The Development of Rhythmic Behavior

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Abstract

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Locomotion is a fundamental component of behavior that is established and refined throughout development into adulthood. The model organism *Caenorhabditis elegans* is a powerful model to study the development of rhythmic locomotion due to its well-defined timecourse of development, powerful genetic tools, a fully resolved connectome of both sexes, and an array of naturalistic behaviors. *C. elegans* perform crawling, swimming and transitions between these gaits, readily adapting to their environmental conditions. Here, I comprehensively investigate the establishment and maintenance of rhythmic locomotor behaviors–swimming and crawling–in wild-type hermaphrodite *C. elegans* across various developmental stages. To do this, I assayed rhythmic locomotor behavior of young L1, late L1, L2, L3, L4, and adult hermaphrodite worms and applied principal component analysis (PCA) to transform both swimming and crawling postural data into a low-dimensional space. My research revealed that, similar to crawling, swimming behavior in *C. elegans* can be distilled into four principal components, referred to as 'eigenworms'. However, the characteristic eigenworm shapes associated with swimming differ from crawling. Further, I analyzed the progression of coordination of locomotor behavior over the course of development.

My findings reveal that young L1 animals can perform adult-like swimming and crawling behaviors but struggle to maintain an organized rhythm. By the time animals reach the late L1 stage, rhythmic locomotion stabilizes for both swimming and crawling. From these discoveries, I speculate that the neural circuits in young L1 stage *C. elegans* are immature, limiting their ability to fully integrate sensorimotor feedback in the rhythmic networks required for stable, smooth motor output. Remarkably, we observed juvenile *C. elegans* generate coordinated rhythmic locomotion during this period of rapid growth into adulthood, managing to withstand substantial restructuring of neuronal networks demonstrating a striking example of circuit degeneracy. To study the emergence of sexual dimorphism in locomotor development, I compared findings in hermaphrodite swimming development to their male counterparts. My analysis revealed differences in adult male swimming, reflected in a more complex higher-dimensional behavior. These differences coincide with the development of neural circuitry and sensory organs in the male-specific tail, potentially revealing a link between swimming and sexual maturation. Overall, these findings lay a groundwork to investigate the molecular mechanisms underlying the neural dynamics of locomotor behaviors across development and between sexes.

Thank you, Pidamaya ye, شكراً

“All that you touch, you change. All that you change, changes you. The only lasting truth is change.”
-Octavia Butler, *Parable of the Sower*

I believe it was my mom or aunt that told me we are lifelong learners; I am sure someone passed that knowledge onto them before they passed it on to me. In this way *We are All Related* or *Mitakye Oyasin*. I am forever grateful to the communities that have shaped how I relate to the world and taught me how to dream transformatively, learn collectively, laugh uncontrollably, and grow endlessly.

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This PhD is dedicated to my late cat, Habibti Xena, you were the sweetest.

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Chapter 1: Introduction

1.1 Development of locomotion

"A process cannot be understood by stopping it. Understanding must move with the flow of the process, must join it and flow with it."
-Frank Herbert, *Dune*

Instrumental to animal survival is the ability to enact locomotor activity as it serves as the basis for so many other animal behaviors. This is evident in the elegance of a hummingbird floating from flower to flower to sip nectar. We can also observe the power of an orca diving back into the water after coming up for air. As well as appreciate how devoted elephant mothers guide their calves for years to life. In fact, our capacity to move allows us to engage fully with life, navigate our surroundings, and meet our daily needs[1,2]. So how does this indispensable component of life develop into its necessary form?

In the nascent phases of life, albeit limited, animals are equipped to move so to survive their environments at the earliest stages of development or risk death. For instance, host nestlings that are unable to fly may meet their demise after being evicted from their nest by brood parasites such as cuckoos[3]. This is an example of a developmental limitation on locomotor behaviors in the wild. Rather, animals can rely on rudimentary movement at birth that will seemingly diversify into coordinated, independent movement across development[4–8]. The onset of coordinated rhythmic locomotion varies across animal species and within invertebrates and vertebrates alike[8,9]. This can be dependent on multiple factors such as ecological and developmental constraints. Some mammals, like ungulates, need to move shortly after birth to avoid predators and can flee with their mother and herd if chased[10,11], such that buffalos and other hoofed animals can stand and walk within hours after birth[12]. Whereas rodents take weeks to start reliable patterned walking once their limbs can support their weight[13–15], but there is evidence showing that the essential

neural circuits can support coordinated locomotor behavior earlier[15–17]. The fruit fly is a classic example of a species undergoing distinct development stages. Yet, *drosophila* larvae are capable of crawling and rolling behaviors[18] and after metamorphosis will develop distinct, refined locomotor behaviors such as walking and flying[19,20]. Shaped from evolutionary forces, species have optimized the pace of their development and have flexibility to adjust within their environment[21].

Development unmistakably allows for the progression of motor behaviors into a sophisticated repertoire. This is readily seen in human locomotor development, where babies can produce stepping movements advancing into independent walking with continuous development into adulthood[6,22–24]. Indeed, the development of the human brain is a life-long process, which begins a few weeks after fertilization and continues into early adulthood, decades later. Rhythmic locomotor behavior and its development is highly conserved across species and provides a lens into the underlying neural circuits supporting the rich diversity of motor behaviors we see within the animal kingdom[7,8,25,26].

The foundation of the human brain emerges during the prenatal period in which neurons are generated and travel to their predetermined site in the brain. Their axons will project towards their destinations to form synapses, with connections undergoing stabilization or extermination. This complex process in early development lays out a global approximation of the neural networks to elaborate further [27–30]. At the time of birth, the human brain has already reached a tremendous achievement, successful differentiation and migration of approximately 86 billion neurons[28,31]. Postnatally, there is continual diversification of neural networks with integration of connections, formation of synapses, and elaboration organization of dendrites and axons. Each neuron can form up to 10,000 synaptic connections, and in toddlers, the developing brain may contain up to 1000 trillion synaptic connections[27,32,33]. This is a critical period of development where neural

circuits undertake heightened plasticity, with the rapid and fluid-like rewiring and pruning of synapses, consolidating to hundreds of trillions of synapses[27]. This continual process integrates internal and external environments to generate a balanced functional output. This is integral to the brain's remarkable ability to support diverse and new functions across development. Neural networks malleability allows for developing circuits to optimize, support, and adapt to a growing body capable of assembling mature rhythmic locomotor outputs[25,29,30].

Mature neural networks develop from cell specification, neuron migration, axon guidance, dendritic growth, synaptic target selection, and synaptogenesis[34–41]. This process of maturation in animal species is dependent on conserved developmental processes that tightly coordinate cell signaling and dynamic gene expression. Extensive research in *Drosophil*a and C*. elegans* studies have characterized cell specification regulation with gene homologues in mammalian species such as Hedgehogs (hhs) and Wnts[42–48]. Underlying molecular mechanisms and genetic regulators across invertebrates and vertebrates specify neuronal cell fate from neural progenitor populations, guided by spatial and temporal cues[32,49–51].

A hallmark of nervous system development is the migration of neurons to their destined locations, where they can form precise circuits and connect with targets to carry out their specific functions effectively[52,53]. This is well recognized in the neuroscience field as "neural wiring" in which neurons can communicate with one another within an organized neural circuit. The complex patterning of neural circuitry is highly directed by specific molecular cues at specific times in development. In fact, spatiotemporal programs expressing canonical cues such as Netrins and Slits uphold dynamic control through attractive and repulsive gradients critical for accurate neuron migration and neural wiring development across species[54–57].

A prerequisite for synaptogenesis, an ultimate step in neural circuit development, is the dovetailing of dendritic growth, axonal target specification, termination of axon outgrowth, and

synaptic target selection. For instance, motor neuron (MN) axonal processes must travel long distances to form synapses with their respective neuromuscular junction (NMJ) targets[58]. This requires integration of axon guidance, growth and termination programs. However, what would happen if MN axons were unable to traverse to their correct target? The neural circuit would be compromised, unable to generate coordinated muscle contraction and relaxation. Fortunately, neurons can extend their axons using growth cones that sense diffusible cues and cell-adhesion molecules (CAMs). Once axons reach their specified targets, synapse formation is initiated through CAMs to localize pre- and post-synaptic formation through players such as SynCAM or Neuroligins and Ephrin signaling, respectively[36,37,59,60].

While the development process might appear complete at this point, however, there is further fine-tuning of neural circuits for optimal output through synaptic rewiring and elimination in a process called remodeling. Looking back, Ramón y Cajal's images from over 100 years ago revealed the remodeling of neural circuits. He identified this in avian Purkinje and mammalian granule cells that undergo a process he described as process resorption[61,62]. One well characterized example of remodeling is at the NMJ. Early in mammalian development, muscle fibers receive synaptic innervations from multiple MNs. Ultimately, one motor axon outcompetes the others through greater glutamatergic transmission, resulting in the withdrawal of the other motor axons at that NMJ synapse[63–65]. This develops a monosynaptic outcome, with one MN capable of innervating a muscle fiber to threshold. MN circuit remodeling is also seen in invertebrates such as *Drosophil*a and C*. elegans*[59,66,67]. *Drosophila* undergo extensive anatomical changes due the nature of metamorphosis with a complexity of MN remodeling events employing distinct developmental mechanisms at the embryo, larva, and adult stages[68–72]. However, once neural circuits have achieved functional stability in development, there are processes to maintain

homeostatic plasticity[73–75]. For instance, ubiquitination has been shown to play a critical role in stabilizing synaptic connectivity during development of invertebrates and vertebrates alike[36,76,77].

Indeed, the harmonized development of gene expression, cellular interactions, neuromuscular connections, whole body system and system-environment interactions are required for continuous sophisticated locomotor output. Although extensive knowledge exists regarding developmental mechanisms in tissue patterning, our comprehension of the progression of rhythmic locomotion and body posture is relatively limited. Nevertheless, there is a need to build a comprehensive understanding of the developmental programs that set up these complicated rhythmic locomotor circuits for stable output within an evolving landscape.

The simple explanation for how motor activity is generated is neurons send signals to activate muscles, causing them to contract and produce movement. However, when adults walk, this is in fact a complex rhythmic locomotor behavior that is assembled from many motor patterns such as stepping down with one foot to pushing off and the stances in between. Yet, our body seamlessly coordinates these actions.

How does a neuronal network generate rhythmic locomotion? This has been an intriguing question to generations of neuroscientists. Up until the early 20th century, the prevailing belief was that locomotor behavior was solely initiated from sensory reflex mechanism[78]. In 1911, Thomas Brown observed cats could still produce stepping behaviors when stimulated even though the cat was decerebrated and nerves deafferenated[79]. This removed the pretense that movement came merely from the periphery. A firm discovery for central pattern generator networks (CPGs) was made 60 years ago, by observing locust flight. Donald M. Wilson found that deafferented nerve cord in an immobilized locust could generate rhythmic motor outputs within a wind tunnel, resembling that of the locust in flight[80]. Indeed, it is thought that in most systems, CPG circuits are composed of pre-motor interneurons and in some cases, motor neurons themselves[81–83].

Through their pioneering work, we now know that rhythmic locomotion requires a complex network of regulation, which coordinates action of CPG networks and sensory feedback systems[7,84,85]. Since then, many studies have identified CPGs in various animal species, showing that CPGs are central oscillators as they can generate intrinsic rhythm without receiving extrinsic sensory feedback[86–92]. However, looking at the behavior, CPGs alone cannot produce smooth muscle movements. Instead, CPGs must integrate feedback signals from descending and sensory circuits to enable flexible, coordinated locomotion[84,90,93–97].

Ultimately, rhythmic locomotor behaviors such as walking are organized from modules of motor outputs[7,90,98–100]. Neuronal networks implement these motor outputs from basic activity of CPGs under the flexible influence of descending and afferent sensory influence[6,101]. However, there are varying theories about the development of these complex locomotor neural networks[95]. One option suggests that immature locomotor networks are supplanted by mature networks later in development. Another suggests that rhythmic CPG circuits are present at birth and secondary sensory and neuromodulatory inputs evolve to support flexible mature motor output. Regardless, there is consensus that organization of CPG networks are inherently complex, even without factoring in their developmental properties[102]. Additionally, reciprocal inhibition of neurons, also called a ‘half-center oscillator’ are key elements of CPG networks, and this is shown from extensive experiments done in lamprey, mollusk, and amphibian tadpoles[7,103–109]. Yet, identifying these neurons and their function as part of a larger network within spinal and nerve cords is far from simple. This has, unfortunately, made dissecting the developmental molecular and cellular identities of CPGs elusive across various animal models. There has been important progress in identifying genetic, neurotransmitter, and circuit components of CPGs. However, studies have been limited due to inaccessibility across development in certain animal models and/or technical difficulty. Continued exploration of rhythmic locomotion and their underlying neural mechanisms

throughout development are necessary and will provide important progress towards understanding how this essential behavior is generated and maintained.

1.2 Exploring developmental biology with *C. elegans*

"Bless the Maker and all His water. Bless the coming and going of Him, May His passing cleanse the world. May He keep the world for His people."
-Frank Herbert, *Dune*

Fundamental to development is body growth in which animals will change in build or shape[110]. Mammals go through a continuous and steady growth in body size into young adulthood. In some instances for invertebrates, such as the metamorphosis of fruit flies, animal geometry undergoes dramatic phase specific changes. For example, the addition of wings or legs abruptly transforms the shape and behaviors from a crawling caterpillar to a versatile flyer as a butterfly. Whereas the body shape alterations during the development of *C. elegans* are less extreme; it undergoes gradual changes, similar to the development of higher animals, including humans. To its advantage, *C. elegans* has a fixed and relatively simple nervous system of 302 neurons, along with a fully reconstructed connectome. This simplicity proves useful for dissecting neural function and behavior. While the human nervous system contains billions of neurons–comparable in number to the stars in our galaxy– highlighting its rich complexity but also presenting obstacles to studying neural activity and function in a living brain. Exploring simpler systems such as the *C. elegans* model can help us to harness the complexity of the development of rhythmic locomotor behavior.

The nematode *C. elegans* is a powerful organismal model that has played a significant role in our understanding of genetic regulation on development and disease, apoptosis, RNA interference, green fluorescent protein (GFP) tracking, and microRNAs[111]. *C. elegans* is one of the most comprehensively understood model organisms. This roundworm was first described as

a soil nematode after observing it in Algeria at the turn of the 20th century[112]. However, it would not come into prominence as a genetic system until the 1970s when Sidney Brenner recognized its simple anatomy, short life cycle, and ability to reproduce quickly in the lab[113,114]. Subsequently, Sulston and Horvitz would map the complete developmental cell lineage tree from fertilized egg to adult revealing how a complex organism develops from a single cell[115,116]. Encouraged by its small nervous system and with extraordinary prescience, Brenner proposed using serial-selection electron microscopy to uncover the circuitry of the nervous system. The full connectome of the *C. elegans* nervous system came to fruition in a landmark study in 1986[117], that would facilitate neuroscience research for decades. In 1998, another major milestone was achieved with the complete sequencing of the *C. elegans* genome, marking it as the first fully sequenced animal genome[118]. Many researchers would go on to study the *C. elegans* in great detail yielding profound impacts in many fields including neurobiology, developmental biology, and genetics.

The *C. elegans* model holds many advantages, in fact, approximately 60-80% of the *C. elegans* proteome has conserved human homologs[119–122] with many implicated in human related diseases[123]. Indeed, worms and humans share a large underlying biology of gene expression, membrane trafficking, cytoskeleton, extracellular matrix, cellular asymmetry, epithelial organization, neuronal connectivity, synapse function, cell signaling pathways, and apoptosis[124]. Thanks to the detailed reconstruction of its 302-neuron nervous system and mapping of neural and muscle connections, *C. elegans* stands out as a well-suited candidate for exploring the connections between neural networks, environment, and behavior[117,125–128]. The worm's simplicity and complete wiring diagram make it especially valuable for examining the neuromechanical basis of locomotion[129].

The adult hermaphrodite worm has 302 neurons, 95 body-wall muscles, 20 pharyngeal muscles, 16 sex muscles, 2 intestinal muscles and 2 anal muscles[127,130]. The 95 striated body-

wall muscles are located along the ventral and dorsal sides of the worm's body and are homologous to vertebrate skeletal muscles. Previous research states that 108 neurons innervate and are functionally responsible for ~91% of input to the body-wall muscles and fall into classes of MNs that organize along the length of the worm[127]. Starting with the head, there are 5 classes of head MNs– URA, RME, RMD, RIV and RMH. There are another 5 classes of head MNs that send their processes within sublateral tracts–SAB, SMD, SMB, SIB and SIA. Moving down the body are motor neurons in the ventral nerve cord (VNC) that innervate the dorsal and ventral body-wall muscles and are composed of 7 classes– AS, DA, VA, DB, VB, DD, VD. Innervating onto the dorsal muscles are the AS, DA, DB, and DD motor neurons. The VA, VB, and VD motor neurons form neuromuscular junctions with the ventral muscles. Not included in these classes are the VC and HSN motor neurons in the ventral nerve cord which primarily function to innervate the vulval muscles in hermaphrodites.

Table of motor neurons innervating body-wall muscles[131]

Class	neurons	location	neurotransmitter	born	locomotor function?
URA	URAD(L/R), URAV(L/R)	head	Acetylcholine		nictation[132]
RME	RMED, RME(L/R), RMEV	head	GABA		head bends, foraging[133]
RMD	RMDD(L/R), RMD(L/R), RMDV(L/R)	head	Acetylcholine		head withdrawal and foraging[134]drive dorsoventral undulations[135] coupling head-body movements[132]
RIV	RIV(L/R)	head	Acetylcholine		ventral bias of turns following a reversal[136]
RMH	RMH(L/R)	head	Acetylcholine	Late L1	exploratory head movements[132]
SAB	SABD, SABV, SABVR	head	Acetylcholine		proprioception[137]
SMD	SMDD(L/R), SMDV(L/R)	head	Acetylcholine		omega turns[136] drive dorsoventral undulations[135]

					coupling head-body movements[132]6/13/25 11:12:00 PM
SMB	SMBD(L/R), SMBV(L/R)	head	Acetylcholine		Amplitude of sinusoidal movement[136]coupling head-body movements
SIB	SIBD(L/R), SIBV(L/R)	head	Acetylcholine		coupling head-body movements[132]
SIA	SIAD(L/R), SIAV(L/R)	head	Acetylcholine		'flipping' behavior during sleep[138]
AS	AS(1-11)	VNC	Acetylcholine	Late L1	body bending propagation, innervate dorsal muscles[139]
DA	DA(1-9)	VNC	Acetylcholine		backward movement[140]
VA	VA(1-12)	VNC	Acetylcholine	Late L1	backward movement[140]
DB	DB(1-7)	VNC	Acetylcholine		forward movement[140]
VB	VB(1-11)	VNC	Acetylcholine	Late L1	forward movement[140]
DD	DD(1-6)	VNC	GABA		inhibits ventral & dorsal muscles at L1[141]
VD	VD(1-13)	VNC	GABA	Late L1	inhibits ventral muscles[142]
VC	VC(1-3,6)	VNC	Acetylcholine	Late L1	egg laying, co-active[142]

During forward locomotion, body bends undulate anterior-to-posterior along the length of the worm through contralateral dorsoventral muscle contractions. For instance, a ventral body bend is generated from the contraction of ventral muscles and relaxation of the dorsal muscles. The underlying subcircuit has cholinergic VB MNs excite ventral muscles and GABAergic DD MNs inhibit opposing dorsal muscles. Its complimentary circuit has cholinergic DB MNs excite dorsal muscles and GABAergic VD MNs inhibit ventral muscles[140,143]. Whereas the A-type MNs (VA and DA) are excitatory cholinergic neurons important for backward locomotion[144]. Body waves alternate along the length of the worm through proprioceptive coupling and stretch activation[143,145]. There are five premotor interneurons–AVA, AVB, AVD, AVE and PVC–that innervate the excitatory

A- and B-type MNs. From ablation experiments, AVB and PVC interneurons are associated with B-type MN forward locomotion. In contrast, AVA, AVD and AVE interneurons are important for A-type MN associated with backward locomotion[117,146,147].

Undoubtedly, various neural processes work together to generate smooth, robust, and coordinated rhythmic patterning for nematodes to move. However, identifying specific neurons involved in central pattern generator networks remains elusive. There are three key models that have been scrutinized in the field. First, it is possible there are CPGs in the head circuit that ignite propagating waves that are maintained through proprioception[148,149]. This model relies on premotor interneurons such as AVB and PVC to couple waves along the body. Although, one question that remains is if there are CPGs in the tail that can initiate backwards locomotion. A second model would be that CPGs are in the VNC and depend on motor neurons and their coordination via chemical synapses, gap junctions[137], or stretch receptors[139,150–153]. The third model proposes that the generation of oscillating waves is maintained entirely from a chain of reflexes relying on stretch receptor feedback, not requiring local, dedicated CPG neurons[143,154–156]. However, it is also likely these models are not mutually exclusive, and it has been proposed rhythm is generated in various parts of the body[144], working together to ensure the robustness that is required for locomotion. Further investigations are required to understand where the underlying rhythm is coming from utilizing the power of the connectome, electrophysiology, imaging, behavior, and computational tools. Less is understood about origins and development of these rhythmic processes. Our extensive knowledge on developmental processes in *C. elegans* offers a unique avenue to explore these questions.

Neurodevelopment mechanisms have been extensively explored using mutagenesis, cell ablation, and gene targeting techniques in the worm. To date, we have acquired a comprehensive understanding of tissue development in *C. elegans*[115,116,157,158]. In fact, researchers have mapped

the *C. elegans* nervous system as it grows across post-embryonic developmental phases through to its fully mature state using reconstructed connectomes from multiple individual animals[128]. This meticulous study revealed the stability and variability across cell connections illustrating the drastic changes of the nervous system during development. Indeed, a larval stage-1 (L1) animal is born with 222 neurons and approximately 1,300 chemical synapses and will go on to consolidate about 8000 synapses. Approximately 1,200 of the 8,000 synapses added during development are newly connected cells that lacked prior connections. Moreover, most connections stabilize and strengthen after hatching.

Intriguingly, there is a major remodeling event that completely rewires locomotor circuitry. This transformation is supported by developmental processes ensuring post-embryonic birth and neurite growth, embryonic motor neurons continual contact with post-embryonic neurons, extrasynaptic signaling within juvenile circuits, initiation of synapse formation, and rewiring once mature circuits are structurally intact[66,141]. Newly hatched worms have 22 VNC motor neurons that lack excitatory AS motor neurons and completely missing are the neurons that innervate ventral muscles in an adult locomotor circuit–VA, VB, and VD. Notably, the immature circuitry is lacking GABAergic VD motor neurons that inhibit ventral muscles for proper relaxation when the opposing dorsal side is contracted in the adult. Despite this, the juvenile locomotor circuit includes the DA and DB excitatory cholinergic motor neurons and relies on GABAergic DD motor neurons to inhibit both the ventral and dorsal muscles through direct and extrasynaptic feedback, respectively. Studies in *C. elegans* have shown that conserved mechanisms, such as transcription factors, are essential for embryonic and post-embryonic motor neuron development and axonal outgrowth post-mitotically[159–162]. After the addition of 53 motor neurons to the VNC by the L2 stage, the asymmetric juvenile neural circuit converts to a symmetric one that is maintained across the remainder of development. There are many instances in which the worm undergoes temporally

coordinated nervous system development guided by genetic and developmental programs[163]. Ultimately, the nematode has advanced our understanding of neurodevelopment and behavior driven by motor circuitry, but the molecular underpinnings of rhythmic locomotion remain unclear.

1.3 Sex differences in *C. elegans*

"I must not fear. Fear is the mind-killer."
-Frank Herbert, *Dune*

A key feature amongst almost all organisms is the existence of sex differences in behavioral development. Sexual dimorphism explains differences seen in phenotype or behavior between sexes of the same species typically playing a fundamental role for the reproduction and subsequent survival of an animal species. Sexual dimorphism can produce differences in appearance, size, and sexual organs as well as differences in behavioral traits related to mating, foraging, and locomotion. In the case of honeybees, *Apis mellifera*, sex is precisely determined for the advancement of their entire hive. The survival of a bee colony depends on three sex-dependent functional roles–a female queen, female workers, and male drones[164–166]. A beehive is made of tens of thousands of bees and 99% of those bees are female non-reproductive worker bees whose entire role is to maintain, tend and forage for the hive. Whereas the male drones' sole role is to breed with a Queen bee from another colony[167] and once that mating behavior is complete, their endophallus remains attached to the queen, ripping the males' abdomen leading to its swift death. Meanwhile the Queen bee holds the fate of her colony's reproduction, storing sperm from multiple males and determining whether or not to fertilize her eggs to produce female workers or male drones, respectively. Honeybees illustrate dramatic yet critical sex differences stemming from the underlying neurogenetic determinants[168,169] that originate from the behavior of a Queen choosing whether to fertilize an egg. However, sex differences impact on the brain has been a complicated question to study. In particular, the degree to which the nervous system varies

between sexes to generate sex specific behaviors has been a contentious and compelling question within the field of neurodevelopment over the past 50 years[170]. Examining the precisely structured nematode with known sexual dimorphisms makes it well-suited to exploring potential impacts of sex on development and behavior.

In their natural environment, *C. elegans* are predominantly self-fertile hermaphrodites characterized as female with approximately 0.2-0.5% of the population made up of males that arise from spontaneous non-disjunction[171]. However, a greater frequency of males can be generated through mating crosses done in the lab making *C. elegans* an amenable genetic model. Multiple advances have been made characterizing genetic, neural, synaptic, neural modulatory, physiological, anatomical and behavioral differences between sexes. Sexually mature *C. elegans* adults exhibit visibly distinct sex-specific morphologies: (1) larger, egg-laying hermaphrodites possessing a vulva, and (2) slimmer males possessing a hooked tail.

The sex of *C. elegans* is determined from the sex chromosomal X/A ratio[172]. Self-fertile hermaphrodites have two X chromosomes (XX) and develop from a high X chromosomal ratio whereas males only have one X chromosome (X0) from a low X/A ratio. This ratio cues the master regulator, *tra-1*, that confers sexual differentiation in development[173,174]. Male nematodes acquire 387 neurons to the hermaphrodites 302 neurons. Cell-specific lineages will undergo sex specification either through cell proliferation, apoptosis, or transdifferentiation[175,176]. This results in 294 neurons common to both hermaphrodites and males, with an additional 8 neurons specific to hermaphrodites and 93 neurons specific to males[115–117,127,177–179].

Dependent on TRA-1, the first indication of sexual dimorphism arises embryonically with the programmed cell death of the pair of HSN, egg-laying specific, serotonergic motor neurons in males[180]. Rather, hermaphrodite embryos maintain HSN neurons but lose four male specific CEM sensory neurons important for detecting hermaphrodite-derived pheromones[116]. Post-

embryonically, while in the early larval stages the hermaphrodite and male worms are visibly indistinguishable yet there are critical sex-specific cell proliferation events. Indeed, in hermaphrodites, postembryonic VC motor neurons important for egg-laying in the ventral nerve cord are born later in the L1 stage. However, in males, those cells are not born and instead proliferate into the CP and CA motor neurons later in the L3 stage[115,116]. The CP neurons are important for generating turning behavior during mating[181,182]. Finally, the majority of male-specific neurons are found in the tail and are born in the last L4 larval stage as the tail materializes. While there are 294 neurons that appear to be shared among both sexes, their neuronal sex-identity can alter through differences in synaptic connectivity[183] or modulation[184].

Sex specific wiring patterns can differentiate the nervous system between the sexes. Indeed, synaptic connections through establishment or pruning lead to sex specific function. For instance, the ADL chemosensory neuron pair is present in both sexes, but by the L4 stage in hermaphrodites, ADL neurons form synaptic connections with AVA neurons, potentially important for avoidance behavior[127,185]. Moreso, synaptic pruning is the predominant mechanism of rewiring sex-differences. Shared phasmid sensory PHB neurons are connected to both AVA and AVG neurons at the early larval stages in both sexes. Synaptic pruning by the L4 stage ensures PHB is not connected to AVG neurons in hermaphrodites. Conversely, PHB to AVA synapses undergo pruning in males resulting in PHB connecting with AVG neurons necessary for downstream connections with tail motor neurons[185].

Circuits can also differentiate through sex specific neural modulation presented as dimorphic neurotransmitter switches[186]. Indeed, AIM interneurons are serotonergic and glutamatergic in hermaphrodites expressing corresponding receptors. Whereas, up until the L3 stage, these neurons express glutamate receptors in males but switch to cholinergic in the L4 stage[187].

Intriguingly, more male-specific neurons co-transmit multiple neurotransmitters compared to shared-sex neurons in line with males presenting greater circuit complexity[186].

Further studies are needed to explore how potential CPG and sensory networks are implicated in sexual dimorphic behavior in *C. elegans*. Previous studies done in the weakly electric fish, *Apteronotus leptorhynchus*, hypothesize that frequency of oscillations are sexually dimorphic and mediated through glia regulation[188]. Ultimately, sexual identity impinges on multiple factors from neuronal fate, neurite branching, synaptic connectivity to neurotransmitter identity. These findings beg the question of how developmental programs in both sexes correspond to the establishment, maintenance and differences in behaviors such as locomotion.

1.4 Quantifying behavior to study development

"Survival is the ability to swim in strange water."
-Frank Herbert, *Dune*

Despite its simple neural anatomy, the small and meticulously observed nematode reveals a remarkable array of behaviors. In a lifetime, a hermaphrodite worm hatches and crawls out of its egg, senses its environment, searches for food, undergoes four stages of larval development, lays approximately 300 eggs and navigates its environment. Add male worms to the mix and behaviors become more complex. *C. elegans* exhibit a richness of behaviors including foraging[189,190], mating[191], escape[192], learning[193], egg-laying[194] and feeding[195]; central to all of these behaviors is locomotion[196]. Locomotion activities such as swimming and crawling are composed of coordinated motor movements that repeat with a characteristic frequency[197]. While these behaviors appear effortless, they require the intricate activation of body muscles, flexible gait transitions, continual maintenance of postures and acute sensation of one's environment. In their natural habitat, *C. elegans* navigate soil, foliage, compost and face various environmental challenges, including rain and other wilderness conditions[198].

Integral to a worm's survival is its ability to navigate its environment. Like humans, *C. elegans* can select locomotor strategies and transit smoothly between locomotor gaits when prompted. *C. elegans* rely on two major types of movement, crawling and swimming[140,199,200]. Specifically, *C. elegans* move by propagating sinuous waves consisting of alternating dorsal and ventral muscle contractions along their body[140,201,202]. On solid surfaces, *C. elegans* engage in a sinusoidal trajectory with low frequency and small amplitudes. Whereas, when in liquid, *C. elegans* swim with a characteristic C-shaped posture with greater frequency and larger amplitudes[199,203]. The exact neural mechanisms that orchestrate smooth transitions between different gaits remain unknown. Prior studies in *C. elegans* have shown that dopamine and serotonin have critical roles in the swimming-to-crawling and crawling-to-swimming transitions, suggesting that conserved mechanisms are used for gait transitions across animal species[204]. Yet, the debate continues over whether crawling and swimming gaits represent the output of distinct or shared neural circuits[199,203,205–208]. Previous research on *C. elegans* locomotion has primarily focused on important yet simple properties such as speed of the center of mass, body bending frequency, and posture amplitude[199,203,204,208]. While these features are valuable, they do not fully encompass the intricate spatiotemporal variability of *C. elegans* body movements during locomotion. Furthermore, the way coordinated locomotion is established throughout development remains an intriguing and unexplored landscape. *C. elegans* remain a compatible model to chart the course for how locomotor behavior precisely develops.

Recent advancements in computational techniques and pose estimations have opened new avenues for fully capturing and analyzing complex behaviors. In a seminal study, *C. elegans* crawling behavior, though complex, was shown to be low dimensional and could be represented by just four simple quantitative descriptors coined "eigenworms"[209,210]. Eigenworm shapes represent the common postures of the worms' shapes during locomotion. In the case of swimming

worms, these produce stereotypical C and S shapes that repeat each other in sequence to construct a full stroke.

One intuitive way to understand eigenpostures would be to analyze the postures of a person dancing the “Y.M.C.A.”. The main eigenpostures from a person doing this dance would be bodies shaped like a Y, M, C, and A. These four principal eigenpostures would repeat in the sequence– Y, M, C, and A. Additionally, these four eigenpostures would make up the majority of the variations of human postures during this dance. This example demonstrates what eigenpostures capture from complex behavior and what it means to say a behavior is low-dimensional.

Principal component analyses labels and evaluates the main components or in our case, postures (eigenworms), that are present within a dataset. There are a small number of principal shapes that make up the majority of shapes that constitute a behavior. Principal component analysis allows us to describe the full repertoire of shapes the animal forms during locomotor behavior from a linear combination of eigenworms. This approach represents a future direction for the field and provides a new means in which to describe locomotor behavior.

The establishment of the *C. elegans* connectome was a paradigm shift for the worm community 40 years ago[117,211,212]. There is no disputing that this discovery helped pave the way for neuroscientists to understand the link between the brain and behavior. Thereafter neuroscientists have and continue to put in profound effort to map the wiring diagram of multiple species from fruit flies to mice in hopes of discovering how the brain works[213–216]. Significant advancements in our knowledge of *C. elegans*[128] and other model nervous systems needs to be complemented with advancements in behavioral analysis in order to achieve a pluralistic understanding of brain function[217,218].

My findings are part of a recent body of work using advances in machine vision and data analysis to study animal behavior under naturalistic conditions[219,220]. My research will provide the

first quantitative descriptions of locomotor postures at early post-embryonic stages and throughout development in *C. elegans*[221]. Taken together, my results have built a behavioral framework necessary for future studies of the modulatory mechanisms essential for establishment, maintenance, and flexibility of rhythmic locomotion in the developing nervous system in both sexes.

Chapter 2: Dimensionality of locomotor behaviors in developing *C. elegans*[221]

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2.1 Abstract

Adult animals display robust locomotion, yet the timeline and mechanisms of how juvenile animals acquire coordinated movements and how these movements evolve during development are not well understood. Recent advances in quantitative behavioral analyses have paved the way for investigating complex natural behaviors like locomotion. In this study, we tracked the swimming and crawling behaviors of the nematode *Caenorhabditis elegans* from postembryonic development through to adulthood. Our principal component analyses revealed that adult *C. elegans* swimming is low dimensional, suggesting that a small number of distinct postures, or eigenworms, account for most of the variance in the body shapes that constitute swimming behavior. Additionally, we found that crawling behavior in adult *C. elegans* is similarly low dimensional, corroborating previous studies. Further, our analysis revealed that swimming and crawling are distinguishable within the eigenworm space. Remarkably, young L1 larvae are capable of producing the postural shapes for swimming and crawling seen in adults, despite

frequent instances of uncoordinated body movements. In contrast, late L1 larvae exhibit robust coordination of locomotion, while many neurons crucial for adult locomotion are still under development. In conclusion, this study establishes a comprehensive quantitative behavioral framework for understanding the neural basis of locomotor development, including distinct gaits such as swimming and crawling in *C. elegans*.

2.2 Author Summary

Locomotion is an indispensable component of life, which develops effortlessly through adolescence. Locomotor strategies such as running, swimming, and flying are composed of coordinated motor patterns that repeat at stereotypical frequencies. However, the mechanisms governing the establishment and progression of locomotor behaviors in juvenile animals through to adulthood are not well understood. Here we use the nematode *C. elegans* well-defined time course of development, fully reconstructed connectome, and repertoire of simple locomotor behaviors to study the development of locomotion. Using recent developments in quantitative behavioral methods, we observed and measured swimming and crawling behaviors in *C. elegans*. We found that swimming and crawling are characterized by rhythmic patterns of distinct sets of body postures, called “eigenworms.” Newly hatched worms are capable of producing adult-like locomotor postures, albeit in unsteady bouts which become more stereotypical across development into adulthood. These improvements in locomotor stability coincide with previously known neurodevelopmental milestones early on in post-embryotic development. Our findings contribute to a growing trend towards leveraging quantitative methods to capture the complexity of naturalistic behaviors and are a point of reference for studying developmental programs important for locomotor development.

2.3 Introduction

Locomotion, including behaviors such as running, flying, swimming, and crawling, is vital for animals to navigate their surroundings. These behaviors emerge early in life, enabling animals to interact with and adapt to their environments. For instance, juvenile worker bees can perform in-hive tasks, tadpoles can swim and feed, and precocial animals like horses can mobilize soon after birth[7,8,164]. However, juvenile animals are often incapable of performing the full repertoire of smooth rhythmic locomotion that they will eventually develop[4–8,164,222,223]. Organized locomotion is subject to developmental regulation, as animals undergo various anatomical changes, requiring a maturing nervous system to continuously accommodate a growing body[128,224–227]. For instance, early development during the prenatal period in humans lays down the primary structure of the brain but neural networks undergo further refinement to stabilize, adapt, and reshape their control of a growing body to produce structured rhythmic behavior[25,29,30].

In both vertebrates and invertebrates, the nervous system relies on a series of cell signaling pathways to generate the rich diversity of neurons and glia required to perform complex behaviors[43,51,228,53,55]. Progressing into adulthood, synapse formation and neuronal remodeling are pivotal steps in development where intricate neural circuits will forge necessary connections for a stable behavioral output[59,60,66,67]. Significant research efforts have led to an understanding of conserved developmental mechanisms that define neuron specification, migration, and wiring at various life stages. However, how developmental pathways set up neural circuits to produce rhythmic locomotion remains unknown.

Rhythmic locomotion is characterized by repetitive, structured postures that enable continuous coordinated movement[197]. This behavior depends on a number of factors, such as neural processing of sensory information and the assembly of central pattern generators (CPGs) for efficient motor control[94,229]. CPGs, named for their intrinsic ability to generate rhythmic activity

of motor neurons, are groups of neurons central to producing rhythmic locomotion[80,84,85,142]. Sensory input, although not essential to rhythmic patterning, plays a vital role in generating appropriate motor commands and ensuring accurate locomotion[94]. Gaining clarity on the maturation of animal locomotor behavior could significantly enhance our understanding of the development of complex neural circuits underlying rhythmic locomotion.

To understand how coordinated rhythmic locomotion matures during development, we took advantage of the nematode *Caenorhabditis elegans* due to its compact and well-defined nervous system, simple anatomy, and minimal yet reliable production of rhythmic locomotor behaviors. Indeed, *C. elegans* development is extensively characterized, including the known lineage of all its cells[113,116,230,231]. This detailed knowledge allows investigations to pinpoint neuron function related to behavioral phenotypes, as early as embryonic stages[219]. Prior research efforts have led to a systematic understanding of the worm's wiring diagrams across postembryonic development[128]. These extensive studies reveal that young L1 larvae have 222 neurons and approximately 1,500 synapses, while the adult *C. elegans* nervous system consolidates 302 neurons and around 8,000 synapses. These findings illustrate the drastic remodeling of the nervous system over the course of development[117,128,232,233].

C. elegans perform two fundamental locomotor behaviors in their natural environment–crawling and swimming[140,199,200]. These movements are facilitated by rhythmic body bends composed of alternating dorsal and ventral muscle contractions along the length of the body[142]. Prior studies on *C. elegans* locomotion have primarily investigated the body bend frequency in adult crawling and swimming[199,203,204,208]. Furthermore, the crawling behavior of adult animals has been quantitatively described at the postural level, revealing stereotypical sinuous movements[209,210]. Yet, the question of whether crawling and swimming share a common gait

remains contested[154,199,201,203–205,207,208]. Moreover, the progression of coordinated locomotion across development is yet to be determined.

Here, we have delineated the diverse locomotor postures exhibited by *C. elegans* during post-embryonic stages through to adulthood, with the aim of unraveling the developmental progression of rhythmic locomotion. Our findings quantitatively show that swimming and crawling behaviors are categorically distinct in frequency, postural, and principal component analyses. We have also assessed the coherence and stability of swimming and crawling postures at different developmental stages to identify the time point at which they mature. Interestingly, we find juvenile *C. elegans* can generate coordinated locomotor rhythms, despite their small body size and the ongoing restructuring of the nervous system. However, this ability only manifests later in the L1 larval stage, indicating a critical developmental event that triggers the acquisition of this capability.

2.4 Results

2.4.1 Adult *C. elegans* swimming is described by mixtures of unique shapes

C. elegans swim by propagating dorsal and ventral waves along the length of their bodies in a movement commonly referred to as thrashing. This behavior exemplifies rhythmic locomotion, as adult wildtype *C. elegans* can continuously bend their bodies for hour-long timescales before transitioning into resting states[234]. Previous studies have described the basic kinematics of these swimming movements by focusing on body bend frequency and curvature [199,204]. Building upon this, we used tracking methods to monitor day-1 adult *C. elegans*, thereby extracting their swimming postures. We divided the worm's body into 11 equal segments, allowing us to measure the angle between adjacent segments (Fig 1a). This approach resulted in a breakdown into 10 distinct angles, offering an in-depth view of the posture dynamics during swimming. Through

quantifying the worm's swimming curvature over a 10-second period, we confirmed the consistent rhythmic pattern produced by adult *C. elegans* (Fig 1b).

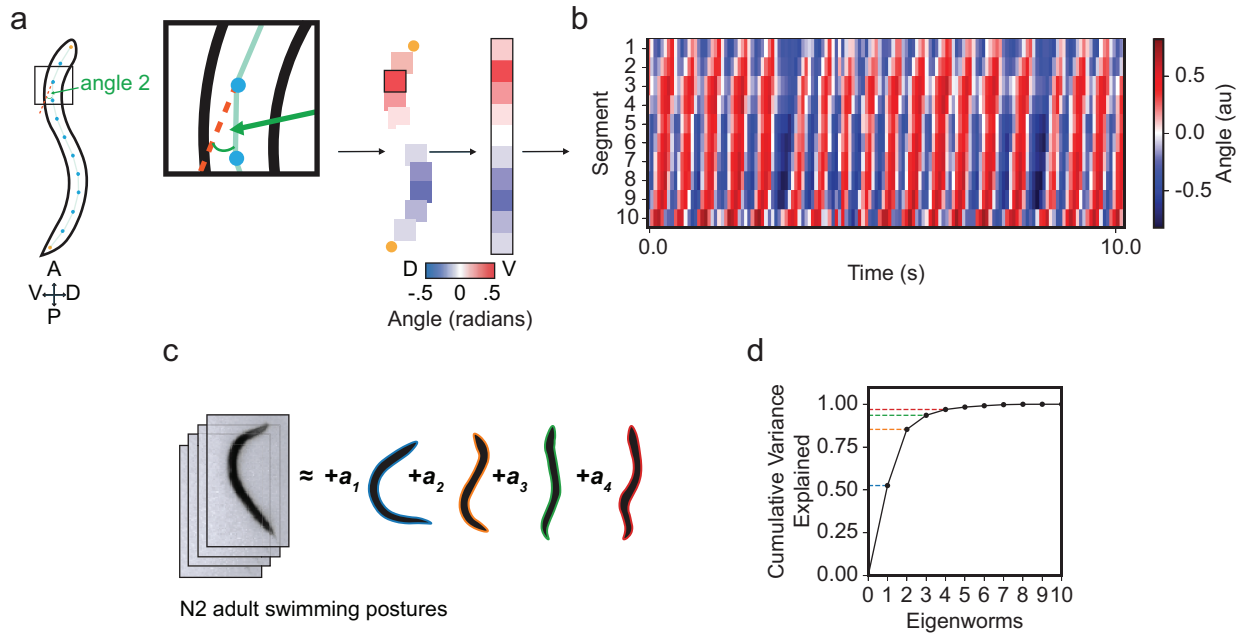

Fig 1. Adult *C. elegans* swimming is low dimensional. (a) Schematic showing the conversion of a worm body posture into curvature angles at a single time point. The shape of the worm is divided into 11 segments along the anterior-posterior axis and angles are calculated between adjacent segments. The 10 angles are converted to a colormap with blue-to-red indicating dorsal-to-ventral deflections. (b) Kymograph of body curvature of a day-1 adult worm during 10-seconds of swimming. Body segment number is plotted on the y-axis and time on the x-axis. (c) The variance in swimming postures of adult worms is largely captured by a linear combination of four eigenworms. The first four eigenworm shapes are reconstructed and are outlined in blue, orange, green, and red, respectively. (d) The fraction of the total variance explained in swimming postures as a function of the number of eigenworms used in reconstruction of adult *C. elegans* (n=43) swimming behavior. Dashed colored lines indicate the cumulative variance explained by the first four eigenworms at 53%, 85%, 94%, and 97%, respectively.

To comprehensively understand the repertoire of swimming postures, we performed principal component analysis on the curvature data of adult swimming worms and found that the continuous body bends can be succinctly described through this approach. This method has previously been used to describe the low dimensional structure of adult crawling [209]. Through this approach, the repertoire of swimming postures can be reconstructed from the linear combination of principal components, or "eigenworms" (Fig 1c and S1 Table). We found that a minimum of four eigenworms can explain 97% of the variance in adult *C. elegans* swimming postures. Furthermore, the first two eigenworms account for a significant portion of the variance in swimming postures, explaining 85% of the total variation (Fig 1d). These data indicate that swimming behavior in adult *C. elegans* has low dimensionality and that a few eigenworms can describe common postures the animals make when swimming.

2.4.2 Distinct eigenworms describe crawling and swimming in adult *C. elegans*

After identifying that swimming behavior in adult worms is low dimensional, we next investigated the structure of these eigenworms and their relationship to crawling. Eigenworms represent correlations between body segment angles, so their shape represents common postures of the worm during locomotion. We found that the first two swimming eigenworms reflect the stereotypical "C-shape" seen in swimming and a sinous, "S-shape", respectively (Fig 2a). Being able to describe the posture of a swimming worm using a linear combination of the eigenworms, we next investigated how the eigenworm amplitudes vary during swimming. A swimming adult worm propels itself through alternating dorsal and ventral muscle contractions generating rhythmic sinuous waves. We found that these oscillations are represented as cycles in the ring-like structure of the first two eigenworm amplitudes during swimming (Fig 2b and S1 File). This ring structure indicates that combinations of the first two eigenworm amplitudes represent the phase of the coordinated dorsal-ventral body oscillations of a swimming worm. The

presence of a ring-like structure in the distribution of the first two eigenworm amplitudes is qualitatively similar to that found in *C. elegans* crawling[209]. However, we discovered that the principal two eigenworms found in swimming locomotion are distinct from the principal two eigenworms discovered in crawling locomotion (Fig 2a and 2c). This demonstrates that crawling and swimming locomotion produce categorically different body postures. Our crawling analyses confirm previous findings that crawling locomotion produces two sinuous eigenworms which form a ring-like structure in the first two crawling eigenworm amplitudes (Fig 2d and S2 File)[209].

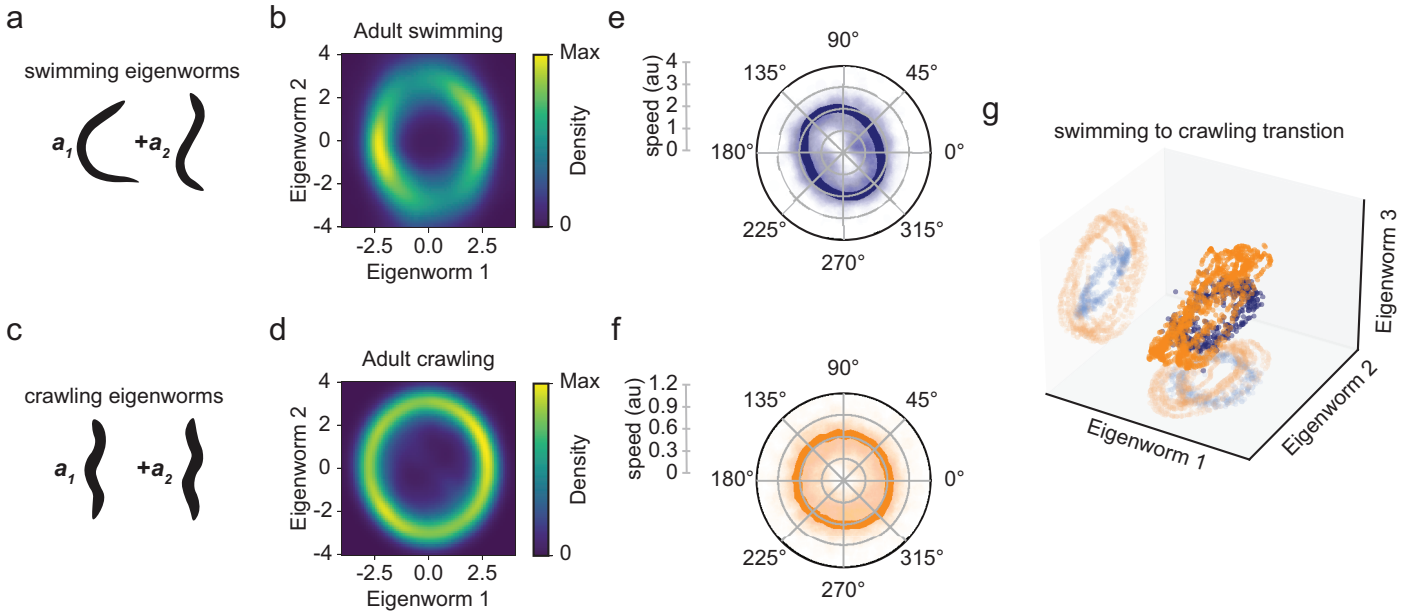

Fig 2. *C. elegans* adult swimming and crawling are distinct gaits. (a) Schematics of the first two principal eigenworms from adult swimming as shown in Fig 1c. (b) Swimming eigenworm amplitude distributions show a stereotyped ring structure which captures the coordination of swimming eigenworms one and two to produce swimming locomotion in adult *C. elegans* (n=43). (c) Schematics of the first two principal eigenworms of adult crawling behavior. (d) Crawling eigenworm amplitude distributions show a stereotyped ring structure of coordination between crawling eigenworms one and two in adult *C. elegans* (n=38). (e and f) Polar plots of eigenworm

amplitude (b and d) speeds as a function of phase in the ring. Speed data across all animals are plotted as scatter points, and the mean is overlayed. (e) In swimming, speed is bimodal and is slowest when in the “C” shape, or eigenworm one, whereas in crawling (f) the speed is constant along the ring. (g) 3D scatter plot of the first three eigenworm amplitudes from a representative worm tracked during a swimming-to-crawling transition. The ring structure of swimming (blue) is distinct from the ring structure associated with crawling (orange).

When comparing swimming and crawling rings, we also found that the swimming ring contains two peaks in density, whereas the crawling ring is essentially uniform in density (Fig 2b and 2d). We hypothesized that this peaked structure in swimming arises due to differences in the speed of oscillations in eigenworm space; if a worm moves slower during a phase of the swimming cycle, we will observe more frames in this posture and vice versa. To test this hypothesis, we calculated the speed of trajectories in eigenworm space and plotted the average speed as a function of phase in the ring. These speed analyses confirm our hypothesis that during swimming locomotion the worm moves slower when eigenworm one is largest in amplitude and faster when eigenworm two is largest in amplitude (Fig 2e and 2f). This indicates that swimming worms spend more time in the stereotypical C-shape and less in the sinuous shape. However, crawling locomotion shows a uniform speed throughout the entire cycle indicating that crawling behavior is a constant propagating wave.

To further demonstrate our finding that swimming and crawling locomotion produce disparate postures, we performed a gait transition assay, where we track an individual adult worm as it transitions from swimming to crawling. When we performed principal component analysis on these postural data, we found that the first four eigenworms are composed of the principal two eigenworms found in swimming and the principal two eigenworms found in crawling. When we plot the first three eigenworm amplitudes from a transition assay, we observed that swimming and

crawling locomotion trace out separate rings. This finding shows that swimming and crawling rings exist in separate regions of posture space (Fig 2g and S1 Figure). These analyses highlight that swimming and crawling locomotion use distinct postures.

2.4.3 Young L1 *C. elegans* generate but cannot reliably maintain swimming rhythmicity

C. elegans, like all animals, undergo dramatic size changes throughout their development. They grow from approximately 0.2 mm in length immediately after hatching to roughly 1 mm as day-1 adults (Fig 3h). Alongside these physical transformations, postembryonic development in *C. elegans* also involves substantial remodeling of the nervous system. To determine the impact of development on rhythmic swimming locomotion in *C. elegans*, we studied the low-dimensional structure of body postures during development. We carried out swimming assays at various developmental larval stages–young L1, late L1, L2, L3, and L4. Interestingly, we found that the first four eigenworms in swimming are highly similar across all developmental stages (Fig 3a). This finding suggests that, even at early post-embryonic developmental stages, worms are capable of executing swimming postures that closely resemble those of fully grown adults.

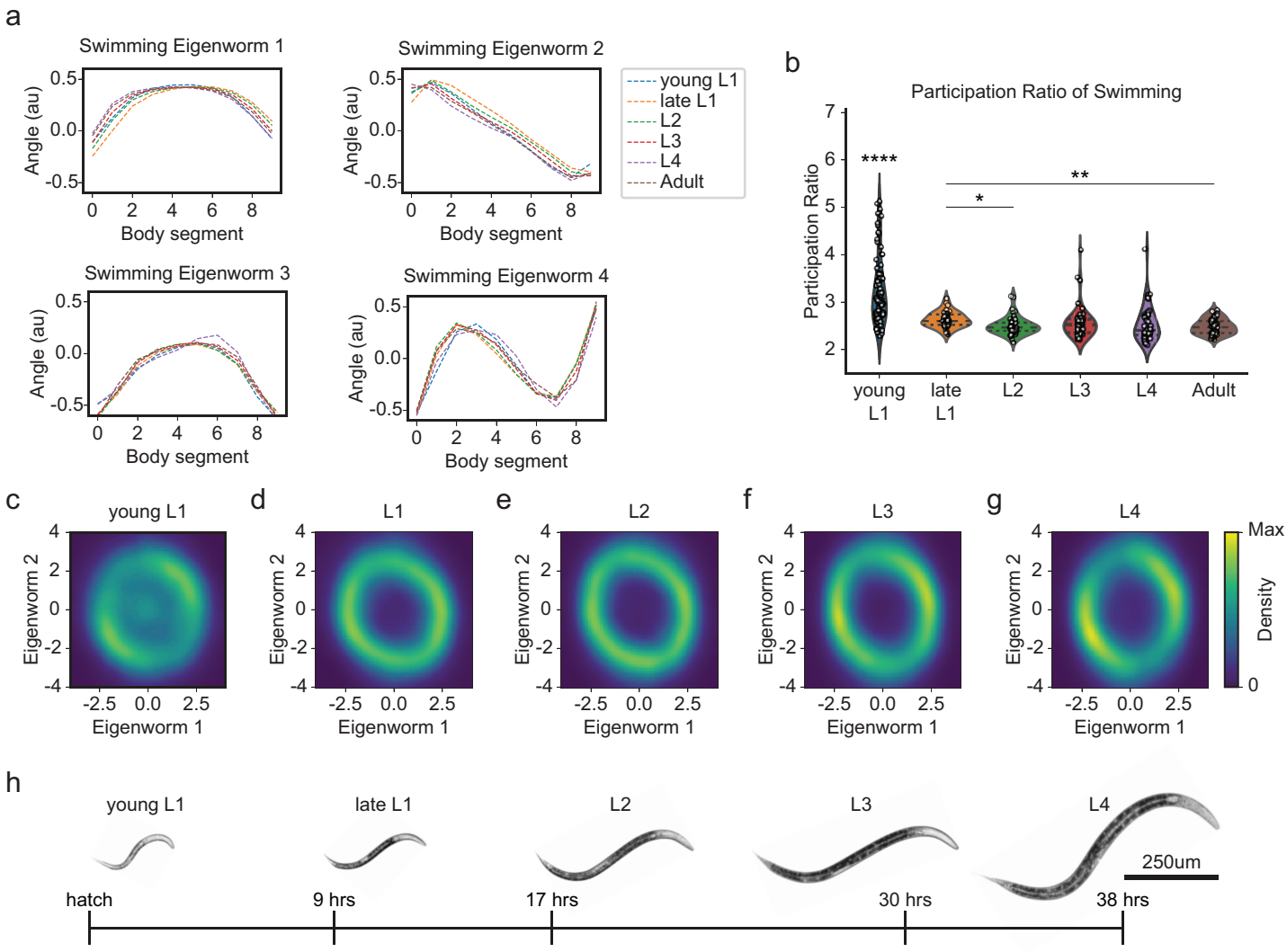

Fig 3. Rhythmic swimming is present at birth and matures throughout development. (a) Swimming eigenworms 1-4 across developmental stages: young L1 (blue), late L1 (orange), L2 (green), L3 (red), L4 (purple), adult (brown). (b) Participation ratios (PRs) representing the dimensionality for each swimming tracking session of young L1 (n=86), late L1 (n=40), L2 (n=47), L3 (n=48), L4 (n=39), and adult (n=43) *C. elegans*. Young L1 and adult *C. elegans* swimming PRs show a significant difference in means (p = 9.07e-08, t-test). (c-g) Swimming locomotion represented by eigenworm one and two amplitude distributions across developmental stages: young L1 (c), late L1 (d), L2 (e), L3 (f), and L4 (g) demonstrate coordination of these eigenworms

is present across development, however young L1 worms also produce uncoordinated postures not represented by the first two eigenworms. (h) The developmental stages, young L1, late L1, L2, L3, L4 of N2 *C. elegans* recorded in this study. Dashed lines in (b) represent means and interquartile range. Statistical significance in (b) was determined using Bonferroni adjusted alpha levels of 0.03 (0.05/15). Young L1 PRs showed ****p statistical significance compared to all other stages. Significance: *p<0.0033, **p<0.00067, ****p<0.0000067.

Furthermore, when we examined eigenworm amplitudes across development, we found that young L1 swimming has a stereotypical ring-like structure, indicating adult-like coordination. However, there is a notable increase in density at the center of the ring when compared to later developmental stages (Fig 3c), corresponding with irregular coordination of postures (S3 File). Thus, while all developmental stages are capable of producing adult-like swimming postures, young L1 animals are unable to sustain rhythmic locomotion for the duration of the recording. This result suggests that the ability to sustain rhythmic locomotion emerges at the late L1 stage and is retained throughout the developmental progression into adulthood (Fig 2b and 3d-g).

To quantify these differences, we calculated the dimensionality of the swimming behavior across different developmental stages using a metric known as the participation ratio (PR). The PR is a continuous number representing the dimensions required to describe approximately 80% to 90% of the variance in a dataset (see Methods and S1 Table)[235]. We found that young L1 swimming has a mean PR of 3.25, which is significantly higher than all other developmental stages with means of 2.63, 2.50, 2.57, 2.54, 2.49 for late L1, L2, L3, L4, adult respectively (Fig 3b). This indicates a higher dimensionality at this stage, corresponding to a less structured locomotor repertoire. In summary, the swimming behavior of young L1 animals, despite its higher-dimensional structure, exhibits the same principal four eigenworms observed in adults. This suggests that young L1 worms are capable of performing, but unable to sustain, adult-like

rhythmic swimming behaviors. Furthermore, postural swimming patterns become more organized during development, indicating potential developmental mechanisms associated with this stabilization of swimming behavior.

2.4.4 Young L1 *C. elegans* have immature crawling postures and locomotor patterns

In light of our finding that adult swimming and crawling constitute distinct gaits, it was crucial to examine if these locomotor behaviors follow similar developmental timelines. Thus, we performed behavioral crawling assays across various developmental stages and conducted principal component analysis on the posture data. Interestingly, we found that young L1 eigenworm shapes in crawling are different from all other developmental stages (Fig 4a). All deviations were observed in the posterior half of the worm, indicating that the posterior region lags behind the anterior half in forming mature crawling postures. While these body postures are distinct, the overall shape still resembles the sinuous postures necessary for the assembly of coordinated crawling behaviors in adult animals. By the late L1 stage, these eigenworm shapes had transitioned into the adult form, suggesting that an immature locomotor circuit in young L1 worms must undergo critical changes in order to support crawling poses across the full length of a worm.

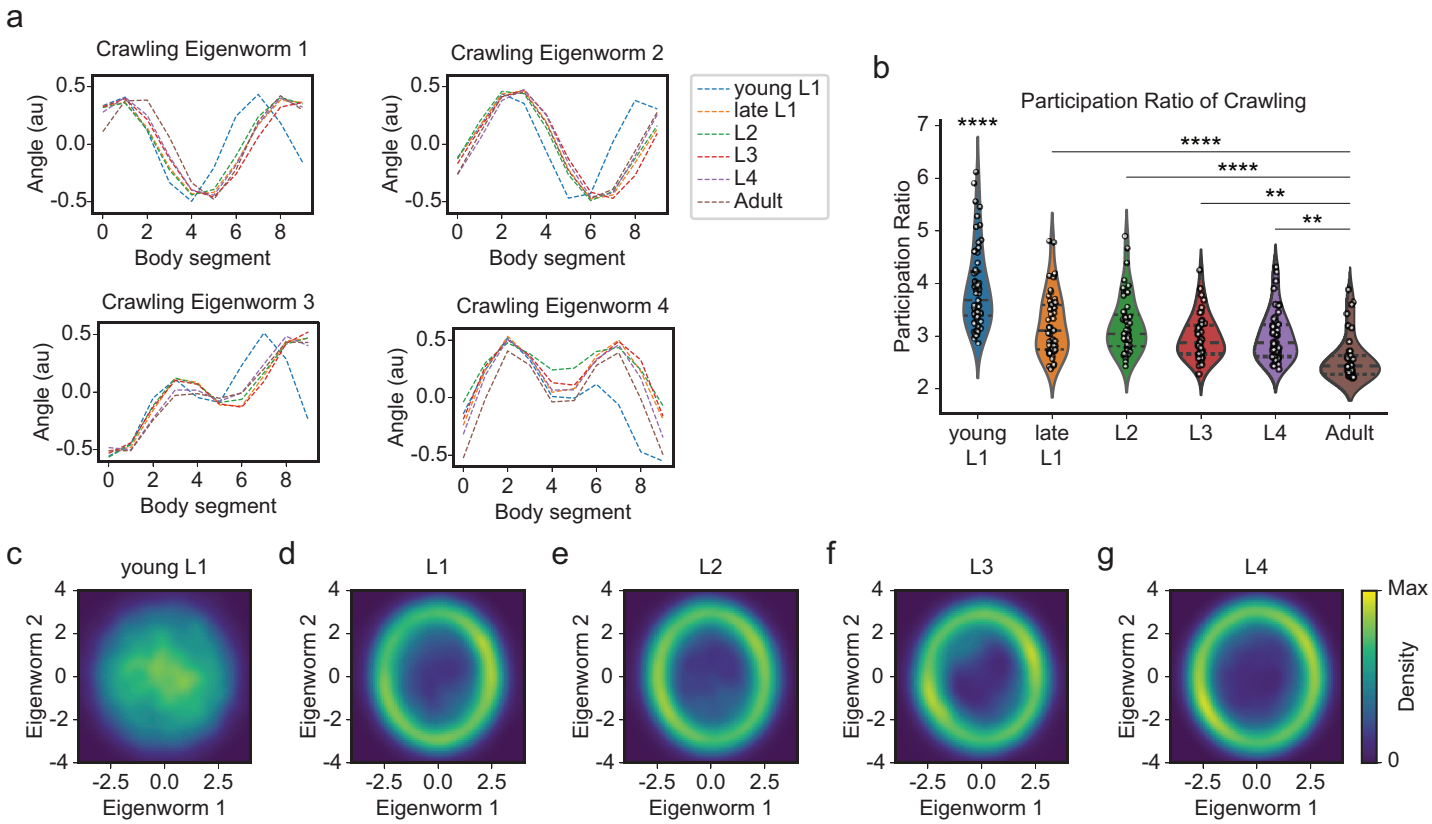

Fig 4. *C. elegans* crawling postures are disrupted at birth and stabilize by late larval 1 stage.

(a) Crawling eigenworms 1-4 are identical across developmental stages: late L1 (orange), L2 (green), L3 (red), L4 (purple), adult (brown), except for posterior deviations along the body at the young L1 stage (blue). (b) Participation ratios (PRs) for each crawling tracking session of young L1 (n=61), late L1 (n=51), L2 (n=51), L3 (n=43), L4 (n=47), and adult (n=38) *C. elegans*. Young L1 and adult *C. elegans* crawling PRs show a significant difference in means (p = 1.87e-14, Welch's t-test). (c-g) Crawling eigenworm one and two amplitude distributions across developmental stages: young L1 (c), late L1 (d), L2 (e), L3 (f), and L4 (g) demonstrate the coordination of these eigenworms is not present at the young L1 stage but develops by the late L1 stage. Dashed lines in (b) represent means and interquartile range. Statistical significance in (b) was determined using Bonferroni adjusted alpha levels of 0.03 (0.05/15). Young L1 PRs

showed ****p statistical significance compared to all other stages. Significance: **p<0.00067, ****p<0.0000067.

Next, we examined whether young L1 worms could assemble rhythmic crawling patterns like those observed in adults. This was accomplished by measuring the distribution of the first two eigenworm amplitudes. Surprisingly, we found that young L1 crawling worms exhibit a complete loss of ring structure in the eigenworm amplitude distributions (Fig 4c). These findings reveal that young L1 animals are unable to produce the synchronous body waves that are characteristic of mature crawling (S4 File). The distinct eigenworms and lack of organized crawling postures in young L1 animals suggest the existence of distinct locomotor circuits for crawling and swimming at this stage. However, similar to swimming, the coordination of crawling postures has become clearly defined by the late L1 stage and preserved throughout subsequent developmental stages (Fig 4d-g). These findings suggest that both crawling and swimming behavior mature during the L1 developmental period. To further understand the variability of postures in crawling data across development, we quantified the dimensionality of crawling behaviors using PR. We found that young L1 crawling has a mean PR of 3.90, significantly higher than all other developmental stages with means of 3.22, 3.21, 2.98, 2.99, 2.58 for late L1, L2, L3, L4, adult respectively (Fig 4b). Our results show that young L1 animals exhibit considerable uncoordinated crawling, but as development progresses, locomotor patterns become more stable by the late L1 stage, highlighting the significant maturation of locomotor circuits that occurs during this critical developmental period.

2.5 Discussion

C. elegans perform two fundamental rhythmic locomotor strategies dependent on their surroundings–swimming and crawling. Building on advances in pose-estimation and behavioral

analysis[220], here we demonstrate that *C. elegans* rely on mixtures of locomotor postures unique to swimming and crawling. These postures are present in swimming young L1 larvae and are maintained into adulthood. However, in crawling young L1 larvae, eigenworms are immature and develop by the late L1 stage. Although these basic postures are present in young L1 larvae, they struggle to sustain coordination. Development leads to a stabilized coordination of locomotor patterns, allowing for the production of robust rhythmic locomotion as early as the late L1 stage. We also extend our analysis to identify features of gait transitions by distinguishing swimming and crawling behaviors in eigenworm space. Together, our findings establish a quantitative basis for unraveling the link between locomotion and the significant remodeling of sensorimotor circuits in a developing nematode.

An innate nature of locomotor patterning

The *C. elegans* locomotor circuit is composed of sensory neurons and interneurons that orchestrate excitatory and inhibitory motor neuron output[117,140,142,145,146,201,206]. Recent research has shown that L1 animals, which have an incomplete motor circuit lacking a subset of motor neurons, depend on extrasynaptic signaling for the facilitation of “adult-like” bending waves[128,141]. In a related fashion, we found that young L1 animals have characteristics of swimming and crawling seen in adult animals. Thus, variable circuits can produce similar behaviors making this a salient example of circuit degeneracy, where diverse neural structures can yield similar functional outcomes. In *C. elegans* natural habitat of diverse decaying plant materials, they are required to navigate soil and liquid after hatching[112]. We speculate that young L1 *C. elegans* survival necessitates the ability to generate rudimentary locomotor postures from immature neural circuitry.

Our results show that crawling and swimming behaviors are highly stereotyped, starting from the late L1 larval stage and continuing into adulthood. This stable pattern of motor execution might be attributed, in part, to the stereotyped nature of neuromuscular connections. Prior connectomics studies across various species have shown a pattern–while other types of neuron connections exhibit high variability, motor neuron connectivity typically maintains a strict stereotypy during development. This dichotomy suggests that the stability and fidelity of motor execution may be reinforced by the lower variability inherent in motor neurons' connections, in contrast with the higher variability observed in the output connections from modulatory neurons[128]. However, despite this stability, the nematode locomotor circuit undergoes significant remodeling at early development. Specifically, within the ventral nerve cord motor neurons, a total of 53 neurons are added during the late L1 stage, resulting in substantial rewiring[115,117,140,236–238]. Despite this intensive period of neuronal network restructuring and rapid growth, *C. elegans* remarkably manage to generate coordinated rhythmic locomotion. Moreover, previous research has shown that *C. elegans* locomotion tolerates the inactivation of key neurons in the locomotor circuit[133,146,147,239–242]. Taken together, these observations underscore the resilience and robust nature of locomotion in *C. elegans*, even amidst significant developmental changes.

The robustness of locomotor coordination is acquired during development

It is generally thought that networks of neurons need to be appropriately assembled into functional circuits to produce smooth motor outputs. For instance, CPG neurons produce the basic rhythm of locomotion[80,84,85,243]. However, without sensory feedback, CPGs alone cannot generate smooth movements despite their intrinsic rhythmic activity[84,93,95,96]. We demonstrate that young L1 animals fail to maintain an organized crawling or swimming rhythm. We hypothesize that immature neural circuits in young L1 worms cannot fully integrate sensorimotor feedback into

rhythmic circuits required for the coordination observed in adult animals. In fact, during the late L1 developmental stage, the nervous system undergoes significant changes with the addition of various neurons and substantial rewiring, potentially compensating for early uncoordinated locomotion[238,115,244,236,117].

Notably, we observe young L1 animals exhibit deviations in crawling postures, specifically in the posterior half of the body and display considerable uncoordinated crawling. We hypothesize that swimming and crawling behaviors are potentially the output of discrete circuits at the young L1 larvae stage. *C. elegans* sense surrounding physical forces prompting distinct crawling and swimming locomotor strategies. It will be informative to investigate the role of neurons born later in development. For instance, post-embryonic sensory neurons, AVM and PVM, emerge during the L1 stage and are thought to play a role in sensing gentle touch in the anterior and posterior regions of the body important for forward and backward locomotion, respectively[115,192,245–247]. It is possible that crawling is limited at this stage due to the absence of sensory neurons that modulate CPGs. Additionally, stable locomotor behavior could also be subject to muscle development[248,249]. Our findings that there is a general decrease in dimensionality across development into adulthood, where locomotor behavior is most stereotypical, will be a useful benchmark for future studies of developmental mechanisms.

Swimming and crawling use different gaits

We find that swimming and crawling gaits are distinct in both their postures and rhythms. Prior studies in *C. elegans* have shown that dopamine and serotonin play critical roles in the swimming-to-crawling and crawling-to-swimming transitions, suggesting that conserved mechanisms are used for gait transitions across animal species[204]. Yet, the debate continues over whether crawling and swimming gaits represent the output of distinct or shared neural

circuits[199,203,205–208]. Our gait transition analysis in adult worms demonstrates that swimming and crawling behaviors are posturally distinct as they trace out separate rings in eigenworm space. Additionally, the analysis of angular speed shows variability in the rhythm underlying swimming and crawling, with a more uniform distribution in crawling. Our findings raise the question of whether animals can transition between gaits across development and if gait selection is primarily a response to physical sensation.

The power of quantitative behavioral analysis

Eigenworm analyses provide a quantitative and reproducible framework which enhances the reliability and comparability of behavioral data across studies. Indeed, when comparing our eigenworm analysis of adult crawling with previously published work[209], we confirmed many of their key findings: we found that four eigenworms describe over 95% of the variance, that these four eigenworms have the same shape, and that the first two eigenworms trace out a ring structure. A thorough quantitative understanding of behavior is an important first step before subsequent studies of neural circuit function. Furthermore, the precise temporal nature of these behavioral analyses are well suited for neural circuit investigations where variation at the sub-second timescale is important[250]. For example, recent research using eigendecomposition of embryonic postures, so called “eigen-embryos”, shows that motor behaviors mature embryonically and are modulated by specific neurodevelopmental processes such as RIS neuron activation[219]. Longer timescale measurements like undulation frequency, distance traveled, and speed, are often incapable of matching these fast neural timescales. It is from this point of behavioral understanding that studies of the neural mechanisms can be launched.

Our results have not only advanced the understanding of locomotor development and gait transitions in *C. elegans* but have also laid a foundational framework for future investigations into

the modulatory mechanisms that drive the establishment, maintenance, and flexibility of rhythmic locomotion during nervous system development.

2.6 Materials and Methods

Preparation of Worms

Wild type hermaphrodite *C. elegans* (N2 Bristol) worms from the CGC (Minneapolis, MN, USA) were used for all assays. The worms were maintained at 15°C on 60mm NGM agarose plates with Escherichia coli OP50 lawns as food. To obtain worms at later stages of development, synchronization procedures were carried out in which 20 gravid hermaphrodites were placed on seeded NGM plates. After one hour, all worms were subsequently removed, leaving only eggs on the plates which were immediately put into a 20° incubator. After 21 hours of incubator growth time for late L1s, 29 hours for L2s, 42 hours for L3s, 50 hours for L4s, and 65 hours for adult worms, the synchronized animals were subjected to assays. Young L1 recently hatched animals were collected by transferring 50 gravid hermaphrodites to a seeded plate 24 hours before experimental assay. On the day of the experiment, the plate with gravid hermaphrodites and laid eggs was washed with M9 buffer until all animals and bacteria were removed leaving only eggs on the plate. Over the course of an hour, plates were closely monitored for hatching and newly hatched young L1 animals were subjected to assays.

Assays

To obtain locomotion data across the stages of development, worms synchronized at each stage were subjected to swimming and crawling assays in which their movements were captured on video. All assays were conducted on NGM plates with no bacterial lawn. For crawling assays, worms were starved for one hour prior to the start of the assay. A platinum wire worm pick was

used with Halocarbon 700 oil to transfer 20 worms onto a 90mm NGM plate in the absence of OP50. We took 1-2 minute long video clips of each crawling worm. For swimming assays, each worm was transferred using a pick into a 5, 10, or 15µl drop of M9 buffer solution placed on the surface of the assay plate for L1-L2, L3, and L4-adult animals, respectively. M9 droplets were flattened with a worm pick in a circular motion beforehand to reduce glare. For each assay, one minute was allowed for the worm to acclimate to swimming conditions before 1-2 minutes of the worm's locomotion was tracked and analyzed. For gait transition assays we followed the crawling protocol for adult animals, however, we added a 1.5µl drop of M9 buffer to the plate in the worm's path following previously reported protocols[204].

Tracking

WormLab imaging stations were used in conjunction with WormLab software (both from MBF Bioscience) to capture videos of the worms and subsequently track the curvature data of each worm. Additionally, for young and late L1 larval assays, a macro lens (LAOWA 25mm F2.8 2.5-5x ULTRA MACRO) at 2.5x magnification was mounted instead of the default lens in order to capture the small worms at high resolution. All videos were taken at 1200x1600 resolution and 14 frames per second. To ensure worms remained in the field of view, assay plates were gently moved if necessary. For each worm, the angles between each of the eleven segments along the length of the animal (as defined by the WormLab software) were used to define the worm's curvature for each video frame. We segmented the animal into 5, 9, 11, 17, and 33 segments and found no significant differences in eigenworm shape analyses with more than 11 segments on a small held-out test data set. Subsequently, we proceeded with 11 segments (10 subsequent segment angles) for the remainder of the study.

Data

All worm segment curvature data is publicly available at https://doi.org/10.5061/dryad.stqjq2c8p [251]. TXT files are available for every worm assayed and tracked in these studies. Files are organized into subfolders by locomotion gait and developmental age. Each TXT file has 11 columns, the first is for time stamps, and the remaining for the 10 segment angles (radians).

Eigenworm Analysis

We denote the worms posture as θ(s) where s denotes the segment number. We perform an eigen-decomposition by first constructing the covariance matrix of the postures as:

$$C(s, s') = \langle (\theta(s) - \langle\theta\rangle)(\theta(s') - \langle\theta\rangle) \rangle$$

Eigenworms $\mu_i(s)$ and eigenvalues λ_i are defined by the eigendecomposition:

$$\sum_{s'} C(s, s')\mu_i(s') = \lambda_i \mu_i(s)$$

The cumulative variance explained is defined by:

$$\sigma_k^2 = \sum_{i=1}^{k} \frac{\lambda_i}{\sigma^2}$$

For all eigenworm amplitude figures, we define the eigenworms from the covariance matrix of posture angles across all developmental stages for that gait type. This choice is justified as the covariance matrix calculated from each separate developmental stage has nearly identical eigenvectors (Figure 3a and 4a), meaning there is no loss of descriptive power by combining stages into a single covariance matrix. This combination allows for comparisons across all developmental stages from the same perspective.

Kernal Density Estimate

We visualize the eigenworm amplitude distributions through a kernel density estimate of the eigenworm amplitudes. A kernel density estimate is a smoothed histogram; data are binned in two dimensions and then smoothed using a kernel. We used a gaussian kernel with a bandwidth determined by Scott's rule, implemented by the Python function *scipy.stats.gaussian_kde.*

Speed Analysis

We calculate speed at each frame by calculating the Euclidean distance between subsequent points in eigenworm space and multiplying them by the sampling frequency. We infer the phase in each frame by taking the Hilbert transform of the first eigenworm amplitude.

Participation Ratio

We utilize a continuous measure of dimensionality derived from the eigenvalues of the posture covariance matrix called the participation ratio (PR). The PR can be thought of as the dimensions required to capture approximately 80% to 90% of the variance of the data [235]. The participation ratio is defined as:

$$PR = \frac{(\sum_i \lambda_i)^2}{\sum_i \lambda_i^2}$$

In the simple case of three-dimensional (N=3) data, if the eigenvalues are 1, 0, 0, the PR is 1. If that same data were evenly distributed with eigenvalues of ⅓, ⅓, ⅓, it would have a PR of 3. Most data will contain some correlational structure that will place the PR somewhere in between the values of 1 and N, with N being the number of features in the data.

We calculated the participation ratio of each recording individually and collected the distribution of participation ratios for each age group. We performed a standard independent two sample t-test assuming equal sample variance using the scipy ttest_ind function in Python. We

corrected alpha values for multiple comparisons using Bonferroni adjustment yielding a significant p-value of 0.05/15 or 0.0033.

Code

All analyses of worm segment data were performed using custom Python scripts relying primarily on the *matplotlib, numpy, scipy* libraries. Code to reproduce figures as well as instructive example notebooks are hosted at https://github.com/ssterrett/wopodyn.

2.6 Acknowledgements

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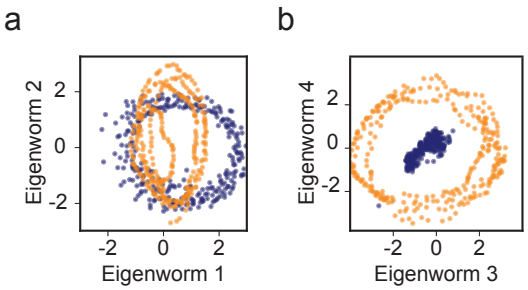
2.7 Supporting Information



S1 Figure. Adult *C. elegans* swimming and crawling are distinct in eigenworm space. (a and b) Scatter plots of swimming and crawling data from a swimming to crawling transitions assay in the plane of (a) eigenworm 1 and eigenworm 2 and (b) eigenworm 3 and eigenworm 4.

S1 File. Adult *C. elegans* rhythmic swimming video. Representative 10 second video of adult *C. elegans* swimming aligned with kymograph and eigenworm amplitude probability density estimates.

S2 File. Adult *C. elegans* rhythmic crawling video. Representative 10 second video of adult *C. elegans* crawling aligned with kymograph and eigenworm amplitude probability density estimates.

S3 File. Young L1 *C. elegans* disrupted swimming video. Representative 10 second video of young L1 *C. elegans* swimming aligned with kymograph and eigenworm amplitude probability density estimates.

S4 File. Young L1 *C. elegans* disrupted crawling video. Representative 10 second video of young L1 *C. elegans* crawling aligned with kymograph and eigenworm amplitude probability density estimates.

S1 Table. Table of definitions.

Table of Definitions	
Kymograph	A graphical representation of postures (y-axis) over time (x-axis), where the angle between segments is represented in a blue-white-red color scheme. Rhythmic locomotion is seen as repeating striped patterns, whereas unstructured flailing lacks stripes.
Eigenworm	Mathematically, these are the eigenvectors of the postural covariance matrix. Their shape represents common postures of the worm during locomotion.
Eigenworm amplitudes	The scalar associated with each eigenworm when reconstructing the original posture.
Eigenvalue	Describes the amount of variance of the original data that is captured by an eigenvector
Dimensionality	The effective number of degrees-of-freedom present in a data set. The lower bound is 1, the upper bound is the number of recorded features (N), with most data containing correlations that reduce the dimensionality below N. The participation ratio (PR) is a continuous estimate of dimensionality derived from the eigenvalues that estimates how many dimensions are required to describe approximately 80% to 90% of the

	variance. [235]
Cumulative Variance Explained	A cumulative sum of the eigenvalues, typically expressed as a ratio or percentage, describing how much variance in the original data is captured by the eigenvectors.
Kernel Density Estimate	An estimate of the probability distribution based on kernel smoothing the histogram of a finite sample of data.
Naturalistic	"behaviors that are representative of actions generated during real-world tasks, like exploring new environments, obtaining food, finding shelter, and identifying mates; naturalistic behaviors … are also largely self-motivated and expressed freely without physical restraint." [220]

Chapter 3: The development of sexually dimorphic swimming behaviors

3.1 Abstract

Sexual dimorphism shapes neural and behavioral adaptations in animals, yet the mechanisms driving these differences in locomotion remain less understood. *Caenorhabditis elegans* serves as a powerful model for investigating sexually dimorphic locomotor behavior, as it is the only organism with fully mapped nervous systems for both sexes and their previously described known differences in rhythmic behavior. In this study, we utilized advances quantitative frameworks to analyze swimming behaviors in *C. elegans* across all larval stages and adulthood in hermaphrodite and male animals. From swimming frequency, curling, and postural analysis, we illustrate the emergence of sex-specific differences in swimming across development. Our dimensionality analyses reveal that while hermaphrodite swimming remains low-dimensional throughout larval L2, L3, L4 development into adulthood. In contrast, adult males swimming becomes more complex, reflecting higher variability in adult motor outputs likely coinciding with the development of more complex neural circuitry. By uncovering the developmental timeline of swimming behaviors, we provide a foundation for understanding potential neuromuscular coordination underlying sexually dimorphic motor behavior in *C. elegans*.

3.2 Introduction

Differences in sex among species is a universal element of animal biology. These features have shaped animal behavior in countless ways throughout evolutionary history. In this way, the variations in nervous system structures and functions have long played a critical role in supporting

animal survival and adaptation since time immemorial. An instrumental feature of behavior that is shared across animal species is locomotion, yet the mechanisms driving sex differences in this shared trait remain less understood. The nematode *C. elegans* provides a useful tool for understanding the sexual dimorphism in locomotor behavior.

C. elegans are the only animal with a fully mapped connectome for both sexes, offering a unique perspective on sexually dimorphic wiring[115,127,177,178]. The male *C. elegans* nervous system consists of 387 neurons compared to the hermaphrodite's 302, making the male neural anatomy approximately 30% larger. Of these neurons, 294 are characterized as sex-shared based on their anatomical, molecular, and lineage properties. The hermaphrodite has eight unique neurons–HSN and VC motor neurons–important for vulva control. In contrast, the male features 93 male-specific neurons, 89 of which develop post-embryonically[252]. At present, the precise timing of these male-specific neurons' differentiation in larval development is not fully understood. Among the male-specific post-embryonic neurons, 19 belong to the CA and CP motor neuron classes, which differentiate along the full length of the ventral nerve cord during the late L3 stage[115,177]. While more research is required to fully understand their function, studies suggest they play an important role in turning and sperm transfer behavior[182].

The majority of male-specific neurons are born in the tail. These 68 neurons are highly interconnected and arise at variable, yet less understood, times during larval development. However, the complex neural circuitry that emerges in sexually mature male *C. elegans* follows a "just-in-time" differentiation process[252]. This research suggests that male-specific neurons don't fully realize their sex-specific functional properties until the mid-to-late L4 stage in which non-neuronal mating structures such as the "fan-shaped" tail and other required end organs are completely developed. This illustrates the complex coordination of neural wiring and molecular factors that enable proper functional control of a sexually dimorphic process, that emerges with

the formation of male-specific organs during a specific window in the L4 stage. Overall, the hermaphrodite and male nervous systems of *C. elegans* share similar locomotor circuitry and muscles but also contain rich differences, many emerging at the L4 stage.

In the context of the sexes in *C. elegans*, motor output varies depending on the animal's environment[196]. For example, male worms are driven by mate-seeking behaviors, prioritizing sexual appetite over food[253,254]. This is driven by multiple dimorphic factors from male-specific neurons in the tail recognizing contact with hermaphrodites to physiological signaling. In contrast, hermaphrodite motor behavior is strongly regulated by their access to food[255–257]. While sex-specific behaviors become evident in sexually mature adults, the developmental evolution of locomotor behavior and the timing of sexual dimorphism remain less understood. A prior study on *C. elegans* locomotor development examined crawling behavior in the L3, L4 and adult stages, revealing that male worms crawl faster, a trait that primarily emerges in adulthood[258]. Prior findings suggests that sex-typical locomotor behavior arises from sex-specific modifications of muscles and neural modulation within sex-shared circuits. However, less is known about how these differences in locomotor behavior emerge and diverge across all developmental larval stages, including right after hatching. Our understanding of sexual differences in locomotor behavior in liquid remain relatively poorly understood.

Overall, the sexually dimorphic nervous system can integrate external signals and enable corresponding, likely sex-specific motor outputs during development. Here, we have explored the emergence of robust swimming behavior between the male and hermaphrodite *C. elegans* and when behaviors diverge between sexes. To do this, we have qualitatively and quantitatively analyzed the development of swimming at the L1, L2, L3, L4, and adult in hermaphrodite and male *C. elegans*. We utilized a robust quantitative framework, implementing principal component analysis, to understand the full complexity of behaviors the worm makes when swimming. Our

findings show that swimming behavior in both sexes stabilizes coordinated output by the L2 larval stage of development. Intriguingly, we observe sexual dimorphism of swimming behavior emerge in adulthood, specifically male swimming showing higher dimensionality. This is reflected in adult male worms exhibiting faster, stereotypical body bends, however punctuated by male-specific coiling behavior. Our studies suggest adult male *C. elegans* swimming differs from the simple coordinated swimming patterns seen in earlier juvenile stages (excluding L1) of both sexes as well as in adult hermaphrodites. Our findings demonstrate how the complex male nervous system, that fully manifests at the latest L4 stage of larval development to confer sexual maturation, provokes a variable swimming pattern that has not been previously studied.

3.3 Results

3.3.1 Adult male *C. elegans* exhibit sex-specific tail curling

C. elegans locomotor output is generated from waves propagating along their bodies in rhythmic dorsal to ventral alternations. Previous studies undercovering sexual dimorphism in the development of locomotor behaviors has focused on crawling in the larval L3, larval L4 and adult stages revealing sex differences emerge primarily in adulthood[258]. *C. elegans* swim in a way that has been described as thrashing, also propagating dorsoventral waves along their body principally generating C-shapes[221,259]. To understand the sex-specific differences in rhythmic swimming, we analyzed hermaphrodite and male *C. elegans*. In adult animals, both sexes primarily make stereotypical alternating C strokes when swimming (Fig 1a and 1b). However, we observed that adult male animals also generate a unique, albeit infrequent, shape when swimming by tightly curling their tail (Fig 1b). Further, to quantify the frequency and amplitude of rhythmic locomotion, representative videos of hermaphrodite and male worms swimming were

converted into kymographs of body curvature (Fig 1c and 1d). Male *C. elegans* observably swam faster than their hermaphrodite counterparts, however, a representative 20-second kymograph illustrates a short period when the animal's tail curls, veering from stereotypical hermaphrodite swimming to a period of paused curling posture for a moment (Fig 1d).

To understand when sexually dimorphic swimming behavior emerges and if the adult male curling phenotype was present throughout development, we quantified the average body-bending frequency of hermaphrodite and male *C. elegans* across development. We analyzed frequency at the earliest post-embryonic stage, soon after hatching in larval L1 animals as well as L2, L3, L4 and adult animals (Fig 1e). Our results indicate that hermaphrodite worms swim with an average body bending rate of 1.84 Hz (adults), 1.79 Hz (L4), 1.72 Hz (L3), 1.67 Hz (L2), and 1.49 Hz (L1) over a one-minute swimming period. Whereas, male worms swim at 2.06 Hz (adults), 1.89 Hz (L4), 1.87 Hz (L3), 1.68 Hz (L2), and 1.42 Hz (L1). In agreement with previous crawling data, we see sex differences in locomotor behavior in adult animals. However, in swimming we find the average body bending frequency significantly increased in male worms starting at the L3 stage and remains significant for the remainder of L4 and adulthood. Furthermore, we wanted to look across development to determine if the male-specific tail-curl phenotype is tied to anatomical changes seen in adults with the emergence of a sexually-mature, fan-shaped tail. We measured the amount of time the worms spent curled, with any part of their body crossing over itself, during one minute of swimming across developmental stages (Fig 1f). Our findings indicate that differences in tail curling are only significant in adult male worms compared to hermaphrodites, and do not arise anywhere else in development. These data indicate that sex-differences emerge as early as the L3 larval stage in swimming frequency with male worms displaying greater frequency. The adult male worms show a specific curled-tail phenotype not seen in hermaphrodites or earlier in male development.

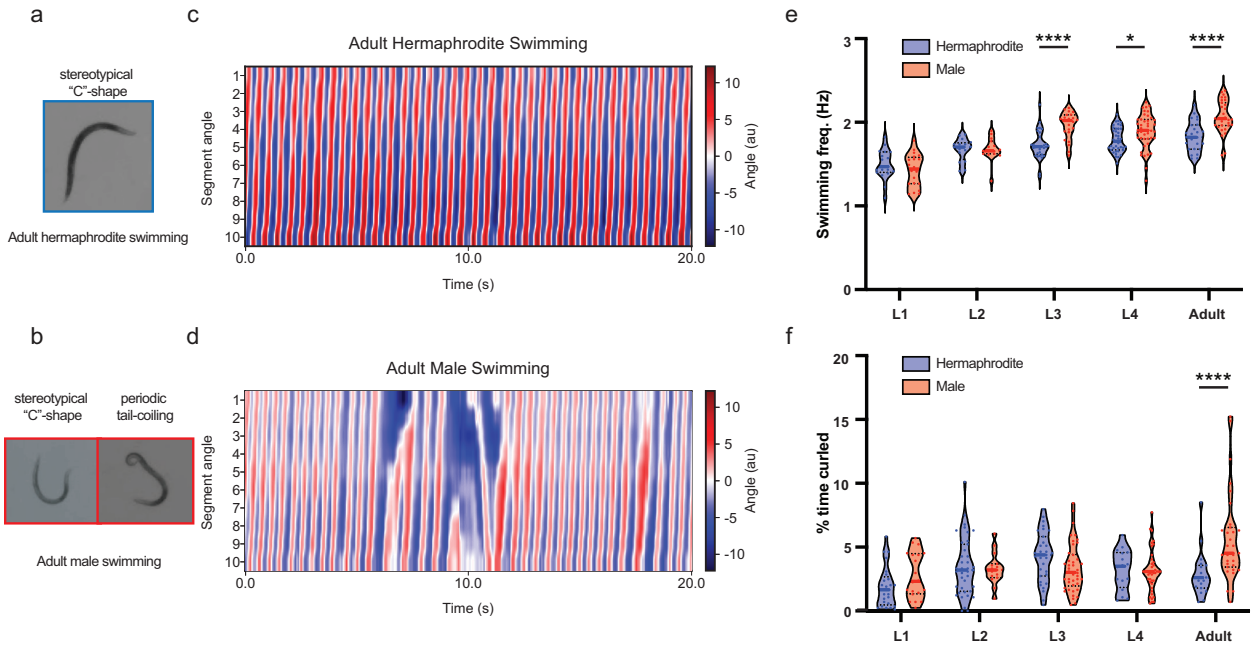


Fig 1. *C. elegans* adults exhibit sex-specific swimming phenotype. (a and b) Representative raw images of adult (a) hermaphrodite and (b) male *C. elegans* swimming in M9 droplet. Adult male *C. elegans* exhibit an infrequent but unique tail curling posture (b, right panel). (c and d) Representative kymographs of body curvature of a day-1 adult (c) hermaphrodite and (d) male worm during 20-seconds of swimming. Body segment number is plotted on the y-axis and time on the x-axis. (e) Violin plot shows the body bending frequency for each swimming tracking session of hermaphrodite L1, L2, L3, L4, and Adult; and male L1, L2, L3, L4, and Adult developmental stages. Hermaphrodite and male *C. elegans* show a significant difference in means at the L3 ($p<0.0001$, 2way ANOVA), L4 ($p=0.0418$, 2way ANOVA) and Adult ($p<0.0001$, 2way ANOVA) stages. (f) Violin plot shows the percentage of time spent curled for each swimming tracking session of hermaphrodite and male *C. elegans* in L1, L2, L3, L4, Adult developmental stages. Sample sizes range from n=14-42. Hermaphrodite and male adult *C. elegans* show a significant difference in means ($p<0.0001$, 2way ANOVA). Statistical significance in (e and f) was

determined using Two-way ANOVA using Bonferroni adjusted alpha levels to correct for multiple comparisons. Significance: *p<0.05, ****p<0.00001.

3.3.2 Basic postures of swimming are sex-shared across development

C. elegans hermaphrodite and male anatomy display clear differences in morphology with hermaphrodite worms possessing a vulva with a larger 1 mm body whereas males possess a distinguished tail and slimmer bodies. This is coupled with sex-specific neural development programs, including significant neural remodeling at the L4 stage in males as the tail develops. Moreover, given our findings of male-specific tail curling, we aimed to determine if this was a prominent posture.

To further determine the impact of sexual dimorphism on animal development for rhythmic locomotor output in *C. elegans*, we conducted principal component analyses (PCA). We previously described the development of postures integral to swimming across hermaphrodite development[221]. To understand the full repertoire of postures necessary for swimming and if those postures were dependent on sex, we extracted the principal eigenworms during swimming at the L1, L2, L3, L4 and adult stages in hermaphrodite and male worms (Fig 2a and 2b). First, our results in hermaphrodites were able to recapitulate our previous findings, pulling out the same eigenworms. Next, our analysis revealed that the main postures for swimming remained the same in both hermaphrodites and male worms. Interestingly, this demonstrates that swimming is generated from the basis of sex-shared postures. Evidently, there wasn't a specific eignenworm associated with adult male tail curling perhaps due to its similarity to the C-shape in swimming. However, this warranted a more targeted analysis of posture to capture the tail-curling phenotype seen in adult males.

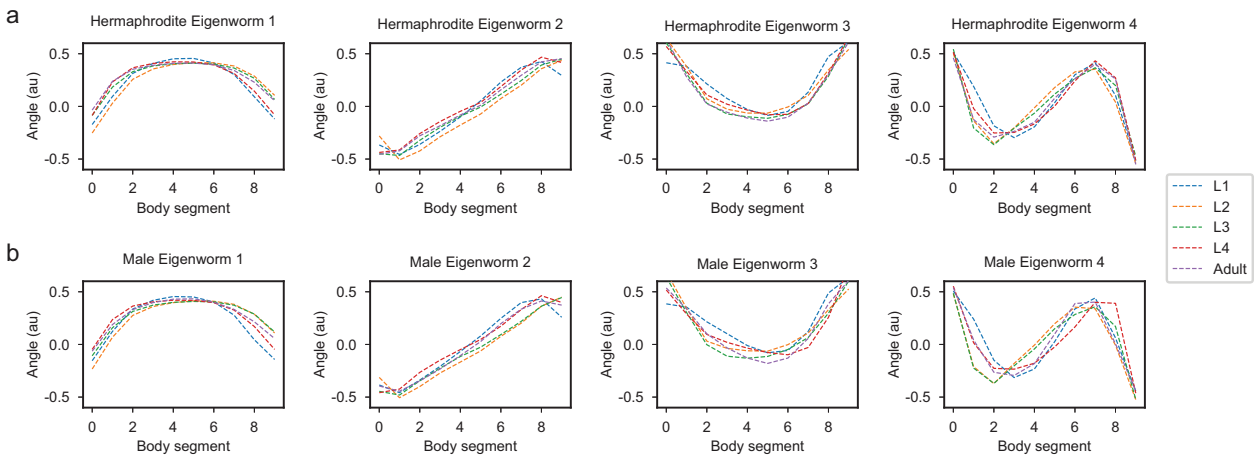


Fig 2. Hermaphrodite and male eigenworms are shared in development. (a and b) Swimming eigenworms 1-4 across developmental stages: L1 (blue), L2 (orange), L3 (green), L4 (red), adult (purple) in (a) hermaphrodites and (b) males.

3.3.3 Adult males exhibit sex-specific higher dimensional swimming patterns

Given our findings that eigenworm shapes necessary for swimming are the same at different developmental stages and between hermaphrodite and male worms, we wanted to know if both sexes coordinate these postures similarly in development. There are wide-ranging sex-differences in neural development starting as early as post-embryonically. We wanted to see if our results would indicate corresponding early sex-differences granted our findings indicated swimming frequency diverged only starting at the L3 stage.

To understand the variability and structure of locomotor patterns, we looked at the dimensionality in our swimming dataset at the different developmental stages. To do this, we quantified the dimensionality in swimming using participation ratio analysis (PR) of hermaphrodite (Fig 3a) and male (Fig 3b) *C. elegans* throughout development to adulthood. The PR defines the number of principal components or in our case, eigenworms, necessary to explain 80-90% of the variability of all the swimming postures within a data set. Indeed, a lower PR describes a data set

with low-dimensionality. Our results indicate the mean PRs are 3.07 (L1), 2.37 (L2), 2.30 (L3), 2.34 (L4), and 2.25 (adult) in hermaphrodites (Fig 3a). In males, the PRs are 3.21 (L1), 2.33 (L2), 2.29 (L3), 2.40 (L4), 2.84 (adult) (Fig 3b). Interestingly, when comparing the differences in age-matched means between hermaphrodites and males, we find male PR is greater at the L4 stage ($p=0.019$, student's t-test) and increases in adult male swimming with a PR of 2.84 compared to hermaphrodite PR of 2.25 (Fig 3b, $p<0.0001$, student's t-test). This indicates male worms have higher dimensionality at the latest stage of development compared to hermaphrodites and more so in adulthood–highlighting a comparatively more complex swimming behavior in male animals.

Our previous findings showed that juvenile L1 hermaphrodite worms are unable to output continuous stable rhythmic swimming. This unstable swimming behavior resolved itself rapidly, by the late L1 stage[221]. To understand if male swimming underwent a similar timeline, we examined whether male worms could assemble rhythmic swimming patterns in development and if this diverged from hermaphrodite stabilization of swimming. To analyze this, we compared the amplitude of the first two eigenworms in L1, L2, L3, L4, and adult hermaphrodites and males (Fig 3c and 3d). Previously, adult hermaphrodite rhythmic swimming was represented by an elliptical structure with two fixed amplitudes. Our prior findings for hermaphrodite swimming coordination were recapitulated here.

At first glance, swimming coordination developmental timeline appears shared between the sexes. However, we found that male L1 swimming has a mean PR of 3.21, significantly greater than all other male developmental stages. Interestingly, we also found that male adult swimming has the second highest mean PR of 2.84, proving significantly higher than L2, L3, and L4 stages of male development. Together, these findings suggest, development of male swimming follows a variable timeline than that of hermaphrodites. Additionally, subtle differences emerge in male L1 worms, including a greater PR compared to hermaphrodites and noticeably disorganized

swimming, as shown by the distinguishable centroid in the eigenworm amplitude plot. Together, these findings suggest sexual dimorphic development of swimming behavior.

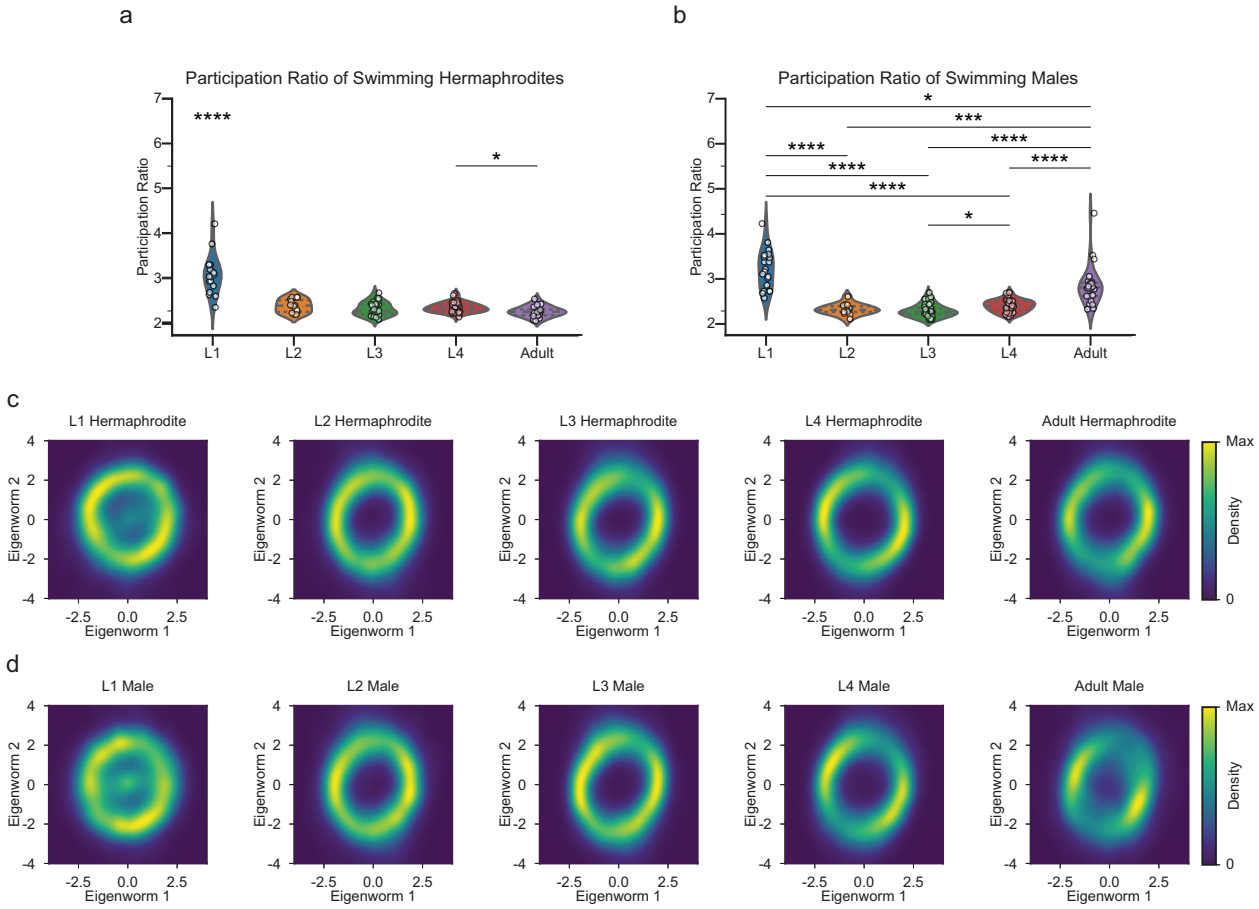


Fig 3. Rhythmic swimming becomes low-dimensional by the larval L2 stage in both sexes, but higher dimensionality reemerges only in adult males. (a and b) Participation ratios (PRs) representing the dimensionality for each swimming tracking session of (a) hermaphrodite and (b) male L1, L2, L3, L4, and adult *C. elegans*. (c and d) Swimming locomotion represented by eigenworm one and two amplitude distributions across developmental stages in (c) hermaphrodite and (d) male: L1, L2, L3, L4 and adult demonstrate coordination of these eigenworms is present across development, however L1 worms also produce uncoordinated postures not represented by the first two eigenworms. Dashed lines in (a and b) represent means and interquartile range.

Statistical significance in (a and b) was determined using t-test Bonferroni adjusted alpha levels to correct for multiple comparisons of 0.005 (0.05/10). In (a) hermaphrodite L1 PRs showed ****p statistical significance compared to all other stages. Significance: *p<0.005, **p<0.001, ***p<0.0001, ****p<0.000001.

3.4 Discussion

C. elegans exist in two sexes–hermaphrodites (putative females) and males–yet most research has traditionally focused on hermaphrodites. Here, we applied advanced behavioral techniques previously described[221] to dissect the full spectrum of swimming behaviors from early post-embryonic stages through to adulthood in both sexes. Our findings reveal that swimming pattern organization stabilizes early in larval development (L2) and remains consistent throughout this period for both sexes. These findings illustrate how shared locomotor and muscular growth during larval development generates sex-shared robust rhythmic outputs. However, our study uncovers higher-dimensional swimming patterns in adult males compared to hermaphrodites, reflecting the greater behavioral variability observed in males. Upon sexual maturation, the emergence of sex-specific organs and neural architecture correlates with a divergence in swimming behavior between males and hermaphrodites. Our results suggest that the development of male-specific tail morphology and complex neural wiring underlies distinct swimming behavior in males. This offers new insights into sexually dimorphic locomotor strategies in *C. elegans* and suggest how males might differ their swimming behaviors to prioritize mate seeking.

We also describe a new swimming tail curling phenotype observed only in adult male *C.* elegans. This posture might be attributed to the highly interconnected sensorimotor circuits supporting motor function in mating. Male *C. elegans* depend on ray sensory neurons for male-

specific mate-searching behavior. The male worm utilizes its tail with 18 ray sensilla to sense hermaphrodites. The maturation of the tail circuitry and anatomical sexual maturation coincides with male-specific mate searching behavior seen in adults[177,253,254]. Mating is a complex behavior that requires a diverse set of neural circuitries to produce tail posture control in males. Studies have shown that the intricate interaction and modulation of A- and B-type motor and ray sensory neurons enable specific tail postures important for mating behavior[260]. We speculate that the growth of male-specific tail sensory neuron functional circuity late in the larval L4 stage introduces an alternative male-specific posture that has not been observed previously with a plate-based, solid surface environment and that our study revealed in the alternative liquid sensory environment. Further circuit-based analyses will be necessary to understand sexual dimorphism in both crawling and swimming behavioral development, and to provide insight into sensory modulation of male-specific locomotor behaviors. Our results provide new ways to describe and quantify sexual dimorphism in development of locomotor strategies between the sexes. Our approach now provides a new lens to understand neuromuscular control of behavior.

3.5 Materials and methods

Preparation of Worms

Wild type hermaphrodite and male *C. elegans* (N2 Bristol) worms from the CGC (Minneapolis, MN, USA) were used for all assays. The worms were maintained at 20°C on 60mm NGM agarose plates with Escherichia coli OP50 lawns as food. Males were obtained for experiments by performing crosses, where four approximately aged L3 hermaphrodites were transferred to seeded NGM plates along with 12 young adult males. Multiple crosses were carried out at the same time. The plates were then stored in a 20° C incubator for 3 days to allow for sufficient time for mating. To obtain males and age matched hermaphrodites at later stages of development,

synchronization procedures were carried out in which parent hermaphrodites (F0) from crossed plates were placed on seeded NGM plates, allowing for egg laying. After one hour, all worms were subsequently removed, leaving only eggs on the plates which were immediately put into a 20° C incubator. After 12 hours of incubator growth time for L1s, 30 hours for L2s, 40 hours for L3s, 50 hours for L4s, and 65 hours for adult worms, the synchronized animals were subjected to assays.

Assays

To obtain locomotion data across the stages of development, worms synchronized at each stage were subjected to swimming assays in which their movements were captured on video. All assays were conducted on NGM plates with no bacterial lawn. Each worm was transferred using a platinum wire worm pick first into an intermediary NGM plate in the absence of OP50, then into a 5, 10, or 15µl drop of M9 buffer solution placed on the surface of the assay plate for L1-L2, L3, and L4-adult animals, respectively. M9 droplets were flattened with a worm pick in a circular motion beforehand to reduce glare. For each assay, one minute was allowed for the worm to acclimate to swimming conditions before 1 minute of the worm's locomotion was tracked. To confirm the sex of L1, L2, and L3 animals, worms were singled after the completion of respective assays onto NGM plates with OP50 present. Then the worms would be allowed to reach sexual maturation and their sex would be recorded.

Tracking

WormLab imaging stations were used in conjunction with WormLab software (both from MBF Bioscience) to capture videos of the worms and subsequently track the curvature data of each worm. Additionally, for L1 and L2 larval assays, a macro lens (LAOWA 25mm F2.8 2.5-5x ULTRA MACRO) at 2.5x magnification was mounted instead of the default lens in order to capture

the small worms at high resolution. All videos were taken at 1200x1600 resolution and 14 frames per second. To ensure worms remained in the field of view, assay plates were gently moved if necessary. For each worm, the angles between each of the eleven segments along the length of the animal (as defined by the WormLab software) were used to define the worm's curvature for each video frame. We segmented the animal into 5, 9, 11, 17, and 33 segments and found no significant differences in eigenworm shape analyses with more than 11 segments on a small held-out test data set. Subsequently, we proceeded with 11 segments (10 subsequent segment angles) for the remainder of the study.

Eigenworm Analysis

We denote the worms posture as θ(s) where s denotes the segment number. We perform an eigen-decomposition by first constructing the covariance matrix of the postures as:

$$C(s, s') = \langle(\theta(s) - \langle\theta\rangle)(\theta(s') - \langle\theta\rangle)\rangle$$

Eigenworms $\mu_i(s)$ and eigenvalues λ_i are defined by the eigendecomposition:

$$\sum_{s'} C(s, s')\mu_i(s') = \lambda_i\mu_i(s)$$

The cumulative variance explained is defined by:

$$\sigma_k^2 = \sum_{i=1}^{k} \frac{\lambda_i}{\sigma^2}$$

For all eigenworm amplitude figures, we define the eigenworms from the covariance matrix of posture angles across all developmental stages for that gait type. This choice is justified as the covariance matrix calculated from each separate developmental stage has nearly identical eigenvectors (Figure 3a and 4a), meaning there is no loss of descriptive power by combining stages into a single covariance matrix. This combination allows for comparisons across all developmental stages from the same perspective.

Participation Ratio

We utilize a continuous measure of dimensionality derived from the eigenvalues of the posture covariance matrix called the participation ratio (PR). The PR can be thought of as the dimensions required to capture approximately 80% to 90% of the variance of the data [235]. The participation ratio is defined as:

$$PR = \frac{(\sum_i \lambda_i)^2}{\sum_i \lambda_i^2}$$

In the simple case of three-dimensional (N=3) data, if the eigenvalues are 1, 0, 0, the PR is 1. If that same data were evenly distributed with eigenvalues of ⅓, ⅓, ⅓, it would have a PR of 3. Most data will contain some correlational structure that will place the PR somewhere in between the values of 1 and N, with N being the number of features in the data.

We calculated the participation ratio of each recording individually and collected the distribution of participation ratios for each age group. We performed a standard independent two sample t-test assuming equal sample variance using the scipy ttest_ind function in Python. We corrected alpha values for multiple comparisons using Bonferroni adjustment yielding a significant p-value of 0.05/15 or 0.0033.

Code

All analyses of worm segment data were performed using custom Python scripts relying primarily on the *matplotlib, numpy, scipy* libraries. Code to reproduce figures as well as instructive example notebooks are hosted at https://github.com/ssterrett/wopodyn.

3.6 Acknowledgements

We would like to thank multiple high-school trainees who helped complete this project. We thank Arush Talasila and Christina Jones for their gathering of data.

Chapter 4: Conclusions and future directions

"There is nothing new under the sun, but there are new suns."
-Octavia Butler, *Recovering Lost Parables*

4.1 Conclusions from this work

Throughout the progression of my thesis, I have identified novel ways to explain complex locomotor behaviors across development. I first set out to comprehensively describe locomotor behaviors of wild type hermaphrodite *C. elegans* across developmental stages through dimensionality reduction techniques, collaborating with computational experts. I examined the major forms of locomotion in *C. elegans* - crawling, swimming, and gait transitions. I found that swimming behavior could be reduced to four principal components or 'eigenworms.' However, the characteristic shapes of eigenworms in swimming and crawling differed significantly, suggesting that they don't necessarily originate from the same circuits. I further analyzed the locomotory patterns of worms at five different developmental stages - young L1, late L1, L2, L3, and L4. I discovered that young, newly-hatched L1 animals could not sustain rhythmic movements, despite displaying basic features of adult swimming eigenworms. Between young and late L1 stages, the locomotory pattern of developing worms is rapidly stabilized. My findings demonstrated that early features of juvenile animals are maintained while the locomotor networks undergo substantial remodeling during development. This suggests that larval and adult *C. elegans* have more than a single orientation of circuitry that mediates rhythmic locomotor behavior. Next, I expanded upon my research techniques and analysis to explore sexually dimorphic locomotor development. I analyzed swimming frequency, curling, postural, and dimensionality across L1, L2, L3, L4, and adult stages in both hermaphrodite and male *C. elegans*. I discovered that adult males have higher-dimensional swimming potentially due to more complex circuitry and different tail

morphology arising from sexual maturation by the late L4 stage. The methods and analysis suite I have designed and developed now provide a framework to examine different neurodevelopmental mechanisms necessary for the establishment, maintenance, and adaptation of rhythmic locomotion during animal growth.

4.2 Future directions

Intuitively, rhythmic circuits for locomotion could be subjects of developmental changes, as locomotory patterns from a child and an adult are different. Moreover, locomotory patterns deteriorate with age. However, we do not know the mechanisms that are used to modify rhythmic circuits during growth and their failure during aging. The complexity of rhythmic circuits makes them difficult to study. Developmental programs carry overlapping functions. Such redundancy can make rhythmic networks resistant to changes of individual genes. Additionally, individual adaptations of rhythmic circuits are unlikely to undergo changes at the same time. While locomotion appears to be an on-off behavioral task, it indeed utilizes a complex system of neurons for rhythm generation and gait selection. Moreover, locomotor behavior needs to be integrated and coordinated with other behaviors, such as mating. Thus, studies on rhythmic circuits require precise dissection of neuronal anatomy, circuit function, role of individual neurons, neural activity and animal behavior at multiple developmental and aged stages in both sexes of living animals. While I have not examined how rhythmic circuits adapt to changes in a growing or ageing brain, this remains a variable but challenging task to explore.

Modulation of gait transition behavior

Healthy people can effortlessly transition between gaits for everyday tasks. Similarly, *C. elegans* can also reliably switch between swimming and crawling behaviors. To quantify gait transitions, I employed PCA analysis and visualized gait switching between swimming and crawling in *C.*

elegans. My analysis identified the features of gait transitions and established a new approach for quantifying *C. elegans* gait transitions by distinguishing behaviors in eigenworm space[221]. Further, characterizing the timing of gait switching in wild-type animals will provide a useful benchmark for future comparisons. Previous studies have identified conserved serotonin and dopamine modulation involved in controlling gait output[204]. Future research might use my analysis tools to examine transition behaviors in wild-type and mutant animals lacking the ability to perform these behavioral tasks. Furthermore, our findings demonstrate organized motor output in wild-type animals beginning in the late larval L1 stage. This raises intriguing questions about how postembryonic neurons born later in larval development contribute to smooth locomotion and gait selection in a growing *C. elegans*. The PVD sensory neuron is of interest due to its extensive processes that span the entire body, playing an important role in mechanosensation[261]. Future studies using our quantitative frameworks could explore the locomotor behavior of swimming, crawling, and gait transitions in animals lacking PVD neurons. These experiments would contribute to our understanding of neurons and neural modulation necessary for smooth locomotor behaviors.

Identifying rhythmic neurons in a simple animal model

Through a collaborative effort with Dr. Julien Dubrulle at the Fred Hutch Cellular Imaging Core, we have designed an imaging paradigm to identify potential neural oscillators for motor regulation. Prior studies on deafferented nerve cords in immobilized locusts discovered that a subset of neurons can produce and maintain oscillating activity in the absence of muscle movements and sensory feedbacks[80,262–264]. Such neurons with intrinsic oscillating activities are known as central pattern generators (CPGs), which are considered fundamental components of the neural networks for producing rhythmic control of locomotion. To identify neurons exhibiting oscillating

activity in the absence of physical movements in *C. elegans*, I conducted fast time-lapse 3D calcium imaging of neurons in restrained, glued down adult worms. Using the fluorescence of the Ca^{2+} probe GCaMP6 and a spinning-disk confocal microscope, I effectively monitored neuronal activity (Fig. 1). Our preliminary efforts led to the successful development of an image analysis pipeline that segments and tracks neurons using a machine learning tool (StarDist3D)[265], extracts their GCaMP intensity, and identifies neurons based on their spatial location with respect to a published reference atlas[266] (Fig 1). A previous studies identified AVF neurons as a potential candidate as these neurons produce oscillating activity[267]. Future studies will test how optogenetic stimulation of sensory neurons alters the behavior of neurons with oscillating activities, confirmed in our analysis. This is crucial because CPGs must work in conjunction with sensory feedback systems to produce smooth locomotion[7,84,197] and to enable gait transitions[93]. These analyses will take advantage of the established *C. elegans* connectome to shed light on how sensory feedback is integrated into the neuronal regulation of rhythmic locomotion. Providing an interdisciplinary understanding of how neural activity supports locomotor behavior.

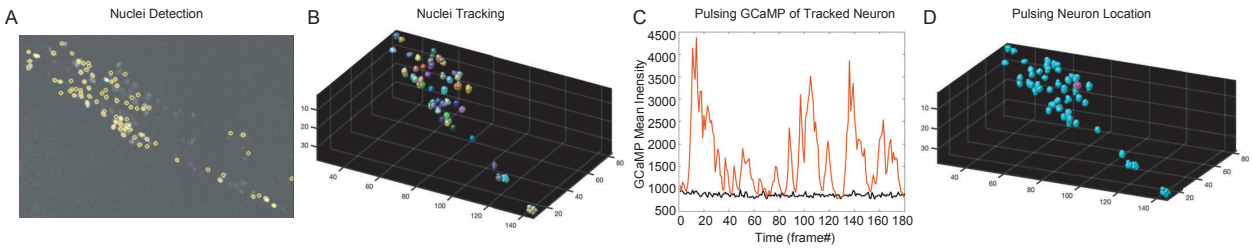


Fig 1. Identification of neurons with oscillating activity. (a) Nuclei segmented using StarDist3D from adult *C. elegans* with RFP expression in nuclei of neurons using the *Prab-3* promoter. (b) Individual nuclei are tracked through space-time. (c) Trace of mean GCaMP intensity of tracked neuron. (d) Neuron is assigned based on a reference atlas.

WNT signaling on locomotor development

Future studies exploring the neural mechanisms underlying the development and maintenance of locomotor behavior in the nematode *C. elegans* is an exciting next step. Utilizing genetic, imaging, and behavioral analyses would allow us to investigate developmental programs that establish and modify locomotory rhythm at distinct developmental stages. The role of Wnt signaling in rhythmic circuits is unknown. I hypothesize that certain Wnt pathways are recruited in juvenile and adult animals to influence neural circuits for locomotion and gait transitions. Wnt pathways are evolutionarily conserved and have a crucial role in the early development of the brain[268–272]. Development and neurological dysfunction arise from defects in Wnt signaling, highlighting the importance of Wnt signaling throughout lifespan[273–275]. In *C. elegans*, there are five genes encoding the Wnts, *cwn-1*, *lin-44*, *egl-20*, *cwn-2*, and *mom-2*[276–280]. I have characterized swimming frequency and patterns of wild type and several *wnt* mutant worms at various developmental stages (Fig 2a-e). I have found that *cwn-2* and *egl-20* play distinct roles in the development of locomotor patterns. Furthermore, I have found that CAM-1[270], a Wnt receptor, as the likely receptor for CWN-2 regulation of swimming due to both *cwn-2* and *cam-1* mutants displaying similar defective sigenworm amplitude patterns during swimming (Fig 2f and 2g). Future cell-specific rescue experiments could delineate where CWN-2 signaling acts to support swimming. These findings suggest Wnt pathways could regulate neural circuitry development and locomotion. Future studies with *wnt* mutants could provide a potential avenue to explaining the molecular underpinnings of CPG and sensory circuits required for smooth locomotion.

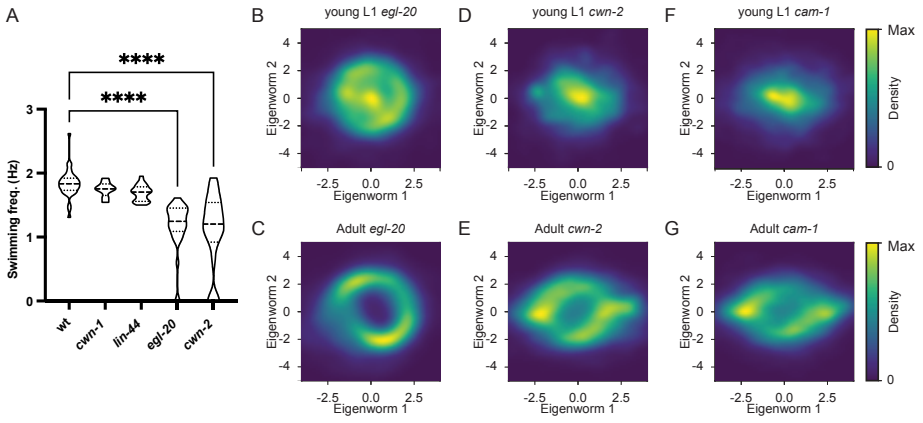


Fig 2. Impact of *wnt* mutations on swimming. (a) Body bending frequency of adult wild type and *wnt* mutant animals was quantified and shown in a violin plot. ****p<0.0001 (one-way ANOVA). (b-g) amplitude distribution for young L1 and adult *egl-20* mutant (b and c), *cwn-2* mutants (d and f), and *cam-1* mutants (g and h) swimming.

Quantitative framework for locomotor behavior in ageing and disease

Gaining a deeper understanding of the formation and organization of locomotor circuits in development can provide valuable insights for how these circuits fail during ageing and disease. Prior research has shown neural circuits including motor neurons synaptic structures deteriorate with age in *C. elegans*[281,282]. Characterizing locomotor function in aged animals with quantitative and postural analysis will provide a framework to understanding how locomotor behavior changes with age corresponding with deterioration of neural circuits. Moreover, we can investigate diseases that impair our ability to move associated with ageing. For example, Parkinson's disease (PD) has a severe impact on motor function and manifests later in life from the degeneration of dopamine neurons[283]. There are well characterized PD mutants in the *C. elegans* model providing a unique opportunity to precisely quantify locomotor defects and test potential therapeutics.

Ultimately, the quantitative behavioral farmwork developed during my thesis work could allow for investigations of locomotor behavior with age and disease-relevant genetic perturbation.

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