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1 **Are *Caenorhabditis elegans* magnetoreceptive?**

2

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12 **Abstract**

13 A diverse array of species on the planet employ the Earth's magnetic field as a
14 navigational aid. As the majority of these animals are migratory, their utility to
15 interrogate the molecular and cellular basis of the magnetic sense is limited. Vidal-
16 Gadea and colleagues recently argued that the worm *C. elegans* possesses a
17 magnetic sense that guides their vertical movement in soil. In making this claim they
18 relied on three different behavioural assays that involved magnetic stimuli. Here, we
19 set out to replicate their results employing blinded protocols and double wrapped
20 coils that control for heat generation. We find no evidence for a magnetic sense in *C.*
21 *elegans*, and demonstrate that iron-contamination from the laboratory setting can
22 result in false positive results. We further show that the Vidal-Gadea hypothesis is
23 problematic as the adoption of a correction angle relative to the Earth's magnetic
24 inclination does not necessarily result in vertical movement.

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30 **Introduction**

31 The ability to sense the Earth's magnetic field is a widespread sensory faculty in the
32 animal kingdom. Magnetic sensation has been shown in migratory birds (Merkel and
33 Wiltschko, 1965; Zapka et al., 2009), mole rats (Nemec et al., 2001), pigeons (Wu
34 and Dickman, 2012), and turtles (Lohmann et al., 2004). While behavioral evidence
35 supporting the existence of a magnetic sense is unequivocal, the underlying sensory
36 mechanisms and neuronal circuitry that transduce and integrate magnetic information
37 are largely unknown. A major impediment to progress in the field is the lack of
38 genetic and molecular tools in magnetosensitive species. One such model system
39 could be the nematode *Caenorhabditis elegans*, which has proved to be a powerful
40 tool to explore a wide variety of senses. It has been claimed by Vidal-Gadea et al.
41 (2015) that *C. elegans* possess a magnetic sense which can easily be exploited for
42 mechanistic investigation (see also Bainbridge et al., 2016). They argue that *C.*
43 *elegans* possess a magnetic sense that is employed for vertical orientation, worms
44 adopting a correction angle relative to the inclination of the Earth's magnetic field.
45 This conclusion was based on results from three assays which they developed: (1) a
46 "vertical burrowing assay"; (2) a "horizontal plate assay"; and (3) a "magnetotaxis
47 assay". Here, we set out to replicate the aforementioned behavioral assays, adopting
48 several critical controls that were absent in the original study.

49

50 **Results**

51

52 **Benzaldehyde Control Experiment**

53 We established a positive control for our experiments employing the odorant
54 benzaldehyde. It has been shown that if worms are placed in the center of a petri-
55 dish and given the choice between 1% benzaldehyde and 100% ethanol they are
56 attracted to the benzaldehyde. Conversely, if worms are pre-exposed to 100%
57 benzaldehyde their preference is disrupted (Nuttley et al., 2001). Employing blinded

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58 protocols we found that worms preferred 1% benzaldehyde (n=11, p<0.005,
59 Wilcoxon signed rank test), which was lost when pre-exposed to 100% benzaldehyde
60 (Figure 1A-B, Figure Supplement 1). These results show that we are able to replicate
61 published *C. elegans* chemotaxis experiments in our laboratory.
62

63 **Infrastructure and double wrapped coils**

64 To perform the magnetic experiments described by Vidal-Gadea and colleagues we
65 built the necessary infrastructure to insure that our experiments were performed in a
66 clean magnetic environment. This consists of 6 double-wrapped Helmholtz coils,
67 within a mu-metal shielded room that is surrounded by a faraday cage (Figure 2A-C).
68 Radio frequency contamination within this room is very low, with intensities below
69 0.1nT between 0.5 to 5MHz (see Figure 2A-B). This infrastructure is critical for
70 applying magnetic stimuli in a controlled fashion.
71

72 **Vertical Burrowing Assay**

73 In the first magnetic assay described by Vidal-Gadea, starved animals were injected
74 into agar-filled plastic pipettes (Figure 3A). Worms were allowed to migrate overnight,
75 and the number on each end of the tube were counted. In the absence of an external
76 field the authors reported that animals preferentially migrated downwards, however,
77 when exposed to an inverted Earth strength magnetic field worms migrated upwards.
78 This preference was reversed in the case of fed animals. We repeated these
79 experiments, but observed no effect of inverting the magnetic field on the burrowing
80 index when the worms were starved (Mann-Whitney U-test, n_1=38, n_2=40, U=681, n.
81 s.) or fed (Mann-Whitney U-test, inclination down: n_1=20, n_2=35, U=300, n. s.) (Figure
82 3B).
83

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84

85 **Horizontal Plate Assay**

86 In their second behavioural assay, Vidal-Gadea placed ≈50 worms in the center of an
87 agar plate (Figure 3C). This plate was placed within a single wrapped Merritt coil
88 system which permitted the generation of either null or horizontal magnetic fields of
89 Earth strength intensity (either 32.5μT or 65μT). They reported that in the absence of
90 magnetic stimuli worms displayed no directional preference, whereas in the presence
91 of a horizontal field worms distributed in a biased direction 60° either side of the
92 imposed vector. We replicated these experiments, treating each plate as an
93 experimental unit. Blind analysis of worm orientation revealed no effect on orientation
94 behavior when applying a 32.5μT stimulus (Rayleigh-test, r=0.20, n=24, n. s) or a
95 65μT stimulus (Rayleigh-test, r=0.25, n=24, n. s., Figure 3D). Nor did we observe any
96 directional preference in our control experiments (32.5μT: Rayleigh-test, r=0.10,
97 n=24, n. s.; 65 μT: Rayleigh-test, r=0.11, n=24).

98

99 **Magnetotaxis Assay**

100 In their third behavior assay worms were placed in the center of a horizontal agar
101 plate between two different goal areas (Figure 3E). An extremely strong neodynium
102 magnet generating a field up to 0.29T (approximately 8,000 times Earth strength),
103 was placed beneath one of the goal areas. Vidal-Gadea reported that in the absence
104 of this magnet worms were distributed evenly between the goal areas, however, if the
105 magnet was present worms migrated towards it. We replicated their set up placing a
106 strong neodynium magnet under one goal area, but added an equally size non-
107 magnetic brass control under the opposing goal area. We observed no preference for
108 the goal area associated with the neodynium magnet (n=49 plates, P>0.5, Wilcoxon
109 signed rank test, Figure 3F). As false-positives in magnetoreception have been
110 associated with contamination of biological material with exogenous iron we asked
111 whether this might influence the behavior of worms (Edelman et al., 2015). We tested

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112 this by growing worms on agar plates spiked with magnetite particles, and repeated
113 the magnetotaxis assay. We found a weak but significant preference for the goal
114 area under which the magnet resided (Wilcoxon signed rank test, n=29, V=670.5,
115 P=0.042, Figure 3F).
116
117 **Discussion**
118 Why are our results different from those of Vidal-Gadea? We have gone to great
119 lengths to employ the same protocols. We have used worms from the same source,
120 we have employed the same neodymium magnets, we have used the same assay
121 plates, and the same synchronization and starvation protocols. There were, however,
122 a number of important differences. First, we have used double wrapped coils for our
123 experiments (Kirschvink, 1992). Our large double wrapped coils allow the application
124 of a magnetic stimulus without generating a change in temperature compared to the
125 control condition. In contrast the small single wrapped coils employed by Vidal-
126 Gadea generate a temperature gradient. This is problematic when dealing with *C.*
127 *elegans* as it is known that they can reliably detect temperature changes that are
128 <0.1°C (Ramot et al., 2008). Second, we used strict blinding procedures in all our
129 assays, assuring an unbiased assessment of the worm responses. While Vidal-
130 Gadea report blinding when comparing different genotypes, they do not report
131 blinding to the magnetic condition. Third, we have applied the appropriate statistical
132 methodology when analysing our data from the horizontal plate assay. Vidal-Gadea
133 placed ≈50 worms on a plate treating each worm as a biological replicate. However,
134 as worms tested on the same plate can interact with each other, they are not true
135 independent biological replicates. The approach adopted by Vidale-Gadea is known
136 as pseudoreplication, as it confuses the number of data points with the number of
137 independent samples, increasing the probability of rejecting the null hypothesis whilst
138 it is actually true (Lazic, 2010).
139

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140
141
142 Moreover, there are a number of conceptual issues that undermine the assertion that
143 *C. elegans* are magnetosensitive. First, the magnetotaxis assay relies on a
144 permanent magnet that (in the absence of a particularly thick layer of agar) generates
145 a magnetic vector that is perpendicular (i.e. 90°) with respect to the plate surface
146 (Figure 4A). As a consequence, neither the polarity nor the inclination, of the field can
147 be employed as a cue for guidance. It is the equivalent of placing worms on the
148 magnetic north pole – all directions are south. Worms would have to rely on variation
149 in magnetic intensity to direct their movement. As *C. elegans* live in a geographically
150 restrained environment in which there is, in effect, no change in magnetic intensity it
151 seems improbable that they would have evolved an intensity-based magnetic sense.
152 An alternative explanation for this "magnetotactic behavior" could be that exogenous
153 iron particles attached to, or ingested by the worm, might, in the presence of an
154 extremely large magnetic field influence the direction of locomotion by applying a
155 force to surface mechanoreceptors.
156
157 More troubling is the underlying hypothesis that nematodes adopt a correction angle
158 (α) relative to the inclination of the field to guide their vertical movement. Imagine a
159 nematode is located in Cairo where the inclination of the Earth's magnetic vector is
160 44° 33'. To migrate vertically (i.e. 90°) it should adopt a correction angle of
161 approximately 45° to the magnetic vector and maintain that trajectory (Figure 4B).
162 Assuming that nematodes cannot distinguish up from down, the adoption of a 45°
163 angle from the inclination of the field is just as likely to result in horizontal movement
164 (180°) as vertical translation (90°). This problem is exacerbated as the correction
165 angle increases (e.g. 60°) (Figure 4C). The concept proposed by Vidal-Gadea is only
166 an efficient strategy if the worms are using the 'correction angle' in relation to an
167 independent reference (i.e. gravity). However, if worms are able to distinguish up

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168 from down based on gravity, why would they rely on a magnetic field vector? In
169 conclusion, we were not able to replicate the findings of Vidal-Gadea and colleagues.
170 It is pertinent to note that other attempts to elicit magnetoreceptive behavior in *C.*
171 *elegans* have also been unsuccessful (Njus et al., 2015). Collectively, these data
172 indicate that *C. elegans* is not a suitable model system to understand the molecular
173 basis of magnetoreception.
174

175 **Methods and Materials**

176 **Animals**

177 Worms (N2 strain, received from Caenorhabditis Genetics Center) were maintained
178 at 20°C on OP50 bacteria. For all assays we used adult hermaphrodite worms that
179 had not previously been starved. Worms were synchronized (bleached) before the
180 tests to make sure animals of the same age were employed for behavioural analysis.
181 Worms referred to as 'fed' were always tested within 10 mins of being removed from
182 the culture plate. 'Starved' animals were kept in liquid Nematode Growth Media
183 (NGM) for ≈30min.
184

185 **Chemotaxis experiments**

186 For our chemotaxis experiments we used 100mm petri dishes filled with 3%
187 chemotaxis agar as test plates . Employing a template we marked each of the test
188 plates with one center release point (see Figure 3E) and two smaller 'scoring' circles
189 (diameter: 3.5cm). Sodium azide (1.5µl of 1M) was applied to the center of each of
190 the scoring circles to immobilize the worms (Nuttley et al., 2001). Worms were picked
191 from the culture plates and collected in a small drop of NGM on a parafilm strip. In
192 order to reduce bacterial contamination we carefully removed liquid containing
193 bacteria and replaced it with new NGM. Worms were pipetted onto the center of the
194 assay plate and 1µl 1% benzaldehyde solution (in ethanol) was applied to one
195 scoring circle and 1µl 100% ethanol was applied to the other scoring circle. The

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196 plates were covered with aluminum foil and placed in the shielded room and left
197 undisturbed for one hour. For our pre-exposure experiments a strip of parafilm with a
198 2µl drop of 100% benzaldehyde was placed on the upper inside lid of a plate. After
199 90 minutes of pre-exposure the worms were tested as described above. For all
200 chemotaxis experiments we tested ≈50 worms per test. A preference index (PI) was
201 calculated by ascertaining the difference between the number of worms reaching the
202 benzaldehyde decision circle (B) and the 100% ethanol decision circle (E) and
203 divided it by the total number of worms scored, PI=(B-E)/(B + E).
204

205 **Magnetic coil set-up and magnetic shielding**

206 For earth-strength magnetic field manipulations we used a double wrapped custom
207 built Helmholtz coil system (Serviciencia, S. L). The coils were located in the center
208 of a 4.4m (long) x 2.9m (wide) x 2.3m (high) shielded room. The room was shielded
209 against static magnetic fields by a 1mm thick layer of Mu-metal and against
210 oscillating electromagnetic fields by an aluminum layer (5mm) (Magnetic Shielding).
211 The 'Inclination down' setting as used in this study comprises a magnetic field vector
212 with a 25µT horizontal component, -42 µT vertical component and an inclination of -
213 59.16°. The vertical component was inverted in the 'inclination up' treatment. Static
214 magnetic fields were measured using a Three-axis Fluxgate Magnetometer
215 (Bartington Instruments). Radio frequencies were measured using an EMI test
216 receiver (Rhode & Schwarz: MNr: LE0056) and an active shielded loop antenna 6507
217 (EMCO: MNr: E0575). The receiver was put on MAXHOLD and measurements were
218 taken for one minute.
219

220 **Burrowing assay**

221 We used 24cm long tubes filled with 3% chemotaxis agar (see Figure 3A), each end
222 was closed with a plastic stopper. The tubes contained three small holes (3mm in
223 diameter), one in the center and two 10cm apart from the center hole on either side.

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224 During filling of the tubes great care was taken to avoid air bubbles at the ends of the
225 tubes. Tubes with air bubbles were discarded. 1.5μl of 1M NaN_3 was added to each
226 end-hole of a test tube and ≈50 were injected into the center-hole (Figure 3A). The
227 test tube was then covered with aluminum foil and placed upright in a holder. The
228 holder was placed in the shielded room inside a smaller copper Faraday cage (Figure
229 2C). Tubes were left undisturbed overnight or alternatively over a day. At the
230 conclusion of the test the tubes were removed from the room and worms on either
231 side (3 cm from the end hole) were counted. The 'Inclination down' setting as used in
232 this study comprises a magnetic field vector with a 25μT horizontal component, 42μT
233 vertical component and an inclination of -59.16°. The vertical component was
234 inverted in the 'inclination up' treatment. These magnetic conditions were identical to
235 those employed by Vidal-Gadea. We calculated the burrowing index (BI) by dividing
236 the difference between worms on either side of the plastic tube (A), (B) by the total
237 number of scoring worms, BI=(A-B)/(A + B).
238

239 **Horizontal plate assay**

240 Non-starved worms (≈50) were placed, with a droplet of NGM, on the center of a
241 100mm style petri dish filled with 3% chemotaxis agar. Sodium azide (0.1 M, 20μl)
242 was applied to the rim of the plate to immobilize the worms once they reached it.
243 Worms were released from the NGM droplet by removing the liquid with a tissue. The
244 plate was then immediately placed in the center of the magnetic coils, described
245 above, and covered with aluminum foil. Animals were tested in one of four magnetic
246 directions (magnetic north pointing towards topographic north, east, south or west),
247 with a field strength of 32.5μT and 65μT (close to the strength of the horizontal
248 component of the Earth's magnetic field). In addition, we used two control conditions
249 where the double wrapped coils were switched to antiparallel currents, which resulted
250 in a zero magnetic field. We performed this control for the 32.5μT and 65μT field
251 settings. Worms were allowed to move freely on the plate for one hour, then the

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252 position and the direction of each worm relative to the center was recorded. Magnetic
253 field conditions were set by a person not involved in the analysis. Treatments and
254 field conditions were revealed after all worms were counted and the angles
255 measured.
256

257 **Magnetotaxis assay**

258 We used 100mm style petri dishes filled with 3% chemotaxis agar as test plates,
259 marked with one center release point and two smaller 'scoring' circles. Sodium azide
260 (1.5μl of 1M) was applied to the center of each of the scoring circles to immobilize
261 the worms. We randomly placed a magnet (N42 Neodymium 3.5-cm diameter
262 magnet 5 mm thick and nickel-plated) under one goal area, and a brass coin with
263 identical dimensions as a control under the opposing goal area. The magnet was
264 placed with the magnetic north pole pointing up in all tests. ≈50 worms were placed
265 in the central release point with a droplet of NGM. After the worms were released by
266 removing the liquid the plate was covered quickly with aluminum foil and placed in
267 the shielded room. After one hour the number of worms in each goal area were
268 counted blind. It should be noted that Vidal-Gadea performed this experiment over
269 30mins, however, our pilot experiments showed that a longer time resulted in a
270 higher percentage of worms in the goal areas. For our iron contamination
271 experiments the OP50 (in solution) was mixed thoroughly with magnetite to create a
272 1% magnetite/OP50 solution. Worms were then synchronized and grown on OP50
273 covered plates until they reached adulthood. Experiments were performed as
274 described above. In order to avoid cross-contamination separate picks were used for
275 the magnetite and non-magnetite trials. To calculate the preference index (PI) the
276 number of worms on the magnetic side (M) were subtracted by the number of worms
277 on the control side (C) and then divided by the total number of scoring worms, PI =
278 (M - C)/ (M + C).
279

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280 **Statistics**

281 In all tests the experimenter was blind to the particular treatment when counting the
282 worms. A one-tailed Wilcoxon test was used to analyse the chemotaxis and
283 magnetotaxis experiments. For the burrowing assay we used a two-tailed Wilcoxon
284 one-sample test to ascertain if worms burrowing preference differed from zero. In
285 order to compare groups we used a Mann–Whitney U test. All linear statistical tests
286 were performed in R (R Development Core Team, 2012). The circular data from the
287 horizontal plate assay were analyzed using Oriana 4. We define a true biological
288 replicate as a treatment application on one independent experimental unit (e.g. plate
289 with worms), in contrast to a technical replicate which simply involves multiple
290 measurements of the same experimental unit. As worms tested together at the same
291 time on the same plate can interact with each other they do not constitute
292 independent experimental units. Therefore, we calculated one mean orientation
293 vector for each test plate, by calculating the vector sum of all worms from this plate.
294 The directions from the plates, relative to the magnetic field and a geographically
295 fixed direction (topographic north), were then tested for a significant unimodal
296 orientation using the Rayleigh test. A summary of the statistics is shown in Figure
297 Supplement 1.
298
299 **Acknowledgements**

300 We wish to thank Boehringer Ingelheim for funding basic research at the Institute for
301 Molecular Pathology. We also wish to acknowledge the IMP-IMBA biooptical facilities
302 and the media kitchen. We are indebted to Manuel Zimmer, Annika Nichols and
303 Harris Kaplan for their assistance with the experiments described in this manuscript.
304 We thank Andres Vidal-Gadea for hosting Lukas Lander in his laboratory. The *C.*
305 *elegans* strain was provided by the CGC which is funded by NIH Office of Research
306 Infrastructure Programs (P40 OD 010440).
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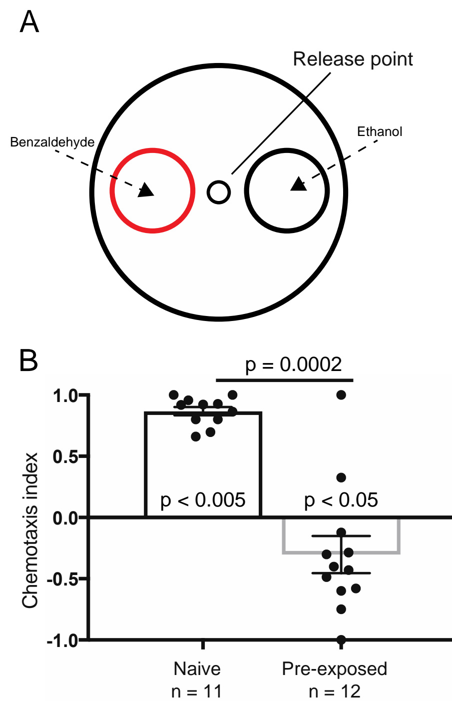
Figure 1



353

354 **Figure 1. Benzaldehyde control experiment**

355 (A) Experiment set up for the benzaldehyde positive control experiments. Worms
356 were placed at the release point and given a choice between 1% benzaldehyde in
357 ethanol, or 100% ethanol. (B) Naïve worms preferentially orientated towards the
358 benzaldehyde (n=11, P<0.005), and away from it if pre-exposed to benzaldehyde
359 (n=12, P<0.05).

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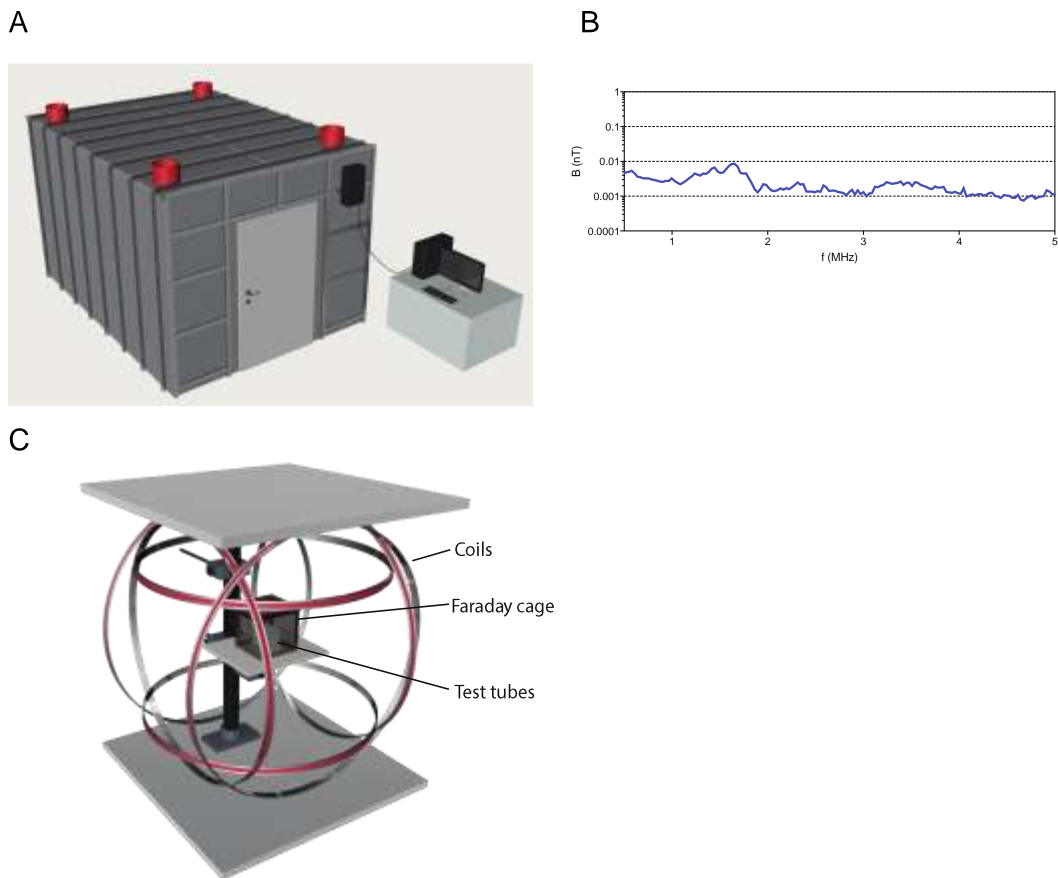
361

Figure 2



362

363 **Figure 2. Infrastructure for magnetic experiments.**
364 (A) All experiments were performed within a mu metal shielded room surrounded by
365 a 5mm aluminum Faraday cage. DC power sources and the computer driving the
366 Helmholtz coils were located outside this shielded room, and cables into the room
367 were filtered for radio frequencies. (B) Graph showing the radio-frequencies present
368 in the shielded room between 0.5 to 5MHz are below 0.01nT, indicative of very low
369 levels of radio frequency contamination. (C) Experimental setup for exposure of
370 worms to magnetic fields. Three pairs of double-wrapped Helmholtz coils surround a
371 plastic stage in the center. Worms were placed on this stage for the vertical
372 burrowing, horizontal plate, and magnetotaxis assays. In the burrowing assay we
373 surrounded the tubes by an additional small Faraday cage.

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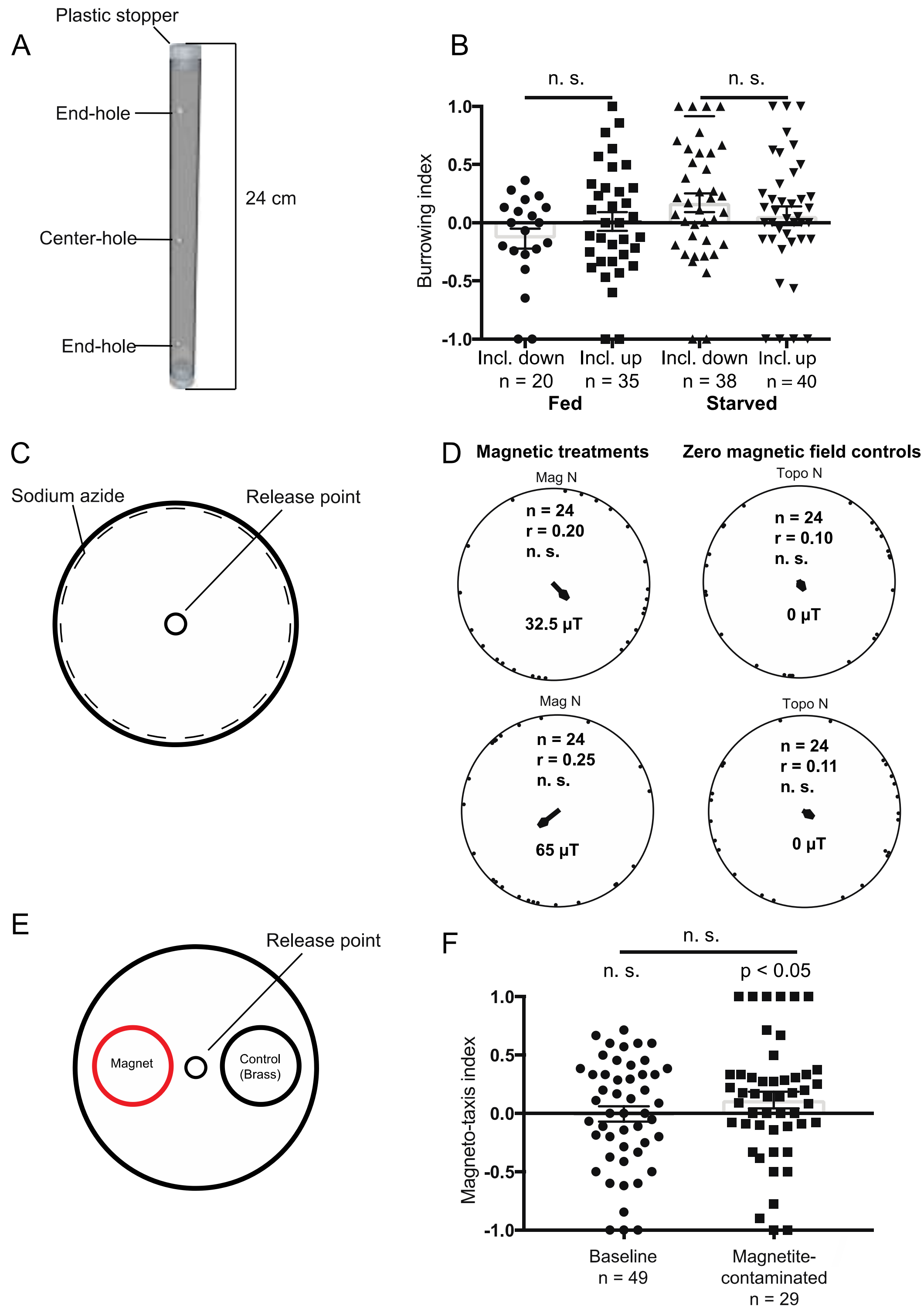

374

375 **Figure 3. Magnetic assays and results**
376 (A) Diagram showing the tubes employed for the vertical burrowing assay. Worms
377 were injected in the center hole, and NaN_3 in the end-holes to immobilize them. Fed
378 or starved worms were allowed to burrow overnight with the inclination of the
379 magnetic field either up (59.16°) or down (-59.16°). At the conclusion of the test the
380 worms on either side (3 cm from the end hole) were counted and a preference index
381 calculated. (B) Results for the vertical burrowing assay. We observed no significant
382 difference in the burrowing index when the inclination of the magnetic field was
383 inverted, whether the worms were fed or starved. (C) Set up for the horizontal plate

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384 assay. Worms were released in the center of the plate and allowed to move freely for
385 one hour before the position and the direction of each worm relative to the center
386 was recorded. Animals were tested in one of four magnetic directions (magnetic
387 north pointing towards either topographic north, east, south and west), with a field
388 strength of 32.5μT and 65μT. Control experiments employed antiparallel currents
389 resulting in a zero magnetic field. We calculated one mean orientation vector for each
390 test plate, by calculating the vector sum of all worms from this plate. (D) Results for
391 the horizontal plate assay. We observed no directional preference when worms were
392 exposed to either 32.5μT or 65μT magnetic stimuli. Each dot represents the mean
393 worm direction for one plate, while the black arrow showing the direction and length
394 (r) of the mean vector (radius of the circle is 1). Mag N indicates the normalized
395 magnetic north and Topo N the topographic north. (E) Set up for the magnetotaxis
396 assay. Worms were released in the center of a testing plate and could choose
397 between two 3.5 cm diameter circles (goal areas) with a strong magnet (0.29T) or a
398 brass control underneath. Worms in each of the goal areas were counted and a
399 preference index calculated. (F) We observed no preference for the area above the
400 magnet, unless worms were fed bacteria contaminated with magnetite particles
401 ($P<0.05$, $n=29$ plates). Error bars show standard error of the means.
402
403

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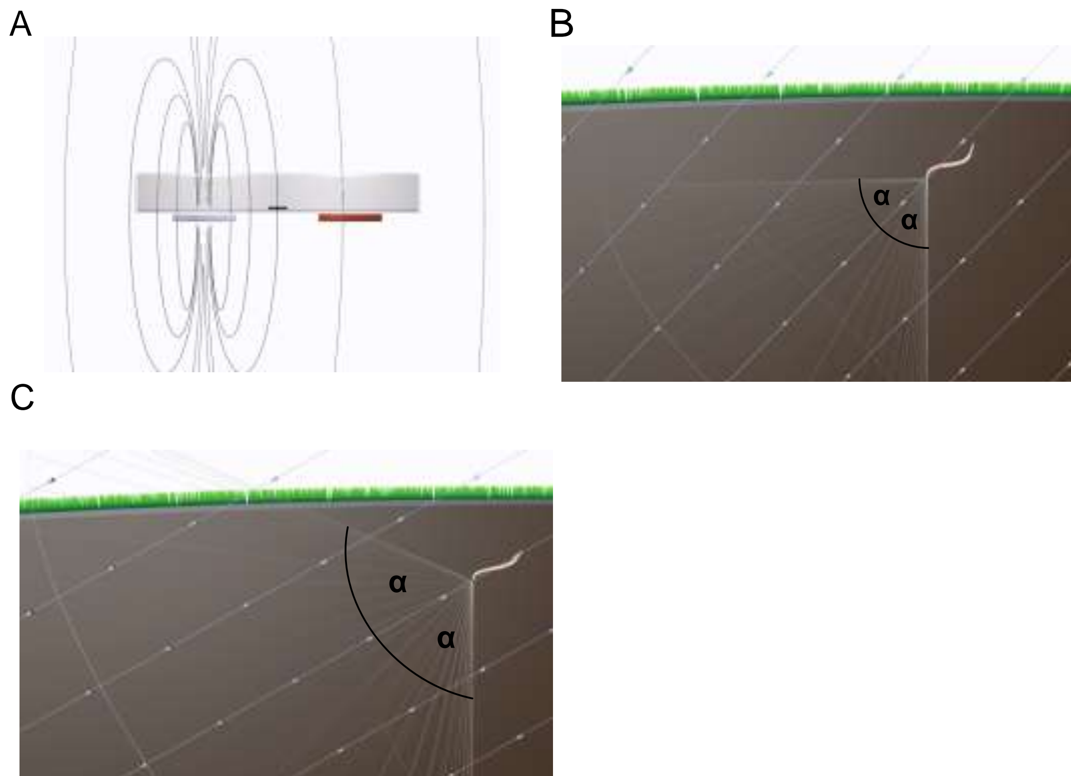
404

Figure 4



405

406 **Figure 4. Conceptual issues with the Vidal-Gadea hypothesis.**
407 (A) The magnetoaxis assay developed by Vidal-Gadea et al. (2015), relies on worms
408 moving towards a very strong neodymium magnet (0.29T) placed beneath an agar
409 testing plate. The magnetic field lines that emerge from this magnet (in the absence
410 of a thick layer of agar) are perpendicular (i.e. 90°) with respect to the plate surface.
411 As this is analogous to standing on the magnetic north pole of the planet (with all
412 directions being southerly), neither the polarity nor the inclination of the field can be
413 employed by nematodes as a guidance cue. The worms could detect the increasing
414 field intensity when moving towards the magnet, however, an intensity based
415 magnetoreceptor that is sensitive to 8,000 times Earth strength fields is unlikely to
416 have evolved on our planet. (B) The hypothesis advanced by Vidal-Gadea and
417 colleagues argues that nematodes exploit the inclination of the Earth's magnetic field
418 to guide vertical movement. They propose that nematodes adopt a correction angle
419 (α, e.g. 45°) relative to the inclination of the field, which varies depending on the
420 latitude. However, in the absence of gravitational information this is as likely to result
421 in a worm that travels horizontally as vertically. (C) As the latitude nears the equator
422 the correction angle increases (e.g. 60°), and consequently a worm is just as likely to
423 translate downwards, or at an oblique angle towards the Earth's surface. The blue
424 lines show the magnetic field vector.
425

Assay	Satiation stat	Sample size naiv	Sample size pre-exposed	p-value	critical value
Chemotaxis	fed	n = 11	n = 12	p = 0.0002	U = 10
Chemotaxis	fed	n = 11	NA	p = 0.002	V = 66
Chemotaxis	fed	NA	n = 12	p = 0.036	V = 15.5

Statistical test

Mann-Whitney U-test (two tailed)

Wilcoxon signed rank test (one sided)

Wilcoxon signed rank test (one sided)