The effect of ethanol concentration on the locomotion speed of *Caenorhabditis elegans*

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Abstract

Ethanol exposure can result in many observable behavioral responses, such as a decrease in speed and lack of coordination. When exposed to ethanol, *Caenorhabditis elegans* (*C. elegans*) may exhibit these behavioral responses. In this study, we examined the effect of ethanol exposure on the speed of locomotion of *C. elegans*. We had four treatments: three solutions containing ethanol at concentrations of 150 mM, 300 mM, 400 mM, and one control comprised solely storage buffer solution. After an exposure time of five minutes in 20 µL of each solution, and an acclimation time of one minute, 30 second videos were recorded with a DinoXcope and used to calculate speed in ImageJ. Our results showed a strong negative correlation ($R^2 = 0.98$) between speed of locomotion and ethanol concentration, with mean speeds of locomotion of *C. elegans* in the 0 mM, 150 mM, 300 mM and 400 mM solutions being 0.50 ± 0.11 mm/s, 0.31 ± 0.17 mm/s, 0.23 ± 0.11 mm/s, and 0.12 ± 0.08 mm/s respectively. A one-way ANOVA test showed a statistically significant difference in the mean speed of locomotion of *C. elegans* in the four treatments: [$F_{(3, 20)} = 9.84$, $p = 1.99 \times 10^{-2}$]. This allowed us to reject our null hypothesis and provide support for our alternate hypothesis that increasing ethanol concentrations decrease speed of locomotion. Ethanol may lead to the increased activation of the SLO-1 protein subunit of the BK potassium channel resulting in the observed lack of coordination and decreased speed of locomotion.

Introduction

Caenorhabditis elegans (*C. elegans*) is a small free-living transparent nematode with a simple nervous system consisting of 302 neurons (De Bono and Maricq 2005). This nervous system has been extensively mapped, which allows *C. elegans* to be used for studies on the neurobiological basis of behaviour in different environments or conditions (Simonetta and Golombek 2007). One such condition is exposure to ethanol, which is a commonly used drug with many well-documented behavioral effects such as lack of coordination and decreased speed (Hull and Bond 1986). It is also known that these behavioral responses are associated with the

effects of ethanol on neuronal proteins; however, the mechanisms behind the action of ethanol on this system are still not fully understood (Ient 2004; Davis *et al.* 2014). A study by Davies *et al.* (2003) on loss-of-function mutants, found that the *slo-1* gene, which derives its name from the *Drosophila* ortholog *slowpoke,* has a central role in the observed behavioural changes following ethanol exposure. This gene, which acts on both neurons and muscles, codes for the SLO-1 protein subunit of the big potassium (BK) channel (Davies *et al.* 2003).

For this experiment, we decided to study the effect of ethanol concentration on the locomotion speed of *C. elegans*. Because ethanol is a depressant, it tends to slow down the movement of organisms, especially at higher concentrations (Graham *et al.* 2008). We modeled our experiment on the above-mentioned study by Davies *et al.* (2003) where the effects of ethanol on various aspects of *C. elegans'* behaviour, including speed of locomotion were studied. In that study, exogenous ethanol concentrations ranging from 0 millimolar (mM) to 500 mM were used. These concentrations were chosen because they correspond to an internal concentration of 22-29 mM, which results in approximately 0.1% blood alcohol, which usually corresponds to intoxication in humans. Davies *et al.* (2003) discuss that the change in movement behaviour is due to the interaction of ethanol and the SLO-1 protein. Ethanol results in an activation of the SLO-1 protein which in turn inhibits voltage dependent calcium channels that limits excitatory neurotransmitter release leading to the inhibition of motor behaviours, including speed of locomotion (Crowder 2004).

Based on the above research, we decided to test the effect of ethanol concentration on the speed of locomotion of *C. elegans* using the following concentrations: 0 mM, 150 mM, 300 mM, and 400 mM. With the above findings in mind, we formed the following hypothesis:

H_0: Increasing ethanol concentrations will increase or have no effect on the speed of locomotion of *C. elegans*.

H_a: Increasing ethanol concentrations will decrease the speed of locomotion of *C. elegans*.

Our proposed biological model can be seen below in **Figure 1.**

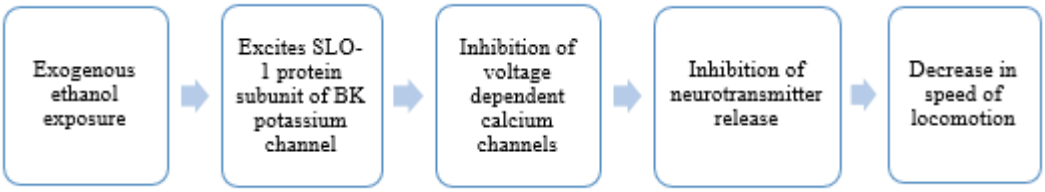

Figure 1. Proposed biological model. Exogenous ethanol exposure excites the SLO-1 protein subunit of the BK channel, which inhibits voltage dependent calcium channels, resulting in inhibition of neurotransmitter release, causing a decrease in speed of locomotion.

Methods

For this experiment, we had a total of four treatments. The control consisted solely of storage buffer solution. The experimental treatments were 150 mM, 300 mM, and 400 mM of ethanol in storage buffer solution. These solutions were prepared for us via serial dilutions using storage buffer to dilute the ethanol. Based on the study by Davies *et al.* (2003) discussed above, we originally planned to use 450 mM as our highest concentration, but were given 400 mM. We used storage buffer for the control and the serial dilutions because we found that *C. elegans* are very sensitive to osmotic pressure changes and hyperosmolarity, which can result in paralysis, and possibly death (Thomas and Wheeler 2006). The storage buffer was the solution used to store the *C. elegans*, so it was prepared with caution to maintain favourable conditions.

During the experiment, we had two Kyowa dissecting microscopes set up. One microscope was set up for transferring *C. elegans* between plates, and the other one was set up with the DinoXcope to record a 30 second video of the nematode after treatment (**Figure 2**).

After setting up, we placed the millimeter side of the ruler under the field of view and took a picture using the DinoXcope. This was done in order to calibrate the number of pixels in one millimeter during analysis.

Figure 2. Kyowa dissecting microscope with the DinoXcope attachment.

Looking through the Kyowa microscope set up to transfer the nematodes, we used a platinum worm pick and proper sterile technique to transfer the nematode from the stock wild type (N2) dish to the clean, *E. coli*-free 60 mm agarose dish. All nematodes chosen were wild-type adults. We attempted to choose ones that had a similar length and width, and ended up with an average length of approximately 1.2 mm, and an average central body width of approximately 0.1 mm. Using a micropipette, we placed 20 μL of the treatment solution on top of the nematode. Using a timer, we exposed the nematode to this solution for five minutes, after which we immediately picked it up using the worm pick and transferred it to a separate 60 mm agarose dish, which we placed under the DinoXcope. Five minutes was chosen, because according to Davies *et al.* (2003), this is the time period it takes in order for the ethanol to reach steady state and have behavioral effects. We then allowed the nematode to acclimate for one minute in the

new agarose dish. We recorded the nematode using the DinoXcope after its acclimation period for 30 seconds. We had six replicates per treatment, for a total of 24 replicates.

For the data analysis, we used an image analysis platform called ImageJ to track the movement of the nematodes. The first thing we did was set the scale for the videos. Using the picture with the ruler, we drew a one millimeter line. The program then calculated the number of pixels in one millimeter.

Next, we used a plugin we had installed into ImageJ called MtrackJ (Meijering *et al.* 2012). This plugin allowed us to track the movement of the nematodes, frame by frame. The head was used as a reference point for each replicate. This gave us the total distance that the nematode moved in millimeters, which was under the column called lens. An example image of the traced path can be seen in **Figure 3**.

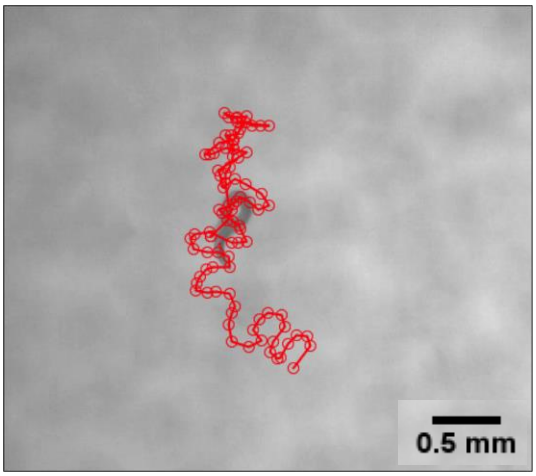

Figure 3. Example of a path traveled by *C. elegans* on agar after exposure to 300mM of ethanol. The path was traced using ImageJ with the MtrackJ plugin.

To calculate the speed, we divided this distance by the length of the video, which ranged from 29-31 seconds. After collecting all of the data for the 24 replicates, we conducted statistical

analysis, by calculating means, standard deviations, 95% confidence intervals, and one-way analysis of variance (ANOVA).

Results

The qualitative observations show that *C. elegans* primarily moved in a sinusoidal S-shape prior to exposure in ethanol. This S-shape movement was also observed after exposure to the control treatment of 0 mM of ethanol and occasionally after exposure to the 150 mM treatment. As the ethanol concentration increased to 150 mM, 300 mM and 400 mM, a more straightened body shape was observed after exposure (**Figure 4**). Also, as the treatment concentrations were increased, a noticeable reduction in total distance travelled by *C. elegans* was observed.

For the two highest concentrations (300 mM and 400 mM) many nematodes appeared to be immobile or moving very minimally after being exposed to the treatment. In the observations, we noted that upon stimulation with a platinum worm pick, the nematodes were in fact able to move, but in an uncoordinated manner and for very short periods of time.

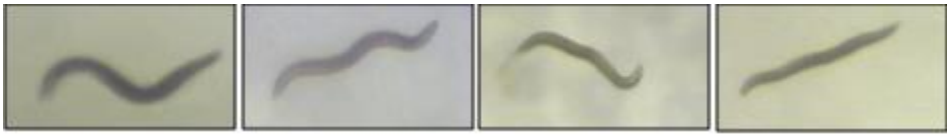

Figure 4. Progressive loss of S-shape posture after exposure to differing concentrations of ethanol. From left to right: 0 mM, 150 mM, 300 mM, and 400 mM ethanol treatments.

In our raw data, some data points were noted as potential outliers. To test for outliers, a Grubbs test was performed and we concluded that there were no outliers in any of our treatments and that a one-way ANOVA could be conducted.

The mean speeds of locomotion of *C. elegans* in 0 mM, 150 mM, 300 mM and 400 mM treatments of ethanol were 0.50 ± 0.11 mm/s, 0.31 ± 0.17 mm/s, 0.23 ± 0.11 mm/s, 0.12 ± 0.08 mm/s respectively (**Figure 5**). These mean speeds were plotted, along with the 95% confidence intervals (CIs), were graphed and a negative linear trend in mean speed as ethanol concentrations increased was observed.

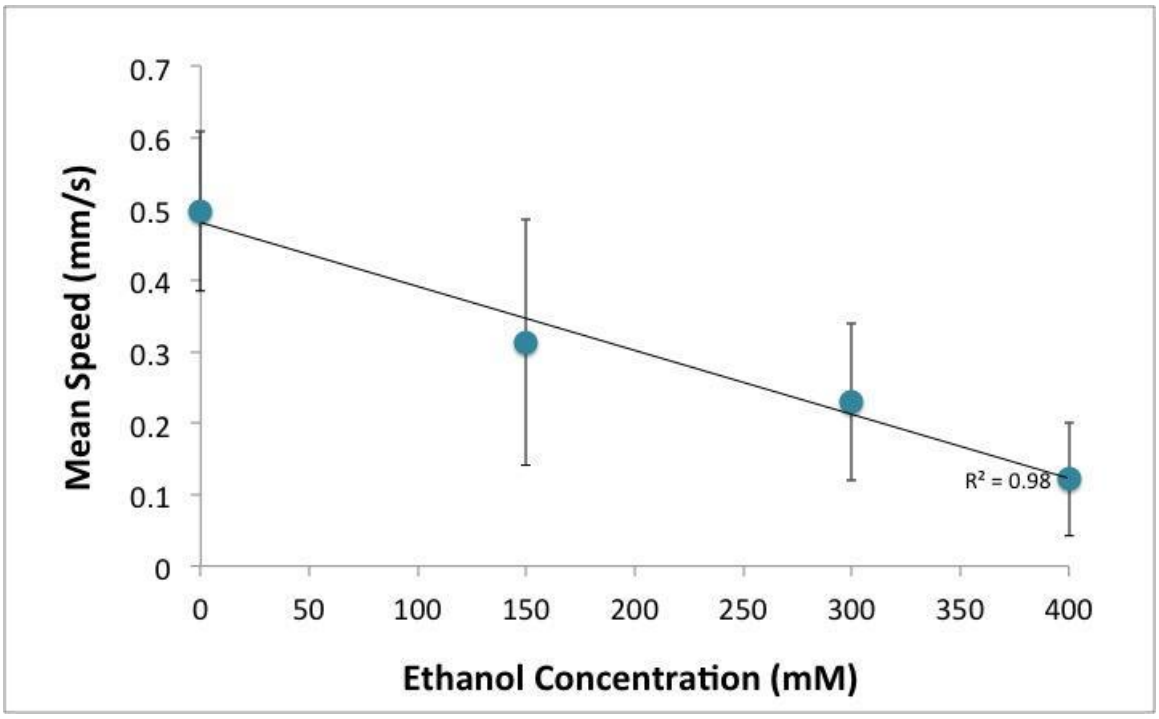

Figure 5. The effect of varying levels of ethanol concentration on the mean speed travelled of *Caenorhabditis elegans* in 30 seconds. The treatments consisted of a 0 mM control with buffer and 150 mM, 300 mM and 400 mM ethanol in buffer treatments. Error bars indicate 95% confidence intervals, n=6. A line of best fit is also shown.

Using a one-way ANOVA test, we found a significant effect of concentration of ethanol on speed of *C. elegans* for the four conditions: [$F_{(3, 20)} = 9.84$, $p = 1.99 \times 10^{-2}$]. With the calculated F-value > F-critical and p-value < 0.05, we concluded that the ANOVA results were significant, meaning that there is a significant difference in the mean speed of locomotion at the different treatment concentrations of ethanol.

To identify which treatments are significantly different, we conducted a Tukey post-hoc test, which compares the means of all treatments in a pairwise manner. This pairwise analysis showed that the mean speed for both the 300 mM and 400 mM treatments were significantly different from the control.

Discussion

After performing a one sided analysis of variance (one-way ANOVA) on mean speed, we were able to reject our null hypothesis and provide support for our alternate hypothesis ($p<0.05$). Based on these statistical results, we were able to confirm that increasing ethanol concentration does result in a lower speed of locomotion in *C. elegans*. When this relationship was graphed, and a line of best fit was fitted into the graph ($R^2 = 0.98$), we found that there is a significant negative correlation between ethanol concentration and speed of locomotion.

When comparing the 95% confidence intervals for the mean speeds in the four treatments, we observed overlap between our 0 mM and 150 mM treatments, as well as our 300 mM and 400 mM treatments. Davies *et al.* (2003) found that *C. elegans* have a low permeability to exogenously applied ethanol. So, a difference of 150 mM and 100 mM between the two low and two high concentrations may not have been large enough to allow significant difference in uptake of ethanol for the different treatments.

After conducting a post hoc Tukey test, the results from the 300 mM and 400 mM treatments were found to be significantly different from the control. Due to the larger exogenous ethanol concentrations, our replicates had higher internal ethanol concentrations, which were observable through behavioral responses such as lack of coordination and slower speed. At 300

mM and 400 mM, body straightening was also observed. The results from both of these responses observed were similar to the results in the study that we modeled our experiment after, where an increase in concentration resulted in a decrease in speed of locomotion, lack of coordination, and increased body straightening (Davies *et al*. 2003).

As mentioned above, our 300 mM and 400 mM treatments resulted in significant internal alcohol concentrations causing intoxication like behavioral responses. Though the exact mechanism and targets for ethanol are not yet fully understood (Mitchell *et al.* 2014), based on the literature searches we conducted we believe that the responses at these concentrations were due to the SLO-1 protein's effect on calcium channels as proposed in our biological model. Davies *et al*. (2003) found that *slo-1* mutations did not affect ethanol uptake and both the *slo-1* mutant and wild type had similar internal concentrations. However, the mutants without the *slo-1* were found to be quite resistant to ethanol. Therefore, based on this information, we believe that lack of coordination and decrease in speed is caused by a mechanism at the molecular level as seen in our biological model in **Figure 1**.

The *slo-1* gene encodes for a protein subunit of the BK channel called SLO-1. This protein is pore-like and works to repolarize presynaptic motor neurons, by pumping potassium (K^+) out of the presynaptic terminal (Crowder 2011). In order to cause neurotransmitter release, voltage gated calcium channels cause a flux of calcium (Ca^{2+}) into the nerve terminal, which can cause the exocytosis of the neurotransmitter (Crowder 2011). However, the SLO-1 subunit causes a negative feedback by causing a flux of K^+ out, which inhibits the influx of Ca^{2+} and also then inhibits the release of the neurotransmitter (Davies *et al*. 2003; Crowder 2011). Davies *et al*. (2003) also found that ethanol seemed to increase the frequency of the SLO-1 channel opening directly in the motor neurons. Ethanol has a similar effect on this conserved ion channel

in species ranging from invertebrates to humans, which allows for *C. elegans* to be used in a variety of studies to better understand how ethanol affects behavior and genetics (Davies *et al*. 2003).

Throughout the experiment, we had some of our replicates die during the transferring process, and some that just did not move. Because of this, we had to discard and replace them with new replicates. Since our control group needed to be the same, all of our replicates and data could only be gathered in one lab session day. For this reason we were only able to record six replicates per treatment. Also, due to the constraint on time, we were not able to allow longer exposure times for our replicates, or a longer acclimation period. Davies *et al.* (2003) had an exposure time of 22 minutes.

Though our results are similar to the Davies *et al.* (2003) experiment, our shorter exposure time could have resulted in a lower intake of ethanol. Other biological variations such as size or age of each replicate nematode, genetic makeup, and tolerance levels among replicates may have resulted in some uncertainty. For instance, larger nematodes may have had a higher tolerance and we selected only obvious adults. To account for this variation our replicates consisted of adult *C. elegans* that were on average approximately 1.2 mm in length.

Conclusion

This experiment analyzed the effect of ethanol concentration on the speed of locomotion of *C. elegans*. Based on our one-way ANOVA results, we are able to reject our null hypothesis and provide support for our alternate hypothesis that increasing ethanol concentration resulted in decreased speed of locomotion. Ethanol acts on the SLO-1 protein subunit of the BK potassium channel, which inhibits behaviours such as speed of locomotion. Ethanol is thought to have a

similar effect on the BK potassium channel in organisms ranging from invertebrates to humans, and therefore *C. elegans* can provide insight into intoxication on both behavioural and molecular levels.

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