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Article

# Measuring effects of caffeine and melatonin on learning trends of Zebrafish juveniles

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## SUMMARY

**As humans navigate a renewed digital age, the use of energy-influencing substances like caffeine and melatonin has risen, especially among adolescents. Many teens consume energy drinks with high caffeine levels and take melatonin to sleep. The effects of these substances on the adolescent brain and learning are not well understood. This study used juvenile zebrafish (*Danio rerio*) to examine how caffeine and melatonin affect learning. We randomly assigned zebrafish to three groups: caffeine-treated, melatonin-treated, and control. To minimize the external stressors caused by human intervention, we developed an automated learning and video-recording system. Over one week, we observed notable differences in learned responses between the groups. We hypothesized that melatonin would increase the amount of time it took for the zebrafish to learn food-associated visual cues, while caffeine would cause more variation in learning time. We found that caffeine led to erratic, fluctuating behaviors, while melatonin slowed learning. Both made learning inconsistent, with caffeine causing more extreme effects. Our results indicate that melatonin and caffeine negatively impact learning trends among zebrafish adolescents in a statistically significant manner, potentially suggesting a similar effect on human adolescents' learning patterns. Further investigation is needed to delve into the physiological effects of these compounds and the mechanisms by which they influence learning patterns, as well as whether these compounds have similar effects in adolescent humans.**

## INTRODUCTION

With the rise of energy drinks and over-the-counter medications, caffeine and melatonin use has increased among younger generations (1,2). While many studies examine short-term effects in adults, fewer focus on long-term impacts on the developing adolescent brain, which continues maturing until age 25 (2). Caffeine use is now routine, with many teens consuming it daily for energy, focus, or to counter sleep deprivation, often exceeding the recommended 100 mg limit through drinks like Celsius, Red Bull, and Monster (1,2). Melatonin is also widely used as a sleep aid, though its long-term effects on adolescent brain development—especially in memory-related regions like the hippocampus—remain underexplored (3,4). To study these effects, we used automated systems to measure how quickly juvenile zebrafish learned food-associated visual cues under caffeine and melatonin exposure. We hypothesized slower learning with melatonin and increased variability with caffeine. Results confirmed that both substances increased variation in learning time and melatonin slowed learning. While these findings cannot be directly applied to humans, they suggest possible impacts on adolescent learning that warrant further study.

## RESULTS

We conducted a controlled behavioral experiment using a between-subjects design. 45 zebrafish adolescents were randomly assigned to one of three tanks: a control tank, a tank exposed to caffeine, and a tank exposed to melatonin. This design allowed us to compare behavioral responses across the three conditions. Levene's test ($p > 0.05$) showed no significant difference in behavioral variances between any two tanks, indicating the individual zebrafish within each tank were behaving homogeneously.

Fish were exposed to green and red lights, which served as visual stimuli. No food was dispensed after the green light turned on, and food was dispensed 15 seconds after the red light turned on. Learning was established when the zebrafish moved towards the side of the tank where food was dispensed upon exposure to the red-light stimulus. Our object-detection algorithm tracked the trajectory of each zebrafish, determining them as "learned" or "not-learned" based on how their pathways differed from their normal movements **(Figure 1)**.

### Control Tank Learning Trend

Using the data collected during the experimental trials, we confirmed that the control tank zebrafish showed expected learning patterns **(Figure 2)**. Specifically, early trials had little response to either red or green light, but as the trials progressed, zebrafish increasingly responded to the red light, following a logarithmic trend ($R^2 = 0.92$) **(Figure 2)**. Green-light responses followed a weaker quadratic trend ($R^2 = 0.25$) **(Figure 2)**. Statistical analysis showed that the distribution of zebrafish reacting to the green light did not follow a normal distribution, while the distribution of zebrafish reacting to the red light did.

### Caffeine Tank Learning Trend

Zebrafish in the caffeine tank showed a slower, more erratic learning pattern **(Figure 3)**. The zebrafish reacting to both the green light and red light followed a normal distribution. It took five trials for five zebrafish to react to the red light, compared to three trials in the control group **(Figures 2 and 3)**. The red-light response followed a logarithmic trend ($R^2 =$

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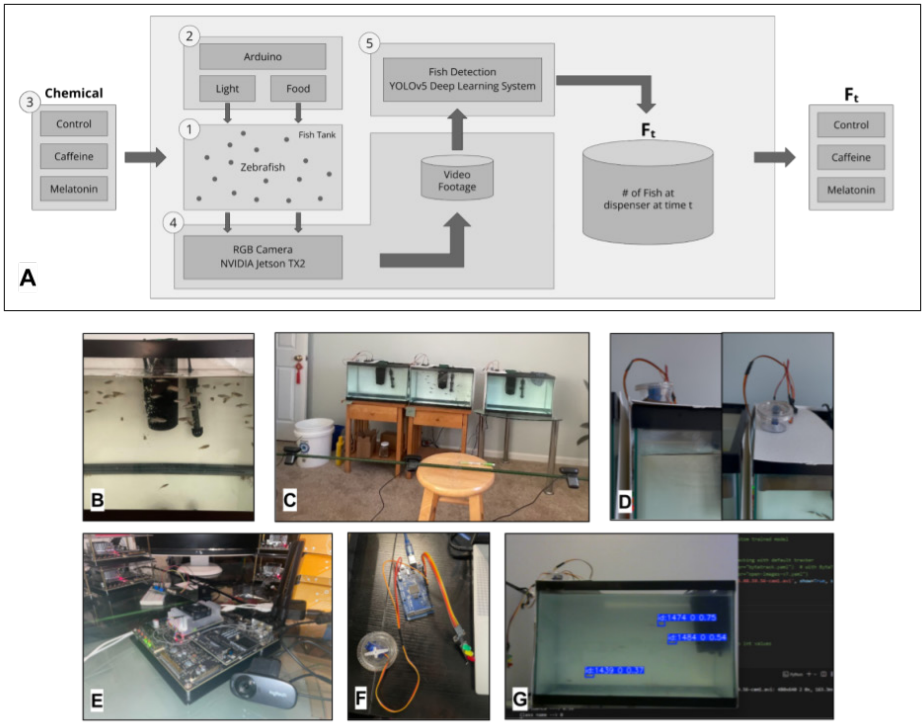


**Figure 1. Experimental Setup and Methodology.** Component A is a flowchart that encapsulates all procedures. Component B represents 15 juvenile zebrafish (*Danio rerio*) in a zebrafish tank, and we used three tanks total (Component C). The left tank was treated with caffeine, the central tank was left untreated, and the right tank was treated with melatonin. Components D and F represent our automated lighting and feeding systems that utilize Arduino boards and their built-in timers to turn on/off LED traffic lights and rotate Mini Servo motors for food dispensing based on the experimental trial schedule. Component E represents the automated video recording system that uses a NVIDIA Jetson TX2 board to control and automate video recording of the trials utilizing the attached cameras to generate video files. Component G represents the zebrafish detection system, which employs image processing and machine learning techniques to automatically recognize and track the movement of zebrafish in the recorded videos, thus facilitating the counting of zebrafish moving towards the food dispenser.

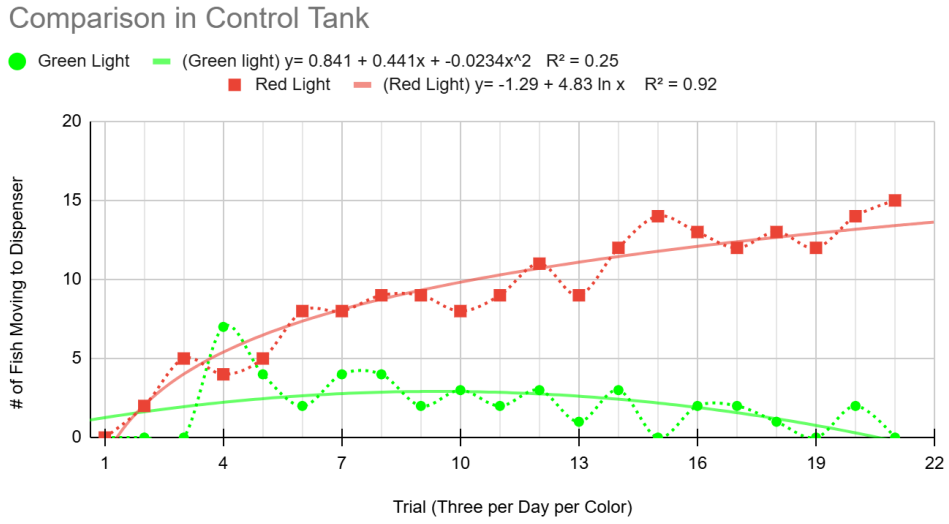


**Figure 2. Red- and green-light learning.** Statistical analysis showed that the distribution of zebrafish reacting to the green light did not follow a normal distribution (Shapiro-Wilk *p-value* < *0.05*), while the distribution of zebrafish reacting to the red light did (Shapiro-Wilk *p-value* > *0.05*). The trial number (the ticks represent different trials, but each marked number marks a new day of experimentation)—tracked progress over time. The number of zebrafish that moved toward the dispenser was used as a proxy for learned behavior, and spanned over the trial length of a week.

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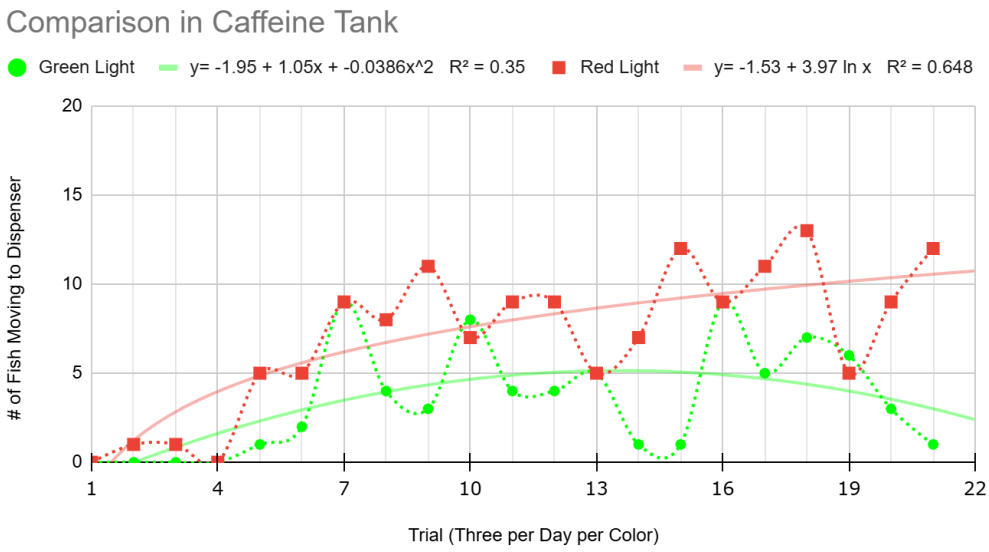


**Figure 3. Caffeine red/green learning comparisons.** Zebrafish move towards the dispenser in response to red or green-light stimuli at each trial in the caffeine-treated (concentration left to cycle for 12 hours prior to fish being placed = 2.5 x $10^{-2}$ g/L; 946.353 mg) tank. The zebrafish reacting to both the green light and red light followed a normal distribution (Shapiro-Wilks p-value > 0.05). The trial number (the ticks represent different trials, but each marked number marks a new day of experimentation)—tracks progress over time. The number of zebrafish that moved toward the dispenser—a proxy for learned behavior.

0.648), showing gradual learning, though the caffeine tank's responses were visually less consistent than the control group **(Figure 3)**. The ADF test value of zebrafish reacting to the red light in the caffeine tank was larger than that of the control tank, indicating that there was weaker evidence of behavior stationarity (i.e., more temporal drifting) in the caffeine tank than the control tank.

Green-light behavior followed a weaker quadratic trend ($R^2$ = 0.35). Performance was inconsistent, with noticeably poorer responses in the mornings, specifically during the 9:00 AM red light trials, despite performing well the night before and the night after **(Figures 3, 4B, and 4E)**. We also observed an increase in reactions to the green light during the earlier morning trials that was not demonstrated further in the day **(Figure 4B)**.

## Melatonin Tank Learning Trend

Zebrafish in the melatonin tank learned more slowly than those in the control and caffeine tanks, taking over seven trials for five zebrafish to respond to the red light **(Figure**

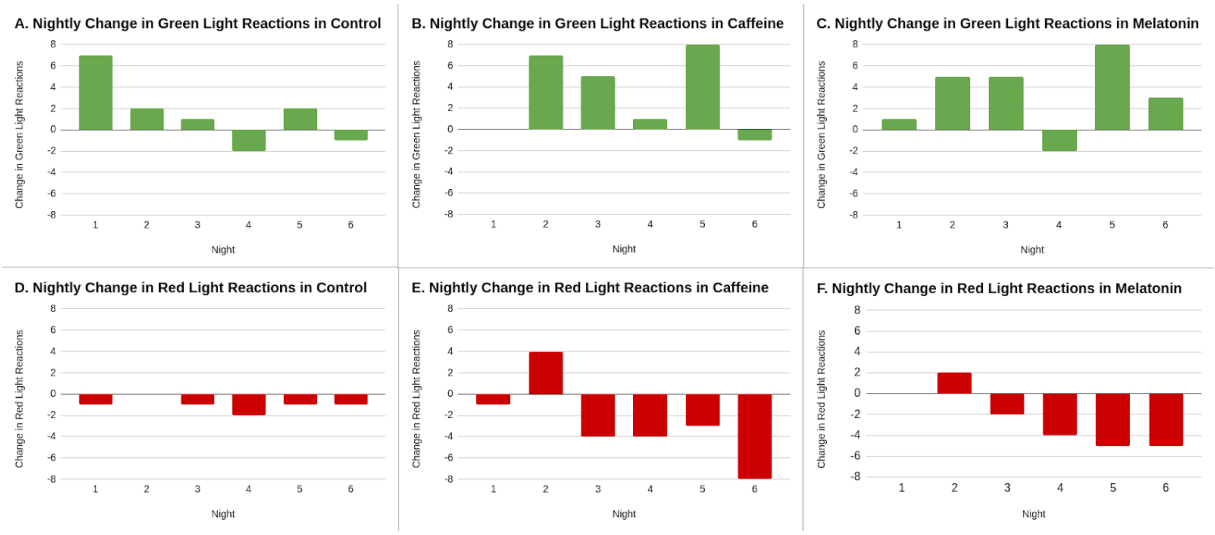


**Figure 4. Loss of learned behavior following overnight breaks.** This exemplifies the loss of behavior learned during the day following overnight breaks in trials, calculated by subtracting the number of fish who reacted to either the red or green lights following the overnight break in trials from the number of fish who reacted to either the green or red lights prior to the overnight break in trials. The plots indicate the relapse in pattern recognition in the caffeine and melatonin treatments in comparison to the control.

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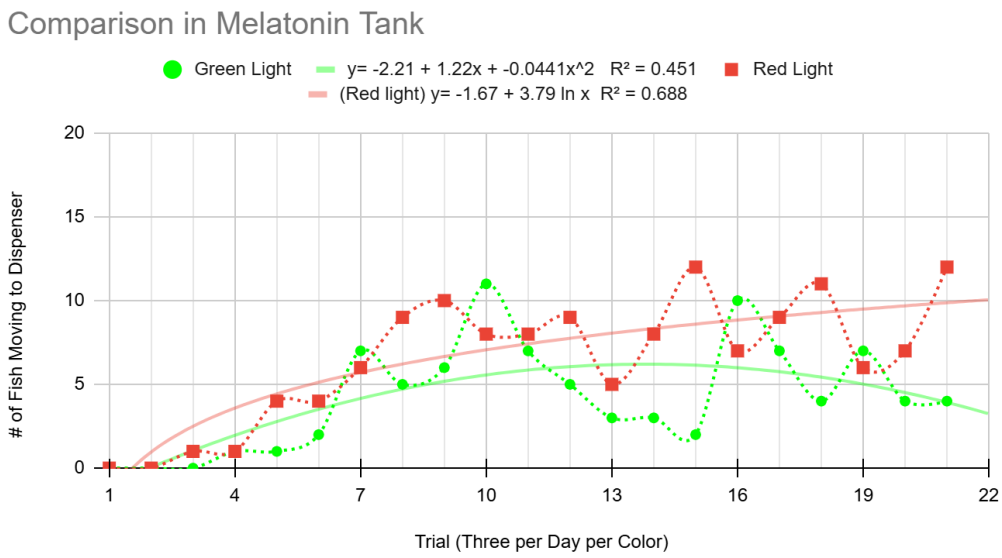


**Figure 5. Melatonin red/green learning comparisons.** Line graph that shows the distribution of zebrafish moving towards the dispenser in response to red- or green-light stimuli at each trial in the melatonin-treated tank. The number of zebrafish reacting to the green light and the number of zebrafish reacting to the red light both followed a normal distribution (Shapiro-Wilks $p$-value > 0.05). The trial number (the ticks represent different trials, but each marked number marks a new day of experimentation)—tracks progress over time. The number of zebrafish that moved toward the dispenser—a proxy for learned behavior.

**5)**. The red-light trend was logarithmic ($R^2$ = 0.688), and the green light followed a quadratic trend ($R^2$ = 0.451) **(Figure 5)**. The zebrafish reacting to both the green light and the red light followed a normal distribution. Similar to the caffeine tank, zebrafish performance declined in the mornings with more zebrafish reacting to the green light and less zebrafish reacting to the red light **(Figure 4C and 4F)**. Overall, melatonin-treated zebrafish performed the worst, with only 12 showing learning compared to 15 in the control tank and 13 in the caffeine tank.

## Direct Red-Light Comparisons

From the graphed comparison between all three tanks, we observed some learning trends of each treatment **(Figure 6)**. Despite the differences between caffeine and melatonin, they both generally showcased a fluctuation in the learning curve relative to the control. Overall, the control tank's zebrafish seemed to have learned faster and at a more consistent rate

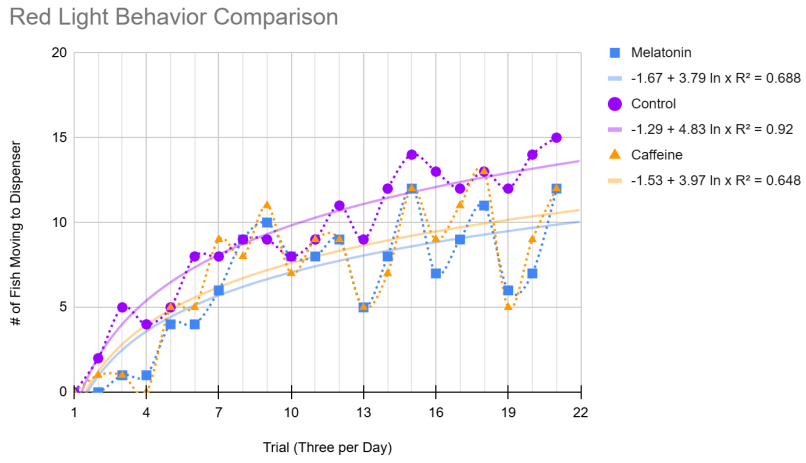


**Figure 6. Direct red light behavior comparison.** The line chart compares the number of zebrafish moving towards the dispenser in response to conditioned stimuli among two experimental tanks and one control tank. Levene's test ($p$ > 0.05) showed no significant difference in behavioral variances between any two tanks. The Augmented Dickey-Fuller (ADF) test value of zebrafish reacting to the red light in the caffeine tank (ADF value = -1.85, $p$-value > 0.05) was larger than that of the control tank (ADF value = -2.15, $p$-value > 0.05), which was larger than that of the melatonin tank (ADF value = -3.85, $p$-value < 0.05). This indicates that there was more variability in behavior in the caffeine tank than the control tank but less variability in the melatonin tank.

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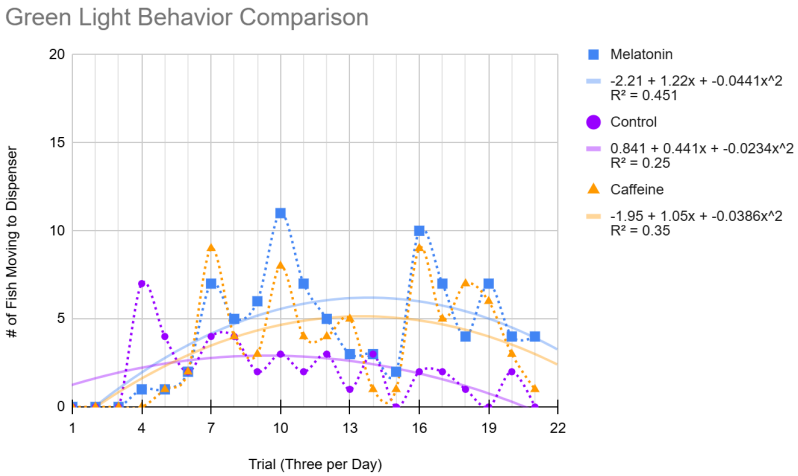


**Figure 7. Direct green light behavioral comparison.** A line graph that compares the number of zebrafish moving towards the dispenser after the green light flashes. The corresponding Augmented Dickey-Fuller (ADF) test demonstrates higher evidence of stationarity on zebrafish learning patterns in the control and melatonin-treated tanks than in the caffeine-treated tank.

than the caffeine tanks, but at a less consistent rate than the melatonin tank (ADF *test statistic* = *-3.85* in the melatonin tank but *-2.15* in the control tank), with higher numbers of zebrafish learning the taught behavior. The trendline seemingly rises much faster than those of the control and melatonin treatments **(Figure 6)**. Learning behavior dropped almost every morning in the caffeinated tank, while it seemed to drop only every couple of days in the melatonin tank **(Figure 4)**.

Notably, the percentages of zebrafish that responded to the red-light stimulus in each experimental group were non-significant in the majority of the trials. Almost all the earliest time points were not significant, but several later trials (19, 20, and 21) were significant. This may suggest that, while the results seen initially could have been seen through random variation, running the experiment for a longer period may yield more concrete results as the varying percentages of reactions within each group continue to increase.

### Direct Green-Light Comparison

Zebrafish were generally more interested in the green light at the start of each day, opposite their response to the red light **(Figure 7)**. Zebrafish in the control tank showed initial interest that quickly dropped as they learned the green light did not signal food, reflected in its early quadratic apex **(Figure 7)**. In contrast, the zebrafish in the caffeine and melatonin tanks took longer to lose interest in the green light, with more fluctuations and regressions **(Figure 7)**. Caffeinated zebrafish relapsed daily, while melatonin zebrafish relapsed every few days **(Figures 4 and 7)**. The ADF test value of zebrafish reacting to the green light within the caffeine tank (-7.47) is less than that of the zebrafish within the melatonin tank (-6.14), indicating that there was more inconsistency within the caffeine tank and more instances of regression, likely reflecting caffeine's stimulating versus melatonin's sedating effects. Most of the early trials had statistically non-significant differences between the percentages of zebrafish responding to the stimulus in each experimental group, which indicated that the results could have been due to external variation. However, the later trials (15, 17, 18, and 20) were statistically significant, which may suggest an increase in significance with an increase in experiment duration.

### Zebrafish Behavior

The zebrafish did not show signs of distress or being unwell and were closely monitored for the duration of the experiment. Visually, the zebrafish in the control tank tended to school in groups, often swimming around the tank. We noticed that the zebrafish exposed to caffeine were generally more active than the others, moving with quick bursts of speed and often gathering in close groups. They also tended to group in the corners. The zebrafish exposed to melatonin were less active and tended to school less than the untreated zebrafish. They moved across the tank less often than those in the control or exposed to caffeine, more often remained still in the water column. We were unable to concretely observe sleep cycles.

## DISCUSSION

Our study investigated how caffeine and melatonin influence learning trends in juvenile zebrafish. Based on caffeine's stimulating properties, we hypothesized that caffeine exposure would produce more erratic and irregular learning behavior, while melatonin's suppressive effects would slow learning. To test this, we used an automated red- and green-light associative learning system to compare control, caffeine-treated, and melatonin-treated zebrafish over a one-week period. Our study suggests that caffeine and melatonin have adverse effects on zebrafish learning behavior, slowing it down and making it more unpredictable. The results suggest that both caffeine and melatonin may have adverse effects on long-term memory and learning retention in zebrafish. Caffeine provided a steeper learning curve than melatonin. However, it also exhibited more inconsistency than melatonin. These compounds resulted in a regression of behavior following long periods of rest, indicating that exposure may impair long-term memory.

Caffeine made zebrafish behavior less consistent, as learning deficits reappeared after sleep each morning. We expected green-light responses to decrease and red-light responses to increase, reflecting learning. However, in the

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caffeine group, green-light values often remained positive while red-light values were negative. This reversal suggests the zebrafish failed to recall prior learning, indicating possible overnight memory loss. More data is needed to confirm this, though the trend may indicate increased sensitivity to stimuli without meaningful learning improvement.

Melatonin, at low dosages, slowed down the learning of zebrafish significantly, which can likely be attributed to its properties as a sedative. This region of the brain that plays a central role in memory formation is the hippocampus (5).
One of the primary mechanisms by which it supports memory is long-term potentiation (LTP)—a process where synaptic connections are strengthened through repeated stimulation (4–8). If LTP is disrupted during this critical developmental period, it can impair both memory and learning. Excessive melatonin may impact these processes, as well as motor function. Current literature has shown that melatonin slows down zebrafish cognitive function and increases their sleep, which may be the underlying cause of the slower learning (3,9). Melatonin also caused fluctuations in the number of zebrafish that exhibited the learned behavior, but it was not as extreme or as frequent as caffeine. Zebrafish treated with low doses of melatonin seemed to forget learned behaviors every two days, around two times the recollection time of caffeine. The promising results shown by the control tank therefore suggest that exposure to melatonin and caffeine resulted in poorer learning in Juvenile zebrafish.

In our statistical analysis, we utilized an ADF test to demonstrate inconsistency in the number of zebrafish exhibiting learned behavior. However, because we only ran the experiment one time, the fluctuations could also be attributed to significant external noise in our methodology and data. If we reran the experiment, we ultimately may not observe as much inconsistency.

A potential future experimental design that more closely reflects real-world patterns of caffeine and melatonin consumption could be performed, particularly among adolescents who often ingest caffeine during the day to promote alertness and melatonin at night to facilitate sleep. This could be achieved by introducing an alternating-treatment group of zebrafish that receive caffeine during the light phase and melatonin during the dark phase, in addition to control, caffeine-only, and melatonin-only groups. Using the established red/green-light associative learning design, training could be conducted in the afternoon with subsequent recall probes in the morning to assess overnight memory consolidation. This design would allow for direct evaluation of whether daytime caffeine enhances memory acquisition while nighttime melatonin alters memory consolidation, as well as whether the combination produces cumulative or interactive effects distinct from single-agent exposure.

We faced several limitations that we aim to address in future studies. The Arduino MEGA2560 boards that we used to control the learning system did not have ways to connect to WiFi and were therefore running on internal clocks. These clocks were often out-of-sync, resulting in each tank accumulating timing discrepancies. As a result, we had to manually restart the learning systems daily to resynchronize their internal clocks and guarantee accurate recording of data. Since the system was launched in the same room as the zebrafish tanks, walking into the room and restarting the system may have impacted the zebrafish's reactions to the earliest trial–6:00 AM, green light.

All three Logitech webcams were mounted onto one NVIDIA Jetson TX2 board because of software difficulties with other Jetson boards. Because all three webcams were connected to one board, video resolutions were limited to a lower quality. This made it more difficult for the YOLOv5 program to track each zebrafish and required us to manually confirm footage and data after the auto-tracing algorithm finished. Multiple-round manual confirmation was required to accurately count the Zebrafish. Moreover, the Jetson board housing the three web cameras occasionally crashed, with each crash occurring between two consecutive trials, although this was quickly detected and manually fixed. Restarting the cameras was a physical process, and the presence of external stimuli like humans may have impacted the performance of some zebrafish.

Arduino boards equipped with WiFi hardware could be utilized in the future, which would prevent the manual restart needed daily and result in less external stimuli for the zebrafish. More NVIDIA boards could also be used, since each camera would then be able to film at higher resolutions, resulting in a higher resolution video frame for the multiple object detection software to analyze. The objective of the selected procedures is to fully automate the behavioral study, which was not possible in this project, but is possible in future studies.
We also faced some limitations with experimental design. The experiment was only run for one week to ensure the safety of all the zebrafish involved, which limited our ability to gather more data points. Therefore, it is likely that some of the variation that we observed was a product of experimental noise. By repeating the experiment and collecting data averages, we can decrease the effect of such variations and gain a more holistic view of caffeine and melatonin's impacts on zebrafish learning behavior.

Another limitation we encountered consisted of linearizing our data to incorporate a semi-log plot fit analysis. We originally planned to apply linearized values using a logarithmic scale to better quantify the relationship between trial progression and response strength. Although we were able to generate best-fit lines and corresponding $R^2$ values, the dataset did not follow a strictly linear trend, and our spreadsheet system did not allow us to produce semi-log plots, which may have more accurately represented the underlying relationships. As a result, the lines provide only an approximate view of the data, and the $R^2$ values should be interpreted with caution since they are based on non-linearized values. Importantly, the statistical tests used in our analysis remain constant, so the overall conclusions are not substantially affected by this limitation. If we had been able to linearize the data, the best-fit lines would have likely shown stronger correlations (higher $R^2$) and a clearer directionality of the trends. In general, this might have emphasized the strength of the observed effects while reducing some of the variability in the data. Nonetheless, organizing results by trial number and retaining the natural data structure allowed for consistent interpretation alongside the time markers referenced throughout the study.

Zebrafish were kept in groups of 15 to stimulate group settings commonly found within adolescents, but the schooling behavior of the zebrafish may have accelerated their behaviors. Since zebrafish tend to follow each other around, a single zebrafish can lead the entire group to exhibit

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the learning behavior, and vice versa. Although the results from Levene's test indicated homogeneity of variance among these three original zebrafish cohorts, the learning trends in this study may be different for smaller groups of zebrafish or individuals. This study can be repeated in the future using fewer zebrafish within each tank (1–3 zebrafish). This may allow for more accurate results, since the zebrafish in these cases are likely not as susceptible to group influence. More trials may also allow for more distinct patterns to develop.

We stuck to lower dosages of each compound to maximize the safety of the zebrafish based on prior studies, which may have limited their effects (9,10). Replication of the experiment with increased dosages may yield faster and more extreme results that highlight the differences between each experimental group.

With the addition of further resources, we may be able to investigate the physiological changes behind this phenomenon, utilizing technologies like electroencephalography and microscopy to monitor the changes happening within the zebrafish neurological system. Utilizing this, we may be able to identify the underlying cause of poorer learning and loss of learned behavior following caffeine and melatonin exposure.

By expanding the range of compounds covered in the project, new software for early detection and prevention may be developed. In the future, compounds like corn syrup and other common ingredients may be added in place of melatonin and caffeine, with the study aiming to explore the effects of these common compounds on learning and developmental behaviors in zebrafish juveniles. Ultimately, the experiment can be extended to rats and other model animals, gradually moving closer towards humans. With the information and results gathered through these organisms, an integrated predictive model can be developed to predict the long-term effects of common compounds on humans, specifically adolescents. Ideally, this would be developed into an online application that users could use as needed, analyzing the risks behind their favorite drinks and serving as a public health tool. By starting with smaller model organisms like zebrafish, our findings on how caffeine and melatonin affect adolescent learning could inform public health guidance on teen consumption of these substances, ultimately promoting safer use and greater awareness of their potential cognitive effects.

## METHODS

### Zebrafish

Forty-five juvenile wild type (WT) zebrafish (*Danio rerio)* were used in the study. We chose to use unsexed specimens of various juvenile ages to mimic the diversity found in adolescents who consume compounds like caffeine and melatonin. Zebrafish were returned to untreated conditions following the experimental period. Zebrafish were regularly monitored to ensure health and safety. Dr. Greg Lewbart, a veterinary professor at North Carolina State University specializing in ornamental fish, provided a guide on warning signs and behaviors, which we used to assess each group daily. A visual BCS (Body Conditioning Scoring) was done before and after the experiment using a guide, since weighing was not feasible (10).

### Zebrafish Housing

Zebrafish were kept in groups of 15 throughout the course of the experiment. Each group was kept in a standard 10-gallon tank. A filter and heater, kept to 78°F, were attached to the back of the tank. Filter medium and partial water changes were done weekly. The sides and back of each tank were covered with opaque white PVC sheets to prevent visual access to other environmental cues and stressors **(Figure 1)**. The zebrafish were kept on a 15-hour light:9-hour dark cycle (lights of the room where the zebrafish were housed turned on at 6:00 AM each day and turned off at 9:00 PM each day). Zebrafish were fed pellets from the automated feeder three times a day, six hours apart. The zebrafish were isolated during the experiment—feeding, learning, camera, and lighting systems were all automated, allowing them to exhibit natural behaviors without the stressors of human presence or interactions.

### Drugs

Laboratory-grade caffeine ($C_8H_{10}N_4O_2$) was purchased from Carolina Biological Supply Co. The caffeine tank was dosed with 946.353 mg, at a concentration of approximately $2.5 \times 10^{-2}$ g/L. Melatonin lozenges were purchased from Neil's Compounding Pharmacy (Charlotte, NC), crushed and weighed, and 8.79 mg of melatonin was added to the treatment tank, at a concentration of approximately $2.3 \times 10^{-4}$ g/L. A partial water change was done shortly before the compounds were added to the water, and filter cartridges were replaced. The addition of these compounds did not change the additional water parameters (such as nitrate, nitrite, chlorine, pH, etc.). Each tank was left to cycle for 12 hours, and the zebrafish were added directly after. The experiment began 12 hours after the zebrafish were added and ran for 1 week.

### Automated Learning Model

We developed the automated feeding and learning model with an Arduino MEGA 2560 board. Attached to the board was a mini traffic light and a small Servo motor. These were connected with Dupont wires controlled with an automated code written in Arduino IDE 2.3.4. A servo motor was glued to the lid of a 2-oz deli cup, which held the food. A hole in the cup allowed the motor to push food into the tank when activated **(Figure 1)**. Both the Arduino and the feeding cup were mounted on an opaque white PVC sheet placed over the tank **(Figure 1)**.

### Automated Camera

Three Logitech c270 webcams were connected to a NVIDIA Jetson TX2 board, with each camera positioned in front of a tank **(Figure 1)**. We programmed in Python 3.8 to automate the video recording of zebrafish activities, facilitated by the OpenCV library. Cameras were set to record for 3 minutes—around 90 seconds before and after each trial. Recorded footage was stored in hard drives connected to the NVIDIA Jetson TX2 and later collected for analysis.

### Automated Learning System

Zebrafish were trained to associate a red light with food and a green light with no food. The system, coded in Arduino IDE 2.3.4, flashed a green light (no food) 3 hours before a red light (food dispensed 15 seconds later), every 6 hours (11). Each light was flashed for 15 seconds, then turned off for 15 seconds before approximately 10–15 food pellets

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were dispensed. Webcams linked to an NVIDIA Jetson TX2 began recording the tanks 60 seconds before the lights began flashing and stopped recording 120 seconds after they flashed, storing footage on an external hard drive. While this system only recorded when trials were occurring, we had an online livestream monitoring all tanks throughout the experimental period.

We trained an Ultralytics YOLOv5 object detection model using Roboflow on a 400-frame custom dataset representative of how the zebrafish looked within our tank setups (12, 13). We manually marked each frame, running the training with 75% of the frames and testing with the remaining 25%. We leveraged the trained model to automatically recognize and track zebrafish **(Figure 1)**. The model could not track individual zebrafish throughout the duration of the experiment (ex. zebrafish ID #1 is always zebrafish #1), only counting the number of zebrafish demonstrating learning behavior.

Our performance metric measured the number of zebrafish that moved towards the red light when it flashed and then remained under the motor until food was dispensed, which strongly indicated the individual zebrafish had learned the correlation between the red light and food. While the YOLOv5 software assisted us with automated capabilities, we also extracted frames from videos and manually tracked the number of zebrafish that displayed this behavior at each trial. In addition to this, we tracked the number of zebrafish that moved towards the green light.

### Statistical Analysis

After collecting behavioral data, we analyzed the number of zebrafish exhibiting learning behavior (e.g., Melatonin–green light, Caffeine–red light, Control–green light) across treatments. Assuming random samples and using a significance threshold of $p < 0.05$, we first applied a Shapiro-Wilks test to assess normality and identify appropriate statistical tests for paired group comparisons. A Levene's test confirmed homogeneity of variance, indicating zebrafish behaviors were consistent. We also conducted an ADF test to examine observed regressions in learning behavior and a Fisher's Exact Test to assess associations between treatment groups and the percentage of zebrafish moving toward the feeding area.

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