What can a Worm Learn? Nonlinear Categorization by *Caenorhabditis elegans* using a Hydrochloric Acid Gradient

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Microorganisms have been utilized to study the human nervous system for decades. However, the computational capabilities of the neural networks of microorganisms are often not well understood. The goal of this experiment was to determine the level of neural complexity in the microorganism, *Caenorhabditis elegans,* by examining whether its neural networks are capable of learning categories that are not linearly separable. *C. elegans* were once thought to possess only a fixed neural network, but then their ability to learn associations and simple linear classifications was discovered. No microorganism is known to have the next level of computational capability, that of performing nonlinear classification. Here, it was hypothesized that *C. elegans* would be able to learn nonlinearly separable classes. This was tested by setting up three levels of an HCl gradient and associating them with food, in a one-dimensional nonlinear classification problem. Positive and negative associations between HCl levels and the presence/absence of food could only be learned through a nonlinear decision boundary. *C. elegans* were put into petri dishes with three distinct HCl concentrations of 100µM, 200µM, and 300µM. During training, 100µM and 300µM values were associated with *E. coli* (positive category), while 200µM was associated with the absence of *E. coli* (negative category). The learning was then tested in separate dishes by examining the movement of *C. elegans* towards positively or negatively trained concentrations. In a second experiment, the reverse association (100µM and 300µM negative; 200µM positive) was also established through training and tested. The results showed that *C. elegans* were able to learn these positive and negative associations, thus acquiring a nonlinear decision boundary (100/300 positive test: x-squared = 24.06, $p < 0.001$; 100/300 negative test: x-squared = 7.5958, $p < 0.023$). This demonstrates, for the first time, that the neural networks of *C. elegans* possess sufficient computational complexity, requiring at least three layers of neurons, to learn nonlinear classification. This advances our understanding of the computational sophistication of the neural networks of even the simplest microorganisms.

Introduction

To understand complex organisms such as humans, it is often useful to first examine organisms such as *Caenorhabditis elegans* or *Escherichia coli*. Since 1989, extensive research has been conducted to identify the structure and capabilities of the *C. elegans* brain[1].

Previous research has demonstrated the ability of C. elegans to learn simple associations[2,3,4,5]. *C. elegans* can be thought of as a linear classifier, where its neural network can separate two categories of sensations with a straight line. Here, it is asked whether the neural networks of *C. elegans* are capable of learning a nonlinear function. Historically, this distinction is very important, as nonlinear neural networks are much more powerful than linear ones. After the basic structure of a neuron was discovered by McCulloch and Pitts[6], psychologist Frank Rosenblatt[7] laid out the foundation of implementing neurons on computers. Rosenblatt[7] discusses a simplified mathematical model in which the computer takes a set of binary inputs, which represent the output of neurons connected to the present neuron, multiplies each input by weighting, and then takes the end value of each input and thresholds to output a 1, in which case the neuron fires, or 0, in which case the neuron does not fire[8]. In 1969, Minsky and Papert demonstrated that the perceptrons in a one-layer network would not be able to complete one of the most basic Boolean functions, the XOR function[9]. The reason for that is that the network above can only differentiate between two subjects when a single line can be drawn to divide them, whereas XOR is a nonlinearly separable function. The third layer of neurons, between the input and output layers, is required to learn this nonlinear classification. Artificial neural networks in use today, such as Deep Neural Networks, have many such layers, which allows them to learn complex nonlinear functions.

XOR is a two-dimensional problem (two inputs, one output). Here, we developed a one-dimensional problem that requires a nonlinear classification for testing the abilities of *C. elegans*, to make the experiment tractable. *C. elegans* were used due to their ability to learn an association[2,3,4,5] and because of the extensive research done on the structure of their brain[1,10]. *Escherichia coli* was chosen as a food source since multiple papers have suggested that *C. elegans* naturally prefer *E. coli* and thus would make it the best bacteria to feed to the *C. elegans*[11,12]. *C. elegans* have also been identified to have good motile movement[13].

The independent variable is exposing *C. elegans* to multiple HCl concentrations, which is known to have a negative (aversive) association[14]. There are three levels of the independent variable 100 µM, 200 µM, and 300 µM[15]. The other component to the variable is the ability of the *C. elegans* to learn the association[2,3,4,5] between the hydrochloric acid gradient and *E. coli*. Previous research has shown that *C. elegans* can learn an association[2,3,4,5]. Ghosh, Jin, and Nitabach[4] (2016) demonstrated that *C. elegans* are capable of detecting pigmented food sources such as *E. coli*. The *C. elegans* would learn that they would be rewarded with food if they would go to the pigmented food and not rewarded otherwise.

The purpose of the experiment was to find the cognitive capabilities of *C. elegans* in regards to linearly separable judgment and nonlinearly separable judgment. The experiment is testing the capability of *C. elegans* to learn a positive association with 100 µM HCl and 300 µM HCl but no association with 200µM HCl concentration. It can be connected to the cognitive capabilities of humans and possibly suggest the basic minimum number of neurons that are required for higher levels of thinking.

It was hypothesized that *C. elegans* could successfully learn a positive correlation with 100 µM HCl and 300 µM HCl then they would gravitate toward the ends of an HCl gradient if offered based on previous studies that have shown that *C. elegans* have an exceptional ability to learn an association[2,3,4,5].

The *C. elegans* were put into Petri dishes with three distinct HCl concentrations and underwent 6 trials of memory, 3 trials with 100 µM and 3 trials with 300 µM. Figure 1 demonstrates the three tests that were used, (a) represents the proof of concept test with only 2 different sections. (b) and (c) represent the experimental test with three different sections and curved learning demonstrated. After the data was recorded, statistical analysis was conducted using Pearson's Chi-squared test to find the p-values of the data.

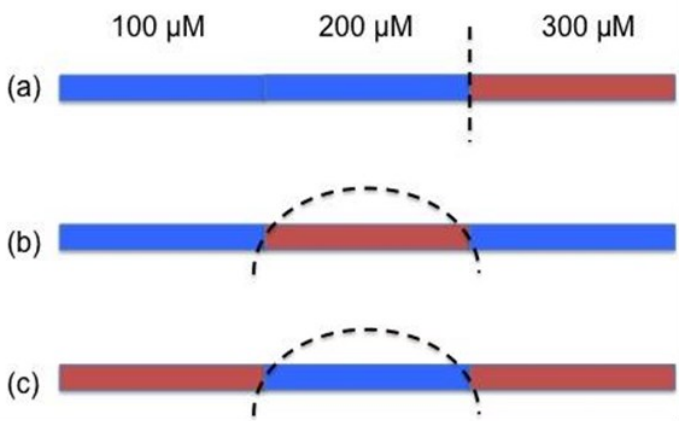


Figure 1. One-dimensional learning problem created with three HCl concentrations. Blue represents positive association category (presence of food) and red is negative association (absence of food) with levels of HCl. (a) Linearly separable case, where the two categories can be separated with a straight line. (b) A nonlinearly separable case, where a nonlinear boundary is needed to separate the two categories. (c) A reverse of the nonlinearly separable case.

Methods

There were two components of experimentation: the initial learning by the *C. elegans* and then testing the learned association using the same *C. elegans*. It was important to keep track of the same *C. elegans* that were used to make sure that the *C.elegans* learned the association. The three HCl concentrations which were incorporated into the Petri dishes were 100 µM, 200 µM, and 300 µM. These three gradients were chosen based upon Gruber et al.´s study[15] which used HCl concentrations ranging from 200 µM to 500 µM. However, it is important to note that 5-fluoro-2′-deoxyuridine (FUdr) Gruber et al.[15] has a higher pH and was more tolerable for the *C. elegans* to survive compared to the HCl (pH of 3.01) they were exposed to in this experiment. First, two different Petri dishes were prepared by elevating one side of the petri dish so that the bottom of one side is level with the top of the opposite side[16]. For one of the Petri dishes, the dish was filled with melted agar with an HCl concentration of 100 µM. The agar was poured in such a way that it formed a triangular wedge of agar[16]. The other petri dish was filled with melted agar with an HCl concentration of 300 µM and looked similar in appearance. Both plates were then laid flatly and melted agar with a concentration of 200 µM was poured on top to provide the chemical gradient. Because agar hardens at a lower temperature (<30°C) than it melts (>60°C) the first triangular wedge (100µM or 300µM) is not disrupted as the second wedge (200µM) is poured[16]. Figure 2 demonstrates this process visually. Each concentration was made in 30 mL batches to fill the entire plate at an angle. The agar is standard Nematode Growth agar but contains 3, 6, or 9 mL of 1 M hydrochloric acid. The 3 mL corresponds to the 100 µM gradient, 6 mL corresponds to 200 µM, and 9 mL corresponds to 300 µM HCl concentration. The natural *E. coli* was ordered from Carolina.com. The *E. coli* was grown on Nematode Growth Medium[17] for both the control group and the experimental group.

Each type of plate was made twice; one plate was used for experimentation, while the other was used for the control group. The *C. elegans* started their training by being soaked in the HCl solution (100 µM for the 100/200 proof of concept test and 300 µM for the 200/300 proof of concept test) for less than one second. 20 µL of the HCl gradient was added each time for the association. The *C. elegans* were then immediately placed in a petri dish seeded with *E. coli* for 1 minute. The *C. elegans* were then taken off of the plate, and the previous steps were repeated two more times to instill association retention[14]. The minimum of three repetitions allowed *C. elegans* to learn the association, but not die before testing the learned association. 20 uL of M9 Buffer was added after the *C. elegans* were fed in to wash off the previous HCl gradient.

The same process was repeated; however, instead of training them for a 100µM HCl solution, they were trained to associate with a 300 µM HCl solution. This training was completed for the two proof of concept tests, which were the 100/200 gradient and the 200/300 gradient. For the experimental/nonlinearly separable association, there were mixed trials of 100 and 300 where there were three trials of 100 and three trials of 300 interspersed. The sequence of learning trials is as follows: 100, 300, 100, 300, 100, 300. Time in the *E. coli* dish, or feeding dish, was shortened to 30 seconds to enable the *C. elegans* to survive through all of the trials and the learning association test. An approximately 1 cm^3 cube of *C. elegans* was used for each experimental test.

The test of learning was done by creating a petri dish in the same way as the process detailed earlier. However, after the dish was poured and the learning trials had been completed by the *C. elegans*, the *C. elegans* were put in a separate 100-200, 200-300, or 100-300 µM HCl gradient petri dish and after 1 minute their positions were recorded. To ensure that the *C. elegans* had equal opportunity to go to 100 or 200 and 200 or 300 in the 100/300 or 200/300 plate, the *C. elegans* population was split in half with 500 uL of *C. elegans* being put approximately a third away from the right edge of the dish, and the other 500 uL being put a third away from the left edge of the dish.

The measure of the nonlinearly separable judgment learning was the direction they traveled in a hydrochloric acid gradient petri dish. The number of *C. elegans* in each gradient was recorded data and corresponded to how well the *C. elegans* learned the nonlinearly separable association. The position at 1 minute after being put in the dish is the recorded position of *C. elegans.* Figure 3 represents the methods information visually.

Results

For the control group of the 100/200 HCl gradient, 5 out of 30 *C. elegans* were found in the 100 µM area. The other 25 were found in the middle of the plate. For the control group of 200/300 HCl gradient, 15 out of 30 *C. elegans* were found in the middle of the plate while the other 15 were found in the 300 µM HCl gradient. For the control group of 100/300 HCl gradient, 20 out of 30 of the *C. elegans* were found in the 200 µM gradient while the other 10 were found in the 300 µM gradient. No *C. elegans* was found in the middle of the plate. This portion of the experiment was repeated because the first time, all of the *C. elegans* died. The control groups are represented by the first three columns in Table 1.

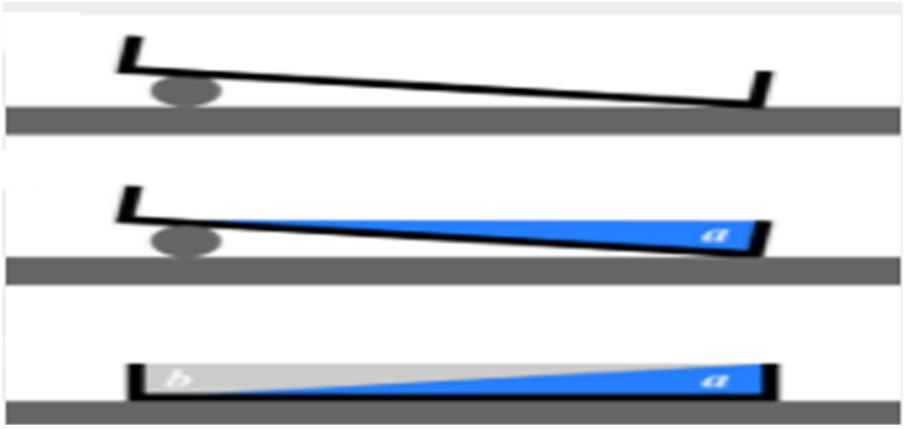


Figure 2. A visual representation of how the agar plate was poured. The blue segment represents 100 µM or 300 µM while the grey segment represents 100 µM (only in the 100/300 plates) and 200 µM (used in both proof of concept plates).

Figure 3. Experimental Design Diagram

Title: The effect of a hydrochloric acid gradient on the evaluation of *Caenorhabditis elegans* nonseparable judgment			
Hypothesis: ● *C. elegans* would demonstrate a nonlinearly separable positive association with the higher and lower values of an HCl gradient and food, because they would be able to grasp the correlation between food and hydrochloric acid gradient learning.			
IV: Molarity of hydrochloric acid			
Levels of IV:	100 µM	200 µM	300 µM
Number of trials (1 trial= 1 *C. elegans*)	~30	~30	~30
DV: The direction traveled by the *C. elegans* (Reported in the number of *C. elegans* in each side of the dish)			
Constants: Amount of light[13], Amount of *E. coli* for consumption (5 mL), Agar gel, petri dish, and same wait period between testing.			
Control: *C. elegans* on Nematode Growth Medium[17] exposed to same hydrochloric acid petri dishes but with no training and put on the same HCl gradient plates.			

Table 1. The effect of an HCl gradient on the evaluation of *C. elegans* nonseparable judgment

Molar Concentration of agar (reported in μM) or state of the *C. elegans* when found	C - 100/200	C - 200/300	C - 100/300	PC - 100/~~200~~	PC - ~~100~~/200	PC - ~~200~~/300	E - 100/~~200~~/300	E - ~~100~~/200/~~300~~	Total
	Number of C. elegans found in each HCl concentration (N = 30 for all groups)								
100	5	0	0	20	7	0	13	5	50
200	0	15	20	0	13	13	4	21	86
300	0	10	10	0	0	17	13	4	54
Dead	0	0	0	10	0	0	0	0	10
None (represents no movement from place put in)	25	5	0	0	10	0	0	0	40
Total	30	30	30	30	30	30	30	30	240

C represents the control group, PC represents the proof of concept trials, and E represents the experimental trials. The gradient striked through was the gradient that had a negative association.

For the proof of concept 100/200 test, 20 out of 30 of the *C. elegans* were traveling in the direction of the 100 μM gradient at the time of death, while approximately 7-10 of the remaining *C. elegans* were found dead. For the opposite proof of concept 100/200 test, 200 μM was the positive stimulus. Seven *C. elegans* was found in the 100 μM gradient, 13 were found in the 200 μM gradient, and 10 did not move from the position they were put in. Graph 1 shows the comparison of both 100/200 proof of concept tests to the 100/200 control group. For the proof of concept 200/300 test, *C. elegans* were located in the 200 μM and 300 μM gradient. Eight of the 30 *C. elegans* were found in the 200 μM gradient and 17 of 30 were found in the 300 μM gradient. Five of the 30 *C elegans* were found dead. Graph 2 shows the 200/300 proof of concept test compared to the 200/300 control group. For the experimental 100/300 test, *C. elegans* were found both in the 100 μM and 300 μM gradients and a few were located in the 200μM gradient. Thirteen of the 30 *C. elegans* were located in the 100 μM gradient; 13 were located in the 300 μM gradient, and 4 were located in the 200 μM gradient. In contrast, where the only positive stimulus was 200 μM, 5 out of 30 *C. elegans* were found in the 100 μM gradient, 21 were found in 200 μM gradient, and 4 were found 300 μM gradient. This test was repeated because only two *C. elegans* survived the first time. Graph 3 shows both 100/300 experimental groups compared to the 100/300 control group. The experimental groups are represented by columns 4-8 in Table 1.

The difference in the results for the proof of concept 100/200 test versus the 100/200 control group was significant, X2 (2, N= 30) = 14, $p < 0.001$. The chi-squared and p-value were achieved by using an online rcode and plugging in the two sets of values; the control group acted as the expected values while the test group was the observed values. A visual representation of the 100/200 proof of concept is as follows:

```
chisq.test(rbind(c(5,0,25), c(20,10,0)))
```

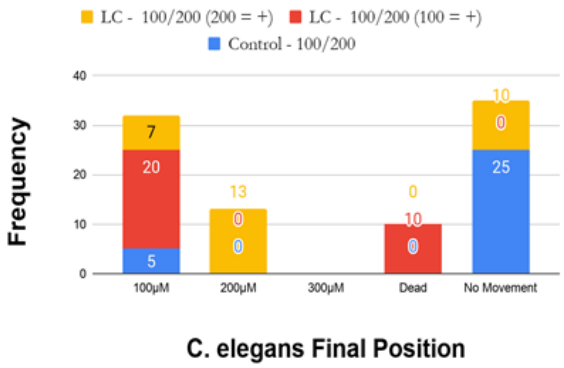
which output the following:

```
data:  rbind(c(5, 0, 25), c(20, 10, 0))
X-squared = 44, df = 2, p-value = 2.789e-10
```

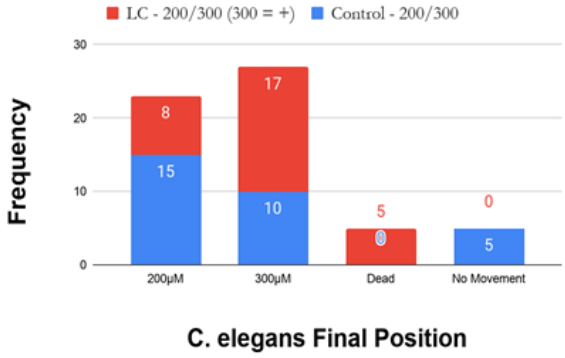
(also shown in Appendix A as Figures 4 and 5). Figure 4 represents the inputs and Figure 5 represents the outputs. The degrees of freedom were found based upon the r code and double-checked by hand. The difference in the results for the opposite proof of concept 100/200 test versus the 100/200 control group was significant, X2 (2, N=30) = 19.76, $p < 0.001$. The difference in the results for the proof of concept 200/300 test versus the 200/300 control group was significant, X2 (2, N=30) = 13.945), $p = 0.002981$. The difference in the results for the experimental 100/300 test versus the 100/300 control group was significant, X2 (2, N=30) = 24.06, $p < 0.001$. The difference in the results for the opposite experimental 100/300 test versus the 100/300 control group was significant, X2(2, N=30) = 7.5958, $p < 0.02242$.

Discussion

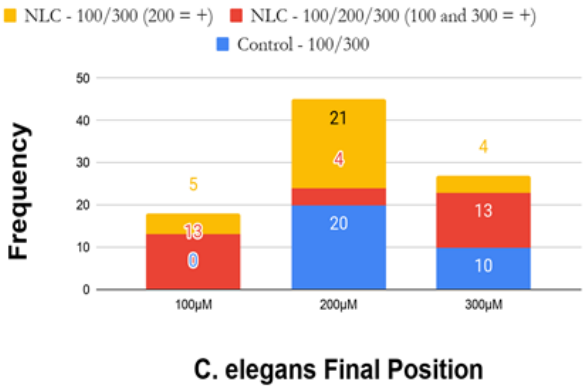
The purpose of this project was to find the cognitive capabilities of *C. elegans* with regards to linearly and nonlinearly separable classification. This was tested by devising a one-dimensional learning problem that required a nonlinear decision boundary. The capability of *C. elegans* to learn a positive association between 100 μM HCl, 300 μM HCl, and *E. coli*, but no association with 200 μM HCl concentration (thus maintaining the



Graph 1: This graph represents the 100/200 proof of concept test. None represents *C. elegans* that did not move from the start location. Dead represents *C. elegans* that died. Each µM each HCl gradient. C represents the control group and PC represents the proof of concept groups. The red PC represents the 100/200 proof of concept test. The green PC represents the opposite proof of concept 100/200 group.



Graph 2: This graph represents the 200/300 proof of concept test. None represents *C. elegans* that did not move from the start location. Dead represents *C. elegans* that died. Each µM each HCl gradient. C represents the control group and PC represents the proof of concept group.



Graph 3: This graph represents the 100/300 experimental test. None represents *C. elegans* that did not move from the start location. Dead represents *C. elegans* that died. Each µM each HCl gradient. C represents the control group and E represents the experimental groups. The red E represents the 100/300 experimental test. The green E represents the opposite 100/300 experimental test.

```
chisq.test(rbind(c(5,0,25), c(20,10,0)))

chisq.test(rbind(c(5,0,25), c(7,13,10)))

chisq.test(rbind(c(15,10,0,5), c(8,17,5,0)))

chisq.test(rbind(c(0,20,10), c(13,4,13)))

chisq.test(rbind(c(0,20,10), c(5,21,4)))
```

Figure 4. Input values for the online Chi-squared test where the first c values are of the control group and the second c values are of the according proof of concept or experimental group.

```
data:  rbind(c(5, 0, 25), c(20, 10, 0))
X-squared = 44, df = 2, p-value = 2.789e-10


        Pearson's Chi-squared test

data:  rbind(c(5, 0, 25), c(7, 13, 10))
X-squared = 19.762, df = 2, p-value = 5.114e-05


        Pearson's Chi-squared test

data:  rbind(c(15, 10, 0, 5), c(8, 17, 5, 0))
X-squared = 13.945, df = 3, p-value = 0.002981

Warning message:
In chisq.test(rbind(c(15, 10, 0, 5), c(8, 17, 5, 0))) :
  Chi-squared approximation may be incorrect

        Pearson's Chi-squared test

data:  rbind(c(0, 20, 10), c(13, 4, 13))
X-squared = 24.058, df = 2, p-value = 5.969e-06


        Pearson's Chi-squared test

data:  rbind(c(0, 20, 10), c(5, 21, 4))
X-squared = 7.5958, df = 2, p-value = 0.02242
```

Figure 5. The output values for the online Chi-squared test where x-squared represents the Chi-squared values, df represents the degrees of freedom, and p-value represents the p-value.

default negative association) was tested. This study investigated the degree of complexity of functions *C. elegans* can learn which provides information regarding what the neural networks of a relatively simple organism, C. elegans, can comprehend. This comprehension can relate to what type of network may be necessary for the human brain, and the type of artificial neural networks that may be required for machine learning of complex problems.

The three proof of concept tests showed the ability of the *C. elegans* to learn a linearly separable association. The purpose of these tests was to see if research suggested by other papers[2,3,4,5] could work with an HCl gradient. These tests are represented in Figure 1 by (a) because only two gradients were available in the tests. The specific tests were 100/200, opposite 100/200, 200/300. The opposite 100/200 test was done to prove that *C. elegans* could learn this association between 200μM and *E. coli*. The test suggested that it was not just the natural affinity of *C. elegans* to be attracted to the lower HCl concentration, as could be falsely shown by the proof of concept 100/200 test, but instead a true two-way proof of association.

The experimental 100/300 test, which tested for nonlinear judgment, suggested that the *C. elegans* can learn a nonlinearly separable judgment as shown in Figure 1, (b), $X2\ (2, N=30) = 24.06$, $p < 0.001$. This test also showed that the *C.elegans* can learn a positive association with two ends of a gradient. It can be further hypothesized that the minimum number of neurons required to have a multi-layered neural network, or capability of non-linearly separable judgment is 202 neurons based upon White et al.[1].

The opposite experimental 100/300 test, where 200μM was the only positive reinforcement, suggested that the *C.elegans* can learn an opposite nonlinearly separable judgment as shown in Figure 1, (c), $X2\ (2, N=30) = 7.5958$, $p < 0.02242$. The main purpose of this specific trial was to identify that the *C. elegans* could also learn a negative nonlinearly separable association, which was demonstrated by having two negative reinforcements of 100 μM and 300 μM and positive reinforcement for 200 μM compared to the 2 positive reinforcements and negative reinforcement which was in the 100/300 test. The two ends of the spectrum were negative, while the middle was positive. This type of learning is the opposite of the 100/300 test and showed that the *C. elegans* are capable of both positive and negative learning of a gradient.

No previous studies have attempted learning of nonlinear classification, and this experiment was unique in that sense. The proof of concept trials, which only required linear classification, were similar to previous experiments. Perhaps the most comparable research study is "Aversive olfactory learning and associative long-term memory in *Caenorhabditis elegans"* by Amano and Maruyama[14]. The major difference was that this experiment used a stronger negative stimulus than in Amano and Maruyama[14] and involved multiple learning trials as opposed to only one in that study. The results were similar in the ability of *C. elegans* to learn a linear association.

Possible sources of error included the *C. elegans* dying after 5 minutes of exposure to the HCl, so the trials were very quick; therefore, the short training times i.e. only being fed for 30 seconds and dipped in the HCl for less than a second, did not show a long-term association. The 200/300 control plate had 300μM HCl agar that did not completely solidify; thus the *C. elegans* were more easily able to travel in it. This poses an error since the media of travel were not the same between the control and proof of concept trials. The control plate had a solid and liquid medium, while the proof of concept group had only a solid medium.

The central finding from this experimentation is the first demonstration that simple neural networks of *C. elegans* are surprisingly powerful and can learn a nonlinear function. Surprised by the ability of *C. elegans* to learn a variety of stimuli and associations, Rankin[18] asked: "What can't a worm learn?" Here, we show that the learning mechanisms of the worms are quite powerful and includes the ability to learn nonlinear functions. This is surprising, given that such nonlinear abilities are generally associated with higher cognitive functions in much more complex animals such as mammals.

Future work could include corrections such as homogeneity of agar in all test plates and using deionized H2O to better rinse off worms after each trial. Additionally, including a pH sensor for each dish to ensure that the HCl concentrations are accurate, especially in the 100/300 plate where 200μM is achieved through the combination of 100μM and 300μM agar dissolving into each other, may improve results. Another key improvement would be to use a negative stimulus which has a more neutral pH to ensure that the *C. elegans* survive the trials to be tested. Future work could also involve computational modeling by building artificial neural networks that can simulate the ability of *C. elegans.*

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