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1 **Potential nematode alarm pheromone induces acute avoidance in *Caenorhabditis*
2 *elegans***

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12 **ABSTRACT**

13 It is crucial for animal survival to detect dangers such as predators. A good indicator of
14 dangers is injury of conspecifics. Here we show that fluids released from injured
15 conspecifics invoke acute avoidance in both free-living and parasitic nematodes.
16 *Caenorhabditis elegans* avoids extracts from closely related nematode species but not
17 fruit fly larvae. The worm extracts have no impact on animal lifespan, suggesting that the
18 worm extract may function as an alarm instead of inflict physical harm. Avoidance of the
19 worm extract requires the function of a cGMP signaling pathway that includes the
20 cGMP-gated channel TAX-2/TAX-4 in the amphid sensory neurons ASI and ASK.
21 Genetic evidence indicates that the avoidance behavior is modulated by the
22 neurotransmitters GABA and serotonin, two common targets of anxiolytic drugs.
23 Together, these data support a model that nematodes use a nematode-specific alarm
24 pheromone to detect conspecific injury.

25

26 **Keywords:** *Caenorhabditis elegans*, pheromone, olfactory, chemotaxis

27 **INTRODUCTION**

28 Detecting danger is crucial for animal survival. Alarm pheromones are used to
29 communicate danger by many animal species such as sea anemones, insects, fishes, and
30 mammals (Wyatt 2003). Even humans have alarm pheromones (Mujica-Parodi *et al.*
31 2009). In these animals, chemical cues are released from injured or stressed animals, and
32 detected by conspecifics or closely-related species to invoke innate alarm responses such
33 as fleeing. Chemical compositions of alarm pheromones are often species specific, e.g.,
34 anthopleurine in sea anemone (Howe and Sheikh 1975), CO_2 in fruit flies (Suh *et al.*
35 2004), chondroitin fragments in zebrafish (Mathuru *et al.* 2012), 2-*sec*-butyl-4,5-
36 dihydrothiazole in mice (Brechbühl *et al.* 2013b). The olfactory pathways that detect
37 alarm pheromones largely consist of odorant receptors, G proteins (e.g., Gαq in flies, Gαi
38 in fish, Gαo and Gαi in mice), and a second messenger (e.g., cAMP in fish, cGMP in
39 mice) (Enjin and Suh 2013).

40 Surprisingly, it remains unclear whether there is an alarm pheromone in
41 nematodes, considering that alarm pheromones exist in a wide variety of animals (Wyatt
42 2003) and that nematodes are the most abundant animals on earth (Lorenzen 1994).
43 Nematodes are known to use a class of small molecules called ascarosides as pheromones
44 to regulate behaviors such as mate-finding and aggregation (Ludewig 2013). However,
45 there is no published report of an alarm pheromone in the nematodes.

46 Here we present evidence of a potential nematode alarm pheromone in the
47 internal fluid released from injured worms. The fluid induces an acute avoidance without
48 inflicting physical harm. This avoidance signal appears ascaroside-independent and
49 conserved among multiple nematode species. In *C. elegans*, detection of this signal

50 requires a cGMP signaling pathway. Together, these data suggest the existence of a
51 nematode alarm signal.

52
METHODS

53
Animal maintenance

54 *C. elegans* strains were cultured on nematode growth medium (NGM) with OP50
55 *E. coli* at 20°C as previously described (Stiernagle 2006). N2 (Bristol) was used as the
56 wild-type strain. All worm strains were obtained from the *Caenorhabditis* Genetics
57 Center (CGC) except *daf-37(ttTi3058)* from the Centre National de la Recherche
58 Scientifique (CNRS), *goa-1(sy192)* from the Sternberg lab, *srbc-64(tm1946)* and *srbc-*
59 *66(tm2943)* from the Sengupta lab, and *Steinernema carpocapsae* from the Hallem lab.
60 Un-outcrossed strains that showed as a hit in chemoavoidance assays were outcrossed six
61 times and tested again. Detailed information of all mutant strains is listed in Table S1.

62 Unless otherwise specified, day-one adult hermaphrodites were used in our
63 behavioral assays. Synchronized L1s were collected by bleaching gravid adults as
64 described (Stiernagle 2006) and cultured on OP50 plates till they reached adulthood.

65 To obtain starved worms, we washed well-fed young adult worms off the plates
66 into M9 buffer. The worms were washed three additional times in M9 and placed in M9
67 at a concentration of 1 worm/μl. Control worms were placed in M9 with 1% OP50. Both
68 groups were incubated at 20°C and tested after 1, 3, and 5 hours of starvation.

69 To collect dauers, *C. elegans* plates were starved for five additional days after the
70 worms cleared the bacterial lawn. Five holes were made on the wall of each plate above
71 the agar level using a flamed needle. Five 100 μl drops of sterile water were placed on

72 each lid where the plate wall would touch. The plates were placed upside down sitting on
73 the lids overnight. The water drops on the lids were then collected and examined for
74 dauers.

75 *Steinernema carpocapsae* were cultured as described (Ehlers and Shapiro-Ilan
76 2005). Five waxworms (PetSmart) were placed in a 60 mm petri dish lined with filter
77 paper (55 mm, Whatman). 200 µl of water containing about 100 infective juveniles (IJs)
78 were dropped on top and around each waxworm. Waxworms were examined 48 hour
79 after infection to ensure they were dead. The Petri dish was kept in dark at room
80 temperature for 5-8 more days until all waxworms flattened and dried.

81 *Steinernema* IJs were harvested using the white trap method (White 1927). A 70
82 mm filter paper (Whatman) was placed on a raised island in a 100 mm Petri dish.
83 Distilled water was added to the level of the filter paper. Dried infected waxworms were
84 placed in the middle of the wet filter paper, and left for 7-10 days. The water containing
85 IJs was then collected. Freshly-collected IJs were used immediately for experiments, or
86 washed three times in water, resuspended in 10ml water in a 25 cm^2 culture bottle
87 (Falcon, cat#353014) and stored at 15°C as stock.

88 **Worm extract**

89 Animals were washed off from NGM plates for small-scale experiments or
90 collected from liquid culture (Stiernagle 2006) for large-scale experiments. Animals were
91 washed more than three times in M9 buffer (0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.5% NaCl,
92 1 mM $MgSO_4$). The wash was to remove the culture media because they are known to
93 repel *C. elegans* hermaphrodites and attract males because they contain ascarosides
94 (Simon and Sternberg 2002). Unless noted otherwise, worms were put in a 100°C water-

95 bath to be instantly killed. They were then homogenized using a pestle or sonication. The
96 mixture was centrifuged and the supernatant was collected as worm extract. The worm
97 extract was filtered using a 0.22 µm syringe filter (Millipore), and stored in aliquots at -
98 20°C. To obtain fly extract, wandering third instar *Drosophila melanogaster* larvae were
99 collected, washed three times in M9 buffer, and homogenized using the same procedure.

100 **Total organic carbon measurement**

101 Total organic carbon was measured using a TOC-VCSH total organic carbon
102 analyzer (Shimadzu, Kyoto, Japan) operating in nonpurgeable organic carbon mode. A
103 five-point calibration curve (0-20 ppm organic carbon) was constructed using potassium
104 phthalate monobasic (Fluka, > 99.5%) as the standard. Prior to measurement, aqueous
105 worm extract samples were filtered through a Millex-GP 0.22 µm-pore-size PES syringe
106 filter (Millipore). Filters were pre-rinsed with ultrapure water before use, and the first
107 few milliliters of sample eluent were discarded. Samples were measured 3-5 times with
108 the machine determining the average and variance values for the data. A 10 mg/l organic
109 carbon standard solution was run with each series of samples to ensure the standard curve
110 remained accurate. In our experiments, 100 ppm TOC was equivalent to aqueous content
111 from about 2.6 mg dry weight of worms dissolved in 1 ml of buffer.

112 **Population assay**

113 Chemotaxis plates were prepared by pouring 8 ml of CTX agar [CTX buffer (5
114 mM KH_2PO_4/K_2HPO_4 at pH 6, 1 mM $CaCl_2$ and 1 mM $MgSO_4$) with 1.6% agar] into 6
115 cm Petri dishes. The plates were spread with 10 µl worm extract (at 100 ppm TOC or an
116 otherwise indicated concentration) on one side and 10 µl M9 buffer on the other side (Fig.
117 1A). Worms were washed three times with CTX buffer and once with water. About 100

118 worms were dropped in the center of each chemotaxis plate. Excess liquid was
119 withdrawn using a Kimwipe. The plates were then placed in a 20°C incubator. After one
120 hour (or otherwise indicated length of time) in the 20°C incubator, chloroform was added
121 to the lid of the plates to instantly immobilize and kill the animals as previously described
122 (Ward 1973). The plates were then scanned using the QuantWorm imaging system (Jung
123 *et al.* 2014) and the images were analyzed using the Java program WormCounter (see
124 Image Processing below). Animals remained in the center 0.5 cm-wide strip were not
125 used in calculation of AI (Fig. 1A) because they may have mobility issues. Plates with
126 fewer than 50 worms counted were considered invalid.

127 **Drop assay**

128 A single animal was placed on a chemotaxis plate at room temperature and
129 allowed to rest for 5-10 minutes. 0.4 µl 100 ppm TOC worm extract or M9 buffer was
130 dropped about 1 mm in front of the head of the moving worm. Once the worm reached
131 the drop, it would either move into the drop or reverse to avoid the drop. A reversal
132 within 3 seconds of contact was counted as an avoidance response. Each animal was
133 tested with worm extract and M9 buffer drops alternatively with an interval of at least
134 one minute between successive drops. Each animal was tested with no more than 15
135 drops.

136 **Trap assay**

137 Young adult animals were collected and washed three times in CTX buffer. Two
138 platinum loops of 5 mm diameters were dipped into M9 buffer and worm extract (200
139 ppm TOC) respectively. The loops were then used to briefly touch the surface of a
140 chemotaxis plate to print two ring-shaped liquid marks. Three worms were placed inside

141 each ring and video-recorded for five minutes. The videos were analyzed using the Java
142 program WormTrap (see Image Processing below).

143 While the three assays (population, drop, trap assays) gave similar results, each
144 had a unique strength. The population assay had the highest throughput and was used as
145 the default method in this study. The other two assays required much fewer animals and
146 were used when the number of animals was limited, e.g., laser-ablated animals, or the
147 animals had certain locomotion defects. For example, the drop assay was used for
148 mutants that crawled slowly; the trap assay was used for male worms that tend to touch
149 other worms and have excessive spontaneous reversals.

150 **Image Processing**

151 Two programs, WormTrap and WormCounter, were developed for automatic
152 image video processing. More details, including source codes, executable files, user
153 manuals, and sample images, are available at www.quantworm.org and
154 figshare.com/articles/Potential_nematode_alarm_pheromone_induces_acute_avoidance_i
155 n_C_elegans_Source_code_executable_files_and_sample_images_/4989776 .

156 WormCounter analyzes images of worm plates from population assays. It
157 assembles tiled images taken by the QuantWorm imaging system to create one image for
158 a plate, and binarizes the image using an empirically determined threshold. Worms are
159 detected by region extraction, and their areas are determined as number of pixels. As
160 most worms do not overlap on the image, the median worm area is used as the size of a
161 single worm to calculate the total number of worms.

162 WormTrap analyzes videos from trap assays. It extracts time-lapse images for
163 every two seconds of a video, then binarizes the images using local adaptive thresholding
164 (Bradley and Roth 2007). Median particle area is used as the size for a single worm. The
165 number of worms in each trap is calculated for each image. The average trapped time, T_r
166 (sec), is calculated as $T_r = \int C(t)dt \approx \sum C(t)\Delta t$, where $C(t)$ is the normalized worm count
167 ($N_t/N_{t=0}$), N_t is the worm count at time t, $N_{t=0}$ is the initial worm count at time = 0, and Δt
168 is the measurement interval (2 sec in this case).

169 **Lifespan assay**

170 Lifespan assays were carried out at 20°C as described (Gandhi *et al.* 1980). 50-70
171 synchronized L1 larvae were dropped onto seeded 60 mm NGM plates. 80µl 2.5 mM 5-
172 fluoro-2'-deoxyuridine (FUdR) (Sigma, Cat# 50-91-9) was added to each plate when the
173 worms reached the L4 stage to prevent progeny from hatching. After the worms reached
174 L4, 80 µl 100 ppm worm extract or M9 were added every other day to each test and
175 control plate, respectively. Two independent trials were performed, with triplicates used
176 in each trial. Dead worms were removed every day and the number of dead worms on
177 each plate was recorded. The first day of adulthood was counted as day one.

178 **Laser ablation of neurons**

179 Cell ablations were done using the standard protocol (Bargmann and Avery 1995).
180 The operation was conducted using a Spectra-Physics VSL-337ND-S Nitrogen Laser
181 (Mountain View, CA) attached to an Olympus BX51 microscope. L1 worms were
182 operated on 5% agar pad containing 0.5 µl of 0.1 µm diameter polystyrene microspheres
183 and covered with a coverglass. The mock-ablated animals were placed on the same agar

184 pad for the same amount of time to rule out the possibility that behavioral changes are
185 due to pressure applied on the worms by the coverglass. Animals were then recovered on
186 regular culture plates and assayed when they were one-day adults.

187 **Transgenic animals**

188 The ASI- and AWC- genetically ablated (via caspase expression) worms (Beverly
189 *et al.* 2011) were kindly provided by Dr. Piali Sengupta. After the drop assay, the animals
190 were mounted on an agar slide and observed under the microscope to confirm the loss of
191 the neurons. *tax-4(p678)* worms with transgenes expressing *tax-4* cDNA sequences under
192 the promoter *ceh-36* or *srbc-65* (Beverly *et al.* 2011) were kindly provided by Dr. Piali
193 Sengupta. Plasmids with wild-type *tax-4* cDNA sequences under the promoter *sra-13*,
194 *str-3*, or *srg-8* (Olofsson 2014) were kindly provided by Dr. Birgitta Olofsson. The
195 plasmids were microinjected into *tax-4(p678)* worms at 50 ng/µl together with 50 ng/µl
196 *Pmyo-2::dsRED* as an injection marker to generate transgenic worms.

197 **Data availability**

198 The authors state that all data necessary for confirming the conclusions presented
199 in the article are represented fully within the article. All strains and plasmids are available
200 upon request. Relevant Java codes for image processing are available at
201 figshare.com/articles/Potential_nematode_alarm_pheromone_induces_acute_avoidance_i
202 n_C_elegans_Source_code_executable_files_and_sample_images_/4989776 .

203 **RESULTS**

204 **Quantitative assays were developed to study nematode alarm response**

205 It was observed that when a *C. elegans* was punctured with a needle, worms
206 within the radius of 1-2 mm would flee from the victim (Thomas and Horvitz, personal
207 communication; Bargmann *et al.* 1990), suggesting that the internal fluid from the injured
208 worms contains a potential alarm signal. We also observed the same phenomenon. To
209 study this, we designed assays to quantify both the signal and the response.

210 To collect a large amount of the signal molecule, we used a pestle or sonication to
211 break the animals, and collected the aqueous content (hereinafter referred to as "worm
212 extract"). As the chemical identity of this avoidance signal is unknown, total organic
213 carbon (TOC) content was used to measure the concentration of worm extracts.

214 We modified three standard chemotaxis assays (Hart 2006) to quantify the worm
215 response to the worm extract (Fig. 1). In the population assay (Fig. 1A), we spread the
216 worm extract on one side of an agar plate and buffer on the other side, placed live worms
217 in the center, and measured the distribution of live worms after a given time. Let A, B
218 denote the number of animals on the buffer side and worm extract side respectively; the
219 avoidance index (AI) is calculated as (A-B) / (A+B). The avoidance index ranges from -1
220 to 1 with 1 being complete repulsion and -1 being complete attraction. In the drop assay
221 (Fig. 1B), a drop of buffer or worm extract was placed in front of a worm, and the
222 percentage of times that the animal reversed its movement was calculated. In the trap
223 assay (Fig. 1C), individual worms were placed inside either a ring drawn with worm
224 extract or a ring drawn with buffer, and the time the worms remained inside the circles
225 was measured. We developed open-source software to automatically analyze images and
226 videos for the population assay and the trap assay.

227 While the three assays gave similar results, each had a unique strength. The
228 population assay had the highest throughput and was used as the default method in this
229 study. The other two assays required much fewer animals and were more tolerant on
230 animals with locomotion defects. We used these two assays for laser-ablated animals,
231 male worms, and mutants that crawled slowly.

232 **Existence of a potential nematode alarm pheromone**

233 All three methods showed that the worm extract induced an acute avoidance
234 behavior in *C. elegans* (Fig. 1, Files S1, S2, S3). The avoidance was dose-dependent of
235 the worm extract (Fig. 1A), and was not due to residual bacterial food (Fig. S1).

236 As *C. elegans* avoids many harmful chemicals, we asked whether the worm
237 extract is harmful to the worms and thus induces nociception rather than an alarm
238 response. We dosed *C. elegans* with the worm extract every other day and found that
239 such constant exposure to the worm extract did not reduce their lifespan (Fig. 2A, another
240 independent experiment was shown in Fig. S2. In both experiments $p > 0.05$ between
241 buffer and extract, log-rank test). These data suggested that the worm extract did not
242 induce any physical damage.

243 Consistent with the importance of an alarm response, avoidance of the worm
244 extract is a very robust behavior in *C. elegans*. In the population assay, the worms
245 remained avoiding for over two hours (Fig. 2B). Both males and hermaphrodites avoided
246 the worm extract (Fig. 2C, males vs. hermaphrodites, $p > 0.05$; buffer vs. worm extract, p
247 < 0.0001, Student's *t*-test). Starvation was known to modulate certain *C. elegans*
248 chemotaxis responses (Hallem and Sternberg 2008), so we tested starved worms for their
249 avoidance of the worm extract. Starved worms were less effective in avoiding the worm

250 extract (Fig. 2D), suggesting that the avoidance response is modulated by feeding status.
251 However, worms starved for up to five hours still strongly avoided the worm extract (AI
252 > 0.6, Fig. 2D), demonstrating the robustness of this behavior.

253 **The avoidance factor is nematode-specific and conserved in multiple nematode**
254 **species**

255 Some animals such as fishes can detect alarm pheromones released by not only
256 conspecifics but also related species (Wyatt 2003). To test the species-specificity of the
257 avoidance factor, we exposed *C. elegans* to worm extracts from other free-living
258 terrestrial nematodes. *C. elegans* strongly avoided not only the conspecific extract, but
259 also extracts from three other nematodes in the *Rhabditis* genus (Fig. 2E). An extract
260 from a more distant nematode, *Panagrellus redivivus*, was also able to invoke a
261 significant ($p < 0.001$, Student's t-test), yet much milder avoidance response from *C.*
262 *elegans* (Fig. 2E). In contrast, despite the fact that *Caenorhabditis* and *Drosophila* often
263 share the same habitat of rotting fruits (Félix and Duveau 2012), extract from the fruit fly
264 larvae had no effects on *C. elegans* (Fig. 2E), suggesting that the avoidance signal is
265 nematode specific.

266 The *Rhabditis* genus also contains families of parasitic nematodes. To examine
267 whether the avoidance factor is also conserved in these parasitic nematodes, we collected
268 extract from the insect parasite *Steinernema carpocapsae* (*Sc*). *C. elegans* avoided both
269 the conspecific and the *Sc* extracts, however, *Sc* infective juveniles (IJs) avoided only the
270 *Sc* (AI > 0, $p < 0.001$, Student's t-test) but not the *C. elegans* extract ($p = 0.75$, Fig. 2F).
271 This difference in the avoidance behaviors is unlikely due to difference in developmental
272 stages, because *C. elegans* dauers (an IJ-equivalent developmental stage) also avoided

273 both *C. elegans* and *Sc* extracts (Fig. 2F). These data suggested that the avoidance signals
274 in different nematode species are similar but not identical, and that parasitic and free-
275 living nematodes have different responses to various avoidance signals.

276 **The avoidance factor is a novel nematode repellent**

277 The avoidance signal is unlikely an ascaroside, the best-known nematode
278 pheromone. Worm extracts from *C. elegans* mutants defective of ascaroside synthesis
279 (e.g., *daf-22*, *maoc-1*, *acox-1* (Ludewig 2013)) functioned effectively as avoidance
280 signals (Fig. 3A). In addition, mutants of known ascaroside receptors (*daf-37*, *srbc-64*,
281 *srbc-66*, *srg-36*, *srg-37* (Ludewig 2013)) successfully avoided the worm extract (Fig. 3B).
282 These results suggested that the avoidance factor is not an ascaroside or at least contains
283 ascaroside-independent factors.

284 The avoidance factor appeared to be none of the known nematode repellents
285 because *C. elegans* mutants defective in avoiding known repellents such as acid,
286 osmolarity, benzaldehyde or quinine, still efficiently avoided the worm extract (Fig. S3A).
287 Glycosaminoglycan chondroitin (GAG) has been reported as the fish alarm pheromone
288 (Mathuru *et al.* 2012). RNAi of *C. elegans* chondroitin synthesis gene *mig-22* or *sqv-5*
289 (Hwang *et al.* 2003; Suzuki *et al.* 2006) produced extracts with normal alarm efficacy
290 (Fig. S3B), suggesting that chondroitin is also not the nematode alarm pheromone.

291 Our preliminary efforts to fractionate the crude extract using reversed phase and
292 size exclusion chromatography indicate that the avoidance signal consists of at least three
293 distinct components of medium polarity. While the chemical identity of the components
294 remains unknown, we have characterized several properties of the avoidance signal.

295 **The avoidance factor is a non-volatile endogenous factor**

296 The avoidance factor appeared non-volatile. In a modified population assay, we
297 poured agar on both lids and plates of Petri dishes, spread the worm extract and buffer on
298 the lid agar, and placed the worms on the plate agar. That way the worms were not in
299 direct contact but a short distance (1-2 mm) away under the signal. Worms showed no
300 avoidance under these conditions even with a fivefold increase in the amount of the worm
301 extract (Fig. 3C), suggesting that the avoidance signal is not volatile.

302 Alarm pheromones can be actively secreted by stressed animals (e.g., flies and
303 mice), or passively diffused from internal cells that become exposed to the environment
304 by tissue damage (e.g., zebrafish) (Enjin and Suh 2013). The nematode avoidance factor
305 likely belongs to the second class because it existed in all developmental stages,
306 including embryos in which secretion to the environment is hindered by egg shells (Fig.
307 3D).

308 We further tested whether the avoidance factor is synthesized when animals are
309 stressed or whether it is an endogenous chemical that constantly exists but is released
310 upon injury. We prepared worm extracts from animals that were killed instantly in
311 boiling water-bath or liquid nitrogen. Extracts from instantly-killed worms induced
312 similar avoidance behaviors as those from living worms (Fig. 3E), suggesting that injury
313 did not induce synthesis of the avoidance factor but rather released an endogenous factor
314 that was already present inside worms.

315 **Worm extract avoidance requires cGMP signaling**

316 Most *C. elegans* sensory neurons signal through the cGMP-gated ion channel
317 encoded by the *tax-2* and *tax-4* genes, and the TRPV (transient receptor potential)
318 channel encoded by the *osm-9* and *ocr-2* genes (Bargmann 2006). We tested mutants of
319 these genes and found that TAX-2 and TAX-4, but not OSM-9 or OCR-2, are required
320 for avoidance of the worm extract (Fig. 4A). Consistent with this observation, mutants of
321 *daf-11* and *odr-1*, two guanylyl cyclases that have been linked to chemosensation
322 (L'Etoile and Bargmann 2000; Birnby *et al.* 2000), also showed defective avoidance of
323 the worm extract.

324 **Worm extract avoidance requires the ASI and ASK neurons**

325 Next we seek to identify the sensing neurons in the neural circuit mediating the
326 avoidance of the worm extract. *C. elegans* has two types of chemosensory organs,
327 amphids in the anterior of the worm and phasmids in the posterior, that have sensory cilia
328 exposed to the environment (Scholey 2007). Mutations that caused structural defects in
329 these cilia (Scholey 2007) abolished the avoidance of the worm extract (Fig. 4B),
330 suggesting that the worm extract is detected through these ciliated neurons.

331 Because TAX-2 and TAX-4 are required for avoidance of the worm extract (Fig.
332 4A), we focused on the 12 neurons where *tax-2* and *tax-4* are expressed: AWC, AFD,
333 ASE, ASG, ASJ, ASI, AWB, ASK, BAG, AQR, PQR, and URX (Coburn and Bargmann
334 1996). We tested the *tax-2* allele *tax-2(p694)*, which has a mutation in *cis*-regulatory
335 elements and only disrupts *tax-2* expression in the AQR, AFD, ASE, and BAG neurons.
336 *tax-2(p694)* mutants showed normal avoidance of the worm extract (Fig. 4C). Therefore,
337 we focused on the remaining eight neurons. Observation from our drop assay and trap
338 assay showed that the worm head could sense the alarm pheromone (Files S2 and S3),

339 indicating that amphid neurons were involved. Among the remaining *tax-2/tax-4*-
340 expressing neurons, six were amphid neurons: ASG, ASI, ASJ, ASK, AWB, and AWC
341 (Bargmann 2006). Two mutants, *lim-4* and *unc-130*, with defects in the development of
342 the AWB and ASG neurons, respectively (Hobert 2005), did not show significant defects
343 in worm extract avoidance (Fig. 4C), leaving four neurons, ASI, ASJ, ASK, and AWC, as
344 candidates.

345 To examine whether the ASI neurons are required for avoidance of the worm
346 extract, we tested strains in which the ASI neurons were genetically ablated using either a
347 mutation of *unc-3*, which encodes a transcription factor required for the ASI neurons
348 (Prasad *et al.* 1998), or ASI-specific expression of caspases (Beverly *et al.* 2011). These
349 strains displayed strong defects in avoiding the worm extract (Fig. 4D). In contrast,
350 AWC-expression of caspases (Beverly *et al.* 2011) did not cause significant defects in
351 worm extract avoidance (Fig. 4D).

352 Laser ablation of the ASI neurons also caused defective avoidance of the worm
353 extract (Fig. 4E), confirming that the ASI neurons are involved in the avoidance of the
354 worm extract. Laser ablation of the ASK neurons caused similar defects (Fig. 4E),
355 suggesting that the ASK neurons are also part of the avoidance neural circuit. In contrast,
356 laser ablation of the ASJ neurons did not produce any avoidance defect (Fig. 4E). We
357 also tested the ADL neurons because they have been reported to be involved in
358 nociception and chemoavoidance (Bargmann 2006). We found that they were not
359 required for avoidance of the worm extract (Fig. 4E), consistent with the fact that ADL
360 neurons do not express TAX-2/TAX-4 (Bargmann 2006) and our observation that TAX-
361 2/TAX-4 are required for the worm extract detection.

cGMP signaling is required in the ASI and ASK neurons for avoidance of the worm extract

The genetic and laser ablation experiments revealed that the ASI and ASK neurons are required for the avoidance of the worm extract. To examine whether TAX-2/TAX-4 function in these neurons to modulate the avoidance behavior, we performed cell-specific rescue experiments with *tax-4* by expressing *tax-4* cDNA under various promoters in *tax-4(p678)* mutants. *tax-4* mutants in which *tax-4* is rescued in the ASI neurons either through the *srbc-65* promoter or the *str-3* promoter (Beverly *et al.* 2011; Olofsson 2014) showed significantly higher avoidance of the worm extract than the mutants without rescue (Fig. 4F). Similar effects were achieved by restoring *tax-4* in the ASK neurons (Fig. 4F). In contrast, *tax-4* expression in the AWC neurons failed to rescue the avoidance defects (Fig. 4F). These data support our model that the ASI and ASK neurons function in direct sensing of the avoidance factor.

Other neurons may also be involved in sensing the avoidance factor. Restoring TAX-4 function in either ASI or ASK neurons did not restore the avoidance to wild-type levels (Fig. 4F, $p < 0.01$ in comparison with wild-type, Student's t-test), suggesting that more than one neurons are needed in wild-type sensing. This is consistent with the genetic and laser ablation experiment showing that missing either ASI or ASK caused avoidance defects (Fig. 4D, 4E). Restoring TAX-4 in both ASI and ASK still did not fully reach wild-type avoidance (Fig. 4F, $p < 0.05$, Student's t-test). This could be a result of varying levels of transgene expression, or may suggest that additional neurons are involved in worm extract sensing.

Worm extract avoidance is modulated by GABA and serotonin

385 Currently there are two major classes of drugs for treating anxiety: 1)
386 benzodiazepines that target the neurotransmitter gamma-aminobutyric acid (GABA), and
387 2) monoamine-altering drugs, which are also antidepressants (Griebel and Holmes 2013;
388 Murrough *et al.* 2015). The second class of drugs includes tricyclic antidepressants
389 (TCAs) that modulate the neurotransmitters serotonin and norepinephrine, monoamine
390 oxidase inhibitors (MAOIs) that modulate monoamine neurotransmitters including
391 dopamine, serotonin, melatonin, epinephrine, and norepinephrine, and selective serotonin
392 re-uptake inhibitors (SSRIs) that modulate serotonin levels (Griebel and Holmes 2013;
393 Murrough *et al.* 2015).

394 As all existing anxiolytic drugs target certain neurotransmitters, we examined
395 whether these neurotransmitters are involved in *C. elegans* avoidance of the worm extract.
396 *C. elegans* has seven types of neurotransmitters: acetylcholine (ACh), serotonin (5-HT),
397 dopamine (DA), tyramine (TA), octopamine (OA), glutamate (Glu), and gamma-
398 aminobutyric acid (GABA) (Loer 2010).

399 We first examined GABA, which is the target of benzodiazepine anxiolytic drugs.
400 Mutants of the GABA biosynthetic enzyme glutamic acid decarboxylase UNC-25 or the
401 membrane GABA transporter UNC-47 displayed reduced avoidance of the worm extract
402 (Fig. 5A, 5B), suggesting that the avoidance is modulated by GABA levels.

403 5-HT is a common target of monoamine-altering drugs. Mutants of the tryptophan
404 hydroxylase TPH-1, an enzyme required for 5-HT biosynthesis, had normal avoidance of
405 the worm extract (Fig. 5C). Mutants of the serotonin reuptake transporter (SERT) MOD-
406 5 displayed mild defects in worm extract avoidance (Fig. 5C). These data suggested that
407 increased but not decreased 5-HT levels have a mild influence on the avoidance behavior.

408 # DISCUSSION

409 We present evidence of a potential nematode alarm pheromone. First, the worm
410 extract does not cause pain or physical harm considering that the worm extract did not
411 reduce animal lifespan (Fig. 2A), and that the nociceptive ADL neurons and the TRPV
412 channels OSM-9/OCR-2 (Bargmann 2006) were not required for worm extract avoidance
413 (Fig. 4). Second, unlike most worm repellents that require the ADL neurons, the acute
414 avoidance of the worm extract is sensed by the ASI and ASK neurons (Fig. 4), two
415 neurons that are also involved in detection of the pheromone ascaroside, suggesting that
416 the worm extract may differ from generic repulsive signals and contain a pheromone.
417 While chemical identification of the avoidance factor is needed to definitively answer
418 whether it is an alarm pheromone, existing data consistently support the model of an
419 alarm pheromone in the worm extract.

420 Avoidance of the worm extract requires the cGMP-gated TAX-2/TAX-4 channels
421 in the amphid ASI and ASK neurons (Fig. 4). The behavior is susceptible to modulation
422 of GABA and serotonin levels (Fig. 5). As our assay does not detect functional
423 redundancy, some molecules and cells that showed no effects in this study may still be
424 involved.

425 There are some similarities between the nematode and the mouse alarm responses.
426 First, the alarm pheromone detecting cells are similar. The mouse alarm pheromone-
427 sensing organ, the Grueneberg ganglion, differs significantly from the canonical olfactory
428 system in both cellular and molecular components (Enjin and Suh 2013), yet showed
429 striking similarity to *C. elegans* amphid neurons in both neuron morphology (Brechbühl
430 *et al.* 2008) and protein expression profiles (Brechbühl *et al.* 2013a). Second, the

431 molecules mediating alarm pheromone detection are also similar between *C. elegans* and
432 mice. Orthologs of TAX-4 and DAF-11 are expressed in mouse Grueneberg ganglion
433 (Brechbühl *et al.* 2013a). Both *C. elegans* and mouse use a cGMP-dependent pathway in
434 alarm pheromone sensing whereas zebrafish use cAMP. Finally, similar to mice, the *C.*
435 *elegans* alarm response is also susceptible to modulation of GABA and 5-HT levels.

436 A likely function for the nematode alarm pheromone is to signal the presence of a
437 nematode-feeding predator so that other nematodes can escape. In their natural habitat, *C.*
438 *elegans* live in large populations in rotting fruits (Félix and Duveau 2012). Because of
439 such high-density aggregation of animals, the alarm pheromone does not need to be
440 volatile to cover a long range. The same rotting vegetal environments are often shared by
441 multiple *Caenorhabditis* species and *Drosophila* (Félix and Duveau 2012). *C. elegans*
442 can distinguish injured nematodes from *Drosophila* larvae (Fig. 2E), enabling them to
443 avoid nematode-specific dangers.

444 Similar to fish alarm pheromones, the nematode alarm pheromone is likely an
445 endogenous signal that is stored and released only upon injury (Fig. 3D), instead of a
446 product of acute synthesis upon stress or injury. For a nematode, an injury that penetrates
447 the cuticle is likely to be fatal, as the worm is under internal hydrostatic pressure, and
448 bursts when its cuticle is punctuated. Therefore, the alarm pheromone has little adaptive
449 advantage for the sender. Using an endogenous factor as the alarm pheromone in this
450 case brings no additional cost to the sender while benefiting the receivers.

451 Ascarosides and the worm alarm pheromone are similar in that both of them are
452 non-volatile, conserved in nematodes, and detected by the amphid neurons ASI and ASK
453 in a cGMP-dependent pathway (Ludewig 2013). However, the alarm pheromone is likely

454 not a member of ascaroside class of pheromones, as dauers synthesize less ascarosides
455 (Kaplan *et al.* 2011) but have abundant alarm pheromone (Fig. 3D); a handful of
456 ascarosides have sexual dimorphic effects at certain concentrations (Srinivasan *et al.*
457 2008), but the alarm pheromone has no sexual dimorphism (Fig. 2C); and the ascaroside
458 C9 is sensed by the ADL neurons in addition to ASI and ASK neurons (Jang *et al.* 2012;
459 Ludewig 2013) whereas ADL does not appear to be required for the alarm pheromone
460 sensing (Fig. 4E). However, because of the diversity of ascarosides, it remains possible
461 that the alarm pheromone is a novel ascaroside that has not been well characterized. We
462 also cannot exclude the possibility that the alarm pheromone contains both ascaroside and
463 non-ascaroside components. These questions can be revealed by future research on the
464 chemical identity of the alarm pheromone.

465

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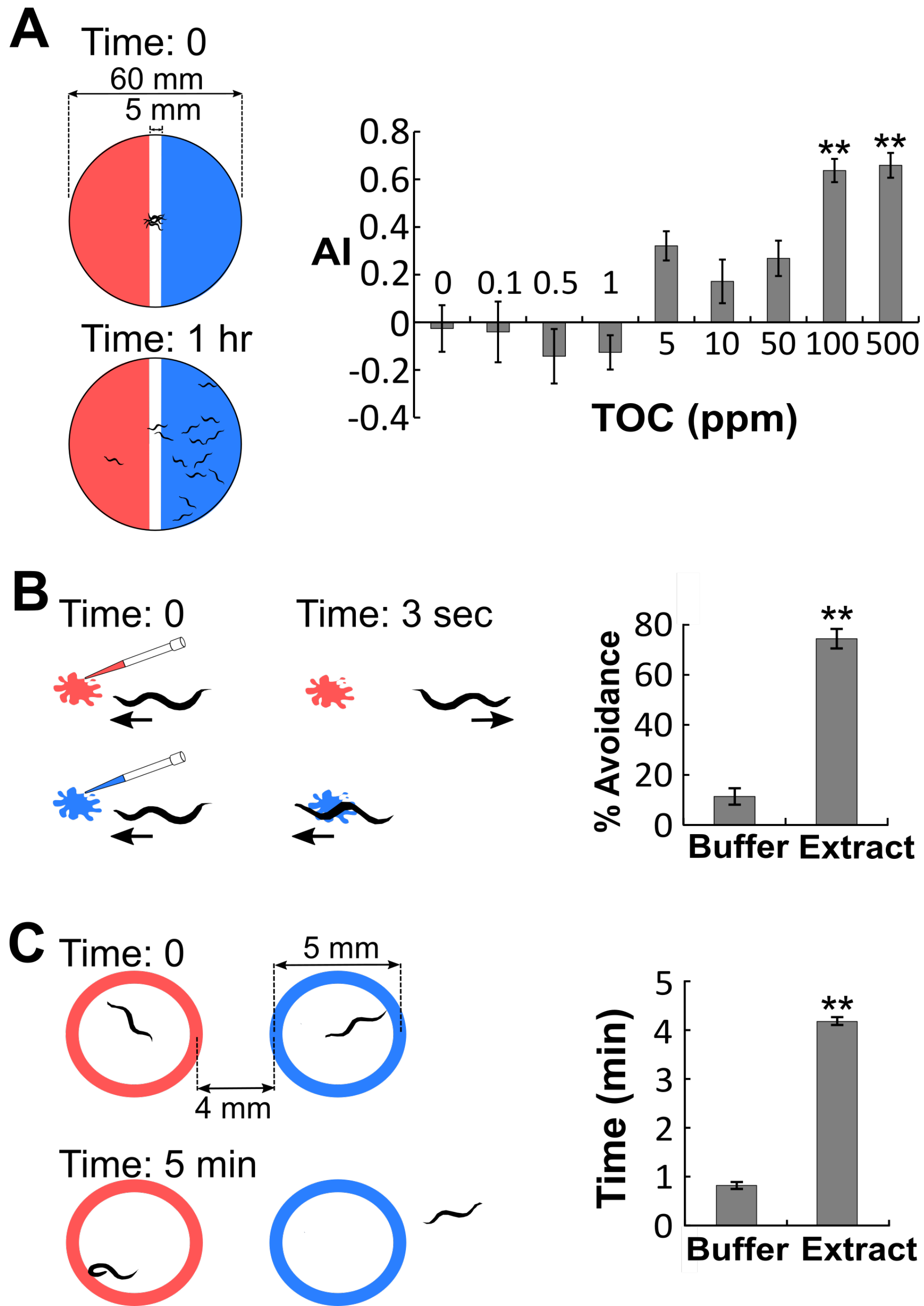
AUTHOR CONTRIBUTIONS

W.Z. designed the research. Y.Z., M.L., Z.L., J.K.N., Y.C., and W.Z. conducted the experiments. Q.S. and R.A.B. performed fractionation on alarm pheromone. B.A.M. and S-K.J. developed analytic tools. W.Z. analyzed and interpreted the data. W.Z. wrote the paper.

579 **FIGURES**



580

581 **Figure 1. Three assays to quantify nematode alarm response**

582 (A) Population test. Plates were spread with worm extract (red) on one side and buffer

583 (blue) on the other. Approximately 100 worms were dropped at the center, immobilized

584 after an hour to evaluate the distribution of worms. In the dose response, 10 µl of

585 different concentrations of worm extracts were tested. AI, avoidance index. n ≥ 10 plates

586 for each data point. TOC, total organic carbon content. (B) Drop assay. A drop of worm

587 extract (red) or buffer (blue) was applied in front of the head of a moving worm. A

588 reversal within 3 seconds indicated avoidance. Percentage avoidance was scored. n = 35

589 worms for each group. (C) Trap assay. Two unfilled circles were drawn using worm

590 extract (red) and buffer (blue). 1-3 worms were placed into each of circle and recorded

591 for 5 minutes to measure the average time each worm stayed inside the circle. n = 26 tests

592 for each group. Bar graphs represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, one-way

593 ANOVA and Scheffé *post hoc* analysis.

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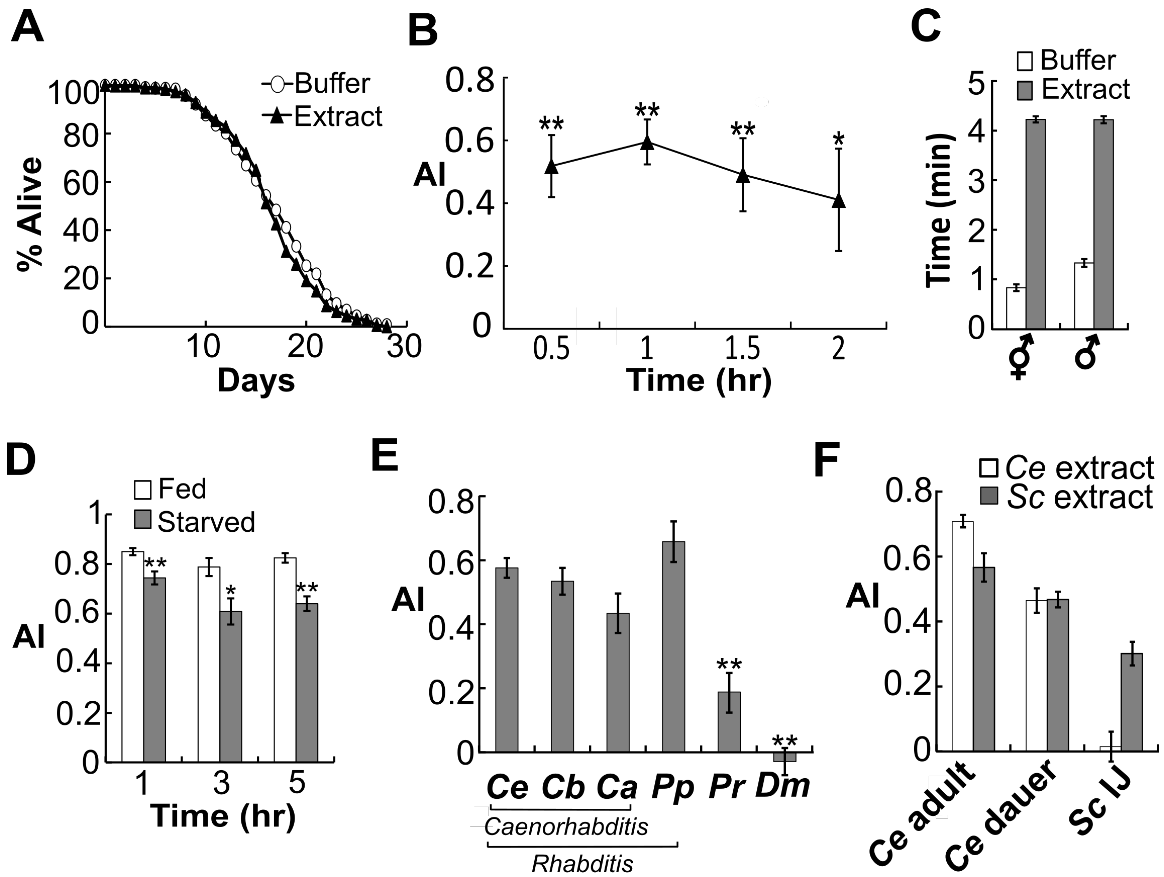

596 **Figure 2. Evidence of a potential nematode alarm pheromone**

597 (A) Worm lifespan was not affected by repeated doses of the worm extract. n ≥ 333

598 worms in each group. (B) Worms avoided the worm extract for over two hours in the

599 population assay. * $p < 0.05$, ** $p < 0.01$ in comparison with AI=0, Student's t-test. n ≥

600 10 plates for each data point. (C) Both males and hermaphrodites avoided the worm

601 extract in the trap assay. n ≥ 21. (D) Worms starved in M9 buffer for 1- 5 hours were less

602 effective but still avoided the worm extract. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test,

603 starved vs. fed. n ≥ 10. (E) *C. elegans* responses to extracts from different nematodes.

604 The labels indicate extracts from the following species. *Ce*: *Caenorhabditis elegans*, *Cb*:

605 *Caenorhabditis briggsae*, *Ca*: *Caenorhabditis angaria*, *Pp*: *Pristionchus pacificus*, *Pr*:

606 *Panagrellus redivivus*, *Dm*: *Drosophila melanogaster*. * $p < 0.05$, ** $p < 0.01$ in

607 comparison with the *Ce* group, one-way ANOVA and Scheffé *post hoc* analysis. n ≥ 10

608 (F) *C. elegans* (*Ce*) and *Steinernema carpocapsae* (*Sc*) responses to *Ce* and *Sc* extracts. n

609 ≥ 10. All bar graphs display mean ± SEM.

610

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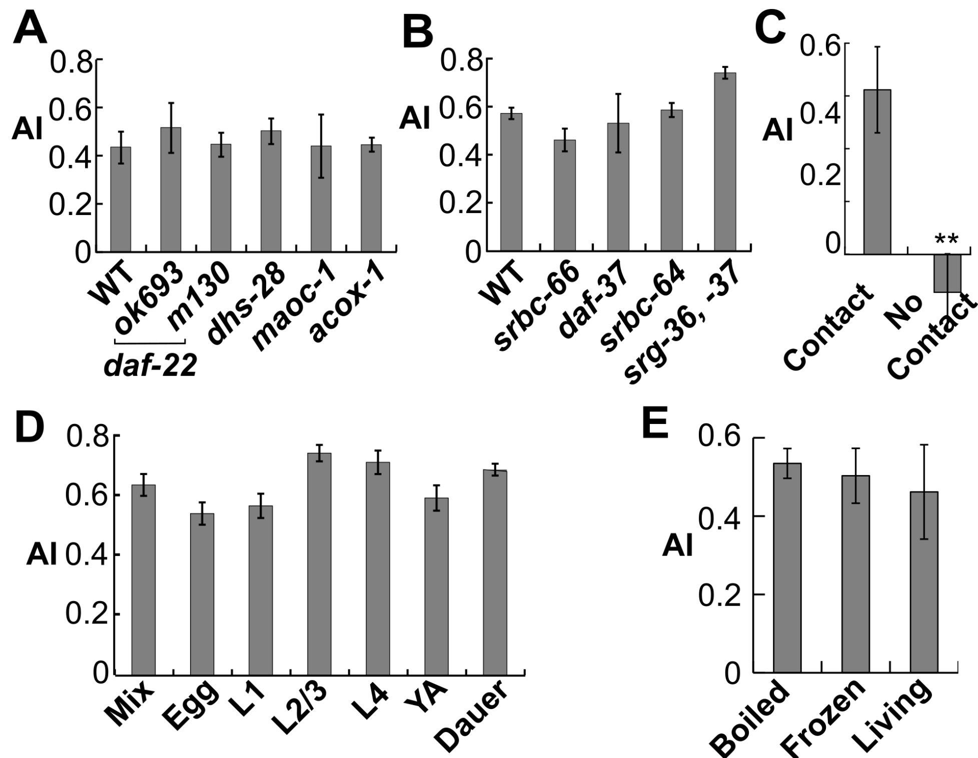
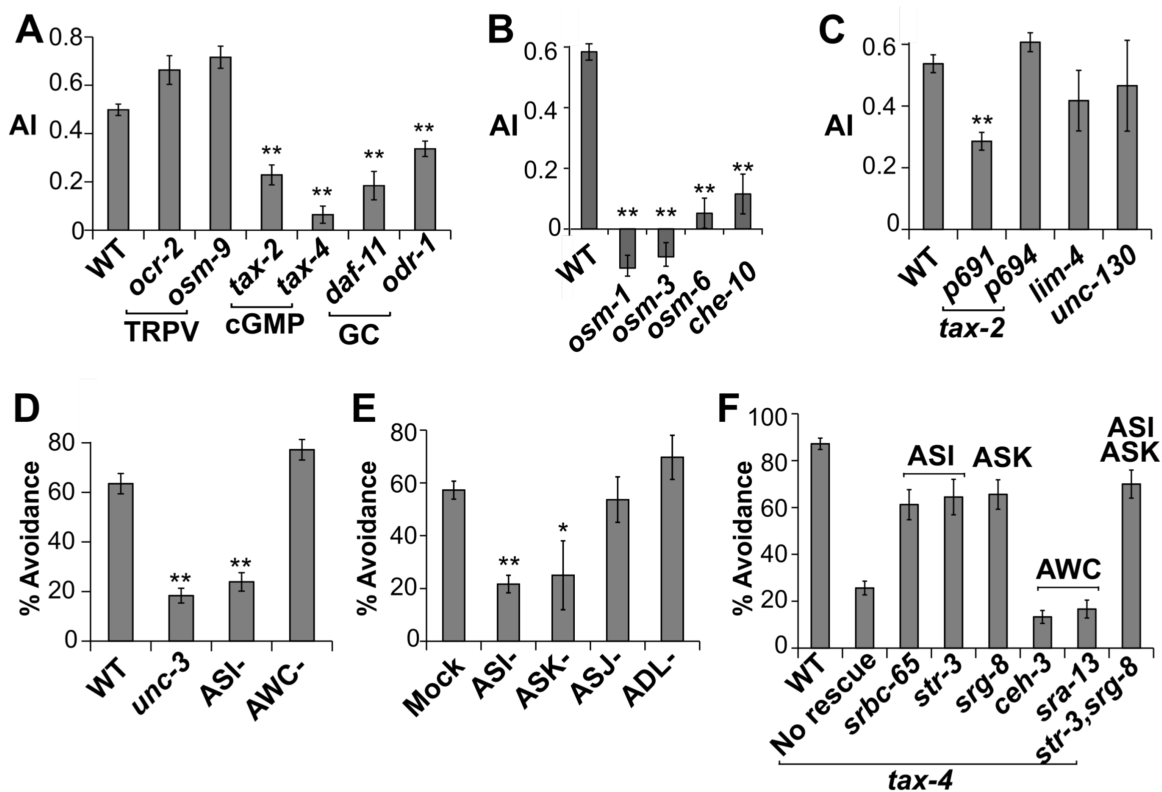


612 **Figure 3. Properties of the nematode alarm pheromone**

613 (A) Worm extracts from ascaroside synthesis mutants repelled *C. elegans*. n ≥ 7. (B)
614 Ascaroside receptor mutants avoided worm extract. n ≥ 9. (C) The alarm pheromone was
615 not volatile. 500 ppm worm extract was used in the "no contact" group whereas the
616 default 100 ppm was used in the "contact" group. n ≥ 10. (D) The alarm chemical existed
617 in different developmental stages of worms. Mix, mixed stages. YA, young adults. n ≥ 10.
618 (E) Effects of differently prepared worm extracts. n ≥ 10. All bar graphs display mean ±
619 SEM. * $p < 0.05$, ** $p < 0.01$ in comparison with the first group, one-way ANOVA and
620 Scheffé *post hoc* analysis.



621

622 **Figure 4. Avoidance of the worm extract requires cGMP signaling in the ASI, ASK**

623 **neurons.**

624 (A) cGMP-channel mutants as well as guanylyl cyclase (GC) mutants were defective in

625 avoiding the worm extract while TRPV-channel mutants exhibited normal avoidance. (B)

626 Mutants with cilia defects showed defective avoidance of worm extract. n ≥ 10. (C, D)

627 Among worm strains with defective neurons, genetic ablation of the ASI neurons showed

628 defective avoidance of the worm extract. ASI- and AWC- indicate cell ablation via

629 caspase expression. *unc-3* mutants were tested using the drop assay because of motor

630 defects. n ≥ 10 plates for population assay in C and n ≥ 10 animals for drop assay in D.

631 (E) Avoidance response of animals after laser ablation of amphid neurons. n ≥ 10 animals.

632 (F) Cell-specific rescue of *tax-4*. Labels indicate promoters used to drive neuron-specific

633 expression. Restoring *tax-4* in the ASI and/or ASK neurons showed significant rescue

634 effects of the avoidance behavior (p<0.01, Student's t-test, in comparison with the no-

635 rescue group). $n \geq 10$ animals for each group. In all bar graphs, bars and error bars

636 represent mean and standard error, respectively. * $p < 0.05$, ** $p < 0.01$, one-way

637 ANOVA and Scheffé *post hoc* analysis. WT, wild-type.

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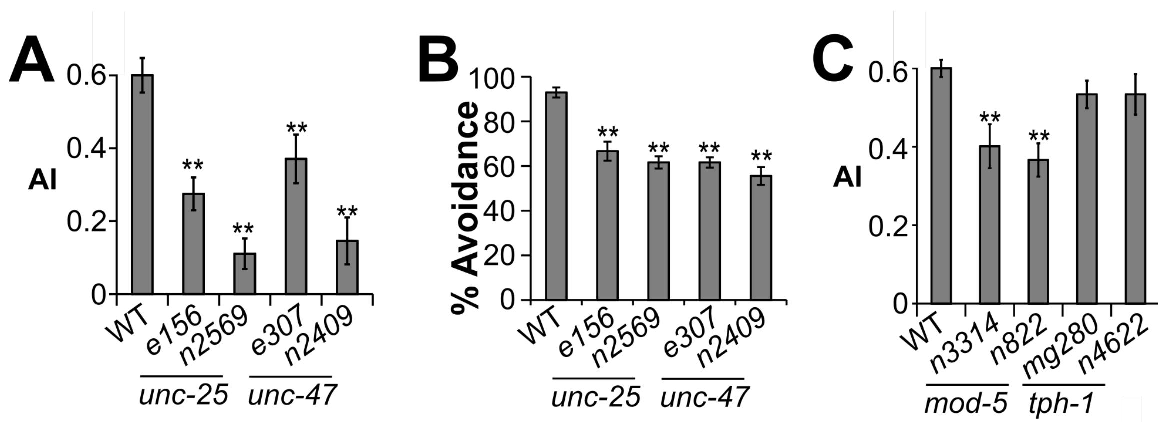

640 **Figure 5. Neurotransmitters modulate avoidance of the worm extract**

641 (A) Avoidance of GABA mutants tested by the population assay. n ≥ 17 (B) Avoidance
642 response of GABA mutants tested by the drop assay. n ≥ 11 (C) Avoidance response of
643 serotonin mutants. n ≥ 16. In all panels, bars and error bars represent mean and SEM,
644 respectively. ** $p < 0.01$, one-way ANOVA and Tukey *post hoc* analysis.