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Large-Scale Gravitaxis Assay of *Caenorhabditis* Dauer Larvae

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Abstract

Gravity sensation is an important and relatively understudied process. Sensing gravity enables animals to navigate their surroundings and facilitates movement. Additionally, gravity sensation, which occurs in the mammalian inner ear, is closely related to hearing - thus, understanding this process has implications for auditory and vestibular research. Gravitaxis assays exist for some model organisms, including *Drosophila*. Single worms have previously been assayed for their orientation preference as they settle in solution. However, a reliable and robust assay for *Caenorhabditis* gravitaxis has not been described. The present protocol outlines a procedure for performing gravitaxis assays that can be used to test hundreds of *Caenorhabditis* dauers at a time. This large-scale, long-distance assay allows for detailed data collection, revealing phenotypes that may be missed on a standard plate-based assay. Dauer movement along the vertical axis is compared with horizontal controls to ensure that directional bias is due to gravity. Gravitactic preference can then be compared between strains or experimental conditions. This method can determine molecular, cellular, and environmental requirements for gravitaxis in worms.

Introduction

Sensing Earth's gravitational pull is crucial for many organisms' orientation, movement, coordination, and balance. However, the molecular mechanisms and neurocircuitry of gravity sensation are poorly understood compared with other senses. In animals, gravity sensation interacts with and can be outcompeted by other stimuli to influence behavior. Visual cues, proprioceptive feedback, and vestibular information can be integrated to generate a sense of body awareness relative to an animal's surroundings[1,2]. Conversely, gravitactic preference can be altered in the presence of other stimuli[3,4,5]. Therefore, gravitactic behavior is ideal for studying gravity sensation and understanding the nervous system's complex sensory integration and decision-making.

C. elegans is an especially useful model organism for studying gravitaxis because of its polyphenic lifecycle. When exposed to stressors during development, including heat, overcrowding, or a lack of food, *C. elegans* larvae develop into dauers, which are highly stress-resistant[6]. As dauers, worms perform characteristic behaviors, such as nictation, in which worms "stand" on their tails and wave their heads, that may facilitate dispersal to

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Disclosures

The authors declare no competing interests.

better habitats[7]. Gravitaxis assays of *C. elegans* and *C. japonica* suggest that dauer larvae negatively gravitax, and that this behavior is more readily observed in dauers than in adults[8,9]. Testing gravitaxis in other *Caenorhabditis* strains may reveal natural variation in gravitactic behavior.

Mechanisms for gravity sensation have been characterized in *Euglena*, *Drosophila*, *Ciona*, and various other species using gravitaxis assays[3,10,11]. Meanwhile, gravitaxis studies in *Caenorhabditis* initially provided mixed results. A study of *C. elegans* orientational preference found that worms orient with their heads down in solution, suggesting positive gravitactic preference[12]. Meanwhile, although *C. japonica* dauers were identified early on as being negatively gravitactic[8], this behavior has only recently been described in *C. elegans*[9]. Several challenges arise in developing a representative gravitaxis assay in worms. *Caenorhabditis* strains are maintained on agar plates; for this reason, behavioral assays typically use agar plates as part of their experimental design[13,14,15]. The earliest reported gravitaxis assay in *Caenorhabditis* was performed by standing a plate on its side at a 90° angle to the horizontal control plate[8]. However, gravitaxis behavior is not always robust under these conditions. While adult worms can be assayed for orientational preference in solution[12], this directional preference may also be context-dependent, leading to different behaviors if the worms are crawling rather than swimming. Additionally, *C. elegans* is sensitive to other stimuli, including light and electromagnetic fields[16,17], which interfere with their responses to gravity[9]. Therefore, an updated gravitaxis assay that shields against other environmental variables is important for dissecting the mechanisms of this sensory process.

In the present protocol, an assay for observing *Caenorhabditis* gravitaxis is described. The setup for this study is based in part on a method developed to study neuromuscular integrity[18,19]. Dauer larvae are cultured and isolated using standard procedures[20]. They are then injected into chambers made from two 5 mL serological pipettes filled with agar. These chambers can be oriented vertically or horizontally and placed within a dark Faraday cage for 12–24 h to shield against light and electromagnetic fields. The location of each worm in the chambers is recorded and compared with the vertical taxis of a reference strain such as *C. elegans* N2.

Protocol

The strains used in the present study are *C. elegans* (N2) and *C. briggsae* (AF16) (see Table of Materials). A mixed-sex population of dauers was used for each assay.

1. Chamber preparation
    1. Work in a fume hood. Set up the workspace with a Bunsen burner, 1–2 razorblades, pliers, tweezers, and a plastic cutting surface (see Table of Materials).
    2. For each chamber, gather two 5 mL serological pipettes. Remove the cotton plug from one pipette with tweezers. Hold a razorblade over the Bunsen burner until hot using pliers. Use the heated blade to slice off

the tapered end of the second pipette so that the entire pipette has a uniform diameter (Figure 1A).

3. Working quickly, bring the two modified pipette ends close to the flame, melting them slightly. Join these ends by pressing them firmly together, ensuring that the walls of both pipettes are continuous. If any gaps are visible after joining, break them apart and repeat this step (Figure 1A,B).

2. Filling the chambers with agar

1. Prepare NGM with 4% agar according to standard worm procedures[21]. While the agar is still molten, fill each chamber by attaching it to a serological pipettor and slowly drawing up the solution. Seal the tip with paraffin film before removing the chamber from the pipettor. Lay the chamber flat (parallel to the benchtop) to cool.

   NOTE: Covering the flask with cling-wrap (see Table of Materials) after autoclaving helps prevent bubbles from forming in the solution[18].

   1. To minimize any variations in the consistency of the agar due to uneven cooling, hold the pipettor (see Table of Materials) parallel to the benchtop while drawing up the agar. Lay the chambers flat on the benchtop and allow the agar to harden before moving.

      NOTE: The agar percentage and media content can be tailored to the experiment. 4% agar is stiffer than the roughly 2% concentration used for plate pouring and prevents worms from burrowing through the medium in our hands. However, other experimenters have observed burrowing behavior by adult worms in up to 9% agar[18,19].

   2. Once cooled, heat a 3 mm hex key (or another metal tool of the same size, see Table of Materials) over a Bunsen burner. Firmly press it into the wall of each chamber about 5 mm to one side of the midline, creating a small opening in the plastic (Figure 1C). Use a heated blade to remove the cotton and tapered ends of the chamber and seal these ends with paraffin film.

      NOTE: Once prepared, each chamber must be used within 1 day or kept at 4 °C for several days. Cover any exposed openings with paraffin film to prevent the agar from drying out.

3. Isolating dauer larvae

1. 10–15 days prior to the experiment, chunk each strain onto 2–3 large NGM plates with OP50 bacteria and wrap with paraffin film. After 10–15 days, each plate must be fully starved with thousands of dauer larva present on the agar, walls, and lids.

NOTE: Make plates ahead of time using a thick OP50 recipe for maximum crowding (see Table of Materials). Dauer formation can also be induced through other methods, including the addition of pheromones or by growing at higher temperatures[22].

2. Collect worms by rinsing the lids and plates with M9 buffer and pipetting the solution into 15 mL centrifuge tubes. Spin at 1,600 × *g* for 30–60 s at room temperature and aspirate most of the M9 buffer with a pipette or vacuum aspirator.

3. Isolate dauers by treating with 1% SDS followed by a 30% sucrose flotation gradient following the previously published report[20].

    1. Add 7 mL 1% SDS solution to each worm pellet. Leave the worms in SDS for 30 min; rotate the tubes continuously during this time to allow for aeration. Rinse 3–5x with M9 to remove the detergent.

    2. Next, add 5 mL M9 followed by 5 mL of cold, filtered 60% sucrose solution. Mix thoroughly, and then centrifuge at 1,600 × *g* for 5 min at room temperature to create a separation gradient.

4. Fill new 15 mL centrifuge tubes with 2 mL of M9 buffer. Crush the ends of glass Pasteur pipettes to widen the bore and use these pipettes to transfer the top layer of the solution (containing isolated dauers) from the sucrose gradient to the new tubes. Rinse the dauers 3–5x with M9.

    NOTE: There will be thousands of isolated dauers in solution at this point. They may be used immediately or kept aerated by rotating in 5–7 mL M9 overnight.

4. Adding dauers to the chamber

    1. Centrifuge dauers at 1,600 × *g* for 5 min at room temperature and aspirate most of the M9 solution with a pipette or vacuum aspirator. To estimate worm density, manually count the number of worms in three separate 1 µL droplets under a dissecting microscope. Use the average of these counts to approximate the number of worms per µL.

        NOTE: Some small volume pipettors may be unable to reach the bottom of a 15 mL centrifuge tube; in this case, a concentrated droplet of worms can be transferred first to a piece of paraffin film.

    2. Cut the end of a 10, 20, or 200 µL pipette tip to widen the bore. Set the micropipette to a slightly larger volume than the intended aspiration volume. Aspirate approximately 1–2 µL of the concentrated worm solution (ideally between 100–300 worms) and allow a small amount of air to enter the tip.

NOTE: Large numbers of worms (1,000+) in one chamber may increase the range of distances traveled due to overcrowding[9].

3. Gently push the pipette tip into the agar while depressing the micropipette. This will create an injection site in the agar without clogging the tip. Release the worms into the agar. Use paraffin film to seal the opening.

5. Running and scoring the assay

NOTE: Gravitaxis can be tested under various conditions that may affect behavior[9].

1. To eliminate as many variables as possible, place the chambers within a dark Faraday cage (see Table of Materials) at room temperature.

2. Label each chamber and hang it vertically within the Faraday cage (perform this using labeling tape). Test horizontal controls concurrently by laying some chambers flat within the same environmental conditions. Test the experimental strains against vertically oriented N2 worms as a positive control. Use horizontally oriented chambers containing N2 dauers as an additional negative control.

   NOTE: Horizontal and vertical assays must be performed in the same setting within the lab to minimize environmental variability between the two conditions. A large incubator may be used to house both Faraday cages.

3. Dauers begin to disperse from the start site after a few hours and take several hours to reach either end of the chamber. Leave the chambers undisturbed during this time and score within 12–24 h following injection.

   NOTE: Do not use a paralytic (such as sodium azide) to immobilize worms that have reached the ends of the chambers. Because the overall distribution of worms is being measured instead of a preference index (as in a two-choice assay), sodium azide is unnecessary and may alter the results.

4. Remove and score gravitaxis chambers one at a time. Look for live dauers under a dissecting microscope and mark their locations in ink (Figure 1C,D). Do not score worms within 2.5 cm to either side of the injection site, as these worms are not likely to be demonstrating a directional preference.

   1. Also, avoid scoring worms that appear dead or are trapped in the liquid. Discard any chambers containing >50% dead or swimming worms.

      NOTE: Once the chamber has been removed from the testing area, it needs to be scored promptly for accuracy. Dauers must

only be visible between the agar's surface and the pipette's wall. If burrowing behavior is observed, consider increasing agar concentration in future assays.

5. To quantify the results, use a marker to divide each half of the chamber into seven 3.5 cm sections starting 2.5 cm away from the injection site (on some pipettes, 3.5 cm = 1 mL volume). Use a manual tally counter (see Table of Materials) to tally the number of worms observed in each section.

   NOTE: There should be seven sections on each side of the origin, which can be numbered from −7 (bottom) to +7 (top). These numbers must correspond to the same relative locations based on how the chambers were constructed; agar is drawn from the −7 end to the +7 end in step 2.

Representative Results

Comparing gravitaxis across species

Following the procedure outlined above, *C. briggsae* dauer gravitaxis can be compared with *C. elegans* gravitaxis and horizontal controls. The vertical distribution (maroon) of *C. briggsae* dauers is skewed toward the tops of the chambers, with a large percentage of worms reaching +7 (Figure 2A). Contrasted with horizontal controls (aqua), in which dauers are distributed in a roughly bell-shaped curve around the center of the chambers, this trend indicates negative gravitactic behavior. These data can be compared with *C. elegans* gravitaxis performed on the same experimental days (Figure 2B).

A Kruskal-Wallis test followed by Dunn's test with Bonferroni correction for multiple comparisons determined any significant differences between assays[9,23]. In this experiment, 1,108 *C. briggsae* dauers were scored across three vertical chambers from two independent experimental days. These worms migrated significantly upward than horizontal controls ($p < 0.001$; 1,639 dauers in three horizontal chambers over 2 experimental days). Moreover, the vertical *C. briggsae* and vertical *C. elegans* distributions did not differ ($p > 0.05$; 386 *C. elegans* dauers in two vertical chambers over 2 experimental days). These results suggest that *C. briggsae* dauers show a negative gravitaxis behavior similar to that of *C. elegans* dauers.

Discussion

Comparison with prior methods

Unlike chemotaxis, gravitaxis in *Caenorhabditis* cannot be reliably observed using a traditional agar plate experimental design. A standard Petri dish is 150 mm in diameter, resulting in only 75 mm available in either direction for dauers to demonstrate gravitaxis preference. Although *C. elegans'* orientational preference can be assayed in solution[12], this method is low throughput as worms must be analyzed one at a time. Additionally, gravitactic preferences and behaviors may differ between worms floating in media versus crawling on a surface. For these reasons, we developed a high throughput assay with enhanced sensitivity that can be used to analyze gravitaxis behavior in crawling or climbing worms. Because

C. elegans are sensitive to light and electromagnetic fields, both of which interfere with gravitactic preference[9], these assays need to be performed without either stimulus. If a homemade Faraday cage is being used, checking the cage's strength and reinforcing it are necessary and recommended.

The assay chambers described in this protocol measure approximately 54 cm in length and are placed within Faraday cages to shield against light and electromagnetic fields. It was found that changing the "arena" for observing gravitaxis behavior has several advantages. First, it allows for detailed quantification and descriptive analysis. The relative distances traveled, and overall distributions of worms can be collected and compared instead of counting the number of negatively versus positively gravitactic worms. Second, the enclosed environment of an agar-filled pipette more closely replicates the conditions that may promote gravitaxis in the wild. As stated above, the dauer stage is a dispersal phase that allows escaping from unfavorable conditions[6]. Because *Caenorhabditis* live in compost, where guiding cues such as light may not be available, gravity may enable worms to navigate to the surface[6,9]. Two-dimensional plate-based assays are unlikely to replicate these conditions even if they are tested without other stimuli. Finally, this larger apparatus tests larger quantities of worms in a single assay.

Limitations and other considerations

While this protocol effectively measures gravitactic preference in large numbers of dauers, it is impractical for small or single worm experiments due to the time and materials required to construct each chamber. By combining counts across trials rather than using a gravitaxis index, statistical power is increased because each worm is treated as an independent event. While this enhanced sensitivity is useful in differentiating gravitactic and non-gravitactic worms, it is important to note that *C. elegans* dauers exhibit social behaviors[7,24] that could affect overall gravitaxis. We found that higher worm densities are correlated with a greater range in the distance traveled over the large-scale assay, though this effect is minimal when the total count is less than approximately 1,000 worms[9]. Interaction effects resulting from factors such as the total worms in each chamber should be monitored when analyzing the data. As with all behavioral assays, scoring should be blinded when possible.

Finding an average density of worms in the solution can minimize variability in the number of worms injected from chamber to chamber. Worms quickly settle in solution, so mixing the worms before pipetting, either by flicking the tubes or pipetting up and down with a wide-bore tip is important. Even when a consistent density is achieved, the number of worms added may vary because worms are likely to stick to the inside surface of the plastic pipette tips. Coating pipette tips with BSA or other solutions could minimize this effect. Worms must be injected with as little solution as possible; if too much liquid is added to the chamber, worms will become trapped in the solution and may even drift. For this reason, only live, crawling worms may be scored, and any chamber containing more than 50% dead or swimming worms must not be used. Chambers are to be removed from the Faraday cage one at a time and scored within 10–15 min for the greatest accuracy.

Potential applications

This assay may be used to test the environmental, genetic, and cellular requirements for gravitaxis in a variety of *Caenorhabditis* strains. So far, light and electromagnetic fields have been identified as stimuli that interfere with gravitaxis[9]. However, *C. elegans* is sensitive to other stimuli, including temperature, volatile and non-volatile chemicals, texture, humidity, and sound, which influence their behavior[25,26,27,28]. Understanding how various sensory inputs are integrated within the nervous system is an outstanding question in neuroscience, particularly when integration occurs at the level of individual neurons[9,29,30,31,32]. Sensory integration is especially important in proprioception, which is itself an integrative modality that draws from multiple sensory cues[1].

Understanding gravity sensation in *Caenorhabditis* has implications for human health. Millions of individuals suffer from vestibular dysfunction in the United States alone[33], and many of these disorders have underlying genetic causes[34,35]. For this reason, the identification of gene candidates and targeted therapies is an active area of research[36]. Additionally, because vertebrate vestibular and auditory systems are closely linked developmentally and evolutionarily[33,36,37], elucidating gravity sensation could also provide insight into hearing and hearing disorders.

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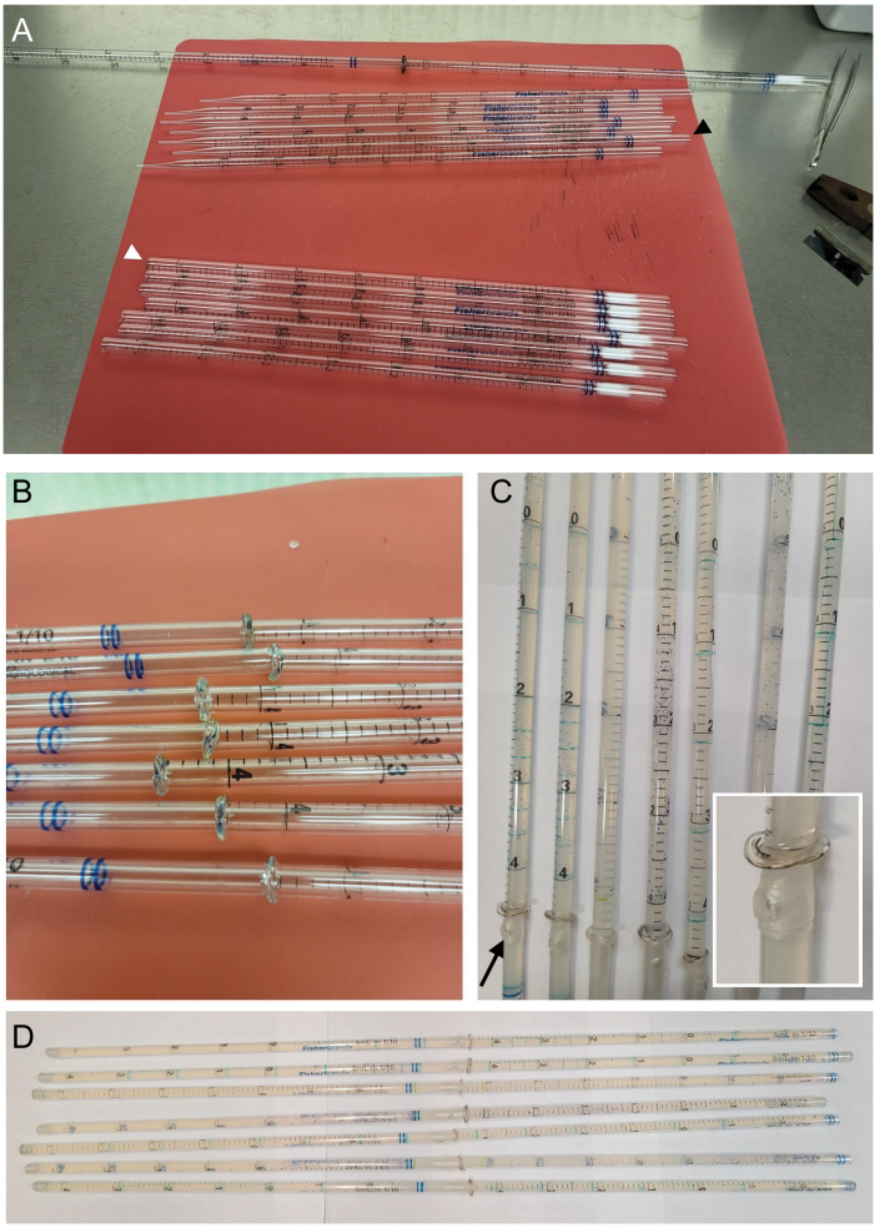

Figure 1: Gravitaxis assay chamber setup.

Photos depicting steps of gravitaxis assay chamber preparation. (**A**) Individual 5 mL pipettes were prepared for fusion into a single chamber. Removal of cotton plug indicated with black arrowhead; removal of tip indicated with a white arrowhead. The tubes used in this study have an inner diameter of 6 mm and are each 34.5 cm long (prior to cutting). (**B**) Completed chamber prior to addition of NGM agar. Pipettes have been fused by first melting over a Bunsen burner. (**C**) Example chambers filled with NGM agar. The injection site is indicated with an arrow and enlarged in the inset. Worms have been marked in ink, and lines are drawn to distinguish distances along with the chamber as well as to facilitate scoring. (**D**) View of chambers in their entirety (taken after scoring).

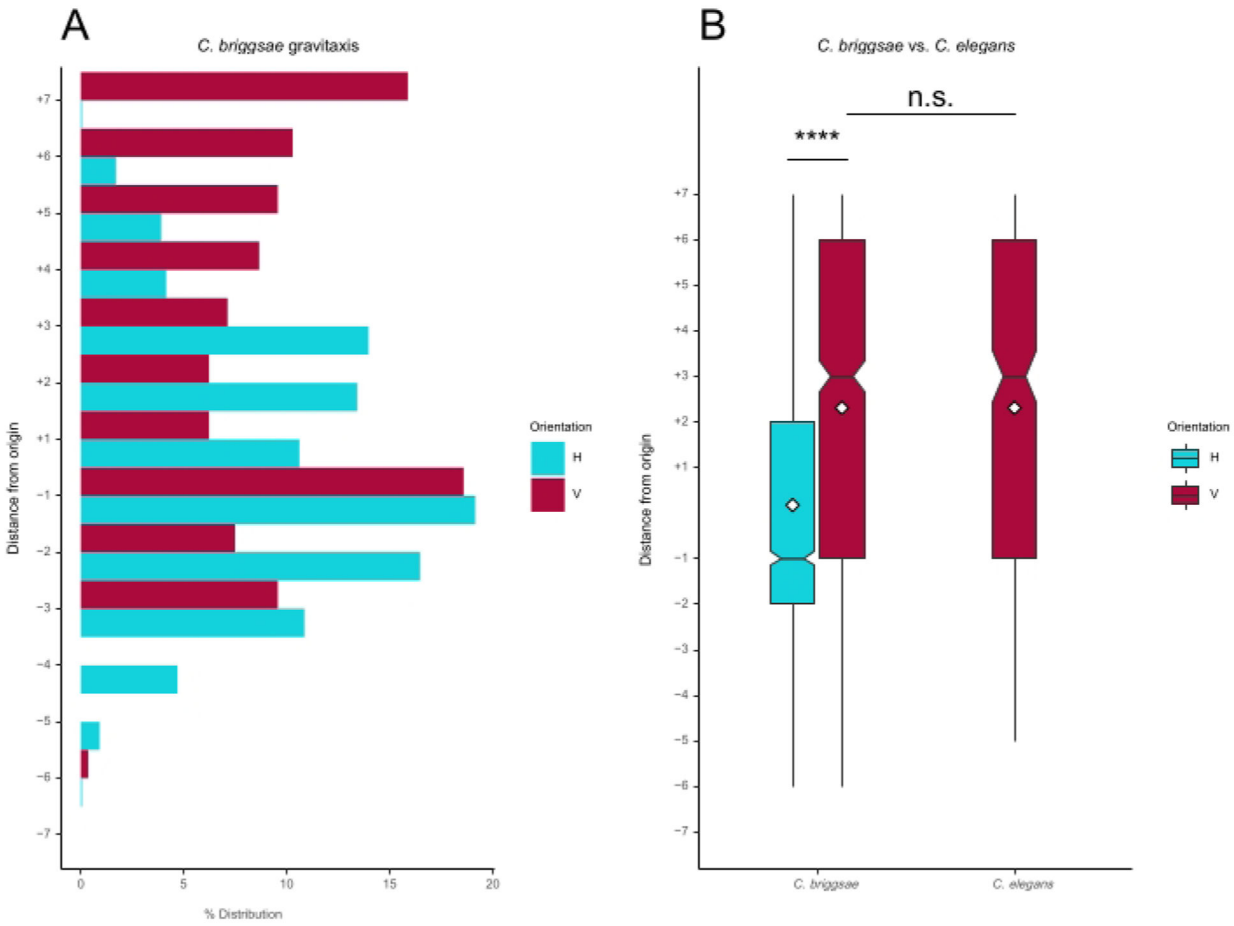

Figure 2: Gravitaxis in *C. briggsae* vs. *C. elegans*.

Results of gravitaxis in *C. briggsae* and *C. elegans*. (**A**) Histogram of *C. briggsae* gravitaxis. Movement in vertical chambers (maroon) is compared with horizontal controls (aqua). Distance from the injection point (−7 being the furthest below, +7 furthest above) is plotted as the percent of total worms assayed (x-axis). No data are plotted for the origin (+0) as worms are not counted within 2.5 cm to either side of the injection site. N = 1,639 worms across three tubes in the horizontal condition; vertical N = 1,108 worms across three tubes. (**B**) Boxplots comparing the distribution of *C. briggsae* horizontal and vertical worms versus *C. elegans* vertical worms. *C. briggsae* sample sizes are given above. *C. elegans* vertical N = 386 worms across two tubes. Means are indicated with white diamonds; vertical conditions are in maroon and are labeled “V”, and horizontal conditions (“H”) are in aqua. Comparisons were made using the Kruskal-Wallis test, followed by the Dunn’s test with Bonferroni correction for multiple comparisons. No stars = $p > 0.05$, **** = $p < 0.0001$.

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Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1% Sodium Dodecyl Sulfate solution			From stock 10% (w/v) SDS in DI water
15 mL Centrifuge tubes	Falcon	14-959-53A	
3 mm Hex key			Other similar sized metal tools may be used
4% Agar in Normal Growth Medium (NGM) - 1 L			Prior to autoclaving: 3 g NaCl, 40 g Agar, 2.5 g Peptone, 2 g Dextrose, 10 mL Uracil (2 mg/mL), 500 µL Cholesterol (10 mg/mL), 1 mL CaCl2, 962 mL DI water; After autoclaving: 24.5 mL Phosphate Buffer, 1 mL 1 MgSO4 (1 M), 1 mL Streptomycin (200 mg/mL)
5 mL Serological pipettes	Fisherbrand	S68228C	Polystyrene, not borosilicate glass
60% Cold sucrose solution			60% sucrose (w/v) in DI water; sterilize by filtration (0.45 µm filter). Keep at 4 °C
AF16 *C. briggsae* or other experimental strain			Available from the CGC (Caenorhabditis Genetics Center)
Bunsen burner			
Cling-wrap	Fisherbrand	22-305654	
Clinical centrifuge			
Disposable razor blades	Fisherbrand	12-640	
Faraday cage			Can be constructed using cardboard and aluminum foil; 30”L × 6”W × 26”H or larger
Ink markers			Sharpie or other brand for marking on plastic
Labeling tape	Carolina	215620	
M9 buffer			22 mM KH2PO4, 42 mM Na2HPO4, 86 mM NaCl
N2 *C. elegans* strain			Available from the CGC (Caenorhabditis Genetics Center)
NGM plates with OP50			1.7% (w/v) agar in NGM (see description: 4% agar in NGM). Seed with OP50
Paraffin film	Bemis	13-374-10	
Plastic cutting board			
Pliers			
Rotating vertical mixer	BTLab SYSTEMS	BT913	With 22 × 15 mL tube bar
Serological pipettor	Corning	357469	
Stereo Microscope	Laxco	S2103LS100	
Tally counter	ULINE	H-7350	
Thick NGM/agar plate media - 1 L			See 4% Agar in NGM recipe; replace 40 g Agar with 20 g Agar
Tweezers			