

1 **Noxious stimulation induces self-protective behaviour in bumblebees**

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5

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18 supervision and writing – review and editing.

19

20 **Abstract**

21 Self-grooming directed towards a noxiously-stimulated body part is one indicator that
22 an animal may feel pain. In insects, the lack of evidence for such behaviour has
23 been widely argued to reflect the absence of pain experiences. Here, we tested
24 whether bumblebees (*Bombus terrestris*) selectively groom one of their antennae
25 that was touched with a noxiously-heated (65 °C) probe. In the first two minutes
26 after being touched with the noxiously-heated probe, bees groomed their touched
27 antenna a) more than their untouched antenna, b) more than bees that were touched
28 on the antenna with an unheated probe, and c) more than control (untouched) bees
29 groomed either of their antennae. Our results clearly show that bumblebees can
30 direct grooming towards a site of noxious stimulation. Our findings thus refute
31 arguments that claim that insects do not feel pain because of their lack of displaying
32 this behaviour.

33

34 **Keywords:**

35 Grooming, insects, nociception, pain, self-protective behaviour

36

37 **Background**

38 Nociception is the detection and processing of noxious stimuli [1], whilst pain is the
39 negative subjective feeling that, at least in humans, typically accompanies
40 nociception [2]. Nociception can be identified from neural activity within nociceptive
41 circuits [3] or from the performance of behaviours that require nociceptive circuits [4].
42 The subjective experience of pain, however, is harder to identify [5]. Even the gold-
43 standard evidence for the subjective experience of pain in humans, verbal self-
44 report, can be unreliable [6,7]. This issue is amplified in other animal species
45 because they cannot verbally describe their pain. However, sets of neural and
46 behavioural indicators to evaluate pain have been developed that attempt to assess
47 whether an animal has the neural capacity and behavioural ability to feel pain [8–
48 10].

49 One behavioural indicator of pain is self-protective behaviour directed towards
50 a site of noxious stimulation [8–11]. Examples include tending to, guarding,
51 grooming, and rubbing a noxiously stimulated body part. Consider, for example, the
52 human response to a stubbed toe or a bump on the head to ‘rub it better’, thereby
53 reducing the feeling of pain [12,13]. This self-touch reduces pain, and not just
54 nociception, since pain can be affected with no effect on nociceptive processing
55 [14,15]. An example of this was demonstrated using the thermal grill illusion, where
56 some fingers are warmed while the other fingers are cooled, and, in doing so, the
57 person perceives noxious heat; self-touch (touching their other hand with the
58 affected hand) reduced perception of heat by 64% compared to controls [14]. This is
59 one reason that the Checklist of Nonverbal Pain Indicators in humans includes
60 ‘massaging or clutching the affected area’ [16]. Therefore, the presence of this
61 behaviour is used to indicate that the individual is feeling pain, because it suggests
62 that the person might both be internally representing the bodily location of the
63 aversive stimulus and trying to reduce the pain [17].

64 Self-protective behaviour is also observed in other vertebrates. For example,
65 rats (*Rattus norvegicus*) rub their face more after their face is injected with a noxious
66 substance [18]. Further, some bird species have been observed grooming a limb that
67 was injected with a noxious substance (e.g. *Pyrrhura molinae*: 19). There are similar
68 findings in fish (*Oncorhynchus mykiss*), where the fish rubbed an area that was

69 treated with a noxious injection into the gravel and the sides of their tank [20]. In
70 these studies, the grooming of the noxiously-stimulated area was taken as evidence
71 for the presence of pain in these non-human animals.

72 Some invertebrates have also been observed performing self-protective
73 behaviour, in the form of grooming a noxiously-stimulated site. For example, injecting
74 formalin into the claw of Asian shore crabs (*Hemigrapsus sanguines*) induced
75 rubbing of the affected claw [21]. Similarly, applying acetic acid to various body parts
76 induces grooming or scratching in shore crabs (*Carcinus maenas*) [22], prawns
77 (*Palaemon elegans*) [23], cuttlefish (*Sepia pharaoensis*) [24] and octopuses
78 (*Octopus bocki*) [25]. The latter also respond with grooming to an area on their arm
79 that was crushed with forceps for up to 20 seconds [26].

80 With regards to insects, however, there are no quantitative studies of self-
81 protective behaviour (such as grooming) towards a noxiously-stimulated site [27]. In
82 addition, anecdotal reports claim that insects do not protect their injury sites, and that
83 insects continue to walk, feed, and mate normally after injury [28; 29]. These reports
84 are often cited as evidence against insects experiencing pain [30–32].

85 Insects are known to self-groom in non-noxious contexts, e.g. during general
86 cleaning [33], and when removing dust particles (e.g. in the *Blattella germanica*
87 German cockroach [34]), pollen grains (e.g. in bees: 35) and parasites such as mites
88 (e.g. in honeybees, *Apis mellifera*: 27). After noxious stimulation, insects may also
89 generally groom more or change their grooming pattern. For example, after having
90 their antenna amputated, red mason bees (*Osmia bicornis*) wipe, or groom, the head
91 and body, although no site-specific measurements were found/taken, nor was there
92 a non-noxious control to compare to [37]. There are also reports that hint insects
93 may self-groom noxiously-stimulated sites, although such reports have not yet been
94 supported by quantitative or statistical analyses [38]. When pinched on the
95 abdominal proleg, *Manduca sexta* moth larvae reportedly turned their heads to the
96 wound, and repeatedly touched the area with their mouthparts, but this behaviour
97 was not measured or compared to a control [39]. *Periplaneta americana*
98 cockroaches appeared to groom their wounds following an abdominal puncture but,
99 again, this behaviour was not measured or compared to a control [40]. Since these
100 observations were not supported by quantitative measurements or analyses [38], a
101 robust, experimental assessment of grooming behaviour in response to noxious
102 stimuli, and as an indicator of pain in insects, is required.

103 In this study, we tested whether *Bombus terrestris* bumblebees selectively
104 groom a noxiously-stimulated antenna. For each bumblebee, we either briefly
105 touched one antenna with a noxious stimulus (a 65°C heat probe), or a non-noxious
106 tactile stimulus (an unheated probe), or we did not touch either antenna (control). We
107 recorded grooming behaviour on both antennae for 25 minutes.

108 If bees direct self-grooming towards a site of noxious stimulation, we would
109 predict more grooming on the noxiously-stimulated antenna than the other antenna.
110 We would not expect this difference in bees touched with an unheated probe, nor by
111 bees that were not touched.

112

113 **Methods**

114

115 *Ethics*

116 The UK does not regulate insect welfare in research. Nonetheless, we followed the
117 3Rs principles [41] in our experimental design and husbandry. In this vein, although
118 some noxious stimulation is required to study pain, we chose a temperature that,
119 when brief, has no long-term effects on the bees (65°C; based on [35]). We also
120 used a power analysis to estimate the minimum required sample size (estimated
121 sample size = 80; alpha: 0.05; power: 80%). According to current best practice, we
122 have followed the ARRIVE guidelines for reporting this research [43].

123

124 *Animals and Housing*

125 We used 82 bees from seven bumblebee colonies (standard hives from Biobest
126 Group, Belgium). The bees were housed in ventilated wooden boxes (56×16×11cm;
127 see Figure 1). Each box comprised four sections, arranged linearly and connected
128 by 1cm-diameter holes. At one end was the section containing the nest, which was
129 covered with plywood. The section at the opposite end contained a 35 ml cylindrical
130 feeder (74.5×31 mm), which dispensed Biogluc sugar solution *ad libitum* (Biobest
131 group, Belgium). To access the food source, the bees had to cross the middle two
132 sections. The middle section adjacent to the feeding section was the observation box
133 during the testing period. The floor of both middle sections was covered with a thin
134 layer of cat litter (Catsan Hygiene Plus, Mars Inc, USA) to absorb waste and debris.
135 Each colony received 7g of pollen (Natupol Pollen, Koppert Biological Systems)
136 every two days, and the laboratory was maintained at 23° C.

137

138

139 **Figure 1.** Housing and testing apparatus. A ventilated wooden box (56x16x11 cm) with four
140 sections. The nest section was covered with plywood. The feeding section contained a feeder
141 with *ab libitum* food. The observation box was adjacent to the feeding section.

142

143 *Treatments*

144 For testing, we removed bees individually from the nest box by letting them walk
145 onto metal forceps and placed them into a marking cage (Thorne, UK). A sponge in
146 the marking cage was used to temporarily immobilize the bees to ensure precisely
147 targeted noxious stimulation. A soldering iron (HAKKO FX-888D; Japan) was either
148 heated to 65°C (noxious condition) or unheated (tactile condition), then touched onto
149 the right or left antenna (counterbalanced across bees) for five seconds. We chose
150 this method of noxious stimulation based on how stimulation of a honeybee's (*Apis*
151 *mellifera*) antenna with a 65°C heat probe causes consistent sting extension reflexes
152 [44] (a defense reflex seen in response to noxious stimuli [45]). Thirty bees were
153 touched with the noxiously-heated probe (noxiously-stimulated; N = 30); 28 were
154 touched with the control unheated probe (tactilely-stimulated; N = 28); and 24 were
155 put in the marking cage but not touched with a probe (control: N = 24). No bees were
156 excluded from the analysis. We used an RST Soldering Iron Tip Thermometer 191
157 (YWBL- WH; China) to test the temperature of the soldering iron. After the treatment,
158 bees were immediately placed in the observation box and filmed with an iPhone 8
159 (Apple; USA) for 25 minutes. We sealed the holes between boxes during the
160 experiment, so bees were confined to the observation box (14x16x11 cm). We
161 sexed each bee visually from the videos, based on the presence (in females) or
162 absence (in males) of a black abdomen tip. There were 40 females and 18 males;
163 sex was then accounted for in the statistical analysis.

164

165 *Behavioural analysis*

166 Four treatment-blind coders recorded the grooming behaviour displayed in the 25-
167 minute videos using BORIS behavioural analysis software (BORIS, version 7.9.15;
168 Italy). Grooming was defined as 'the right or left front, middle, or hind leg moves over
169 the left or right antenna either in one direction or in a repeated back and forth
170 motion'. To measure inter-rater reliability, all four raters recorded grooming

171 behaviour for two bees (corresponding to two 25-minute videos: one noxiously-
172 stimulated bee and one tactilely-stimulated bee). Because the rating scale was
173 continuous, we calculated the intra-class correlation coefficient. The correlation
174 compared the total grooming duration of the right and left antenna across the four
175 raters. The coefficient was 0.86, on a scale of 0-1, indicating a 'good' reliability [46].
176

177 *Statistical analysis*

178 We analysed the data in R (R Core Team, Cran-r-project, Vienna, Austria, version
179 2022.12.0+353), using generalised linear mixed effect models (GLMMs; packages:
180 'lme4' (Bates et al., 2015) and 'car' (Fox et al., 2021)) and Wilcoxon tests. We
181 checked model assumptions using histograms and 'Q-Q plots', and corrected for
182 multiple testing using the Holm-Bonferroni correction [48]. We considered $p < 0.05$
183 significant.

184 To test for a difference between the grooming duration on the touched versus
185 untouched antenna in noxiously-stimulated and tactilely-stimulated bees, we ran a
186 GLMM. The response variable was the duration of antennal grooming for each
187 antenna per bee. The fixed effects were stimulation type (noxious or tactile), whether
188 the antenna was touched or untouched, the sex of the bee and their interaction. The
189 random effect was the bee identity. We ran this model for the whole observation
190 period (25 minutes), as well as individual time bins 0-1, 0-2, 0-3, 0-4 0-5, 6-10, 11-
191 15, 16-20 and 21-25 minutes. We tested the individual time bins because some
192 previous invertebrate studies have only detected self-grooming within the first few
193 minutes after stimulation [21–23]. The only time bin with a significant interaction
194 effect (after applying the Holm-Bonferroni correction for multiple testing) was 0-2
195 minutes, so this is the only time bin we ran the other GLMM and Wilcoxon tests on
196 (described below).

197 We used unpaired two-sample Wilcoxon tests (as our data did not meet the
198 criteria for parametric analysis) to test the difference between the grooming durations
199 on the touched or untouched antenna in the tactile and noxious treatment groups in
200 the first two minutes after stimulation.

201 To test for a difference between grooming durations on either the touched or
202 untouched antenna in the noxiously-stimulated and tactilely-stimulated bees, and the
203 mean grooming duration for both antennae in bees in the control condition in the first
204 two minutes, we ran another GLMM. The response variable was either the duration

205 of touched antennal grooming per bee or the duration of untouched antennal
206 grooming per bee (for control bees, the mean grooming on one antenna was used,
207 because neither antenna was touched in this condition). The fixed effects were the
208 stimulation type (noxious, tactile, control) and the sex of the bee. The random effect
209 was bee identity.

210

211

212 **Results**

213 We first tested whether there was a difference between grooming durations on the
214 touched and untouched antennae, and, if so, whether this difference was larger
215 when the probe was noxiously heated. For the whole 25-minute observation period,
216 bees groomed their touched antenna significantly more (touched: 18.11 ± 26.79
217 seconds; untouched: 2.22 ± 3.57 seconds; $t_{5792} = 5.922$; $p < 0.001$; $N = 40$), regardless
218 of whether the stimulation was noxious or non-noxious tactile (no significant effect:
219 $t_{5792} = 0.056$, $p = 0.955$; $N = 40$; no significant interaction: $t_{5792} = -0.224$, $p = 0.822$; $N =$
220 40 ; Figure 2). Therefore, over the 25 minutes, grooming was directed towards the
221 touched antenna, but not the noxiously-stimulated antenna specifically.

222 We also observed a significant interaction effect of sex on the total grooming
223 duration over the 25 minutes, with females grooming their touched antenna (and not
224 their untouched antenna) significantly longer than males (females: $N = 40$; touched
225 antenna: 22.89 ± 30.29 seconds; untouched antenna: 2.60 ± 3.78 seconds; males: $N =$
226 18 ; touched antenna: 7.59 ± 11.33 seconds; untouched antenna: 1.36 ± 2.96 seconds;
227 $t_{5792} = -2.665$; $p < 0.01$).

228

229

230 **Figure 2.** Duration of grooming for the untouched and touched antenna per each minute after
231 noxious or tactile stimulation. P-values < 0.001 are represented by “**”; non-significant p-values
232 are not represented in this graph.

233

234 However, in the 0-2 minute time bin (the only time bin with a significant p-value after
235 applying the Holm-Bonferroni correction), bees groomed the touched antenna more
236 than the untouched antenna when the touch was noxious (significant interaction: t_{459}
237 $= 3.069$, $p < 0.005$; $N = 40$). This result was clarified by Wilcoxon tests: in this time
238 bin, noxiously-stimulated bees groomed their touched antenna (6.65 ± 8.8 seconds)

239 significantly more than their untouched antenna (0.75 ± 1.95 seconds; $W = 249.5$, $p <$
240 0.001 ; $N = 30$; Figure 3). By contrast, for tactilely-stimulated bees, there was no
241 difference in grooming between the touched antenna (1.19 ± 2.23) and the untouched
242 antenna (0.55 ± 1.57 seconds; $W = 324$, $p = 0.159$; $N = 28$; Figure 3). There was no
243 difference in the antennal grooming durations for male and female bees ($t_{459} = -0.851$,
244 $p = 0.395$; $N = 40$).

245

246 We then tested whether the duration of antennal grooming was greater for either the
247 noxiously-stimulated or the tactilely-stimulated bees compared to the control bees.
248 Noxiously-stimulated bees groomed their touched, and not their untouched, antenna
249 for longer than the control bees groomed either antenna (touched: 2.85 ± 5.48
250 seconds; $t_{75} = 2.55$, $p = 0.0127$; $N = 54$; untouched: 0.50 ± 1.64 ; $t_{75} = -0.318$, $p =$
251 0.752 ; $N = 54$; either antenna: 0.57 ± 2.14 ; Figure 3). There was no significant effect
252 of sex on either the grooming in touched or untouched conditions (touched: $t_{75} = -$
253 0.111 , $p = 9.117$; $N = 54$; untouched: $t_{75} = -1.493$; $p = 0.140$; $N = 54$). By contrast,
254 tactilely-stimulated bees did not groom either their touched or untouched antennae
255 significantly more than the control bees groomed either antenna (touched:
256 0.77 ± 1.84 ; $t_{73} = -0.404$, $p = 0.689$; $N = 52$; untouched: 0.48 ± 1.54 ; $t_{73} = 0.228$, $p =$
257 0.821 ; $N = 52$; Figure 3; either antenna: 0.57 ± 2.14). There was no significant effect
258 of sex on either the grooming in touched or untouched conditions (touched: $t_{75} = -$
259 0.127 , $p = 0.210$; $N = 52$; untouched: $t_{75} = -1.875$; $p = 0.065$; $N = 52$). Similarly,
260 noxiously-stimulated bees groomed significantly more than the tactilely-stimulated
261 bees on the touched antenna ($t_{83} = 2.885$, $p < 0.005$; $N = 40$; Figure 3), but not on the
262 untouched antenna ($t_{83} = 0.647$, $p = 0.519$; $N = 40$; Figure 3). There was also no
263 significant effect of sex on either the grooming in touched or untouched conditions
264 (touched: $t_{83} = 0.253$, $p = 0.800$; $N = 40$; untouched: $t_{83} = -1.273$; $p = 0.207$; $N = 40$).

265

266

267 **Figure 3.** Duration of grooming each antenna for each treatment group. Box plot boundaries
268 indicate the 25th and 75th percentiles; the whiskers indicate the minimum and maximum values
269 within 1.5 times the interquartile range. Crosses indicate values outside this range (boxplot
270 outliers); triangles indicate the mean; lines indicate the median. P-values < 0.001 are
271 represented by ‘**’, and p-values > 0.1 are represented by ‘NS’.

272

274 **Discussion**

275 No previous studies have experimentally tested and quantifiably measured whether
276 insects groom noxiously stimulated sites. This lack of evidence, as well as anecdotal
277 reports of insects not demonstrating self-protective behaviour in other ways, has
278 been used to argue that insects do not experience pain [30–32]. Our results provide
279 clear, quantitative evidence of self-protective behaviour in insects.

280 In the first two minutes after stimulation, bees targeted grooming towards a
281 noxiously-stimulated antenna, but not towards a tactilely-stimulated one. Noxiously-
282 stimulated bees groomed their touched antenna more than their untouched antenna,
283 more than tactilely-stimulated bees groomed their touched antenna, and more than
284 control bees groomed either antenna. The same results were not found in tactilely-
285 stimulated bees. These results were also only found in the first two minutes.
286 Although there is an increase in grooming duration after noxious stimulation
287 compared to tactile stimulation in the first minute (Figure 2), this increase is not
288 significant after correction for multiple comparisons. This could be explained by the
289 use of the Holm-Bonferroni correction, which has a high risk of false negatives [49].
290 There was a significant effect of sex on grooming in the first 25 minutes, with
291 females grooming their touched antenna more than males. This is a potentially
292 interesting result that could pave the way for some future research into sex
293 differences in insects' nociceptive responses. In this study, however, it does not
294 affect the main result, so will not be discussed further.

295 Our results may hint at a potential control mechanism for nociception in
296 insects. In mammals, rubbing a noxiously-stimulated site activates A-beta fibers [50].
297 The gate control theory posits that these fibers activate inhibitory interneurons in the
298 dorsal horn of the spinal cord, which can inhibit the nociceptive signal's progression
299 to the brain [51,52]. This gated control means that 'rubbing it better' reduces
300 nociceptive processing [12,13]. Self-grooming bumblebees may activate a similar
301 mechanism. In honeybee antennae, thermo-sensory neurons detect the nociceptive
302 stimulus and carry the information to the antennal lobe [53], and then, possibly, to a
303 nociceptive thermal center in the brain [44]. The 'gate' could involve activating
304 campaniform sensilla neurons in the antenna, although it is, so far, unclear how this
305 might inhibit nociceptive processing. Further, some human experiments suggest that
306 self-touch reduces the conscious aspect of pain, rather than just nociceptive
307 processing [14,15]. Therefore, in particular, because of our results and other

308 accumulating evidence for pain in insects, another mechanistic possibility is that
309 grooming acts as a control mechanism for pain in bumblebees. Again, though, it is
310 currently unclear how this might happen in insects.

311 Directed grooming of the noxiously-stimulated antenna only occurred in the 0-
312 2 minute time bin. This timing is consistent with some studies on other invertebrates,
313 which describe self-grooming in the first few minutes after noxious stimulation (17–
314 19). A reason for this timing might be that the nociceptive processing of the heat
315 stimulation ceased after around two minutes; this would likely depend on the
316 intensity of the noxious stimulus used. An association between grooming and the
317 cessation or onset of nociceptive processing has been previously seen in mice, in
318 response to formalin injection. There is an acute grooming phase, which apparently
319 relates to the injection itself and lasts 3 minutes, then no grooming is seen for
320 another 3 minutes, followed by a tonic phase that is longer-lasting and appears to
321 correspond to formalin's inflammatory effects [54–56]. By analogy, we suggest that,
322 in our study, the first two minutes corresponded to an acute phase of grooming in
323 response to the noxious heat stimulation. Based on this evidence, future research
324 should investigate the neural processing of noxious heat stimulation in insects, and
325 how the temporal characteristics of the grooming compare to the neural processing.

326 The experiment contained multiple novel and/or potentially stressful
327 experiences and environments for the bees. For example, the stimulation itself
328 involved them climbing onto metal forceps, being lifted out of the nest box, and
329 immobilized during the stimulation - all potential stressors. Further, bees were
330 isolated from the nest and other colony members during testing, and their normal
331 route back to the nest was blocked. This means that the grooming we observed with
332 this set-up may only be a fraction of the bees' natural response, when not under
333 stress or in a novel environment. Stress and novel contexts reduce the expression of
334 behaviours after noxious stimulation in insects (honeybees: 57), similarly to other
335 taxa (humans: 58; rodents: 59, fish: 60, birds: 61 and snails: 62). In future
336 experiments, observing bees in the nest post-stimulation may reflect a more
337 naturalistic behaviour.

338 Our study alone does not prove pain in insects, as there are multiple criteria
339 for the experience of pain [8,9]. However, evidence for many of these other criteria
340 has been shown in insects (see 37 for review). Importantly, our results provide clear
341 evidence for a strong indicator of pain, self-protective behaviour [8], and undermine

342 the claim that insects do not feel pain because they fail to show this behaviour. It is
343 important to note that similar self-protective behaviour has been considered to be an
344 indicator of pain in many other taxa, including crustaceans (21; molluscs (24; 25; 26),
345 rodents [18], birds (19) and fish [20]. Excluding bumblebees from the same
346 interpretation would be logically inconsistent.

347

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356

357

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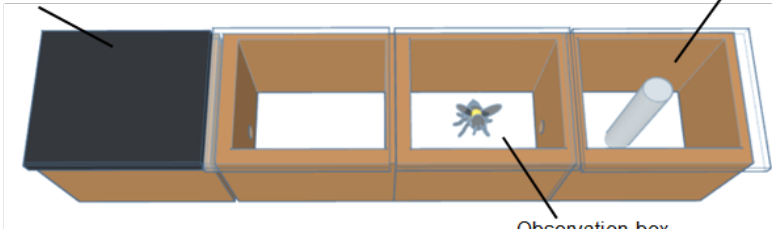
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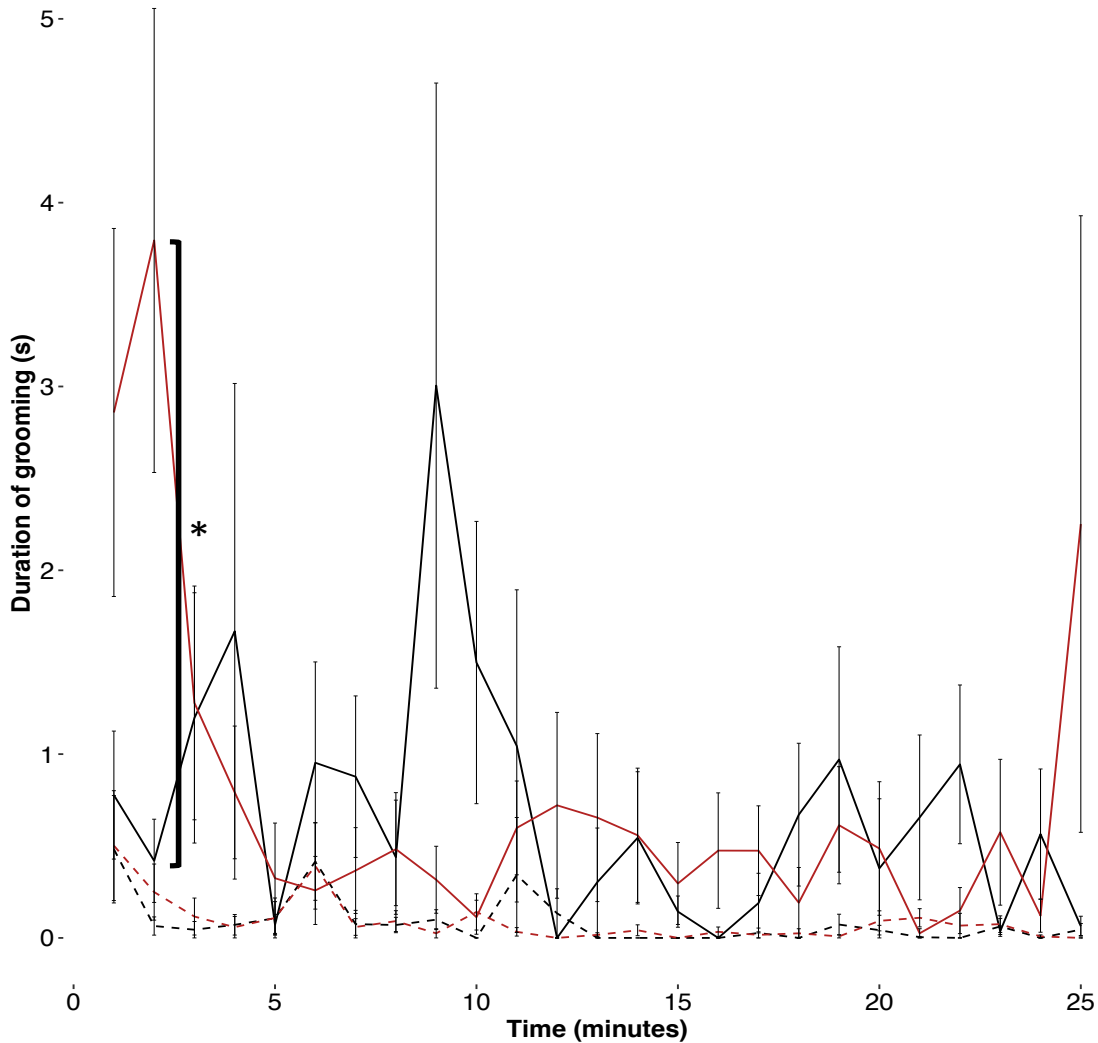
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Nest section

Feeding section





- Tactile stimulation, touched antenna
- Noxious stimulation, touched antenna
- - - Tactile stimulation, untouched antenna
- - - Noxious stimulation, untouched antenna

