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A learning assay for the color discrimination skills of the Amazon molly

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Abstract

In an ever-changing world, animals must constantly adjust their behavior and learn new associations. These can be learned from trial-and-error or observing conspecifics and/or other species. In this study, the color discrimination and learning abilities of the clonal Amazon molly (*Poecilia formosa*) were assessed in a simple binary choice assay. Two groups of genetically identical individuals were tested on alternate days and were required to learn to form a connection between a certain color and a hidden food reward inside the colored compartment (Red or Blue). The experimental results indicated that the fish potentially learned to make associations with color and food as they appeared to improve in accuracy over time. The average proportion of trials with the blue colored cup entered first increased from 25% (on the trial day 1) to 54% (day 5), with a correlation of $r = 0.962$ ($p = 0.009$). This finding however suggests a day 5 accuracy of no better than random chance. We also found that the fish who were assigned the focal color red performed significantly better compared to those assigned to blue (89% and 44% as the accuracy, respectively; 2-sample t test $p < 0.001$). Overall, the results suggested that this assay was unsuccessful in providing evidence for the color discriminating abilities - and hence, of the changes in learning abilities - of the clonal Amazon molly. The unsuitability of this assay may be due to its inability to discount a preference for the red color; being similar to the color of the diet of the Amazon molly.

Keywords

Amazon molly, Behavioral variation, Clonal fish, Color discrimination, Learning ability, *Poecilia formosa,* Binary choice assay, Food reward, Adaptation, Predator stress

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Introduction

For animals in a changing environment, learning is important for survival. For example, animals need to learn whether a certain location is safe, or whether a particular pattern indicates a poisonous substance or prey (1). They may also acquire skills from conspecifics, such as a fledgling learning to take flight from its parents. These require the animal to learn from trial and error or from observing others. This is apparent even in humans, because humans are not born with the ability to do vital tasks, such as walking and talking. However, individuals, even of the same species, have varying cognitive and learning abilities (2). This difference can be caused by many factors, including an individual's past experiences which may influence their present behavior. For example, stress from predation or hunger has been shown to influence the learning ability of juvenile Mulloway fish (*Argyrosomus japonicus*) in a two-sided compartment apparatus (3). In one side of the apparatus, food was scheduled to be released periodically. The fish were put into groups based on how much stress they had previously experienced, indicated by the presence of the stress hormone cortisol. In the first week of the experiment, the fish with lower levels of stress were more active and improved their accuracy (time spent in rewarded area) in the apparatus. During this experiment, fish with higher stress levels did not improve their accuracy, showing that stress variation can result in different expressions of behavior and learning (3). Another factor that can result in behavioral differences is genetic variability in a species. Even when raised under similar environmental conditions, animals of the same species can still have a very wide range of behavioral differences (4-6). Since underlying genetic variation can contribute to behavioral variation, it is difficult to determine how much behavioral variation is caused by extraneous factors, such as differences in rearing environment or past encounters with stress. In order to isolate and better understand the other factors driving behavioral variation, it is necessary to minimize the effect of genetic variation by working with clonal individuals.

Therefore, we used the Amazon molly (*Poecilia formosa*) (Figure 1), a clonal and unisexual female fish species that is native to the Southern United States and Mexico (7). It is a natural hybrid of the two *Poecilia* species *P. latipinna* and *P. mexicana* (8). Since it is a clonal species, using this system reduces the effect of genetic variation on behavioral variation. However, before the Amazon molly system can be utilized to isolate and understand the factors influencing cognitive and behavioral variation, it is necessary to create a learning apparatus that we know the Amazon molly is able to succeed at. This ensures that whenever a variable of interest is manipulated, (i.e., exposure to stress), any resulting change in the learning ability is because of this outside manipulation, and not because the fish is not able to solve the task at baseline.

The color discrimination ability of fish has been demonstrated in previous studies of species in the *Poecilia* genus, which the Amazon molly belongs to. Similar *Poecilia* fish (guppies) have been shown to more effectively learn differences in color than in shape (9), which indicates that a color-discrimination task may be effective for the Amazon molly. In female *Poecilia reticulata*, fish were able to associate a certain color as an indicator of food

by interacting with colored disks (10). The disks were set up in a way such that a food reward was hidden underneath the disk in a small well/indent. The disks were also designed to be easily dislodged by the fish. The results of this study indicated that the fish learned the color association task as evidenced by their approaching and displacing the correct (food-rewarded) disk more often over time (10). When both sexes of *P. latipinna, P. mexicana,* and female *P. reticulata* were exposed to similar color discrimination training with colored disks and food hidden underneath, they also remembered a certain color disk as a food indicator (11). After learning the initial association between color and food, they were even able to perform reversal learning, where the focal color (color of the disk with food inside) was changed (11). These studies indicate that species closely related to the Amazon molly are able to perform color discrimination tests, which suggests that the Amazon molly may be similarly successful in these tests.

Figure 1. Amazon molly of the clonal line AM11

Previously, when the Amazon mollies in our lab were tested with a color discrimination maze apparatus, they did not seem to improve their learning across trial days. These previous failures may be explained by a different system of perception of the Amazon molly, which has a different visual system compared to its parent species, the Atlantic and Sailfin mollies (12). Another potential reason for not achieving proficiency in a color discrimination task could be that the apparatus used previously was not appropriate for the fish in some manner. The experiences and considerations from previous efforts were incorporated in this study.

In order to assess the learning skills of the Amazon molly, we constructed a different learning apparatus. The experimental setup consisted of two cups, or compartments, of different colors (Red or Blue), and as the fish were released into the setup, the fish were required to decide which cup contained a food reward. Over time, we evaluated if the fish learned the connection between target color and reward by measuring their choice accuracy. In this experiment, we attempted to answer the question: “Can the Amazon molly learn an association between a color cue and the location of a food reward?” We hypothesized that the fish would use the different colors of

blue and red to learn which cup indicated the presence of a food reward. We predicted that the fish would deliberately approach their assigned color cup repeatedly as the first choice, which would indicate that they had formed such an association.

Materials and Methods

Experimental setup

Two groups of four Amazon mollies, for a total of eight fish, were obtained from a lab stock of the AM 11 clonal line (Figure 1). Prior to the experiment, on April 11, 2024 these fish were separated into eight individual, clear compartments and labeled with a three-number code. These codes were 1-1-1, 1-2-1, 1-2-2, 2-2-1 in group 1, and 1-1-2, 2-2-1, 2-1-2, 2-1-3 in group 2. They were fed daily *ad libitum*., with San Francisco Bay Brand bloodworms (*Chironomus sp.*), live, freshly hatched Brine shrimp (*Artemia sp.*) from Brine Shrimp Direct, and Tetra brand TetraMin Tropical Flakes. Clean, reverse-osmosis treated water was allowed to flow between the perforated containers continuously. Periodically, the holding containers of the fish were cleaned and the water quality of the tank water were tested, in order to determine if any care changes needed to be made.

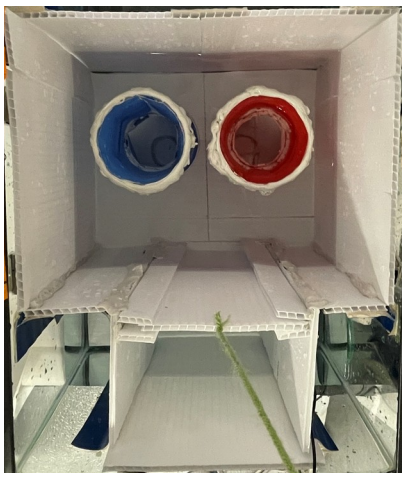

Figure 2. Experimental setup of the two-cup assay (top view)

A 10-gallon tank was modified into the experimental apparatus, and two plastic cups for the dichotomous choice compartments (Figure 2) were added. After the initial two weeks, the tank design was changed slightly to allow more room for the fish to navigate in the arena. The tank consisted of two sections, a ~13 by ~7 cm holding start area, and a larger ~15 by ~22 cm experimental area. The tank was lined with Coroplast®, a porous, waterproof, opaque white plastic board material. Coroplast® was also used for all the main experimental tank compartments. Connecting the starting area and the experimental area was a guillotine door system, operated by a string-pulley, and held in place with a Velcro clamp, to minimize the amount of human contact during the experiment. Two

modified plastic cups were designed to serve as food reward compartments. The cups had a diameter of ~8 cm, and had an opening on the side, measured from the bottom rim, of ~4 cm high by ~3 cm wide. This size was chosen for the opening, as it was large enough that the fish were able to enter the compartment easily, but also small enough that the fish could not see all the way inside. Before modifications were made to the experimental setup after the first two weeks, the cups were the same color on the inside and outside, in order for the fish to attend to the color cue when inside the cup as well. Before each trial, the experimental cups were placed ~7 cm from the start area, and ~3 cm from the long side of the experimental area wall during trials. In conjunction with the cups, two circular plastic feeding platforms were constructed to contain the food reward. This food reward consisted of a segment of mini bloodworm (midge fly larvae), *Chironomus sp.,* which were roughly cut into even thirds. This food reward was used successfully in other experiments (13). The feeding platforms were made of the same material as the cups and had the same color as their respective cups on the outside rim. On the inside, the feeding platforms were a white color, in order to achieve contrast with the red colored bloodworm. They were ~3 cm in diameter, and the walls were ~1 cm high, in order reduce the likelihood that the fish could see the food inside the container before fully entering the cup. During all experiments, the food was adhered to the feeding platform with a small amount of Vaseline. Periodically, the wall of the feeding platform was replaced. Construction of the setup, cups, and platforms was done with waterproof Butyl tape.

After the initial two weeks, the experimental area was increased in length, and the colored cups were modified to be white on the inside. The colored cups were changed to have only white on the inside as opposed to their respective colors, because the entrance to the cups may have been obscured due to lack of contrast between the inside and the outside. The experimental area was increased by ~8 cm in length, which resulted in the experimental area being ~23 by ~22 cm, allowing the fish more space to explore and view the cups. The exposed butyl tape on the inside of the apparatus was also lined with white Coroplast® so the fish would not be distracted by inspecting it during the trials. The placement of the cups was not changed.

During the trials, the water level was kept ~ 13 cm from the bottom of the tank. After every set of trials (6 for each fish, each day), the water was drained to ~7 cm from the bottom of the tank and refilled with new water (to 13 cm), in order to ensure cleanliness for the next fish, and to maintain water temperature. The temperature in the room the experiment took place was a little lower than the usual temperature of the room the fish were housed in. The water used in this experiment was all reverse osmosis, deionized water, kept at constant conditions of pH 7.2-7.4, conductivity 850-1000 µS/cm, and temperature ~26-28 °C. Outside of the trials, the fish were maintained in comparable water conditions.

Experimental trials

Fish were tested primarily every other weekday. Each group of fish rested for at least one day between trials, on which they were not fed in order to increase their motivation to find

food during testing. Before the operation of the trials, the four fish in each group were randomly ordered. This ensured that any scent cues left from the previous trial would have an even effect on the overall fish behavior, as they were not always in the same order. The placement of the cups was also randomized for every trial. For example, for a total of 6 trials, 3 trials were randomly assigned for the focal cup (which contained the food reward) to be on the right side, and for the other 3 trials, the focal cup was on the left side. This eliminated the possibility that the fish could learn using the relative location of the food reward (*i.e.*, left or right), and ensured that the decision was primarily driven by color. For all trials, no more than two consecutive trials had the same arrangement of cups (e.g., red on the left side).

Before the start of the trials, the fish were carefully transferred from their holding tank to the experimental apparatus. The individual compartment of the fish was poured into the apparatus, in order to minimize the time out of water for the fish. For the first half of the experiment, after each set of trials the fish were removed from the setup with a net and placed back in their individual compartments. This later changed to coercing the fish back to its original compartment within the testing apparatus and moving it back to its home tank. For each trial, 5-minute videos were taken with a Raspberry PI 4 with a camera extension and managed on a personal computer.

The fish were pre-trained to enter the cups in order to prepare for the experimental training. For one week, each fish had two sets of 6 individual trials each. Prior to this experiment, five fish underwent some pre-training with the cups, and these fish were spread evenly among the two groups. The pre-training followed the previously discussed protocol with the randomized fish order and cup order, but food was placed in *both* cups. The goal of the pre-training was to get the fish acquainted with entering the cups when they were turned entirely away from them with food hidden inside of them. For the first day (6 trials) of pre-training, the feeding platform was initially placed entirely outside of the colored cups (Figure 3, Panel a), and the entrance of the cups remained facing the front for the remainder of the first set of trials. After the fish were transferred into the holding start area, the 5-minute recording started, and they were allowed two minutes to rest and acclimate before they were allowed into the experimental setup with the cups for a period of three minutes. After the allotted trial time ended, the fish were coerced back to the start holding area. The cycle of recording the 2-3 minutes of exploration was repeated for each trial. If the fish was successful in finding the food reward near the cups within the 3-minute trial time, the food compartment was then gradually moved inside the cup for all 6 trials, until it was fully inside of the cup. On the second day of pre-training, the fish were subjected to the same trial times, however the cup was gradually turned away from the fish, until it was pointing towards its respective back corner of the experimental area (Figure 3, Panels b and c). This was because during training trials, it was important that the fish could not directly see into the cup to ensure they were making a conscious decision to approach and enter a certain color cup. With this adjustment, it was evident which cup the fish approached and entered first.

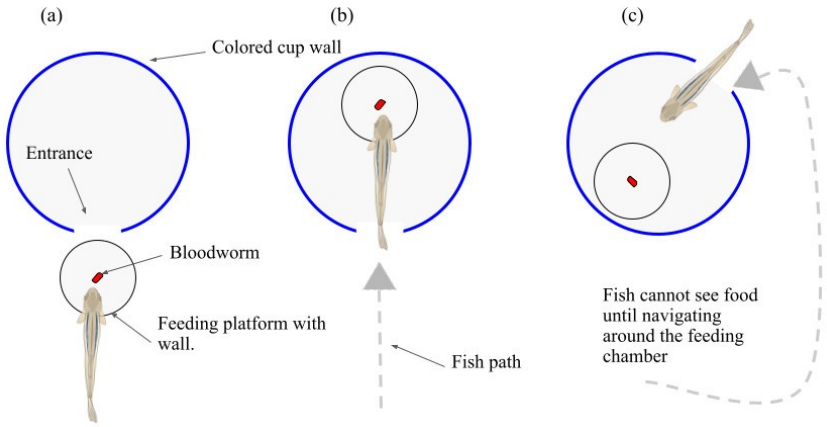

Figure 3. Diagram of feeding platform location and fish path change over pre-training

Formal training of the fish included food in only one container; as opposed to two during the pre-training. For each fish, this consisted of 5 days of training with 6 trials each day, for a total of 30 training trials. Before training, each fish was randomly assigned red or blue as a focal color. The focal color was the color of the cup that contained the food reward for the remaining trials. Data was collected on which cup the fish entered first for each trial, in order to determine if the fish improved in their accuracy over time (frequency of selecting the cup with the food reward). There was an equal amount of blue-focal and red-focal fish in each group, for a total of four red and four blue in the entire experimental cohort. The recording schedule was the same as during pre-training; two minutes of acclimation time after being added to the tank or corralled to the start between trials, and three minutes of exploration time in the main experimental area for each trial. The orientation of the cups was the same as that during pre-training, however, the placement of the segment of bloodworm on the feeding platform was different. In order to ensure that the fish was not able to directly see the food reward before entering the cup, the food was placed near the side of the cup wall on the feeding platform (Figure 4). The feeding wall was intended to block visual contact with the food reward, unless the focal fish entered the cup and swam to the center of the cup. This reduced the likelihood that the decision of the focal fish was based on direct visual contact with the food. Before each trial, a few drops of bloodworm scent cues (present in the liquid used to thaw frozen bloodworms) were placed in the non-target cup. This reduced the likelihood that the fish were using olfactory cues to make their decisions. The amount of scent cues added varied based on the concentration of scent in the thawing water (if the water was more concentrated, fewer drops were added, and vice versa). Trials took place over eleven days, with one day skipped, and each group of fish was tested every alternate day.

Timestamps of the five-minute videos were recorded during data processing. This included when the fish left the start area after the door was opened, which functioned as a measure of

how eager/willing the fish was to enter the experimental chamber. We also recorded the timestamp of when the food was found, which was indicated by the moment when the fish's mouth made contact with the food. This allowed us to determine if the fish had become more efficient at locating the food over time, by tracking whether this time decreased across trials. Binary variables were recorded for which cup the fish entered first (red or blue), and whether the fish ate the food (yes or no). This was used to determine, if the fish improved in their accuracy when locating food and whether they appeared to form a connection between the color of the cup and the reward (frequency of the correct cup entered first and frequency of the fish obtaining food successfully).

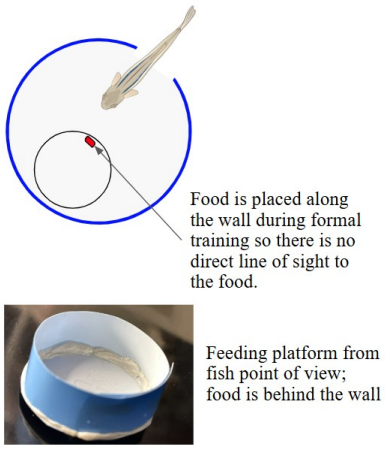

Figure 4. Placement of food during formal training to enforce color learning in fish

Results

The proportion of trials with the correct cup entered first was graphed for each fish over the five trial days (Figure 5). The behavior of each of the eight focal fish can be categorized into three general types. The first of these include fish that seemed to have near-perfect scores, as in they entered the correct cup first from the outset of training trials. This group consists of fish "111" (trained with blue as the target color), "211" (red), and "221" (red). This is shown in Figure 5, as some lines have a constant proportion of trials with a correct first choice of above 50% for the entire duration of trials. This means that these fish were potentially able to make associations with color and food at a faster rate than others. The second group included fish that gradually improved, as predicted, including the fish "121" (red), "212" (blue), and "213" (red). These fish increased in their accuracy from below 50% for the first relative half of trials to above 50% for the remainder of trials as depicted in figure 5. These fish initially entered the correct cup first a low number of times, but as the experiment progressed, the proportion of trials with the correct cup entered first increased. This indicates that these fish may be learning gradually, albeit slower than those fish mentioned in the first behavioral type. The last behavioral type observed were fish that consistently did not enter the correct cup first

throughout the whole week of trials, including the fish "112" and "122" which were both trained with blue as their target color. As shown in Figure 5, these fish had a proportion of trials with the correct first choice of 50% or below for the entire duration of training. This indicates that these fish most likely did not learn to make associations between the color and food.

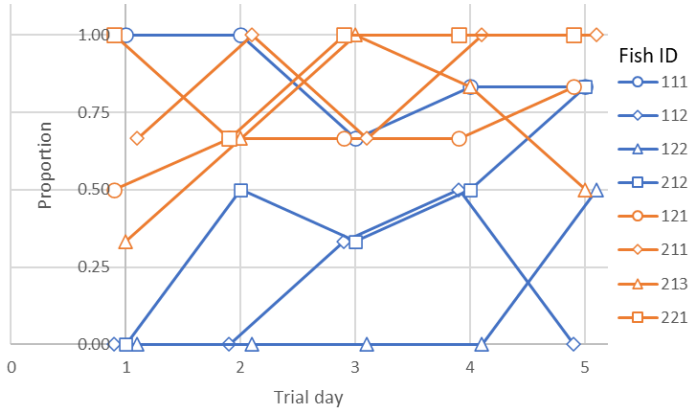

Figure 5. Proportion of trials with the correct cup entered first vs. trial day to assess fish learning (the x values were slightly shifted to better illustrate the otherwise overlapped data points)

There was a general increasing trend between the average proportion of trials with correct first cup choice over trial days (Figure 6). This proportion increased from 44% (day 1) to 69% (day 5), with an average of 59%. It is noteworthy that due to the small sample size in this experiment, these percentages are not appreciably different from random chance (i.e., 50%) when the data from the blue and red assigned fish are combined. The average proportions of trials with correct first cup choice were significantly correlated with the trial days (r = 0.962, p = 0.009), which was mainly contributed by the increasing trend identified for the fish trained for blue cups (r = 0.936, p = 0.019), while the fish for red cups showed high but stable proportions (p = 0.061). The red assigned fish were always associated with high accuracy across all days (63% to 88%, Figure 6). However, the fish assigned blue increased from a very low proportion (25% on day 1) to the level only slightly higher than random change (54% on day 5), which could suggest that blue is a deterrent color to the Amazon molly.

We also evaluated whether the position of the target cup (left or right) had any impact on whether the fish chose correctly. The overall proportion of trials with correct first cup choice was 61% and 57%, for the left and right positions, respectively. The results of two-sample t-test indicate that the proportions for the two positions were not significantly different from each other (p = 0.630) (Figure 7). This is as expected, because the target cup position was randomized across trials.

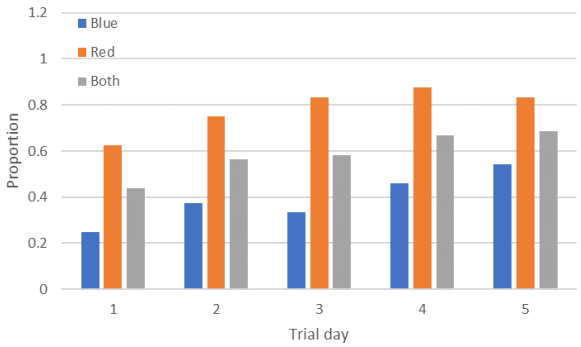

Figure 6. Average proportion of trials with the correct cup entered first vs. trial day to assess fish learning

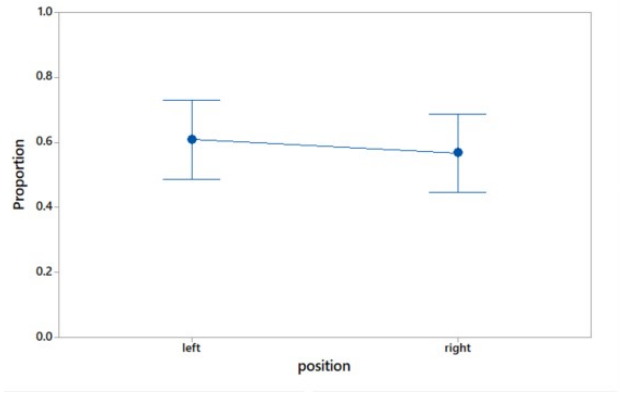

Figure 7. Interval plot of the proportion of trials with the correct cup entered first vs. position (left or right) of correct cup (showing 95% confidence interval for the mean)

Finally, the "overall foraging success" was characterized by the proportion of trials with food eaten, i.e., the ratio of trials where the food was found and eaten by a fish. For all trials, the average proportion of food eaten was 67%, suggesting that a fish had about a two-thirds chance of successfully finding and eating the food. In successful trials, the average time fish took to find food was 2.8 minutes, which did not vary across trial days or target colors.

Similar to the proportion of trials with the correct cup entered first, there was a general increasing trend between the average proportion of food eaten and the trial day, perhaps indicating that the fish were able to locate the food better over time (Figure 8). For all trials together (blue and red cups), the possibility of food eaten increased from 52% (day 1) to 77% (day 5), with a correlation of $r = 0.978$ ($p = 0.004$). The fish assigned red as their target color had a higher likelihood of eating the food than those assigned blue, with averages of 89% and 44%, respectively.

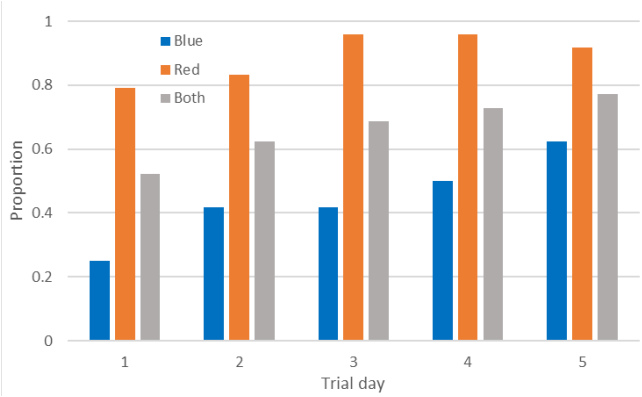

Figure 8. Average proportion of trials with food eaten vs. trial day

Discussion

The aim of this experiment was to evaluate how proficiently the Amazon molly could learn a color discrimination task. Generally, the fish learned to enter their rewarded cup first, since the frequency of entering that cup first increased over time. However, there was both between and within individual variation in accuracy, and not all fish were able to achieve or maintain a high level of accuracy across trials. As stated earlier, some fish were proficient at forming the association quickly, others seemed to improve a little over time. But some fish displayed minimal motivation to enter the cups and locate the food reward.

Specifically, two fish assigned the focal color blue displayed significantly less motivation to enter the blue cup (where the reward was located). Rather, they chose to enter the red cup or no cup at all. This could be due to an innate color preference for red in these two fish, or a preference developed during the pre-training stage. In an experiment conducted by Culbert et al. (14), the color of a disk was shown to influence the number of interactions by a fish. In the first trial of this experiment, cichlid fish were presented with colored disks with no reward, and disk color significantly affected the number of interactions with that disk (e.g., fish interacted significantly more with blue as compared to yellow) (14). As this study was done with no motivation for the animal to interact with a certain color, it shows that other animals, such as the Amazon molly, also possibly have an innate preference for certain colors over others. Similar *Poecilia* fish (*Poecilia reticulata)* also show an innate attraction to orange, independent of their reproductive preferences. Orange, a visually similar color to red, is shown to drive innate color preferences in guppies since their diet is comprised of fruit of this color (15). Since the Amazon molly is similar to guppies, it may be that they also have innate preference for certain or similar colors. As shown in a study done on *Poecilia reticulata,* fish displayed less interest in approaching colors that were different from their natural food source. Guppies in this study interacted with green significantly more than blue. Guppies' natural food (algae) is green, and there are less foods that display a blue color, so there would be no benefit evolutionarily to have an innate attraction

towards a color that did not provide a reward (16). This was reflected in our study as one of the food sources readily given to our fish reared in the lab were bloodworms, which are a reddish color. As the fish are familiar with this food, they may have developed a cognitive connection between the color red and food, influencing them to approach the red cup more often. A preference developed during pre-training could be explained as the fish "training" itself to only go into the red cup, without realizing that the other cup (Blue cup) also had food. This may cause an individual to habitually ignore or disregard the blue cup when searching for food, and result in a "wrong" choice. Lastly, certain fish assigned blue as a focal color could have had low motivation in general, which would account for them not actively seeking out and entering cups.

The behavior of individual focal fish was still visibly different, potentially due to small differences in environment or past experience. The fish were subject to the most standardized environment possible, however there were noticeable differences in fish learning ability, even as clonal organisms. This aspect of personality has also been explored in previous research done on two strains of artificially cloned red-spotted cherry salmon (*Oncorhynchus masou macrostomus)* (6). When these two strains of fish were observed in four different states: tranquil (resting), feeding, alert, and threat, they were shown to behave significantly differently in some of the stages. Differences were evident in their boldness, activity, and greediness, which were determined based on different interactions during the trials (e.g., hiding during "tranquil" trials) (6). This research shows that clonal fish can have different behavior, despite being genetically identical. In addition, previous studies in the Laskowski Lab (4, 5) have indicated that the Amazon molly could develop behavioral individuality in the absence of genetic and environmental variations. These findings show that there is significant variation in learning behavior across individuals.

Knowing possible explanations to problems encountered during the experimental process, future experimental setups can be changed. Since some fish still seemed unsure of the rewards existing in the cups, the number of the pre-training trials can be increased until it can be determined that the fish recognize both cups as a food source, thus reducing bias in the fish. In order to determine innate color preferences in our fish, another supplementary experiment can be conducted where the fish are tasked to explore and enter a variety of colors of cups. The number of times the fish enter the cups can be used to determine if they have a preference or natural dislike for a certain color, and these colors can be eliminated before training begins. The remaining colors which the fish have neutral preference for can be used during later pre-training and training trials when the reward is present.

As this method seems promising in evaluating the learning of the Amazon molly, steps can be taken to further illustrate the effects of different environmental factors on Amazon molly learning. These can include exposing fish to predator stress or housing fish in different environments to determine how these factors specifically affect learning. Fish can be exposed to predator stress by visually seeing a

predator or adding the olfactory cues of natural predators to the holding chambers of fish for a significant period of time. Different environments that fish can be exposed to include keeping them in varying-colored tanks, placing them in a range of social environments, or feeding the fish more vs. less.

Conclusion

A binary choice assay was developed in this study to assess the color discrimination abilities and associate learning progress of the clonal Amazon molly. We used the Amazon molly fish in order to eliminate the effects of genetic variation on learning behavior.

The findings suggest that color preferences, possibly linked to dietary cues such as attraction to red due to familiarity with bloodworms, may influence learning performance. As a result, the fish who were assigned the focal color red performed significantly better compared to those assigned to blue. With the small sample size in this experiment, the average accuracy with blue color was not appreciably different from random chance (i.e., 50%). Overall, the results suggested that this assay was unsuccessful in providing sufficient evidence for the color discriminating abilities - and hence, the changes in learning abilities - of the clonal Amazon molly. The unsuitability of this assay may be due to its inability to discount a preference for the red color; being similar to the color of the diet. It is also noteworthy that the performance for the fish trained for blue cups was increased with the trial days from 25% (day 1) to 54% (day 5) as the proportion of correct fish cup choice (Figure 6), or from 25% to 63% as the proportion of food eaten (Figure 8). This suggests that, even if blue is a deterrent color to the Amazon molly, the fish still showed learning progress to approach food under blue cups throughout the trail; although; as stated earlier, the average accuracy was no better than that obtainable due to pure chance.

With appropriate modifications, the assay developed in this study may be a promising avenue to pursue and may serve as a viable option for observing potential changes in learning ability in response to a variety of factors. Future research is proposed to explore environmental factors including a variety of colors of cups influencing color discrimination and learning outcomes for the Amazon Molly.

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