Precise sensorimotor control impacts reproductive fitness of *C. elegans* in 3D environments

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The ability of animals to sense and navigate towards relevant cues in complex and elaborate habitats is paramount for their survival and reproductive success. The nematode *Caenorhabditis elegans* uses a simple and elegant sensorimotor program to track odors in its environments. Whether this allows the worm to effectively navigate a complex environment and increase its evolutionary success has not been tested yet. We designed an assay to test whether *C. elegans* can track odors in a complex 3D environment. We then used a previously established 3D cultivation system to test whether defect in tracking odors to find food in a complex environment affected their brood size. We found that wild-type worms can accurately migrate toward a variety of odors in 3D. However, mutants of the muscarinic acetylcholine receptor GAR-3 which have a sensorimotor integration defect that results in a subtle navigational defect steering towards attractive odors, display decreased chemotaxis to the odor butanone not seen in the traditional 2D assay. We also show that the decreased ability to locate appetitive stimuli in 3D leads to reduced brood size not observed in the standard 2D culture conditions. Our study shows that mutations in genes previously overlooked in 2D conditions can have a significant impact in the natural habitat, and highlights the importance of considering the evolutionary selective pressures that have shaped the behavior, as well as the underlying genes and neural circuits. *NeuroReport* 35: 123–128 Copyright © 2023 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Decades of research using the nematode *C. elegans*, a tractable genetic model with a simple nervous system, have produced seminal discoveries that have contributed to our understanding of fundamental biological processes in development, physiology, and aging. In particular, due to its invariant cell lineage development, the hermaphrodite *C. elegans* nervous system consists of exactly 302 neurons and over 7000 synapses that coordinate its movement and a plethora of behaviors [1,2]. The knowledge of the full *C. elegans* connectome allows us to understand the behavioral circuitry from the sensory level to the motor output level. However, behaviors are also understood at the evolutionary level as adaptations in a specific ecological environment that increases reproductive fitness of the animal. While establishing standard laboratory culture conditions that are easy to maintain and amenable to observation was a critical step in establishing *C. elegans* as one of the major model organisms [3], it has limited our imagination in understanding the relevance of the discoveries in relation to the animals' natural ecology. Research in the last decade or so has shed light on many aspects of the natural habitat and ecology of *C. elegans*, leading to studies that revealed the ecological significance of previously studied behaviors, such as odor- and pheromone-related behaviors, and behaviors exhibited by dauer stage worms [4–9].

Navigation behavior in a complex habitat requires both intact sensory function and intact motor function to migrate through the environment. Normal 2D laboratory culture of *C. elegans* is a simple environment that even animals with defective sensory and motor function can survive and reproduce competently [10–12]. However, *C. elegans* natural habitat is a 3D complex environment that requires an intact sensory system to survive in [10]. Whether navigation in 3D environments can occur normally when sensorimotor control is compromised has not been investigated.

In the 2D environment, *C. elegans* navigates toward attractive odor cues by relaying odor information from sensory neurons to head motor neurons that control head muscles to steer the worm toward the sensory cue. This requires a specific interneuron called the RIA neuron that encodes head movement through compartmentalized calcium

dynamics in the axons [13]. When an attractive odor is nearby, signals from odor sensory neurons are also relayed to the RIA, where the two inputs are integrated to activate SMD head motor neurons and effectively steer the worm towards the odor [14]. However, animals defective in sensorimotor control via the RIA neuron are not necessarily compromised in their odor attraction behavior in 2D. This implies that sensorimotor function is dispensable for navigation, counterintuitive to the idea that specific sensorimotor circuitry has evolved in the nematode to impart selective fitness advantages in its natural habitat.

To rectify the role that sensorimotor function may have on *C. elegans* navigation behavior, we analyzed steering behavior in a 3D environment that closely simulates the nematode's spatially complex natural habitat. In this study, we first designed a 3D chemotaxis assay where *C. elegans* are required to navigate a 3D agar environment to reach an odor, and then asked whether attraction behavior to odors occurs normally in 3D environments. We then determined whether proper sensorimotor control was necessary for migration toward an appetitive odor in 3D. Finally, we assessed whether sensorimotor control was necessary for normal reproductive fitness in the 3D environment.

Materials and methods

C. elegans growth and maintenance

Worms were cultivated and kept at 20 °C on Nematode Growth Medicum plates seeded with E. coli OP50 as previously described [15]. Strains used for this study, N2, *gar-3(vu78)*, and *tax-2(p671)* were obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA).

3D cultivation chamber - NGT-3D

Nematode growth media tube-3D (NGT-3D) was prepared using methods previously described [16].

Chemotaxis assay

Standard 2D chemotaxis assay was carried out using previously established methods [17]. 1 µl of odors dissolved in ethanol were added at the following dilutions: 1:1000 for 2-butanone (Sigma), 1:100 for diacetyl (Waco), 1:200 for benzaldehyde (Sigma), and 1:100 for isoamyl alcohol (Sigma).

The composition of the 3D chemotaxis assay agar was identical to the 2D media, except 0.5% Difco agar was used instead of 1.6% (3 g NaCl, and 5 g Difco Granulated Agar in 1 liter distilled water, after heating and cooling to below 60 °C, add 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM KPO_4 was added) [10]. To embed odors underneath the agar arena, Odor was mixed with the cooled, but still molten, agar at the same dilution described above. 5 µl of the molten agar containing odors was placed on one side of a 9 cm plate as shown in Fig. 1c. Molten agar without any odor was placed on the other side as a control. After the odor gels hardened, 50 mL of molten agar was poured on top, creating a 1 cm thick layer of agar. Before the gel completely hardened, a 5 mm deep divot was created in the middle of the plate by gently aspirating the agar using a pasteur pipet. Approximately 30 min after pouring the agar, when the agar is sufficiently hardened, day 1 adult worms were collected from their culture plate and washed three times with S-basal. Worms were added to the divot and buffer was gently removed with a kimwipe. Plates were used immediately after they were made, to keep the level of odor diffusion consistent between trials. Worms in each area were counted after 1 h to calculate the 3D chemotaxis index (Fig. 1c).

Relative brood size

Relative brood size was measured as previously described [10]. Briefly, 96 h after L4 worm was placed into 2D or 3D culture, progeny worms that were L3 larval stage and older were counted. This was to ensure that only the first-generation worms were counted. To count progeny on 2D plates, worms were immobilized by lightly spraying 0.1 M sodium azide solution to the surface of the plate. To count progeny on NGT-3D, the whole tube containing the worm culture was immersed in 88 °C water bath to melt the agar. Melted agar with the dead but intact worms was poured into a 9 cm plate, where worms L3 and older could be counted.

Results

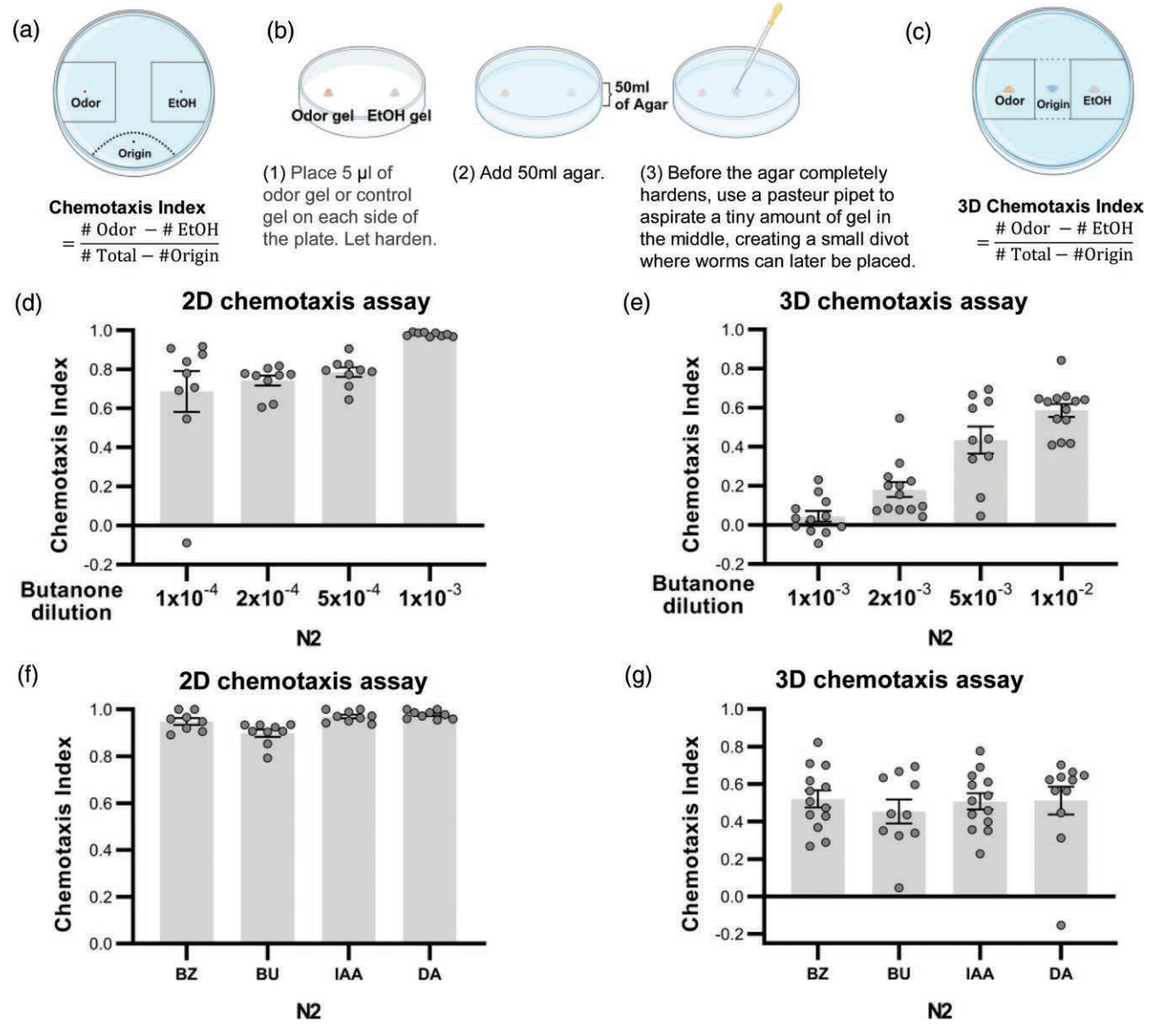
Worms can track and migrate toward attractive odors in 3D

In order to more closely simulate *C. elegans* sensory behavior in its natural habitat, we designed a 3D chemotaxis assay where worms have to navigate a 3D agar gel matrix to reach an attractive odor (Fig. 1a–c). Instead of odors placed on the surface of the agar as with the standard chemotaxis assays in 2D (Fig. 1a), odor was embedded underneath a thick 1 cm-deep agar (Fig. 1b). Soon after the plate was set, worms were added to the middle of the assay plate, where they must move not only horizontally but vertically within the thick agar gel to reach the odor. After 1 h, a chemotaxis index is calculated to ascertain attraction behavior (Fig. 1a and c).

To compare odor attraction behavior in the 2D and 3D chemotaxis assays, we tested wild-type N2 strain animals for the ability to track an attractive odor. The odor butanone is strongly attractive to *C. elegans* in a range of concentrations in 2D (Fig. 1d). We found that wild-type animals required a higher concentration of odor to effectively track the odor in 3D; although *C. elegans* easily migrates to butanone at 10^{-3} dilution in 2D, they are unable to sense the odor at the same concentration in 3D. At dilutions 5 to 10 times higher, however, they can track the odor rather well (Fig. 1e). We therefore used a dilution of 5×10^{-3} of odor for all further experiments.

We next tested whether the 3D assay was effective to determine attraction behavior to other odors. Since butanone concentration was increased 5-fold from what is used in the standard 2D assay, we increased the standard concentration of all other odors by the same amount.

Fig. 1



Design of 3D chemotaxis assay. (a) Diagram of the standard chemotaxis assay plate. (b) Schematic representation describing the steps to make the 3D chemotaxis assay plate. (c) Diagram of 3D chemotaxis assay plate. (d–e) Chemotaxis index in various dilutions of 2-butanone in 2D and 3D, respectively. (f–g) Chemotaxis index of various odors in 2D and 3D, respectively. BU, 2-Butanone; BZ, Benzaldehyde; DA, Diacetyl; IAA, Isoamyl alcohol. Data are represented as mean ± SEM.

Diacetyl, benzaldehyde, and isoamyl alcohol are all attractive odorants commonly used to test chemotaxis behavior (Fig. 1f). We found that in 3D, worms displayed chemotaxis to all odors at a comparable chemotaxis index to butanone (Fig. 1g). Thus, *C. elegans* odor sensory ability is intact in the 3D environment, and worms are able to navigate towards a variety of attractive odors in 3D.

Proper control of head movement is required for effective navigation towards odor in 3D

Our previous study demonstrated that the 3D environment presented navigational challenges for *C. elegans* in finding bacterial food that is absent in the standard 2D environment [10]. We wondered, then, whether mutant animals with subtle navigational defects that are tolerated in 2D may display impaired odor chemotaxis in 3D. To test this, we chose the *gar-3* mutant, which is defective for a G-protein-coupled muscarinic acetylcholine receptor [13]. GAR-3 is expressed in the RIA interneuron, which makes reciprocal connections with head motor neurons to modulate head bending. *C. elegans* locomotion is characterized by sinusoidal body movement steered by the head bend angle. Without *gar-3*, worms cannot receive cholinergic input from the head motor neurons, leading them to display exaggerated head bends and higher amplitude gait. Interestingly, RIA interneuron

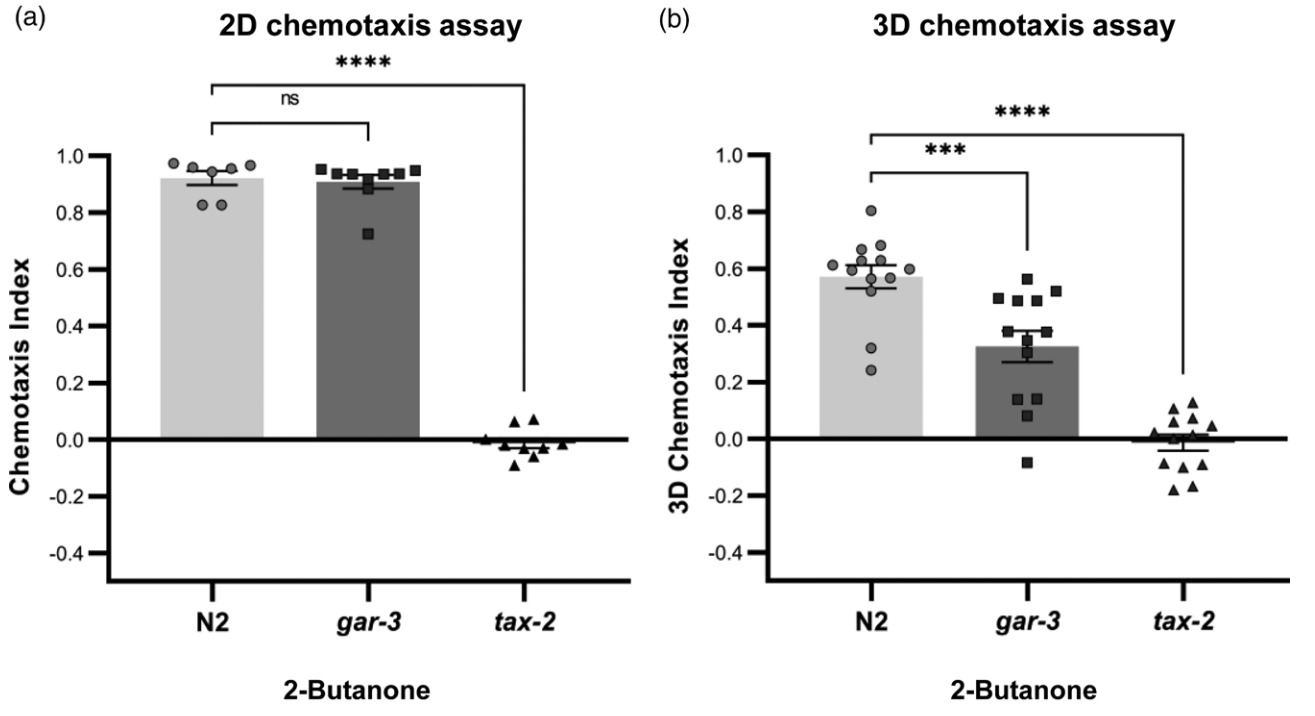
also lies downstream of the glutamatergic olfactory circuit and integrates the two signals to produce appropriate head bends to steer the worm towards the direction of an attractive odor [13]. Without *gar-3*, mutants were less efficient in steering towards the odor, thereby having to travel a longer distance to reach it. We wondered whether this minor inefficiency in 2D would become a significant impediment in 3D navigation, in which more sophisticated and precise steering is required.

When we tested *gar-3* mutants in the 2D assay, they showed normal odor attraction behavior, displaying a chemotaxis index as high as N2 worms (Fig. 2a). This indicates that *gar-3* mutants have no difficulty in traveling to the odor source in a 2D environment. This was in contrast to *tax-2* mutants, which are defective for the cGMP-gated calcium channels required for sensing odors such as butanone, which could not navigate towards the odor at all, as previously established [11]. Interestingly, when tested in the 3D assay, *gar-3* worms showed a significantly decreased chemotaxis index compared to N2 (Fig. 2b). The lower chemotaxis index was not due to defective motility in 3D; mutant worms were found throughout the plate, with some reaching the walls of the plate, in the same way as the wild type. The odor sensing-defective *tax-2* mutants were unable to navigate towards the odor whether in 2D or 3D. Thus, subtle navigation defects such as one seen in *gar-3* mutants become a significant disadvantage when worms need to navigate the 3D environment.

Defective 3D navigation negatively impacts reproductive fitness

In normal 2D culture, C. elegans wild-type adults navigate and feed on a large bacterial lawn of about 30-40 mm in size and lay about 250–300 eggs for several days. In 3D culture, C. elegans must navigate a much smaller bacteria colony of about 1 mm in size. However, wild-type adults do not appear to have difficulty navigating, redding, and reproducing in 3D as their brood size and brood development in 2D and 3D are similar [10]. Previously, we showed that a mutant with severe sensory defects that survived and reproduced normally in 2D culture could not reproduce in 3D [10]. Furthermore, we demonstrated that even in 2D, the sensory mutant could no longer reproduce when bacteria colonies were smaller and sparser, demonstrating that full sensory capabilities were necessary to locate food and reproduce in challenging environments [10]. We wondered whether the gar-3 mutant that has intact sensory function but compromised steering abilities could navigate, feed, and reproduce normally in the challenging 3D environment.

Fig. 2



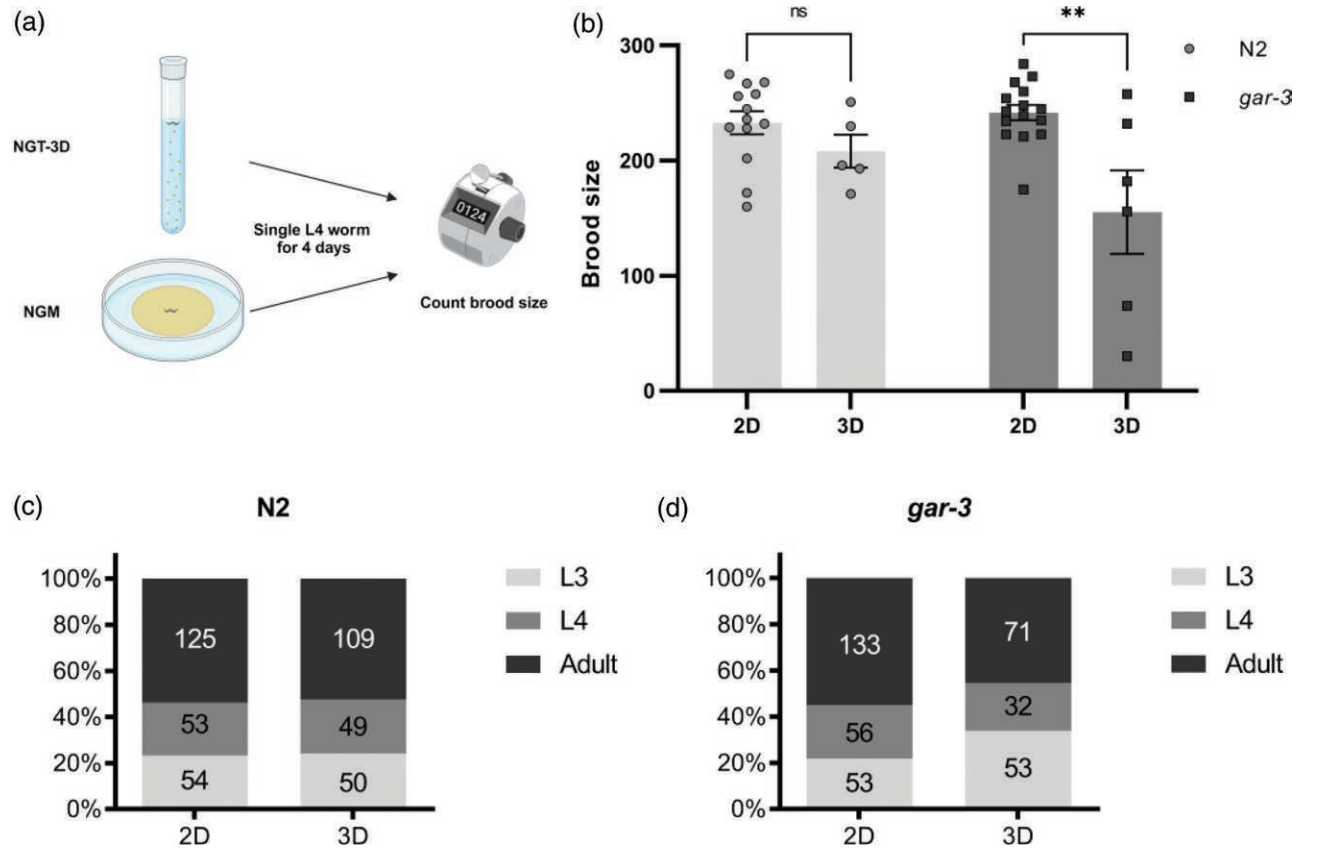
3D chemotaxis in different *C. elegans* mutant strains. (a) Chemotaxis index in the standard 2D assay. (b) Chemotaxis index in 3D chemotaxis assay. Data are represented as mean ± SEM. Statistical analysis was conducted using PRISM with ordinary one-way ANOVA, followed by Dunnett's multiple comparison test for between-group comparisons. Asterisks indicate $P \leq 0.001$ (***) and $P \leq 0.0001$ (****).

To test this, we placed single worms into either 2D or 3D culture, and counted the number of progeny after 96 h, following previously established protocol [10] (Fig. 3a). Because by then the second-generation eggs begin to hatch, we limited our counting to adult, L4, and L3 larval stage worms to ensure that only the first-generation progeny is considered. We call this 'relative brood size' to contrast it with total brood size [10]. Because relative brood size measures progeny within a limited time frame, it tends to depend on how soon they encounter food after being placed in culture, as starved worms stop laying eggs [10]. As reported previously, wild-type N2 worms show similar relative brood sizes whether in 2D or 3D (Fig. 3b). While *gar-3* mutants showed brood size indistinguishable from the wild type in 2D, they had significantly lower brood size in NGT-3D (Fig. 3b). In addition, when we analyzed the larval stages of the progeny, wild-type N2 worms showed similar distribution of each larval stage whether in 2D or 3D (Fig. 3c). In *gar-3* mutants, however, progeny in 3D tended to be younger, which indicates that mutants laid eggs at a delayed pace than wild type, which corresponds to the decrease in relative brood size (Fig. 3d). We also noted considerable variation of relative brood size between the individual trials, which may be a result of variable success in finding food. Thus, the subtle navigational defect of *gar-3* due to defective sensorimotor integration leads to a significant decrease in reproductive fitness in a more complex 3D environment.

Discussion

Although a leading genetic model organism, *C. elegans* is first and foremost a free-living nematode that occupies a specific ecological niche and is shaped by evolutionary pressures. The more recent efforts in studying the impact of different bacterial diets, natural predators and infectious agents, as well as strategies employed to overcome limited resources, have revealed the functions of genes whose importance had been previously missed [4,6,18]. Furthermore, decades of cultivating the Bristol N2 strain in the laboratory have led to the loss of gene functions and behaviors that are still found in wild isolates of *C. elegans*, such as oxygen-related behaviors and foraging

Fig. 3



Relative brood size in 2D standard NGM plate and NGT-3D. (a) Schematic representation of comparing relative brood size between 2D and 3D. (b) Comparison of relative brood size between N2 and *gar-3*. (c and d) Distribution of larval stages in the progenies of N2 and *gar-3* in percentages. Numbers inside bars indicate total number of worms in each larval stages. Data are represented as mean ±SEM. Statistical analysis was conducted using IBM SPSS Statistics with Student's *t*-test. Asterisks (**) indicate ** $P \leq 0.01$.

[19,20]. This highlights the fact that genomes of organisms are always being shaped by selective pressure.

In this study, we have shown that 3D conditions require higher precision in sensorimotor control and navigation and that subtle defects in navigation are greatly magnified in 3D and therefore detrimental to survival. The head movement of *C. elegans* is distinct from the rest of the body in that it can move in all directions while the rest of the body can only move in the dorso/ventral plane [21]. However, because of 2D laboratory culture conditions and difficulty in tracking and imaging worms in a 3D environment, deeper studies into the neural mechanism and significance of the 3D steering have been lacking.

Nevertheless, a number of studies have designed various novel culture conditions that allowed them to observe a richer variety of worm behaviors. These studies observed that the 3D environment requires more diverse muscle functions than the 2D environment [22,23], and that worms can discriminate and prefer certain spatial patterns of their surroundings [24,25]. In Lee *et al*., the authors designed agar plates with an uneven surface to observe nictation, dispersal behavior of dauer stage worms, because the behavior is not displayed on a flat surface [6]. Similar future efforts, combined with the powerful tools available in *C. elegans*, will lead to a fuller understanding of its genome and a fuller understanding of our own.

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Conflicts of interest

There are no conflicts of interest.

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