Chapter 4

Caffeine Dose-Response Relationship and Behavioral Screening in Zebrafish

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Additional information is available at the end of the chapter

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Abstract

It has been centuries since humans consume coffee and get the benefits of this bean. Many researches worldwide continue to show healthful properties of coffee, while others suggest a number of side effects. In fact, anything consumed in excess may cause disturbance of the body functioning, whereas caffeine is a central nervous system stimulant that increases focus and improves performance, its high concentration can cause insomnia, dizziness, and vomiting. Thus, the question is: which coffee dose promotes benefits and prevents risks? To answer it, we used the zebrafish, a popular animal model that is at the vanguard of psychopharmacological research due to its unique combination of complexity and simplicity, translational relevance and applicability to high throughput behavioral drug screens. In the current study, we examine time-course and dose-dependent changes in zebrafish following exposure to caffeine. Our data show an inverted U-shaped path for the locomotor parameters and crescent path for the anxiety-like parameters. High doses are harmful to the individual, because the stimulating effect disappears and anxiogenic effects take place. We conclude that temporal analysis of zebrafish behavior is a sensitive method for the study of acute caffeine exposure–induced functional changes in the vertebrate brain.

Keywords: *Danio rerio*, anxiety-like, drug therapy, pharmacology, biphasic effect

1. Introduction

Caffeine is a psychostimulant substance worldwide used, which can potentially increase alertness and decrease fatigue and drowsiness [1–5]. Coffee, the beverage in which caffeine is most representative, is known to be rich in biologically active compounds that possess a

variety of therapeutic and functional effects. However, heavy coffee consumption may provoke systemic damages such as irregular heart rate, increased ventilation, anxiety, and due to its psychoactive properties, caffeine is likely to have addictive properties [6, 7]. Unlike other psychoactive drugs, caffeine consumption is legal and does not present any form of regulation. Moreover, caffeine consumption is not restricted to coffee and tea, but it has commonly been combined with other food products, such as chocolates, sodas, potato chips, and also bottled water. Even more risky, caffeine has been associated with other psychoactive drugs, as alcohol (i.e., energy drinks). While there is no specific recommendation on the amount of caffeine used or an indication of a critical value that may cause health problems, the U.S. Food and Drug Administration (FDA) has suggested 400 mg of caffeine/day for health adults [8]. However, there is not a clear picture of the overtime and dose-dependent effects of caffeine, demanding attention on behavioral pharmacological studies on this issue.

During the past 2 decades, numerous studies have approached the effects of psychoactive compounds used by humans in other mammals (rodents) [9–11]. Caffeine has gained attention because of its multiple targets in the brain. For instance, adenosine, ryanodine, and γ-aminobutyric acid receptors and cyclic nucleotide phosphodiesterase isoenzymes [12] seem to be related to stimulant effects of caffeine. However, as other drugs, caffeine empowers the central nervous system functioning when it reaches the therapeutic range; otherwise, it is too low concentration to cause an effect or too high concentration that causes intoxication. In this sense, several studies show contradictory behavioral effects following caffeine exposure: both increase in locomotor activity [13] and decrease in motor response [14, 15] were observed. The divergences on results may be related to caffeine dose and observation period in each study. Therefore, it is urgent to present effect of different doses and a short time-scale evaluation of caffeine induced changes in order to establish its therapeutic range, and then, how it can be properly used when in combination with other stimulant or depressant drugs.

Instead of using the most common animal model in pharmacological research, we propose the use of zebrafish to fill this gap in caffeine research. This small vertebrate is at the vanguard of neuroethological research and has been suggested for behavioral screening of drugs. The zebrafish has gained attention in behavioral brain research due to its ideal balance between the complexity of the physiological system and the simplicity of the biological model. It includes the fact that the zebrafish presents several molecular pathways, proteins, and protein products also found in mammals [16–22], besides the genome homology of about 70–80% [23]. Also, its brain structure [24, 25] and neurochemistry [26] offer translational relevance to humans [23] and allow exploring the model for a thorough understanding of the effects of substances used/abused by humans. In fact, various studies have shown that zebrafish respond similar to mammals when treated with many pharmacological compounds [27–29]. For example, benzodiazepine medication causes sedative effects in mouse [30, 31] and zebrafish [32]. For caffeine, it is not different: both rodents and zebrafish present anxiety-like behavior following caffeine exposure [33–36]. Furthermore, caffeine is water soluble and can be delivered to the fish via noninvasive method.

Zebrafish is not only an ideal model for behavioral screening, but also the majority of the genes identified in this species is conserved and has homologs in mammals [23, 37], which

allows for the examination of brain function and the development of brain diseases [27]. The zebrafish is an important model for research on psychoactive substances; in this sense, to know the effect of different doses of caffeine in their behavior is an important step for the development of methodologies to assess the effect of the substance in physiology and cognition. Our overtime dose-response analyses are one of the most detailed studies of caffeine in zebrafish and serves as a behavioral screen for future studies on the neural effects of caffeine or its effects when combined with other drugs.

2. Materials and methods

2.1. Animals and housing

Adult zebrafish (wild-type, both sexes) was obtained from a local fish farm (Natal-RN) and held in 50 L tanks forming a closed recirculating high-density system at the vivarium of the Fish Laboratory (Physiology Department—UFRN). The system maintained water quality by a multistage filtration, in which four filters processed the water in a flow of 3200 L/h, including a mechanical, a biological, activated carbon, and a UV light sterilizing filter. Water temperature was maintained at 28 C, pH in 7.1. Photoperiod was set at 12:12 light:dark cycle, with light intensity of 250 lx.

Fishes were fed two times a day with live brine shrimp and flaked food. Experimental procedures were revised and approved by the Ethical Committee for Animal Use of Federal University of Rio Grande do Norte (CEUA 045/2015).

2.2. Caffeine exposure

To determine overtime effect of caffeine doses in zebrafish, 144 animals (both sexes; 4.87 ± 1.35 g) were randomly assigned to different experimental groups that corresponded to each caffeine concentration (n = 12 for each group). This experimental design utilized 12 acute challenge doses: 0.0 (control), 0.5, 1.0, 5.0, 10.0, 15.0, 25.0, 50.0, 65.0, 75.0, 100.0, and 150.0 mg/L caffeine (Sigma Aldrich, 1,3,7-trimethylxanthine, Cat#C0750).

Fishes were initially held in groups of 12 in glass tanks (50 cm × 30 cm × 25 cm, width × depth × height; 37 L) for 7 days to acclimatize the fish to the test room. The bottom and back side of the holding tanks were covered with white paper to provide a uniform environment. During this period, water quality was kept the same as in the stock condition, with filtration and oxygen renovation given by a 140 Bio Wheel power filters. Food was offered twice daily.

For the behavioral assay, smaller tanks (40 cm × 20 cm × 25 cm, 15 L) were used. Caffeine was added directly to the testing water, to achieve each testing dose. Fishes were individually transferred to the testing tanks and behavior was recorded during 60 min using an HD camcorder (Sony Digital Video Camera Recorder; DCR-SX45) positioned 1 m away and in front of the tanks.

Fish behavior records were tracked using the Zebtrack software developed in MatLab [38]. The behavioral variables measured were average swimming speed, total distance travelled, duration of immobility (freezing), and time spent at the bottom of the tank (up to 5 cm from the bottom).

2.3. Statistical analysis

Data were first evaluated in search for outliers, homogeneity, normality, zero trouble, collinearity, and variables independency by inferential statistics [39]. After that, a Mixed Effects Model Analysis was applied considering the behavioral response as the response variable and the time (60 min records) and caffeine dose as the explanatory variables. The repeated measures characteristic of the data (over time data sampling) required longitudinal data analysis [39].

The exploratory analysis showed abnormal distribution and over dispersion of the residuals, and thus, a glmmPQL command (MASS package [40]) was used to develop the mixed model in the R program [41]. The mixed model showed random effect factors, which was the variation in behavior between groups, fixed effect factors, which was the caffeine doses used, and error.

The response variables were positive continuous quantitative data, not including zero ($Y > 0$); thus, a Goodness-of-fittest was performed to verify the best distribution function. If the response variable is continuous, then the normal and gamma are the best options [39]. Our response variable data best distribution function fitted gamma distribution (link function = inverse). The post-hoc comparisons between treatments, of each model, were made using the Tukey test in "lsmeans" package [42].

Average speed, distance traveled, freezing, and time at the bottom of the tank were also compared between the groups (caffeine doses) using One-Way ANOVA. For all comparison, the probability level considered for significance was $p < 0.05$.

3. Results

Figure 1 depicts zebrafish average swimming speed during acute caffeine exposure. Graphs referent to the very small doses (0.5 and 1.0 mg/L) are not presented to make it clear and due to its similarity to the doses below (0 mg/L) and above (5 mg/L). The mixed model comparison showed that average speed changed over time (GLMM, $\chi^2 = 7.33$, df = 1, $p < 0.006$; **Table 1**) and due to the caffeine dose used (GLMM, $\chi^2 = 651.49$, df = 10, $p < 0.001$; **Table 1**). The post-hoc comparison test (lsmeans) between caffeine doses is shown in **Table 1**: 10 and 25 mg/L of caffeine lead to an increase in average speed, while higher doses (50–150 mg/L) cause a decrease in swimming speed compared to low doses (0–5 mg/L).

Figure 2 shows zebrafish total distance traveled along the 60-min period of caffeine exposure. Graphs denoting the behavior for 0.5 and 1.0 m/L are not shown. The mixed model

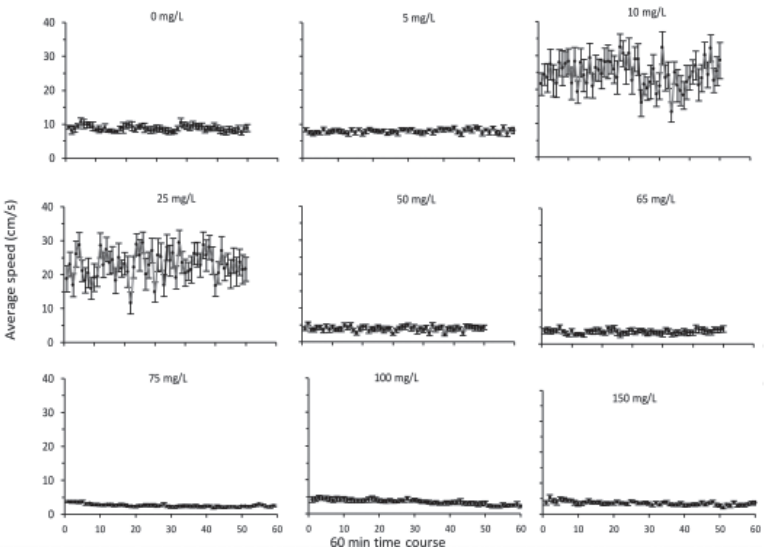

Figure 1. Time-course path of the average swimming speed during 60-min caffeine exposure in zebrafish. Mean ± SEM are shown for every 1-min intervals of the total 60-min recording. The caffeine doses (group designations) are shown above the graphs. Sample sizes (n) were 12 for each dose. Note the elevated activity in the group of fish exposed to 10 and 25 mg/L caffeine as compared to control. Also note the decreased activity in the fish that was exposed to doses of 50 mg/L caffeine and above it. For statistical analysis see Section 3 and **Table 1**.

comparison showed that total distance traveled changed over time (GLMM, $\chi^2 = 11.68$, df = 1, $p < 0.0006$; **Table 1**) and due to the caffeine dose used (GLMM, $\chi^2 = 271.49$, df = 10, $p < 0.001$; **Table 1**). The post-hoc comparison test (lsmeans) between caffeine doses is shown in **Table 1**. Caffeine doses higher than 50 mg/L decreased distance traveled compared to doses from 0 to 25 mg/L.

Figure 3 illustrates freezing behavior presented by zebrafish during caffeine challenge, a behavior related to fear and anxiety. Graphs referent to doses 0.5 and 1.0 mg/L are omitted. The mixed model comparison showed that freezing did not change over time (GLMM, $\chi^2 = 2.13$, df = 1, $p < 0.14$; **Table 1**) but changed according to the caffeine dose used (GLMM, $\chi^2 = 214.66$, df = 10, $p < 0.001$; **Table 1**). The post-hoc comparison test (lsmeans) between caffeine doses is shown in **Table 1**. Caffeine doses higher than 50 mg/L increased freezing behavior in zebrafish compared to doses from 0 to 25 mg/L.

Figure 4 presents zebrafish time spent at the bottom of the testing tank, another behavior associated to fear and anxiety response. Again, graphs representing doses 0.1 and 1.0 mg/L were not presented. The mixed model comparison showed that distance from the bottom did not change over time (GLMM, $\chi^2 = 0.18$, df = 1, $p < 0.66$; **Table 1**) but changed due to the caffeine exposure (GLMM, $\chi^2 = 170.91$, df = 10, $p < 0.001$; **Table 1**). The post-hoc comparison test (lsmeans) between caffeine doses is shown in **Table 1**. Caffeine doses of 50 mg/L and higher increased the time fish spent at the bottom of the tank.

	Behavioral parameters									
	Average speed			Total distance travelled			Freezing			Distance from bottom		
Explanatory variable	**Chi-squared**		***p*-Value**	**Chi-squared**		***p*-Value**	**Chi-squared**		***p*-Value**	**Chi-squared**		***p*-Value**
60 min time course	7.33		0.006	11.68		0.0006	2.12		0.14	0.18		0.66
Doses	651.49		>0.001	271.49		<0.001	214.66		<0.001	170.91		<0.001
Pairwise comparison	**lsmeans** ± SEM	***t*-Value**	***p*-Value**	**lsmeans** ± SEM	***t*-Value**	***p*-Value**	**lsmeans** ± SEM	***t*-value**	***p*-value**	**lsmeans** ± SEM	***t*-value**	***p*-value**
Dose 0.0 vs. dose 0.5	−0.00 ± 0.01	−0.22	1.0	−4.17 ± 0.00	−0.83	0.99	0.01 ± 0.02	0.73	0.99	−0.01 ± 0.01	−0.84	0.99
Dose 0.0 vs. dose 1.0	−0.00 ± 0.01	−0.28	1.0	−3.11 ± 0.00	−0.62	0.99	0.01 ± 0.02	0.42	1.00	−0.01 ± 0.01	−0.80	0.99
Dose 0.0 vs. dose 5.0	−0.00 ± 0.01	−0.40	1.0	−3.45 ± 0.00	−0.68	0.99	0.03 ± 0.02	1.34	0.95	−0.00 ± 0.01	−0.33	1.00
Dose 0.0 vs. dose 10	0.06 ± 0.01	4.52	0.00	7.32 ± 0.00	1.46	0.92	−0.06 ± 0.02	−2.45	0.34	−0.00 ± 0.01	−0.64	0.99
Dose 0.0 vs. dose 25	0.06 ± 0.01	4.29	0.00	4.17 ± 0.00	0.83	0.99	−0.06 ± 0.02	−2.43	0.45	−0.00 ± 0.01	−0.07	1.00
Dose 0.0 vs. dose 50	−0.09 ± 0.01	−0.55	<0.00	−3.08 ± 0.00	−6.03	<0.00	0.14 ± 0.02	5.34	<0.00	0.04 ± 0.01	3.38	0.03
Dose 0.0 vs. dose 65	−0.13 ± 0.02	−8.07	<0.00	−3.74 ± 0.00	−6.69	<0.00	0.14 ± 0.02	5.25	<0.00	0.06 ± 0.01	4.50	0.00
Dose 0.0 vs. dose 75	−0.18 ± 0.01	−12.26	<0.00	−4.78 ± 0.00	−9.19	<0.00	0.15 ± 0.02	5.87	<0.00	0.08 ± 0.01	6.34	<0.00
Dose 0.0 vs. dose 100	−0.13 ± 0.01	−9.20	<0.00	−3.29 ± 0.00	−6.42	<0.00	0.07 ± 0.02	5.80	0.01	0.06 ± 0.01	5.08	0.00
Dose 0.0 vs. dose 150	−0.12 ± 0.01	−8.18	<0.00	−2.64 ± 0.00	−5.18	<0.00	0.17 ± 0.02	6.72	<0.00	0.07 ± 0.01	5.32	<0.00
Dose 0.5 vs. dose 1.0	−0.00 ± 0.01	−0.06	1.0	1.05 ± 0.00	0.20	1.00	−0.00 ± 0.02	0.30	1.00	0.00 ± 0.01	0.03	1.00

	Behavioral parameters											
	Average speed			Total distance travelled			Freezing			Distance from bottom		
Dose 0.5 vs. dose 5.0	−0.00 ± 0.01	−0.18	1.0	7.18 ± 0.00	0.14	1.00	0.01 ± 0.02	0.61	0.99	0.00 ± 0.01	0.50	1.00
Dose 0.5 vs. dose 10	0.07 ± 0.01	4.74	0.00	1.14 ± 0.00	2.29	0.44	−0.08 ± 0.02	−3.18	0.06	0.00 ± 0.01	0.19	1.00
Dose 0.5 vs. dose 25	0.06 ± 0.01	4.51	0.00	8.35 ± 0.00	1.66	0.85	−0.08 ± 0.02	−3.17	0.06	0.01 ± 0.00	0.76	0.99
Dose 0.5 vs. dose 50	−0.09 ± 0.01	−6.33	<0.00	−2.67 ± 0.00	−5.21	<0.00	0.12 ± 0.02	4.61	0.00	0.05 ± 0.01	4.23	0.00
Dose 0.5 vs. dose 65	−0.12 ± 0.02	−7.88	<0.00	−4.36 ± 0.01	−8.38	<0.00	0.12 ± 0.02	4.57	0.00	0.07 ± 0.01	5.29	<0.00
Dose 0.5 vs. dose 75	−0.18 ± 0.01	−12.04	<0.00	−2.87 ± 0.01	−5.60	<0.00	0.13 ± 0.02	5.13	0.00	0.09 ± 0.01	7.19	<0.00
Dose 0.5 vs. dose 100	−0.13 ± 0.01	−8.98	<0.00	−2.22 ± 0.01	−4.35	0.00	0.05 ± 0.02	2.07	0.60	0.08 ± 0.01	5.93	<0.00
Dose 0.5 vs. dose 150	−0.12 ± 0.01	−7.96	<0.00	−3.35 ± 0.01	−0.06	0.00	0.15 ± 0.02	5.99	<0.00	0.08 ± 0.01	6.17	<0.00
Dose 1.0 vs. dose 5.0	−0.00 ± 0.01	−0.12	1.0	1.04 ± 0.01	2.08	0.59	0.02 ± 0.01	0.92	0.99	0.00 ± 0.01	0.46	1.00
Dose 1.0 vs. dose 10	0.07 ± 0.01	4.80	0.00	7.29 ± 0.01	1.45	0.93	−0.07 ± 0.01	−2.87	0.14	0.00 ± 0.01	0.16	1.00
Dose 1.0 vs. dose 25	0.06 ± 0.01	4.57	0.00	−2.77 ± 0.01	−5.42	<0.00	−0.07 ± 0.01	−2.06	0.14	0.01 ± 0.01	0.72	0.99
Dose 1.0 vs. dose 50	−0.09 ± 0.01	−6.27	<0.00	−3.43 ± 0.01	−6.12	<0.00	0.12 ± 0.02	4.92	0.00	0.05 ± 0.01	4.19	0.00
Dose 1.0 vs. dose 65	−0.12 ± 0.02	−7.82	<0.00	−4.47 ± 0.01	−8.58	<0.00	0.13 ± 0.02	4.85	0.00	0.07 ± 0.01	5.26	<0.00
Dose 1.0 vs. dose 75	−0.18 ± 0.01	−11.98	<0.00	−2.98 ± 0.00	−5.80	<0.00	0.14 ± 0.01	5.44	<0.00	0.09 ± 0.01	7.16	<0.00
Dose 1.0 vs. dose 100	−0.13 ± 0.01	−8.92	<0.00	−2.33 ± 0.00	−4.56	0.00	0.06 ± 0.02	2.38	0.38	0.08 ± 0.01	5.90	<0.00

	Behavioral parameters											
	Average speed			Total distance travelled			Freezing			Distance from bottom		
Dose 1.0 vs. dose 150	−0.11 ± 0.01	−7.90	<0.00	1.07 ± 0.00	2.15	0.04	0.16 ± 0.01	6.30	<0.00	0.08 ± 0.01	6.14	<0.00
Dose 5.0 vs. dose 10	0.07 ± 0.01	4.93	0.00	1.07 ± 0.00	2.15	0.54	−0.10 ± 0.00	−3.80	0.01	−0.00 ± 0.01	−0.30	1.00
Dose 5.0 vs. dose 25	0.06 ± 0.01	4.69	0.00	7.63 ± 0.00	1.52	0.90	−0.09 ± 0.01	−3.78	0.01	0.00 ± 0.01	0.25	1.00
Dose 5.0 vs. dose 50	−0.09 ± 0.01	−6.14	<0.00	−2.74 ± 0.00	−5.35	<0.00	0.10 ± 0.01	4.00	0.00	0.05 ± 0.01	3.73	0.01
Dose 5.0 vs. dose 65	−0.12 ± 0.02	−7.71	<0.00	−3.39 ± 0.00	−6.06	<0.00	0.11 ± 0.02	4.00	0.00	0.07 ± 0.01	4.83	0.00
Dose 5.0 vs. dose 75	−0.18 ± 0.01	−11.86	<0.00	−4.43 ± 0.00	−8.52	<0.00	0.11 ± 0.02	4.52	0.00	0.09 ± 0.01	6.70	<0.00
Dose 5.0 vs. dose 100	−0.13 ± 0.01	−8.80	<0.00	−2.94 ± 0.00	−5.74	<0.00	0.03 ± 0.00	1.45	0.93	0.07 ± 0.01	5.43	<0.00
Dose 5.0 vs. dose 150	−0.11 ± 0.01	−7.78	<0.00	−2.29 ± 0.01	−4.49	0.00	0.14 ± 0.02	5.38	<0.00	0.07 ± 0.01	5.68	<0.00
Dose 10 vs. dose 25	0.00 ± 0.01	−0.23	1.00	−3.14 ± 0.00	−0.63	0.99	0.00 ± 0.00	0.01	1.00	0.00 ± 0.01	0.56	1.00
Dose 10 vs. dose 50	−0.16 ± 0.01	−11.04	<0.00	−3.82 ± 0.00	−7.48	<0.00	0.20 ± 0.02	7.79	<0.00	0.05 ± 0.01	4.03	0.00
Dose 10 vs. dose 65	−0.19 ± 0.02	−12.19	<0.00	−4.47 ± 0.00	−8.01	<0.00	0.21 ± 0.02	7.52	<0.00	0.07 ± 0.01	5.11	0.00
Dose 10 vs. dose 75	−0.25 ± 0.01	−16.68	<0.00	−5.51 ± 0.01	−10.62	<0.00	0.21 ± 0.02	8.32	<0.00	0.09 ± 0.01	7.00	<0.00
Dose 10 vs. dose 100	−0.20 ± 0.01	−13.66	<0.00	−4.02 ± 0.00	−7.87	<0.00	0.13 ± 0.00	5.25	<0.00	0.07 ± 0.01	5.74	<0.00
Dose 10 vs. dose 150	−0.19 ± 0.01	−12.66	<0.00	−3.37 ± 0.00	−6.63	<0.00	0.24 ± 0.02	9.17	<0.00	0.08 ± 0.01	5.98	<0.00
Dose 25 vs. dose 50	−0.16 ± 0.01	−10.81	<0.00	−3.50 ± 0.01	−6.86	<0.00	0.20 ± 0.02	7.78	<0.00	0.04 ± 0.01	3.47	0.02

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	Behavioral parameters										
	Average speed			Total distance travelled			Freezing			Distance from bottom		
Dose 25 vs. dose 65	−0.19 ± 0.02	−11.98	<0.00	−4.16 ± 0.00	−7.44	<0.00	0.21 ± 0.02	7.50	<0.00	0.06 ± 0.01	4.59	0.00
Dose 25 vs. dose 75	−0.25 ± 0.01	−16.45	<0.00	−5.20 ± 0.01	−10.00	<0.00	0.21 ± 0.00	8.30	<0.00	0.08 ± 0.01	6.44	<0.00
Dose 25 vs. dose 100	−0.20 ± 0.01	−13.43	<0.00	−3.71 ± 0.00	−7.24	<0.00	0.13 ± 0.01	5.24	<0.00	0.07 ± 0.01	5.18	<0.00
Dose 25 vs. dose 150	−0.18 ± 0.01	−12.42	<0.00	−3.05 ± 0.00	−6.00	<0.00	0.24 ± 0.01	9.16	<0.00	0.17 ± 0.01	5.42	<0.00
Dose 50 vs. dose 65	−0.03 ± 0.02	−2.05	0.60	−6.55 ± 0.00	−1.15	0.98	0.00 ± 0.02	0.29	1.00	0.01 ± 0.01	1.35	0.95
Dose 50 vs. dose 75	−0.08 ± 0.01	−5.76	0.08	−1.69 ± 0.00	−3.19	0.06	0.01 ± 0.00	0.52	1.00	0.03 ± 0.01	2.98	0.11
Dose 50 vs. dose 100	−0.04 ± 0.01	−2.67	0.22	−2.05 ± 0.00	−0.39	1.00	−0.06 ± 0.02	−2.54	0.29	0.02 ± 0.01	1.70	0.82
Dose 50 vs. dose 150	−0.02 ± 0.01	−1.64	0.85	4.47 ± 0.01	0.86	0.99	0.03 ± 0.02	1.38	0.95	0.02 ± 0.01	1.95	0.67
Dose 65 vs. dose 75	−0.05 ± 0.02	−3.28	0.05	−1.03 ± 0.01	−1.80	0.77	0.00 ± 0.02	0.18	1.00	0.02 ± 0.01	1.40	0.94
Dose 65 vs. dose 100	−0.00 ± 0.02	−0.41	1.00	4.50 ± 0.00	0.79	0.99	−0.07 ± 0.02	−2.65	0.23	0.00 ± 0.01	0.22	1.00
Dose 65 vs. dose 150	0.00 ± 0.02	0.53	1.00	1.10 ± 0.01	1.94	0.68	0.02 ± 0.02	0.98	0.99	0.00 ± 0.01	0.45	1.00
Dose 75 vs. dose 100	0.04 ± 0.01	3.09	0.08	1.48 ± 0.00	2.80	0.16	−0.08 ± 0.01	−3.06	0.09	−0.01 ± 0.01	−1.28	0.97
Dose 75 vs. dose 150	0.06 ± 0.01	4.12	0.07	2.14 ± 0.00	4.05	0.07	0.02 ± 0.02	0.85	0.99	−0.01 ± 0.01	−1.02	0.99
Dose 100 vs. dose 150	0.01 ± 0.01	1.03	0.99	6.52 ± 0.00	1.25	0.97	0.10 ± 0.02	3.92	0.00	0.00 ± 0.01	0.25	1.00

Dose values are expressed in mg/L.

Table 1. Estimates of mixed effect model for the behavioral parameters measured during caffeine exposure.

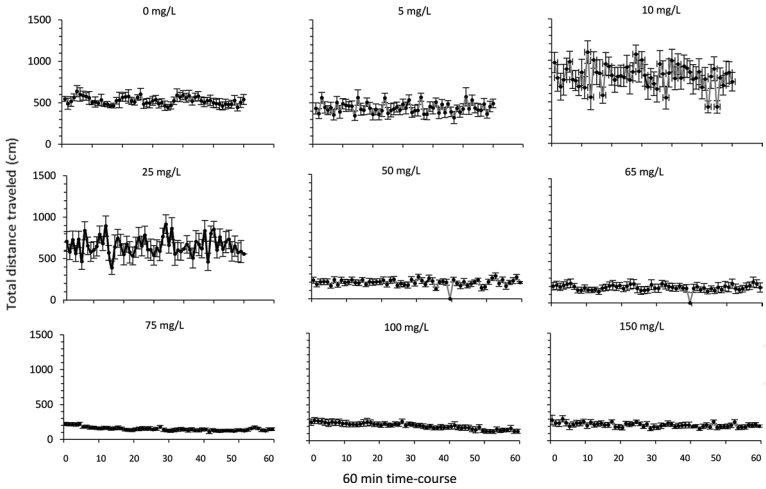

Figure 2. Time-course path of the total distance traveled during 60-min caffeine exposure in zebrafish. Mean ± SEM are shown for every 1-min intervals of the total 60 min recording. The caffeine doses (group designations) are shown above the graphs. Sample sizes (*n*) were 12 for each dose. Note the elevated activity in the group of fish exposed to 10 and 25 mg/L caffeine as compared to control. Also note the decreased activity in the fish that was exposed to doses of 50 mg/L caffeine and above it. For statistical analysis see Section 3 and **Table 1**.

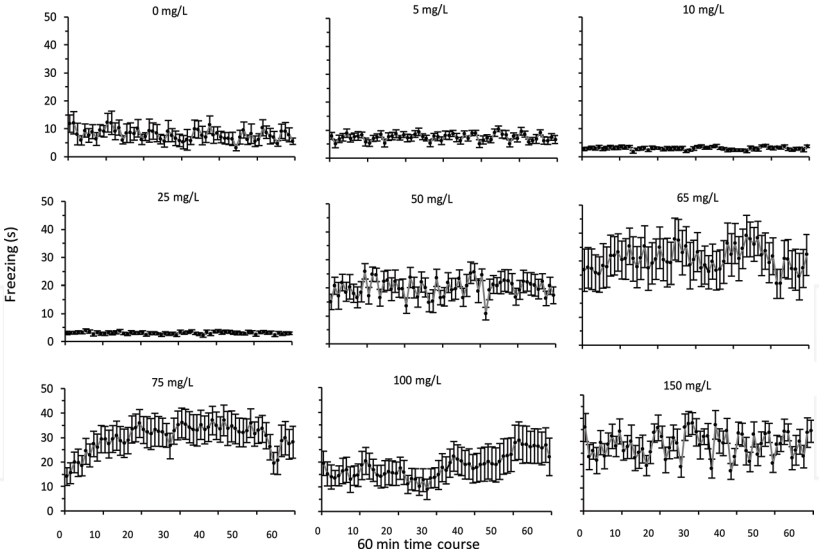

Figure 3. Time-course path of the freezing behavior during 60-min caffeine exposure in zebrafish. Mean ± SEM are shown for every 1-min intervals of the total 60 min recording. The caffeine doses (group designations) are shown above the graphs. Sample sizes (*n*) were 12 for each dose. Note the decreased freezing in the group of fish exposed to 5, 10 and 25 mg/L caffeine as compared to control. On the contrary, note the increased freezing in the fish that was exposed to doses above 50 mg/L caffeine. For statistical analysis, see Section 3 and **Table 1**.

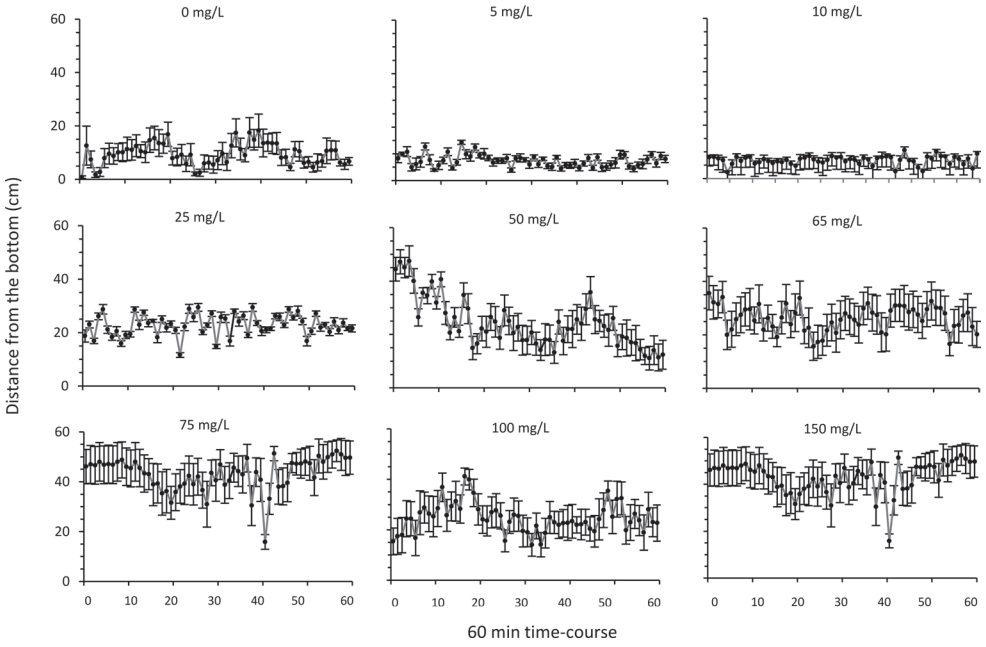

Figure 4. Time-course path of the time spent at the bottom of the tank (up to 5 cm from the bottom) during 60-min caffeine exposure in zebrafish. Mean ± SEM are shown for every 1-min intervals of the total 60 min recording. The caffeine doses (group designations) are shown above the graphs. Sample sizes (*n*) were 12 for each dose. Note the decreased time at the bottom in the group of fish exposed to 5, 10, and 25 mg/L caffeine as compared to control. Also note the increased time at the bottom in the fish exposed to doses above 50 mg/L caffeine. For statistical analysis, see Section 3 and **Table 1**.

Figure 5 displays the median and range of variation of the 60-min caffeine exposure in zebrafish, both for the parameters related to locomotion (**Figure 5a** and **b**) and the parameters related to anxiety-like behavior (**Figure 5c** and **d**). One-Way ANOVA between groups (caffeine doses) showed that average speed did not differ from the control condition when fish is exposed to doses up to 5 mg/L caffeine, but it is increased with doses of 10 and 25 mg/L and decreased with doses above 50 mg/L ($F = 1087.97$, df = 10, $p < 0.001$, **Figure 5a**) indicating an inverted U shape. The same patterns were observed for total distance travelled, in which the lower doses (0.5–5 mg/L) did not differ from the control, doses of 10 and 25 mg/L increased distance traveled and doses above 50 mg/L decreased distance traveled (One-Way ANOVA, $F = 374.82$, df = 10, $p < 0.001$, **Figure 5b**). For the freezing behavior, One-Way ANOVA showed a slight decrease with increasing doses, with the lowest values of freezing registered at 10 and 25 mg/L, and a sharp increase with doses above 50 mg/L ($F = 462.15$, df = 10, $P < 0.001$, **Figure 5c**), suggesting the anxiogenic effect of high caffeine doses. The time fish spent at the bottom of the tank was reduced by doses from 0.5 to 25 mg/L and highly increased by doses above 50 mg/L (One-Way ANOVA, $F = 427.27$, df = 10, $p < 0.001$). **Figure 5d** depicts the comparison between caffeine doses and the tendency line indicating an increasing in time at the bottom concomitant to the increase in caffeine dose.

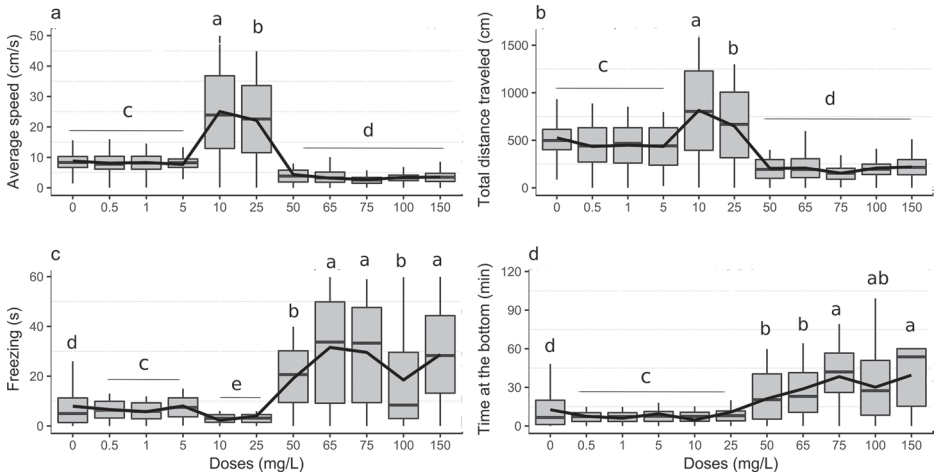

Figure 5. Box plot shows median and interquartile range of locomotor behavior: (a) average swimming speed, (b) total distance traveled, (c) freezing, and (d) time spent at the bottom of the tank, whiskers represent the range. Tendency line indicates the inverted U shape observed for increasing doses of caffeine on locomotor behavioral parameters (swimming speed and distance traveled), and the ascendant pattern observed for increase doses of caffeine on anxiety-like behavioral parameters (freezing and time at the bottom of the tank). For statistical analysis, see Section 3.

4. Discussion

This study characterizes variations in the locomotor pattern and anxiety-like behavior derived from acute exposure to caffeine in zebrafish. We evaluated a wide spectrum of caffeine doses, from 0.5 to 150 mg/L, every 1-min interval along 60-min period of the drug exposure. This detailed analysis of different doses overtime allowed to the observation that caffeine has a biphasic effect, stimulating locomotion and decreasing anxiety at low levels, and diminishing activity and increasing anxiety-like behavior at doses above 50 mg/L.

While caffeine is accepted to act as a stimulant on the central nervous system [43], it is worth noting that in fact this drug exerts distinct responses depending on the amount used. Initially, caffeine has little or no impact on behavior, followed by the most evident effect caused by an intermediate dose, and then a remarkable suppression at the level of behavioral activity, as observed in **Figure 5**. High caffeine doses (50 and 100 mg/L) were previously shown to depress locomotor activity in zebrafish [15, 44], but our study is the first to present a time-course analyses of the effects of several doses of caffeine in adult zebrafish. The same dose-dependent effects observed herein in rodents, suggesting the models similarity in terms of the mechanism by which caffeine produces behavioral effects [45].

Adenosine receptor blockade seems to be the prevalent action of caffeine in the brain. The increase on locomotor activity after caffeine exposure derives from the blockade of A_{2A} adenosine receptors, preventing the inhibitory action of adenosine on the nervous system [46]. The antagonist role of caffeine usually stimulates the central nervous system and also activates dopaminergic transmission [47–49], which is consistent with the drug reinforcing properties. Another event that occurs at the time of caffeine ingestion and which causes increased

locomotor activity is the inhibition of phosphodiesterase (an enzyme that hydrolyses cAMP), which promotes the release of calcium from intracellular reserves and interferes with the sensitivity of GABA receptors [50].

Caffeine is a substance widely used by the society [1] and, if used in moderation (up to 200 mg/day/person on average), it may lead to several benefits, such as improved performance on tasks that require attention and focus [51, 52]. For instance, it was observed that zebrafish improves object discrimination when treated with moderate doses of caffeine [53]. The most notable effects of low-to-moderate doses of caffeine include increased alertness, energy, and ability to concentrate [54]. On the other hand, high and abusive use of caffeine may inhibit these effects [55]. At high concentrations, caffeine is suggested to increase glucose utilization in the CNS, what also seems to be related to its stimulatory effects [56]. The elevated sugar level (main CSN subtract) together with the blockage of adenosine inhibitory effects, in turn, is responsible to the caffeine side-effects on the motor system and sleep-wake cycle, two functions highly susceptible to caffeine.

Moreover, higher caffeine doses induce negative effects such as increased sympathetic response (tachycardia, higher ventilation), restlessness, insomnia, and anxiety [57]. The high acute dose of caffeine exacerbates anxiety-like behavior, reduces locomotion, and in many cases, causes behavior similar to seizure [58], very high doses of caffeine may also cause intoxication and death of the individuals [54, 59]. The same pattern of effect was observed in zebrafish larvae under the action of high doses of caffeine [60]. It is known that adenosine regulates the activity of several neurons, such as glutamatergic; thus, if the effect of adenosine is blocked, glutamatergic transmission increases and may turn to an extremely high excitatory response [12, 56].

Finally, our results reinforce the zebrafish as a valuable model organism for throughput screening of behavioral-related drugs. While caffeine is a legal and widely consumed substance, the amount of caffeine ingested should be taken into consideration since negative effects are observed after high doses consumption. We found that moderate caffeine intake ameliorates performance, but a robust anxiety-related response occurs following exposure to high doses of caffeine. Taken together, these results confirm zebrafish as an accurate, reliable, and efficient model for basic translational research of psychoactive drugs on physiology and behavior.

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