The effect of levamisole hydrochloride on the rate of anterior-lateral movement in *Caenorhabditis elegans*

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I. Introduction

A member of perhaps the most ubiquitous Phylum found on planet Earth (*Nematoda*), *Caenorhabditis elegans* is a non-parasitic nematode, approximately 1mm in length upon full maturation, which exists naturally in the soil of temperate regions of the world (Hope, 1999; Brown, 2003). *C. elegans* has been utilized as a model organism by biological researchers for many years due to its easily-recognizable morphology, brief gestation period, easy maintenance, and lack of need for invasive techniques to observe cellular behavior due to the organism's complete transparency (Hope 1999). In addition to its physiology and cell biology, recent interest in the species' phylogeny and biochemistry has also soared due to the success of the *C. elegans* genome project, completed in 1998. Although it is itself non-parasitic, the study of *C. elegans* has been of great value to the medical world in treating pathologies caused by its genetically- and anatomically- similar relatives (Hope, 1999).

Locomotion in *C. elegans* is produced by 95 body-wall muscle cells arranged longitudinally in the organism's body in two dorsal and two ventral quadrants (Hope, 1999). The activity of these muscle cells is controlled by excitatory and inhibitory nerves that stimulate muscle contraction on one side of the body, while simultaneously inhibiting muscle contraction on the other side of the body (Hope, 1999). These cell-cell interactions result in a bent appearance and sinusoidal movement of the organism; forward motions are the result of posterially-directed waves, and backward locomotion is the result of anteriorally-directed waves.

When the neurotransmitter acetylcholine and drug nicotine are applied to nematodes, muscle contraction occurs (Lee, 1965). The anthelminitic drug Piperazine reduces the muscular response to acetylcholine by interfering with neuromuscular junctions and nerves, effectively paralyzing the worm (Lee, 1965). Nematodes' muscles exhibit a similar response to an anesthetic called levamisole hydrochloride, or levamisole, which is used to treat nematode parasite infections in humans by flushing them from the body (Robertson et al., 1999). At a molecular level, levamisole is a nicotinic agonist, targeting and binding to the nicotine/acetylcholine receptor, a specific class of nematode somatic ligand-gated ion channels called nicotinic acetylcholine receptors (nAChRs). Because it interacts with this receptor differently than acetylcholine and nicotine, levamisole prolongs the duration of the ion channel's opening effectively paralyzing the muscle cells (Robertson et al., 1999).

In the past, researchers have gauged the effects of levamisole on the motility of *C. elegans* by counting the

number of times the body bends per minute (Culetto, et al., 2004). In this study, the effects of the levamisole anesthetic will be assessed by calculating the average rate of lateral (from side-to-side) motility of the anterior end of the organism *in vitro*. This is a behavior easily observed with the aid of time-lapse photography and real-time movie imaging. Based on the fact that levamisole is known to impair the activity of muscle cells through its interaction with nAChRs, it is expected that the worms that are exposed to the levamisole hydrochloride solution will exhibit a measurably decreased rate of lateral motility, compared to those worms that are not. Because levamisole interacts with the nAChRs found in muscle and nerve cells, it is expected that other cellular activity not involving these receptors should not be affected or impaired. It has been qualitatively observed by Bri Jeffrey and I that levamisole is an effective method for immobilizing *C. elegans* at high magnifications for observation of cellular detail and physiological behavior; this report aims to provide quantitative support for this observation.

II. Materials and Methods

- Live *C. elegans* from Ward's and Carolina Biological Supply Companies in Nematode Growth Medium
- Ward's "The Four 'L's' of *C. elegans*" seeding kit
- Levamisole hydrochloride solution, 10 mM
- Flame-drawn Pasteur pipettes
- Bunsen burner
- Transfer pipettes, non-sterile
- Poly-lysine-treated glass coverslips
- Glass microscope slides
- Valap (1:1:1 w/w/w mixture of Vaseline :lanolin: paraffin wax) to seal coverslips to slide
- Kimwipes
- Nikon SMZ645 dissecting microscope
- Nikon Eclipse E400, E200 microscopes for bright field microscopy
- Spot Insight QE Camera
- Sony Digital Interface DFW-X700 Camera
- Spot Advanced imaging software
- BTV Pro imaging software
- Apple G4 Computer with 17" monitor
- Adobe Photoshop

Preparation of Materials

Poly-lysine-treated coverslips were created by placing glass coverslips on several drops of poly-lysine solution in a Petri dish and putting several more drops of the solution on top of the coverslips to completely immerse them in the solution. The cover was placed on the Petri dish and the coverslips were treated with the solution for at least several hours. They were often prepared as much as twenty-four hours before use. Prior to placing them on a double-distilled-water-washed glass slide and the specimen, the coverslips were removed from the poly-lysine solution and rinsed thoroughly with double-distilled water. Excess fluid was removed by placing the corner of a Kimwipe to the coverslip and allowing it to absorb into the fibers; special care was taken not to wipe the slides with the Kimwipes as this would remove the poly-lysine residue.

Flame-drawn Pasteur pipettes were created by placing the tips of standard Pasteur pipettes in a Bunsen burner and pulling on either end. Additional *C. elegans* growth plates were created per instructions and materials in Ward's "The Four 'L's' of *C. elegans*" seeding kit.

Microscope slide mounting of *C. elegans*

To remove the worms from the growth medium, the nematode-containing Petri dishes were viewed at 4x magnification beneath a Nikon SMZ660 dissecting microscope. Using the field of view, flame-drawn Pasteur pipettes were utilized to draw up live worms. Larvae, eggs, any fungal growth, and excess agar were avoided. To ensure that *C. elegans* were successfully removed from the medium, the pipette was examined for the presence of live worms, also

at 4x magnification, beneath the dissecting microscope.

The content of the worm-containing Pasteur pipette was released onto a double-distilled water-cleaned microscope slide covered with at least four small (~1mm x 1mm) coverslip chips arranged so that they were situated at approximately the same height and width of the dimensions of the coverslip. 2-3 drops of 10 mM levamisole (Signor et al., 1999) were added to the microscope slide with a transfer pipette, and mixed with the worm-containing growth medium removed from the Petri dish by simultaneously drawing both solutions up into the Pasteur pipette and releasing them back onto the slide. A double-distilled-water-rinsed poly-lysine-treated coverslip was placed on top of the fluid and glass coverslip chips so that the chips rested between the coverslip and the glass slide, creating a chamber in which the worms were not crushed by the glass. Any excess water was absorbed with the corner of a Kimwipe, and the sides of the chamber were sealed using Valap.

The same procedure was followed for control worms, except 2-3 drops of water were substituted for the anesthetic solution.

Time lapse recording of anterior organismal movement

Worms were selected using bright field microscopy on a Nikon Eclipse E200 microscope. Movies were taken of worm movement using BTV Pro imaging software and Sony Digital Interface DFW-X700 camera at 640 x 480 pixel resolution. Images of unanesthetized worms were taken at 10x and, because the movements of paralyzed worms were much slighter, images of anesthetized worms were taken at 4x.

Before we developed this technique, time-lapse photography, Spot imaging software, Spot Insight QE Camera, and a Nikon Eclipse E400 microscope were used to take images of a paralyzed but twitching worm at 10x magnification.

All cameras, microscopes and software were operated per the protocol on the Imaging Center for Undergraduate Collaboration website.

Measurement analysis and calculation protocol

To measure movement, a straight line was drawn on sheets of white paper, creating a single axis on which to mark the position of the organism. The sheets were then taped on the Apple 17" monitor directly on top of the image to be measured (Borkowski, 2003). For the time lapse-photography of the paralyzed, twitching worm, I observed the movement of the organism over the course of 7 frames at 1 frame/3 seconds, or 21 seconds of movement. For the movies of the paralyzed untwitching worm and the un-anesthetized worm, a twenty-one second clip of video was taken and measurements of change in position were taken as before. To stop the video at exactly three-second intervals, a measurement of the length of the progress bar was taken and divided into 7 equal segments and taped on top of the actual progress bar on the computer screen.

All measurements were taken in millimeters to one decimal place. The position of the worm was determined by measuring the position of the mouth, which is positioned centrally at the anterior end of the organism, relative to the bi-lateral axis drawn on the sheet of paper. The absolute value of the distance between consecutive frames was measured and the average rate of lateral motion per second of the anterior end of the organism with the mouth as a point of reference was calculated. In the higher-magnification image (100x) of the anesthetized twitching worm, where the mouth was magnified to larger than one point on the screen, the organism was measured laterally prior to plotting its position on the bilateral axis to accurately determine the exact position of its center.

Conversions from mm to µm were performed by calculating the actual dimensions of the field of view in mm, measuring the length of the image on the computer screen, and using the ratio of dimensions to determine the real value of the movement of the organism over the time period.

Adobe Photoshop was used to crop images and create scalebars.

III. Results

In all of the collected images of worms, cellular movement inside the organisms' bodies was visible. The average lateral motility of the anterior end of the three organisms studied over the course of twenty-one seconds is displayed below.

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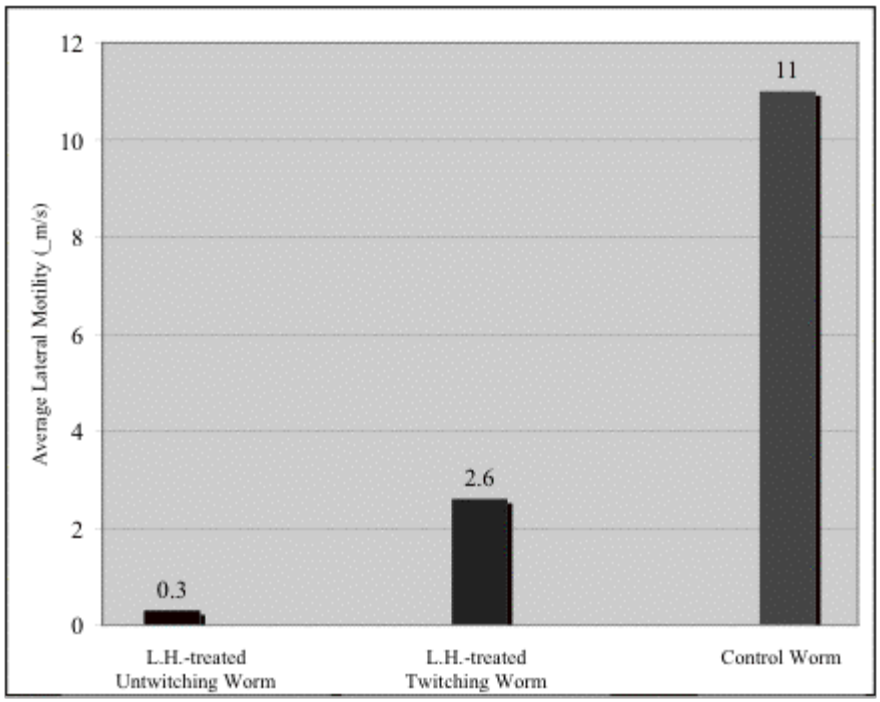
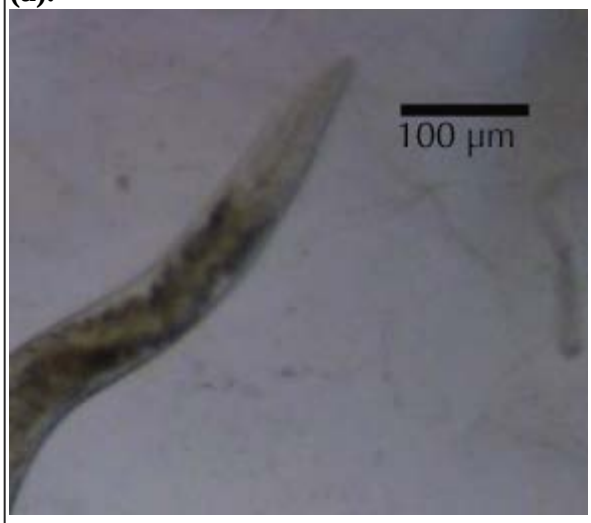


<>**Figure 2.** Average Anterior Lateral Motility Rate in *C. elegans* Treated with Levamisole Hydrochloride (LH) in µm/s

Graphical representation of average rate of anterior lateral motility over a twenty-one second time period, where change in position was measured every 3 seconds. Values are rounded to the lowest degree of uncertainty for the ruler used to measure distances. The graph demonstrates the differences in average (n=7) anterior lateral motility over the twenty-one-second time period, where the control reading was 11 µm/s, the anesthetized twitching worm was 2.6 µm/s, and the anesthetized untwitching worm was 0.3µm/s .

The unanaesthetized control worm showed the greatest rate of anterior lateral motility over the 21-second time frame, 11 µm/s; that is, it moved, on average, the greatest distance between the three-second increments in which measurements were taken out of the three worms. The below pair of images illustrates the difference in position of the control worm at 4x between Frame 5 and Frame 6, where one frame correlates to a 3-second interval. (These particular frames show the most dramatic difference in position of the data set.)

(a).



(b).

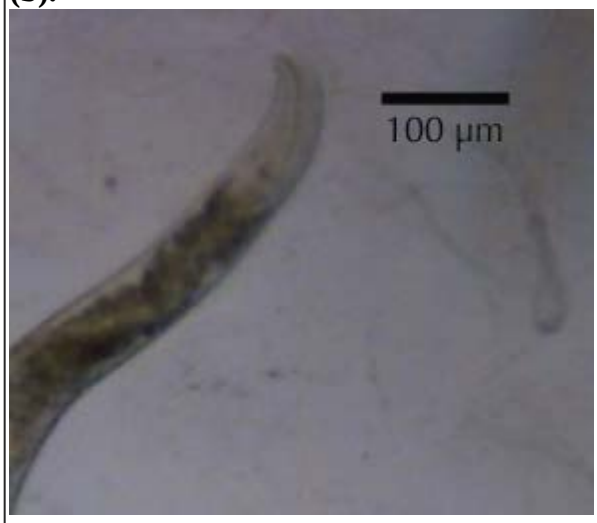


Figure 3.
(a). Position of control worm in Frame 5
(b). Position of control worm in Frame 6
(scale bar = 100 µm; magnification = 4x)

The anesthetized untwitching worm showed the least amount of measurable average rate of lateral anterior motility over the 21-second time frame of 0.3 µ/s; that is, it moved, on average, the least distance between the three-second increments in which measurements were taken out of the three worms. The pair of images below illustrates the difference in position of the anesthetized worms at 10x between Frame 4 and Frame 5, where a frame equals a 3-second interval. (These particular frames show the most dramatic difference in position of the data set. The fact that this anesthetized worm exhibited the lowest overall rate of lateral motility is reflected in the similarity between these images.)

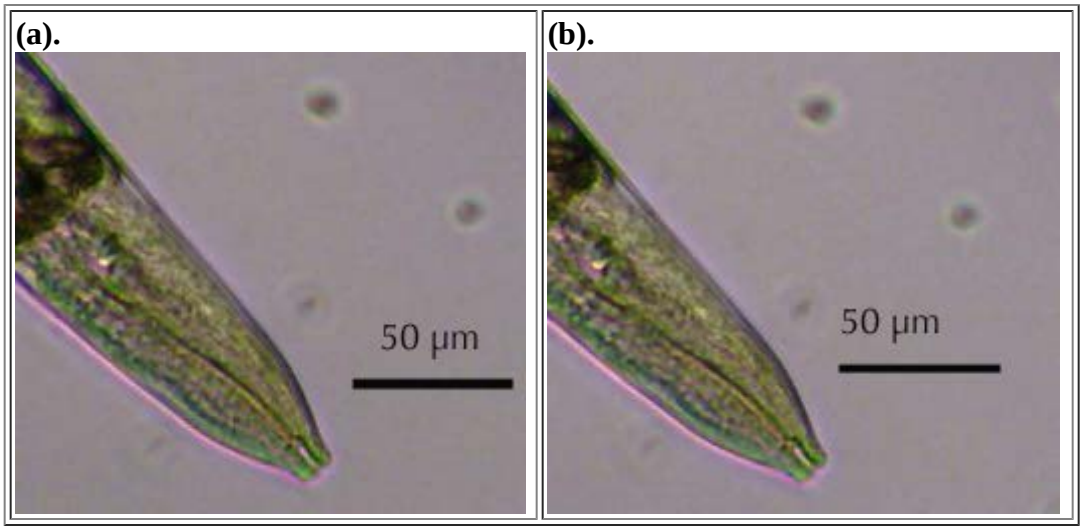


Figure 4.
(a). Position of the anesthetized untwitching worm in Frame 4
(b). Position of the anesthetized untwitching worm in Frame 5
(scalebar = 50 µm; magnification = 10x)

Qualitative observation of anesthetized worms prior to the time that the above images were collected showed that some worms were not as affected by the anesthetic as others, and exhibited a twitching behavior in the levamisole solution on the coverslip. Because this was prior to the data collection developed for the above displayed images, a different imaging system and microscope were used to capture time-lapse images of a twitching worm over the course of a minute. By looking at Figure 2 it can be observed that the anesthetized twitching worm displayed an average anterior lateral motility rate of 2.6 µm/s, a value between that of the anesthetized un-twitching worm and the un-anaesthetized worm.

IV. Discussion and Conclusions

The results of this study support the initial hypothesis that *C. elegans* immersed in a levamisole hydrochloride solution will exhibit a measurably decreased rate of lateral motility compared to worms that are not. By observing the distance that the anterior end of the organism traveled in a lateral fashion over a defined time period and determining the average rate of movement per second from these figures, we see that there is a measurable correlation between exposure to levamisole and a decreased rate of lateral motility *in vitro*. The anesthetized non-twitching worm had the lowest rate of lateral motility, followed by the anesthetized twitching worm, and the un-anesthetized control worm had the highest rate of lateral motility of the anterior end of its body. In terms of percentages, there was a 99.7% difference between the rates of motility in the un-anesthetized worm and the anesthetized non-twitching worm, and a 76.4% difference between the rates of motility in the un-anesthetized worm and the anesthetized twitching worm.

Despite the administration of the anesthetic, *C. elegans* clearly remains alive, as cellular activity was qualitatively observable in all of the images collected. The preservation of cellular activity and simultaneous inhibition

of muscle activity can most likely be attributed to the fact that levamisole specifically targets and impairs the activity of the nAChRs, which are known to exist in neuromuscular junctions and nerves (Lee, 1965). Since it is the stimulation of dorsal and ventral excitatory nerves that causes muscle contraction in *C. elegans* (Hope 1999), it can be speculated that these nerves, specifically, contain nAChRs and were thus specifically targeted and affected.

The rate of anterior lateral motility in the anesthetized twitching worm was 76.4% less than that of the un-anesthetized worm, but its rate of lateral motility was still 88.5% greater than that of the anesthetized un-twitching worm. The behavior of the worm twitching in response to levamisole is an area that requires further investigation. Several recent studies have examined the cellular and molecular explanation for a resistance to levamisole as a therapeutic agent in other nematodes, and have attributed this resistance to a difference in the arrangement of the subunits of the acetylcholine/nicotinic receptor (Robertson et al., 1999). A future area of research could be to investigate the possible connection between the twitching behavior and resistance to levamisole.

Although this study supports the hypothesis that there is a correlation between the rate of anterior lateral movement of *C. elegans*, there is ample room for experimental procedure refinement. Firstly, the sample sizes of one organism per experimental category are insufficient. In order to obtain a more accurate idea of the correlation between the rates of anterior lateral motility, the movements of many worms over much longer period of time should be measured. Additionally, the time period over which the movements of the worms that were studied was somewhat arbitrary; a more comprehensive data set could trace the activities of the worms over several minutes rather than just twenty-one seconds. Various concentrations of levamisole should be experimented with as well to determine what constitutes a lethal dosage, and how long it takes for it to become ineffective. It should be noted that much smaller concentrations were used in the study conducted by Robertson than the one 10 mM concentration used in this procedure; our base concentration was obtained from a study by Signor et al., 1999. In addition, using a standard magnification for all experimental samples would provide a greater degree of accuracy in measuring the movements of the organisms. Although great care was taken to measure the organism exactly in the center, this method almost certainly introduced error into the calculated rates of movement. Finally, variables other than the rate of lateral movement of the anterior end of the organism could be used to asses the effects of levamisole. For example, Culetto et al (2003) counted the number of body bends per minute.

The purpose of this paper was to conduct an in-depth investigation of the cellular mechanisms associated with the paralysis of *C. elegans* from levamisole hydrochloride, and to support a qualitative observation with quantifiable data. From our experience in working with these organisms for over a month, my lab partner and I have both been anecdotally assured of the effectiveness of levamisole as an anesthetic; however the results of this study confirm its efficiency. The non-twitching worms barely move at all in real-time, but certain cellular and physiological processes and movements (e.g. peristalsis) are still observable and appear to be undisrupted by the use of the anesthetic.

The work of my lab partner Bri Jeffrey investigates the effect of levamisole on the contractions of the radial muscles surrounding the pharynx, a process involved in the digestion of food (Jeffery, 2005).

V. Works Cited

Brown, Andrew. (2003). *In the Beginning Was the Worm: Finding the Secrets of Life in a Tiny Hermaphrodite*. New York: Columbia University Press.

Borkowski, R. (2003). *The cell-motility-lowering effects of Microtubule depolymerization in* Amoebae Proteus. <http://icuc.wheatonma.edu:16080/%7Ebio219/2003/rborkows/>

Culetto, E., Baylis, H., Richmond, J.E., Jones, A.K, Fleming, J.T., Squire, M.D. (2003). The *Caenorhabditis elegans* unc-63 Gene Encodes a Levamisole-sensitive Nicotinic Acetylcholine Receptor Subunit. *The Journal of Biological Chemistry*. 279(41): 42476-42483.

Hope, I.A. (1999). C. elegans*: A Practical Approach*. Oxford University Press.

Jeffrey, B.A. (2005). *Muscle contraction in the pharyngeal portion of* Caenorhabditis elegans *is effectively*

anesthetized by (-)-tetramisole hydrochloride. <http://icuc.wheatonma.edu:16080/%7Ebio219/2005/bjeffrey/>

Lee, D.L. (1965). *The Physiology of Nematodes*. Edinburgh: Oliver & Boyd.

Robertson, A.P., Bjorn, H.E., Martin, R.J. (1999). Resistance to levamisole resolved at the single-channel level. *The FASEB Journal*. 13:749-760.

Signor D., Wedaman, K.P., Orozco, J.T., Dwyer, N.D., Bargmann, C.L., Rose, L.S. (1999). Role of a Class DHC1b Dynein in Retrograde Transport of IFT Motors and IFT Raft Particles Along Cilia, but Not Dendrites, in Chemsensory Neurons of Living Caenorhabditis Elegans. *The Journal of Cell Biology*. 147(3): 519-530.