The Journal of Experimental Biology 216, 1470-1474

doi:10.1242/jeb.075739

## RESEARCH ARTICLE

# Negative gravitactic behavior of *Caenorhabditis japonica* dauer larvae

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### SUMMARY

**Gravity on Earth is a constant stimulus and many organisms are able to perceive and respond to it. However, there is no clear evidence that nematodes respond to gravity. In this study, we demonstrated negative gravitaxis in a nematode using dauer larvae (DL) of *Caenorhabditis japonica*, which form an association with their carrier insect *Parastrachia japonensis*. *Caenorhabditis japonica* DL demonstrating nictation, a typical host-finding behavior, had a negative gravitactic behavior, whereas non-nictating *C. japonica* and *C. elegans* DL did not. The negative gravitactic index of nictating DL collected from younger nematode cultures was higher than that from older cultures. After a 24 h incubation in M9 buffer, nictating DL did not alter their negative gravitactic behavior, but a longer incubation resulted in less pronounced negative gravitaxis. These results are indicative of negative gravitaxis in nictating *C. japonica* DL, which is maintained once initiated, seems to be affected by the age of DL and does not appear to be a simple passive mechanism.**

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/8/1470/DC1

Key words: graviperception, geotaxis, phoresy, nictation, waving.

Received 6 June 2012; Accepted 20 December 2012

## INTRODUCTION

Gravity is a constant stimulus for life on Earth. Most organisms, including animals, are able to sense the gravitational force and their behavior is influenced by gravity. Although nematodes are able to perceive and respond to different kinds of stimuli, such as chemical, mechanical and thermal stimuli, as well as light, magnetic fields and electric currents (Riga, 2004), information on graviperception and response to gravity in nematodes has been controversial (Croll, 1970). Early studies on nematode behaviors showed a phenomenon of upward movement in plant and animal parasitic nematodes, which was considered to be a negative gravitactic response (Lees, 1953; Croll, 1970). However, after additional experiments, it was concluded that those responses were instead consistent with unbiased random migration (Crofton, 1954; Barraclough and French, 1965). The only known example of negative gravitaxis in nematodes is from the vinegar eelworm, *Turbatrix aceti*. This species swims upward in vinegar culture medium because of the dragging effect of its heavy tail (Peters, 1952; Croll, 1970): the gravitaxis is passive in origin. To date, no study has experimentally demonstrated that nematodes actively respond to gravity and show negative gravitaxis.

In addition to negative gravitactic behavior, a typical behavior that is often observed in parasitic and phoretic nematodes, is nictation. Nictation, also known as waving, is a behavior in which the nematodes lift their anterior part (or more) of their body up in the air and wave it around (Croll and Matthews, 1977). This upward movement apparently increases the opportunity to infect or attach to hosts that move on the soil, but information on the mechanisms of induction and regulation of nictation is limited (Lee, 2002). Furthermore, there is no information on the physiological condition of nematodes that nictate.

*Caenorhabditis japonica* is a bacterial-feeding nematode that was discovered from the burrower insect *Parastrachia japonensis* (Kiontke et al., 2002). *Caenorhabditis japonica* dauer larvae (DL), a non-feeding third larval stage, specialized for surviving unfavorable conditions, are exclusively found on the body surface of adult female *P. japonensis*. *Caenorhabditis japonica* DL are able to recognize *P. japonensis*, which acts as their carrier insect, by their chemicals and embarks onto the carrier (Okumura et al., 2012). *Caenorhabditis japonica* DL disembark and the nematodes propagate in the nests of *P. japonensis* where they feed on the remains of eggs and nymph carcasses of *P. japonensis*. This indicates that *C. japonica* has a unique phoretic and necromenic association with *P. japonensis* (Okumura et al., in press).

During *in vitro* culture of *C. japonica* on artificial medium, newly produced DL show active upward migration and nictation (Tanaka et al., 2010) (supplementary material Movies 1, 2), and these behaviors seem to be useful for the nematodes to increase their opportunities of encountering and embarking onto host insects wandering on leaf litter. Because nictating DL migrate up, against the direction of gravity, onto their host insects and accumulate readily on pipette tips, we hypothesized that nictating DL could show negative gravitactic behavior. In this study, we demonstrated negative gravitaxis of *C. japonica* DL, and the influence of physiological conditions on this phenomenon.

## MATERIALS AND METHODS

### Nematodes

*Caenorhabditis japonica* strain H1 was isolated from an adult female of *P. japonensis* collected from Hinokuma Mountain Prefectural Park, Kanzaki City, Saga Prefecture, Japan. *Caenorhabditis elegans* N2 were obtained from the Caenorhabditis Genetics Center

(University of Minnesota, Minneapolis/St Paul, MN, USA). Nematodes were maintained on nematode growth medium (NGM) plates (1.7% agar) seeded with *Escherichia coli* strain OP50 (Stiernagle, 1999) at 25°C. DL of *C. elegans* and *C. japonica* were collected from NGM plates 20 and 10 days, respectively, after starting nematode culture depending on the appearance of the DL. Nematodes were treated with 2% sodium dodecyl sulfate (SDS) for 15 min and then washed with distilled water five to six times before use to eliminate non-dauer-stage nematodes. Dog food agar (DFA) medium (Hara et al., 1981) seeded with *E. coli* OP50 was used as a nutrient-rich medium to obtain a large number of DL. Nictating *C. japonica* DL were exclusively collected as described by Tanaka et al. (Tanaka et al., 2010). Briefly, a sterile yellow 200 µl pipet tip (Watson, Fukaekasei Co., Tokyo, Japan) was vertically placed, such that the tapered side was up, at the center of a 100 ml culture bottle of DFA medium, and *E. coli* OP50 and the nematodes were cultured at 25°C. Approximately 5 days after inoculation, the DL started to move upward towards the yellow tip and masses of nictating DL were picked up from the top of the tip using a fine needle, washed with distilled water three times, and used for subsequent experiments. DL from DFA medium, on days 6 and 7 after nematode inoculation, were used as otherwise mentioned. Because *C. elegans* DL did not move upward to the pipet tip, we were unable to use *C. elegans* DL for further assays.

To examine the effect of incubation medium on negative gravitaxis, nictating DL were soaked in M9 buffer (Stiernagle, 1999) for up to 120 h. Because nematode mortality increased dramatically thereafter, incubation was terminated at 120 h. Nematodes were collected from M9 buffer at 24 h intervals and used for gravitaxis assay.

Non-nictating *C. japonica* DL were isolated from 5-day-old nematode culture. Mixed-stage nematodes collected from DFA medium 5 days after starting the nematode culture were treated with 2% SDS solution for 15 min and then washed with distilled water five times.

To understand the effect of SDS treatment, which is used for killing non-DL and preparing DL for behavior experiments, nictating DL from day 7–9 cultures were treated with 2% SDS solution for 15 min and then washed with distilled water five to six times. Negative gravitaxis of SDS-treated or untreated DL was compared.

*Turbatrix aceti* obtained from a pet shop was cultured in apple vinegar solution (apple vinegar:water, 2:1) with small pieces of apple at 25°C in the dark.

### Negative gravitaxis assay

Nearly 3 µl of nematode suspension containing ~20 DL were inoculated onto the center of a 9 cm NGM plate (1.7% agar). Subsequently, water around the inoculated nematodes was absorbed by NGM and the DL started moving on the plate; hence we placed the plate vertically. As a control, we prepared the plates that were placed horizontally. The assay was repeated 20, 5, 37 and 10 times for non-nictating *C. elegans* DL, non-nictating *C. japonica* DL, nictating *C. japonica* DL and nictating *C. japonica* DL (horizontal control), respectively. All assays were performed in a closed room, to avoid the influence of air current, under the same conditions at 25°C. One hour later, we counted the number of nematodes that moved upward and downward from the inoculation point. Nematodes between the two horizontal lines, 1 cm above and below a horizontal central line (see Fig. 2A), were considered immobile and were not counted. The negative gravitaxis index (NGI) was calculated as follows: [(number of nematodes migrating upward)−(number of nematodes migrating downward)]/(total number of nematodes inoculated).

To confirm negative gravitaxis, the DL that had migrated upward beyond the line 1 cm above the horizontal center line were washed with distilled water and harvested by centrifugation at 3000 ***g*** for 1 min at room temperature. The collected DL were used to perform a new negative gravitaxis assay as described above.

Negative gravitaxis of a single nematode was also analyzed in the same manner described above using a single larva to eliminate any collective effects on nematode migration. The assay was repeated 10, 13 and 29 times for non-nictating *C. elegans* DL, non-nictating *C. japonica* DL and nictating *C. japonica* DL, respectively, using different individuals. The distribution of nematodes on the assay plate during the negative gravitaxis assay was recorded 1 h after nematode inoculation. Control plates that were placed horizontally were similarly analyzed.

### Center of gravity of the nematode body

The DL center of gravity was estimated qualitatively as follows. Individual nematodes were heat-killed at 60°C for 1 min. This treatment does not influence the morphology of nematodes and it is commonly used before fixation of the sample for morphological identification of nematodes. Heat-killed nematodes were placed just below the water surface in a 6 cm Petri dish, and they were allowed to sink according to Peters (Peters, 1952). These nematodes were categorized into three groups (head, body and tail), according to the nematode body part that first touched the bottom of the plates after free sinking.

### Statistical analysis

An ANOVA with the Bonferroni/Dunn test was used for the statistical analysis of negative gravitaxis (StatView version 4.54, Abacus Concepts, Piscataway, NJ, USA). A $P$-value $<0.05$ was considered significant. Statistical analysis of single nematode migration was performed using the chi-square test.

## RESULTS

### Nictating DL showed negative gravitaxis

When non-nictating DL collected from NGM plates were used, no difference between the number of upward and downward migrations of either *C. japonica* or *C. elegans* were observed, which resulted in low NGI values (Fig. 1A). In contrast, significantly larger numbers of nictating *C. japonica* DL migrated upward than downward ($P<0.0001$), and the NGI value was significantly higher than that of non-nictating DL. When we used a single DL for the assay, ~70% of the nictating DL migrated upward, which was a significantly larger percentage than those that moved downward (Fig. 1B). A comparison of the distribution of nictating and non-nictating DL on the plate showed that the nictating DL moved up higher than non-nictating DL (Fig. 2), which resulted in a difference in the NGI value between nictating (0.4) and non-nictating DL (0). To confirm the upward movement, the DL that migrated upward on the assay plates were collected and used for a second negative gravity assay. Larger numbers of DL moved upward, and the NGI value increased to ~0.6.

When plates were placed horizontally, nictating *C. japonica* DL equally dispersed to all directions on the plate (Fig. 2B) and the NGI value for 20 DL was –0.06 (see Fig. 1A).

### Effects of nematode condition on negative gravitaxis

Negative gravitaxis of nictating DL collected from cultures incubated for different periods was compared (Fig. 3). The NGI of nictating DL from a younger culture (days 6 and 7) was significantly higher than that from an older culture (day 9). After

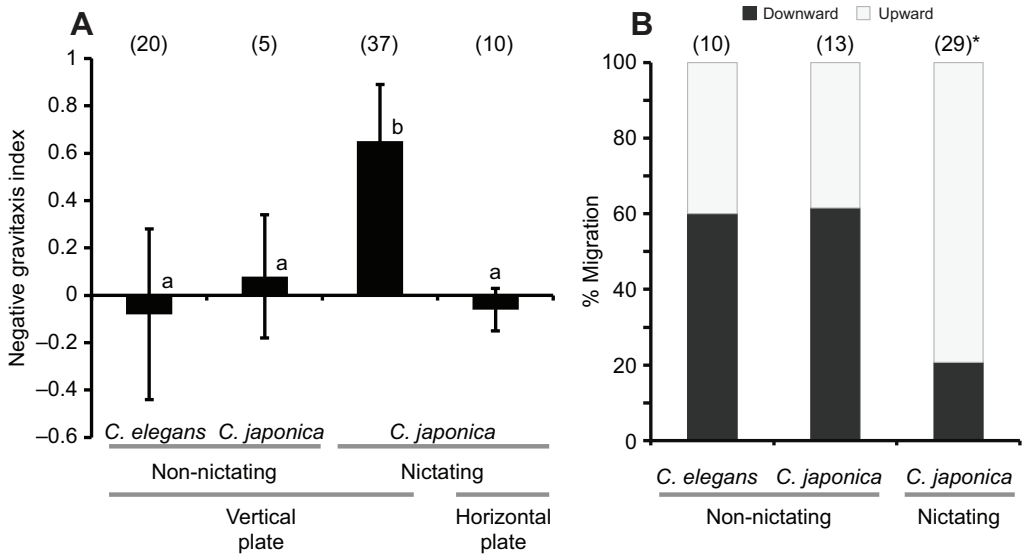


Fig. 1. Comparison of negative gravitaxis between *Caenorhabditis japonica* and *C. elegans* dauer larvae (DL). The distribution of nematodes on the assay plate during the negative gravitaxis assay was recorded 1 h after nematode inoculation. (A) Negative gravitaxis index; approximately 20 DL were used for each assay. Plates were set vertically or horizontally. Error bars indicate ±s.d. Different letters above the columns indicate a statistically significant difference detected by ANOVA with the Bonferroni/Dunn test ($P$<0.0001). (B) Percent migration upward and downward; a single DL was used for this assay. Asterisk indicates significant difference by the chi-square test ($P$<0.0001). Values in parentheses indicate the number of replicates.

day 9, the number of nictating DL often decreased and collecting DL was difficult.

The effect of SDS treatment, which was used to kill non-DL and collect DL, was tested. No significant difference was observed in the NGI values between SDS-treated and untreated *C. japonica* DL from day 7 and day 9 cultures (data not shown).

In a separate test to study the influence of incubation medium, nictating DL were incubated at the bottom of a flat Petri dish in M9 buffer for up to 120 h and then tested for negative gravitaxis. An incubation period of up to 24 h did not affect negative gravitaxis, but further incubation resulted in decreased negative gravitaxis (Fig. 4).

### Gravity center of body

To examine the possibility that gravitaxis is caused by a passive heavy tail dragging mechanism, as for *T. aceti*, we investigated the approximate center of gravity location of *C. japonica* using a sinking experiment. For this, we compared the center of gravity of body of the nematodes. *Turbatrix aceti*, which has a heavy tail, was used as a control and sank in the water from the posterior part, whereas *C. japonica* DL sank as if the center of gravity was located in the middle of the body, and no difference was observed between nictating and non-nictating conditions (Fig. 5).

## DISCUSSION

Positive and negative gravitactic behaviors have been reported in many nematodes. However, based on further experiments, it has been concluded that these behaviors were either unbiased or passively biased by dragging a heavy tail (Croll, 1970). Since then, no studies have reported evidence of active gravitaxis in nematodes. In the present study, we demonstrated a negative gravitactic behavior in nictating *C. japonica* DL. Our results indicate that the negative gravitactic behavior in *C. japonica* DL continues for several days once started, it is affected by the age of DL, and it does not appear

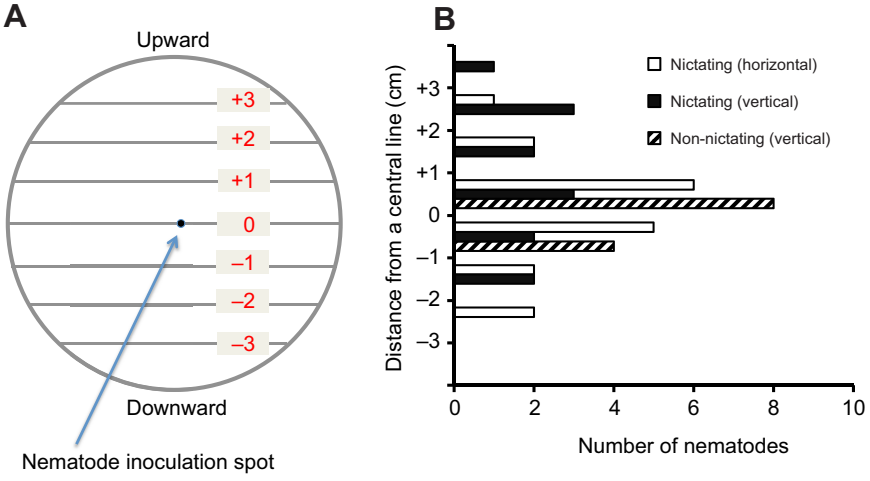


Fig. 2. Comparison of distribution between nictating and non-nictating *C. japonica* DL on a nematode growth medium (NGM) plate. (A) Diagram of the NGM plate used for the distribution assay. Numbers (–3, –2, –1, +1, +2 and +3) indicate the distance (cm) from the horizontal center line. (B) Distribution of DL on the assay plate 1 h after starting the experiment. The results of three different experiments were combined. Larger numbers of nictating DL moved upward while non-nictating DL did not on the plates were set vertically. When plates were set horizontally as a control, nematode distribution was unbiased.

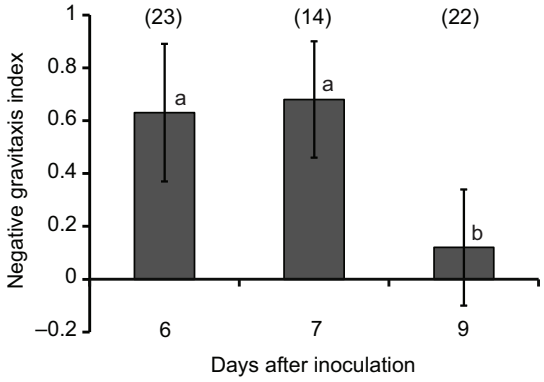


Fig. 3. Comparison of negative gravitaxis index of nictating *C. japonica* DL collected on different days. The negative gravitaxis index of nictating DL from a younger culture (days 6 and 7) was significantly higher than that from an older culture (day 9). After day 9, the number of nictating DL often decreased and collecting DL was difficult. Error bars indicate ±s.d. Different letters above the columns indicate a statistically significant difference as detected by ANOVA with the Bonferroni/Dunn test. Values in parentheses indicate the number of replicates.

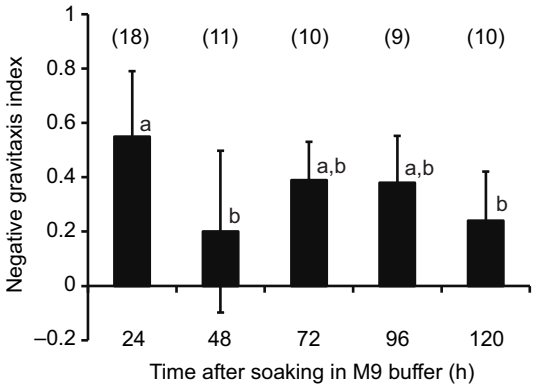


Fig. 4. Effect of the period of incubation in buffer on negative gravitaxis of *C. japonica* DL. Negative gravitaxis was observed until 120 h incubation in M9 buffer. Incubation of nematodes was stopped at 120 h because of the high mortality thereafter (see Tanaka et al., 2012). Error bars indicate ±s.d. Different letters above the columns indicate a statistically significant difference as detected by ANOVA with the Bonferroni/Dunn test. Values in parentheses indicate the number of replicates.

to be a simple passive behavior. This is the first experimental demonstration and characterization of negative gravitactic behavior in nematodes.

Nictation is a movement that continues for seconds to several minutes or longer (see supplementary material Movies 1, 2). It is unlikely that nictation *per se* is a response to gravity. Rather it is a behavior that allows nematodes to sense and respond gravity. Upward migration and nictation apparently increase the opportunity for DL to attach to hosts in the field. Thus, it is plausible that nictating *C. japonica* DL show a negative gravitactic behavior. But the mechanism of inducing negative gravitactic behaviors remained unclear. We assumed that negative gravitactic behavior of nictating DL might be caused by a change in the center of gravity from a central balance to a heavy tail through the accumulation of body fluid and/or hemocytes in the posterior part of the body during upward movement. To test this possibility, we performed a sinking assay. *Caenorhabditis japonica* DL seems to have a central balance and no difference was observed between nematode conditions, indicating that there was no physical change in the center of balance in *C. japonica* DL. In addition, nictating DL showed negative gravitactic behavior, whereas non-nictating DL collected from the same DFA medium did not. These results suggest that negative gravitactic behaviors were induced and influenced by the age of DL rather than by genetic and nutritional differences. It seems that nictating DL lift their body up on end and wave around (see supplementary material Movies 1, 2). When DL stop lifting themselves, gravity causes the lifted portion of the body to fall, perhaps allowing them to sense the way up and migrate in that direction (on average). Further studies on inducible conditions of nictation and negative gravitaxis will help elucidate host-finding behaviors and the relationships and consequences of these behaviors.

We demonstrated a change in negative gravitaxis based on the condition of the nematodes. NGI values of waving DL collected from older plates were lower than those collected from younger plates. Furthermore, NGI values of nictating DL that were incubated for 48 and 120 h in solution were lower than those of nictating DL incubated for 24 h. Although there was no significant difference between 24, 72 and 96 h incubation times, there was a tendency towards lower NGI values with longer incubation times. We are uncertain why the NGI value was lower after incubation for 48 h. Among the 11 plates used for experiments at 48 h, NGI values from two plates were negative with unknown reasons, which may have caused lower NGI values. However, NGI values were significantly lower after incubation for 120 h, and all plates showed similar tendencies. Longer incubation times caused nematode death, and nematode mortality was higher for incubation times greater than 120 h (data not shown) (see Tanaka et al., 2012). Swimming in solution as well as nictation appear to consume a large amount of energy because of the vigorous movement involved. The movement of *C. japonica* DL was much faster than that of other DL such as *C. elegans*. Although dauer is the survival stage under unfavorable conditions, *C. japonica* DL have a shorter survival time than *C. elegans* (Tanaka et al., 2012). The decrease in negative gravitaxis may be related to physiological changes, including depletion of the

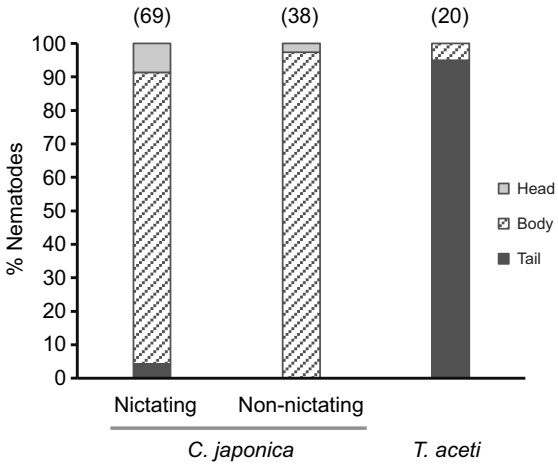


Fig. 5. Qualitative estimation of the center of gravity offset. Nematodes were categorized into three groups (head, body and tail) according to the nematode body part that first touched the bottom of the plates after free sinking. *Caenorhabditis japonica* DL sank from the middle of the body, and no difference was observed between nictating and non-nictating conditions, whereas *Turbatrix aceti* sank from the posterior part of the body.

energy reservoir, and fatigue after vigorous host-finding behavior. In a previous study, we found that the amount of triacyl glycerol did not significantly decrease, but protein carbonyl concentration, an indicator of oxidative damage and aging, significantly increased during the 6 day incubation of *C. japonica* DL (Tanaka et al., 2012). This suggests that oxidative stress could lower the survival rate of *C. japonica* DL and may be related to the decrease of negative gravitactic behavior. Very little information is available on negative gravitaxis in invertebrates. In *Drosophila*, negative gravitaxis is influenced by aging because of age-related decline in locomotor speed (Gargano et al., 2005; Simon et al., 2006; Rhodenizer et al., 2008). Our results, together with these data, indicate that aging may have a great influence on locomotion and negative gravitactic behavior in invertebrates including nematodes.

*Caenorhabditis japonica* DL have to locate *P. japonensis* wandering on the ground, and they must embark onto the insect after encountering the insect to form a phoretic association. Without an association with their carrier insect, the survival of *C. japonica* dramatically decreases (Tanaka et al., 2012). In addition, we found that nictating DL are able to recognize *P. japonensis* by their chemicals and embark specifically onto the carrier (Okumura et al., 2012). This results in their transfer to another place for propagation (Okumura et al., in press). Thus, both nictation and upward migration by negative gravitaxis appear to be important for the formation of a phoretic association with the host insect, and *C. japonica* seems to have acquired and developed these behaviors.

Because nictation is a typical host-finding behavior often observed in parasitic and phoretic nematode species (Croll and Matthews, 1977), it is possible that other nematodes also perceive and respond to gravity. The ability to detect and respond to gravity occurs through the action of mechanoreceptors in many animals (Barbercheck and Duncan, 2004). In *Drosophila*, negative gravitaxis requires a Johnston's organ, a mechanosensory structure located in the antenna that also detects near-field sound (Sun et al., 2009; Kamikouchi et al., 2009). Although the existence of graviperception mechanisms in *C. elegans* has been reported (Beckingham et al., 2005), there is no information about where and how gravity is perceived in nematodes. Lee et al. (Lee et al., 2012) found that nictation is regulated by IL2 neurons in *C. elegans*. Thus, IL2 neurons may be related to graviperception in nematodes. In the present study, we tried collecting nictating *C. elegans* DL. However, it was difficult to obtain a sufficient number of *C. elegans* DL for analysis because *C. elegans* DL are not as active as *C. japonica* DL. Therefore, we need to establish a method to collect a sufficient number of nictating *C. elegans* DL for further analysis. Further studies on gravitactic behaviors in other nematodes including *C. elegans* are necessary to understand the mechanisms of graviperception and response to gravity in nematodes.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| DFA | dog food agar |
| DL | dauer larvae |
| NGI | negative gravitaxis index |
| NGM | nematode growth medium |
| SDS | sodium dodecyl sulfate |

## ACKNOWLEDGEMENTS

*Caenorhabditis elegans* and *Escherichia coli* OP50 strains were supplied by the Caenorhabditis Genetics Center.

## AUTHOR CONTRIBUTIONS

E.O. performed the experiments and data analyses, and drafted the article. R.T. contributed to the design of the experiments. T.Y. contributed to the conception and design of the experiments, and the drafting and revising of the article.

## COMPETING INTERESTS

No competing interests declared.

## FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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**Movie 1.** Top view of nictating *Caenorhabditis japonica* dauer larvae (DL). DL lift and wave their anterior body part in the air. Nictation is supported by the posterior body part attached to the soil surface.


**Movie 2.** Side view of nictating *C. japonica* DL on plant residues in potting compost.