

1 DEET as a feeding deterrent

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31 Abstract

32 The insect repellent *N,N*-diethyl-3-methylbenzamide (DEET), is a multimodal compound that
33 acts as a spatial repellent as well as an irritant (contact repellent), thus being perceived by the
34 insect's olfactory and gustatory systems as an odorant and a tastant, respectively. Soon after
35 DEET was developed, almost 6 decades ago, it was reported that it reduced mosquito feeding on
36 blood mixed with this repellent. It is now known that the mosquito proboscis senses contact
37 repellents with the tips (labella) of the labium, which remain in direct contact with the outer
38 layers of the skin, while the stylets, including the feeding deterrent sensor (labrum), penetrate the
39 skin. We designed a behavioral assay that allowed us to tease apart contact repellency from
40 feeding deterrence. First, we demonstrate here that when DEET was mixed with blood and
41 covered by Parafilm® layers, it did not leak to the outer surface. In our assays, the mean number
42 of landings and duration of contacts with surfaces covering blood mixed with DEET or blood
43 plus solvent (dimethyl sulfoxide) did not differ significantly. The feeding times, however, were
44 significantly different. When blood was mixed either with 0.1 or 1% DEET, female southern
45 house mosquitoes spent significantly less time feeding than the time spent feeding on blood
46 mixed only with the solvent. By contrast, there were no significant differences in the mean times
47 of feeding on blood containing 1% picaridin and blood plus solvent. Like DEET, the contact
48 repellent and insecticide, permethrin, caused a significant reduction in feeding time. We,
49 therefore, concluded, that in this context, DEET and permethrin act as feeding deterrents.

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58 Introduction

59 Chemicals used to reduce mosquito bites are not only repellents *sensu stricto*, ie, compounds that
60 cause the responder to steer away from the source, but are also excitorepellents or irritants, ie,
61 chemicals eliciting increased locomotor activity after an insect makes contact with the source
62 (Obermayr 2015). From a strict mechanistic viewpoint, these 2 groups should be named
63 noncontact and contact disengagers, respectively (Miller et al. 2009). From a more pragmatic
64 perspective, the end result is the same, ie, mosquitoes are kept at bay by sensing odorants in the
65 vapor phase (spatial repellents) and/or by detecting non-volatile tastants (contact repellents) upon
66 direct contact with these chemicals (on a skin surface, for example). Although its complete mode
67 of action is still a matter of considerable debate, DEET (=N,N-diethyl-3-methylbenzamide) is
68 undoubtedly a multimodal compound (DeGennaro 2015), which is perceived by both the
69 olfactory and gustatory systems as an odorant and a tastant, respectively. Additionally, evidence
70 in the literature suggests that DEET also acts as a feeding deterrent (Barzeev & Smith 1959). The
71 pioneering findings by Bar-Zeek and Schmidt (Barzeev & Smith 1959) that blood-feeding was
72 prevented when samples were spiked with DEET has been overlooked most probably because of
73 the difficulty in teasing apart feeding deterrence from contact repellency.

74 Mosquitoes sense the environment with their antennae, maxillary palps, proboscis, tarsi, and
75 ovipositors. Whereas the antennae and maxillary palps are involved in the reception of odorants
76 (eg, spatial repellents), the proboscis is involved in the reception of contact repellents and other
77 tastants. This sophisticated “microneedle system” (Kong & Wu 2010) comprises a gutter-like
78 labium that encloses a fascicle. There are 2 lobes (labella) at the tip of the labium, and the
79 fascicle contains 6 stylets: a pair of teeth-bearing maxillae, a pair of mandibles, a hypopharynx
80 with its salivary canal, and a labrum that carries sense organs on its tip (Wahid et al. 2003).
81 During feeding, the fascicle penetrates the host’s skin while the labium bends and the labella
82 remain in direct contact with the outer layer of the skin (Choo et al. 2015). Although it has been
83 demonstrated that labral apical sensilla respond to phagostimulants (Liscia et al. 1999; Werner-
84 Reiss et al. 1999) and feeding deterrents (Kessler et al. 2014), it remains difficult to
85 unambiguously determine whether reduced feeding on DEET-spiked blood is mediated by
86 “contact repellency” or “deterrence.” Indeed, Bar-Zeek and Schmidt (Barzeev & Smith 1959)
87 suggested that “repellency” was caused by low concentrations of DEET (then named
88 diethyltoluamide) in the blood.

89 To address whether reduced feeding on DEET-spiked blood was due to repellency or deterrence,
90 we devised a modified version of our surface landing and feeding assay (Fig. 1) (Leal et al.
91 2017). We lured mosquitoes to feed on 2 cotton rolls covered with dual layers of Parafilm®
92 sealing film and loaded with blood, one spiked with DEET and the other with solvent, and
93 meticulously measured feeding times in the 2 parts of the arena. Here, we report that mosquitoes
94 spend significantly less time feeding on DEET-spiked blood than on the control. Likewise,
95 permethrin also acted as a feeding deterrent, but the time spent feeding on blood spiked with
96 picaridin was not significantly different from the time spent on feeding on the control side of the
97 arena.

98

99 **Materials and methods**

100 **Mosquitoes**

101 *Culex quinquefasciatus* mosquitoes used in this study were originally from a laboratory colony
102 initiated with mosquitoes collected in the 1950s in Merced, California and currently kept by Dr.
103 Anthony Cornel (Kearney Agricultural Center, University of California-Davis). The Davis
104 colony has been maintained separately for more than 6 years under 12:12 (L:D), 27±1°C, and
105 75% relative humidity.

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108 **Behavioral arena**

109 Feeding behavior was measured using a modified surface landing and feeding assay (Leal et al.
110 2017). In brief, the device consisted of a base and a detachable assay cage (Fig. 1B). The frame
111 of the base was made from an aluminum collapsible field cage (Bioquip, 30.5 × 30.5 × 30.5 cm)
112 with a wooden board (30 × 30 cm) attached to the front of the cage and covered with red
113 cardstock (The Country Porch, GX-CF-1) and red lab tape. Three openings were drilled through
114 the wooden board to accommodate one 50-mL Dudley bubbling tube (Fisherbrand, 40356) and
115 two 16-gauge syringe needles (Sigma-Aldrich, Z108782), orientations of which are illustrated on
116 Fig. 1A. The Dudley tube painted internally with black hobby and craft enamel (Krylon, SCB-
117 028) was attached to a water bath circulator with the temperature set at 38°C. The 2 syringe
118 needles were connected to a CO₂ tank through a bubbler to deliver CO₂ at 50 mL/min. The frame
119 of the detachable assay cage was made with the same aluminum collapsible field cage. Red

120 cardstock was taped internally at 1 face of the cage, 1 circular opening, and 2 small holes were
121 made in the cardstock to allow the Dudley tube and CO₂ needles to project into the mosquito
122 cage. The cage was completed with a field cage cover (Bioquip, 30.5 × 30.5 × 76.2 cm). One
123 square, sealable opening (7 × 7 cm) was made at the backside of the field cage cover, allowing
124 the Dudley tube and CO₂ needles to insert into the cage. A slit was made on the top of the cage,
125 and a zipper (10 cm) was sewn on to the slit for an easily accessible opening. A camera-
126 accessible opening (d=5 cm) with a drawstring was made at the front of the field cage (Fig. 1B).

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128 **Chemicals**

129 DEET and permethrin (mixture of cis and trans isomers) were acquired from Sigma-Aldrich
130 (PESTANAL[®], analytical standards); picaridin was a gift from Dr. Kamal Chauhan (USDA-
131 ARS, Beltsville) (Leal et al. 2017). Stock solutions (10% m/v) were prepared in dimethyl
132 sulfoxide (DMSO) and diluted to 1% when needed. The blood mixtures were prepared by mixing
133 180 μL of defibrinated sheep blood (UCD, VetMed) with 20 μL of a 10% solution (of DEET,
134 picaridin, or permethrin) to give a final concentration of 1%. The control was prepared in the
135 same manner but using only DMSO.

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138 **Behavioral measurements**

139 Fifty female mosquitoes (6 days after emergence) were aspirated and transferred to the arena 2
140 hours before each experiment. All openings were sealed, and the cage was kept near the base of
141 the arena. Thirty minutes after the water started circulating, the assay cage was then inserted into
142 the base (Fig. 1). Aliquots (200 μL) of blood mixed with DMSO only or DEET in DMSO were
143 gently pipetted onto one end of a piece of dental cotton (Primo Dental Products, #2 Medium) to
144 make a blood circle on the cotton. A strip of Parafilm sealing film (ca. 8 x 5 cm) was stretched
145 fully along the length and then wrapped around the cotton roll, covering the surface twice. To
146 distinguish the treatment from the control group, a snipped insect pin (BioQuip, black enameled
147 No.5) was tagged at the back of the cotton by a small piece of Parafilm. The sealed cotton rolls
148 were placed in between the CO₂ dispensing needles and the Dudley tube. Five microliters (the
149 amount of 1 blood meal (Nikbakhtzadeh et al. 2016)) of defibrinated sheep blood were smeared
150 onto the surface of the Parafilm (to prime mosquitoes to start feeding). CO₂ flow was initiated,

151 and the assay was recorded with a camcorder equipped with a Super NightShot Plus infrared
152 system (Sony Digital Handycam, DCR-DVD 910). After 30 min, insects were gently removed
153 from the cotton rolls, and the assays were reinitiated with fresh sealed cotton rolls with switched
154 positions. For each group of tested mosquitoes, test and control were placed at least twice on
155 each side of the arena.

156

157 **Statistical analysis**

158 Behavioral observations were not done in real time, but rather by retrieving the recorded videos.
159 Mosquito-feeding duration was counted only after the blood used for priming was already dried.
160 For measuring feeding time, we selected mosquitoes that clearly pierced the membrane by
161 forcing its head down towards blood, stopped movement of the head and the body, and started
162 waving the hind leg while the stylets were inserted. Once all these steps were observed, we
163 rewound the tape and started counting the feeding time. End of feeding was determined when the
164 proboscis was removed and mosquitoes walked away. We preferred mosquitoes that were
165 feeding solitarily rather than in groups so as to avoid interruption of feeding by other
166 mosquitoes' interference. We limited observations to at most 10 mosquitoes per assay, but each
167 experiment was replicated 3-9 times and comparisons were made at least 30 times. Treatments
168 and their controls were compared by 2-tailed Wilcoxon matched-pairs signed rank tests using
169 Prism 7 (GraphPad, La Jolla, CA).

170

171 **Results and discussion**

172 **Behavioral responses**

173 Upon retrieving the videos, it became clear that contact repellency was not involved. Indeed, the
174 mean duration of landings on the treatment side of the arena did not differ significantly
175 (Wilcoxon 2-tailed, matched-pairs signed rank test, $n=3$, $P<0.05$) from the mean duration of
176 landings on the control side (Fig. 2A). Additionally, the mean time that mosquitoes spent on the
177 Parafilm-covered blood spiked with DEET did not differ from the mean time spent on the
178 surface covering blood devoid of DEET (Fig. 2B). Of note, this "residence time" on the Parafilm
179 surfaces was recorded from the time mosquitoes landed and before feeding was initiated. As far
180 as contact is concerned, mosquitoes behaved similarly when landing on the surfaces covering

181 blood spiked with DEET or loaded with blood plus solvent. These observations suggest that
182 DEET did not leak from the blood to the outer surface of the paraffin film. Therefore, the feeding
183 times we measured next were not influenced by repellency upon contact with the surfaces. We
184 observed that mosquitoes probed similarly on both sides of the arena; the difference in behavior
185 was observed once they had initiated a blood meal (Video 1). Mosquitoes spent significantly
186 more time (91.8 ± 12.1 s) feeding on the control side of the arena than on cotton rolls loaded with
187 0.1% DEET-spiked blood (32.7 ± 4.2 s, $n=30$; $P < 0.0001$, Prism notation: *****) (Fig. 3A).
188 Likewise, they spent significantly less time feeding on 1% DEET-spiked blood (30.8 ± 2.1 s,
189 $n=90$) than on blood with solvent only (78.6 ± 8.2 s, $n=90$; $P < 0.0001$, *****) (Fig. 3B).
190 Surprisingly, there was no significant difference in the time feeding on blood spiked with 1%
191 picaridin (76.6 ± 11.2 s) compared with its control (89.0 ± 7.2 s, $n = 60$; $P = 0.0364$) (Fig. 3C).
192 Although all samples were freshly prepared and tested, we cannot rule out the possibility that
193 picaridin degraded more rapidly upon being mixed with blood.
194 It has been demonstrated that a DEET-sensitive odorant receptor from the southern house
195 mosquito, CquiOR136, (Xu et al. 2014) is also expressed in the tip of the labrum (Choo et al.
196 2015). Therefore, we initially surmised that mosquitoes detected DEET in the blood samples by
197 activating this receptor. The fact that this receptor is sensitive to both DEET and picaridin
198 coupled with the lack of feeding deterrence elicited by picaridin does not support this
199 assumption. It is, therefore, likely that mosquitoes detect DEET in the blood with their gustatory
200 system. Next, we tested the effect of permethrin, a compound commonly used in long-lasting
201 insecticidal nets (Kawada et al. 2014) given its dual property as an insecticide and
202 excitorepellent (Zaim et al. 2000). Of note, permethrin is neither a spatial repellent nor a ligand
203 for CquiOR136 (Xu et al. 2014). Like DEET, permethrin had a significant deterrent effect, with
204 mosquitoes feeding significantly less on permethrin-spiked blood (21.8 ± 2.8 s) than on blood
205 containing only DMSO (79.6 ± 8.8 s, $n= 60$; $P < 0.0001$, *****) (Fig. 3D).

206

207 Conclusions

208 With a modified version of the surface landing and feeding assay (Leal et al. 2017), we were able
209 to demonstrate that reduced feeding on blood spiked with DEET was due to a deterrent rather
210 than contact repellency effect. In this experimental setup, we provided blood on cotton rolls,
211 which were covered with 2 layers of Parafilm. DEET did not leak and, consequently, contact

212 repellency was not at play. This is demonstrated by the fact that mosquitoes landed randomly on
213 the various surfaces of the arena (Video 1) and that the number and duration of the landings on
214 the surface covering blood spiked with DEET did not differ from the similar data recorded for
215 the side covering blood with solvent only (Fig. 2). Upon direct contact of the stylets with blood,
216 mosquitoes prematurely terminated feeding on blood spiked with DEET and permethrin, but not
217 with picaridin. Our findings suggest that the earlier observation of “repellency” by the presence
218 of DEET (Barzeev & Smith 1959) in blood is due to “feeding deterrence.” In addition to being a
219 spatial and a contact repellent, DEET is also a feeding deterrent. Previously, it has been
220 suggested that DEET is a feeding deterrent due to contacts with treated surfaces (Klun et al.
221 2006). By contrast, our findings show that feeding is deterred by direct contact with a blood
222 meal. Whereas the 2 well-known properties of DEET are essential for reducing mosquito bites
223 and, consequently, transmission of diseases, “feeding deterrence” is of less importance in
224 medical entomology given that once mosquitoes are already in contact with the blood they may
225 have already transmitted arbovirus.

226

227 **Additional Information and Declarations**

228

229 **Competing interests**

230 No author has competing interests to disclose.

231

232 **Author contributions**

233 WSL designed the experiments and constructed the behavioral arena. WL, JH, and FZ carried
234 out the research. WL, JH, FZ, and WSL analyzed the data. WSL and WL wrote the manuscript.

235 All authors have agreed to the final content of the manuscript.

236

237 **Data Availability**

238 All raw data are provided as Supplementary Information.

239

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249 used in this research.

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292 Figure Legends

293

294 **Figure 1. Illustration of the modified arena.** (A) A Dudley tube painted black from inside was
295 flanked by 2 cotton rolls secured in place by syringe needles that delivered CO₂. Samples of
296 defibrinated sheep blood mixed with solvent only or spiked with DEET were loaded on these
297 cotton rolls, which were subsequently covered with Parafilm. (B) An aerial view of the arena.
298 Mosquitoes were placed on a mosquito cage accessible from the top and having a camera (not
299 shown) attached to the left. The Dudley tube was connected to a water bath (not shown) and the
300 syringe needles to a CO₂ tank (not shown).

301

302 **Figure 2. Measurements of landings and duration of contact with the surfaces prior to**
303 **feeding.** (A) The mean number of mosquitoes landing on the control and DEET sides of the
304 arena in 15 min did not differ significantly (Wilcoxon matched-pairs signed rank test, n=3). (B)
305 The contact times measured from the time the mosquitoes landed until they started feeding were
306 not significantly different (Wilcoxon matched-pairs signed rank test, n=7).

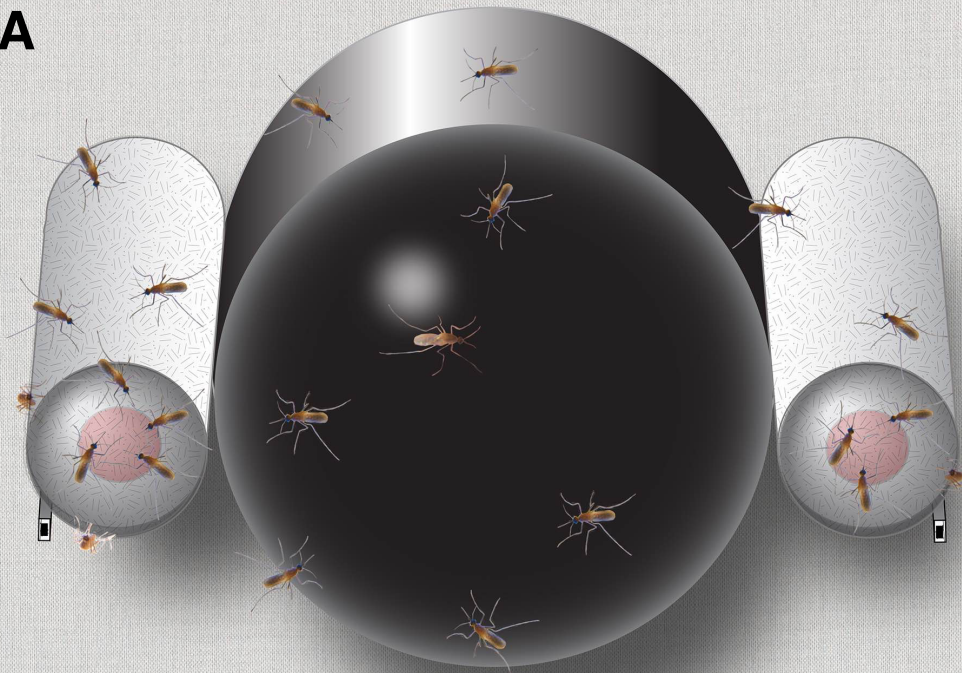
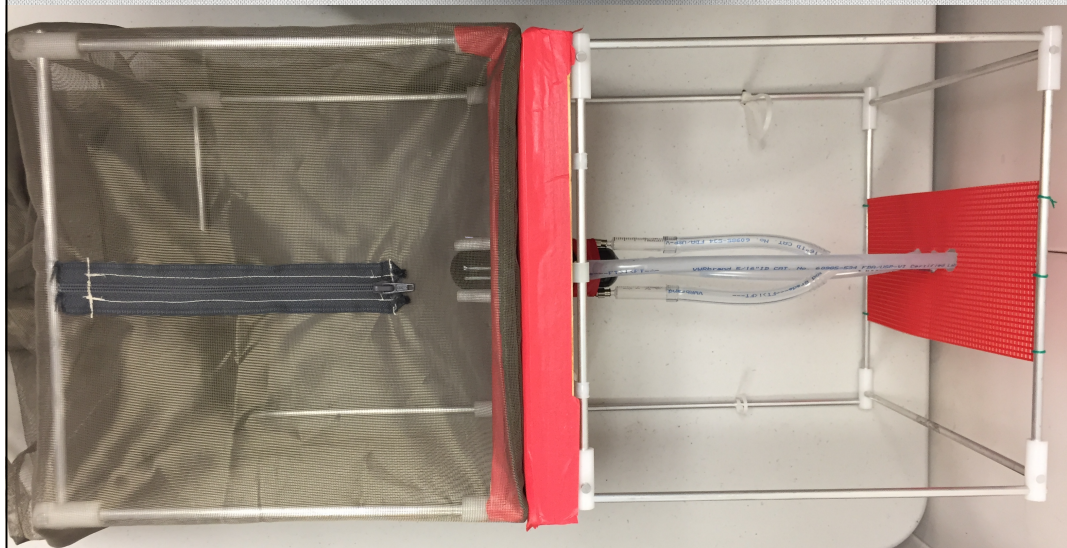
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308 **Figure 3. Comparative feeding times on blood mixed with solvent or test repellents.** (A)
309 0.1% DEET, (B) 1% DEET, (C) 1% picaridin, and (D) 1% permethrin.

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A**B****Figure 1**

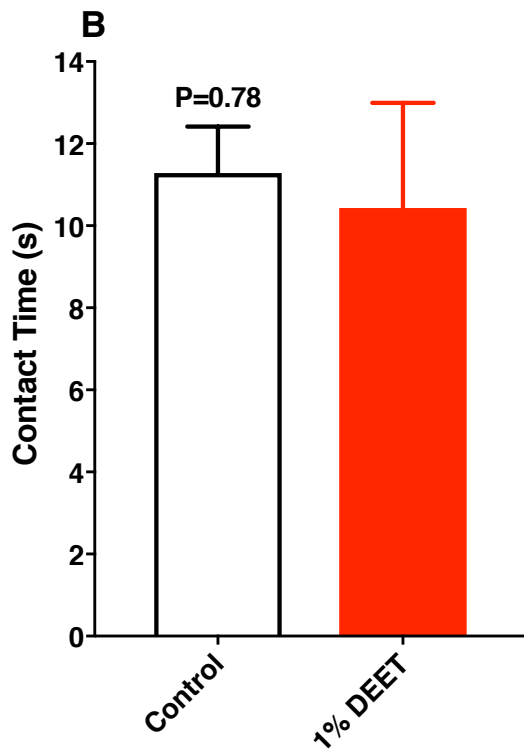
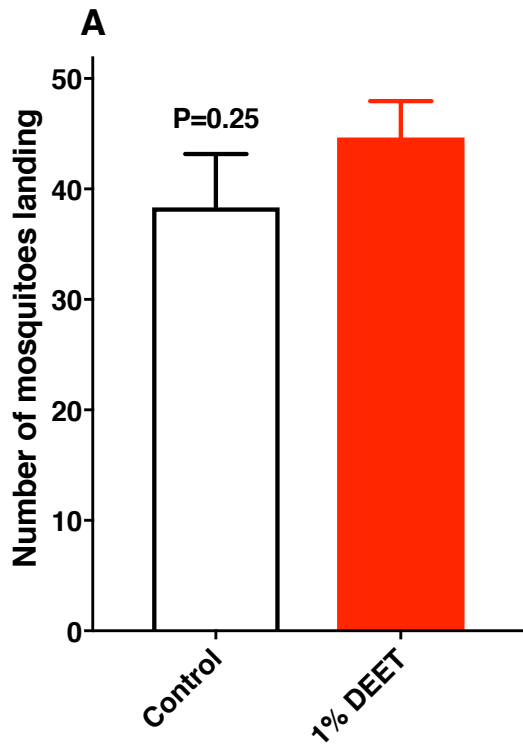


Figure 2

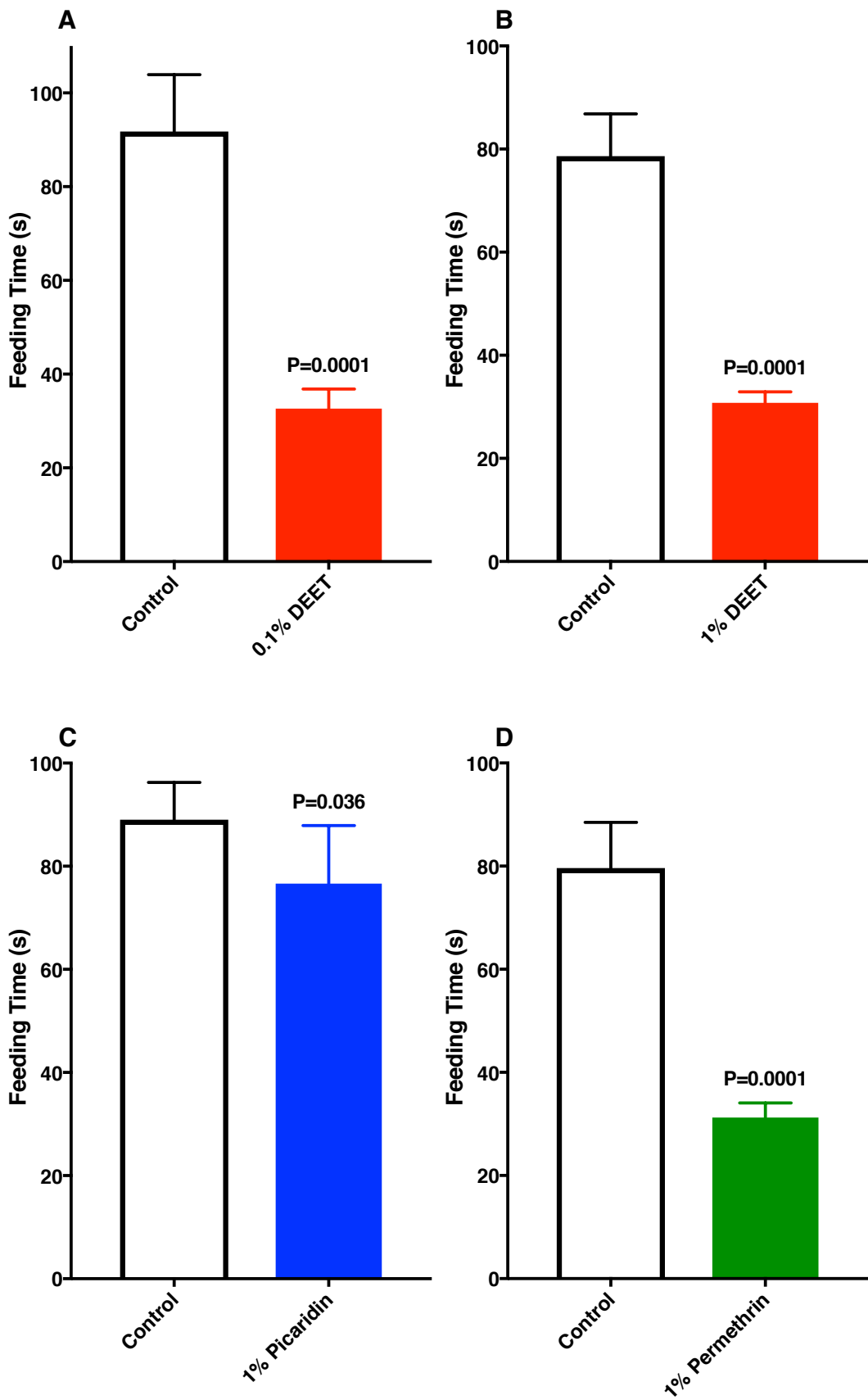


Figure 3