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Article

Stress-induced genetic memory inheritance and retention in Planarian biological model

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SUMMARY

Memory transfer has been widely studied at the neuronal level, yet the possibility of memory persistence through regeneration or non-neural mechanisms remains less understood. This study aims to systematically investigate whether planaria, freshwater flatworms capable of regenerating their entire bodies within 14 days, can acquire and retain memory through training, whether such memory persists after central nervous system regeneration, and how exposure to stressors like alcohol affects memory retention. We established a reliable and reproducible system for measuring memory inheritance and retention using an associative memory task, with semi-automated video tracking to quantify retention in planaria, improving upon previous observational methods. We hypothesized that planaria could acquire and retain memory through training, that memory could be transferred genetically through regeneration, and that alcohol would transiently impair memory. Four experiments were conducted: 1) development of associative memory task for training planaria; 2) analysis of the correlation between training trials and memory retention; 3) examination of the effects of regeneration on memory; and 4) assessment of alcohol exposure on memory performance. We found that increasing the number of training trials led to progressive improvement in memory retention. The results also implied that the regenerated head and tail retained memory, with no significant difference observed between the head and tail segments. Trained intact planaria exposed to 0.1% (v/v) ethanol for 5 minutes showed temporary impairment of learning and memory; however, they regained normal memory within 24 hours. Collectively, these findings help to understand the memory persistence in a regenerating model and how environmental stressors modulate learned behavior.

INTRODUCTION

Memory loss is a hallmark of many debilitating neurological disorders, including Alzheimer's disease and other forms of dementia, affecting millions of individuals worldwide and posing significant challenges to healthcare systems (1). Understanding how memory is formed, stored, and preserved is central to both basic neuroscience and the development of therapeutic strategies. A key question in this field is whether memory can persist despite alterations to the brain, such as damage, disease, or regeneration (2). This question becomes more complex when considering the possibility of memory transfer across generations, that is, whether experiences or learned behaviors might influence offspring.

Several evolutionary models have been proposed to explain the discontinuity in memory transfer across generations, particularly between parents and offspring (3). Three notable examples are the Weismann barrier, which holds that changes or traits acquired by somatic cells during an individual's lifetime cannot be inherited by their offspring; Lamarckian inheritance, which posits that acquired characteristics can be passed to the next generation; and the hologenome theory, which suggests that heritable changes can also occur through the transmission of symbiotic microorganisms that influence host traits (4). While it has traditionally been accepted that memory is encoded through neuronal changes in synaptic connectivity, growing evidence suggests that epigenetic modifications may also contribute to encoding and preserving information within biological systems (5). Some studies have reported evidence for transgenerational effects of learned experiences, such as in mice, where parental exposure to an odorant paired with a fear stimulus led to altered behavioral responses and neuroanatomical changes in offspring (6). Together, these findings broaden understanding of how biological systems might retain learned information independently of direct neural encoding.

Previous studies have proposed several potential mechanisms for transgenerational memory transfer. One study demonstrated that environmental information can be inherited transgenerationally in Caenorhabditis elegans (C. elegans), a nematode worm widely used as a model organism in genetic and neurobiological research, through epigenetic modifications, supporting the idea that learned experiences can influence biological systems beyond the nervous system (7). Another investigation found that different histone methyltransferases target distinct genomic loci, influencing transcriptional regulation linked to neural plasticity and memory formation (8). Research has also examined the RNA memory transfer hypothesis, suggesting that RNA interference (RNAi) may play a role in memory-related brain functions, although direct evidence was limited when the article was published in 2001 (9). Additional work has emphasized the importance of epigenetic regulation in the formation and maintenance of memory within neural tissues (10).

To investigate whether such transgenerational or regeneration-related memory mechanisms can be observed in behavior, we used planaria as the model

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organism. Planaria are flatworms belonging to the phylum Platyhelminthes and class Rhabditophora (11). They inhabit freshwater environments and possess an elongated, flat tail with a distinct head region, along with basic sensory organs, such as eyespots, which detect changes in light intensity. Due to their ability to regenerate within 14 days and their neural system's similarity to that of vertebrates, planaria are well suited for investigating memory persistence across regeneration (12). Their neurons exhibit dendritic spines and vertebrate-like neural proteins, and their central nervous system contains synaptic structures that support plasticity (13). They can regenerate from tail segments to produce genetically identical individuals, and their cephalic ganglia, nerve cells, and neurotransmitter systems closely resemble those found in vertebrates, further validating their use as a simplified model for studying fundamental neural processes and memory mechanisms (14, 15). Planaria also exhibit associative learning, offering insights into the neural mechanisms underlying memory processes in humans (16).

McConnell produced one of the first reports on conditioning protocols to create learned memories in planaria in 1959 (17). He conditioned planaria with paired light and mild electric shock, regenerated head and tail fragments, and found that tail-derived regenerates showed more conditioned responses to light than untrained controls. Previous research indicates that long-term associative memory, such as conditioned light-response behaviors, can be retained in regenerated planaria for at least 14 days using a classical conditioning model, showing the potential for memory storage in planaria (18). However, the precise mechanisms of memory retention in planaria, whether neural, epigenetic, or otherwise, remain largely unclear.

A major challenge in studying memory in planaria has been the lack of quantifiable, standardized systems for training and testing. Previous studies often relied on manual observation and lacked consistent conditions (19). To address this, we implemented an associative memory task in which planaria were trained to navigate toward a food source while enduring an aversive light stimulus. We chose to pair an illuminated food source with training because planaria naturally exhibit negative phototaxis, meaning they tend to avoid bright light (20). By associating a normally aversive stimulus (light) with a positive reinforcement (food), we created a robust and measurable learning challenge. Successful navigation toward the illuminated food source would therefore serve as a clear indicator of memory formation and behavioral adaptation, as planaria must overcome their innate avoidance response through learned association.

Automated tracking of planarian movement presents unique challenges. Planarian behavior is highly sensitive to environmental factors such as light and temperature, often causing the animals to cluster near the edges of test chambers. This behavior can interfere with video tracking due to overlapping shadows (21). Furthermore, the planarian's irregular and constantly shifting tail morphology can lead to recognition errors in computer-assisted analysis. To mitigate these issues, we used the ImageJ MTrackJ plugin for semi-automated tracking, enabling precise measurement of movement trajectories and behavioral responses. By improving the objectivity and reproducibility of behavioral tracking, our method provides a more reliable framework for evaluating learning and memory formation in planaria.

In addition to regeneration, few studies have examined how external stressors may impact memory retention in planaria. This is an important gap, as stress has been shown in other organisms to disrupt memory consolidation, retrieval, and synaptic plasticity (22). In vertebrates, alcohol (ethanol) exposure reduces hippocampal long-term potentiation and impairs spatial and associative memory, and in humans it is associated with memory blackouts (23, 24). In planaria, prior work has focused on ethanol-related morphological changes during regeneration, with little attention to learned behavior (25). To investigate the potential for memory retention under stress conditions, this study evaluated the impact of alcohol as a negative stressor on planarian learning and memory.

This study aims to systematically investigate whether planaria can acquire and retain memory through training, whether such memory persists after central nervous system regeneration, and how alcohol affects memory retention in trained intact (non-regenerated) planaria. Our study does not attempt to isolate the underlying mechanism of memory retention, and we do not claim to distinguish between genetic inheritance, neuronal memory, synaptic plasticity, or other physiological processes. We hypothesized that planaria could acquire and retain memory through training, memory could be preserved after central nervous system regeneration, and

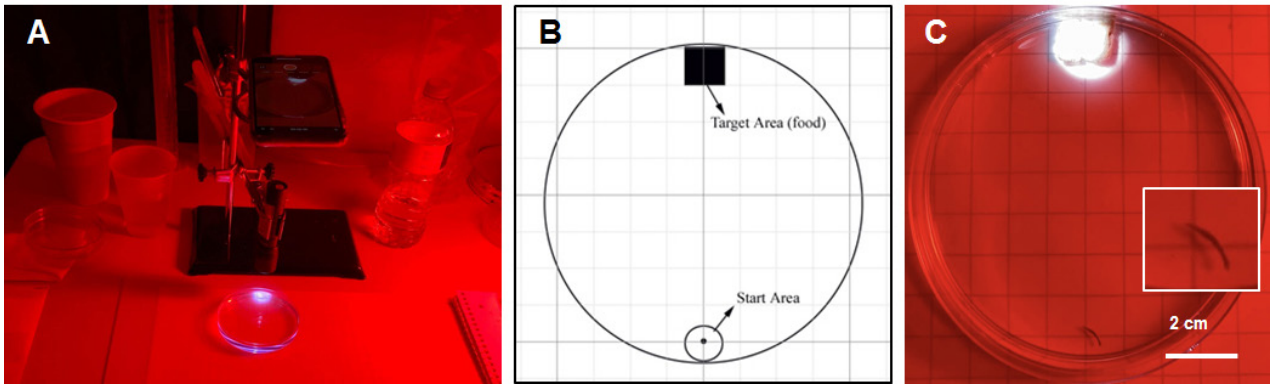

Figure 1: Setup of planaria training and testing system. (A) System setup using an A19 LED red light bulb for planaria testing and training. The white-light endoscope was placed approximately 2.5 cm above the petri dish, and the recording system was placed approximately 16 cm above the petri dish. (B) Diagram of start area and target area for planaria training. (C) Detailed view of petri dish with planaria and food source with white light stimulus. The inset is the zoomed-in view of planaria at the start area.

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with exposure to alcohol would transiently impair memory. To test this hypothesis, we conducted four experimental procedures: (1) development of a system for monitoring and optimizing planarian training using an associative memory task; (2) examination of the relationship between training duration and memory retention; (3) analysis of the effects of regeneration on memory persistence; and (4) assessment of the impact of alcohol exposure on memory retention.

RESULTS

To investigate whether planaria are capable of learning and memory retention, we developed a behavioral training and testing system (**Figure 1A**). The setup included a start area and a target area containing a food stimulus paired with a white-light aversive stimulus **(Figure 1B).** We conducted all experiments in a red-light environment to minimize external stress. Once a week for four consecutive weeks, we trained planaria with illuminated food, recorded and analyzed their ability to reach the target area **(Figure 1C)**. These measurements provided indicators of learning and memory development over the course of the training period.

A single cohort of 20 planaria was followed across four consecutive training sessions (Training 1–4), with Training 1 used as the baseline for evaluating memory and learning (no separate untrained control group). The trajectory of an individual planarian approaching the target area is shown **(Figure 2A–F)**. Early in training, planaria exhibited exploratory movement patterns, navigating in various directions before locating the food source. Despite their innate negative phototaxis in response to white light, planaria gradually demonstrated a preference for the food stimulus, indicating their ability to prioritize rewards over environmental stressors, a key aspect of associative learning. The input videos were calibrated in ImageJ to prepare for tracking (**Figure 2G**). After four training sessions, the planaria exhibited a reduction in the time taken to reach the light source as the number of training sessions increased (**Figure 3**). By the fourth training session, the time to locate the food decreased by approximately 6 minutes. One-way ANOVA showed a significant effect of training session on time to reach the light, $F(3, 76) = 156.16$, $p < 0.0001$, and Tukey's HSD indicated that all consecutive training groups differed significantly (Training 1 vs. 2, 2 vs. 3, 3 vs. 4; all $p < 0.001$). Beyond the fourth training session, no significant further improvement in learning and memory was observed. These results provide direct evidence that planaria are capable of learning and memory retention. Additionally, the variation in response times across individual planaria became narrower with each successive session, indicating that the planaria were beginning to develop and retain memory. To our knowledge, this is the first report to quantitatively demonstrate a learning curve in planaria using a reproducible tracking system, in contrast to earlier studies that relied primarily on observational methods.

To visualize learning-related changes in behavior, we tracked the x–y position of 10 randomly selected planaria (from the 20 trained animals) over time and plotted their movement relative to the illuminated food target **(Figure 4)**. Notably, the trajectories became increasingly direct and shorter as training progressed, indicating that the planaria were refining their ability to locate the food with greater efficiency. These visual representations clearly demonstrate that with each successive training session, planaria exhibited faster and more direct movements, reflecting an improvement in learning and memory function.

Furthermore, the average speed of the planaria increased, and the variability decreased over time, providing further evidence of memory consolidation (**Figure 5**). Increased average speed and decreased variability further support memory formation. One-way ANOVA showed a significant effect of training session on swimming speed ($F(2, 27) = 8.73$, $p = 0.0012$), and Tukey's HSD indicated that average swimming speeds were significantly higher after Training 4 compared to Training 2 ($p = 0.002$), with smaller but still significant increases from Training 2 to 3 ($p = 0.038$) and from Training 3 to 4 ($p = 0.042$).

To test whether regenerated planaria could retain memory, we bisected the 20 trained planaria into head and tail segments using sterile scalpels **(Figure 6)**. Following

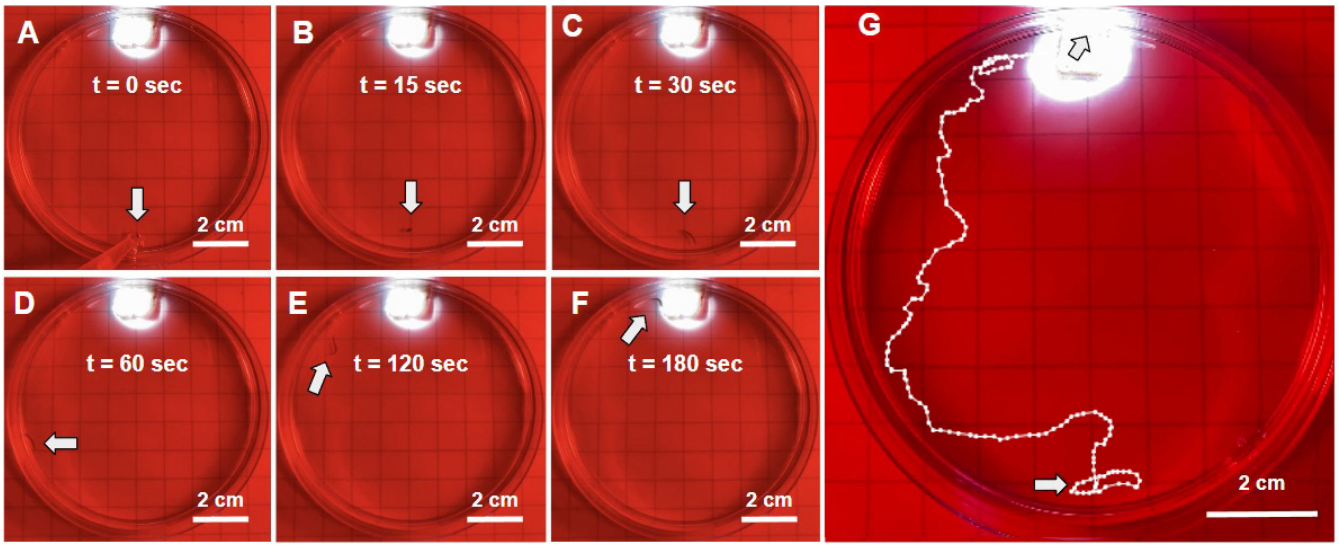

Figure 2: Training and tracking the planaria using ImageJ. (A-F) Photos of planaria movement from the start area to target area over 180 seconds. These images show that planaria prefer food over potential white light stress, which aligns with the associative memory task procedure. (G) Photo of separate planaria movement, traced by ImageJ application over 180 seconds.

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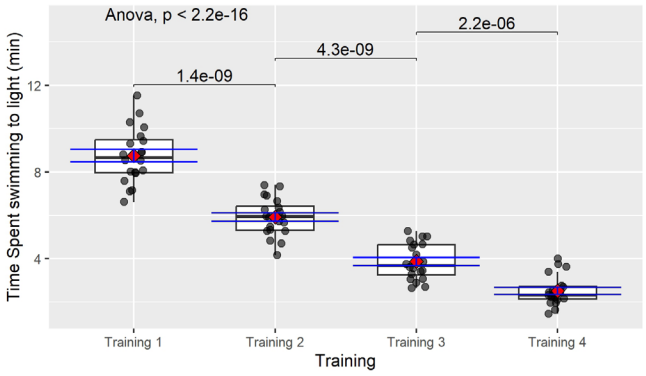

Figure 3: Swimming time as a function of the number of training sessions. Intact planaria displayed progressively decreased time swimming to the light as training number increased over four training sessions. Variation became tighter over the training time. Each dot represents the time to reach the light (in minutes) for an individual planarian (n = 20 per group). In the box plot, the box represents the interquartile range (IQR; 25th to 75th percentile), the thick black line within the box represents the median, the red dot indicates the mean, and the blue lines indicate the standard error of the mean (SEM) for each group.Statistical significance was determined by one-way ANOVA followed by Tukey's HSD post-hoc tests; all consecutive groups differed significantly ($p < 0.001$).

a 14-day regeneration period, the regenerated head and tail groups were assessed using the same memory-testing protocol. Swimming times to reach the light were compared between the regenerated groups and the swimming times recorded during Training 4, prior to bisection **(Figure 7)**. One-way ANOVA showed no significant difference in swimming times between the three groups ($F(2, 57) = 0.71$, $p = 0.55$). These results suggest comparable memory performance across all groups, implying that memory retention in planaria may not solely depend on the original CNS.

To assess the effect of alcohol as a negative stressor on memory retention, we used a separate cohort of 20 planaria that completed the same four-session training protocol. Training 4 served as the within-subject baseline for evaluating ethanol effects, and no separate control group was used. We chose n = 20 to match the previous experiments for consistency. For exposure, the trained intact (non-regenerated) planaria were briefly immersed in 0.1% (v/v) ethanol solution for 5 minutes, rinsed in fresh spring water, and retested immediately, then retested again 1 day later. The ethanol immersed planaria demonstrated a substantial increase in the time required to reach the target area, with durations approaching six minutes. By 1 day after alcohol exposure, the planaria exhibited times comparable to the baseline levels prior to ethanol treatment,

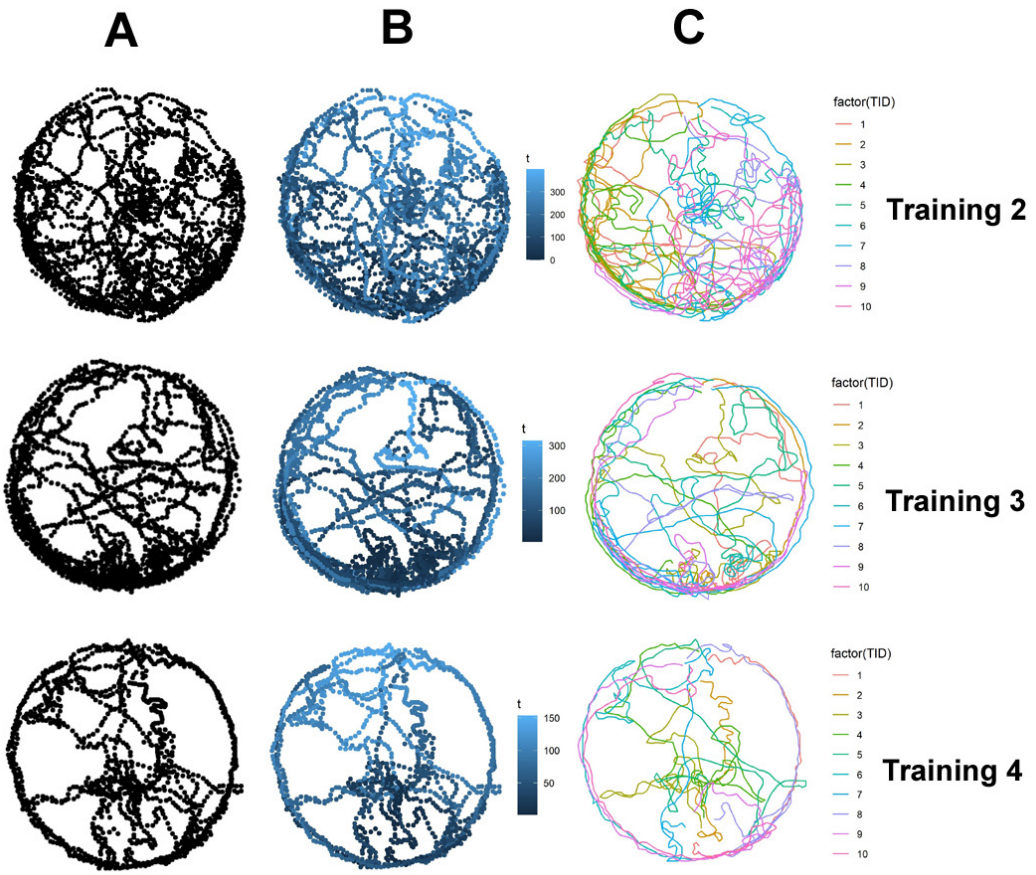

Figure 4: Visualization of tracked data of 10 planaria from start area to target area. The first row is the second training, the second row is the third training, and the last row is the fourth training. (A) Spatial positions of planaria (one point per frame), shown in black. (B) Time-resolved positions (one point per frame) colored by elapsed time t (s) from trial start (0 = placement; larger values = later in the trial). (C) Trajectories of individual planaria; each path is colored by Individual ID (TID) to distinguish individuals. Paths became much more straightforward and less kinesis-based over time. They retained the memory of finding the food in the simplest way over training time.

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suggesting a recovery of memory function (**Figure 8**). One-way ANOVA showed a significant difference in time to light between the groups, $F(2, 57) = 140.64$, $p < 0.0001$, and Tukey's HSD indicated that the time to reach the light was significantly increased 5 minutes after drug exposure compared to both before drug exposure ($p < 0.001$) and 1 day after exposure ($p < 0.001$), with no significant difference between the before and 1 day after groups ($p = 0.29$). These results indicate that although acute alcohol exposure temporarily impairs memory (and potentially motor function), planaria are capable of regaining normal memory function within 24 hours. Because ethanol can also alter motor activity, which we did not quantify here, we observed that exposed planaria maintained forward locomotion with typical body movement, showed no obvious loss of coordination or prolonged pauses. Therefore, the increased time to reach the target area is more likely due to impaired memory rather than reduced motor function.

DISCUSSION

This study established a robust monitoring system to analyze memory retention and potential genetic memory transfer in planaria using an associative memory task. Results demonstrated that planaria can acquire and retain memory through repeated training and that this memory may persist following regeneration. In the associative training experiment, planaria displayed a progressive decrease in the time required to reach the illuminated food source across four training sessions, along with reduced variability and increased swimming speed, indicating that their movements became more direct and efficient as learning progressed. These trends collectively suggest the emergence of a learning curve.

Both regenerated head and tail segments demonstrated memory task performance comparable to the control group. However, this similarity does not necessarily indicate genetic transmission of memory. Given the presence of distributed neural networks and known synaptic plasticity in planaria, it is possible that retained memory may instead be due to neural or muscular mechanisms rather than heredity. This is consistent with earlier studies

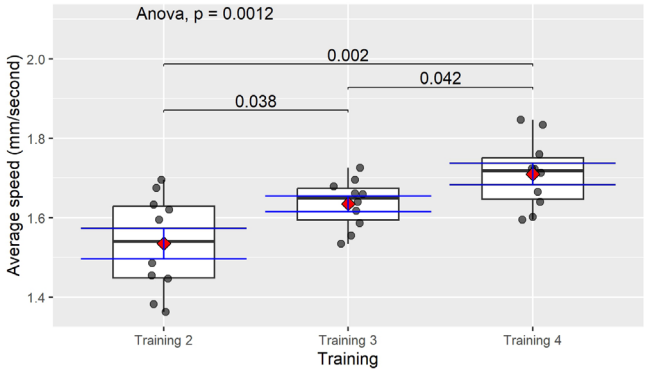

Figure 5: Graph of average swimming speed (mm/second) of planaria during training 2, 3, and 4. Planaria exhibited progressively faster average swimming speeds by the fourth training session, and variation became tighter, with mean speed increased from 1.53 ± 0.04 mm/s at Training 2 to 1.71 ± 0.03 mm/s at Training 4. Each dot represents the average speed of an individual planarian (n = 10 per group). In the box plot, the box represents the interquartile range (IQR; 25th to 75th percentile), the thick black line within the box represents the median, the red dot indicates the mean, and the blue lines indicate the standard error of the mean (SEM) for each group. Statistical significance was determined by one-way ANOVA followed by Tukey's HSD post-hoc tests; swimming speeds were significantly higher in Training 4 compared to earlier sessions.

suggesting memory-related changes can occur at the level of synaptic strength rather than genetic transmission. Furthermore, the study shows that memory performance is sensitive to external stress. Ethanol treatment was chosen based on previous studies demonstrating reversible immobilization of planaria without permanent damage (26). Exposure to 0.1% v/v ethanol (0.1mL ethanol in 100 mL water, a very low concentration comparable to almost negligible blood alcohol level in humans) for 5 minutes significantly impaired task performance, with planaria exhibiting increased times to reach the target following treatment. However, this effect was temporary: planaria recovered to baseline performance levels within 24 hours, suggesting a reversible disruption of memory retrieval or expression rather than permanent damage.

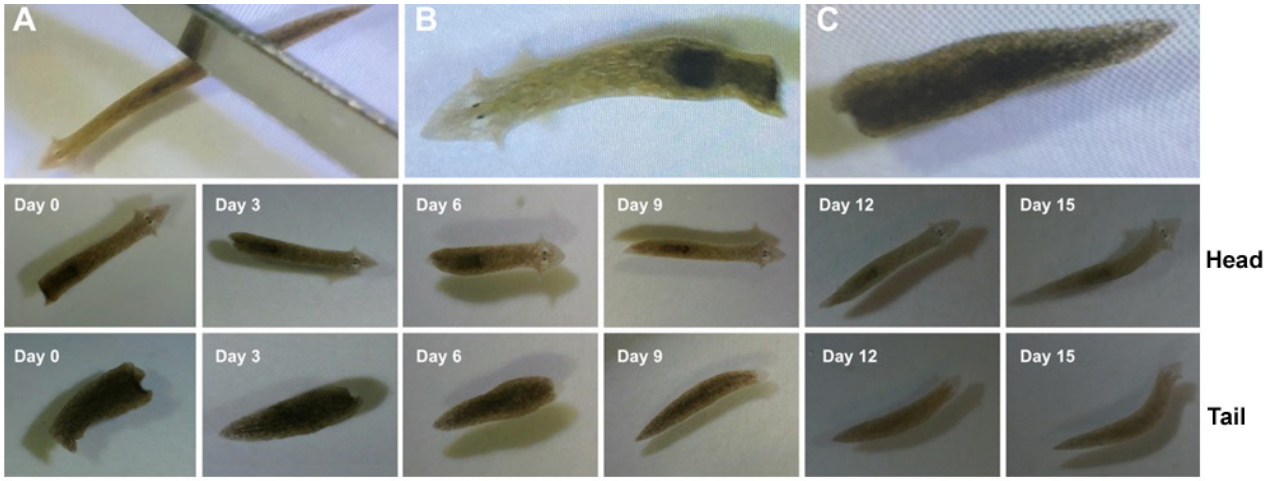

Figure 6: Sequence of photos depicting planaria cutting before regeneration. (A) Before severing planaria head and tail. (B) Severed planaria head. (C) Severed planaria tail. (Days 0-15) Photographs under an optical microscope showing progression of planarian regeneration after 15 days. Each segment grows into an individual organism but maintains the same DNA. The upper row is the head, and the bottom row is the tail.

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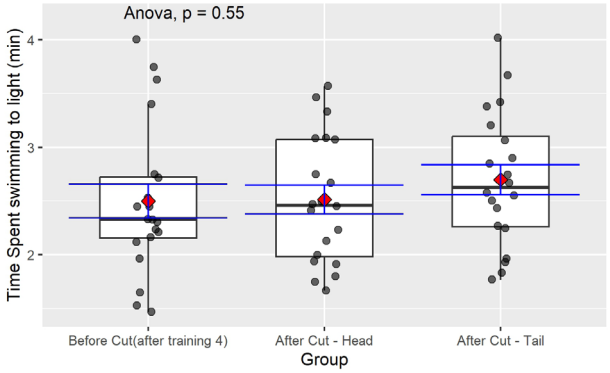

Figure 7: Graph of time to light across three groups: before and after generation (head and trail). While planaria before regeneration swam slightly faster on average, both regenerated head and tail groups exhibited similar swimming times post-regeneration. Each black dot represents the time to light (in minutes) of an individual planarian (n = 20 per group). In the box plot, the box represents the interquartile range (IQR; 25th to 75th percentile), the thick black line within the box represents the median, the red dot indicates the mean, and the blue lines indicate the standard error of the mean (SEM) for each group. Statistical analysis using one-way ANOVA showed no significant difference in the time to reach the light between the groups.

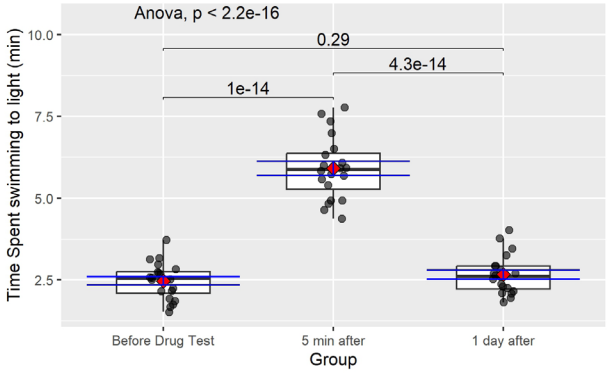

Figure 8: The effect of alcohol on planarian learning and memory. Trained intact (non-regenerated) planaria were tested before ethanol exposure, 5 minutes after exposure to 0.1% ethanol for 5 minutes, and again 1 day later. Each black dot represents the time to light (in minutes) of an individual planarian (n = 20 per group). In the box plot, the box represents the interquartile range (IQR; 25th to 75th percentile), the thick black line within the box represents the median, the red dot indicates the mean, and the blue lines indicate the standard error of the mean (SEM) for each group. Statistical significance was determined by one-way ANOVA followed by Tukey's HSD post-hoc tests; the 5-min post ethanol group took significantly longer to reach the light than the other groups.

Previous research has demonstrated that planaria are highly sensitive to white light and shorter wavelengths, which induce light stress (27). Consequently, a white light source was used during testing to mimic light-induced stress conditions. Studies indicate that planaria exhibit no adverse reactions to red wavelengths of light, thus validating the use of red light for handling (20).

To quantify behavior, we developed a semi-automated video tracking system using ImageJ's MTrackJ plugin and RStudio. This system extracted key metrics such as X/Y coordinates, velocity, and movement trajectories. Compared to manual observation, our approach improved consistency, reduced bias, and enabled analysis of subtle behavioral changes. Visualizations such as layered scatterplots provided clear evidence of learning patterns over time. Although MTrackJ has been used in other systems, its application in planarian memory studies is rare. Our experimental setup, which incorporated a calibrated grid and red-light control environment, provided precise tracking results. The speed and trajectory data aligned with known locomotion patterns in planaria, indirectly validating the method (28). While challenges such as tail shape variability and shadow interference occasionally affected tracking, these were mitigated through video filtering and brightness adjustments. Future improvements could include integrating artificial intelligence (AI) and machine learning algorithms to automate object detection and movement classification, which would reduce human workload and enable higher-throughput experiments.

To further control for the potential influence of motor function, the same ImageJ-based tracking protocol used to measure swimming times could also be applied to assess swimming speed and distance traveled after alcohol exposure. By reanalyzing existing videos or applying this method in future experiments, it would be possible to systematically evaluate whether alcohol or other stressors affect mobility independently of memory function. Incorporating these additional mobility metrics would strengthen the ability to distinguish true cognitive impairments from general motor effects, providing a more comprehensive understanding of how different stressors influence behavior in planaria.

This study had several limitations. Sample sizes were relatively small due to the time-intensive nature of manual training and care. Alcohol exposure was limited to a single concentration and time point (0.1% v/v), which was selected based on previous studies showing that low-dose ethanol produces behavioral effects in planaria without inducing lethality or severe toxicity (29). Future studies could explore multiple concentrations and exposure durations, as well as the long-term effects of neurotoxic stress on memory retention in both intact and regenerated planaria. It would also be valuable to test regenerated planaria under alcohol treatment to examine whether memory retention remains equally resilient post-regeneration.

Additionally, future studies could also consider each individual planarian's performance before and after ethanol exposure as a within-subject control in the future. This can provide a more sensitive comparison of behavioral change relative to baseline and further strengthen conclusions about memory impairment and recovery dynamics.

Overall, this study contributes to our understanding of memory mechanisms in regenerating organisms and highlights the potential of planaria as a model for investigating neural plasticity, stress response, and memory persistence under both normal and altered conditions.

MATERIALS AND METHODS

Planarian Culture

This study utilized an asexual strain of planaria, obtained from Carolina Biological Supply (Item#: 132956), chosen for

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their larger size and regenerative capabilities. The planaria were evenly distributed into 100 mL glass beakers containing room-temperature spring water maintained at 25°C. To optimize conditions for negative phototaxis, an essential behavioral response for this study, the planaria were housed in complete darkness throughout the experimental period. Water changes and transfers between resting beakers and the testing arena were conducted under red light illumination (A19 LED red light bulb, 9 W, approximately 700 nm) using plastic disposable transfer pipettes.

To maintain the cleanliness of the planaria's environment, water changes were performed daily. Spent spring water was replaced with fresh, room-temperature spring water. Weekly feeding was conducted using beef liver as the primary nutrient source, irrespective of the training schedule (30). Based on experimental outcomes, the planaria were subsequently transferred into separate beakers containing room-temperature spring water, to facilitate further testing.

Associative Memory Task and Learning System Setup

An experimental system based on an associative memory task was developed, modeled after Pavlovian classical conditioning principles. The setup used LED red light bulbs for background lighting and a white LED flashlight (approximately 10,000 lumens) as an aversive light stimulus, positioned to directly illuminate the target food location.

Individual planaria were transferred into 90 × 15 mm plastic Petri dishes containing room-temperature spring water. A small piece of beef liver was placed 8 cm from a standardized starting point near the edge of the dish. Planaria were consistently placed at this starting point, always oriented in the same direction to ensure experimental uniformity.

To ensure precise movement tracking, a 1 cm grid paper was placed beneath each transparent Petri dish. A white-light endoscope was positioned 2.5 cm above the food area to deliver the light stimulus, and a video camera mounted 16 cm above the testing site continuously recorded the trials.

Each trial began when the planarian was placed at the starting point and ended when it reached the illuminated food target. Trials were not time-limited. All trial durations were determined retrospectively by analyzing the recorded video footage using ImageJ software. Using Fiji (ImageJ distribution) with the MTrackJ plugin v1.5.1, the X and Y coordinates, velocity, and distance traveled by the planaria in each frame were analyzed to quantify their behavioral responses over time (31, 32). We extracted data including total time to reach the food, average swimming speed, and movement path. These quantitative metrics were processed and visualized using RStudio (33).

Data were collected from groups of 20 planaria to establish baseline behavioral performance and assess the strength of the learned association. These data also served as a reference point for comparisons following regeneration and ethanol exposure.

Regeneration Effects on Planaria Learning and Memory

Following the four-session training protocol, the group of 20 planaria were bisected into head and tail halves using sterile disposable scalpels. The specimens were then allowed to regenerate for 14 days in room-temperature spring water under dark conditions. Regeneration progress was monitored daily with an optical microscope. On day 15, both regenerated head and tail fragments were tested using the same associative memory task described above to evaluate memory retention post-regeneration.

Alcohol Effect on Memory Retention and Learning

In parallel experiments, trained intact (non-regenerated) planaria were exposed to ethanol to assess the impact of stress on learning and memory. Following the four-session training protocol, each of the 20 planaria were immersed in a premixed 0.1% (v/v) ethanol solution for 5 minutes individually. After exposure, planaria were rinsed in fresh spring water and immediately subjected to the associative memory task to assess changes in swimming behavior and time to reach the food source.

Statistical Analysis

All analyses were performed in R language using RStudio. One-way analysis of variance (ANOVA) was used to assess the effects of training and experimental manipulation on planarian behavioral performance. A significance threshold (alpha level) of 0.05 was set for all tests. When significant main effects were observed, Tukey's Honestly Significant Difference (HSD) post-hoc tests were conducted to assess pairwise differences between groups.

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