Neuromuscular Basis of *Drosophila* larval escape behavior

Patricia C. Cooney[1,2], Wenze Li[3,4,§], Yuhan Huang [5,6,§], Richard Hormigo[1], Tanya Tabachnik[1], Elizabeth M.C. Hillman[3,7,8,9], Wesley B. Grueber[1,2,10,*], Aref A Zarin[5,6,*,#]

[1] Mortimer B. Zuckerman Mind Brain Behavior Institute, Jerome L. Greene Science Center, 3227 Broadway, L9-007, New York, NY 10027, USA

[2] Department of Neuroscience, Jerome L. Greene Science Center, 3227 Broadway, L9-007, New York, NY 10027, USA

[3] Laboratory for Functional Optical Imaging, Mortimer B. Zuckerman Mind Brain Behavior Institute, Columbia University, New York, NY 10027, USA

[4] Department of Electrical Engineering, Columbia University, New York, NY 10027, USA

[5] Department of Biology, Texas A&M University, College Station, TX 77843, USA

[6] Texas A&M Institute for Neuroscience, Texas A&M University, College Station, TX 77843, USA.

[7] Department of Biomedical Engineering, Columbia University, New York, NY 10027, USA

[8] Department of Radiology, Columbia University Irving Medical Center, New York, NY 10032, USA
[9] Kavli Institute for Brain Science, Columbia University, New York, NY 10032, USA

[10] Department of Physiology and Cellular Biophysics, Jerome L. Greene Science Center, 3227 Broadway, L9-007, New York, NY 10027, USA

[§] equal contributors

[#] Lead Contact
*Correspondence: azarin@bio.tamu.edu (A.A.Z.), wg2135@columbia.edu (W.B.G.)

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Abstract

When threatened by dangerous or harmful stimuli, animals engage in diverse forms of rapid escape behavior. In *Drosophila* larvae, escape behavior is characterized by C-shaped bending and lateral rolling, followed by rapid forward crawling. The sensory circuitry that promotes escape has been extensively characterized, but the motor programs underlying escape are unknown. Here, we characterize the neuromuscular basis of escape. We use high-speed, volumetric, Swept Confocally-Aligned Planar Excitation (SCAPE) microscopy to image muscle activity during larval rolling. Unlike the sequential peristaltic muscle contractions from segment to segment that underlie crawling, muscle activity progresses in a circumferential sequence

during bending and rolling. Certain muscle subgroups show functional antagonism during bending and rolling. We use EM connectome data to identify premotor to motor connectivity patterns that could drive rolling behavior and test the necessity of specific groups of motor neurons in rolling using neural silencing approaches. Our data reveal body-wide muscle activity patterns and putative premotor circuit organization for escape.

INTRODUCTION

Early in the evolution of animals, nervous systems specialized to permit locomotion[1]. While locomotion supports multiple aspects of evolutionary success (*e.g.* allocating resources, finding mates), one of the most critical of these is escape: the transformation of sensory input into motor output to avoid imminent danger[2–4]. Escape behaviors are rapid and stereotyped yet must be flexible enough to allow animals to evade multiple sources of harm and readjust when danger subsides[3,5,6]. Escape behaviors across species often differ fundamentally from exploratory locomotion.[3,7–910,11]. This specificity suggests that dedicated neural circuits or unique activity patterns within shared locomotor circuits are employed during escape. While many studies have investigated how sensory input promotes escape[2,3], the neuromuscular activities that generate escape movements have been characterized in few model organisms[2,3,5,7,12,13,12,13]. Furthermore, we have limited understanding of how the sensory circuits that promote escape drive motor circuits. By characterizing escape motor circuits in the *Drosophila* model, with its well-studied sensory system and nearly complete connectome, we aim to understand how sensory input is transformed into motor output during escape.

The *Drosophila* larval body consists of twelve segments, with abdominal segments containing up to 60 different muscles[14]. Forward crawling consists of sequential segmental contractions that propagate from posterior to anterior segments and engages all muscles[14–16]. Upon experiencing harmful mechanical touch or heat, larvae initiate a nocifensive escape consisting of C-shaped bending, rolling, and rapid forward crawling[17]. Rolling causes fast lateral motion—which is faster than escape crawling alone, and can dislodge attacking parasitoid wasps[18,19]. This behavior is initiated by activity of class IV (cIV) dendritic arborization neurons, polymodal nociceptors that tile the body wall[18,20]. Several downstream partners of cIV neurons have been identified and reconstructed using serial transmission electron microscopy[21–25]. Activation of any of a number of interneurons that are downstream of cIV neurons is sufficient to evoke escape[21–23].

Despite progress in understanding nociceptive circuitry, characterizing neural and muscular activity during escape behavior presents challenges. In contrast to crawling, rolling behavior is asymmetric, with larvae rolling laterally in one direction. However, both the larval body and central nervous system are symmetric on either side of the dorsal and ventral midlines. The hemisegment unit is important to consider during rolling behavior, since the bilaterally symmetric neural and muscle activity that occurs during crawling must be broken during rolling, setting up a fundamental difference between these two behaviors.

In this study, we examine the muscle activities and motor circuits responsible for escape bending and rolling using a combination of high-speed 3D imaging of fluorescent calcium indicators expressed in muscles, functional manipulations of motor circuits, and connectomics approaches. We compare our findings to the motor activity that drives crawling to identify shared and distinct features of these two modes of locomotion. Both behaviors involve sequential motor activities and antagonistic drive of distinct muscle groups, but our results highlight fundamental differences in the motor patterns. In particular, muscle contractions progress circumferentially around the larva during rolling, in contrast to the anteroposterior progression of muscle contractions during crawling. These data provide a foundational view of motor activity during escape, narrowing in on a full sensory to motor understanding of an escape behavior.

RESULTS

Muscle activity patterns in rolling escape

Drosophila larval escape is comprised of C-shaped bending followed by lateral rolling[17]. Rolling can be triggered experimentally by activation of nociceptive sensory neurons or by central neurons including the Goro command neuron[21]. We confirmed that in response to optogenetic activation of Goro or a global heat nociceptive stimulus, larvae engage in bending and rolling behavior. We found that larvae can bend to the left or right, and, independent of bend direction, may roll in a clockwise or counterclockwise direction (**Figure 1A; Video S1**). Thus, upon optogenetic activation of nociceptive circuitry, larvae can engage four distinct, yet related, escape motor patterns.

Escape circuitry is well-studied, but how circuitry converges on premotor and motor systems is not known. We therefore sought to determine the muscle activities that underlie the escape rolling motor pattern. We imaged larvae using Swept Confocally-Aligned Planar Excitation (SCAPE) microscopy, a volumetric imaging technique that permits high-speed, high-resolution, 3D imaging of behaving animals[26–33]. We induced rolling using Goro activation in

larvae expressing mCherry and GCaMP6f in all body wall muscles. We resolved activity of individual muscles along the entire length of the larva and approximately half of the body thickness, at 10 volumes per second (**Figure 1B**; **Figure S1A,B; Video S2)**. We predicted that as GCaMP6f/mCherry ratios increased, muscle length would decrease, reflecting muscle contraction upon activation. Indeed, these two measurements showed an inverse relationship, suggesting that GCaMP6f/mCherry ratios can be used as an indicator of muscle contraction (**Figure S1C**). We focused on muscles in mid-segments A2-A4 since activity in these showed the greatest dynamic range (**Figure S1C**). We analyzed roughly 19 muscles per hemisegment in A2-A4 across multiple roll events, totaling over 370 muscle measurements.

SCAPE movies revealed that muscles are most active along the bent side of the larva, consistent with a role for asymmetric muscle contractions in C-shaped bending (**Video S3**; **Figure 1B**). Ratiometric calcium signals for many muscles tended to decrease as muscles moved out of the bend (**Video S3; Figure S2B-D**) and to increase as muscles rotated into the bend (**Video S3; Figure S2E**). To contrast escape rolling and crawling at the level of individual muscles, we compared SCAPE imaging data collected during rolling to previously acquired confocal data on muscle activity during crawling[16] (**Figure 1B,C**). As expected, measurements of muscle peak activity during crawling revealed a delay between muscle contraction in neighboring segments during peristalsis (**Figure 1C,D**). By contrast, during rolling, segmentally homologous muscles showed synchronous contractions, primarily on the side of the larva entering the bend. Also, in contrast to peristalsis, sequential muscle activity traveled around the circumference of the larva during rolling (**Figure 1 B,E,F, Figure S3**). Notably, we found that while dorsal (D) and ventral (V) longitudinal and oblique muscles demonstrated significantly greater activity along the bent side than the stretched side of the larva, lateral transverse (LTs) and ventral acute (VAs) muscles show roughly equivalent activity on bent and stretched sides on average (**Figure 2A-C**). Therefore, LT and VA muscles do not follow the typical circumferential wave of activity seen in other body wall muscles.

Altogether, these data demonstrate crucial distinctions between motor patterns during rolling and crawling: 1) muscle activity during rolling is synchronous across segments but is segmentally asynchronous during crawling; 2) muscle activity is left-right asymmetric during rolling but is left-right symmetric during crawling; 3) rolling involves progression of muscle contractions around the circumference of the larva, while crawling involves progression of muscle contractions along the anteroposterior axis. As an important exception to (2) above, we predict that as larvae roll, there are periods of L-R hemisegmental symmetry when homologous muscles along the dorsal and ventral midline enter the bend and co-contract. However, rolling

and crawling are similar in that the within-segment muscle activity patterns both demonstrate opposing functions of longitudinally-spanning versus transverse-spanning (LT and VA) muscles.

A potential circuit basis for circumferential muscle contraction sequences

We next examined electron microscopic (EM) connectome data[22] to determine whether MN structure and PMN-MN connectivity could provide insights into the basis of bending and rolling. Activity measurements indicated two requirements for the escape motor pattern: 1) left-right asymmetric muscle contraction during bending, and 2) circumferential propagation of this contraction during rolling. To first gain insight into asymmetric muscle contraction, we focused on the spatial distribution of postsynaptic fields on MN dendrites. We found that postsynaptic fields on MNs innervating the lateral muscles are located in the ipsilateral side only, hence there is no overlap between the fields of left and right MN counterparts (**Figure S4A,B)**. By contrast, the left and right MNs innervating the dorsal muscles show partial overlap in their postsynaptic fields (**Figure S4C)**. This partial overlap of fields could support the momentary bilateral symmetry that occurs as the dorsal midlines rotate into the bend. Such partial overlap is also seen for the left and right MNs innervating the ventral muscles (**Figure S4D,E)**, which could support the transient synchronous activity of left and right ventral muscles while passing through the bend side. This organization of synapses is consistent with previous studies showing that MNs innervating lateral muscles only have ipsilateral dendritic arborizations, while some of the MNs innervating dorsal or longitudinal muscles have both ipsilateral and contralateral dendritic arborizations[16,34–36], a feature that, could be a crucial feature of motor circuit structure in driving escape muscle activities.

Next, we examined the PMN-MN connectome for circuits that could support circumferential propagation of contraction. Notably, we identified multiple PMNs that synapse in a spatially defined pattern onto MNs that correlates with neighboring regions around the circumference of the larva, such as dorsolateral muscles (i.e., DLs, DOs, and LTs), ventrolateral muscles (i.e., VOs, VLs, and LTs) (**Figure 3A**), and/or muscles flanking dorsal midline (i.e., left-right DLs) (**Figure 3B**). On the other hand, MNs that innervate spatially distant muscles (i.e., lateral transverse muscles LTs on the left and right side) receive inputs from exclusive set of PMNs (i.e., right PMNs synapsing with right LTs and vice versa) (**Figure 3C**). As an example for the first group of PMNs, we found that two presumptive excitatory PMNs in the right hemisgment (A03a1_a1r and A03a3_a1r PMNs) are presynaptic to MNs innervating dorsolateral muscles (DLs and DOs) on animal's right side, while they also make a smaller

number of synapses with MNs innervating DL muscles on the left side (**Figure 3D**). Thus, activity of these right A03 PMNs should strongly activate the right dorsolateral muscles and weakly activate the DL muscles on the left side. Then, activation of the left counterparts of these PMNs should have a mirror effect and strongly activate the left dorsolateral muscles while weakly activating the DL muscles on the right side. Sequential activation patterns of left and right A03 PMNs should therefore facilitate the circumferential progression of dorsolateral muscle contractions from right to left hemisegments, a pattern that is seen during clockwise rolling (**Figure 3E**). Further analysis of connectome data revealed that two inhibitory PMNs (A23a and A31k) have connectivity patterns similar to the excitatory A03a1 and A03a3 PMNs (**Figure 3D**). Such a synaptic organization raises the possibility that circumferential propagation of MN activity could be shadowed by circumferential inhibition of muscles. As A23a and A31k inhibit dorsolateral muscles, other excitatory PMNs may activate lateral and/or ventral muscles, thereby enforcing wave progression (**Figure 3 E**). Taken together, the PMN-MN-Muscle connectome is structured in way that sequential firing excitatory and then inhibitory PMNs in one side followed by the activation of their counterparts on the other side could underlie the progression of muscle contraction waves around the circumference of the larva. In **Figure 3 E and F,** we propose a model describing how circumferential progression of muscle activity enables larvae to produce the four different patterns of rolling presented in this study. Notably, this model suggests that a counterclockwise roll involves clockwise progression of muscle contraction, and vice versa.

MN activity and muscles important for rolling execution.

We next sought insight into the involvement of different MNs and muscles for rolling. Our analysis of muscle activity indicated that longitudinally-spanning muscles and transversely-spanning muscles are activated in distinct phases during rolling (**Figure 2**) raising the possibility that coordination of the activity of these muscles groups is critical for rolling. To test this possibility, and determine the roles of different muscle groups in rolling, we optogenetically activated Goro command neurons while chronically silencing subsets of MNs using Kir2.1. Many larval body muscles are co-innervated by type Is and a single type Ib excitatory MNs **Table-1**. We found that silencing the type Ib MNs innervating ventral longitudinal (VL) and dorsal oblique (DO) muscles blocked bending and rolling (**Figure 4A**). These data are consistent with a key role for ventral longitudinal muscles in nociceptive escape. Interestingly, other groups of muscles appear to have different specific functions during rolling. Notably, larvae with silenced LT-innervating MNs could bend but were unable to roll more than 90° (i.e., from their ventral to lateral side) (**Figure 4B, Video S4**). Dorsal longitudinal (DL) muscles are also important for rolling performance, since silencing of DL-innervating MNs reduced rolling, but not to the extent as type Ib MNs innervating LT or VL muscles (**Figure 4A,B**). Selective silencing of the type Is MNs had little effect on rolling performance; however combinatorial silencing of type Is and different subsets of type Ib MNs led to more severe rolling defects than when either type Ib or type Is MNs were silenced alone (**Figure 4A,B**).

Table-1. Larval MNs and their target muscles

Motor neurons	Target Muscles
(Ib) MN1	1
(Ib) MN2	2
(Ib) MN3	3
(Ib) MN4	4
(Ib) MN9	9
(Ib) MN10	10
(Ib) MN11	11
(Ib) MN19	19
(Ib) MN20	20
(Ib) MN5	5
(Ib) MN6/7	6/7
(Ib) MN12	12
(Ib) MN13	13
(Ib) MN14	14
(Ib) MN30	30
(Ib) MN26	26
(Ib) MN27	27
(Ib) MN29	29
(Ib) MN15/16	15/16
(Ib) MN15/16/17	15/16/17
(Ib) MN28	28
(Ib) MN8	8
(Ib) MN21/22	21/22
(Ib) MN22/23	22/23
(Ib) MN23/24	23/24
(Ib) MN18	18
(Ib) MN25	25
(Is) MNISN	1/2/3/4/9/10/11/19/20
(Is) MNISNb/d	6/7/12/13/14/15/16/30

Taken together, we conclude that, while all different muscle types contribute to efficient rolling performance, the ventral and lateral muscles are the most crucial groups of muscles for initiation and execution of escape rolling. Furthermore, type Is input may act in synergy with type Ib inputs to generate fast and continuous rolling escape behavior.

DISCUSSION

Escape is a fundamental form of locomotion and critical for the survival of all animals. To understand the neural mechanisms of an escape behavior, we have performed live imaging of muscle activity across animals as they perform escape, analyzed a connectome for motor circuits that could support the unique muscle propagation wave that coordinates this escape motor program, and performed MN silencing experiments to determine muscle groups whose activity is necessary for escape.

Enhancements of SCAPE microscopy to permit studies of rolling behavior

Our ability to identify patterns of muscle activity during rolling was aided by the further development of dual-color SCAPE. High-speed, high-resolution SCAPE microscopy has previously captured muscles in behaving larvae[37] and dual-color proprioceptor activity during crawling behavior[28]. Expanding dual-color SCAPE imaging here enabled ratiometric quantification of muscle activity and discrimination of activity signals from passive changes in fluorophore density within muscles. This imaging method also allowed measurement across muscles at different focal depths during freely moving behavior, including muscles within the C-shaped bend during escape. We expect that further development of SCAPE, for example wider field of view optics and simultaneous collection of signals from multiple angles, will allow coincident imaging in the CNS and muscles and test some of the hypotheses about PMN to MN transformations that underlie rolling, and a number of other behaviors, in simple model organisms.

Separable sequences of muscle activity during rolling

Animal movement universally involves coordinated sequential muscle activity. We find that sequential circumferential muscle contractions define escape rolling behavior in *Drosophila* larvae. This sequence of activity progresses in a clockwise or counterclockwise manner, which determines the direction of rolling. Whether this circumferential sequence is the primary motor activity that promotes rolling, or whether other independent patterns are involved, is so far unclear. Muscle imaging revealed evidence that LT muscles contract as part of a separate, out-of-phase sequence relative to the major wave of longitudinal muscle activity. Notably, LT muscles begin to contract after D and V longitudinal muscles also during crawling, suggesting special roles for these muscles in both forms of locomotion. During forward crawling, contraction of LT muscles shortens the dorsoventral axis, providing a force that is thought to push the neighboring internal organs to the anterior side[15,38]. We speculate that during rolling LT muscle

activity provides a circumferential pulling force that is essential for body rotation. A major difference between sequential LT muscle activity during rolling and crawling is that the sequence progresses within the segment during rolling and along adjacent segments during crawling. Interestingly, while silencing the LT innervating MNs substantially compromises rolling behavior, it has no effect on forward crawling (Figure 4; Yoshino *et al.*, 2017[23]).

A major future goal will be to understand how circumferential activity sequences are initiated and then how activity is propagated in a precise sequence essential for rolling. The analogies in LT activity in muscle contraction sequences during crawling and rolling raise the question of how translatable our knowledge of premotor crawling circuits will be for understanding rolling. It will be especially important to determine whether excitatory and inhibitory microcircuit motifs that coordinate crawling[39] also coordinate rolling muscle contractions, and if so, how the crawling pattern is transformed to a rolling one.

Insights into motor circuits critical for rolling behavior

Our functional manipulations of MNs innervating distinct muscle subgroups highlight the modularity of motor control in larval escape. Larvae can exclusively bend, or perform bending and rolling. These behavioral components occur probabilistically and are dependent on the precise nature of sensory input to the larva[32]. Some sensory interneurons are necessary and sufficient for escape bending to occur but are not necessary for escape rolling[37]. Our muscle activity imaging in conjunction with our MN silencing experiments further validate the modularity of the escape motor program. Namely, we see that a circumferential wave of bend-promoting contractions occurs in longitudinally-spanning muscles, while counteracting “pulling” contractions occur in transverse muscles. Our MN silencing experiments demonstrate that longitudinally-spanning muscles are essential for larval bending and rolling altogether, while transverse muscles are essential for rolling to take place. These findings are congruent with previous work demonstrating that neurons driving transverse muscles are essential for body rotation in self-righting[72] and rolling[38]. Uncovering the premotor circuit modules that drive the bend and the roll could provide insight into how the larval nervous system generates many motor components of its behavioral repertoire.

We uncovered MN post-synapse distributions and patterns of PMN-MN connectivity that could support propagation of muscle contractions around the circumference of the larva during rolling behavior. Namely, separate inputs to lateral muscles permit their asymmetric drive escape, a feature not required for left-right symmetric contractions of lateral muscles during crawling. The structural features that support circumferential propagation of excitation and inhibition provide a

neural substrate for how this precise and rapid muscle activity sequence could be coordinated. Studies of inhibitory circuit components in crawling have revealed the importance of inhibition in allowing efficient contractions of antagonistic muscle groups[40,41]. In addition to characterizing specific microcircuits that contribute to rolling, future work could determine the general importance of precisely timed excitation and inhibition in promoting sustained rolling as an efficient and robust behavioral response to danger.

STAR Methods:

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (*Drosophila melanogaster*)	w1118	BDSC		Global heat + vibration assay line, crossed as control for MN silencing experiments
Genetic reagent (*Drosophila melanogaster*)	R69F06-Gal4	BDSC	# 39497	Goro line
Genetic reagent (*Drosophila melanogaster*)	44H10-LexA, Aop-GCaMP6f; Aop-mcherry, UAS-Chrimson-mcherry	Aref Zarin		Optogenetics line
Genetic reagent (*Drosophila melanogaster*)	44H10-LexA, Aop-mcherry; 44H10::GCaMP6f,UAS-Chrimson-mcherry	Aref Zarin		SCAPE ratiometric muscle imaging
Genetic reagent (*Drosophila melanogaster*)	69E06-LexA; Aop-Chrimson-mCherry, UAS-Kir2.1-eGFP	Aref Zarin		Goro activation and MN silencing
Genetic reagent (*Drosophila melanogaster*)	27E09-Gal4	BDSC		MN silencing
Genetic reagent (*Drosophila melanogaster*)	RRa-Gal4	Albert Cardona		MN silencing
Genetic reagent (*Drosophila melanogaster*)	BarH1-Gal4 (w[*]P{w[+mW.hs]=GawB}NP4099 / FM7c)	Kyoto Stock Center	DGRC# 104-601	MN silencing
Genetic reagent (*Drosophila melanogaster*)	CQ-Gal4	Aref Zarin		MN silencing

Contact for Reagent and Resource Sharing:

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Aref Zarin (azarin@bio.tamu.edu).

Experimental Model and Subject Details:

Drosophila melanogaster strains were reared on standard molasses food (agar, cornmeal, yeast, molasses, methylparaben, ethanol, propionic acid, penicillin, streptomycin) at 25°C, 60% humidity. Animals of either sex were used. For behavior experiments, third instar larvae were used. For muscle imaging experiments, late first and early second instar larvae were used.

Method Details:

Behavior Experiments:

For behavior experiments, at least two sessions, performed on separate days, were performed for each genotype. Larvae were only tested once. Sample sizes for global heat + vibration and optogenetic Goro activation escape assays were designed to replicate recently published larval escape assay data[67]. Escape pattern frequency and initial kinematic characterization experiments were conducted using the FIM (Frustrated total internal reflection Imaging Method) (Risse *et al.*, 2013). Videos were acquired at 50 frames per second with a Basler ACE acA2040-90uc four megapixel near infrared sensitivity enhanced camera equipped with CMOSIS CMV4000 CMOS sensor. Camera was equipped with LM16HC-SW lens (Kowa), and BN880-35.5 filter (Visionlighttech). IR diodes (875 nm, Conrad) were used for FTIR imaging and images were acquired using Pylon camera software (Basler). Animals were placed on 0.8% agar surface ~2 mm thick (Molecular grade, Fisher Scientific).

For the global heat and vibration assay, we developed a novel implementation of FIM, where the agar surface was uniformly heated and temperature was read out using a Elitech STC-1000 and two 10 kOhm temperature probes. These probes measured temperature in the FIM table chamber, which was heated by four LUBAN Mini Hot Air Guns, and the agar substrate itself. Third instar w^{1118} larvae were rinsed and transferred to a petri dish with 0.8% agar at least 10 minutes prior to experimentation. Then, larvae were gently transferred with a paintbrush to the agar surface. Vibration was generated using Logitech Multimedia Speakers Z200 with Stereo Sound for Multiple Devices. The composite 1000Hz and wasp sound, played at 100dB, was previously published in Wreden *et al.*, 2017. Behavior was recorded immediately.

For optogenetic Goro activation experiments, larvae were raised in the dark on molasses food with 1 mM all-*trans*-retinal (ATR). These larvae were offspring of 44H10-LexA, Aop-

GCaMP6f; Aop-mcherry, UAS-Chrimson-mcherry female and R69F06-Gal4 male flies. Larval care prior to experimentation was the same as in the global heat assay, above. Two rings of Blue (470 nm) LED lights (WFLS-G30 × 3 WHT, SuperBright LEDs) of 5 inches and 8 inches diameter were placed approximately 5 inches underneath the FIM table. LED brightness was programmed for experiments using custom code in ARDUINO, as the LEDs' pulse width modulation was controlled by an ARDUINO Mega 2560 board. For Goro activation experiments, blue LEDs were active at 2230 uW/mm^2. Larvae that failed to move at all during trials were excluded from any analysis. Bend and roll pattern frequencies were quantified manually by evaluating TIFF stacks in FIJI (https://imagej.net/Fiji).

To assess the roles of different MNs on escape, we performed functional silencing of MN-Gal4 lines. Rolling was induced and imaged in larvae carrying the LexA driver for Goro neurons and Chrimson. Constitutive silencing of MNs was achieved via the expression of the inward-rectifying potassium channel, Kir. 69E06-LexA; Aop-Chrimson-mCherry, UAS-Kir2.1-eGFP females with MN-Gal4 males were crossed for experimental groups, and 69E06-LexA; Aop-Chrimson-mCherry, UAS-Kir2.1-eGFP males with w1118 females were crossed for control groups. For the experimental and control groups involving CQ-Gal4 and BH1-Gal4, L1 larvae were used due to early lethality and reduced transgene expression in later instars, respectively. For other crosses, L3 larvae of the experimental and control groups were used. Kir2.1 lethality when crossed to BH1-Gal4 or decreased expression of CQ-Gal4 in L3 animals. Larvae were raised on molasses food in dark and fed with 1mM all-trans-retinal (ATR) 24 h prior to the experiment. Larvae were picked from food, rinsed, and given a 30-second rest before imaging to avoid any effect of the minimum light exposure during the process. The animals were then placed onto a 1.5% agarose pad in a 10cm round Petri dish and imaged with an iPhone SE (second generation, Apple Inc.) camera at 30fps. For L1 animals, the larvae and agarose gel were placed under a Stemi 508 dissection scope (Zeiss) and imaged by the iPhone SE camera through a microscope phone adapter (Gosky) with 16X built-in eyepiece (the original 10X eyepiece was removed from the dissection scope). Optogenetic activation of rolling was induced with 30 seconds of intense, uniformly applied white light from beneath the agarose gel (Oeegoo 18W square LED ceiling light) after a 10-second dark period.

Rolling performance was scored manually after blinding data. Rolling success was defined as a larva completing at least one 360° roll (after body bending, able to rotate in a single direction from dorsal side up to dorsal side up). Video processing was done with freeware FFmpegtool (https://ffmpeg.org/) and ImageJ (https://imagej.net/, National Institutes of Health, Bethesda, Maryland).

Confocal Image Acquisition:

Muscle activity during crawling was initially measured in Zarin *et al.*, 2019[16]. Briefly, images were acquired using a 10x objective on an upright Zeiss LSM800 microscope. GCaMP6f and mCherry fluorescence values were extracted from a previously acquired forward crawling video and ratiometric values from muscle ROIs (see SCAPE analysis) were subsequently compared to ratiometric muscle ROI values from SCAPE escape images.

SCAPE Image Acquisition:

A custom-built Swept Confocally Aligned Planar Excitation (SCAPE) microscope, extended from that described in Voleti *et al.*, 2019[30] & Vaadia *et al.*, 2019[28], was used to acquire high-speed volumetric imaging of rolling larvae. The system utilizes a custom-made water chamber between the second and third objective lens to accommodate higher collection efficiency over a large field of view with 1.0 NA water immersion objectives throughout. 488nm and 561nm lasers were used to excite GCaMP and mCherry simultaneously, while also activating Chrimson for optogenetics. An image splitter with 561nm long pass dichroic mirror was used with 525/50 and 618/50 filters to split the image into two sCMOS cameras (Andor Zyla 4.2 plus) which enables acquisition of both green and red channels simultaneously with a field of view larger than 1.1mm in the lateral dimension. A 3D field of view with the size of the agar arena was acquired at 10 volumes per second to minimize the motion blur from the larva rolling behavior.

Larvae were raised and fed ATR for optogenetic activation as mentioned above. Larvae were imaged while in a thin 2% agarose chamber filled with distilled water. Each larva was assessed for brightness of muscle fluorophore expression and likelihood of rolling through epifluorescent screening, and the best overall larvae were placed into agar chambers using a fine paint brush. Each chamber was made using 3D-printed molds of different dimensions for late L1 and early L2 larvae of slightly different sizes.

SCAPE Image Processing & Analysis:

SCAPE volumetric data are inherently three-dimensional. To overcome 3D-segmentation difficulties but keep the richness of volumetric muscle data, we developed a custom MATLAB script to generate a 3D-averaged projection of all SCAPE data. Our 3D-averaging method generates separate GCaMP and mCherry 2D images dynamically based on fluorescence histograms of the z-depth at each x,y pixel location, allowing us to isolate and

average signal exclusively from muscle tissue. 3D-averaged projection images were then analyzed using modified versions of the custom MATLAB script from Zarin *et al.*, 2019 and a custom MATLAB script for measuring ratiometric signal and muscle length rapidly using interactive linearly interpolated linescans. For ratiometric signal vs. length measurements, lines were drawn on select frames for example muscles segments A1-A6 on two rolling SCAPE ratiometric movies, and linear interpolations of linescans on intermediate frames were checked frame-by-frame for each movie. Ratio values and muscle lengths were quantified using custom MATLAB scripts. For ROI-based rolling muscle activity analysis, ROIs were drawn manually on non-overlapping portions of each muscle for each segment from A2-A4 on a frame-by-frame basis. Because puncta were present throughout muscles in the mCherry images, pixels with a fluorescence value greater than 90% of the distribution of fluorescence values within the mCherry ROI were discarded in both the mCherry and GCaMP channels to prevent biased ratiometric signal. The ratio of fluorescence between the ROI pixels of the mean depth-projected GCaMP image and the mean-depth projected mCherry image was calculated. The mean of this ratiometric value was extracted for each time point of the ROI, generating individual muscle activity traces. Four roll bouts (one complete revolution based on muscle locations) from four larvae were selected for rolling muscle activity analysis. Subsequent analyses were performed using custom MATLAB scripts.

For comparisons between ratiometric muscle activity values of crawling and rolling, normalized time at maximum ratiometric values were extracted. Statistical analyses were performed as described below. For bend vs. stretch side comparisons of individual muscle activity during rolling, ROI centroids were tracked and, according to the bend and rotation directions of the larvae, divided into four quartiles based on spatial position within the larva's body at each time point. Ratiometric values from the most bent quartile and ratiometric values from the most stretched quartile were extracted and averaged for each muscle. These values were then compiled, z-scored, and averaged across segments and across three roll videos, providing a "bend" and "stretch" ratiometric values for each muscle, shown in the boxplots in **Figure 2A**. Statistical analyses were performed using custom programs written in MATLAB. For muscle activity peak differences, Wilcoxon Rank Sum tests were used.

Electron microscopy and CATMAID reconstructions:

PMN-MN connectome previously reconstructed in Zarin *et al.*, 2019[16] were used to generate **Figure 3 and Figure S4.**

Data and Software Availability:

Behavioral analysis, 3D averaging, signal extraction, and subsequent muscle activity analysis were performed using custom MATLAB scripts that have been included with manuscript submission and are also available here: https://github.com/cooneypc4/larval_escape_manuscript. The source data that support the findings of this study are available from the corresponding authors upon request.

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Figure 1: Muscles demonstrate a segmentally synchronous, circumferential wave of activity during escape (**A**) Schematic illustrating four patterns of escape observed, based on combination of which side larva bends to (left or right) and which direction larva rotates (clockwise or counterclockwise). Translation direction is determined by direction of rotation. Histogram shows frequency of escape patterns observed via sustained optogenetic Goro activation (light gray, n = 64 rolls) and via global heat + vibration stimulus (dark gray, n = 47 rolls). (**B**) SCAPE dual-color stills from single roll bout, showing same muscle appearing in focal plane simultaneously across segments, primarily with increased GCaMP brightness when muscles are on the bent side of the animal. Arrow indicates direction of larva's rotation. (**C**) Confocal dual-color stills from single crawl bout in larva, demonstrating increase in GCaMP brightness from posterior to anterior muscles during single crawl bout. (**D**) Schematic of muscle arrangement in three neighboring hemisegments, color coded by general dorsal, lateral and ventral muscle groups. Blue arrow

indicates posterior-to-anterior propagation of muscle activity. Peak ratiometric muscle fluorescence times during single crawl bout across three segments. During forward crawling, muscles of segment A4 reach peak activity before A3, and muscles of segment A3 reach peak activity before A2 segment, demonstrating the propagation of peristaltic contraction from posterior to anterior segments. Representative homologous muscle across hemisegments and peak activity lines in green for clarity of segmental propagation of activity during crawling. (**E**) Three-segment schematic of hemisegments, color coded by general dorsal, lateral and ventral muscle groups. Blue arrow indicates circumferential propagation of muscle activity. Highest observed ratiometric muscle fluorescence times during single roll cycle with SCAPE across segments A2-A4. Same muscle types across segment a2 to a4 simultaneously reach their peak activity. Muscles are color-coded according to panel D, demonstrating dorsal to ventral to dorsal (circumferential) propagation of muscle contraction. Representative homologous muscle across hemisegments and peak activity lines in green for clarity of segmental synchrony of activity during escape. (**F**) Comparison between time difference of muscles in segments A2-A4 for forward crawling versus rolling (crawl: n = 2 crawls, 2 larvae, 86 muscles; roll: n = 4 rolls, 3 larvae, 372 muscles). Negative values indicate that muscles in A4 are active before muscles in A2 and "0" indicates synchronous contraction. Mann-Whitney U tests were performed between intersegmental roll lag values and intersegmental crawl lag values. P values are indicated as ***p<0.001. Scale bars = 100um.

Figure 2: Intrasegmental muscle activity patterns demonstrate functional antagonism during escape. (**A**) Hemisegment schematic of example measured muscles in heatmap in **B**, color-coded according to activity pattern. Boxplot showing mean z-scored ratio signal for individual muscles from frames when muscles were along the bent side of the larva (black) vs. along the stretched side of the larva (gray), grouped and color-coded along x-axis to correspond with general activity patterns demonstrated in panel **C**. Orange muscles (dorsal longitudinal, DL; dorsal oblique, DO; ventral longitudinal, VL; ventral oblique, VO) show increased ratio signal while along the bent side of the larva, while magenta (lateral transverse, LT) muscles show equivalent ratio signal on average between bent and stretched sides. Purple (ventral acute, VA) muscles show elevated activity on bent side, but the difference between activity in bent and stretched sides is insignificant. (n = 3 rolls, 3 larvae, 280 muscles). (**B**) Example ratiometric SCAPE stills of larval escape. Magenta asterisks highlight lateral transverse (LT) muscles, demonstrating low ratio signal while rotating out of the bend and increased ratio signal while rotating toward the stretched side of the larva. (**C**) Heatmap of z-scored ratio signal across

individually measured example muscles, organized from dorsal (top row) to ventral (bottom row). As in **B**, LT ratiometric traces exhibit an activity pattern counter to other muscles by increasing activity while rotating toward the bend. Color bar to the right of shows range of z-scored ratio signal values. Black indicates frames when muscles were out of the FOV and not measured.

Figure 3: Premotor circuit organization supports circumferential progression of muscle activity observed during escape. (**A-C**) Heat maps representing the normalized weighted-synaptic output of left PMN (rows) onto different subsets of left MNs (**A**), left and right DL MNs (**B**), or left and right lateral/dorsolateral MNs (**C**). Grayed sketches at the bottom of heatmaps indicate the target muscles of MNs in heatmap. (**A**) PMNs presynaptic to DL MNs also establish synapses with MNs innervating the neighboring DO muscles. (**B**) Left PMNs presynaptic to left DL MNs also establish significant number of synapses with right DL MNs. Left and right DL muscles span neighboring regions along the dorsal body midline. (**C**) Left PMNs presynaptic to left lateral/dorsolateral MNs establish negligible number of synapses with right DL MNs. Lateral/dorsolateral on the left and right side are spatially distant from each other. **(D)** Individual PMNs tend to synapse with MNs that correlate with spatially proximal muscles. **(E)** Schematic model showing that to generate clockwise rolling (top panel), muscle contraction progresses counterclockwise (low panel). Circles are cross-section depictions of a larva at different time points. Rectangular shapes around the circumference indicate the body wall muscles. Dark and light green indicate the fully and half contracted muscle, respectively. Purple arrowheads indicate the fixated bend sides in the top panel. The dorsoventral axis rotates clockwise in the top panel, while it is fixated at the bottom panel. The green and magenta oval shapes indicate the excitatory and inhibitory inputs from active PMNs, respectively. Gray oval shapes indicate inactive PMNs. (F) Schematic showing the opposite direction of contraction progression (green arrow) and body rotation (black arrow) in three different roll types not described in panel (E). Note the different location of bend side (purple arrow) in left and right CCW rolls.

Figure 4: Silencing different MN groups leads to different forms of defects in rolling. (**A**) The proportion of L3 animals able to complete at least one complete roll. Silencing Ib MNs innervating ventral longitudinal and dorsal oblique muscles (HB9>Kir) leads to complete failure to perform rolling. Silencing Is MNs alone (27E09>Kir) or in combination with a single Ib MN (RRa>Kir and 27E09+RRa>Kir) had little or no effect on rolling performance. Control animals are 69E06-LexA/+; Aop-Chrimson-mCherry, UAS-Kir2.1/+, while each MN silencing group carries

one or two MN-Gal4 components. N = 23 animals for control, 13 for 27E09>Kir, 16 for RRa>Kir, 16 for 27E09+RRa>Kir, 29 for HB9>Kir. The cartoon below each histogram bar shows the muscles groups whose Ib (light gray), Is (dark gray), or both Ib and Is inputs are silenced.

(**B**) Silencing lateral transverse muscles with BH1>Kir greatly impaired rolling, as 12 out of 14 BH1>Kir animals were unable to complete even a single roll. Silencing Ib MNs innervating dorsal muscles (CQ>Kir) lead to a milder reduction in rolling success rate than combinatorial silencing of Ib and a single Is MN (CQ+RRA>Kir). N = 16 animals for control, 14 for BH1>Kir, 13 for CQ>Kir, 16 for CQ+RRa>Kir, 29 for HB9>Kir.

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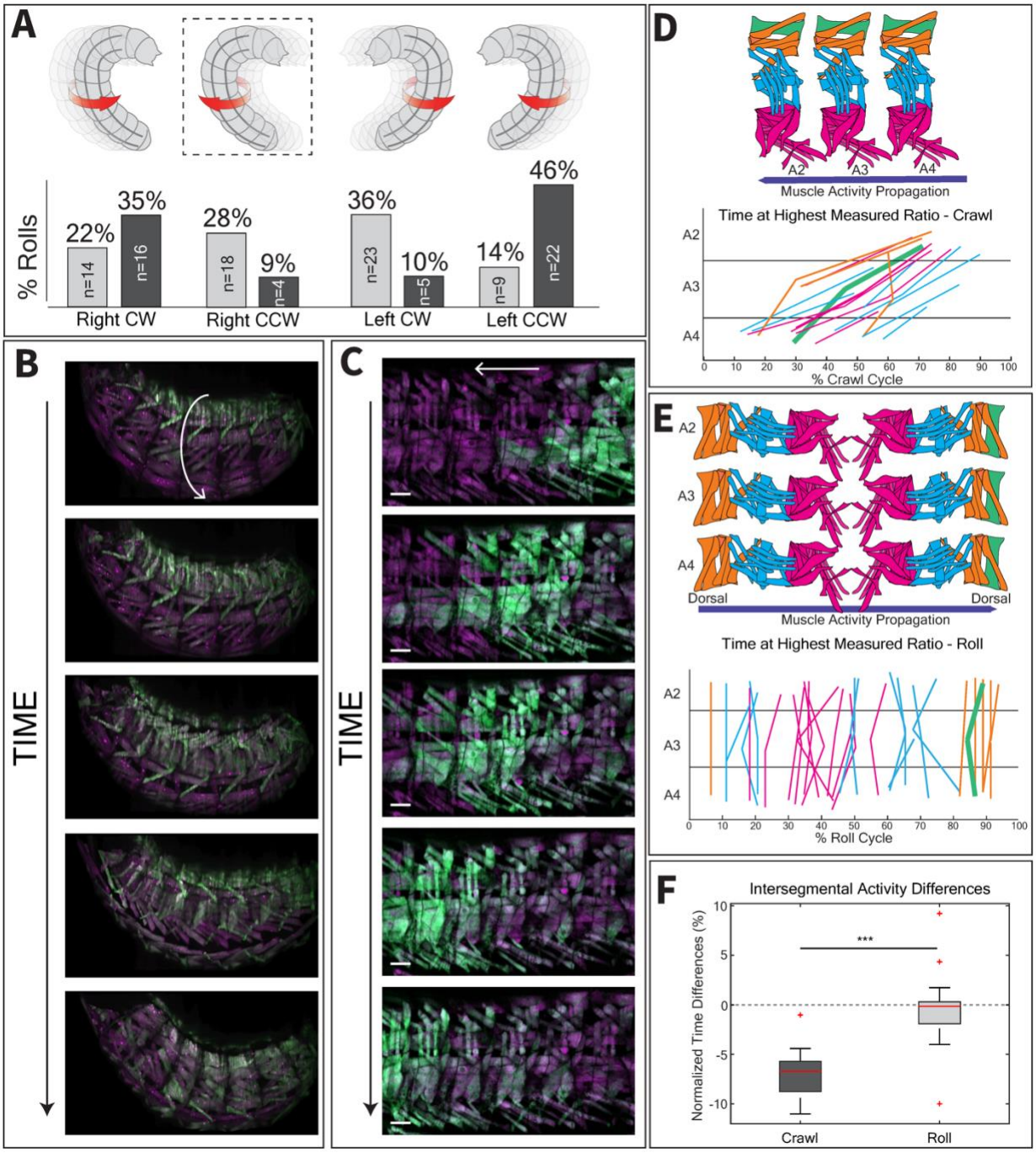

Figure 1: Muscles demonstrate a segmentally synchronous, circumferential wave of activity during escape

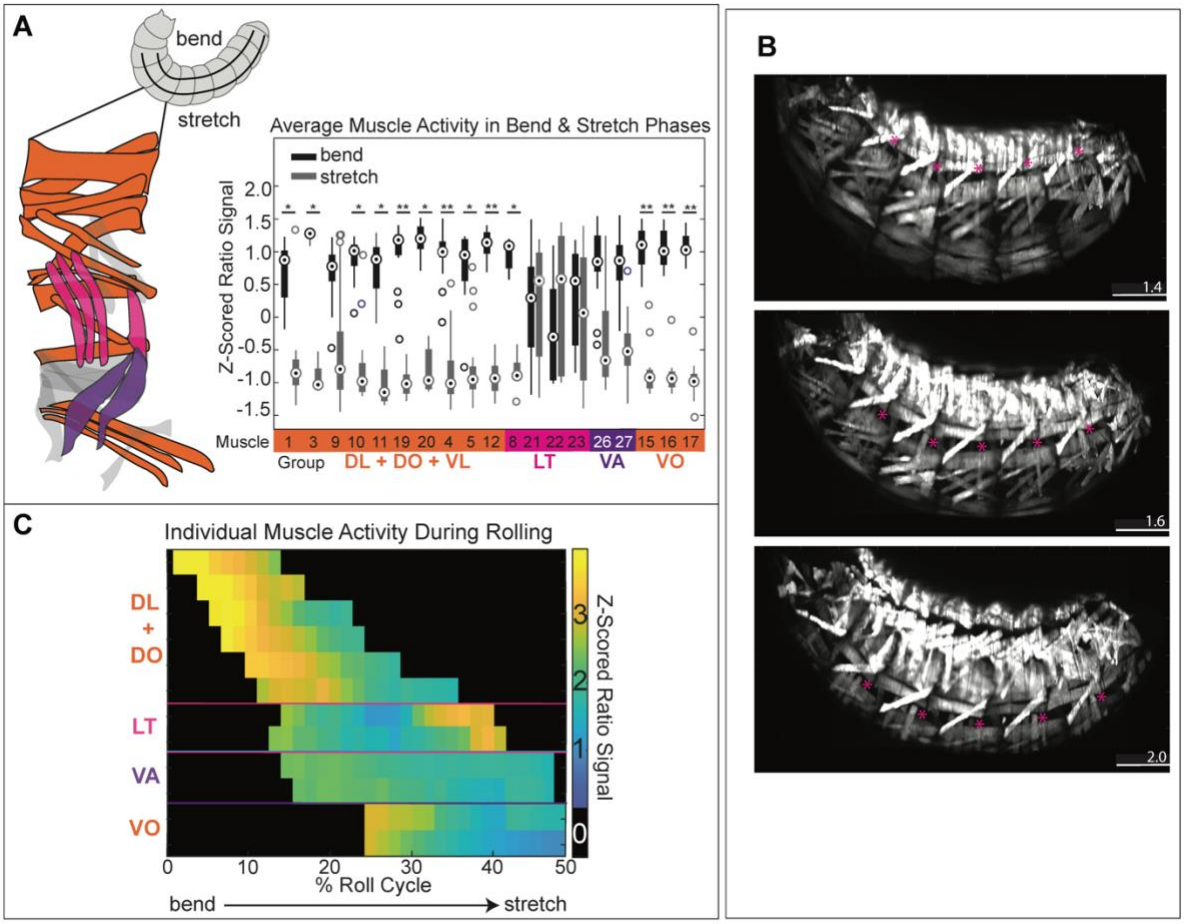

Figure 2: Intrasegmental muscle activity patterns demonstrate functional antagonism during escape.

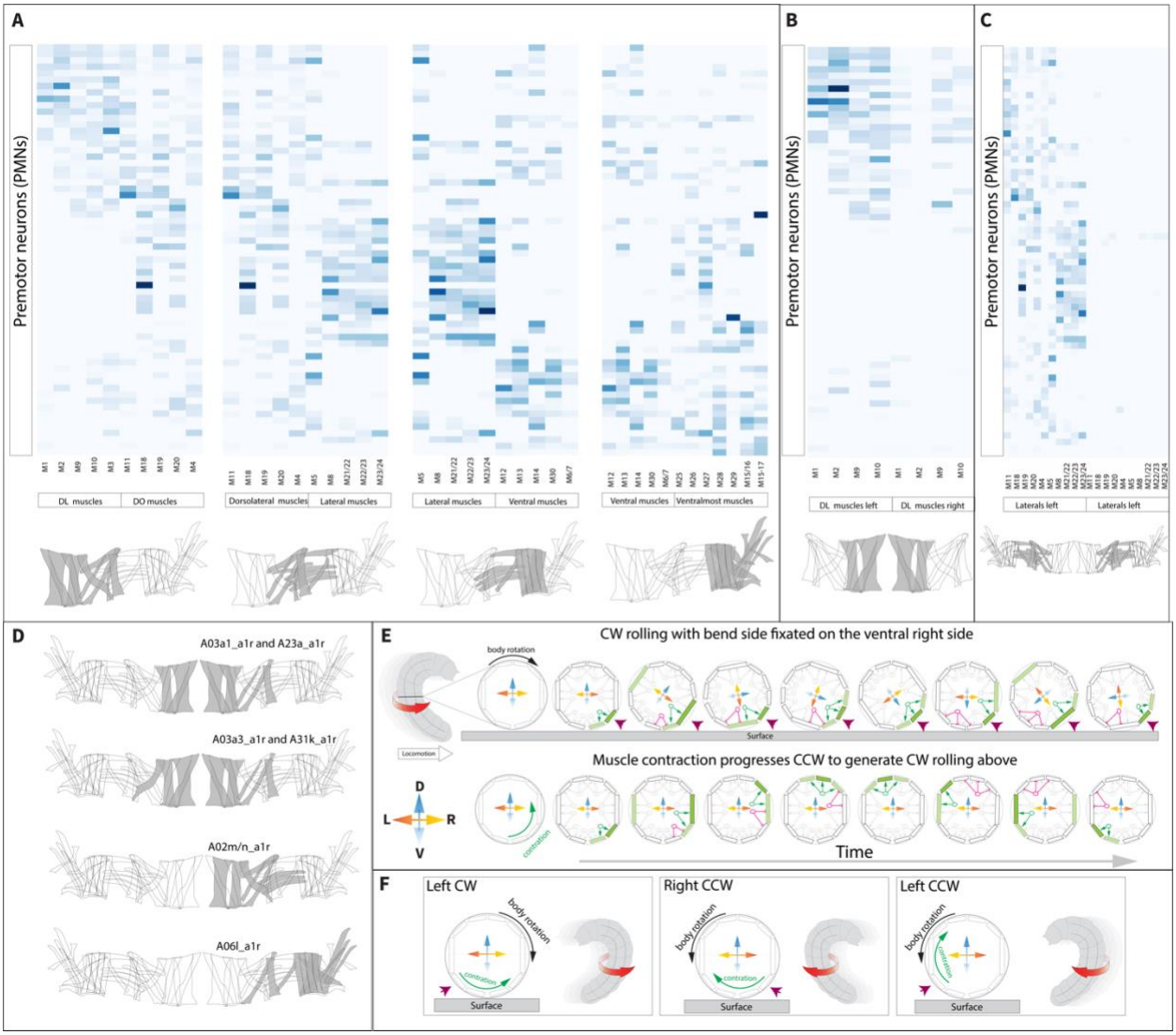

Figure 3: Premotor circuit organization supports circumferential progression of muscle activity observed during escape.

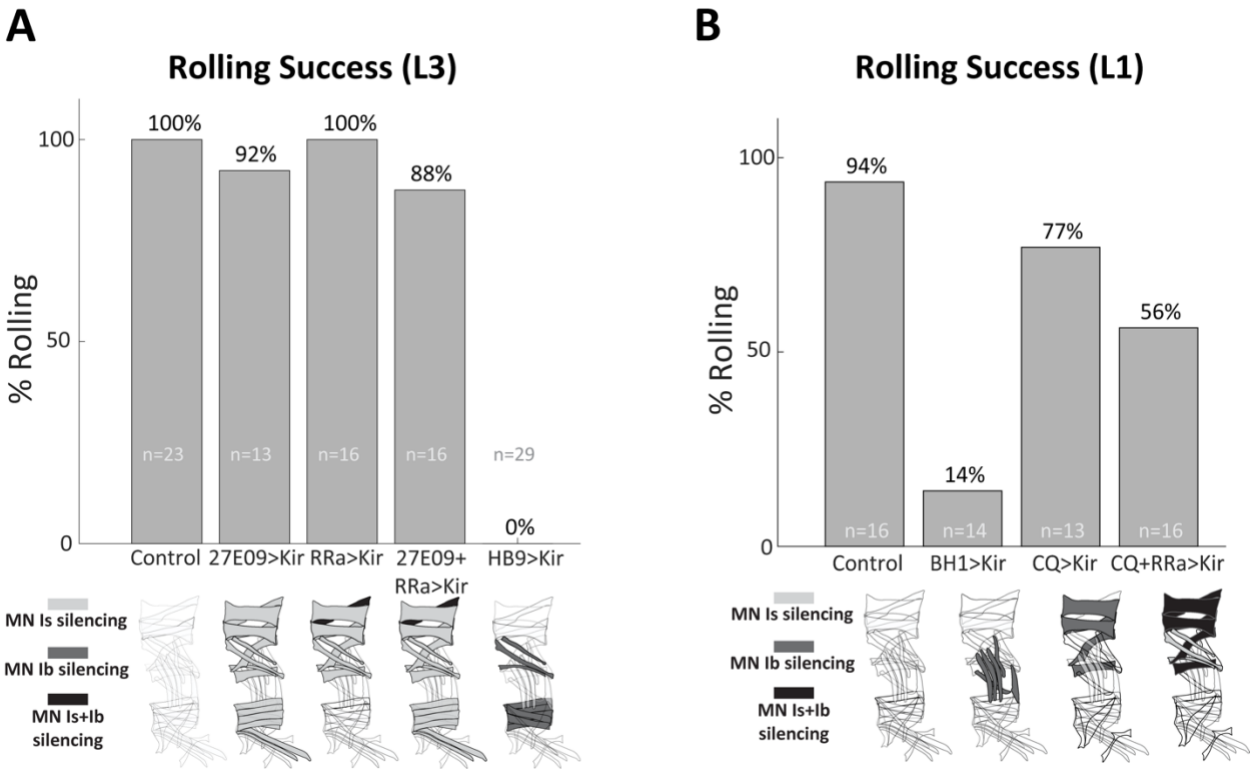

Figure 4: Silencing different MN groups leads to different forms of defects in rolling.