The use of classical conditioning in planaria to investigate a non-neuronal memory mechanism

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Abstract

The concepts of learning and memory have fascinated man since before the advent of science. The evolutionary significance of these mechanisms is clear. If an organism is able to learn and remember information about its environment, it will have a distinct advantage in survival. Biologically, learning is described as the inherent process in which an organism senses an environmental stimulus and undergoes adaptive changes in behavior as a response. Psychologists study learning in laboratories using simple organisms as model systems for humans, and observe specific behavioral changes which indicate the occurrence of learning. As of yet, the complete physiological mechanism of learning is unknown, and so the behavioral approach to learning research is the most commonly accepted. In this study, the planarian is used as the model organism in an investigation of the nature and location of the memory mechanism. Classical conditioning is utilized to train the organism, and the regeneration process is used to research the physiological aspects of the memory mechanism. Ultimately, an understanding of this mechanism in the planarian may hold implications for an understanding of learning and memory in higher organisms, such as humans.

1 Introduction

Planaria, or free-living flatworms, are one of the most useful organisms to serve as a model system in learning and memory studies. Although they are relatively primitive organisms, several factors make them ideal subjects. First, they are the first organisms to have a true synaptic nervous system, definite encephalization, and bilateral symmetry. [7] Because of the early evolutionary position and advanced nervous system found in planaria, it is possible that mechanisms of learning and memory in higher species may be traced back to processes inherent in this organism. In addition to their neurological significance, planaria are capable of regeneration through fission, developing into two or more complete individuals after this process by means of stem cells called neoblasts which are capable of forming any tissue type. [8]

Various training methods have been used in learning experiments, the most popular being classical conditioning, which exposes a test subject repeatedly to a Conditioning Stimulus, or CS, followed immediately by an Unconditioned Stimulus, or UCS. For example, in this study the CS is a change in overhead illumination, and the UCS is a weak electric shock. The UCS induces a consistent reaction in the subject called the Conditioned Response, or CR, in this case, a longitudinal body contraction. Eventually, after many trials, the subject begins to anticipate the UCS by sensing the CS. A subject has “learned” when it has expressed a CR following a CS, but before the onset of a UCS. [9]

Past research has shown that this method is effective in training planaria. [2][10][11] After a planarian has been successfully trained, it can cut transversely into anterior and posterior segments, both of which regenerate into separate individuals. It has been shown that both segments retain the memory of their predecessor. Additionally, it has been observed that planaria regenerated from both anterior and posterior regions retain the same amount of knowledge, suggesting that cephalization is not necessary for memory retention.[12]

This is a noteworthy finding because it refutes most of the commonly accepted theories held today asserting that memory storage is a purely neurological mechanism [1]. The purpose of this particular study is to first reaffirm the assertion that planaria are able to learn, and then to investigate the nature of the planarian memory mechanism. Inquiries into this mechanism may hold vast implications for understanding learning and memory on a broader level.

2 Materials and Methods

2.1 Basic Planaria Care

Three species of planaria were studied in this experiment: *Dugesia dorotocephala*, *Dugesia japonica*, and *Phagocata gracilis*. The *D. dorotocephala* and the *P. gracilis* were obtained from Ward's Biological Supply. All *D. japonica* were derived from one planarian transferred from Japan, and were therefore genetically identical. The planaria cultures were stored in rectangular plastic containers measuring 22 cm × 22 cm × 7 cm filled with Poland Spring water and incubated at 18°C. The culture was fed organic chicken liver twice a week. Excess meat was removed with pipettes after three hours, and the water was exchanged twice to remove residual waste and debris. Planaria were observed regularly under a dissecting microscope to monitor health. Unhealthy worms were removed from the general culture and treated with various antibiotic treatments.

2.2 Species Selection

The three species of planaria were screened to select an optimal experimental subject. First, a descriptive comparison chart was compiled based on literature and qualitative observation to distinguish between the appearance and basic behavior of the three species. Next, 25 baseline trials were performed for each species, in which one subject was placed in the training trough and observed. Any visible movement other than normal gliding motion was recorded every thirty seconds as baseline behavior. A second set of trials was executed in order to determine the Naive Response Rate for each species. Each trial set consisted of 25 administrations of the CS, or strong increase in illumination, with 30 second rest periods between trials. As in the baseline trial set, any motion other than normal gliding was recorded as a reaction to the CS. 25 preliminary classical conditioning trials were performed on three subjects of each species, following the protocol explained in Section 2.3. Lastly, the regeneration abilities of each species were tested by inducing fission with a sterile razor blade in five subjects from each species and making qualitative observations of the regeneration process over a seven day period.

2.3 Classical Conditioning

Twenty *D. dorotocephala* subjects of similar size and appearance were isolated from the general culture into cylindrical glass vials measuring 2 cm × 8 cm filled with approximately 20 mL of Poland Spring water. Eight of these planarians were labeled A-1 through A-8, and two were controls (B-1 and B-2). The remaining ten flatworms were kept as possible replacement subjects for the labeled worms if any underwent spontaneous fission or became unhealthy.

After isolation, the labeled worms began their behavioral training. During the training period, worms were neither fed nor allowed contact with each other. The classical conditioning technique involved repetitive application of an increase in overhead illumination from an 11W full-spectrum fluorescent light (CS). This was followed by an electric shock generated by a 6V DC power source (UCS). This UCS induced a longitudinal body contraction in the subject (CR). With repetition of the of the CS-UCS sequence, subjects began to demonstrate knowledge acquisition and retention by anticipating the UCS after the CS had been administered but before actual administration of the UCS. This anticipation was shown through premature expression of the CR.

The worms were trained individually in an 8 cm × 1 cm × 1 cm Lucite trough filled with Poland Spring water. A V-shaped cross section facilitated continuous movement of the worms. Two copper wires were attached to either ends of the trough and served as the anode and cathode. The electrodes were connected in series with a 6V source, and a "shock" was applied by closing a switch, completing the circuit (Figure 1).

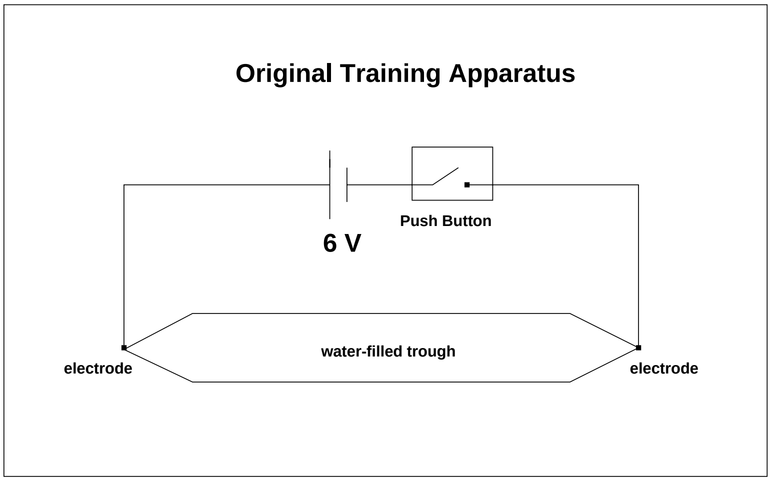


Figure 1: Apparatus for executing the original classical conditioning experiment.

Training was completed in sets of twenty-five trials twice a day, with two to four hours between the two trial sets. A trial consisted of a three second application of the CS, during the last second of which the UCS was administered. A resting period of thirty seconds was allowed between each trial. Prior to each trial set, three non-experimental flatworms were placed in the trough for three minutes to accumulate mucus secretions. This served to familiarize the environment for the test planarian, an effect which has been shown to encourage behavioral cooperation.[6] Once the test worm was placed in the water, it was allowed a one-minute acclimation period to adjust to the new environment. After the twenty-five trials were completed, the worm was returned to its vial and the water was emptied from the trough and replaced with new water to eliminate any voltage gradient between the electrodes.

For each trial, contraction prior to UCS, orientation of the anterior region of the worm relative to the anode or cathode, and induced moving by prodding (with a camel hair paintbrush or pipette) were recorded. Criterion of Learning was defined as follows: when the flatworm demonstrated memory of the UCS by contracting before its administration in nine out of ten consecutive trials, it was determined to have been successfully trained.

2.4 Retention Testing

Once trained, the worms were divided into two groups: an Experimental Group (A-1 through A-8) and a Control Group (B-1 and B-2). The eight members of the Experimental Group were cut transversely with a razor into two equal parts and allowed five days to regenerate. This group was then further divided into two groups: those regenerated from the original anterior region and those regenerated from the original posterior region. The members of the Control Group were not cut, but instead were allowed to rest for the same amount of time. After the period, all three groups were given ten "retest" trials following the same protocol described above. The number of conditioned contractions in the ten trials was recorded, and the CR expression rates were compared with those of the subjects before undergoing fission.

3 Results

The first problem investigated in this study involved selecting a suitable planarian species for experimentation. This was analyzed using five methods. First, a descriptive comparison chart was compiled based on relevant literature [2] and qualitative observation, as shown in Table 1.

Secondly, the baseline behavior of the three species was observed, and it was found that the test subject of the species *P. gracilis* exhibited explicit movement in 13 out of 25 trials, or 52% of the time. The test subject of the species *D. japonica* exhibited explicit movement in 6 out of 25 trials, or 24% of the time. Lastly, the subject of the species *D. dorotocephala* exhibited explicit movement in 3 out of 25 trials, or 12% of the time.

Next, the Naive Response Rates of the three species were determined, and it was found

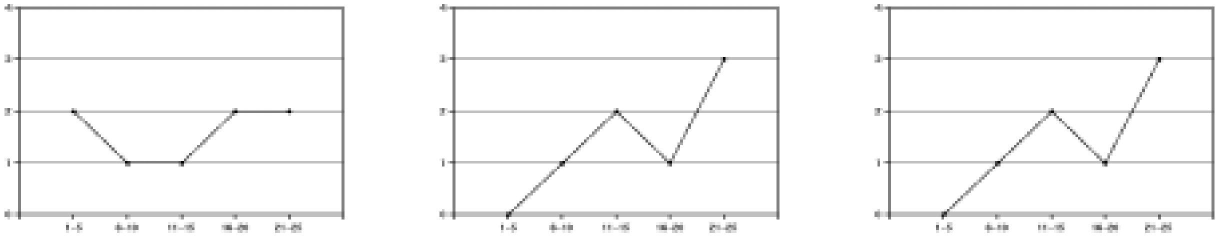
	D. dorotocephala	***P. gracilis***	***D. japonica***
Size	5–15 mm	10–20 mm	5–10 mm
Appearance	Orange-brown in color, pigment ranging from smooth to speckled, translucent	Black in color, square-shaped head, opaque	Light brown in color, smooth pigment, slightly translucent
Reproduction	Asexual, except for seasonal sexuality induced by temperature change	Asexual	Asexual and sexual strains
Spontaneous Fission	Occasional, stress-induced	Very rare	Fairly common
Regeneration Abilities	Regenerates quickly	Regenerates slowly	Regenerates quickly
Cannibalism	Cannibalizes very readily	Does not cannibalize	Cannibalistic traits unknown
Isolation Behavior	Often undergoes spontaneous fission after several days	Stress-induced mortality common	Often undergoes spontaneous fission after several days

Table 1: Qualitative comparison of basic physical and behavioral traits of three species of planaria.

that the test subject of the species *P. gracilis* reacted to the CS in 9 out of 25 trials, or 36% of the time. The test subject of the species *D. japonica* reacted to the CS in 7 out of 25 trials, or 28% of the time. Lastly, the subject of the species *D. dorotocephala* reacted to the CS in 3 out of 25 trials, or 12% of the time.

Fourth, the same three subjects of each species underwent one trial set (25 trials) of classical conditioning, and a preliminary evaluation of their learning ability was determined and compared in Table 2. The graphs show the change in the frequency of CR expression over 25 trials, and it can be seen that *D. dorotocephala* exhibited no net change, *D. japonica* exhibited a decrease in frequency, and *P. gracilis* exhibited an increase in frequency. Also, the total frequencies of CR contractions for each species were also recorded. *D. dorotocephala* had 8 contractions, *D. japonica* had 4, and *P. gracilis* had 9.

Consequently, *D. japonica* was eliminated due to its poor performance in the previous



A **B** **C**

Table 2: These graphs show the results for preliminary classical conditioning trials for three different planarian species. In each graph, the y-axis shows the frequency of CR expression and the x-axis represents grouped sets of trials. Graph A shows the change in frequency for *D. dorotocephala* and indicates that no significant increase or decrease occurred over the 25 trials. Graph B shows the change in frequency for *D. japonica* and indicates that there was a decrease in the frequency of CR expression over the 25 trials. Graph C shows the increase in frequency of CR expression for *P. gracilis* over 25 trials.

screens. *D. dorotocephala* and *P. gracilis* were screened for regeneration abilities (Table 3).

	D. dorotocephala	***P. gracilis***
Day 1	Induced fission of five flatworms	Induced fission of five flatworms
Day 5	12 pieces (5 heads/7 tails); heads almost completely regenerated; tails with pointed anterior regions and beginnings of eyes and auricles	10 pieces (5 heads/5 tails); heads with still visible wounds on posterior regions; tails non-mobile with no new heads formed
Day 7	13 pieces (9 complete organisms, 4 tails); complete flatworms swimming rapidly, healthy but slightly small and transparent; tails with almost completely developed heads moving normally	10 pieces (5 heads/5 tails) heads with tails almost formed, lighter pigment; tails non-mobile and without new heads

Table 3: Qualitative comparison of the regeneration abilities of two planarian species.

After these preliminary investigations, *D. dorotocephala* was chosen for classical conditioning. Ten subjects each underwent eight sets of 25 trials, and the frequency of CR expression was recorded (Table 7). A paired t-test comparing mean frequencies of the first two trial sets with those of the last two trial sets demonstrated a statistically significant increase at a 0.05 level of significance (p=0.001935). A Chi squared test was performed on each planarian to determine whether learning occurred (Table 4). CR frequencies are graphed over eight trials for each worm in Figure 2, showing the mean frequencies in bold.

Worm	P Value	Significance
A-1	0.30617	No
A-2	0.02354	Yes
A-3	0.15522	No
A-4	0.017221	Yes
A-5	0.017221	Yes
A-6	0.09731	No
A-7	0.00035	Yes
A-8	0.40709	No
B-1	0.09731	No
B-2	0.77424	No

Table 4: Significance of the differences in beginning and final trial sets for each planarian in the original classical conditioning study.

The subjects did not reach the predetermined criterion of 9 Conditioned Responses out of 10 trials However, because six out of the ten subjects underwent spontaneous fission after eight trial sets, they were allowed to regenerate with the four non-fissioned organisms serving as controls. After seven days, they were each given a ten-trial retest and their performances and comparisons were recorded (Table 5). It is interesting to note that four out of five of the head/tail comparisons were were statistically the same, while only four out of nine of the comparisons between the original trained subjects and their successors were statistically the same. Also, many of the successors exhibited a higher level of CR expression than their predecessor, as shown in the comparative graph in Figure 3.

The classical conditioning experiment produced other noteworthy results as well relating to electro-polarity. The results in Table 8 show that approximately 68% of the pooled instances of CR expression occurred when the subject's anterior region was oriented toward the cathode as opposed to the anode. This difference was shown to be significant using a Chi squared test (p=0.00032). Figure 4 shows this result graphically. Out of the 2000 trials performed in total, 1030 occurred during cathode orientation, and 970 occurred during anode

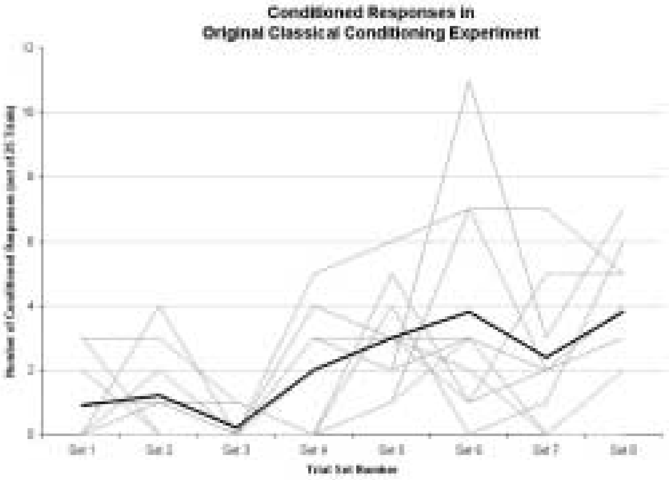


Figure 2: Shows the overall increase in CR expression frequency over a series of eight trial sets, with the mean frequency in bold.

	Trained	Head	Tail	Trained/Head	Trained/Tail	Head/Tail
A-1	12%	30%	20%	different	same	same
A-2	10%	60%	10%	different	same	different
A-4	20%	10%	10%	different	different	same
A-5	20%	60%	50%	different	different	same
A-7	24%	40%	30%	different	same	same
B-2	14%	20%	N/A	same	N/A	N/A

Table 5: Shows the rate of CR expression in the original “trained”subject and compares it to the rates of CR expression in the succeeding anterior-based and posterior-based organisms. A Chi squared test was used to determine the statistical significance of each comparison. B-2 Tail was not tested due to mortality.

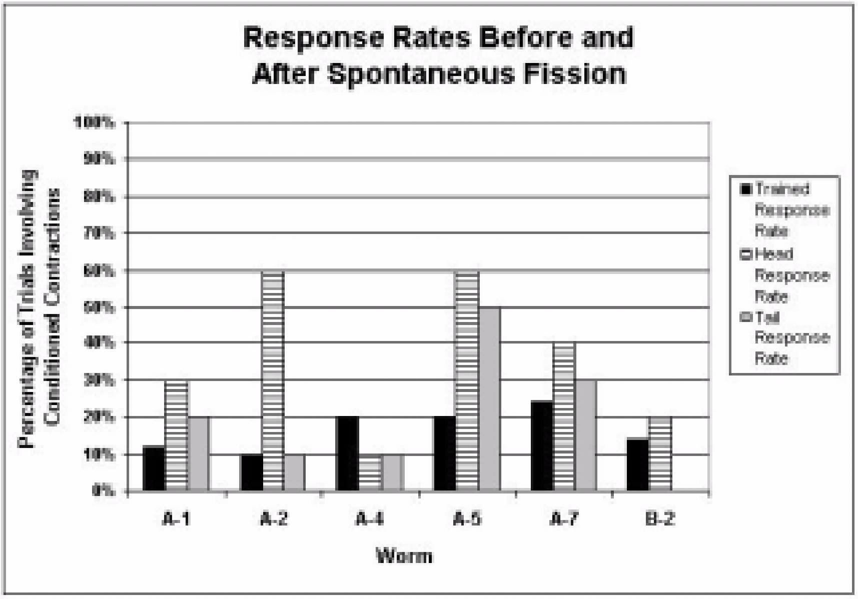


Figure 3: Compares each original worm with the organisms derived from its anterior and posterior regions.

orientation. A Chi squared test shows that these two values are not significantly different (p=0.34273).

In order to confirm this dependence on orientation, a second classical conditioning experiment with slightly modified parameters was executed using ten new subjects. A double-pole, double-throw switch was introduced in the training apparatus (Figure 5). This switch allowed facile alteration of electro-polarity, so that the planarian's anterior orientation would always face the cathode. Based on the results from the original classical conditioning experiment, it was hypothesized that this change in protocol would allow the planarians to learn more efficiently and more effectively. The results of this experiment are shown in Table 9. Also, the significance of the change in CR expression frequency for each worm was calculated (Table 6). The change in CR expression for each individual subject is shown in the graph in Figure 6 with the mean frequency in bold.

A paired t-test showed that the results from the cathode specific study were not statistically different from those of the original classical conditioning study (p=0.37887). The graph

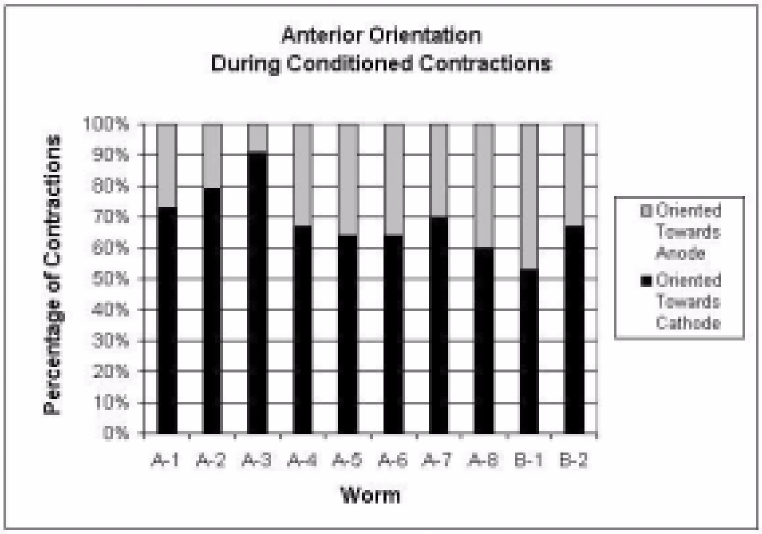


Figure 4: Shows the cathode and anode orientation percentages during CR expression in each subject.

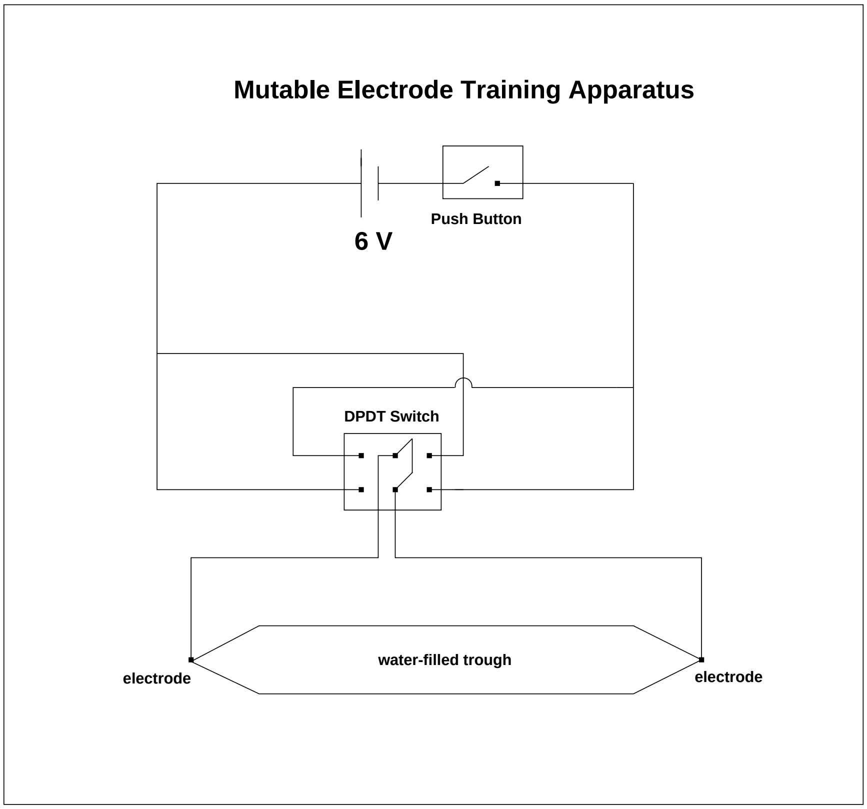


Figure 5: Apparatus used for the cathode specific classical conditioning experiment.

Worm	**P Value**	**Significance**
C-1	0.79984	No
C-2	0.55152	No
C-3	0.04808	Yes
C-4	0.06021	No
C-5	1.00000	No
C-6	0.24838	No
C-7	0.47048	No
C-8	0.08810	No
D-1	0.00098	Yes
D-2	0.00088	Yes

Table 6: Significance of the differences in beginning and final trial sets for each planarian in the cathode specific study.

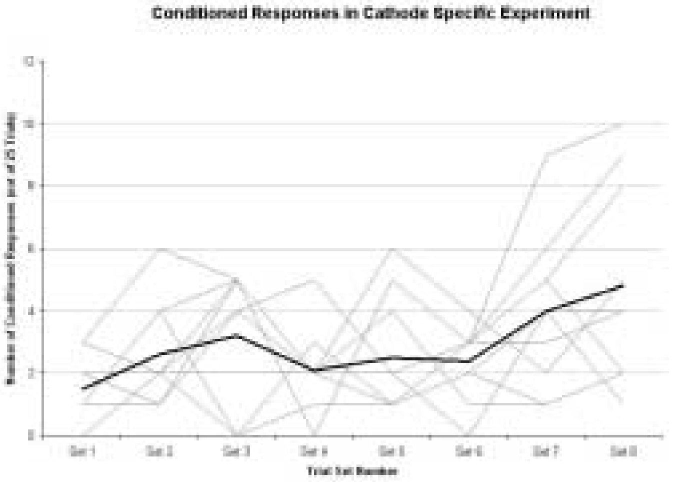


Figure 6: Shows the overall increase in CR expression frequency over a series of eight trial sets, with the mean frequency in bold.

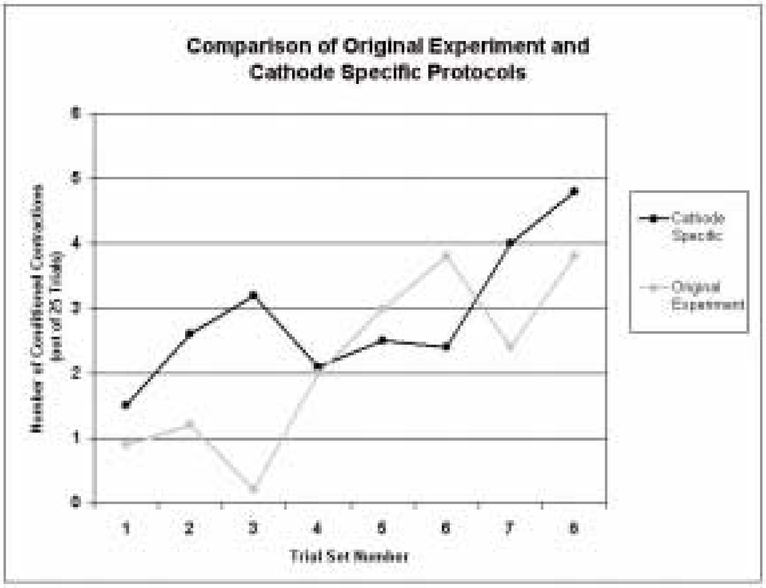


Figure 7: The changes in mean frequency of CR expression over eight 25-trial sets in the two protocols.

in Figure 7 also shows the similarities between the change in mean frequencies. However, it is notable that only three out of the ten planarians in the cathode specific experiment exhibited significant learning as shown in Table 6. In the original classical conditioning experiment, four out of ten subjects exhibited significant learning (Table 4.

4 Discussion

This study provides preliminary evidence of a non-neuronal mechanism of memory in planaria and suggests guidelines for designing efficient classical conditioning paradigms for training planaria. Through the series of experiments described above, several advances were made in understanding conditional learning in planaria.

Based on the results from the preliminary screens, the species *D. dorotocephala* was selected for experimentation. This decision was based on the species' qualitative traits, low Baseline and Naive Response Rates, preliminary classical conditioning results, and regeneration abilities. Because *D. dorotocephala* exhibited a Naive Response Rate identical to its Baseline Response Rate, the reactions observed in the Naive Response Rate trial can be attributed to normal baseline behavior instead of the increase in illumination (CS). This ensured that an increase in illumination would serve as an effective conditional stimulus in classical conditioning without overly sensitizing the subject. Also, the Naive Response Rate of 12% adheres to commonly accepted classical conditioning standards, which require that the Naive Response Rate be between 10% and 20%. [6] Lastly, the rapid regeneration rate of the *D. dorotocephala* indicated that the species would be optimal for regeneration portion of the study. This species selection is in accordance with many other planarian classical conditioning studies. [6]

The original classical conditioning experiment in this study reaffirmed previous literature asserting that simple associative learning occurs in the planarian. The change in the frequency of CR expression was shown to be significant over the course of 200 trials. Although the subjects did not reach the learning criterion as originally intended due to spontaneous fission, the significant increase indicates that if more trials were performed, the criterion would be met, as has been shown in other research. In future studies, more rest time between trials may be needed, as stress from over training may cause spontaneous fission in the planarians. Also, the large differences in results of the individual subjects can be attributed to genetic variations found within the population (wild-caught).

The retest of the subjects from the original experiment after spontaneous fission also provided important results. In four out of the five anterior/posterior pairs, the CR rate was shown to be significantly the same. This supports past research, and indicates that cephalization may not be necessary for memory. Instead, a non-neuronal mechanism may be responsible. Several studies have suggested a molecular mechanism for memory storage may occur throughout the body. For example, it has been hypothesized that RNA modification is involved in this process. Specifically in the planarian, this theory can be applied to the neoblast, which contains a high volume of genetic information. [13]

The classical conditioning study also revealed a strong preference for CR expression when the planarians' anteriors were oriented towards the cathode. The cathode and anode orientations for the total trials performed were statistically the same, showing that the preference in CR expression was not arbitrary. This finding is supported by previous studies, but is of yet unexplainable. [14]

The cathode specific experiment attempted to further investigate this, and was designed based on the hypothesis that the planarians were able to learn more efficiently when receiving a shock with anterior orientation facing the cathode. The results show that enhanced learning did not occur using the new protocol. The increase in CR expression frequency was statistically the same as that observed in the original classical conditioning trials, and fewer individual worms exhibited significant learning.

Although the new training method was not successful, the anterior orientation of the planarian is clearly somehow a factor in the learning process. It has been noted that when placed in an electromagnetic field, planaria express a preference for cathode orientation. [15] Planaria have been shown to possess electro-polarity sensory abilities which may have an unexplained physiological influence on the rate of learning.

5 Conclusion

In this study, methods for improving classical conditioning training techniques in planaria were investigated in order to study the memory mechanism in the organism. It was found that the species *D. dorotocephala* was the optimal subject for classical conditioning. It was also shown that planaria are capable of learning via the classical conditioning technique. The finding that organisms derived from the anterior and posterior regions or a trained organism retained the same amount of memory was significant because it supported the hypothesis that memory is non-neuronal. Lastly, it was also observed that anterior orientation is involved in the learning process, and that in traditional classical conditioning, planarians express learning more when facing the cathode. Although this study's attempts to design a more effective training protocol based on this finding were unsuccessful, important information has been gained about the location and nature of the planarian memory mechanism that will be useful in future classical conditioning studies.

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A Additional Data

Subject	**Set 1**	**Set 2**	**Set 3**	**Set 4**	**Set 5**	**Set 6**	**Set 7**	**Set 8**
A-1	3	0	0	2	3	1	2	4
A-2	0	0	0	3	3	3	2	3
A-3	2	0	0	4	3	2	0	0
A-4	1	1	0	0	5	1	5	5
A-5	0	2	0	3	2	11	3	7
A-6	0	1	0	3	2	3	2	3
A-7	0	0	0	5	6	7	7	5
A-8	0	4	0	0	1	3	0	2
B-1	0	1	1	0	1	7	2	3
B-2	3	3	1	0	4	0	1	6
Mean	0.9	1.2	0.2	2	3	3.8	2.4	3.8

Table 7: CR expression in ten subjects over eight 25-trial sets in the original classical conditioning study.

	Cathode		**Anode**	
	Fraction	**Percent**	**Fraction**	**Percent**
A-1	$\frac{11}{15}$	73%	$\frac{4}{15}$	27%
A-2	$\frac{11}{14}$	79%	$\frac{3}{14}$	21%
A-3	$\frac{10}{11}$	91%	$\frac{1}{11}$	9%
A-4	$\frac{12}{18}$	67%	$\frac{6}{18}$	33%
A-5	$\frac{18}{28}$	64%	$\frac{10}{28}$	36%
A-6	$\frac{9}{14}$	64%	$\frac{5}{14}$	36%
A-7	$\frac{21}{30}$	70%	$\frac{9}{30}$	30%
A-8	$\frac{6}{10}$	60%	$\frac{4}{10}$	40%
B-1	$\frac{8}{15}$	53%	$\frac{7}{15}$	47%
B-2	$\frac{12}{18}$	67%	$\frac{6}{18}$	33%
Total	$\frac{118}{173}$	**68%**	$\frac{55}{173}$	**32%**

Table 8: Anterior region orientation during CR expression.

Subject	**Set 1**	**Set 2**	**Set 3**	**Set 4**	**Set 5**	**Set 6**	**Set 7**	**Set 8**
C-1	3	6	5	2	1	2	4	4
C-2	3	2	2	2	1	3	5	2
C-3	1	4	5	0	5	3	5	8
C-4	2	2	5	2	6	4	2	5
C-5	2	1	4	2	4	2	2	2
C-6	1	1	5	2	2	0	4	1
C-7	1	4	0	1	1	2	1	2
C-8	0	2	0	3	1	3	3	4
D-1	0	2	4	5	2	3	6	9
D-2	2	2	2	2	2	3	9	10
Mean	1.5	2.6	3.2	2.1	2.5	2.4	4.0	4.8

Table 9: CR expression in ten subjects over eight 25-trial sets in the cathode specific study.