrews, 2010) to assess overall sequence quality. CLC Genomics Workbench (v10.0.1; CLC Bio, Aarhus, Denmark) was used for trimming of adapter indexes and poly (A) tails (Ambiguous Limit = 2, Quality Limit = 0.05). Reads were assembled *de novo* into contigs using the following optimized parameters: Word Size = 64, Bubble Size = 500, Length Fraction = 0.6 and Similarity Fraction = 0.95; default settings herein. Contigs with ≥90% sequence similarity were collapsed into clusters and the longest contig was retrieved using CD-HIT-EST (Huang et al. 2010). Microbial and nematode contamination was identified and removed from the transcriptome using desktop-downloaded BLASTn against the NCBI bacteria non-redundant (nr) database (E-value $< 1 \times 10^{-50}$) and a GC content threshold of 45%. Only contigs of ≥250 bp and > 10 reads were represented in the final assembly, and are herein referred to as "transcripts".

2.3.4. Differential expression analysis

Reads were mapped to the final transcript assembly using the RNA-Seq Analysis 2.16 tool in CLC (Similarity Fraction = 0.6, Length Fraction = 0.95; default herein). Only reads that mapped uniquely (i.e., unambiguously) to one transcript in the transcriptome were included in the analysis. Broken pairs were included and paired-end reads were treated independently. Transcripts differentially expressed between DEET-exposed and control *D. variabilis* were identified using the DESeq package (Anders and Huber, 2010) in Bioconductor (Gentleman et al., 2004). This analysis software quantifies differences in mapped reads counts based on a negative binomial distribution model. Significance was defined at $P < 0.05$ for this and all analyses unless otherwise stated.

The Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8) annotation clustering module (Huang et al., 2007, 2009) was used to classify differentially expressed transcripts into functional groups. Transcripts were first converted to *Ixodes scapularis* IDs derived from VectorBase (gene set IscaW1.6) using tBLASTx (E-value $< 1 \times 10^{-10}$). Enrichment of GO, INTERPRO, KEGG and other annotation terms in the sublists were then explored using the functional annotation clustering tool. Significant clusters were defined as having more than ten *I. scapularis* gene matches and using the following parameters: similarity term overlap = 3; similarity threshold = 0.5; initial group membership = 3; final group membership = 3; multiple linkage threshold = 0.5; EASE enrichment scores = 1.3 (equivalent to $P \leq 0.05$).

2.4. Cytochrome P450 enzymatic assays

Relative oxidase activity, a proxy of cytochrome P450 activity, was measured in tick homogenates in the presence and absence of DEET using an established assay developed for the detection of heme peroxidases (Brogdon et al., 1997), and adapted for ticks (Yessinou et al., 2018). Each sample consisted of five adult *D. variabilis* and assays were run in duplicate in 162.5 µL reactions. A total of six paired sampled were quantified in this experiment. In brief, ticks were flash frozen and pulverized in LN_2 with mortar and pestle. The powdered tissue was homogenized in 1.25 mL of 0.0625 M $KHPO_4$ buffer (pH 7.2) and further diluted 1:5 in $KHPO_4$. A total of 10 µL of tick homogenate was placed in randomly assigned "Control" or "DEET" microtiter plate wells. To the DEET wells, 37 µl $KHPO_4$, 100 µl TMB (0.012 g 3, 3′, 5, 5′ tetramethylbenzidine dissolved in 6 mL of sodium acetate 0.25 M pH 5.0 and 6 mL of methanol), 12.5 µL of 3% hydrogen peroxide and 3 µL of > 98% DEET were added. The control wells were set up similarly with the only difference being 3 µL of $KHPO_4$ or 3 µL grapefruit juice were added to their respective wells instead of DEET. Grapefruit juice served as the positive control because it is known to cause inhibitory effects on human CYPs (Mrudula et al.,2017). Kinetic tests were carried out for 35 min at 630 nm on a SpectraMax Plus384 spectrophotometer (Molecular Devices, San Jose, CA, USA). The absorbance (OD) was recorded at the onset of the assay (T0) and at the end of the assay as final OD based on peak enzyme activity (T35). Absolute changes in OD were calculated for each well and relative differences between the control and DEET samples were assessed using a repeated measures ANOVA in SigmaPlot 11.

3. Results

3.1. American dog ticks are effectively repelled by DEET

To explore the efficacy of DEET to deter *D. variabilis*, behavioral trials were carried out on 100 ticks. Ticks were partitioned into tubes containing DEET and/or ethyl alcohol treated adhesive bands, and the capabilities of ticks to navigate through each band were recorded. Table S1 displays the results of each trial, which include the number of ticks challenging versus successfully bypassing the treated bands. For the control, the vast majority of *D. variabilis* (85%) passed through both Ethyl Alcohol bands, with no effects of repellence observed. On the other hand, 64% of eligible ticks challenged DEET and only 3% successfully passed it. After factoring in band treatment and relative position of the treatment (top or bottom), the repeated measures ANOVA revealed DEET significantly deterred *D. variabilis* ($P < 0.001$). Observations of symptom development for ticks that made contact with DEET indicated loss of coordination, muscle spasms and "cleaning" of the forelegs (C.K. personal observation), consistent with the hot-foot response (Eisen et al., 2017).

3.2. Assembly of the whole body D. variabilis transcriptome

A total of 24 cDNA libraries were sequenced, which generated approximately 392 million paired-end reads. *De novo* assembly of the transcriptome resulted in 127,632 contigs of > 250 bp and coverage of > 10. Subsequent removal of redundant sequences and contamination yielded 92,190 putative "*D. variabilis* transcripts". This number is almost certainly an overestimate; it is unlikely we were able to remove all non-tick sequences, and is a limitation of using samples collected in nature. Pairwise BLASTn comparisons of these transcripts to the NCBI non-redundant database found that the greatest number of top matches were to the deer tick, *Ixodes scapularis* (n = 16,529), brown ear tick, *Rhipicephalus appendiculatus* (n = 4440) and Asian blue tick *Rhipicephalus microplus* (n = 2859). Only 1188 transcript had a most significant hit to a species in the genus *Dermacentor*, including 702 for *D. variabilis,* presumably due to the lack of genetic resources currently available. The Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GGTZ00000000 The version described in this manuscript is the first version, GGTZ01000000.

3.3. A small proportion of D. variabilis transcripts show altered expression due to DEET exposure

Between 82% and 85% of reads mapped uniquely to the *de novo* transcriptome, indicating good consistency and mapping efficiencies among treatments/time points (Table S2). DeSeq analysis revealed that less than 4% of *D. variabilis* transcripts were differentially expressed at each time point (0.25 h: 2.8%, n = 2575; 4 h: 2.4%, n = 2174; 24 h: 3.1%, n = 2899). While there was a slight preponderance of transcripts downregulated in the DEET-exposed ticks at 0.25 h (52.4%), the opposite was true at both 4 h (45.6%) and 24 h (39.9%). The Venn diagrams show that only 36 transcripts were either upregulated (12) or downregulated (24) across all three time points, indicative of the plastic nature of DEET's effects on ticks at the transcriptome level (Fig. S3).

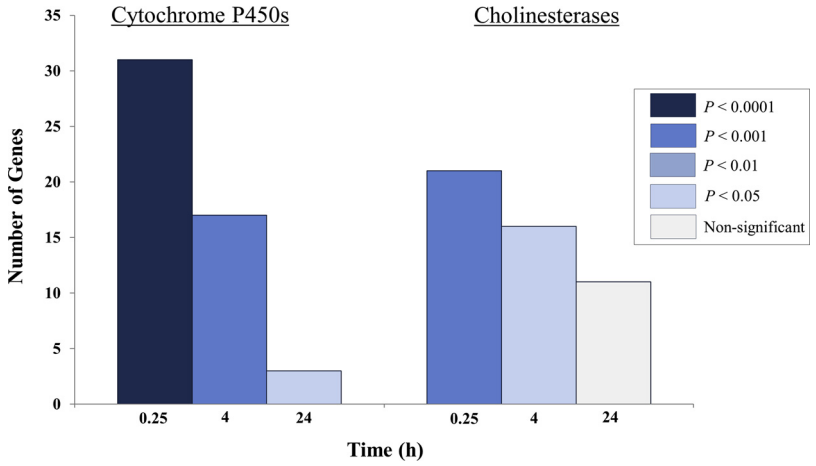

Fig. 1. Functional clustering of downregulated cytochrome P450 s and cholinesterases in *Dermacentor variabilis* in response to DEET exposure. Transcripts with *I. scapularis* orthologs were identified and clusters of functionally related transcripts were identified using DAVID.

3.4. Exposure to DEET inhibits expression of cytochrome P450 s and acetylcholinesterases

The *D. variabilis* transcriptome is in its initial stages of annotation; thus, no formal quantitative efforts were made for functional analysis of differentially expressed transcripts. In lieu of this, we opted for an exploratory approach, which inferred tick functional groups significantly impacted by DEET exposure based on comparisons to the *I. scapularis* genome. *D. variabilis* transcripts were first converted to their ortholog *I. scapularis* ID (where possible) and then partitioned into six lists: up or downregulated in ticks at 0.25 h, 4 h or 24 h post-exposure to DEET. Since only about 28% of *D. variabilis* transcripts had an ortholog match, this analysis presumably identified only a subset of the processes altered by the repellent. Thus, there are many additional unannotated genes that respond to DEET but their functional roles cannot yet be inferred.

From the downregulated lists, a total of nine annotation clusters were identified, three per time point. Most noteworthy were the cytochrome P450 s, which were inhibited by DEET at all three time points, and cholinesterases, which were significant at 0.25 h and 4 h (Fig. 1). The heat maps show the differential expression of individual transcripts encoding P450 s (Fig. 2) and cholinesterases (Fig. 3) across the three time points. The other annotation clusters identified were linoleic acid metabolism at 0.25 h ($P = 0.0046$, n = 15), immunoglobulin-like at 4 h ($P = 0.023$, n = 14), transcription factor activity ($P = 0.0041$, n = 15) and epidermal growth factor ($P = 0.016$, n = 13) at 24 h. From the upregulated lists, only two annotation cluster were significant: metallopeptidase at 4 h ($P = 0.026$, n = 11) and phosphatidylinositol 3/4-kinase at 24 h ($P = 0.015$, n = 12).

3.5. Enzyme kinetics validates DEET impact on oxidases activity

To ensure validity of our assays, we included grapefruit juice and ethyl alcohol as controls with each of our microplate assays. As expected, grapefruit juice acted as an effective negative control, inhibiting oxidase/P450 activity by 46 ± 10% when compared to ethyl alcohol treated wells. Furthermore, across our six paired replicates, *in vitro* exposure to DEET caused a rapid and highly significant 28 ± 4% reduction in *D. variabilis* oxidases activity ($P < 0.001$). The results of the enzyme kinetics assays are displayed in Table S3.

4. Discussion

Despite the global reliance on DEET to repel a myriad of invertebrate pests and prevent vector-borne diseases, its mode of action and genetic underpinnings remain unresolved. The American dog tick, *D. variabilis*, was chosen for these studies because of its abundance in the study area, importance as a vector of human pathogens and the fact that virtually nothing is known about the molecular responses of ticks to DEET. Our experiments indicated that ticks are effectively deterred by DEET, and that exposure can induce muscle spasms and uncoordinated movements. Underlying these behaviors was a small genetic signature, characterized by an immediate and dramatic reduction in the accumulation of transcripts encoding cytochrome P450 s and acetylcholinesterases. In addition, relative oxidases enzyme activity of *D. variabilis* was impeded nearly 30% in the presence of DEET. This is in agreement with recent toxicological and biochemical studies of mosquitoes, which show inhibition of these enzymes, particularly CYP450, in the presence of DEET (Bonnet et al., 2009; Corbel et al., 2009; Ramirez et al., 2012). The transcriptional downregulation was most pronounced within the first 15 min post DEET exposure and gradually recovered over the 24 h time period. Such abrupt changes to only a small number of specific gene groups prompt speculation into their mechanistic roles in DEET avoidance by ticks and provide unique insights into the repellent's mode of action.

The two most commonly accepted theories for DEET's mode of action – "confusion" and "smell and avoid" – are both fundamentally based on odor perception. Both vertebrates and insects use a variety of families of transmembrane receptor proteins to detect and discriminate odors, though their molecular mechanisms of chemosensory signalling are radically different (Pellegrino and Nakagawa, 2009; Silbering and Benton, 2010; Spehr and Munger, 2009; Touhara and Vosshall, 2009). Insects (and perhaps all arthropods) predominately use ionotropic receptors, which form ion channels that are generally closed until a ligand (e.g., odor molecule) binds. On the other hand, vertebrates use metabotropic receptors, which do not form channels. Instead, binding of the ligand indirectly activates ion channels through secondary messengers. From a functional standpoint, ionotropic receptors respond rapidly, whereas metabotropic receptors tend to have slower but broader effects, depending on the number and types of secondary messengers involved in the signaling cascade. From *Drosophila* studies, it has been speculated that an odor molecule binding to a specific chemosensory receptor stimulates an immediate ionotropic pathway followed by a slower forming metabotropic response (Hansson et al., 2010; Wicher et al., 2008). Ramirez et al. (2012) proposed that the metabotropic pathway requires cytochrome P450 activity at some point in the signalling cascade. If this were the case, DEET may initially trigger an attraction via ionotropic receptors and subsequent repellency due to an impediment of the P450 s along the metabotropic pathway, as discussed below in the context of *D. variabilis* and other ticks.

ID	ISCW0	0.25 DEET	4 DEET	24 DEET
35234	24473	*	*	
79613	01527	*		
14084	07953	*	*	
20076	05196	*		
38605	09797	*		
52968	11997	*		*
1302	07349	*	*	*
1480	03279	*	*	
26237	19880	*	*	
63940	01241	*		*
11640	01241	*		
14790	19880	*		
17995	00235	*		
21062	07226	*		
23077	19198	*		
24308	06560	*		
26020	19880	*		
28603	07225	*		
31019	22075	*		
36459	11029	*		
39326	02138	*		
4512	19198	*		
53131	05544	*		
55332	17673	*		
6509	16385	*		
67445	22071	*		
6792	18383	*		
8114	07349	*		
9419	17306	*		
9858	22076	*		
36586	01621		*	
40564	02138		*	
52388	01473		*	
10942	22706		*	
11759	19198		*	
18481	19198		*	
18812	19880		*	
39963	01241		*	
6409	07226		*	
146930	02138			*
39526	19198			*
13196	06560			*
52463	10787			*
12728	17084			*
13463	11997			*
15700	01241			*
21657	01418			*
23003	11998			*
4093	22077			*
5824	18383			*
16261	22948			*

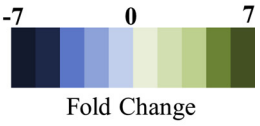

Fig. 2. Heat map showing altered expression of transcripts encoding putative cytochrome P450 s in *Dermacentor variabilis* at 0.25 h, 4 h and 24 h post exposure to DEET. Transcripts/time points denoted * are significantly differentially expressed.

Located on the fore-tarsals of the front pair of legs, the Haller's organ is presumed central to tick chemosensation. Recent genomic, transcriptomic and proteomic investigations have identified several families of transmembrane proteins in the forelegs of ticks that are likely involved in olfactory processes, including numerous ionotropic receptors, odorant-binding (like) proteins and other chemoreception-related proteins (Josek et al., 2018; Carr et al., 2017; Gulia-Nuss et al., 2016; Renthal et al., 2017). Cytochrome P450 s were also well documented in these tissues, as these enzymes are involved in a variety of functions including the metabolism of odor ligands (Feyereisen, 1999, 2012). Of particular interest, Carr et al. (2017) identified a possible olfactory G-protein coupled receptor (GPCR) signal cascade in *D. variabilis* that is seemingly exclusive to the Haller's organ. Since GPCRs can serve as secondary messengers in the metabotropic pathway, it lends support to a coupling of the ionotropic and metabotropic cascade for tick olfaction and highlights a possible mechanistic basis for tick repellence by DEET through P450 inhibition. A suite of over 120 putative GPCRs were identified in our whole-body *D. variabilis* transcriptome, though tissue specificity to the Haller's organ could not be inferred.

In terms of mode of action, inhibition of cytochrome P450 s by DEET could provide a viable explanation for the confusion hypothesis (Ramirez et al., 2012). Carr et al. (2017) identified putatively Haller's organ-specific *D. variabilis* P450 transcripts, suggesting that they function as odorant degrading enzymes (ODEs) within the olfactory sensilla. ODEs are not well understood (Durand et al., 2011; Leal, 2013) but are proposed to maintain olfactory system sensitivity by rapidly removing the receptor-bound odor molecules (Leal, 2013; Vogt, 2005; Younus et al., 2017). In lieu of functional ODEs, the odorant would continue to induce neural activity leading to sensory adaptation, thereby impairing the tick's ability to quickly respond to changes in volatiles in its environment (Vogt and Riddiford, 1981; Younus et al., 2014). Therefore if

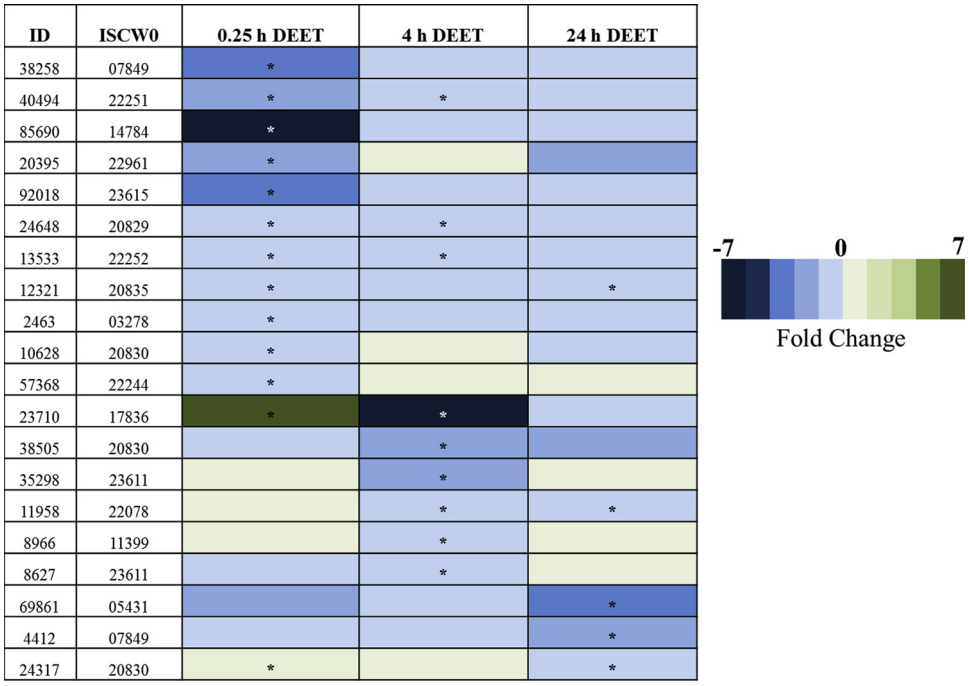

Fig. 3. Heat map showing altered expression of transcripts encoding putative acetylcholinesterases in *Dermacentor variabilis* at 0.25 h, 4 h and 24 h post exposure to DEET. Transcripts/time points denoted * are significantly differentially expressed.

DEET acts as a P450 inhibitor, as our molecular data indicates, the tick's inability to effectively breakdown odor ligands could cause "confusion" and sensory overload, which would impact host-seeking capabilities (Ramirez et al., 2012).

The second major finding of our experiments was a reduction in the expression of transcripts encoding acetylcholinesterase (AChE), which akin to P450 s, was immediate and tapered over time. AChE is a type-B carboxylesterase enzyme involved in the hydrolysis of acetylcholine into acetic acid and choline (Taylor et al., 2009). Acetylcholine is an essential neurotransmitter whose core function is to carry signals from in cholinergic synapses, including at neuromuscular junctions. In these synapses, once the signal has been successfully passed, AChE breaks down the neurotransmitter, effectively halting the signaling and allowing the by-products to be recycled into new neurotransmitters. Given its vital role, many pesticides inhibit AChE, preventing it from breaking down acetylcholine. The resultant increased half-life of the neurotransmitter prolongs its post-synaptic effects and can cause hyperactivity, uncoordinated movements, tremors, convulsions and/or paralysis of the insect (Colović et al., 2013; Fukuto, 1990; Lang et al., 2012). In line with our molecular dataset, Corbel et al. (2009) found in *Aedes* that acetylcholine is not efficiently hydrolyzed by AChE in the presence of DEET, indicating that the repellent can operate as an AChE inhibitor. Thus, if DEET impedes AChE production, as our experiments suggest, it could act as a mild pesticide to further deter ticks from coming into contact with the repellent or from remaining in its vicinity.

In addition to P450 s and AChEs, several other gene groups were also suppressed by DEET. Noteworthy was an immediate down-regulation of transcripts related to linoleic acid metabolism. In humans, linoleic acid synthesis is converted to mono-hydroxyl products by certain P450 s (Ruparel et al., 2013); thus, the activity of transcripts in this pathway appear coupled to that of P450 s. At later time points, transcription factors, immunoglobulin-like transcripts and epidermal growth factors were all repressed. These groups have diverse functions and therefore further studies are needed to shed light into their role (if any) in DEET repellence. On the other hand, both metallopeptidase and phosphatidylinositol 3/4-kinase were induced by DEET exposure. Phosphatidylinositol 3/4-kinase is implicated in the regulation of a broad range of cellular activities (Funaki et al., 2000). Metallopeptidases are associated with neural repair/regeneration after damage in humans and the degradation of the extracellular matrix (Chuang et al., 2010; Estrada-Pena and Mans, 2014; Kiryu-Seo et al., 2000). It is possible that these transcripts are involved in activation of mechanisms aimed to repair damage caused by DEET exposure, including the potential detrimental effects of neural hyperactivity due to AChEs inhibition (see above).

To our knowledge, this study is the first to construct a whole-body transcriptome of *D. variabilis*. Most of the data currently accessible in NCBI are derived from a midgut EST database consisting of 1679 transcripts (Anderson et al., 2008) and about 20,000 mRNA sequences from the testis-vas deferens-male accessory gland. Illumina read data is also available for ticks starved at different durations. First and fourth leg transcriptomes have been described (Carr et al., 2017); however, the sequence information is currently unavailable and inaccessible. Nonetheless, our transcriptome expands on the molecular resources for future genetic studies of the American dog tick.

In conclusion, this study begins to characterize the molecular basis for DEET avoidance by ticks. Although the modes of action and underlying mechanisms have not been fully disentangled, we present transcriptomic data indicating that, at the molecular level, DEET acts as an abrupt inhibitor of cytochrome P450 s and AChE. We propose that DEET may induce impaired host-seeking in ticks through its effects as both a confusant and mild pesticide; however, further targeted studies are needed to substantiate this model. Our results lend some support to recent studies in *Aedes,* thereby suggesting the mode of action could in fact be similar among ticks and insects. Future studies are now needed to elucidate the specific genes, pathways, proteins and cell types involved in P450 and AChE inhibition. Future experiments will help determine the sensory tissues involved in the repellence, as well as the relative role of DEET as a spatial and tactile repellent and may uncover sex-specific differences.

Conflicts of interest

The authors declare there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.12.006.

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