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Feeling the weight of the world: Gravity sensation and sensory integration in *C. elegans*

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Doctor of Philosophy
in
Molecular, Cellular, and Developmental Biology

by

Caroline Rose Ackley

Committee in charge:

Professor Joel Rothman, Chair
Professor Julie Simpson
Professor William Smith
Professor Craig Montell

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The Dissertation of Caroline Rose Ackley is approved.

Professor Julie Simpson

Professor William Smith

Professor Craig Montell

Professor Joel Rothman, Committee Chair

May 2022

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Dedicated to my parents

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Curriculum Vitæ

Caroline Rose Ackley

Education

2022	Ph.D. in Molecular, Cellular, and Developmental Biology (Expected), University of California, Santa Barbara.
2014	B.Sc. in Biopsychology, University of California, Santa Barbara.

Research Experience

2016—present **Graduate student researcher**
University of California, Santa Barbara
Principle Investigator: Joel Rothman
Research Area: Neurodevelopment and behavior in the model organisms *C. elegans* and *H. exemplaris*
1st Rotation PI: Julie Simpson
Altered grooming patterns across species of *Drosophila* and following amputation
2nd Rotation PI: William Smith
miRNA expression profiling of *C. robusta* larvae with neural tube closure defects
3rd Rotation PI: Joel Rothman
Bulk sequencing of sorted mutant embryos and *H. exemplaris* protocol development

2015—2016 **Laboratory Assistant II**
Neuroscience Research Institute
University of California, Santa Barbara
Principle Investigator: Benjamin Reese
Research Area: Mouse retinal development

2014—2015 **Laboratory Assistant I**
Neuroscience Research Institute
University of California, Santa Barbara
Principle Investigator: Benjamin Reese
Research Area: Mouse retinal development

2013—2014 **Undergraduate Research Assistant**
Deparment of Psychological & Brain Sciences
University of California, Santa Barbara
Principle Investigator: Tod Kippin
Research Area: Neural plasticity and addiction

2012—2013 **Undergraduate Research Assistant**
Deparment of Psychological & Brain Sciences
University of California, Santa Barbara
Principle Investigator: James Roney
Research Area: Human behavioral endocrinology

Teaching Experience

Instructor on Record
INT93LS (1 quarter): Blueprints of the Brain:
Genetic tools in neurobiology
Teaching Assistant
MCDB 110 (4 quarters): Principles of Biochemistry
MCDB 101L (1 quarter): Introduction to Molecular Genetics
MCDB 6 (1 quarter): Principles of Molecular and Cellular Biology for Brain Sciences
MCDB 111 (2 quarters): Human Physiology
MCDB 112L (4 quarters): Laboratory in Developmental Biology
MCDB 101A (4 quarters): Molecular Genetics
MCDB 151 (2 quarters): Neurobiology I

Public Outreach

2020—present	President, Graduate Biology Mentorship Association (GBMA)
2017—present	ScienceLine Contributor
2019	Nepris Virtual Classroom Volunteer
2019	Curie-osity Project Innovator/Host
Fall 2018	Partners in Education Science Fair Project Advisor at Santa Barbara Junior High

Grants and Fellowships

Summer 2020 **Summer Institutes Scholarship**
UW Biostatistics Summer Institute in Statistical Genetics (SISG)

Fall 2019 **Graduate Division Dissertation Year Fellowship**
University of California, Santa Barbara

2017 MCDB Graduate Student Research Fellowship

Honors and Awards

2018, 2019	ScienceLine Award in Life Science
2014	The Morgan Award for Academic Excellence in Psychology

2014	Distinction in the Major
2014	Exceptional Academic Performance

Presentations

Ackley CR, Ziaei Kajbaf N, Washiashi L, Krishnamurthy R, Rothman J (2021). "Gravitaxis in *C. elegans* requires TRN tubulins and TRPA-1". Poster presented at the GSA International C. Elegans Conference Online.
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Ackley CR (2014). "Homer2 Regulation of Adult Neurogenesis in the Dentate Gyrus". Poster presented at the Undergraduate Research Colloquium at the University of California Santa Barbara.

Publications

Ackley CR, Ziaei Kajbaf N, Washiashi L, Krishnamurthy R, Joshi P, and Rothman JH (2022). *Parallel Mechanosensory Systems are Required for Negative Gravitaxis in C. elegans.* BioRxiv, 2022.03.03.482913. Kautzman AG, Keeley PW, **Ackley CR**, Leong S, Whitney IE, and Reese BE (2018). *Xkr8 Modulates Bipolar Cell Number in the Mouse Retina.* Front. Neurosci. 12:876. doi: 10.3389/fnins.2018.00876
Kautzman AG, Keeley PW, Borhanian S, **Ackley CR** and Reese BE (2018).*Genetic Control of Rod Bipolar Cell Number in the Mouse Retina.* Front. Neurosci. 12:285. doi: 10.3389/fnins.2018.00285
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Abstract

Feeling the weight of the world: Gravity sensation and sensory integration in *C. elegans*

by

Caroline Rose Ackley

All life on Earth, from the smallest microbe to the largest blue whale, is subject to Earth's gravitational pull. This force may be the only environmental variable that has remained constant for all organisms since the origin of life. Because of this, the ability to sense gravity is present in many species and is often critical for survival. Plants use gravity as a cue for directing root growth. Animals, including humans, sense gravity to facilitate movements and to build spatial awareness. Additionally, gravity sensation is one of many modalities that are integrated by cells and nervous systems to make decisions about behavior. Little is known about gravity sensation compared with other sensory systems. Likewise, polymodality and sensory integration are relatively new and understudied areas of research within sensory biology. To investigate gravity sensation, I developed a novel, large-scale assay for observing gravitactic behavior in *C. elegans*. I found that the worms negatively gravitax — a behavior that has not previously been observed in this species — and that gravitaxis is altered in the presence of light and electromagnetic fields. A screen of known DEG/ENaC mechanosensory components revealed that MEC-7 and MEC-12, which form specialized microtubules required for gentle touch, are required for negative gravitaxis. However, mutations affecting MEC-4 and MEC-10 — the subunits of gentle-touch transducing ion channels — did not impede worms' ability to gravitax. Instead, I found that negative gravitaxis depends on the polymodal TRPA-1 channel protein. These findings suggest a previously unidentified connection between DEG/ENaC and TRPA-1 in mechanosensation. I then assayed worms that, through genetic ablation, lacked either

the gentle-touch sensitive touch receptor neurons (TRNs) or a pair of proprioceptive PVD neurons, which express MEC-7/12 and TRPA-1. While TRN- worms exhibited behavior similar to N2 controls, worms lacking PVD neurons failed to show negative gravitactic preference. This work contribute to an understanding of gravity sensation and sensory integration in *C. elegans* that can provide insight into vestibular, auditory, and cognitive disorders.

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Chapter 1

Introduction

1.1 Gravity sensation and its role in proprioception

Organisms across the tree of life evolved mechanisms for detecting both internal states and external stimuli. This is true even for single-celled organisms that lack nervous systems, such as bacteria and protists (Cox et al., 2018; Häder and Hemmersbach, 2017). Earth's gravitational field is perhaps the only external factor that has remained constant for all species since the origin of life (Adamopoulos et al., 2021). Unsurprisingly then, the ability to sense gravity is common throughout biology and is critical for movement and orientation in many organisms. Plant roots utilize gravitational forces to direct roots down into the Earth (Su et al., 2017), while animals detect gravity to build a sense of body and spatial awareness.

Sensory transduction is the process of converting a signal, such as gravity, into a biological message that can be interpreted by an organism. The traditional five senses in humans can be described by the kind of signal they transduce. Vision and photosensitivity are products of light transduction. Chemoreceptors are used by our noses and tongues to convey smell and taste, respectively. Hearing and touch require the transduction of

mechanical forces. However, this narrow categorization belies the variety of stimuli that can be detected. Touch, for example, encompasses a range of sensory modalities, including texture, pain, heat, and pressure sensation. Smooth muscle cells are sensitive to stretch forces, which are important cues for regulating internal states such as blood pressure. Additionally, humans and other animals have a well-known, but little appreciated, "sixth" sense, commonly referred to as proprioception.

This sense of self-awareness (proprio- meaning "own") is generated through integrating several sensory inputs. In mammals, proprioceptive neurons with processes in the joints, tendons, and muscles relay information about limb extension and muscle movement (Tuthill and Azim, 2018). Some of these signals are inherently gravity sensitive; picking up a heavy object, for example, communicates the need to increase muscle tension proportionately. Meanwhile, vestibular organs in the inner ear transduce inertial forces and gravitational pull to convey a sense of head movement. Proprioceptive and vestibular inputs are combined with visual information as well as visceral sensation to build an overall awareness of self, which is necessary for balance and coordination.

1.2 Disorders related to gravity sensation and sensory integration

Research into gravity sensation and sensory integration has implications for human health. Autism spectrum disorder (ASD) is associated with both vestibular dysfunction and difficulties with sensory integration. Patients with autism experience difficulty with auditory and vestibular processing, which affects their posture, gait, gaze, and attention (Mansour et al., 2021). Children with autism are also hypersensitive to external stimuli, which can be attributed in part to impairments in multimodal sensory process-

ing (Marco et al., 2011). Other disorders associated with abnormal sensory integration include ADHD (Ning and Wang, 2021), schizophrenia (Uhlhaas and Singer, 2010), and mood disorders (Hilber, 2022; Mackowetzky et al., 2021).

Vestibular disorders — including Meniere's disease, superior canal dehiscence, motion sickness, vestibular migraines, Pendred syndrome, and Usher syndrome, and many other conditions — are often debilitating. In young children, these disorders are also likely to delay motor development (Mackowetzky et al., 2021; Roman-Naranjo et al., 2018; Santos et al., 2015). Difficulties with balance and coordination are observed in 5.2% of children between the ages of 2–17 in the United States (Mackowetzky et al., 2021). Meanwhile, approximately 30% of adults suffer from periodic motion sickness (Roman-Naranjo et al., 2018). Vestibular symptoms are frequently comorbid with auditory disorders: 70% of children diagnosed with sensorineural hearing loss (SNHL) — the most prevalent class of hearing disorders in development — experience difficulties with vestibular sensation (Santos et al., 2015). Many of these disorders have known or predicted associations with genes required for inner ear development and function (Mackowetzky et al., 2021). Uncovering the genes and mechanisms involved in vestibular sensation, which transduces gravitational force, is important for understanding both vestibular and auditory systems.

1.3 Vestibular sensation in mammals

The bony vestibular apparatus in the mammalian inner ear consists of the cochlea, which transduces sound, as well as three semi-circular canals attached to a 'vestibule'. The three canals, which sit at right angles to each other, enable transduction of angular acceleration in three dimensions, corresponding to "roll", "pitch" and "yaw". Linear acceleration is detected by two organs in the vestibule — the utricle, which conveys horizontal movements, and the saccule, which conveys vertical movements. Within these

bony compartments are soft-tissue structures containing the hair cell neurons that are capable of converting forces into electrical signals (Fig. 1.1).

The semi-circular canals, utricle, and saccule employ similar strategies to detect acceleration. At the base of each semi-circular canal is a widened 'ampulla', which contains sensory hair cell neurons. The base of these neurons forms an epithelial layer with afferent connections to the brain. The ciliated apical tips of these cells are embedded in a gelatinous membrane (known as the 'cupula') that bisects the hollow ampulla. As the head rotates, endolymph fluid within the semi-circular canals pushes against the cupulae due to inertia. The resulting deformation bends the hair cell cilia, causing membrane depolarization (Glover, 2004). This structure is remarkably similar to the tectorial membrane inside the nearby cochlea, which is deflected by sound-generating pressure waves. Likewise, hair cell processes in the utricle and saccule are ensconced in a gelatinous membrane. However, displacement of these "maculae" is caused by gravitational and inertial pull on otoliths — dense, calcium carbonate deposits which lay on top of the membrane and create drag — instead of by fluid pressure.

The use of statoliths (literally 'standing rock' in Greek) in gravity sensation is not limited to vertebrates, or even the animal kingdom. In plant roots, starch filled amyoplasts, which are denser than the surrounding cytoplasm, sink to the bottom of the cell (called a "statocyte") and cause the plasma membrane to stretch. This triggers a signaling cascade that guides root growth (Kolesnikov et al., 2016). Remarkably, other organisms can detect gravity without mineral deposits or dense organelles. The single-celled protist *Euglena* was found to use its own cytoplasm as a kind of statolith; because it's cytoplasm is denser than the surrounding aqueous environment, it causes the plasma membrane to stretch as the organism rotates in solution (Häder and Hemmersbach, 2017). Diverse mechanisms for detecting orientation with respect to gravity can be found across species and throughout evolutionary history.

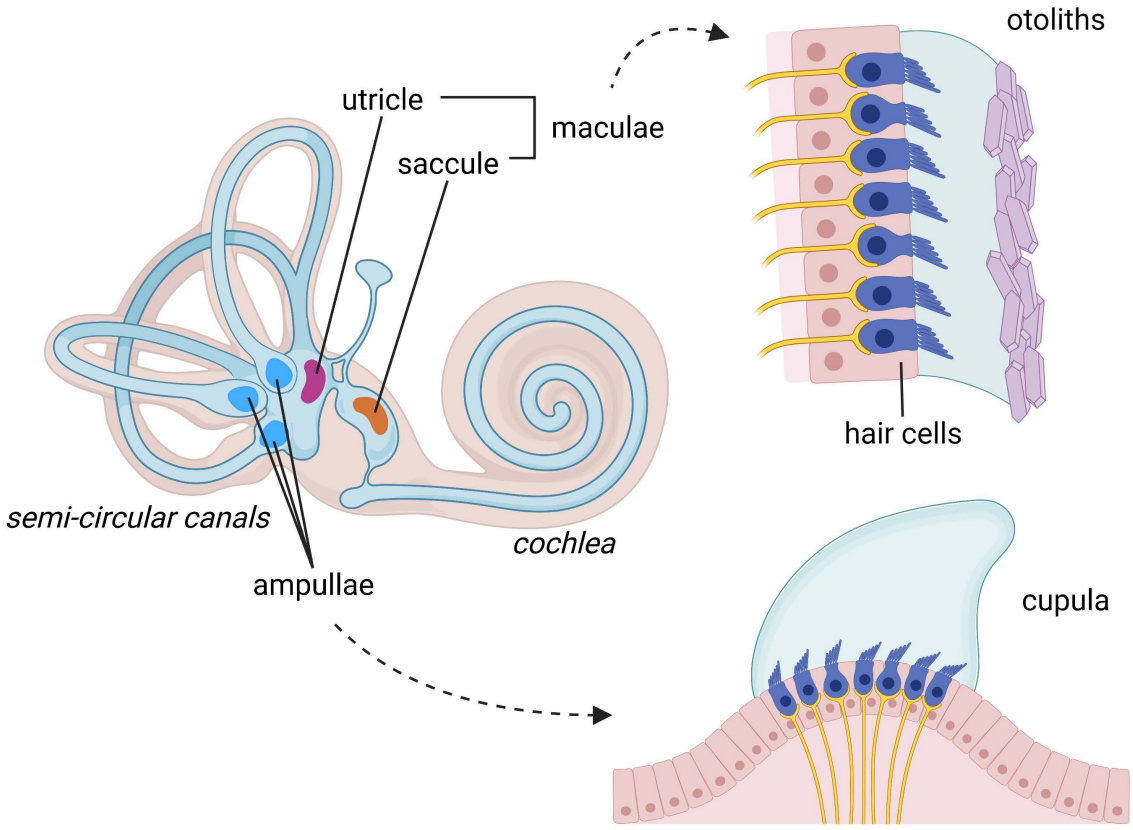

Figure 1.1: Anatomy of the mammalian vestibular aparatus
Created with BioRender.com.

1.4 Tracing vestibular sensation through evolution

The use of statoliths in animals predates bilaterian evolution, appearing first in jellyfish 635 million years ago (Jékely et al., 2021; Ramos de Miguel et al., 2020). Meanwhile, semi-circular canals are present in all extant vertebrates, with the macular organs arising during the divergence of jawed and jawless animals (Ramos de Miguel et al., 2020; Fritzsch et al., 2014). Several lines of evidence suggest that the vestibular organs evolved before the auditory cochlear organ, likely because of the different sound-conducting properties of water and air; although fish use hair cells in lateral line organs to detect pressure changes in the water, defined cochlear structures first appear in the transition from water to land (Fritzsch et al., 2014; Mackowetzky et al., 2021; Maoiléidigh and Ricci, 2019; Lipovsek and Wingate, 2018).

While hair cells themselves are unique to vertebrates, homologous cells are present across bilaterians, and may even have a pre-bilaterian origin (Duncan and Fritzsch, 2012; Fritzsch and Beisel, 2001). Ciliated neurons in *Drosophila* and *C. elegans* share functional, developmental, and genetic characteristsics in common with vertebrate hair cells. As in hair cells, worm and fly ciliated neurons require intracellular cytoskeletal anchors, extracellular matrix components, and structural support cells to transduce mechanical force (Fritzsch and Beisel, 2001). Their development depends on homologs of bHLH transcription factor Atoh1, which guides inner ear hair cell differentiation (Duncan and Fritzsch, 2012; Beisel and Fritzsch, 2004; Fritzsch and Beisel, 2001; Mackowetzky et al., 2021). Orthologs of required channel proteins (voltage gated potassium channels, L-type like calcium channels, and potassium rectifiers) can also be found in fly and worm genomes (Fritzsch and Beisel, 2001).

As mentioned above, mechanosensation exists across the tree of life. Consequently, mechanoreceptors have been found in prokaryotes and archaea as well as eukaryotes

(Delmas et al., 2011). Homologs of bacterial MscL proteins, which are stretch-activated, have been found in all three kingdoms; however, no homologs have been identified yet in animals (Katta et al., 2015; Fritzsch et al., 2020). Five classes of mechanoelectric transducer (MeT) proteins are common to bilateria: TMC, TRP, Piezo, DEG/ENac (aka ENaC/BNac or ASIC), and OSCA/TMEM63 (Katta et al., 2015; Beisel and Fritzsch, 2004). While TMC, TRP, and DEG/ENaC homologs can be found in cnidarians, Piezo and OSCA/TMEM63 proteins are even more highly conserved, with well-known homologs in plants (Katta et al., 2015; Delmas et al., 2011). Despite their genetic and structural characterization (Jabba et al., 2014; Gaudet, 2008; Montell, 2005; Katta et al., 2015), how MeTs transduce mechanical force is still under investigation.

1.5 *C. elegans* as a model for mechanosensation

The 1 mm long roundworm *C. elegans* is an ideal model organism for studying sensation in general, and mechanosensation in particular. Adult hermaphrodites have 302 neurons; of these, between 70-85 are sensory neurons, at least 30 (and possibly up to 46) of which are mechanoreceptive (Hobert, 2013; Metaxakis et al., 2018; Schafer, 2015; Goodman and Sengupta, 2019). *C. elegans* was the first animal for which an entire connectome was completed (White et al., 1986). Since then, worm connectomes have been mapped for multiple hermaphroditic adults as well as males (Cook et al., 2019). Additionally, A 'molecular topology' of each neuron has recently been compiled using single-cell RNA sequencing (Taylor et al., 2021).

Because of the ease of working with worms and studying their behavior, *C. elegans* have been used extensively as a model organism in the field of sensory biology. *C. elegans* respond to a number of stimuli including light, volatile and non-volatile chemicals, touch, temperature, sound, humidity, gas concentrations, Earth's magnetic field, and

proprioceptive cues (Ward et al., 2008; Ward, 1973; Bargmann et al., 1993; Bargmann, 2006; Gu et al., 1996; Chatzigeorgiou et al., 2010; Iliff et al., 2021; Russell et al., 2014; Carrillo et al., 2013; Bretscher et al., 2008; Gabel et al., 2007; Vidal-Gadea et al., 2015; Schafer, 2006). Of these, gentle touch sensation has been especially well characterized. Avoidance behavior in response to gentle touch was first observed by stroking worms with an eyebrow hair attached to a toothpick (Chalfie and Sulston, 1981). Since then, devices such as piezoresistive microcantilevers have been developed to apply and measure precise mechanical forces and to record physiological responses in TRNs (Nekimken et al., 2017). These tools have advanced our understanding of the molecular and physical requirements for gentle touch sensation.

1.6 Mechanosensory receptors and pathways

Gentle touch is sensed through the DEG/ENaC pathway, which was first elucidated through forward genetic screens in *C. elegans* (Gu et al., 1996; Goodman and Schwarz, 2003; Goodman et al., 2002; Chelur et al., 2002). These studies implicated two ion channel proteins, MEC-4 and MEC-10, as well as intracellular and extracellular matrix components in a model for gentle force transduction. According to this model, MEC-4 and MEC-10 subunits form a heterotrimeric sodium channel that is linked intracellularly via MEC-2 to specialized microtubules comprised of MEC-12 and MEC-7 α and β subunits, respectively (Ernstrom and Chalfie, 2002; Shi et al., 2018). Gentle touch transduction is restricted to six touch receptor neurons (TRNs) which span the length of the worm: ALML, ALMR, and AVM detect anterior touch, while PLML and PLMR are essential for posterior touch (the sixth, PVM, is not required, but has many of the same components) (Ernstrom and Chalfie, 2002; Chalfie et al., 1985). Extracellular matrix proteins between the neuron and cuticle convey forces made by indentations along the

body wall to the cell membrane (Ernstrom and Chalfie, 2002). Other proteins, such as the neuronal transcription factor MEC-3, play a developmental or regulatory role in the DEG/ENaC system. Details of the DEG/ENaC pathway will be explored in Chapter 2 and are visualized in 2.4.

In addition to DEG/ENaC proteins, members of the transient receptor potential (TRP), Piezo, and TMC families have also been described in *C. elegans* with putative and known roles in mechanosensation. The first *trp* gene was identified in *Drosophila* (Montell and Rubin, 1989); since then, multiple sub-families of TRP proteins have been described with similar characteristics. TRPs are typically non-selective, tetrameric cation channels. Each protein subunit has six membrane-spanning regions, intracellular N- and C- termini, and often contain ankyrin repeats among other common domains (Montell, 2005) 1.2. TRPs are unique because of their versatility — they are involved in several sensory modalities and behaviors in both worms and flies, including light detection, chemosensation, mechanosensation, sociality, addiction-like behaviors, and mating (Venkatachalam et al., 2014). As further evidence of their polymodality, homologs of a same TRP channel can have divergent and even opposing roles in other organisms (Saito et al., 2011).

The flexibility of TRP channels is exemplified by TRPA1, a receptor which has special relevance for this work. Between flies and worms, TRPA1 homologs are known to participate in chemosensation, photosensation, and thermosensation across a wide range of temperatures (Venkatachalam et al., 2014; Chatzigeorgiou et al., 2010). Critically, *trpa* genes *painless* and *pyrexia* are also required for gravity sensation in flies (Sun et al., 2009). In worms, TRPA-1 is necessary for harsh cold sensation by the posterior PVDL and PVDR neurons (Chatzigeorgiou et al., 2010). Mechanosensory functions for TRPA-1 (outside of thermoception) have been observed in *C. elegans* (Kindt et al., 2007; Han et al., 2013). However, TRPA-1 and other TRP receptors have not been as extensively

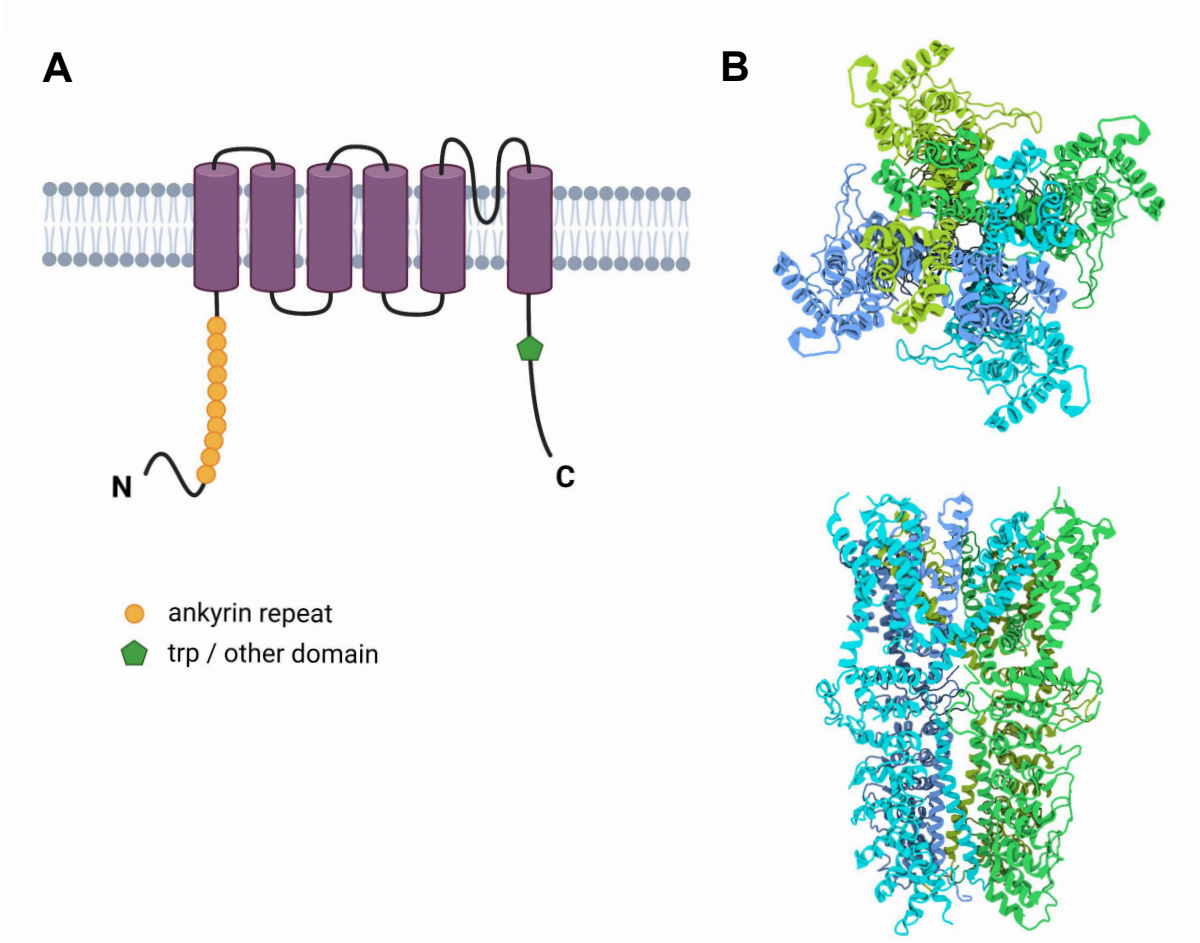

Figure 1.2: TRP channel structure
A) A representative diagram, based on the structure of TRPA-1, of a TRP protein subunit. TRPA-1 in particular contains multiple ankyrin repeats within the intracellular N-terminus. **B)** 3D structural representation (top: top down; below: side view) of human TRPA-1 channel complex. Both figures were created with BioRender.com.

characterized as the DEG/ENaC system in worms.

1.7 Multimodal sensory processing and integration

The polymodality of TRP and other receptor classes, including opsins (Leung, 2019), raises the importance of studying sensory biology from a multisensory perspective. For generations of neuroscience research, the five classical senses (touch, sight, smell, hearing, and taste) were considered to function independently, leading to various "labeled-line" theories (Lacquaniti et al., 2014). These theories proposed that each sense has a dedicated neural pathway consisting of specialized receptors and cell types. However, increasing evidence suggests that different sensory modalities can be integrated at multiple levels, from higher order processing in the vertebrate central nervous system (Taube, 2007) to small, localized circuits (Mellem et al., 2002; Krzyzanowski et al., 2016; Bostwick et al., 2020), and occasionally, to integration within sensory neurons themselves (Tao et al., 2019; Stockand and Eaton, 2013).

Sensory integration is critically important for behavior. Animals are constantly exposed to a barrage of stimuli that can have competing effects on decision-making (Zhang et al., 2020; Metaxakis et al., 2018). Internal states, such as satiety (Chen and Chalfie, 2014; Sengupta, 2013), and prior experiences, which lead to neuroplastic changes (Byrne Rodgers and Ryu, 2020), also provide important context that can alter the likelihood of a behavior. Additionally, some senses, such as proprioception, are necessarily multimodal. Sensory information from neurons in the muscles, tendons, gut, vestibular system, and retina all contribute to a sense of body and spatial awareness in mammals (Lacquaniti et al., 2014). Therefore, uncovering the mechanisms behind sensory integration is essential for understanding how senses are perceived by the nervous sytem.

Expanding connectome data in flies, worms, and other model organisms enable re-

searchers to pinpoint exact synapses that participate in sensory integration within a neural network. How different sensory inputs may be integrated within a single neuron is less understood. The PVD neurons in *C. elegans*, which detect body movement, harsh touch, and harsh cold, provide some insight into this question. In these neurons, harsh touch was found to generate a signal along the axon, while proprioceptive cues led to depolarization only in dendrites (Tao et al., 2019). This compartmentalization allows for selective neurotransmitter release depending on the type of input received. Another mechanism for single cell sensory integration has been proposed in *Drosophila* md neurons, which distinguish between noxious chemical, mechanical, and thermal stimuli. The receptors targeted by each stimulus produce either slow, intermediate, or rapidly adapting currents, which may lead to differential signaling downstream (Stockand and Eaton, 2013). Polymodal neurons are not uncommon in invertebrates; however, few mechanisms for distinguishing sensory inputs within a cell have been described.

Chapter 2

Mechanosensory Systems and Sensory Integration Mediate *C. elegans* Negative Gravitaxis

2.1 Abstract

The ability to sense Earth's gravitational pull is essential for orientation, navigation, and proprioception in most organisms. We report here that C. elegans dauer larvae and adults exhibit pronounced negative gravitaxis. This behavior is antagonized by light and electromagnetic fields, suggesting that it is integrated with other sensory inputs. We found that the MEC-7 and MEC-12 microtubule components of the mechanosensory transduction system involved in gentle touch sensation are essential for negative gravitaxis. Further, TRPA-1, a channel protein associated with cold and mechanosensation in both mammals and invertebrates, is required for this behavior. However, the MEC-4/10 DEG/ENaC channels and other components that transduce gentle touch sensation are not required, suggesting that the sensory system for detecting and responding to gravity

is separable from the touch sensation system. We found that the PVD neurons, which use TRPA-1 to detect harsh cold, but neither their quaternary processes nor the touch receptor neurons (TRNs), are essential for negative gravitaxis. These findings implicate an interconnected mechanism for gravity sensation involving an ion channel that is also present in the mammalian vestibular system, suggesting possible homology in gravity sensing across animal phylogeny.

2.2 Introduction

Gravity sensation is a common trait among most Eukaryotes. Members of the protists, fungi, plants, and animals depend on gravity sensation for survival. Small changes in the position or orientation of these organisms result in a mechanical force that is transduced by graviperceptive organelles or organs. This force is often conveyed through dense organelles or mineral-rich structures whose displacement triggers a signaling pathway that ultimately results in a behavioral output (Ross et al., 1984). A common characteristic of gravity transduction pathways in animals is the use of ciliated neurons, in which deflection of hair-like "stereocilia" opens mechanically gated ion channels (Bezares-Calderón et al., 2020; Lacquaniti et al., 2014). Although this general mechanism has been well characterized, less is known about how mechanoreceptors and mechanosensory cells transduce such minute forces – estimated to be as small as 0.57-1.13 pN in *Euglena*, for example – into a robust signal (Häder and Hemmersbach, 2017).

Given the availability of a detailed connectome of the entire nervous system and powerful genetic and optogenetic tools, *C. elegans* has been a highly effective model organism for elucidating the neural circuitry and molecular mechanisms governing sensory perception, learning, memory, and behavior (Bargmann et al., 1993; Bretscher et al., 2008; Gray et al., 2004; Ramot et al., 2008; Riddle et al., 1997; Russell et al., 2014; Ward et al.,

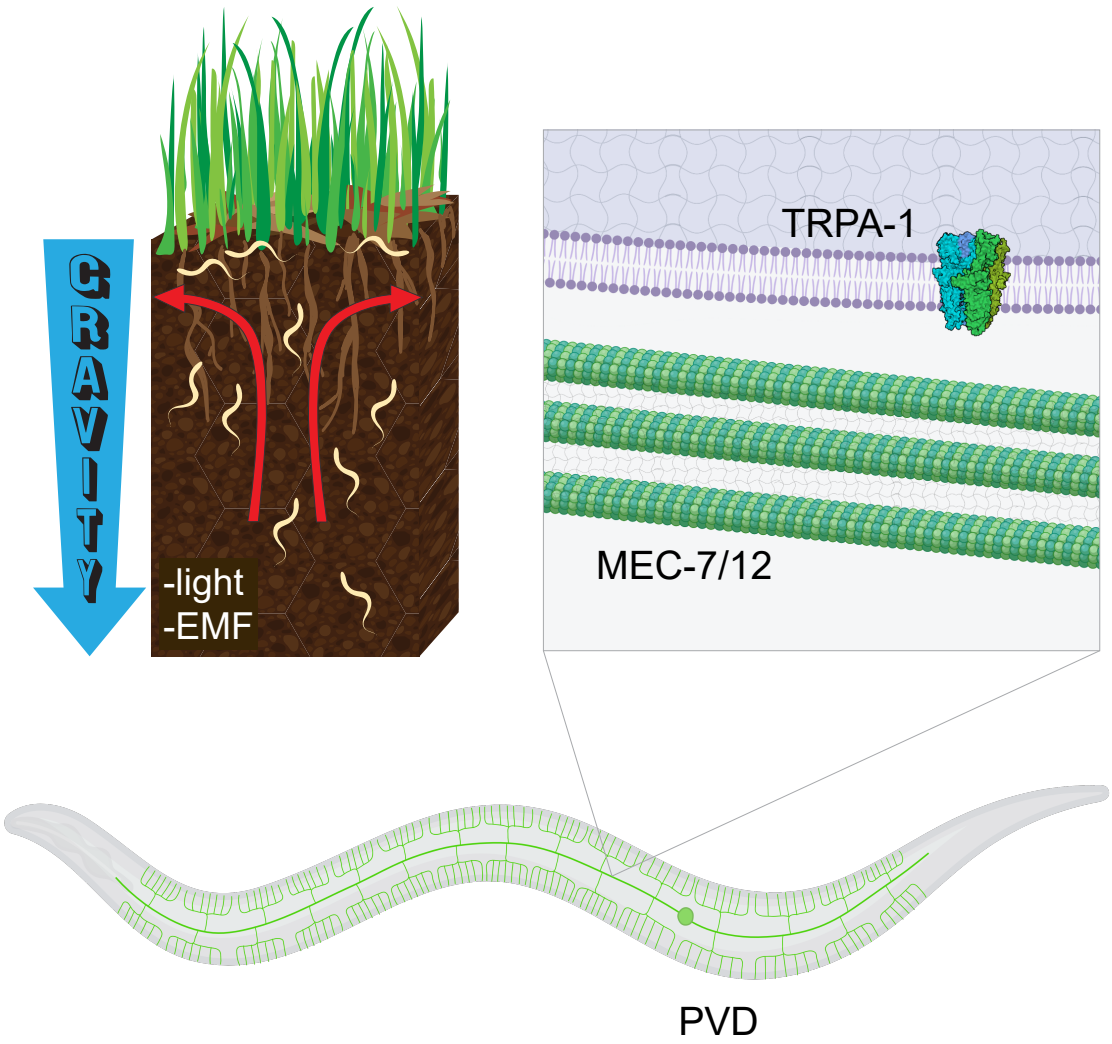

Figure 2.1: Graphical Abstract

2008; Ward, 1973). However, because they are traditionally studied in a two-dimensional environment on the surface of Petri dishes containing agar, perpendicular to the vector of gravity, few studies have examined complex behavior exhibited by *C. elegans* in three-dimensions. In the wild, *C. elegans* is typically found in moist compost, such as under shrubs or along riverbanks, which can change drastically from season to season (Kodama-Namba et al., 2013; Frézal and Félix, 2015). These animals have evolved an adaptive alternative larval phase, the dauer larvae, that prioritizes dispersal over reproduction during adverse environmental conditions. Dauer larvae exhibit characteristic nictation, in which they "stand" on their tails and wave their heads. This behavior is believed to facilitate dispersal, possibly by allowing worms to "hitch a ride" on larger animals that pass by, such as isopods (Lee et al., 2012). Therefore, gravitational force may be a critical input that *C. elegans* use in combination with other cues to navigate to the surface before traveling longer distances (Fig. 2.2A).

In previous studies, *C. elegans* dauer larvae showed no gravitactic preference, in contrast with *C. japonica*, which negatively gravitax on vertically oriented Petri plates (Okumura et al., 2013). Other studies reported that *C. elegans* adults show a tendancy to orient downwards when swimming in liquid, suggesting potential positive gravitaxis (Chen et al., 2021). However, as in *Drosophila* and the ascidian *Ciona*, the ability of nematodes to undergo gravitaxis is likely context-dependent (Bae et al., 2016; Bostwick et al., 2020). *C. elegans* shows a strong aversive response to light (Ward et al., 2008) and also responds to electromagnetic fields (Landler et al., 2018; Vidal-Gadea et al., 2018, 2015). Integration with these or other sensory modalities may influence or mask gravitactic behavior and experiments to test for gravitaxis in the absence of either stimulus have not been reported with *C. elegans*. In this study we report that in the absence of light, *C. elegans* dauers exhibit pronounced negative gravitaxis, which is strongly enhanced when they are shielded from ambient electromagnetic fields and light.

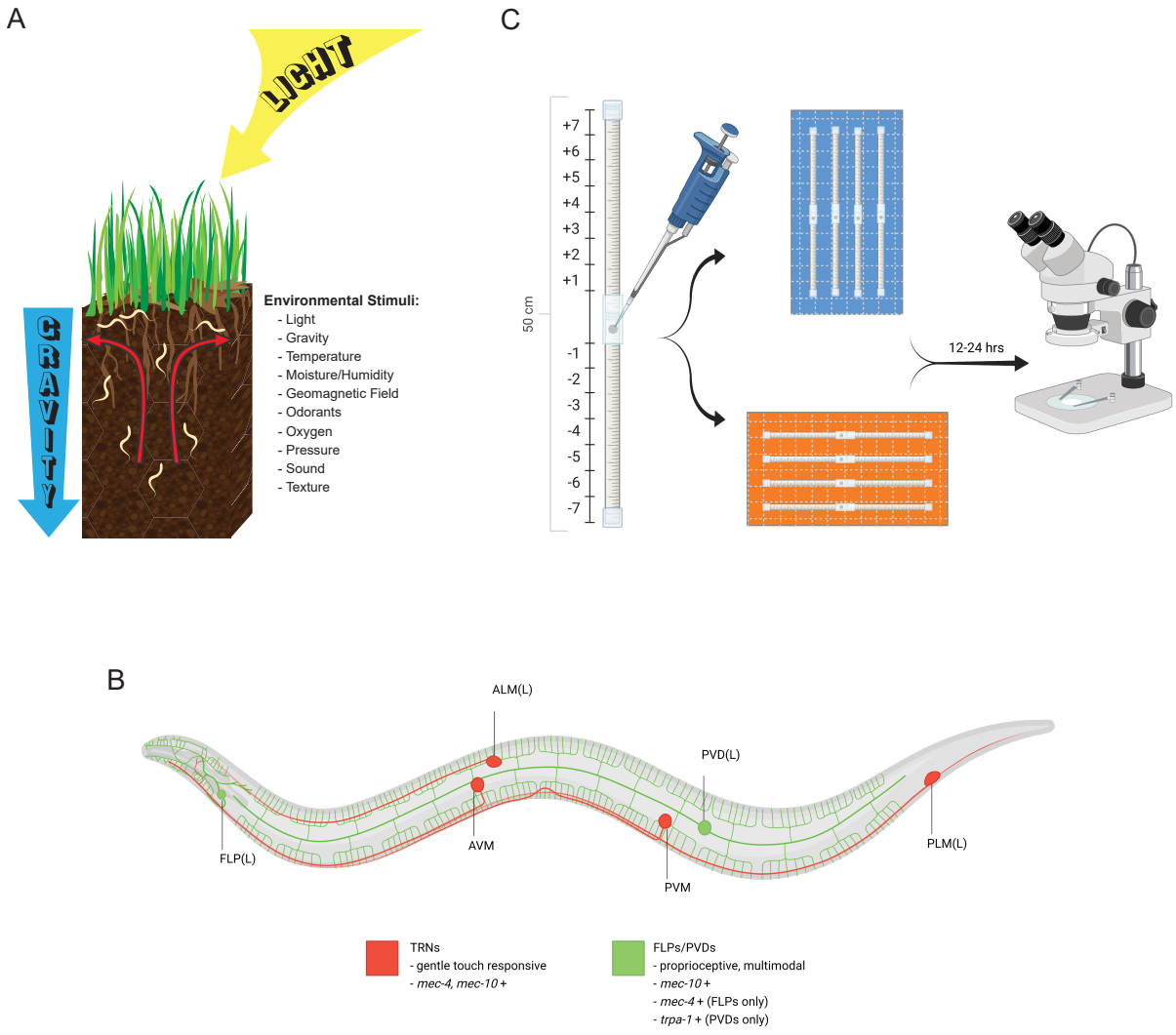

Figure 2.2: Behavioral model, wiring diagram, and gravitaxis assay design
A) Graphic depicting environmental stimuli that inform *C. elegans* behavior in a natural setting. **B)** Diagram of key neurons mentioned in this study. TRNs are labeled in red, while FLP and PVD neurons are green (for neurons with left and right pairs, only the left neuron is displayed). Adapted from individual neuron illustrations available on WormAtlas (Altun, 2022). **C)** Gravitaxis chamber and experimental design used in this study. Figures **B)** and **C)** created with BioRender.com.

While little is known about their behavioral responses to gravity, the effects of hyper- and microgravity on *C. elegans* physiology has been documented (Gao et al., 2015, 2017; Kalichamy et al., 2016; Saldanha et al., 2016; Xu et al., 2014). In response to hypergravitational forces, signaling by the mechanosensory DEG/ENaC sodium channel proteins, MEC-4/MEC-10, leads to nuclear localization of the DAF-16 FoxO transcription factor, which also transduces insulin-like growth factor signaling and stress responses (Kim et al., 2007). The *mec-4/10* genes in *C. elegans*, which encode channel proteins, and several other "mec" genes involved in gentle touch sensation, are expressed in the six touch receptive neurons (TRNs), which specifically mediate gentle touch sensation (Chalfie and Sulston, 1981). While the MEC-4 and -10 proteins function together in the TRNs, they also show non-overlapping expression in some cells: MEC-10 in the proprioceptive/multimodal neurons PVDL/R and FLPL/R and MEC-4 in the FLP neuron pair (Fig. 2.2B). Mechanosensation in many animals is also mediated by TRP (transient receptor potential) cation channels, which are common across metazoan phylogeny (Chatzigeorgiou et al., 2010; Han et al., 2013; Kindt et al., 2007; Montell, 2003). The TRPA-1 receptor is a polymodal sensor, capable of conveying either high or low temperatures as well as light and noxious stimuli (Venkatachalam et al., 2014). In worms, TRPA-1 confers sensitivity to noxious cold in PVD neurons as well as mechanosensation in OLQ and IL1 neurons (Han et al., 2013). The *trpa-1* homologs *pyx* and *pain* are necessary for gravitaxis in *Drosophila* (Sun et al., 2009).

In this study, we sought to determine whether *C. elegans* possesses a system for detecting normal gravitational force and whether known mechanosensory molecular components and neurons participate in response to gravity. We discovered that *C. elegans* prefer to migrate vertically against the force of gravity — ie., to undergo negative gravitaxis. Further, we found that gravitaxis is profoundly influenced by environmental cues of light and background electromagnetic fields, revealing sensory integration in the behav-

ior. We also report that the MEC-7/12 microtubule components, but not the associated MEC-4/10 DEG/ENaC channel proteins or several other components involved in TRN function and touch sensation, are required for gravitactic behavior. Further, we found that the TRPA-1 channel is essential for negative gravitaxis in *C. elegans*. Finally, we identified the polymodal PVD neurons, which span the length of the worm, but not their quartenary processes, as essential for this directional bias. Based on these findings, we propose a model of gravity transduction that involves the unique action of MEC-7/12, TRPA-1, and PVD neurons. Our findings suggest that even in a diminutive animal with low mass, in which the force of gravity is much weaker than many other forces it experiences, a homologous system for gravity sensing integrated with other sensory inputs operates to optimize responses to environmental cues and its orientation on the planet.

2.3 Results

C. elegans exhibits pronounced negative gravitactic behavior

We investigated whether *C. elegans* can detect and respond to the force of gravity by initially focusing on the behavior of the dispersal state, the dauer larva. When first stage (L1) larvae experience stressful conditions, including overcrowding, lack of resources, and extreme temperatures, they subsequently develop into an alternative third stage larva, the dauer larva. Through pronounced physical, metabolic, and behavioral changes, dauers become efficient vectors for dispersal that can survive for months without food (Wang et al., 2009). Dauer larvae of many nematodes show nictation behavior, in which they raise their heads at a 90° angle to the surface plane, raising the possibility that they can orient in the gravitational field, though this behavior may simply reflect orientation perpendicular to the surface.

Under normal laboratory growth conditions in which *C. elegans* are cultivated on

flat agar surfaces in Petri dishes, dauers of the laboratory N2 strain of *C. elegans* do not nictate unless contaminated by fungi or when grown on three dimensional habitable scaffolds (Guisnet et al., 2021); however, 3-D scaffolds are not convenient for studying migratory behavior. To address this issue, we adapted a setup used to study neuromuscular integrity in *C. elegans* (Bainbridge et al., 2016) to investigate whether *C. elegans* dauers exhibit directional bias in response to gravity (see methods). We injected dauers into the gravitaxis assay chambers comprised of two serological pipettes that, when stacked end-to-end, allow for ∼25 cm of movement in either direction from the injection site (Fig. 2.2C). Chambers loaded with worms were oriented either vertically to test for movement in the gravitational field or horizontally as a control for general migratory behavior that is not influenced by gravitational force. The migration of the populations was scored 12–24 hours after loading. Horizontal and vertical chambers were constructed and generally assayed simultaneously to control for any directional preference attributable to the construction and design of the chamber itself; in the absence of other cues, worms are not expected to exhibit a migratory bias in either direction in such chambers. However, if worms perceive and respond to the force of gravity, they would be expected to exhibit a directional bias specifically in the vertically oriented chamber, showing a migratory bias toward the bottom with positive gravitaxis (average migration value of <0 to -7.0) or th top with negative gravitaxis (average migration value of >0 to 7.0; see Materials and Methods).

In contrast to prior assays conducted on Petri plates, which failed to reveal gravitactic preference with *C. elegans* (Okumura et al., 2013), we found that N2 dauers larvae showed a weak but significant directional bias in migration toward the top of the vertical chamber under normal lab conditions when compared to the horizontal controls (average vertical location = 0.89, as defined in Materials and Methods, n = 2,222 worms over 10 trials; horizontal = 0.47, n = 1,594 worms over 4 trials; p <0.0001, Kruskal-Wallis

test) (Fig. 2.3A, B). This difference was not statistically significant when the results were analyzed using a "Gravitaxis Index" (GI; analogous to the Chemotaxis Index (Srinivasan et al., 2012; Larsch et al., 2015)) in which the fraction of worms moving to either side is compared (GI vertical = 0.43 ±0.17, SEM; GI horizontal 0.20 ±0.16; p >0.05, two-tailed t test). These observations suggest that *C. elegans* dauer larvae show a mild preference for upward migration under normal laboratory conditions when the migration field is >5x larger than that of previous studies (Okumura et al., 2013).

Evidence for sensory integration: negative gravitaxis is attenuated by light and electromagnetic fields

External and internal sensory cues that provide important context that alters the likelihood of a behavioral response in animals (Chen and Chalfie, 2014; Sengupta, 2013)). As *C. elegans* exhibits strong negative phototactic behavior (Ward et al., 2008), we posited that negative gravitaxis might be adversely influenced by this response to light (Fig. 2.2A). To address this possibility, we repeated the gravitaxis assay in the dark using a light-blocking blackout cloth. In sharp contrast to the lack of discernible directional preference in the horizontal chambers, we found that N2 dauer larvae demonstrated an enhanced and highly significant preference for upward migration in the vertical chambers under these conditions (average vertical location = 1.42, n 4,276, 11 trials; horizontal = 0.008, n = 1,939, 6 trials; p <0.0001) (Fig. 2.3A, B). This striking preference for upward migration was significantly greater (p <0.01) than was seen under normal laboratory lighting conditions (Fig. 2.3B), suggesting that light attenuates the ability of the animals to sense or respond to gravity.

C. elegans has also been found to respond to electromagnetic fields (EMF) (Vidal-Gadea et al., 2015). To test whether EMF, as with light, influences the gravitactic

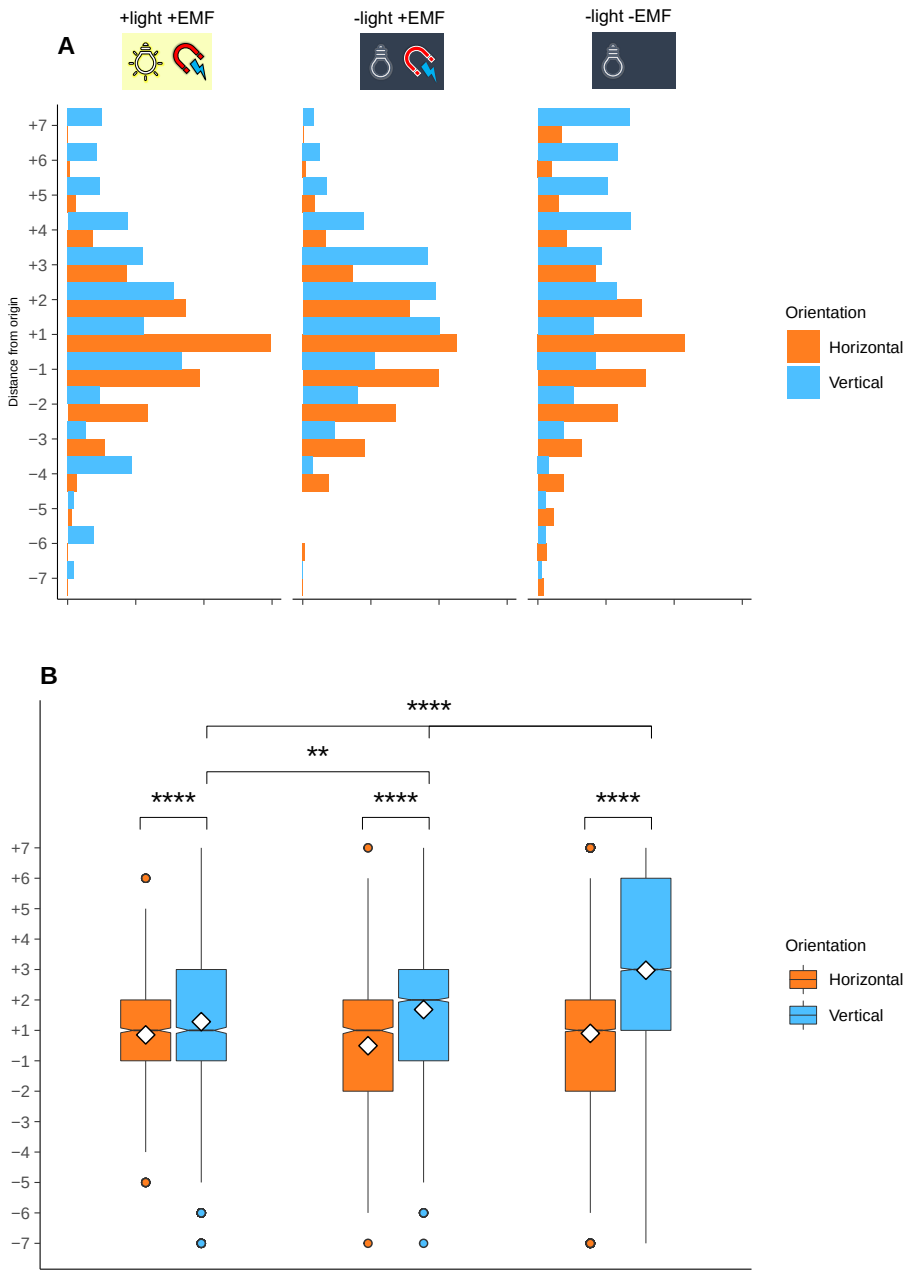

Figure 2.3: *C. elegans* negatively gravitax under different environmental conditions **A)** Histograms depicting cumulative distribution of *C. elegans* worms over multiple trials in vertical (blue) and horizontal (orange) assays. Presence or absence of normal overhead light and background EMF (no Faraday cage) are denoted with +/-. **B)** Boxplots depicting data shown in A. Dauers tested in a dark Faraday cage are used in future comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n.s. is not significant using Kruskal-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

behavior of dauers, we analyzed their movement in chambers that were shielded from the ambient EMF present in the lab using a Faraday cage (see Materials and Methods). We found that shielding the chambers from both EMF and light resulted in dramatically increased negative gravitactic behavior: in such chambers, dauers showed strong negative gravitaxis, with highly significant directional bias compared to horizontal controls (average vertical position = 2.76, n = 28,011, 76 trials; horizontal = 0.48, n = 11,626, 33 trials; $p < 0.0001$) (Fig. 2.3A, B). Further, the animals showed significantly stronger negative gravitaxis under these conditions than either the -light +EMF or +light +EMF conditions ($p < 0.0001$ in both cases). Over the course of several months, we noticed that gravitaxis in our Faraday cage gradually decreased as a result of diminished EMF blocking, as confirmed by restoration of cell phone reception (see Materials and Methods) Strong negative gravitactic behavior was restored after reinforcing the cage (Fig. A.1). Thus, the ability of *C. elegans* to undergo negative gravitaxis is inhibited by both light and EMF, suggesting that gravitaxis behavior in the animal is integrated with other sensory inputs. In all subsequent experiments, we used a Faraday cage in the dark to block these other sensory inputs.

Our findings that *C. elegans* dauer larvae exhibit pronounced negative gravitaxis contrasts with prior studies which found no gravitactic behavior in dauers, or that reported positive reorientation toward the gravitational vector in adults (Okumura et al., 2013; Chen et al., 2021). These differences are likely attributable to several parameters of assay design. The previous experiments were performed on Petri dishes, which limit the distance worms can travel. Owing to the long distances traversed in our chambers, we were able to find robust and highly significant differences between horizontal and vertically oriented chambers. In addition, the environment of a worm crawling in the thin space between the agar substrate and the wall of the chamber may be more similar to the act of crawling through a column of soil than the experience of moving along the agar

surface at the interface with the atmosphere on a plate. Finally, earlier experiments on gravitaxis in *C. elegans* made no mention of lighting conditions used or shielding against EMF, leading us to propose that signal integration with gravity and these other sensory inputs may have attenuated the behavior in those studies, as we have demonstrated here.

Elimination of neural-expressed MEC-7/12 microtubule subunits, but not other components involved in gentle touch sensation, abolishes negative gravitaxis

Gravitational force is exceedingly weak compared with other mechanical stimuli, and particularly so in organisms with low mass. Diminutive organisms must therefore evolve extremely sensitive mechanisms for perceiving this force. In some small invertebrates, stretch receptors in the cuticle generate an ion influx upon deformation in a manner analogous to stretch and pressure sensors in mammals (Bender and Frye, 2009). The comprehensive set of molecular components that mediate gentle touch sensation in *C. elegans* have been identified through genetic analysis (Chalfie and Sulston, 1981). Because the gentle touch mechanosensory channel proteins MEC-4 and MEC-10 are also essential for transducing hypergravitational force in worms, we sought to determine whether these known mechanosensory components function in sensing Earth's gravity.

We found that mutations that eliminate most components in the gentle touch response (Fig. 2.4) did not prevent gravitaxis in dauer larvae, demonstrating that gravity sensing is separable from touch reception. Gentle touch in *C. elegans* is perceived by the mechanosensory DEG/ENaC channels MEC-4 and MEC-10 (Chalfie and Sulston, 1981), which form a pressure-sensitive channel in the set of Touch Receptor Neurons (TRNs). We found that neither *mec-4(-)* nor *mec-10(-)* mutants were significantly defective for gravitaxis compared with wildtype controls (Fig. 2.5A,B): in both mutants, animals in

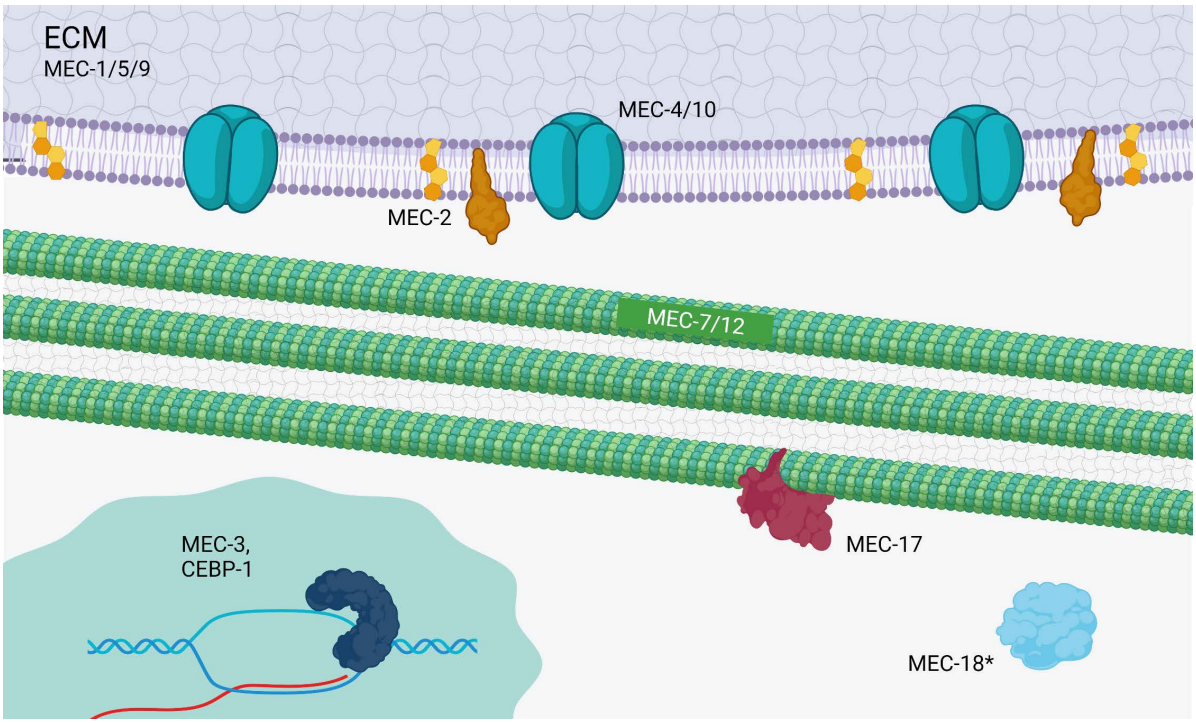

Figure 2.4: Gentle touch mechanosensory system
MEC-4 and MEC-10 subunits form a sodium channel that is required for gentle touch sensation in TRNs. MEC-1, -5, and -9 components of the extracellular matrix (ECM) form a structure between TRNs and the cuticle (Emtage et al., 2004). MEC-7/12 microtubules form 15 protofilament structures in the TRNs only; however, these β and α subunits (respectively) are found in other neuronal 11 protofilament structures (Bounoutas et al., 2009). MEC-2 proteins associate with both the DEG/ENaC channels and TRN microtubules and enhance channel activity, likely through interactions with cholesterol in the plasma membrane (Brown et al., 2008; Chen et al., 2015; Goodman and Schwarz, 2003). (Adapted from images by (Tavernarakis and and Driscoll, 1997; Tavernarakis and Driscoll, 2001) and others). Image was created with BioRender.com.

vertically oriented chambers exhibited a strong directional bias compared to the horizontal controls (average vertical location = 2.96, n = 1,340, 7 trials; horizontal = 0.83, n = 407, 4 trials; <0.0001 for the *mec-4(e1339)* missense mutation and average vertical location = 2.77, n = 2,110, 12 trials; horizontal = 0.81, n = 2,292, 9 trials; p <0.0001 for the *mec-10(tm1552)* null mutation (Fig. 2.5A, B), implying that the MEC-4/MEC-10 mechanosensory channels are not required for response to gravitational fields. Analysis of *mec-4(-)* mutants is complicated by the observation that the *mec-4(tu253)* null mutant animals are sluggish (Zhang and Chalfie, 2002); this impaired movement could confound conclusions about the requirement for MEC-4 in gravitaxis. Indeed, when tested in our assay, mec-4(tu253) showed severely defective movement and did not show clear gravitaxis (Fig. A.2). Nonetheless, our findings with the *mec-4(e1339* missense mutant, which is touch defective, and the *mec-10(tm1552)* null mutant suggest that the MEC-4/10 channel are not required for gravitaxis.

We found that several other genes in the canonical gentle touch sensing system are similarly largely dispensable for gravitaxis. *mec-2* encodes a stomatin-like protein required for MEC-4/MEC-10 channel activity (Brown et al., 2008). MEC-2 binds cholesterol (Huber et al., 2006) and likely facilitates gentle touch transduction by altering the composition of the plasma membrane surrounding MEC-4/MEC-10 ion channels (Brown et al., 2008). We found that *mec-2(e75)* dauers were not significantly impaired in their ability to gravitax (average vertical location = 2.29, n = 2,621, 10 trials; horizontal = -1.13, n = 1,526, 10 trials; p <0.0001) (Fig. 2.5A, B). MEC-18 is required for gentle touch sensation, although its function has not been well characterized (Topalidou et al., 2012; Neumann and Hilliard, 2014). *mec-18(u228)* mutants showed a strong gravitactic preference compared to horizontal controls (average vertical location = 1.11, n = 1,645, 4 trials; horizontal = -0.19, n =4,175, 5 trials; p <0.0001). While both mutants showed a significantly reduced vertical preference compared to N2, these experiments point to at

most a minor role for these components in gravitaxis.

The specialized MEC-12 alpha-tubulin and MEC-7 beta-tubulin proteins form unique 15-protofilament microtubules that are found only in the six TRNs that are essential for mechanosensation. These microtubule bundles are thicker than the typical 11 protofilament microtubules found in most cells, including in other neurons (Gu et al., 1996). These unique 15-protofilament structures may provide intracellular resistance required for mechanotransduction; however, the exact function in the process is unknown (Bounoutas et al., 2009; Krieg et al., 2015). In contrast to our findings with other gentle touch sensation components, we found the removal of either MEC-7 or MEC-12 abolished negative gravitaxis, resulting in a random distribution in the chambers similar to that seen in the horizontal controls (average vertical location = 0.60, n = 1,034, 4 trials; horizontal = 0.26, n = 407, 4 trials; $\gg 0.05$ for *mec-7(ok2152)* and average vertical location = 0.83, n = 5,063, 10 trials; horizontal = 0.92, n = 5,058, 10 trials; $p \gg 0.05$ for *mec-12(e1605)*) (Fig. 2.5A,B).

MEC-7 and MEC-12 are required for normal axonal outgrowth (Chalfie and Thomson, 1982; Bounoutas et al., 2009, 2011) and it is therefore conceivable that the *mec-7(-)* and *mec-12(-)* mutations might block gravitaxis by altering neuronal development or structure. We were able to separate the role of MEC-7/12 in neuronal structure from its other functions by taking advantage of the *mec-12(1605)* allele, an H129Y missense mutation that eliminates gentle touch sensation without detectably altering TRN development or structure (Lockhead et al., 2016; Chen et al., 2014). We found that gravitaxis is abolished in *mec-12(e1605)* mutants (Fig. 2.5A,B; $p \gg 0.05$), suggesting that MEC-12 requirement in gravitaxis is separable from its role in neuronal structure.

MEC-12 is the only *C. elegans* alpha-tubulin subunit known to be acetylated at K40 (Lockhead et al., 2016; Akella et al., 2010). Mutations that modify K40 or that eliminate the MEC-17 transacetylase required for microtubule acetylation also prevent

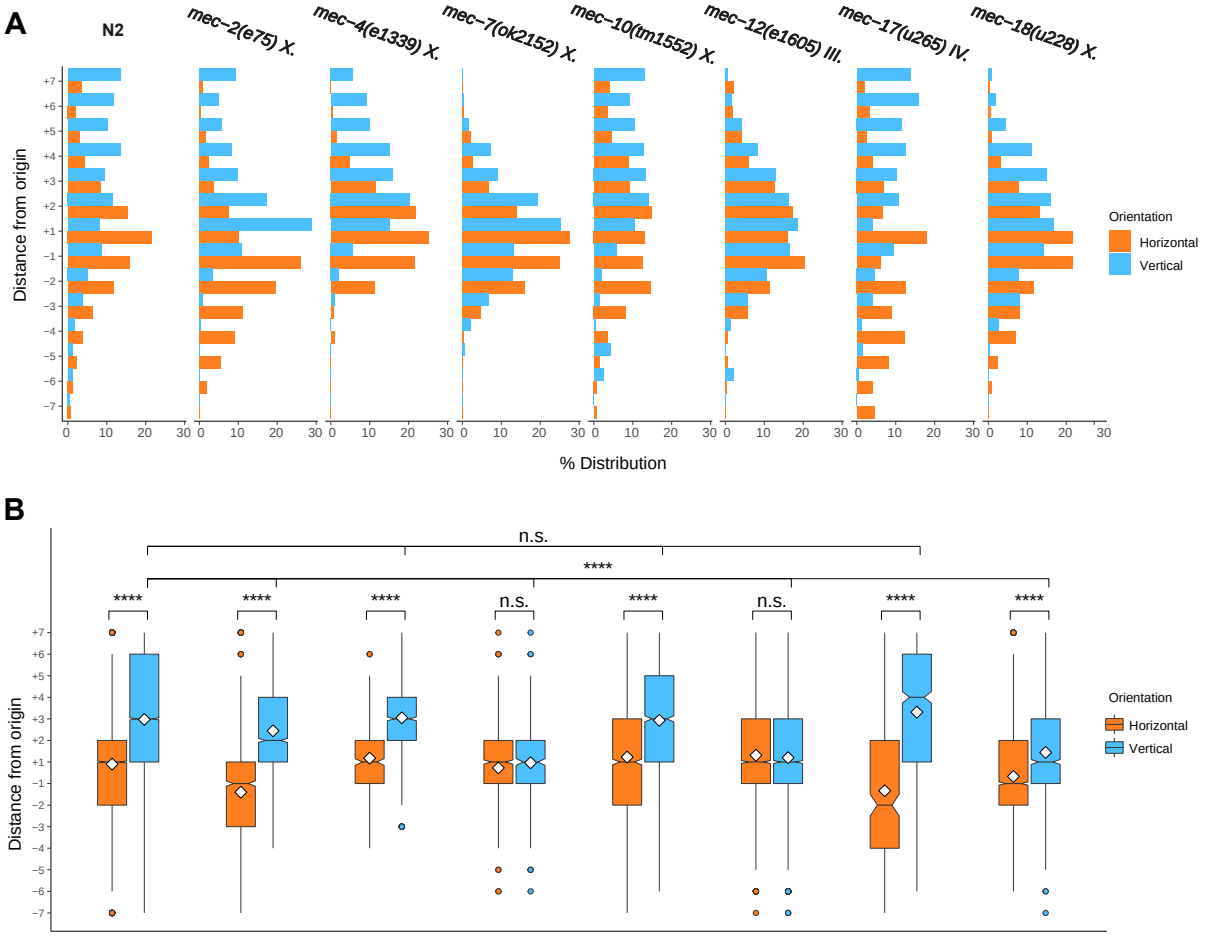

Figure 2.5: MEC-7/12 microtubule subunits, but not several other components in the gentle touch mechanosensory system, are necessary for gravitaxis

Gravitaxis assays of N2 dauers and dauers carrying mutations in genes involved mechanosensory transduction. **(A)** Histograms comparing horizontal (orange) and vertical (blue) distributions of each strain over multiple trials. **B)** Boxplots depicting data shown in A. Dauers tested in a dark Faraday cage are used in future comparisons. * p <0.05, ** p <0.01, *** p <0.001 , **** p <0.0001; n.s. is not significant using Kruskal-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

mechanosensation (Shida et al., 2010; Topalidou et al., 2012; Neumann and Hilliard, 2014). We found that *mec-17(u265)* mutants, which lack functional MEC-17, undergo normal gravitaxis behavior (average vertical location = 3.10, n = 856, 5 trials; horizontal = -0.90, n = 245, 2 trials; $p < 0.001$) (Fig. 2.5A,B) that did not significantly differ ($p \gg 0.05$) from that of N2 animals, indicating that this modification, which is essential for stabilizing the MEC-7/12 microtubules in TRNs, is not required for sensation or response to gravity.

Taken together, these results support the notion that MEC-7/12 microtubules perform a role in gravity perception that is distinct from their structural roles or action in conferring gentle touch sensitivity.

The TRPA-1 channel is essential for gravity sensation

Our finding that MEC-4 and MEC-10 are not required for gravitaxis suggests that other types of mechanosensory channels may be involved in gravity perception. Prime candidates for such channels are members of the superfamily of TRP (transient receptor potential) proteins, which are implicated in many sensory modalities including sensitivity to touch, hot and cold temperatures, noxious chemicals, and light (Chatzigeorgiou et al., 2010; Venkatachalam et al., 2014). Orthologs of these channels have been found across metazoan phylogeny, including in all triploblast and diploblast animals, sponges, and even unicellular choanoflagellates, which are believed to be the closes surviving relatives of all metazoans (Carr et al., 2008). Mouse TRPA-1 is expressed in the vestibular system, the primary organ where gravity sensation occurs in mammals (Kamakura et al., 2013). In *C. elegans*, *trpa-1* is expressed in the proprioceptive/nociceptive PVDL/R neuron pair, among others. Although TRPA-1 is known to play a role in noxious cold sensation in these neurons, *Drosophila* homologs of TRPA channels, *pain* and *pyx*, are required for

gravity sensation (Sun et al., 2009). Additionally, *trpa-1* knockout mice show impaired mechanosensation and perception of noxious cold (Kindt et al., 2007). *C. elegans* TRPA-1 is likely to perform mechanosensory functions in addition to its known role in cold sensation (Sun et al., 2009).

We found that removal of TRPA-1 in the *trpa-1(ok999)* null mutant abolishes gravitaxis in dauer larvae (average vertical location = 0.93, n = 1,392, 10 trials; horizontal = 0.84, n = 1,300, 7 trials; $p \gg 0.05$) (Fig. 2.6A, B). As *trpa-1(ok999)* mutant adult worms exhibit several movement defects, including decreased forward locomotion and several variations in the sinusoidal movement typical of wildtype worms (Yemini et al., 2013), it was conceivable that the elimination of net upward biased movement of the animals might reflect diminished locomotory capacity rather than defects in gravity sensing *per se*. However, we found that *trpa-1(ok999)* dauer larvae distributed broadly across the chambers comparable to that observed with N2 animals in both the horizontal and vertical conditions, demonstrating that these mutants are capable of traveling long distances regardless of orientation. These findings suggest that TRPA-1 channels are essential for, and may mediate, *C. elegans* gravitaxis.

PVD neurons, but not TRNs, are required for gravitaxis

Our observations that apart from the TRN-expressed MEC-7/12 microtubules many components that are essential for TRN function in gentle touch sensation are not required for gravitaxis led us to ask whether TRNs themselves are required in the response. The LIM homeodomain transcription factor MEC-3 mediates differentiation of several sensory neurons including the TRNs by activating a battery of genes required for touch sensation. TRNs in *mec-3(-)* mutants have smaller-than-normal cell bodies and shorter processes, leading to touch insensitivity (Chalfie and Sulston, 1981). We found that elimination

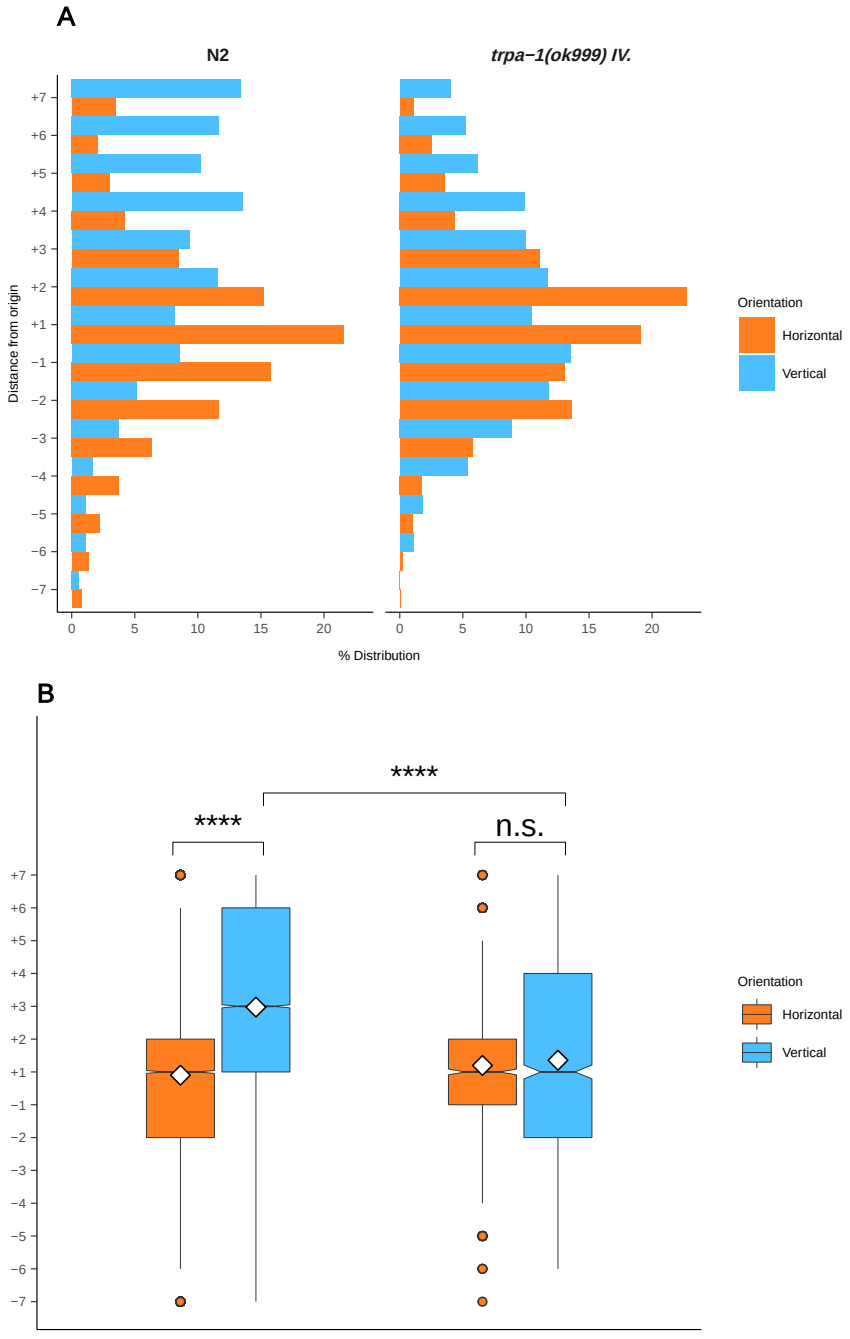

Figure 2.6: TRPA-1 channels are required for gravitaxis
(A-B) *trpa-1* mutants in vertical (blue) and horizontal (assays) compared with controls. * p <0.05, ** p <0.01, *** p <0.001 , **** p <0.0001; n.s. is not significant using Kruskall-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

of MEC-3 function in a *mec-3(e1338)* mutant did not significantly alter the ability of the animals to undergo gravitaxis (average vertical location = 3.8, n = 1,225, 5 trials; horizontal = 2.47, n = 1,657, 7 trials; $p < 0.0001$). Thus, normal TRN differentiation is not essential for negative gravitaxis.

The requirement in gravitaxis for *trpa-1*, *mec-7*, and *mec-12*, but not *mec-4/10*, other genes required for gentle touch response, or normal TRN differentiation, suggested that neurons other than the TRNs are required for this behavior. Although MEC-7 and MEC-12 are present in many neurons, their function beyond the TRNs is poorly understood. Further, there is no reported connection between MEC-7/12 microtubules and TRPA-1 function. Of the six TRNs, only one expresses *trpa-1* (Taylor et al., 2021); however, available expression data (Chatzigeorgiou et al., 2010; Taylor et al., 2021) indicates that PVDs, which detect muscle movement in addition to a host of other stimuli, express all three genes. We hypothesized, therefore, that PVD neurons might be integration centers for two proprioceptive inputs: muscle movement and position relative to the vector of Earth's gravitational field.

To test this hypothesis, we first investigated the requirement for TRNs in gravity sensing by genetically ablating them. The *mec-4(u253d)* gain-of-function mutation results in constitutive MEC-4/10 activation, which triggers degeneration and necrotic death specifically of the TRNs. We confirmed loss of TRNs using a TRN-expressed *mec-3p*::RFP reporter (Fig. 2.7A,C,E). Ablation of the TRNs did not alter the ability of the animals to perceive and respond to gravity: the gravitactic bheavior of worms lacking TRNs was not significantly different from that of N2 animals in our gravitaxis assay ($p \gg 0.05$)(average vertical location = 2.68, n = 1,955, 12 trials; horizontal = -0.57, n = 711, 5 trials; $p < 0.0001$) (Fig. 2.9A, B); hence, efficient gravitaxis of dauer larvae does not require the TRNs.

We then ablated the PVD neurons by expressing a constitutively active version of

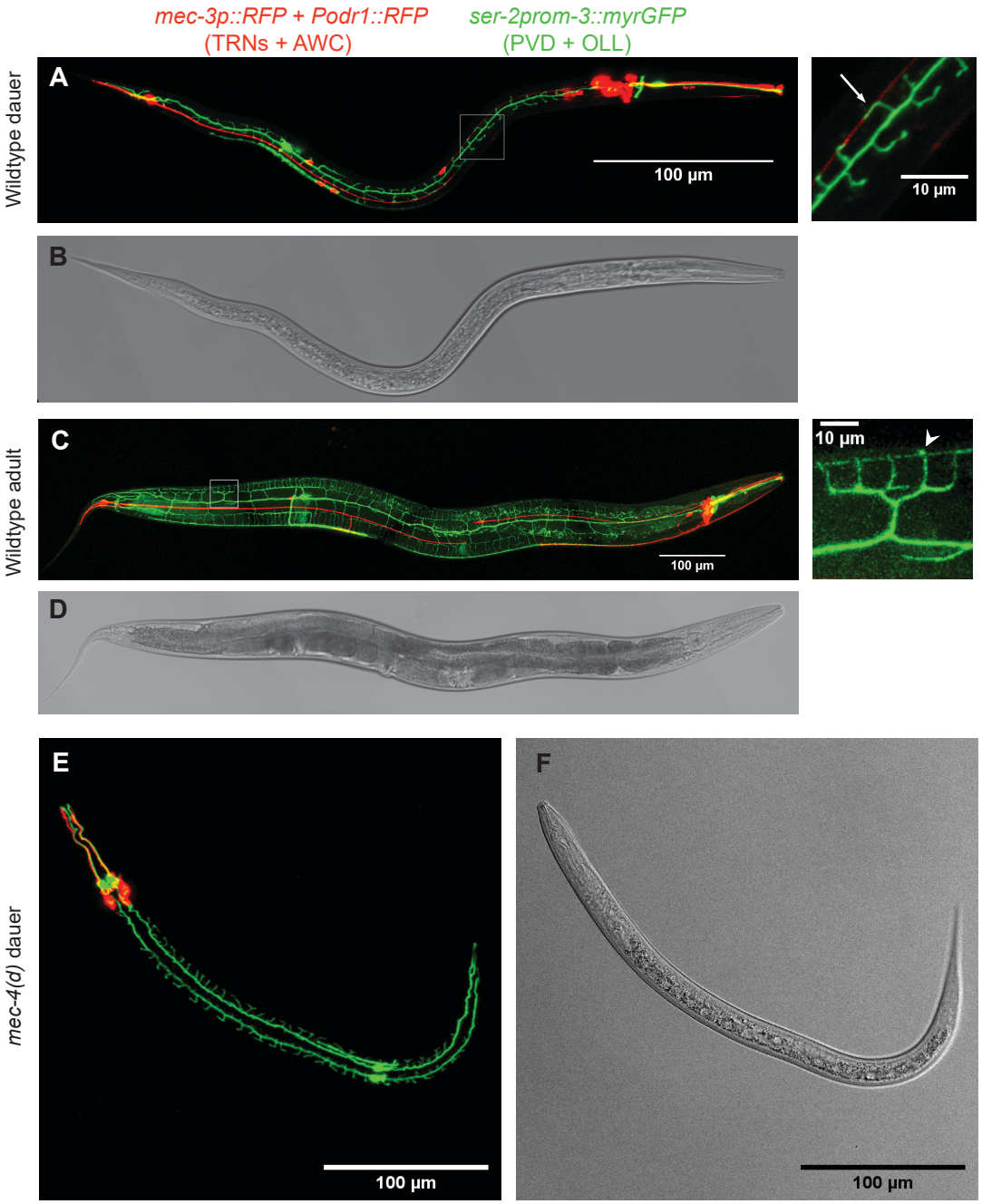

Figure 2.7: PVDs and TRNs in dauer and adult worms
(A,C and E) Confocal fluorescent micrographs of *mec-3p::RFP* and *ser-2prom-3::GFP* expression labelling the TRN and AWC neurons and PVD and OLL neurons, respectively. Brightfield images shown in B, D, and F. **(A-B)** Wildtype dauer worms; developing 3° processes of PVD neurons are indicated with a white arrow in inset. **(C-D)** Wildtype adult hermaphrodites; 4° processes are indicated with white arrowhead in inset. **(E-F)** *mec-4(e1611)* gain of function dauers, in which TRNs are ablated. Scale bar 100μm, 10μm in insets.

the nicotinic acetylcholine receptor (nAChR) channel subunit, *deg-3(u662)*/DEG-3, under the *ser2prom3* promoter (Albeg et al., 2011). Elimination of PVD neurons using this construct has been confirmed with a PVD-specific fluorescent reporter (Albeg et al., 2011). We found that animals lacking PVD neurons completely failed to undergo negative gravitaxis, showing a relatively even distribution between upward and downward movement, with a slight downward bias (average vertical location = -0.14, n = 2,323, 9 trials; horizontal = 0.95, n = 2532, 6 trials; $p < 0.0001$) (Fig. 2.9A, B). Based on this finding it is conceivable that removal of PVDs results in a reversal of gravitactic preference, albeit with a weak effect. This behavior is likely attributable to *bona fide* inability of the PVD-ablated animals to undergo negative gravitaxis, rather than defects in motility, as they are capable of effectively reaching both ends of he chamber in either the horizontal or vertical orientations. These findings strongly support an essential requirement for PVD neurons in sensing and responding to the gravitational fields.

Our observation that *mec-3(e1338)* mutants exhibit negative gravitaxis, while dauers lacking PVD neurons do not, led us to probe how PVDs may be responding to gravity. MEC-3 expression is required for TRN development (Smith et al., 2013; Wang and Way, 1996) and low levels of this transcription factor are required for specification of PVD neurons (Smith et al., 2013, 2010). In adulthood, PVD neurons are highly branched, with 2°, 3°, and 4° processes that have been described as "menorahs" (Fig. 2.7). In the absence of MEC-3, PVD neurons develop 1° axons, but lack 2°-4° branches (Smith et al., 2010; Tsalik et al., 2003). The observation that PVD neurons, but not MEC-3, are required in gravitaxis may be explained by the role of these processes in sensing polymodal information. Harsh touch and proprioceptive signaling are compartmentalized to the axon and 3° branches, respectively, within PVDs (Tao et al., 2019). Thus, the finding that mec-3 mutants are not impaired in their gravitational response implies that 2°-4° processes are not essential for this behavior. Moreover, while 1°-3° processes are

observed in dauer larvae (Fig. 2.7A, inset) (Richardson et al., 2019), 4° processes do not appear until L4 or adulthood (Fig. 2.7C, inset) (Sundararajan et al., 2019), further substantiating that 4° branches are not involved in gravity sensation.

The difference in PVD architecture between dauers and adults led us to ask whether adults behave differently in response to gravity. Changes in gravitational response – or even in the ability to sense gravity – throughout development could also be important for the worms' ecology, particularly given the importance of the dauer stage, but not adults, in dispersal of the animal. We found that, like dauer larvae, N2 adults also undergo significant negative gravitaxis in the absence of light and EMF (average vertical location = 2.00, n = 520, 5 trials; horizontal = -0.50; n = 777, 6 trials; $p < 0.0001$) (Fig. 2.10). While overall gravitactic preference was reduced somewhat in adults compared with dauers in our vertical assay ($p < 0.0001$), we note that the distances travelled by adults and dauers may not be directly comparable as adults are significantly larger, and therefore travel shorter distances relative to their body length in this assay. Evidence of gravitactic behavior by adults suggests that gravity sensation may have a strong influence on behavior throughout *C. elegans* development.

2.4 Discussion

In this study, we report four major advances. 1) We discovered that *C. elegans* dauer larvae and adults show pronounced negative gravitaxis. This drive toward movement away from the center of the earth may direct the animals twoard food sources, typically decomposing vegetative matter at the surface (Frézal and Félix, 2015). 2) We found that the ability of the animals to sense and respond to the force of gravity is attenuated by light and electromagnetic fields, suggesting that they integrate sensation of these other influences to make decisions about whether to undergo negative gravitaxis. 3) The ability

of the animals to sense and/or respond to gravity does not require DEG/ENaC channels or other factors involved in touch sensation but does require the MEC-7/12 microtubule components and TRPA-1 channels. Thus gravity sensing involves a previously unknown system involving specialized microtubules, which also participate in touch sensation and may function with TRP sensory channels. 4) Both immature PVD neurons in dauer larvae, and those in adults, but not TRNs, are essential for response to gravity. Morever, adult PVDs lacking quartenary process ("menorahs") appear fully functional for this response.

Underground habitats are generally ill-suited for *C. elegans* and such environments may cause dauer larvae to find their way to the surface where decaying vegetation might be present. However, the animals also avoid light (Ward et al., 2008). Further, some studies suggest that they may navigate using Earth's magnetic field (Vidal-Gadea et al., 2015; Landler et al., 2018; Vidal-Gadea et al., 2018). Our finding that ambient light and EMF in the laboratory setting strongly attenuate gravitactic behavior – which may explain why negative gravitaxis has not been reported previously – suggests that the animals integrate sensory inputs from a variety of environmental signals, including the force of gravity, to optimize behavioral decisions (Metaxakis et al., 2018). The extent to which light, EM fields, and potentially other inputs override the response to gravity, as well as the circuits required to calculate their respective influence on behavior, are important outstanding questions.

Although it was not detected in earlier studies, our altered assay revealed that both dauers and adults of *C. elegans* exhibit pronounced negative gravitaxis when properly shielded from other environmental stimuli. The reduced ability of N2 dauers to exhibit negative gravitaxis compared to that observed with *C. japonica* dauers, might be an outcome of myriad adaptations to laboratory cultivation, as has been observed for several other traits, including social feeding, egg laying behavior, oxygen tolerance, and nictation

(Gray et al., 2004; Frézal and Félix, 2015; Lee et al., 2012; Félix and Braendle, 2010; de Bono and Bargmann, 1998; Large et al., 2017). We found that negative gravitactic behavior, albeit weakened, occurs in the LSJ1 sister strain, which has been raised in liquid media over many decades, and is also observed in *C. briggsae* (Ackley et al., 2022b). It remains an open question as to whether these strains show negative gravitaxis when shielded from light and EMF when swimming in liquid rather than crawling on solid media.

demonstrate a strong negative gravitactic preference especially in the dispersal dauer phase, and less prominently in adults. In response to stressors such as overcrowding, lack of food, and poor environmental conditions, *C. elegans* enter an altered developmental dauer pathway that facilitates survival and enables them to seek a better habitat (Frézal and Félix, 2015; Ow and Hall, 2015). Therefore, if a habitat underground becomes poor, it is crucial that dauers find their way to the surface and nictate in the absence of other guiding cues. Recent studies have also found that worms are sensitive to the earth's magnetic fields (Landler et al., 2018; Vidal-Gadea et al., 2018, 2015) in addition to photosensitivity (Ward et al., 2008). We found that the normal lighting conditions and EM field in our laboratory setting was enough to alter gravitactic behavior. This supports our understanding of signal integration in the nervous system, in which several sensory inputs are constantly compared when making decisions (Metaxakis et al., 2018).

The extent to which light, EM fields, and other inputs override gravity sensation, as well as the circuits required to calculate their respective influence on behavior, are important questions for future studies. Previous assays for gravitaxis by *C. elegans* dauers conducted on plates did not detect any directional preference while *C. japonica* dauers under similar assay conditions were strongly negatively gravitactic (Okumura et al., 2013). Our altered assay has shown that *C. elegans* dauers as well as adults also exhibit negative gravitaxis. The reduced ability of N2 dauers to exhibit negative

gravitaxis might be an outcome of myriad adaptations to laboratory cultivation as has been observed for several other traits including social feeding, egg laying behavior, oxygen tolerance, as well as nictation (de Bono and Bargmann, 1998; Félix and Braendle, 2010; Frézal and Félix, 2015; Gray et al., 2004; Large et al., 2017; Lee et al., 2012). Interestingly, our results suggest that negative gravitaxis is maintained (albeit weakened) in the LSJ1 sister strain, which has been raised in liquid media over several generations (Fig. A.3). It would be interesting to see how these two strains compare gravitactically when swimming rather than crawling on solid media.

Our finding that *trpa-1* and MEC-7/12 microtubule functions are both required for gravitaxis reveals an unexplored mechanism for transducing gravitational force. Unlike the DEG/ENaC channels, TRPA-1 receptors do not require internal or external machinery to function (Kindt et al., 2007). Additionally, *trpa-1* is expressed in only one of six TRNs, which are the only neurons containing the 15 protofilament structures formed by MEC-7/MEC-12 tubulins. MEC-7/MEC-12 microtubules may perform more than a structural role in mechanosensation; whether this function is independent of their assembly into 15 protofilament structures has not been determined.

We obtained evidence pointing to a pair of neurons, PVDL/R, as primary gravity-sensors. Unlike animals lacking TRN function or cells, which show normal behavior in our gravitaxis assay, those lacking PVDs show no detectable response to gravity. These results, in conjunction with our *trpa-1* findings, suggest a sketch of how gravity may be transduced at a molecular and cellular level. PVD neurons may use TRPA-1 to provide information about the direction of gravity. However, in the absence of additional information, it is not clear whether TRPA-1 is the primary gravity receptor in worms. As with TRP channels generally, TRPA-1 is known to have primary and secondary roles in a variety of sensory pathways (Montell, 2003). It is not clear from our studies, or other reports, how MEC-7 and MEC-12 microtubule subunits function in mechanosensation.

While these tubulins may perform a vital function within PVD neurons that is required to transmit a signal, it is also possible tha they play a role in other neurons involved in the response. It is noteworthy that ciliated neurons are frequently used in gravity sensation including in humans (Bezares-Calderón et al., 2020; Lacquaniti et al., 2014). There are 60 ciliated neurons in *C. elegans* (Albeg et al., 2011), 38 of which express MEC-12 and TRPA-1 (Hammarlund et al., 2018). Of these, 3 (OLQ, AQR, and PHA) also express an ortholog of the likely gravity-sensitive mechanoreceptor in mammals, TMC-1(Corey et al., 2004; Nist-Lund et al., 2019; Chatzigeorgiou et al., 2013). Thus, it is conceivable that PVDs may be secondary neurons that act within a neural circuit to make decisions about movements in response to the direction of the gravitational field.

Understanding how *C. elegans* perceives gravity has implications for both invertebrate and mammalian biology. *C. elegans* has been used for decades as a model for understanding the effects of microgravity on animal physiology in space (Gao et al., 2015, 2017; Honda et al., 2012, 2014; Qiao et al., 2013; Selch et al., 2008; Xu et al., 2014; Zhao et al., 2017), and yet scant information is available regarding their ability to sense gravity. Moreover, gravity perception is linked evolutionarily and developmentally with hearing (Fritzsch et al., 2014; Lipovsek and Wingate, 2018): the vestibular and auditory systems in mammals share similar sensory structures, transduction machinery, and gene expression (Scheffer et al., 2015). A role for TRPA1 has already been identified in sensory transduction within inner ear hair cells in mice (Corey et al., 2004). The similarities between these systems makes the study of gravity sensation important not only for understanding mammalian vestibular sensation, but also auditory sensation. A recent study showed that *C. elegans* can sense sound through mechanical perturbations of the epidermis and that this sense involves the multimodal PVD neurons and the FLP mechanosensory neurons (Iliff et al., 2021). Thus, uncovering the role of TRPA-1 as a mechanoreceptor raises the possibility that *C. elegans* could be used as a model for

studying both vestibular sensation and audition.

2.5 Materials and Methods

Worm rearing

C. elegans worms were grown and maintained on OP50 E.coli at room temperature (23°C) using standard methods (Stiernagle, 2006). Dauer formation was induced by over-crowding, which occurred 8-12 days after chunking onto seeded NGM plates. Adults used for gravitaxis assays and imaging were taken from synchronized populations 2 days after hatching. Crosses were performed as described previously (Fay, 2006), using fluorescence microscopy and/or PCR to confirm the outcome of each cross. All strains used in this study are included in Table A.1.

Dauer isolation

Dauer larvae were isolated from 1-5 starved NGM plates using 1% SDS and a 30% sucrose gradient as described previously (Karp, 2018; Ow and Hall, 2015). Briefly, worms were rinsed and collected in M9, then pelleted and resuspended in 7mL 1% SDS in a 15 mL falcon tube. Worms were left rotating for 30 minutes in SDS to kill all non-dauer lifestages. After 3-5 rinses with M9, worms were resuspended in 10 mL 30% sucrose solution and centrifuged for 5 minutes. Surviving dauers were collected at the interface of the water-sucrose gradient with a large bore glass Pasteur pipette and rinsed 3-5X in M9. Worm were used immediately or after rotating in solution overnight.

Synchronization

Mixed stage worms were rinsed from confluent plates with M9 and collected in 1.5 mL tubes. Worms were then rinsed and pelleted. A 1 mL bleach solution containing 150 μL bleach and 50 μL 5N KOH was added and worms were lysed in this solution for 3 minutes, or until most corpses were dissolved. The embryo pellet was then rinsed 3-5X

in M9 and plated onto OP50 NGM plates until adulthood (2 days at 23°C).

Assay preparation

Gravitaxis assay chambers were created using 5 mL serological pipettes and 4% NGM agar. To assemble each chamber, a heated blade was used to cut the tapered end of one pipette and the cotton plug was removed from the end of a second pipette. The two pipettes were then joined end-to-end by melting the two ends slightly over a Bunsen burner and fusing them with moderate pressure, ensuring that no gaps remained. The NGM media was then autoclaved and then taken up by each double pipette using a standard serological pipettor while the agar was still molten. Best results were achieved by orienting each pipette parallel with the benchtop as much as possible during the procedure and as the agar solidified. Pipettes were used within 24 hours of construction or stored at 4°C to be used within 48 hrs.

Once solidified, a heated 3mm Allen key was used to punch a hole through one wall of the pipette about 5mm below the fusion point (for reference, the cotton-plug end is the "top"). Each end of the pipette was removed using a heated blade and sealed with parafilm.

Gravitaxis assay

Worms were pelleted in M9 and were either extracted directly from the pellet or pipetted onto a square of parafilm. After cutting the tip to increase the bore, a pipette was used to aspirate 0.5-2 μL of the concentrated worm pellet, letting in a small volume of air at the end to facilitate injection. Worms were injected into the agar at the opening created by the Allen key. This opening was then sealed with parafilm, and the chamber was immediately oriented vertically or horizontally. Worms were allowed to gravitax overnight and were scored the following day (typically 12-24 hrs after injection).

Pipettes were examined under a dissecting microscope and a marker was used to mark the location of each worm. Pipettes were scored immediately after removal from

the assay location. Worms were only scored if they appeared alive, healthy, and were not swimming (chambers were only scored if a majority of worms were alive and crawling). Worms were not scored within 2.5 cm to either side of the injection point.

To tally the location of each worm, pipettes were divided into 7 sections on each side of the 5 cm injection site. Each section was 3.5 cm long (the same length as a 1 mL volume indicated on the pipettes). The number of marker points in each section was then recorded.

Imaging

Fluorescent micrographs were taken using a Leica SP8 Resonant Scanning Confocal microscope at 10-40x. Brightness and contrast were adjusted and overlays added using ImageJ.

Quantification and Statistical Analysis

Data were collected in Excel and analyzed using RStudio. Statistical tests, p values, sample sizes, and replicates are detailed in the results and figure legends.

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2.7 Declaration of Interests

The authors declare no competing interests

2.8 Author Contributions

Caroline Ackley: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization, Supervision. Neda Ziaei Kajbaf: Methodology, Validation, Investigation. Lindsey Washiashi: Methodology, Validation, Investigation. Ruchira Krishnamurthy: Investigation, Formal Analysis. Pradeep Joshi: Conceptualization, Writing – Review & Editing. Joel Rothman: Conceptualization, Writing – Review & Editing, Project administration, Funding acquisition.

2.9 Supplemental data

Supplemental figures and tables can be found in Appendix A.

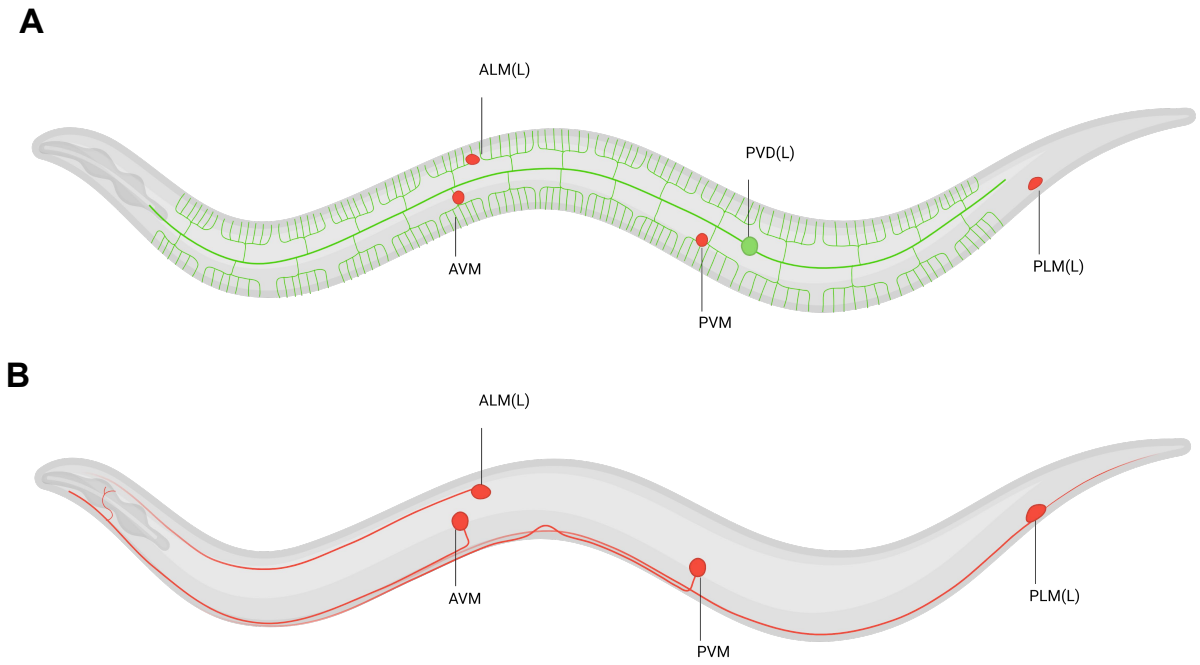

Figure 2.8: *mec-3* mutants and PVD- worms have altered neuronal morphology **(A)** *mec-3(e1338)* mutant worms have small TRN cell bodies that lack axons. **(B)** PVD neurons were ablated genetically, leaving TRNs intact.

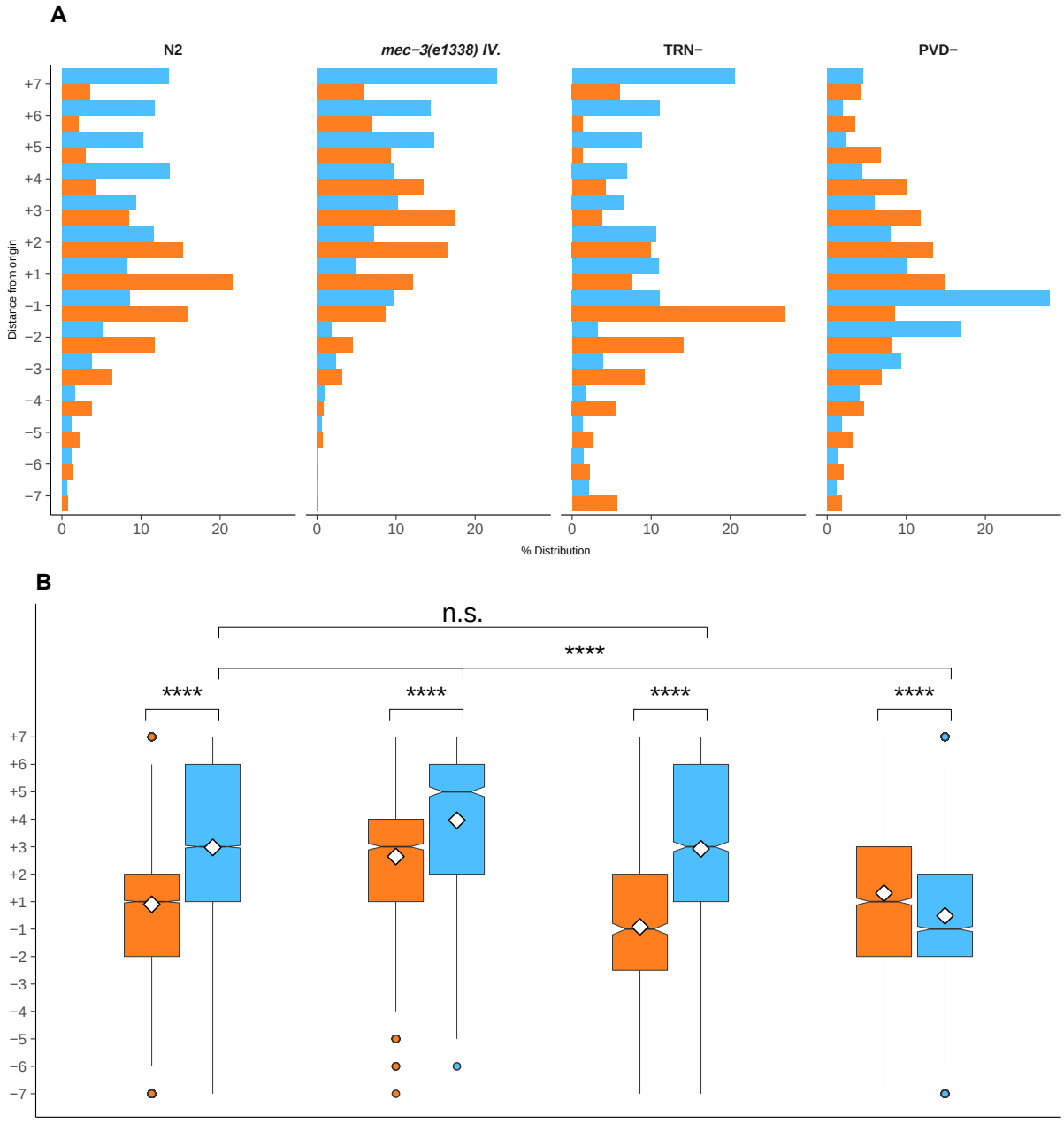

Figure 2.9: Genetic ablation of PVDs but not TRNs affects gravitaxis
(A-B) Gravitaxis behavior of *mec-3(e1338)* dauers, dauers lacking TRNs only (*mec-4* gof). dauers lacking PVD neurons only (*ser2prom-3::deg-3*), and N2 dauer controls. * p <0.05, ** p <0.01, *** p <0.001 , **** p <0.0001; n.s. is not significant using Kruskall-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

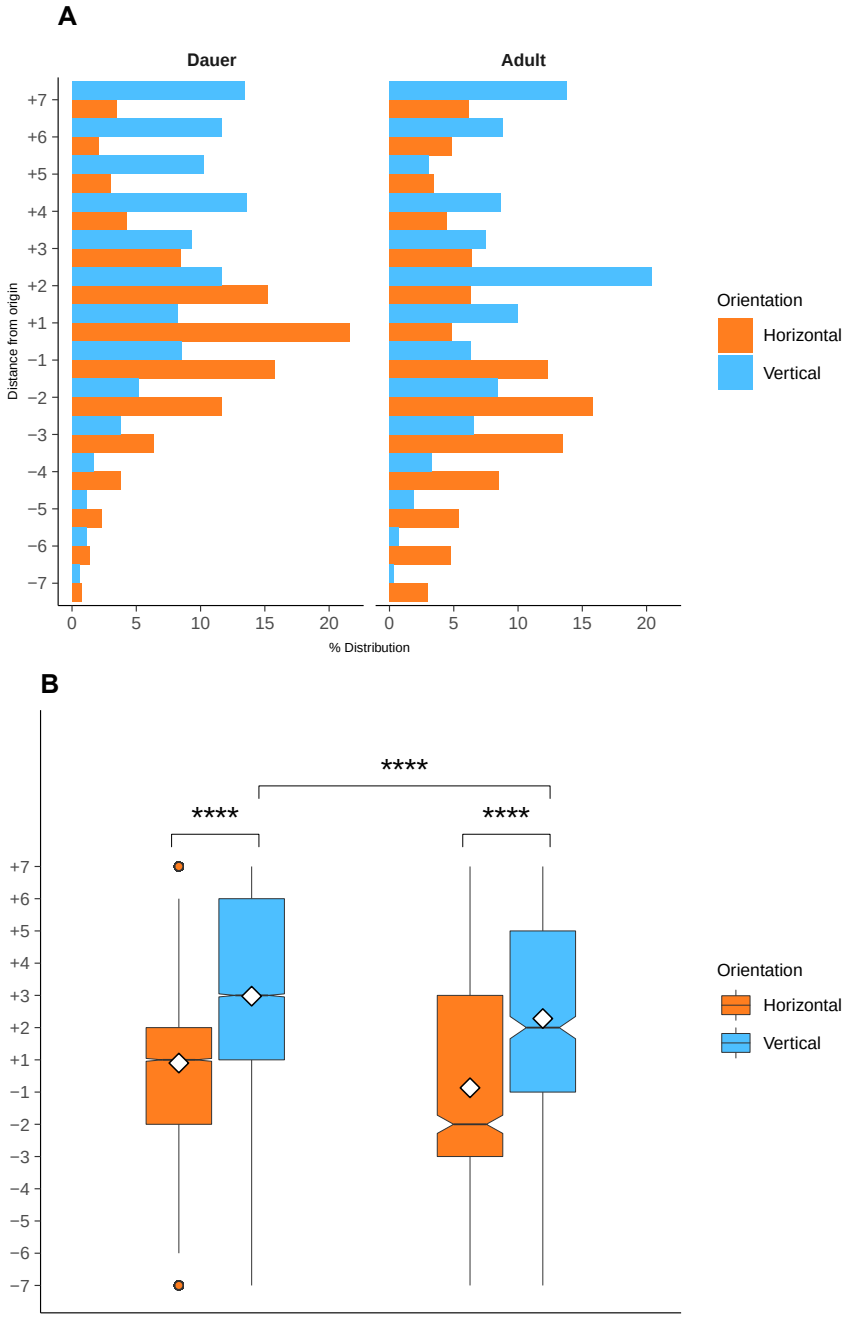

Figure 2.10: Adult worms negatively

(A-B) Adult worms demonstrate a strong negative gravitaxis preference similar to dauers. * p <0.05, ** p <0.01, *** p <0.001 , **** p <0.0001; n.s. is not significant using Kruskall-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

Chapter 3

Large-scale gravitaxis assay of *Caenorhabditis* dauer larvae

3.1 Summary

This chapter outlines methods for conducting a large-scale gravitaxis assay with *Caenorhabditis* dauer larvae. This protocol allows for better detection of gravitaxis behavior compared with a plate-based assay.

3.2 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 takes place in the inner ear in mammals, is closely related to hearing — thus, understanding this process has implications for auditory as well as 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.

3.3 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 (Lacquaniti et al., 2014; Peterka, 2018). Conversely, gravitactic preference can be altered in the presence of other stimuli (Bostwick et al., 2020; Fedele et al., 2014; Ntefidou et al., 2002). Therefore, gravitactic behavior is an ideal system 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 (Frézal and Félix, 2015). 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 better habitats (Lee et al., 2012). Gravitaxis assays of *C. elegans* and *C. japonica* suggest that dauer larvae negatively gravitaxis, and that this behavior is more readily observed in dauers than in adults (Ackley et al., 2022a; Okumura et al., 2013). 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 (Bostwick et al., 2020; Häder and Hemmersbach, 2017; Sun et al., 2009). 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 (Chen et al., 2021). Meanwhile, although *C. japonica* dauers were identified early on as being negatively gravitactic (Okumura et al., 2013), this behavior has only recently been described in *C. elegans*. 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 (Bargmann et al., 1993; Margie et al., 2013; Ward, 1973). The earliest reported gravitaxis assay in *Caenorhabditis* was performed by standing the plate on its side at a 90° angle to the horizontal control plate (Okumura et al., 2013). However, gravitaxis behavior is not always robust under these conditions. While adult worms can be assayed for orientational preference in solution (Chen et al., 2015), this directional preference may be context-dependent, leading to different behaviors if the worms are crawling rather than swimming. Additionally, *C. elegans* are known to be sensitive to other stimuli, including light and electromagnetic fields (Vidal-Gadea et al., 2015; Ward et al., 2008), which interfere with their responses to gravity (Ackley et al., 2022a). 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 protocol is based in part on a method developed to study neuromuscular integrity (Bainbridge et al., 2016; Beron et al., 2015). Dauer larvae are cultured and isolated using standard procedures (Ow and Hall, 2015). 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 hours 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.

3.4 Protocol

The strains used in the present study are *C. elegans* (N2) and *C. briggsae* (AF16) (see Table B.1). A mixed-sex population of dauers was used for each assay.

1. Chamber preparation

    1.1 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 B.1.

    1.2 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 (Fig. 3.1A).

    1.3 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 (Fig. 3.1A,B).

2. Filling the chambers with agar

   2.1 Prepare NGM with 4% agar according to standard worm protocols (Chaudhuri et al., 2011). 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 after autoclaving helps prevent bubbles from forming in the solution (Bainbridge et al., 2016).

   2.2 To minimize any variations in the consistency of the agar due to uneven cooling, hold the pipettor (see Table B.1) parallel to the benchtop while drawing up 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 digging through the medium in our hands. However, other experimenters have observed burrowing behavior by adult worms in up to 9% agar (Bainbridge et al., 2016; Beron et al., 2015).

   2.3 Once cooled, heat a 3 mm hex key (or another metal tool of the same size, see Table B.1) 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 (Fig. 3.1C). 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 should be used within 1 day or may be 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

3.1 10–15 days prior to the start of the experiment, chunk each strain onto 2–3 large NGM plates with OP50 bacteria ad wrap with paraffin film. After 10–15 days, each plate must be fully starved with thousands of dauer larva present on the agar, wallls, and lids.

NOTE: Make plates ahead of time using a thick OP50 recipe for maximum crowding (see Table B.1. Dauer formation can also be induced through other methods, including the addition of pheromones or by growing at higher temperatures (Karp, 2018).

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

3.3 Isolate dauers by treating with 1% SDS followed by a 30% sucrose flotation gradient following the previously published report (Ow and Hall, 2015).

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

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

3.6 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. Add dauers to the chamber

4.1 Centrifuge dauers at 1,600 x g for 5 minutes 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 μ droplets under a dissecting microscope. Use the average of these counts to approximate the number of worms per μ.

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.

4.2 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 (1000+) in one chamber may increase the range of distances travelled due to overcrowding (Ackley et al., 2022a).

4.3 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 (Ackley et al., 2022a)

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

5.3 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.

5.4 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 hours 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.

5.5 Remove and score gravitaxis chambers one at a time. Look for live dauers under a dissecting microscope and mark their locations in ink (Fig. 3.1C,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.

5.6 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.

5.7 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 B.1) 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.

3.5 Representative Results

Comparing gravitaxis across species

Following the procedure outlined above, *C. briggsae* dauer gravitaxis can be compared with *C. elegans* gravitaxis as well as with horizontal controls. The vertical distribution (maroon) of *C. briggsae* dauers is skewed towards the tops of the chambers, with a large percentage of worms reaching +7 (Fig. 3.2A). Contrasted with horizontal controls (aqua), in which dauers are distributed in a roughly bell-shaped curve around the center

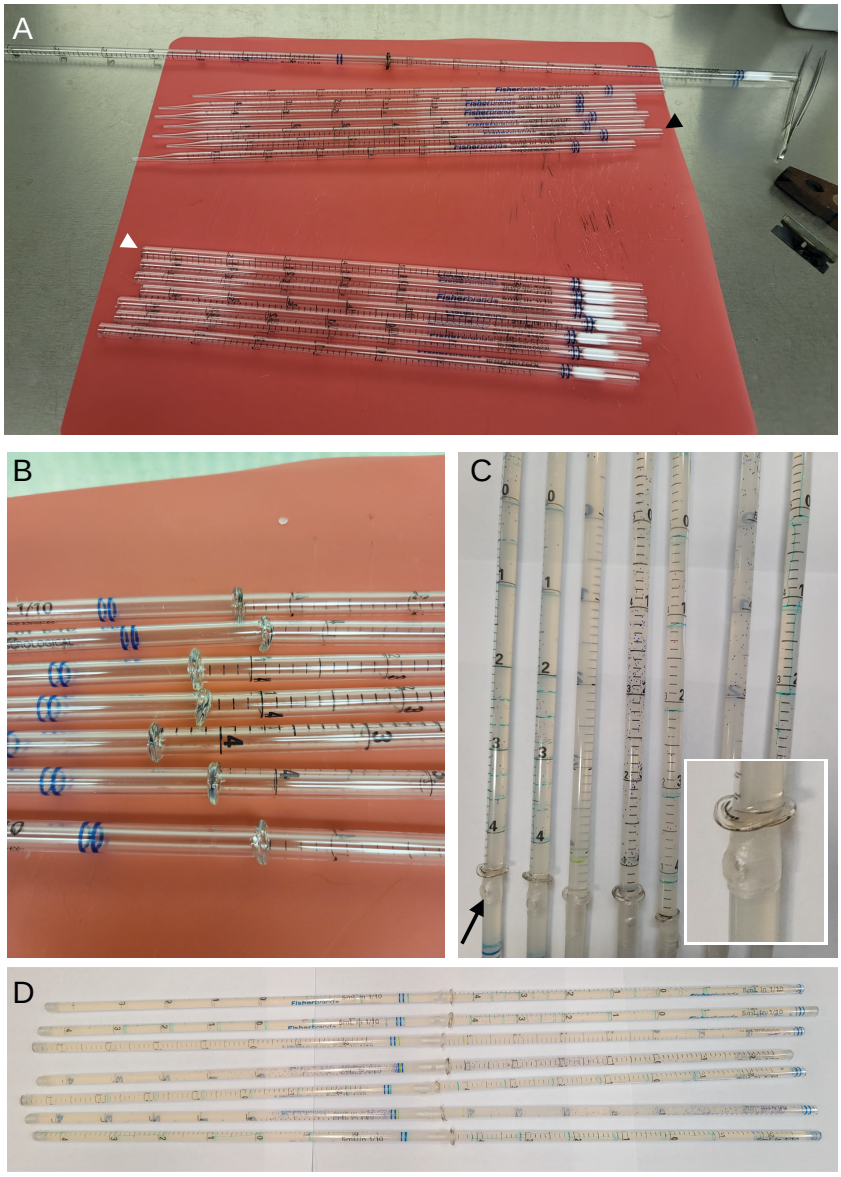

Figure 3.1: Gravitaxis assay chamber setup

Photos depicting steps of gravitaxis assay chamber preparation. **A)** Individual 5 mL pipettes prepared for fusion into single chamber. Removal of cotton plug indicated with black arrowhead; removal of tip indicated with white arrowhead. The tubes used in this study have an inner diamter 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 the chamber as well as to facilitate scoring. **D)** View of chambers in their entirety (taken after scoring).

of the chambers, this trend is indicative of negative gravitactic behavior. These data can be compared with *C. elegans* gravitaxis performed on the same experimental days (Fig. 3.2B).

A Kruskal-Wallis test followed by Dunn's test with Bonferroni correction for multiple comparisons reveals any significant differences between assays (Ackley et al., 2022a; Dinno, 2015). In this experiment, 1,108 *C. briggsae* dauers were scored across three vertical chambers from two independent experimental days. These worms migrated significantly upward compared with horizontal controls (p value <0.001; 1,639 dauers in three horizontal chambers over two 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 two experimental days). These results suggest that *C. briggsae* dauers show a negative gravitaxis behavior that is similar to that of *C. elegans* dauers.

3.6 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 (Chen et al., 2021), 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

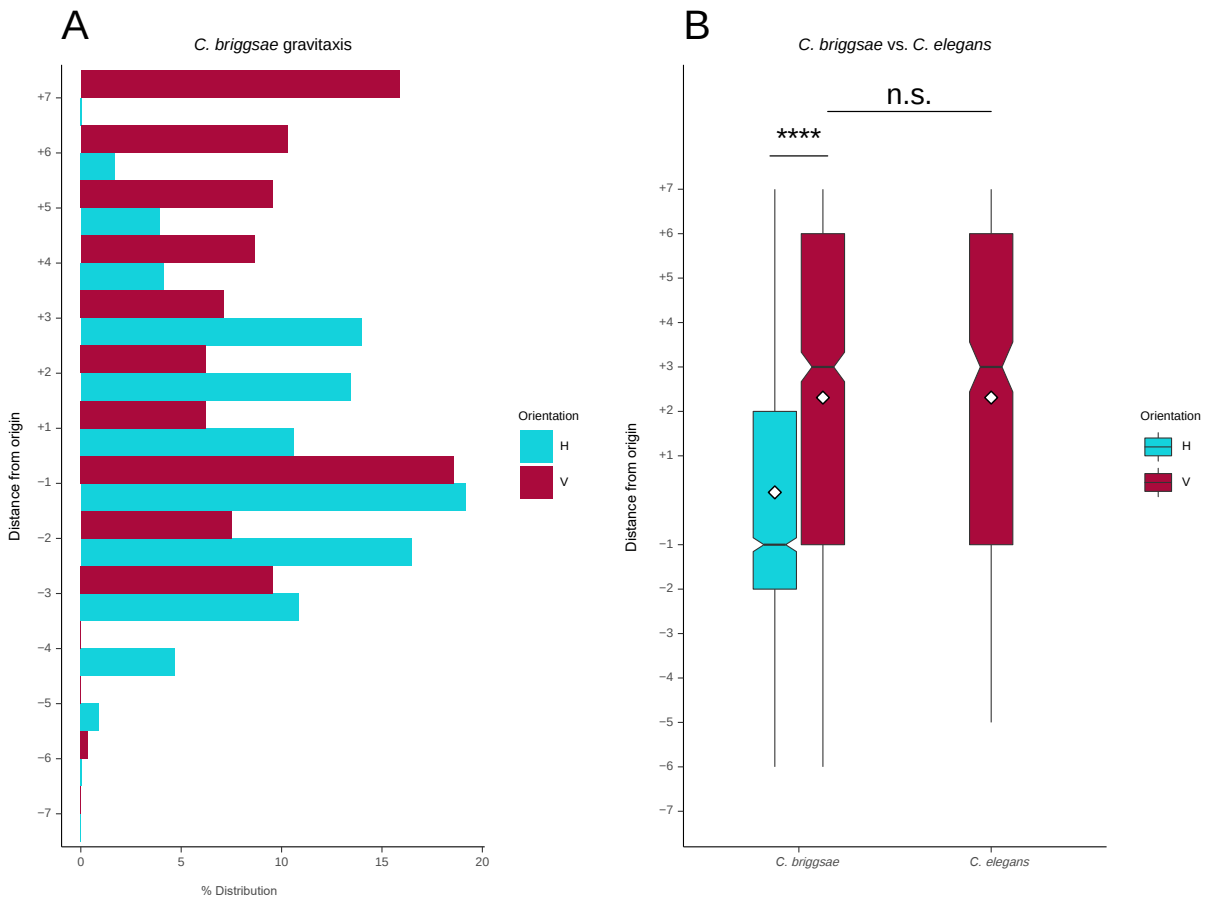

Figure 3.2: Gravitaxis in *C. briggsae* vs. *C. elegans*

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 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 **B)** Boxplots comparing 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 Dunn's test with Bonferroni correction for multiple comparisons. No stars = p >0.05, **** = p <0.0001.

electromagnetic fields, both of which interfere with gravitactic preference (Ackley et al., 2022a), 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 recommend.

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 (Frézal and Félix, 2015). Because *Caenorhabditis* live in compost, where guiding cues such as light may not be available, gravity may enable worms to navigate to the surface (Frézal and Félix, 2015; Ackley et al., 2022a). Two-dimensional plate-based assays are unlikely to replicate these conditions even if they are tested in the absence of other stimuli. Finally, this larger apparatus allows for testing 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 (Lee et al., 2012; Gray et al., 2004) 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 (Ackley et al., 2022a). 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 (Ackley et al., 2022a). However, *C. elegans* is sensitive to of other stimuli, including temperature, volatile and non-volatile chemicals, texture, humidity, and sound, which influence their behavior (Goodman and Sengupta, 2019; Iliff et al., 2021; Iliff and Xu, 2020; Russell et al., 2014). 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 (Ackley et al., 2022a; Chen and Chalfie, 2014; Metaxakis et al., 2018; Stockand and Eaton, 2013; Wicks and Rankin, 1995). Sensory integration is especially important in the context of proprioception, which is itself an integrative modality that draws from multiple sensory cues (Peterka, 2018).

Understanding gravity sensation in *Caenorhabditis* has implications for human health. Millions of individuals suffer from vestibular dysfunction in the United States alone (Mackowetzky et al., 2021) , and many of these disorders have underlying genetic causes (Eppsteiner and Smith, 2011; Roman-Naranjo et al., 2018). For this reason, the identification of gene candidates and targeted therapies is an active area of research (Mei et al., 2021). Additionally, because vertebrate vestibular and auditory systems are closely linked developmentally and evolutionarily (Mackowetzky et al., 2021; Mei et al., 2021; Weghorst and Cramer, 2019), elucidating gravity sensation could also provide insight into hearing and hearing disorders.

3.7 Acknowledgements

This research was supported by research grants from the National Institutes of Health to JHR (#R01 5R01HD081266 and #R01GM141493). Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). We would like to acknowledge Pradeep Joshi (UCSB) for his editorial input. Statistical consultation provided by the UCSB DATALAB.

3.8 Disclosures

The authors declare no competing interests.

3.9 Author contributions

Lindsey Washiashi, Ruchira Krishnamurthy, and Joel Rothman all contributed to this work.

3.10 Supplemental data

A table of materials can be found in Appendix B (Table B.1)

Chapter 4

Conclusion

4.1 Summary and future directions

Gravity sensation is critical for movement, coordination, and balance, and contributes to overall proprioception in animals. Studying gravitactic behavior in *C. elegans* can uncover new genes and pathways involved in gravity sensation that can be extrapolated to other mechanosensory systems. Through observing negative gravitaxis in worms, I identified three genes — MEC-7, MEC-12, and TRPA-1 — that are required for this behavior. This is the first known connection between MEC-7/12 tubulins and TRPA-1, which opens several avenues for future research. While the role of MEC-7/12 microtubules has been well-established for gentle touch sensation, little is known about their function outside of the TRNs. Both proteins are expressed in other neurons but only form 15 protofilament structures in TRNs (Chalfie and Thomson, 1979). Additionally, MEC-12 likely has multiple functions in mechanosensation besides anchoring MEC-4/10 channels and forming TRN axons; the *mec-12* allele used in this research, *e1605*, affects gentle touch sensation and gravitaxis without disrupting TRN development (Bounoutas et al., 2009, 2011; Chalfie and Au, 1989). Although an association between TRPA-1 and

these microtubule subunits has been found, how these proteins interact as well as other components involved in this pathway have not yet been identified.

Likewise, while a pair of neurons, the proprioceptive PVDL and PVDR neurons, were identified as essential for negative gravitaxis, other neurons should be investigated for their role in this behavior. PVDL/R could be the primary graviperceptive neurons because they express MEC-7, MEC-12, and TRPA-1. However, they could also serve as integration centers for multiple proprioceptive cues. Ciliated neurons, such as the IL2 neurons required for nictation (Lee et al., 2012), should be examined because of the prevalence of ciliated neurons in other mechanosensory systems (Bezares-Calderón et al., 2020; Sun et al., 2009). Additionally, it would be of interest to investigate candidate genes that would explain how minute gravitational forces are transduced in an animal as small as *C. elegans*, which has no known statoliths or similar organelles.

Appendix A

Chapter 2 Supplemental

A.1 Supplemental Figures

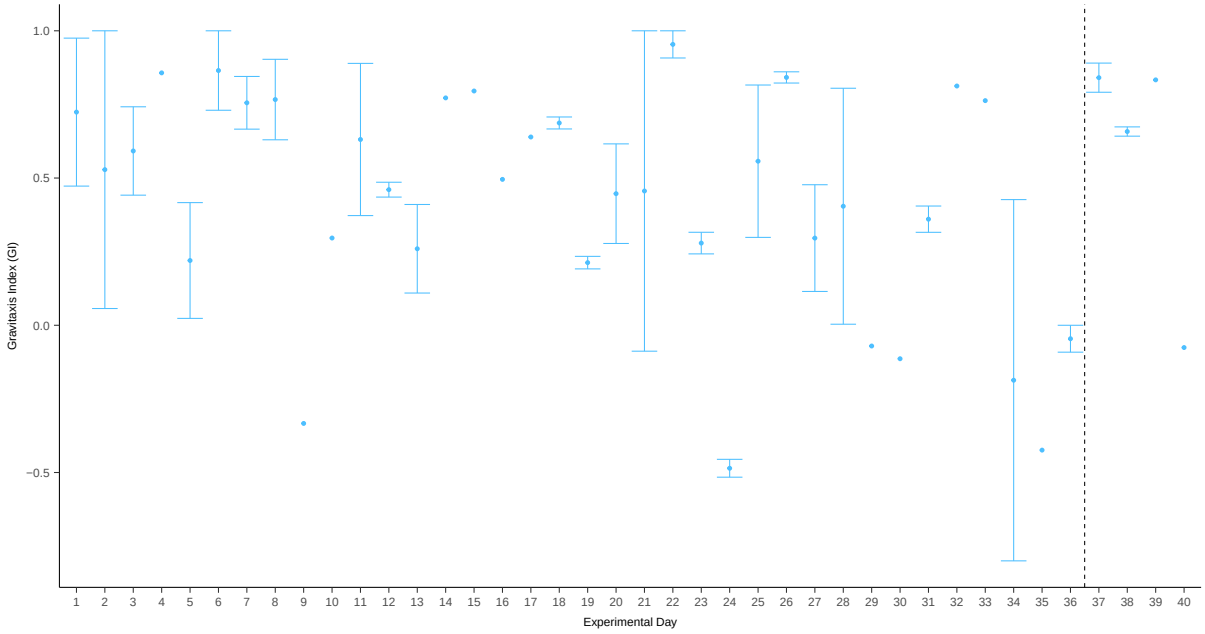

Figure A.1: Faraday cage integrity influences gravitactic behavior

Plot of Gravitaxis Index (GI) — calculated as the sum of worms in the top half of the chamber (segments +1 to +7) minus worms in the bottom half (-1 to -7) divided by the total — for all chambers across different experimental days. Dashed line indicates reinforcement of the Faraday cage.

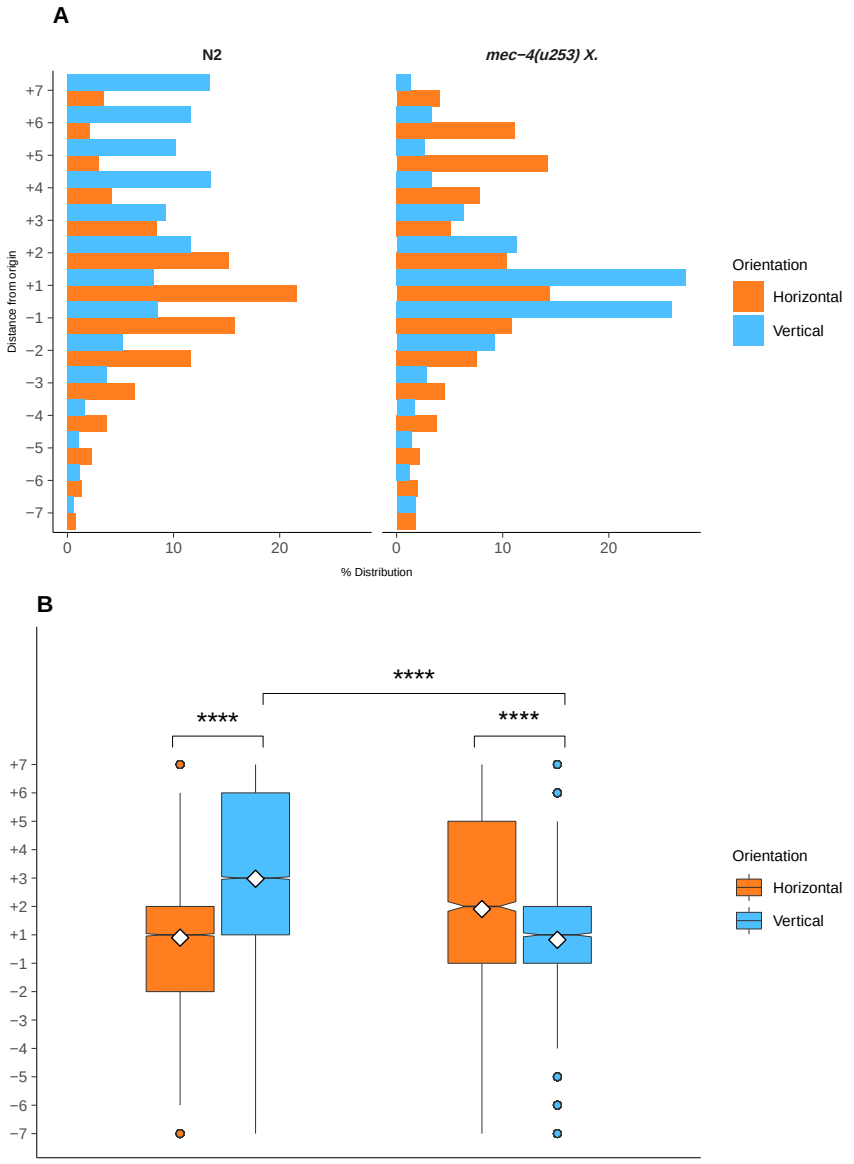

Figure A.2: *mec-4* null mutants do not gravitax

(A) Histograms depicting horizontal and vertical movement of 2 and *mec-4(u253) dauers.* **(B)** Box plots summarizing data shown in A. *mec-4(u253)* vertical: n = 2,075 worms over 7 trials; horizontal n = 2,130 worms over 4 trials. * p <0.05, ** p <0.01, *** p <0.001 , **** p <0.0001; n.s. is not significant using Kruskall-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

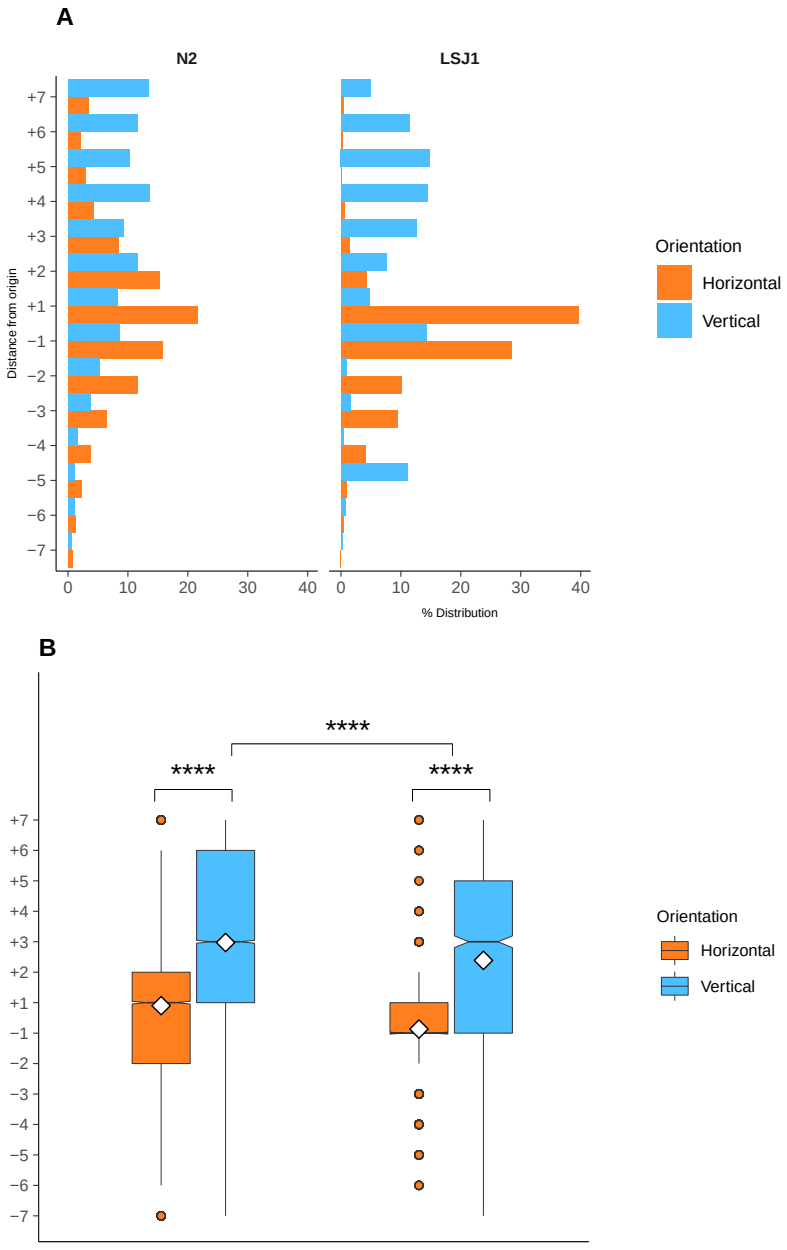

Figure A.3: LSJ1 dauers negatively gravitax to a lesser extent compared with N2 **(A)** Histograms depicting horizontal and vertical movements of N2 and LSJ1 dauers. **(B)** LSJ1 vertical n = 1,666 worms over 7 trials; horizontal n = 2,510 worms over 4 trials; horizontal n = 2,130 worms over 4 trials. * p <0.05, ** p <0.01, *** p <0.001 , **** p <0.0001; n.s. is not significant using Kruskall-Wallis followed by Dunn's test with Bonferroni correction. Notches on boxplots represent 95% confidence intervals; mean values are indicated with a diamond.

A.2 Supplemental Table

Table A.1: List of strains used in this study

Strain	Genotype	Source
N2	wildtype strain	CGC
LSJ1	wildtype strain	CGC
CB75	*mec-2(e75) X.*	CGC
CB1338	*mec-3(e1338) IV.*	CGC
CB1339	*mec-4(e1339) X.*	CGC
CB1611	*mec-4(e1611) X.*	CGC
TU253	*mec-4(u253) X.*	CGC
RB1708	*mec-7(ok2152) X.*	CGC
ZB2551	*mec-10(tm1552) X.*	CGC
CB3284	*mec-12(e1605) III.*	CGC
TU265	*mec-17(u265) IV.*	CGC
TU228	*mec-18(u228) X.*	CGC
CZ8920	*cebp-1(tm2807) X.*	CGC
TQ233	*trpa-1(ok999) IV.*	CGC
TU4295	*uIS152[mec-3p::RFP]*	Chalfie Lab
TV16911	*wyIs592[ser-2prom-3::myr-GFP + Podr-1::RFP] III.*	Shen Lab
MF288	*ser-2prom-3::DEG-3-N293I]*	Treinin Lab
JR4448	*uIS152[mec-3p::RFP];* *wyIs592[ser-2prom-3::myr-GFP + Podr-1::RFP] III.*	TU4295 X TV16911
JR4460	*mec-4(e1611) X; uIS152[mec-3p::RFP];* *wyIs592[ser-2prom-3::myr-GFP + Podr-1::RFP] III.*	JR4448 X CB1611

Appendix B

Chapter 3 Supplemental

B.1 Supplemental Table

Table B.1: List of Materials

Material	Comments
1% Sodium Dodecyl Sulfate solution	From stock 10% (w/v) SDS in DI water
15 mL Centrifuge tubes	
3 mm Metal tool (e.g, hex key)	
4% Agar in Normal Growth Medium (NGM) - 1L	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	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	
Clinical centrifuge	
Disposable razor blades	
Faraday cage	Can be constructed using cardboard and aluminum foil
Ink markers	Sharpie brand or others for marking on plastic

(Continued on next page)

Table B.2: List of Materials (continued)

Material	Comments
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 above)
Paraffin film	
Plastic cutting boards	
Pliers	
Rotating vertical mixer	With 22 x 15 mL tube bar
Serological pipettor	
Stereomicroscope	
Tally counter	
Thick NGM/agar plate media - 1 L	See 4% Agar in NGM recipe; replace 40 g Agar with 20 g Agar
Tweezers	

Appendix C

Future Directions: Video Tracking

C.1 Adult Gravitaxis in Pluoronic Acid

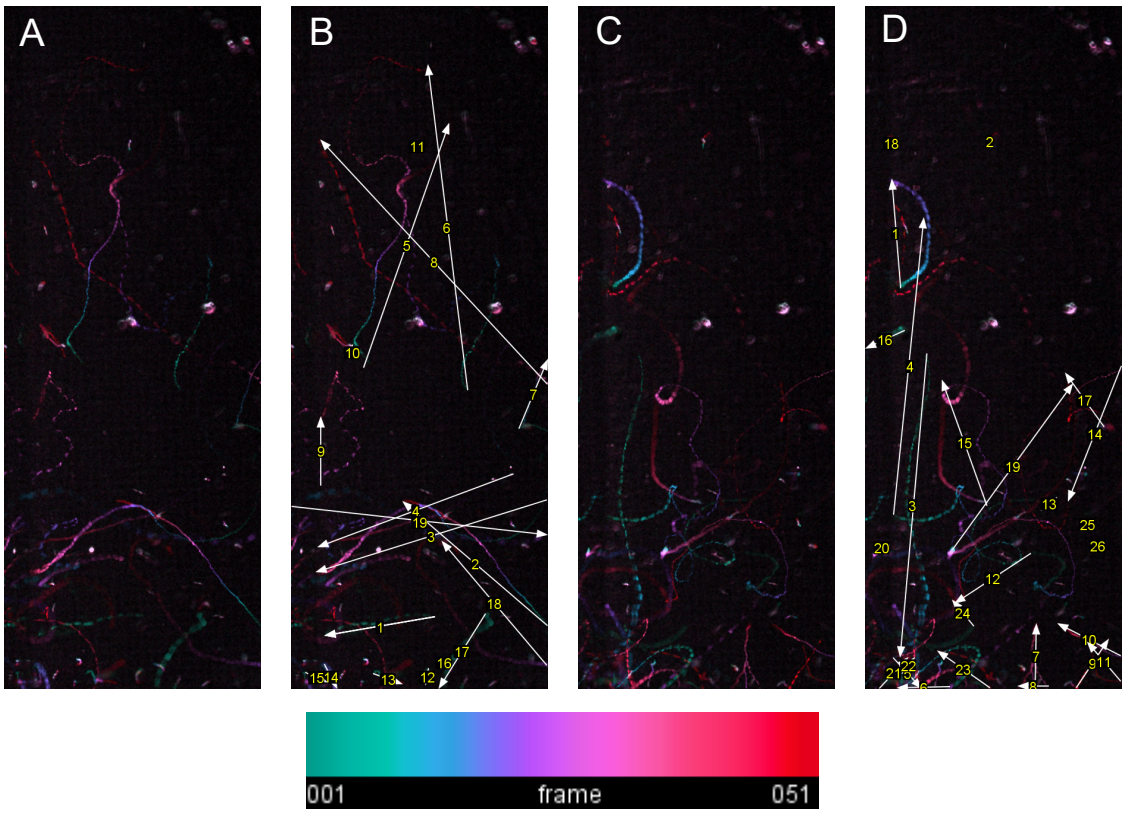

Figure C.1: Adult gravitaxis in pluoronic acid

N2 adult worms were embedded in 20% pluoronic acid in square vials. Recordings taken in a dark room with a Hamamatsu Orca-ER camera at 1 frame/10 seconds using 700 nm light. To enable processing, videos were first converted to 8-bit, then adjusted for brightness and contrast in FIJI. A Z-projection of minimum intensity was created and subtracted from the stack to remove background noise. (Continued on following page)

The video was then split into 100-frame segments and every other frame was removed to reduce the file size. The Temporal-Color Code plugin in FIJI was then applied to create time-lapse tracks of worm movement. **(B,D)** Vectors tracing overall worm trajectories were drawn manually in FIJI. **(A,B)** 16-32 minutes **(C,D)** 33-49 min.

C.2 Dauer Gravitaxis in Pluoronic Acid

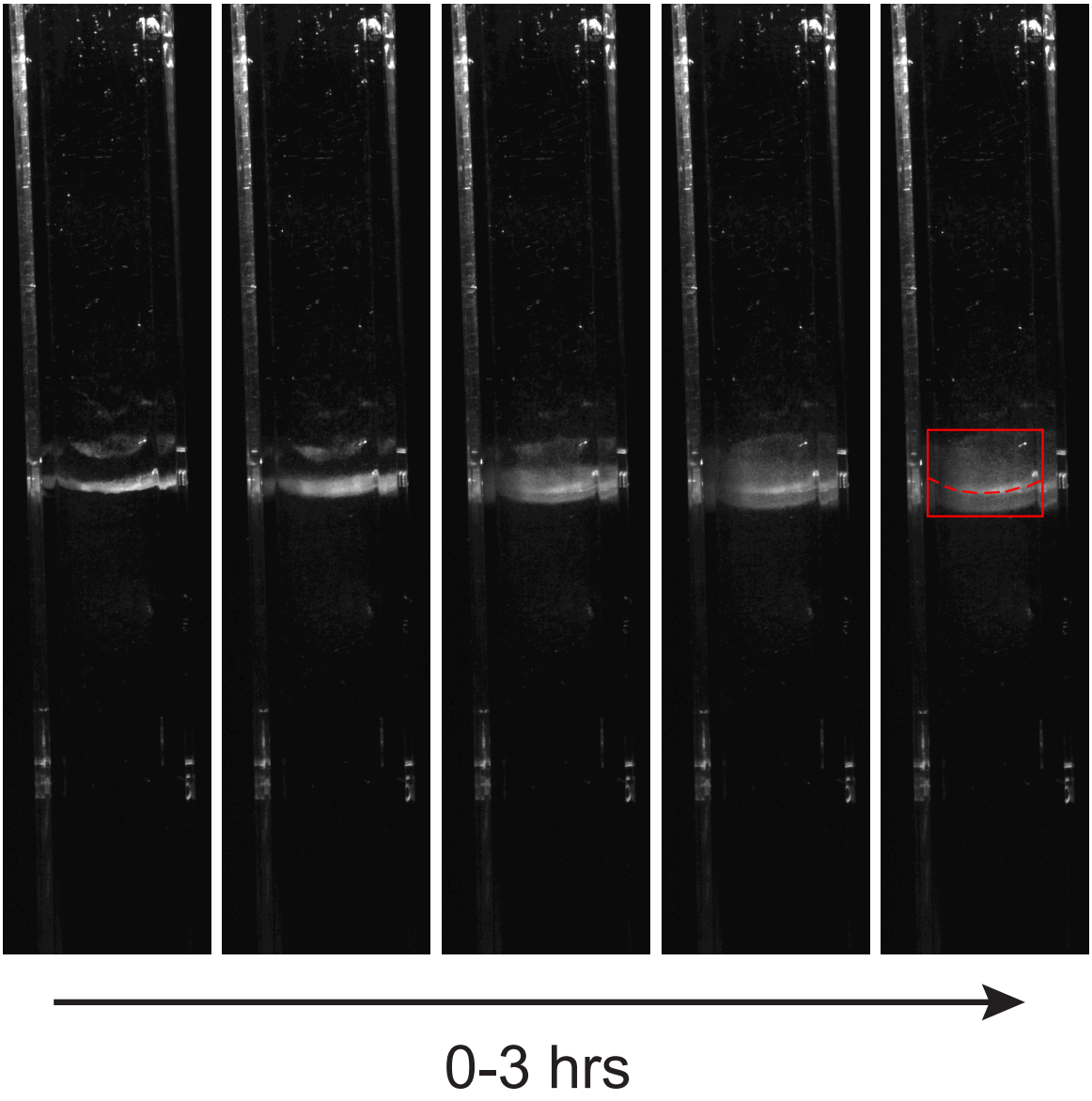

Figure C.2: Dauer gravitaxis in pluoronic acid

N2 dauer worms were embedded in 30% pluoronic acid and recorded as described above. Images shown here were taken at frame 1, 270, 540, 810, and 1080. Dispersal from the center is outlined in red at frame 1080 and the starting position is marked with a dashed line.

C.3 Adult Gravitaxis on Glass Slides (example 1)

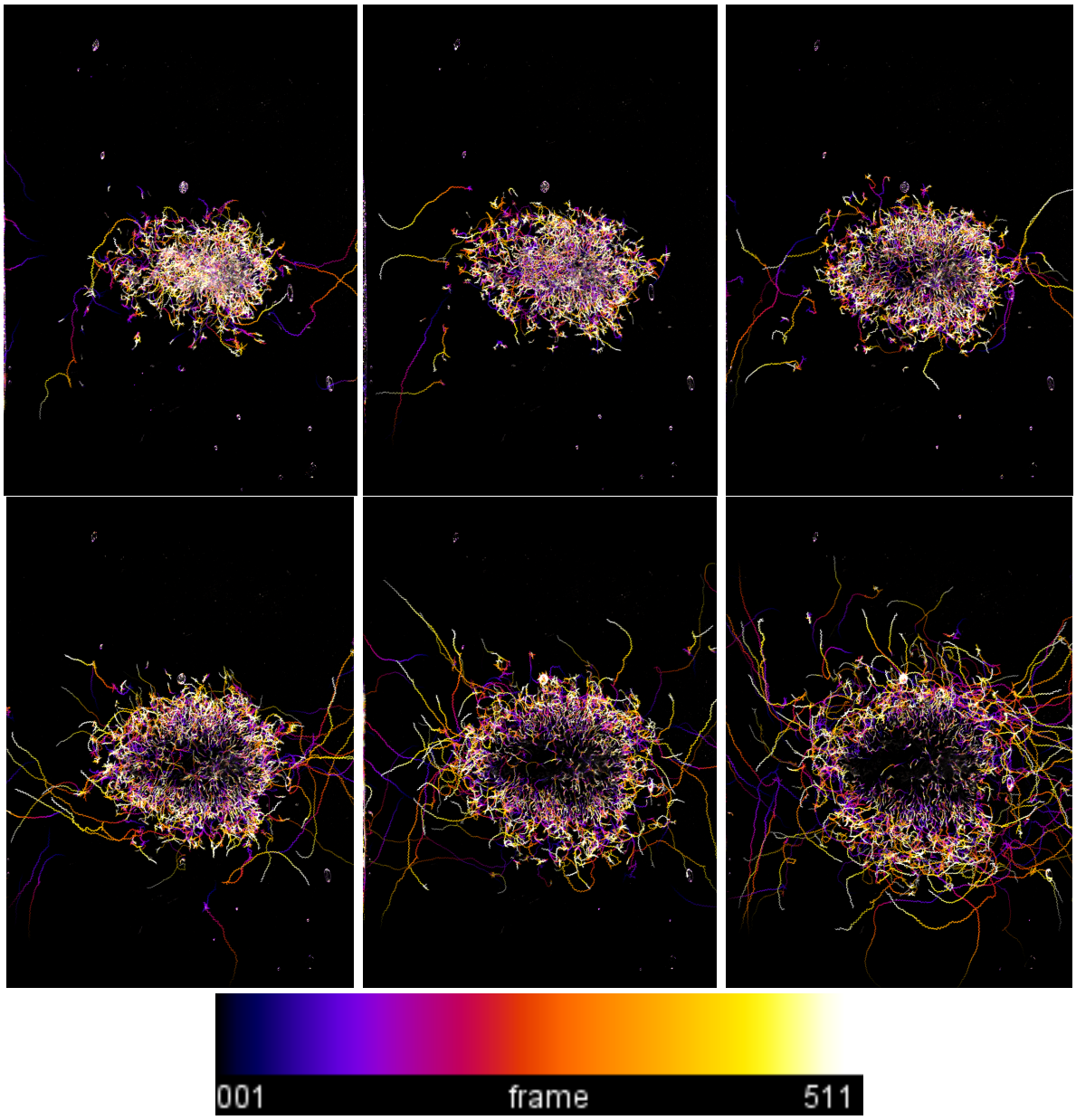

Figure C.3: Adult gravitaxis on glass slides (example 1)

(Caption on following page)

Worms expressing red fluorescent protein in muscle cells were mounted on a 1% agarose pad on a glass slide in M9 buffer and the slide was sealed using a coverslip and Valap. The slide was then held vertically using a ring stand and clamp, and a recording was taken at 1 frame/sec for 1 hour under 700 nm light. For processing, the resulting video was converted to 8-bit and adjusted in FIJI as described previously. The video was then split into 511 frame segments, and worm movements were traced using the Temporal-Color Code plugin in FIJI.

C.4 Adult Gravitaxis on Glass Slides (example 2)

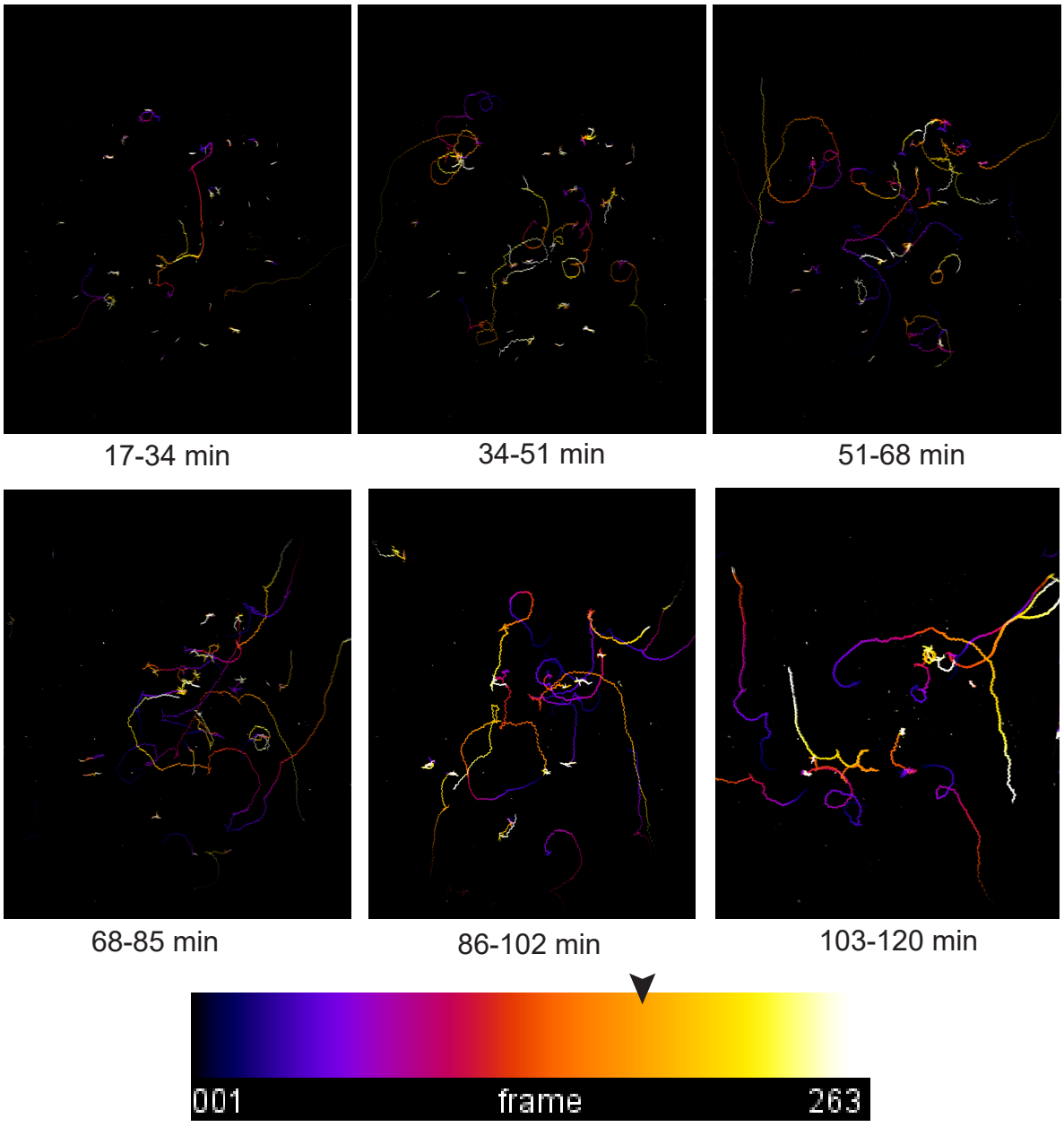

Figure C.4: Adult gravitaxis on glass slides (example 2)

(Caption on following page)

Worms were first rinsed in chemotaxis buffer before being mounted on a 2% agarose pad as described above. A recording was taken at 1 frame/sec for 2 hours. Overhead white light was turned on at frame 6855 (1.9 hours) to assess phototaxis behavior. The resulting video was processed in FIJI using Temporal-Color Code on 263-frame segments. The black arrowhead denotes approximate white-light ON point in the final segment.

C.5 Manual Worm Tracking

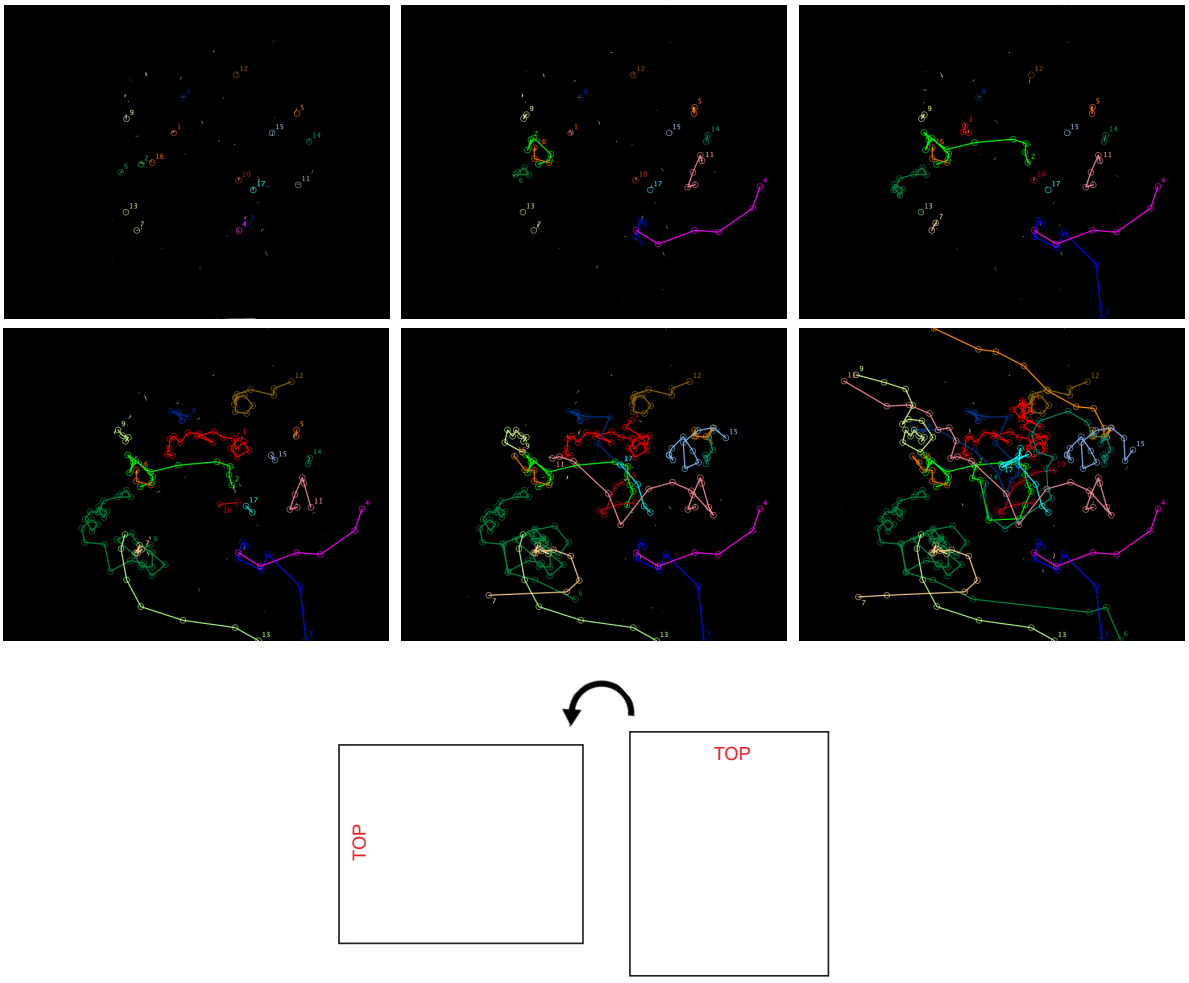

Figure C.5: Example of manual worm tracking using MTrackJ

The video from the previous figure was rotated 90 deg counter-clockwise for blinding. Worms were then tracked manually using MTrackJ (Meijering et al., 2012) in FIJI. A full be video can be found using the following DOI: 10.6084/m9.figshare.21568479.

Appendix D

Future Directions: Motility assays

D.1 Assessing vertical motility

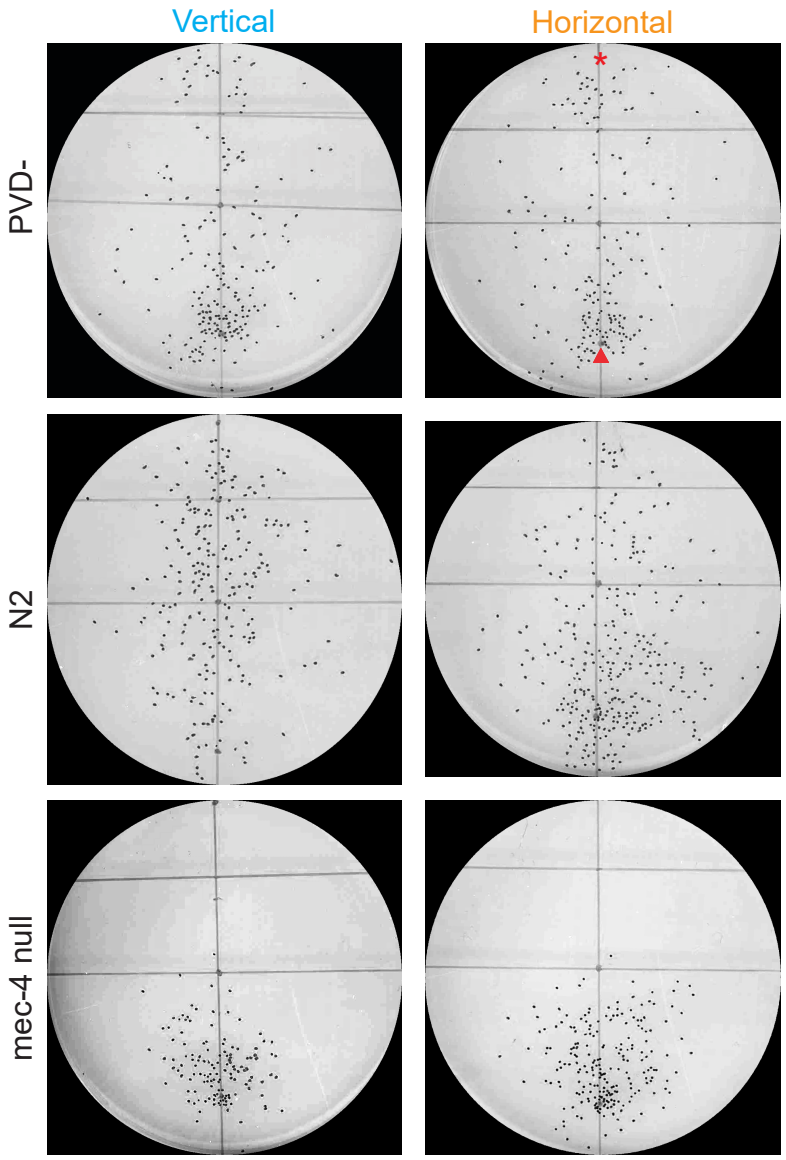

Figure D.1: A chemotaxis vs. gravitaxis assay to asses vertical motility

(Caption on following page)

Chemotaxis plates positioned vertically and horizontally were used to determine if strains showed motility deficits depending on orientation. A chemoattractant (red asterisk) is plated 0.5 cm from the top of a 15 cm plate and isolated dauers were plated 6.5 cm from this point (red arrowhead). The plates were then oriented either vertically (with the chemoattractant at the top) or horizontally in the dark for 30 minutes, then scored using a marker. Plates were then imaged using a desktop scanner and worm movement can be quantified using FIJI.

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