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Coffee time: Low caffeine dose promotes attention and focus in zebrafish

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Abstract

In this study we investigated the ability of zebrafish to discriminate visual signs and associate them with a reward in an associative-learning protocol including distractors. Moreover, we studied the effects of caffeine on animal performance in the task. After being trained to associate a specific image pattern with a reward (food) in the presence of other, distractor images, the fish were challenged to locate the exact cue associated with the reward. The distractors were same-colored pattern images similar to the target. Both the target and distractors were continually moved around the tank. Fish were exposed to three caffeine concentrations for 14 days: 0 mg/L (control, $n = 12$), 10 mg/L ($n = 14$), and 50 mg/L ($n = 14$). Zebrafish spent most of the time close to the target (where the reward was offered) under the effects of 0 and 10 mg/L caffeine, and the shortest latency to reach the target was observed for the 10-mg/L caffeine group. Both caffeine treatments (10 and 50 mg/L) increased the average speed and distance traveled when compared to the control group. This study confirms previous results showing that zebrafish demonstrate conditioned learning ability; however, low-dose caffeine exposure seems to favor visual cue discrimination and to increase zebrafish performance in a multicue discrimination task, in which primarily focus and attention are required in order to obtain the reward.

Keywords Adenosine antagonist · Vision · Conditioned learning · Associative learning

Caffeine is one of the most consumed stimulants in the world (Ferré, 2008; Lieberman, 1992). It is present in a wide range of products, including coffee, energy drinks, teas, and chocolate. The popularity of this substance lies in its beneficial effects, such as heightened attention and alertness and decreased fatigue (Brunyé, Mahoney, Lieberman, & Taylor, 2010; Einöther & Giesbrecht, 2013; Smith, 2002). It is believed to affect reaction time and accuracy in a variety of tasks (Einöther & Giesbrecht, 2013), increasing consumer productivity (Dagan & Doljansky, 2006; Einöther & Giesbrecht, 2013; Franke, Bagusat, Rust, Engel, & Lieb, 2014; Johnson et al., 2016; Souissi, Chtourou, Abedelmalek, Ghozlane, & Sahnoun, 2014).

Caffeine is almost completely absorbed by the body in the gastrointestinal system, rapidly reaching the brain, where it promotes its effects. The drug is a nonspecific antagonist of adenosine receptors, especially A1 and A2A, which are dispersed throughout the brain (Einöther & Giesbrecht, 2013). Because the inhibitory properties of adenosine are blocked, a number of neurotransmitters, such as dopamine, glutamate, acetylcholine, and noradrenaline, increase postsynaptic potential in a large number of neural pathways, usually increasing brain activity (Brunyé et al., 2010; Einöther & Giesbrecht, 2013). However, caffeine exerts its effect in a dose-dependent manner: Moderate amounts increase arousal, whereas large doses have anxiogenic effects (Lieberman, 1992). Furthermore, depending on caffeine dosage, locomotor behavior has exhibited a biphasic response: Low to medium doses increase locomotor activity, but high doses decrease it (Marin et al., 2011).

In the modern world we are constantly bombarded with information in a multitasking work environment, making it important to focus one's attention even in the face of distractors, a valuable asset for enhanced learning. In this respect, studies have investigated the effects of caffeine on cognition, primarily on attention and learning (Angelucci, Cesario, Hiroi, Rosalen, & Cunha, 2002; Santos, Oliveira, Oliveira, Silva, & Luchiari, 2016).

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To combine the effects of distractors and caffeine in a discriminating task, with translational relevance to humans, we used the zebrafish, an animal model at the vanguard of neuroethological research. Zebrafish (*Danio rerio*) are becoming more widely used for neurobehavioral studies because they share psychopharmacologic, anatomic, and genetic characteristics with mice and humans (Barbazuk et al., 2000; Caramillo, Khan, Collier, & Echevarria, 2015). Moreover, several recent studies have used zebrafish to approach behavioral functions such as learning, memory, and anxiety-like responses, in addition to a number of genetic, embryological, and behavioral tools. Zebrafish are also considered a model for assessing drug effects because of the ease of diluting substances in water (Gerlai, Lahav, Guo, & Rosenthal, 2000) and the similarity of the fishes' genetics (more than 70%) with humans, resulting in a highly translational model. As such, in the present study we aimed to test the effect of low and high doses of caffeine on zebrafish performance at locating a target in the middle of several distractors in order to obtain a reward.

Method

Subjects

Zebrafish (four months old, wild type, both sexes) were acquired from a local breeding farm (Natal-RN) and kept in stock tanks (80 × 25 × 40 cm, 50 L) in the vivarium of the Fish Laboratory (Physiology Department of UFRN). The tanks were kept in a closed system using water recirculation with mechanical, biological, and chemical filtering. The water temperature was maintained at 28 °C on a 12/12-h light/dark cycle photoperiod. The fish were fed commercial food (38% protein and 4% lipids, Nutricom Pet) and frozen *Artemia salina* twice a day.

All the experimental procedures were evaluated and approved by the Animal Ethics Committee of Universidade Federal do Rio Grande do Norte (CEUA: 037/2018).

Caffeine exposure

Five days before the beginning of substance exposure, the animals were transferred from the stock tanks into three experimental tanks (40 × 25 × 30 cm) with constant aeration and daily water changes to maintain quality. The following groups were tested: control (0 mg/L caffeine; $n = 12$), chronic 10 mg/L ($n = 14$), and chronic 50 mg/L ($n = 14$). The caffeine concentrations used were based on the behavioral characterization of caffeine effects by Santos et al. (2016). To obtain these concentrations, the specific amount of caffeine powder (Sigma-Aldrich #cat C0507) was diluted in the system water. The doses were gradually increased in order to prevent animal deaths (Tran & Gerlai, 2014), starting with 5 mg/L and increasing by 50% every two days until the desired dosage was reached (10 or 50 mg/L). Caffeine exposure occurred for 60 min before and during the training/test sessions. Fish were individually transferred to a 2-L tank containing the substance and then to the training/test tank, where the caffeine concentration was kept constant.

Discrimination task

The learning task took place in three phases: (1) tank acclimation, (2) training, and (3) test. The three groups (control, caffeine 10 mg/L, and caffeine 50 mg/L) were submitted to all the phases for a total of 20 days. The experimental phases occurred in a 70 × 70 × 15 cm tank (40 L), which had walls covered with white paper in order to avoid external interference (Fig. 1).

The acclimation phase (1) lasted five days. Fish were placed in the tank in groups to prevent isolation stress, and they were allowed to explore the tank for 15 min per day. On the following days, the size of the group was gradually reduced until a single fish explored the tank for 15 min on the last day (5th day). This procedure allowed fish to become familiar with the experimental arena and avoid any novelty effect. After the 15-min period, each fish was returned to its home tank.

The training phase (2) started on the sixth day, following the acclimation phase, and lasted 14 days, with two training trials per day (for a total of 28 training trials). Fish were always alone in the experimental arena. During the training trials, a different figure was placed on each side of the tank (set of figures shown in Fig. 1), one of which was the target. The target was the figure that indicated the reward, and although it was moved every training trial, it was always paired with the reward (*Artemia salina*), whereas the others were distractors. All figures were randomized on each training trial. The reward was only available when the fish entered the target area. A silicon tube connected to a syringe was used to deliver two units of artemia to the fish as soon as it entered the target area. All four of the areas had a silicon tube, so that no other cue than the figures could be used to learn the task. Fish behavior was recorded from above using a handycam (Sony DCR-SX45 Digital Video Camera Recorder). Fish were allowed to explore the arena for 15 min, after which they were returned to their home tank.

The test phase (3) was applied after the 20th day (i.e., after 14 days of training). All procedures were the same as in the training phase, except that individuals received no reward, even when they entered the target area. Fish explored the arena for 15 min. The test was filmed and later analyzed using the Zebtrack tracking program (Pinheiro-da-Silva, Silva, Nogueira, & Luchiari, 2017). To determine whether the animal chose either the target or the distractors, we marked an

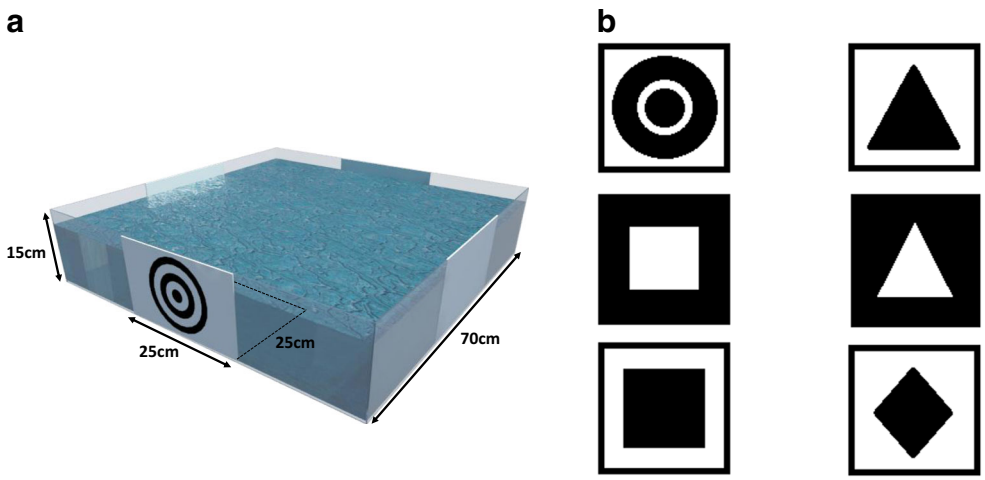

Fig. 1 (**a**) Schematic view of the arena used for the associative-learning task, and (**b**) visual cues (target and distractors) used during the training and test trials

area around each figure, and the tracking software calculated the latency to enter each area and the time a fish spent in each area. The tank (4,900 cm^2) was divided into four equal areas located around each visual cue (500 cm^2 each), plus the central and corner areas (2,900 cm^2). We also measured average and maximum swimming speed and freezing behavior.

Statistical analysis

All data were analyzed using the R program (R Core Team, 2015). A result of $p < .05$ was considered statistically significant for all tests.

First, we evaluated data normality and homoscedasticity using Kolmogorov–Smirnov and Levene tests, respectively. We used one-way analysis of variance (ANOVA) to compare parameters such as intergroup freezing behavior, average swimming speed, and maximum speed. For post-hoc analyses, Tukey's honestly significant difference (HSD) test was used to explore all possible pairwise comparisons of means.

The data for latency to enter the target and distractor areas and residence time in the target and distractor areas needed to be transformed for normality so that a linear mixed model (LMM) could be applied. Thus, we used the maximum-likelihood-like approach of Box and Cox (1964) to select a transformation index using the powerTransform command (R Core Team, 2015). For the latency data, we found the coefficient λ to be .192, and for the time data, the coefficient λ was .585. After transformation, the data showed a Gaussian distribution, and we used the lmer command from the lme4 package (Bates, Mächler, Bolker, & Walker, 2015) to analyze them. In all cases, post-hoc comparisons between the treatments of each model were made using the Tukey post-hoc test (lsmeans package; Lenth & Hervé, 2014).

Results

Figure 2 shows the time fish spent in each area of the arena during the test trial, and Fig. 3 presents the latency to enter the target or each distractor area during the test. Mixed model comparisons showed that the time spent in each area showed statistical significance for area of the tank (target or Distractors 1, 2, and 3) (LMM: $\chi^2 = 9.29$, $df = 3$, $p = .02$) but was not significantly related to treatment (control, caffeine 10 mg/L, and caffeine 50 mg/L) (LMM: $\chi^2 = 4.58$, $df = 2$, $p = .10$). The interaction term treatment by area of the tank was statistically significant (LMM: $\chi^2 = 21.88$, $df = 6$, $p = .001$). The post-hoc comparison tests (Tukey) indicated that time spent in the target area was higher for control and caffeine 10 mg/L than for caffeine 50 mg/L. The fish treated with caffeine 50 mg/L spent statistically similar amounts of time in the target and Distractor 1 and 2 areas, but less time at the Distractor 3 area ($p < .05$; Fig. 2).

The mixed model for latencies to enter each area showed statistical significance among the treatment groups (control, caffeine 10 mg/L, and caffeine 50 mg/L) (LMM: $\chi^2 = 28.16$, $df = 2$, $p < .001$), but no statistical significance was related to the areas of the tank (target or Distractors 1, 2, and 3) (LMM: $\chi^2 = 5.01$, $df = 3$, $p = .17$). The interaction term treatment by area of the tank was statistically significant (LMM: $\chi^2 = 46.58$, $df = 6$, $p < .001$). Tukey post-hoc comparison tests indicated that shorter latencies were shown by the control group to enter the Distractor 1 area, the caffeine 10-mg/L group to enter the target area, and the caffeine 50-mg/L group to enter the Distractor 1 and 2 areas ($p < .05$) (Fig. 3).

The values for average speed, maximum speed, and freezing behavior are presented in Fig. 4. A one-way ANOVA showed statistical significance for average swimming speed [$F(2, 40) = 6.70$, $p = .003$], and the post-hoc Tukey HSD test indicated that the caffeine 10-mg/L group

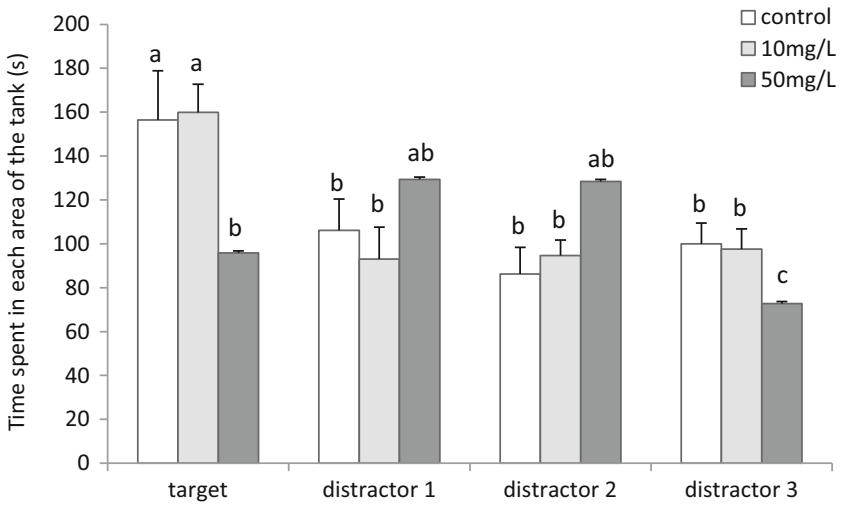

Fig. 2 Zebrafish time spent in each area of the tank during the test trial. The data (means + *SEM*s) show the time spent in each area of the tank for the control (0 mg/L caffeine; $n = 12$), 10-mg/L caffeine ($n = 14$), and 50-mg/L caffeine ($n = 14$) groups. Each area of the tank was assigned a different figure (visual cues; three distractors) and the reward (food) was given in the target area. The fish were allowed to explore the tank for 15 min per day, and no reward was offered during the test trial. Zebrafish showed a strong preference for staying in the target zone throughout the test session (LMM, $p < .05$). Different letters indicate statistical significance for the interaction term treatment by area of the tank (Tukey post-hoc tests, $p < .05$)

had a higher average speed than the other groups ($p < .05$; left panel). Maximum speed was not statistically significant across groups [one-way ANOVA: $F(2, 40) = 0.89$, $p = .42$] (middle panel). Freezing behavior, a trait related to anxiety response, was shown to be statistically significance across groups [one-way ANOVA: $F(2, 40) = 8.60$, $p < .001$]; Tukey HSD tests indicated that the caffeine 10-mg/L group had the lowest freezing response, as compared to the other groups ($p < .05$; right panel).

Discussion

In this study, we evaluated the effect of caffeine on zebrafish performance in a task requiring focus and attention. Zebrafish display a natural tendency to explore and the ability to associate an unconditioned stimulus (food) with a previously neutral cue (the target) in order to process it as a conditioned stimulus. We added distractors—that is, objects resembling the target, which could confuse the fish and impair

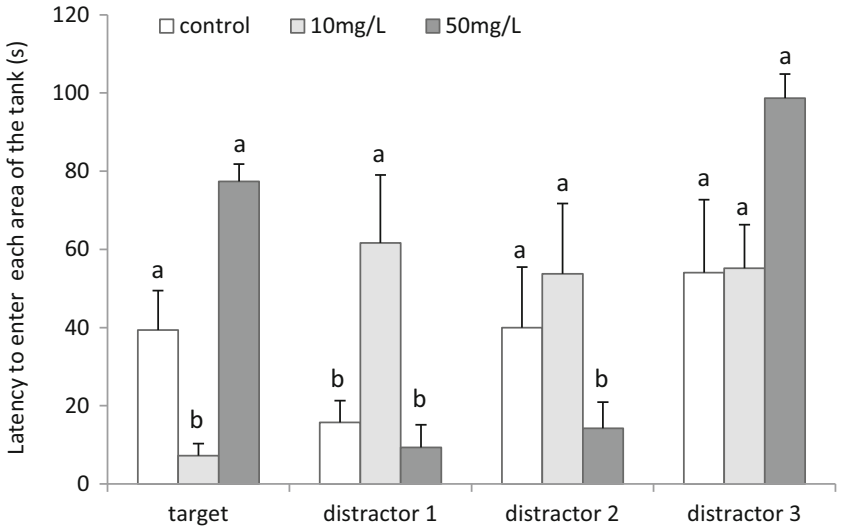

Fig. 3 Zebrafish latencies to enter each area of the tank during the test trial. The data (means + *SEM*s) show the time spent before entering each area of the tank for the control (0 mg/L caffeine; $n = 12$), 10-mg/L caffeine ($n = 14$), and 50-mg/L caffeine ($n = 14$) groups. A different figure (visual cues, distractors) was placed in each area, and the fish received the reward (food) only in the target area. Fish were allowed to explore the tank for 15 min per day, and no reward was given during the test trial. Zebrafish showed a statistically significant latency result for the interaction term treatment by area of the tank (LMM, $p < .05$). Different letters indicate statistical significance between groups (Tukey post-hoc tests, $p < .05$)

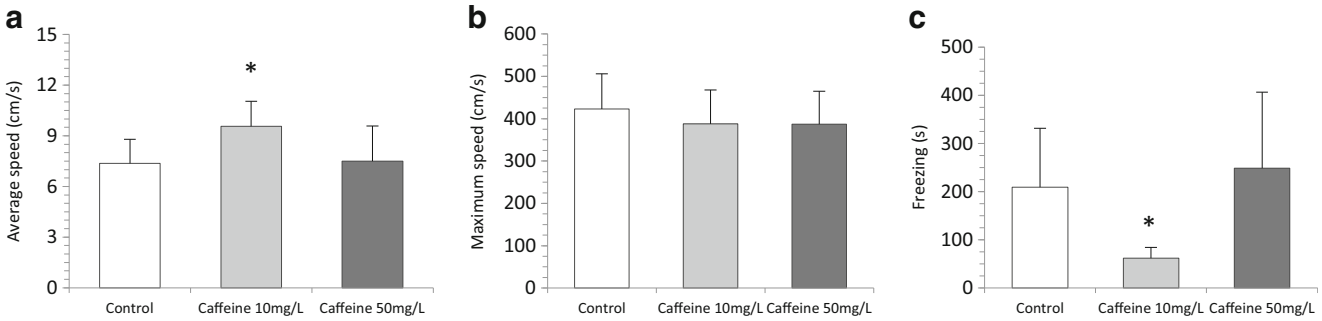

Fig. 4 Behavioral parameters analyzed for (from left to right) average swimming speed (**a**), maximum speed (**b**), and freezing behavior (**c**) exhibited by the zebrafish from the control (0 mg/L caffeine, $n = 12$), 10-mg/L caffeine ($n = 14$), and 50-mg/L caffeine ($n = 14$) groups. The data correspond to 15 min of the test trial, analyzed using tracking software (ZebTrack). * indicates statistical significance at $p < .05$ (one-way ANOVA followed by a Tukey test)

conditioning. Our results showed the associative-learning ability of zebrafish, corroborating other literature studies (Al-Imari & Gerlai, 2008; Braubach, Wood, Gadbois, Fine, & Croll, 2009; Chacon & Luchiari, 2014; Gómez-Laplaza & Gerlai, 2010; Karnik & Gerlai, 2012; Luchiari & Chacon, 2013). In addition, we showed that fish can discriminate a visual target in the presence of distractors and that their performance in terms of time to reach the correct choice improved at a low dose of caffeine (10 mg/L).

Although a number of studies have investigated distractors in fish decision-making (i. e. Silveira et al., 2015), and a few others in zebrafish under the effect of caffeine, none have studied these subjects in tandem. Apart from caffeine's effect of preventing fatigue, society also uses it to maintain focus on certain activities, such as studying (Hameleers et al., 2000), driving (Liu, Yao, & Spence, 2014), and similar attention and vigilance tasks (Foxe et al., 2012). In an environment filled with stimuli, attention allows individuals to process and respond only to what is relevant (Thiele & Bellgrove, 2018).

The increased attentional performance provoked by caffeine is related to its effects on adenosine receptors. In fact, during prolonged alertness and attention, firing neurons accumulate a byproduct called adenosine, which acts by binding to adenosine receptors and signaling that brain activity should decrease, such as when the body needs rest (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). However, when caffeine is available, it binds to the adenosine receptors (antagonist), and the brain's own stimulants, such as glutamate and dopamine, are more likely to function (Fredholm et al., 1999). Another neuromodulatory effect of caffeine is to regulate the brain levels of acetylcholine (Carter, O'Connor, Carter, & Ungerstedt, 1995; Murray, Blaker, Cheney, & Costa, 1982). Methylxanthines such as caffeine increase acetylcholine metabolism and activity (Acquas, Tanda, & Di Chiara, 2002; Murray et al., 1982). Activation of the cholinergic system has been associated with different cognitive functions, including attention, memory, and learning (Herlenius & Lagercrantz, 2004).

These positive caffeine effects occur only with controlled amounts, since high caffeine levels increase receptor binding in many parts of the brain and body, raise heart rate and blood pressure, and release hormones such as epinephrine and cortisol (Benowitz, 2008; Butt & Sultan, 2011; Franco, Oñatibia-Astibia, & Martínez-Pinilla, 2013; Rosa et al., 2018). In this respect, high amounts of caffeine are usually related to stress and anxiety (Wood, Sage, Shuman, & Anagnostaras, 2014).

In the present study, the low caffeine dose seems to have ameliorated the ability of fish to discriminate the cues and reach the target, whereas the higher dose, instead of further enhancing performance, impaired their ability to find the target, and they may have also demonstrated a side effect of the substance—namely, increased anxiety (Lieberman, 1992). This biphasic effect of caffeine on zebrafish behavior has been reported in other studies, showing that high doses negate the stimulant's beneficial effects, giving rise to learning impairment and increased anxiety (Santos et al., 2016; Santos, Ruiz-Oliveira, Silva, & Luchiari, 2017).

It is important to underscore that in our study caffeine also affected locomotor parameters, increasing average speed and decreasing freezing behavior in the group treated with 10 mg/L. The increase in zebrafish swimming could have led to this group having the shortest time to reach the target (Fig. 3); however, this response would induce fish to continue exploring the tank, regardless of the presence of the visual cue, which we did not observe (Fig. 2). In fact, after reaching the target area, fish stayed there longer (as did the control group; Fig. 2). Also, the longer time in the same place could have been interpreted as higher freezing behavior, which also was not observed for the 10-mg/L caffeine group, suggesting that bursts of locomotion might have been caused by a decrease in fatigue (Claghorn, Thompson, Wi, Van, & Garland, 2017), rather than by an anxiogenic response. The possible decrease in fatigue, together with improved focus to find the area of interest, confirms the positive effect of a low caffeine dose, suggesting that caffeine acts mainly in areas related to attention and alertness at this dose. On the other hand, the high dose

(50 mg/L caffeine) may act on other areas of the brain as well, thereby augmenting stress. Rosa et al. (2018) found that 50 mg/L of caffeine increases whole-body cortisol levels in zebrafish. In this regard, we could expect a similar alteration in our experimental fish. However, we cannot confirm this hypothesis, since the levels of freezing and locomotor behavior were the same for the 50-mg/L caffeine and control groups. Therefore, new tests will be required for us to thoroughly understand how 50 mg/L of caffeine impact on fish cognitive ability.

Caffeine is a widely used psychostimulant (De Luca, Bassareo, Bauer, & Di Chiara, 2007), consumed daily by a large part of the population and drunk excessively by people seeking improved physical or cognitive performance. We demonstrated that a low concentration of caffeine helps fish select what is important in their environment in order to obtain a reward. On the other hand, high concentrations seem to create a stress response, preventing individuals from learning the task. However, these effects were not observed for locomotor behavior. In this respect, studies using techniques to show changes in the brain (neurotransmitters, proteins, neuroplasticity) and body (cortisol levels) caused by different doses of caffeine will be crucial for a better understanding of the effects of caffeine on attention and learning shown here.

Finally, our study confirms the importance of zebrafish as a model for drug screening and cognition studies. We showed that low caffeine consumption may help performance on tasks demanding focus and attention, but chronic consumption of high amounts may have the opposite effect. For future studies, we suggest investigating the effects of different concentrations in order to determine the most appropriate dose and regime, in terms of promoting focus and attention and avoiding negative consequences.

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