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Irish coffee: Effects of alcohol and caffeine on object discrimination in zebrafish

Luana C. Santos, Julia Ruiz-Oliveira, Jéssica J. Oliveira, Priscila F. Silva, Ana C. Luchiari

Departamento de Fisiologia, Centro de Biociências, Universidade Federal do Rio Grande do Norte, PO BOX 1510, 59078-970 Natal, Rio Grande do Norte, Brazil

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ABSTRACT

Many studies regarding the effects of drugs investigate the acute and chronic use of alcohol, but only a few address the effects of caffeine and alcohol combined to the performance of the zebrafish in cognitive tasks. The zebrafish is an important model for studying the effects of drugs on learning, because it has large genetic similarities to humans and the non-invasive administration of the substances favors translational bias of research. In this study, we observed the effects of alcohol and caffeine on zebrafish cognition through an object discrimination test. We noticed that animals subjected to acute alcohol dose and those under alcohol or caffeine withdrawal did not show discrimination. When fish were treated with associated alcohol and caffeine, those chronically treated with alcohol and subjected to moderate acute dose of caffeine showed learning of the task. Our results reinforce the harmful effects of the alcohol use on cognitive tasks, and suggest that continued use of high doses of caffeine cause cognitive impairment during withdrawal of the substance. However, the acute use of caffeine appears to reverse the harmful effects of alcohol withdrawal, allowing discriminative performance equivalent to control fish. Finally, we reiterate the use of zebrafish as a model for drug effects screening and search for active compounds that modulate the alcohol and caffeine effects.

1. Introduction

The zebrafish has gained increasing popularity in behavioral brain research due to its practical simplicity, elaborated brain structure (Klee et al., 2012; Kolb and Whishaw, 1998) and neurochemistry (Gerlai et al., 2009) that offers translational relevance to humans (Crollius and Weissenbach, 2005). This species is a very prolific small vertebrate that, due to its size and the social nature, can be held in large numbers in small rack systems (up to 4000 fish in 250 L water volume), demanding little cost and technical maintenance. In addition, the zebrafish shares many molecular pathways, genes and protein products with mammals (Faraco et al., 2006; Holzschuh et al., 2001; Kaslin, 2004; Kaslin and Panula, 2001; McLean and Fetcho, 2004; Mueller et al., 2004; Prober et al., 2006). Moreover, the majority of the genes already identified in this species is conserved and have homologs in mammals (Cerdà et al., 1998; Crollius and Weissenbach, 2005), which makes it an ideal model organism for embryology, development and disease studies (Sison and Gerlai, 2010). A large number of genetic tools have been produced for the zebrafish and genetic knowledge has been accumulated (Ackerman et al., 2009; Welsh et al., 2009). These materials have been successfully used for the examination of brain function and the development of brain diseases (Kalueff et al., 2014), and the zebrafish has been accepted as one of the best research animals for high throughput screening in many areas of study (Gebauer et al., 2011; Gerlai, 2011; Holzschuh et al., 2001). In the past decade, many studies approached the genetics of behavior and brain function of the zebrafish, but only a few attempted to study learning processes (Fernandes et al., 2014; Sison and Gerlai, 2010).

Learning is an important feature that allows the acquisition of new skills or concepts from experience, possibly due to neuronal plasticity (Gould, 2010; Kolb and Whishaw, 1998). Learning capacity is mainly affected by physiological and neural changes due to psychoactive drug use (Gould, 2010). Amongst licit drugs, alcohol and caffeine are the psychoactive substances most widely used by the society (Fredholm et al., 1999; Frances and Garfild, 2006). The alcohol develops a biphasic response, excitatory in the beginning and then depressive; the alcohol effects depend on the dose and exposure regime (Tran and Gerlai, 2013). For instance, low doses of alcohol cause increase on locomotor activity in zebrafish while higher doses lead to the opposite response (Gerlai et al., 2000). Also the effects of alcohol on the cognitive processes seem to be benefic in low doses and harmful in higher doses (Chacon and Luchiari, 2014). Moreover, while acute doses may cause sensitivity (Blaser et al., 2010), chronic exposure to alcohol may develop tolerance to the substance (Luchiari et al., 2015a, b; Tran and Gerlai, 2013; Tran et al., 2015) and throughout long time can cause irreversible dementia known as Wernicke–Korsakoff syndrome (Savage et al., 2000).

Caffeine, as alcohol, shows a biphasic effect depending on the dosage. Low and medium doses increase locomotor activity while high

E-mail address: analuchiari@yahoo.com.br (A.C. Luchiari).

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doses result in robust anxiogenic effects that includes decreased locomotion and increased freezing and erratic movements (Egan et al., 2009; Marin et al., 2011). Although many studies regard the behavioral effects of caffeine (Cachat et al., 2010; Marin et al., 2011; Steenbergen et al., 2011), there are a lack in the literature concerning the chronic and acute consumption effects on memory acquisition and consolidation. Amongst them, some suggests that acute low doses of caffeine might improve memory retention in rodents (Angelucci et al., 2002), however the results are still inconclusive and deserve more studies.

Therefore, while few studies have investigated learning and memory under the effects of alcohol (Chacon and Luchiari, 2014; Fernandes et al., 2014; Luchiari et al., 2015a, b) and caffeine (Collier et al., 2014; Steenbergen et al., 2011), there are no reports of works that address the effects of both drugs combined on the cognitive responses. Knowing that these drugs are indiscriminately used and, many times, used in association for the pursuit of potentializing the excitatory effects of alcohol or lessening its later depressive effects (Heinz et al., 2013; Spinetta et al., 2008), this study proposes to test the influence of the acute and chronic alcohol and caffeine exposure in the performance of zebrafish on a cognitive task. For this aim, we used a one-trial-learning protocol, because the memory formed after a single exposure to an event is more sensible and easily disrupted by the use of psychostimulant substances than memories formed by sequential exposure to stimuli (conditioning) (Oliveira et al., 2015).

2. Materials and methods

2.1. General procedures

For the study, 182 adult zebrafish *Danio rerio* (4 to 5 months; wild-type) were acquired from a local breeding farm (Natal-RN) and kept in high-density system tanks in the vivarium of the Ornamental Fish Laboratory (Physiology Department — UFRN).

Each four 50 L-tanks formed a recirculating system with multi-stage filtration including a mechanical filter, a biological filter, activated carbon filter, and a UV light sterilizing unit. The animals were kept in the tanks (one fish/L), with aerated and filtered water, and temperature, pH and oxygen measured regularly. Photoperiod was set on 12 L:12D (Light:Dark) cycle and light intensity was around 250–30 lx. Feeding frequency was twice a day ad libitum with brine shrimp and commercial flake diet. The Ethical Committee for Animal Use of Federal University of Rio Grande do Norte gave permission for all animal procedures (CEUA 046/2015).

2.2. Alcohol and caffeine exposure

Seven days before the beginning of the drugs exposure, animals were transferred from the stock tanks to 30 L tanks (50 × 30 × 30 cm, width × height × length) composing the groups for drugs treatments. The tanks were kept with constant aeration given by air stones and the entire volume was exchanged in 30% every day to assure quality conditions.

The drugs exposure followed the 2 × 3 experimental design proposed by Tran and Gerlai (2013) with two chronic and three acute doses. For the alcohol treatments, we used 0.00% and 0.50% for the chronic doses and 0.00%, 0.50% and 1.00% for the acute doses (Table 1). For the caffeine treatments, we used 00 mg/L and 50 mg/L for the chronic doses and 00 mg/L, 50 mg/L and 100 mg/L for the acute doses (Table 1). For the groups exposed to alcohol and caffeine combined, we used a 2 × 4 protocol, two chronic doses (0.50% alcohol or 50 mg/L caffeine) and four acute doses (0.50 and 1.00% alcohol or 50 and 100 mg/L caffeine), in which two groups received chronic caffeine and acute alcohol doses and two other groups received chronic alcohol and acute caffeine doses (Table 1).

To achieve the final concentration of 0.50% alcohol and 50 mg/L caffeine, we applied a drug escalation procedure that minimizes the mortality and allows the fish to acclimatize to the drug condition (Tran and Gerlai, 2013). The dosage was progressively increased along the days: 1/4 of the dose for the first four days, after which the dose was increased to 1/2 for days 5–8, reached 3/4 for days 9–12 and then was increased to the final concentration of 0.50% alcohol or 50 mg/L caffeine for the remaining 15 days. The chronic exposure lasted 27 days, 24 h per day. On the last 5 days (23rd to 27th day) fish were transferred to a smaller tank for the habituation phase of the discrimination task. During these days, the drug concentration in the habituation tank corresponded to the chronic dose for each particular group.

On the 28th and 29th days, fish were individually exposed to one of the acute doses for 1 h in a 2 L tank. After the acute exposure on the 28th day, fish returned to the previous chronic dose where it was held before until the 29th day. On these two days, zebrafish were individually tested for the objects discrimination (28th day: memorization phase and 29th day: discrimination phase). During the cognitive tests, drugs concentration in the tank corresponded to the acute dose for each particular fish.

To name each experimental group from 2 × 3 alcohol treatment, 2 × 3 caffeine treatment and 2 × 4 alcohol + caffeine treatment, we used C to refer to the chronic concentration and A to refer to the acute concentration. For the alcohol treatment, animals were divided into the following groups: C0.00A0.00 (control group, n = 17), C0.00A0.50 (acute 0.5% group, n = 18), C0.00A1.00 (acute 1.0% group, n = 12),

Table 1
Summary of the alcohol and caffeine exposure treatments used for zebrafish.

Alcohol exposure		Acute doses (28th and 29th days)		
		0.00%	0.50%	1.00%
Chronic doses (1st to 27th days)	0.00% 0.50%	C0.00A0.00 (n = 17) C0.50 A0.00 (n = 9)	C0.00A0.50 (n = 17) C0.50A0.50 (n = 13)	C0.00A1.00 (n = 9) C0.50 A1.00 (n = 13)
Caffeine exposure		**Acute doses (28th and 29th days)**		
		00 mg/L	50 mg/L	100 mg/L
Chronic doses (1st to 27th days)	00 mg/L 50 mg/L	C00A00 (n = 17) C50A00 (n = 12)	C00A50 (n = 11) C50A50 (n = 10)	C00A100 (n = 11) C50A100 (n = 7)
Alcohol and caffeine exposure		**Acute doses (28th and 29th days)**		
		Alcohol 1.00% (A_a)	Caffeine 100 mg/L (A_c)	
Chronic doses (1st to 27th days)	Alcohol 0.50% (C_a)	–	$C_a0.50A_c50$ (n = 9) $C_a0.50A_c100$ (n = 11)	
	Caffeine 50 mg/L (C_c)	$C_c50A_a0.50$ (n = 13) $C_c50A_a1.00$ (n = 13)	–	

C0.50 A0.00 (withdrawal group, n = 9), C0.50A0.50 (chronic group, n = 13), C0.50 A1.00 (increased dose group, n = 13).

For the caffeine treatment, fish were divided into the following groups: C00A00 (control group, n = 17), C00A50 (acute 50 mg/L, n = 12), C00A100 (acute 100 mg/L, n = 11), C50A00 (withdrawal group, n = 12), C50A50 (chronic group, n = 11), C50A100 (increased dose group, n = 8).

The alcohol and caffeine combined design produced four treatment groups we refer to according to the chronic concentration of alcohol (Ca) and caffeine (Cc), and the acute concentration of alcohol (Aa) and caffeine (Ac) as follows: $C_a0.50A_c50$ (n = 9), $C_a0.50A_c100$ (n = 11), $C_c50A_a0.50$ (n = 13), $C_c50A_c1.00$ (n = 13).

2.3. Object discrimination test

The object discrimination test was based on the experimental design proposed by Oliveira et al. (2015) and consisted in three phases: habituation (23rd to 27th day of the drugs treatment), memorization (28th day) and discrimination (29th day).

The habituation phase lasted 5 days. The animals were transferred to the testing tank (40 × 20 × 25 cm) and were allowed to explore it for 20 min (drugs doses were kept according to the chronic treatment). At first, fish were placed in the tank in groups of 6 to minimize isolation stress. On the following days, the number of fish allowed to explore the tank was progressively reduced to reach 1 fish/tank on the last day of habituation. After each habituation period fish were placed back in each particular chronic treatment tank.

The drugs acute doses were administrated on the 28th and 29th days for 40 min before (in a 2 L tank) and 20 min during the test (in the testing tank), totalizing 1 h exposure. After the end of the memorization phase the animals were transferred back to the chronic treatment tank until the discrimination phase.

On the memorization phase (day 28) two 3D objects named A and B with same color, size and shape, were introduced in the tank, each one positioned next to each smaller wall and around 30 cm away from each other. Fish were individually allowed to explore the tank with the two objects for 20 min. Behavior was recorded from above using a handy cam (Sony Digital Video Camera Recorder; DCR-SX45). After that, fish returned to its chronic treatment tank.

On the discrimination phase (day 29), object B was replaced by a new one named object C. The new object showed the same size and shape but different color from the former. Fish were first exposed to the acute dose for 40 min and then transferred to the tank to explored the objects for 20 min. Behavior was registered in video. In both phases, we considered that animal's permanence in a 10 cm area around the objects, characterizes exploration (Lucon-Xiccato and Dadda, 2014).

2.4. Data and statistical analysis

The behavioral data were analyzed using a tracking software developed in MatLab. The following parameters were evaluated: time fish spent exploring each object, total distance traveled, freezing and average swimming speed.

We compared the objects exploration time in memorization and discrimination phases, and also between the two phases using Student t test. All the locomotor parameters were statistically compared using One Way Anova. We considered the probability level of $p < 0.05$ for statistical significance.

3. Results

3.1. Alcohol treatment

In the memorization phase, the control group (C0.00A0.00) did not show any difference between object A and B exploration time (Student's t test, $t = -1.29$, $p = 0.22$; Fig. 1a). In the discrimination phase, control fish (C0.00A0.00) explore more the new object (object C) than the known one (object A) (Student's t test, $t = 2.78$, $p = 0.01$). Fish explored more object C in the discrimination phase than object B in the memorization phase (Student's t test, $t = 1.94$, $p = 0.05$).

The acute 0.50% alcohol group (C0.00A0.50) explored significantly more object B than object A in the memorization phase (Student's t test, $t = -2.51$, $p = 0.02$; Fig. 1b). In the discrimination phase, both objects were explored equally (Student's t test, $t = -2.00$, $p = 0.05$). Fish explored more object C in the discrimination phase than the object B in the memorization phase (Student's t test, $t = -2.17$; $p = 0.04$, Fig. 1b).

Both acute 1.00% alcohol group (C0.00A1.00; Fig. 1c) and withdrawal group (C0.50 A0.00; Fig. 1d) did not show differences in the exploration time in the memorization phase (Student's t test, acute 1.00%: $t = -0.62$, $p = 0.67$; withdrawal: $t = -0.52$, $p = 0.62$). When the new object was displayed, the groups also did not differ in the exploration (Student's t test, acute: $t = 1.40$, $p = 0.20$; withdrawal: $t = -0.62$, $p = 0.56$).

The animals in the chronic group (C0.50A0.50) did not show difference in the exploration of the objects in the memorization phase (Student's t test, $t = -0.58$, $p = 0.58$; Fig. 1e). In the discrimination phase, fish showed higher exploration of object C than object A (Student's t test, $t = -6.60$, $p < 0.001$). Object A exploration was higher in the memorization than in the discrimination phase (Student's t test, $t = 2.91$, $p = 0.01$).

The increased dose group (C0.50 A1.00) did not show difference in exploration of objects A and B in the memorization phase (Student's t test, $t = 0.34$, $p = 0.71$; Fig. 1f). There was higher exploration of object C than object A in the discrimination phase (Student's t test, $t = 2.24$, $p = 0.04$). This group explored more object C in the discrimination phase than object B in the memorization phase (Student's t test, $t = -2.10$, $p = 0.05$).

The highest values of average swimming speed were observed for C0.50 A1.00 and C0.00A1.00 groups (One Way Anova, memorization phase: $F = 4.88$, $p < 0.001$; discrimination phase: $F = 3.41$, $p = 0.01$; Fig. 2a). Both groups C0.00A1.00 and C0.50 A1.00 showed difference in average speed between the memorization and discrimination phases (Student's t test, C0.00A1.00: $t = 2.65$, $p = 0.05$; C0.50 A1.00: $t = 3.0.5$, $p = 0.01$). Regarding the total distance traveled, groups C0.00A1.00 and C0.50 A00 showed the lowest values, while C0.5 A1.00 showed the highest (One Way Anova, memorization phase: $F = 15.73$, $p < 0.001$; discrimination phase: $F = 9.13$, $p < 0.001$; Fig. 2b). The alcohol withdrawal and the chronic 0.50% alcohol treatment group differed in the total distance traveled between the memorization and discrimination phases (Student's t test, C0.5 A0.00: $t = -2.34$, $p = 0.04$; C0.50A0.50: $t = 2.47$, $p = 0.03$; C0.50 A1.00: $t = 0.37$, $p = 0.71$). The groups that showed the higher freezing values were C0.00A1.00 and C0.50 A0.00 (One Way Anova, memorization phase: $F = 14.56$, $p < 0.001$; discrimination phase: $F = 6.16$, $p < 0.001$; Fig. 2c). Freezing differed between the memorization and discrimination phases only in C0.00A1.00 group (Student's t test, $t = 2.70$, $p = 0.03$).

3.2. Caffeine treatment

Fig. 3 shows objects exploration of animals exposed to chronic and acute caffeine treatment. A single control group (C00A00) was used for the caffeine and the alcohol treatments (results described above), and explored mainly the new object in the memorization phases.

The acute 50 mg/L caffeine group (C00A50) did not show any difference in exploration time of the objects in the memorization phase (Student's t test, $t = 0.28$, $p = 0.78$; Fig. 3b). Fish explored more object C than A in the discrimination phase (Student's t test, $t = 3.01$, $p = 0.01$). This group explored more object B in the memorization phase than object C in the discrimination phase (Student t test, $t = -3.07$, $p = 0.01$).

The acute 100 mg/L caffeine group (C00A100) did not show differences in exploration in the memorization phase (Student t test, $t =$

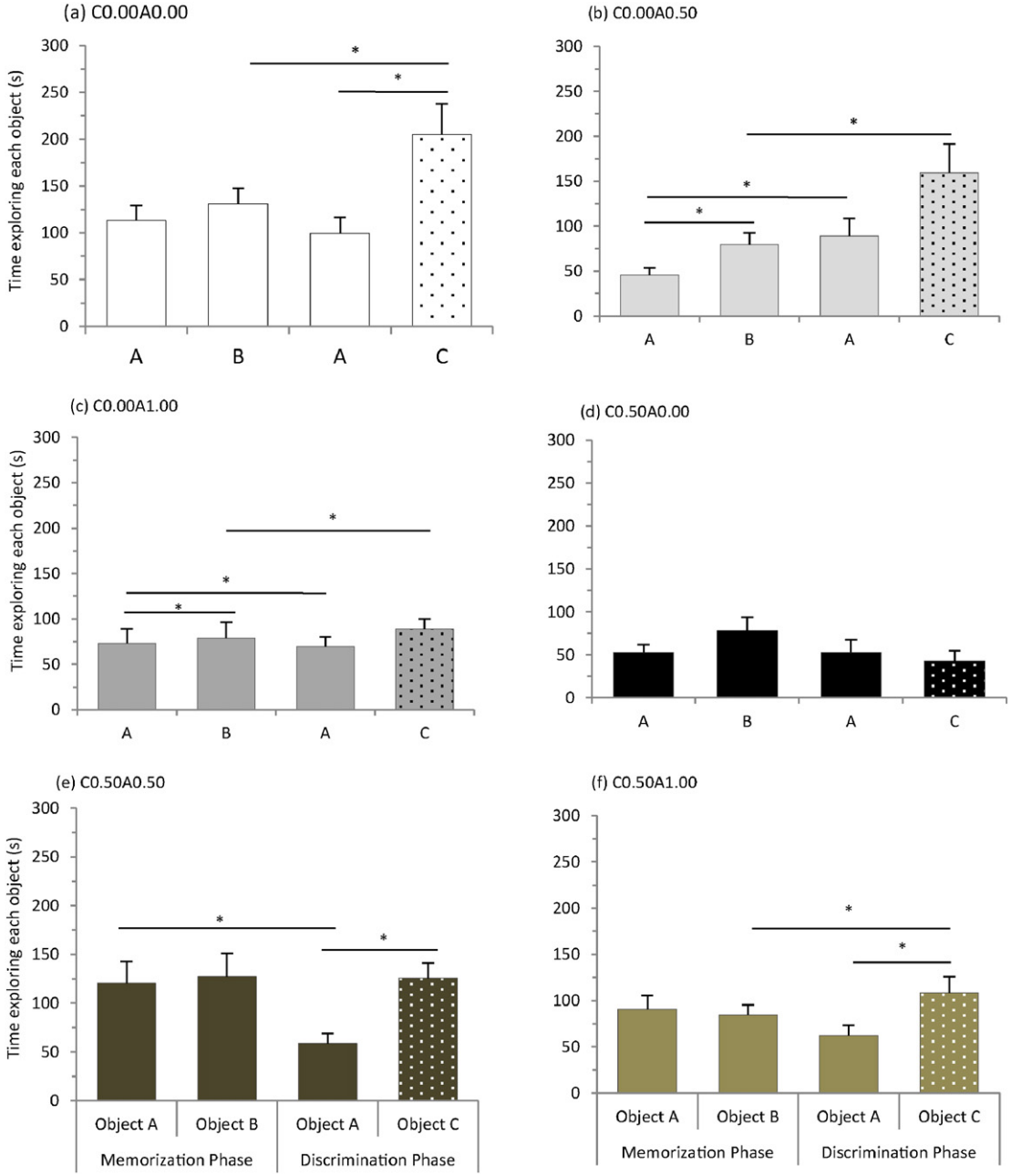

Fig. 1. Zebrafish exploration time for objects A vs. B (memorization phase), or A vs. C (discrimination phase) for the alcohol exposure regimes: (a) C0.00A0.00 –Control (n = 17), (b) C0.00A0.50 – acute 0.50% alcohol (n = 18), (c) C0.00A1.00 – acute 1.00% alcohol (n = 12), (d) C0.50 A0.00 – withdrawal (n = 9), (e) C0.50A0.50 – chronic (n = 13), and (e) C0.50 A1.00 – increased dose (n = 13). Bars mean exploration time + SEM in each object, in the memorization and discrimination phases. Fish were observed for 20 min and analyzed using video-tracking software (ZebTrack). Asterisk indicates statistical difference between fish exploration in each pair of objects marked with a bracket (Student t test, $p < 0.05$).

0.28, $p = 0.78$; Fig. 3c). There was more exploration of object C than A in the discrimination phase (Student t test, $t = -3.04$, $p = 0.01$). This group explored more object A in the discrimination than the same object A in the memorization phase (Student t test, $t = -2.34$, $p = 0.04$), and also explore more object C in the discrimination phase than object B in the memorization phase (Student t test, $t = -6.96$, $p < 0.001$).

The withdrawal group (C50A00) did not present exploration differences either in the memorization phase (Student's t test, $t = -1.04$, $p = 0.32$; Fig. 3d) or in the discrimination phase (Student's t test, $t = 1.92$, $p = 0.08$).

The chronic group (C50A50) did not show differences in exploration time between objects in the memorization phase (Student's t test, $t = 0.27$, $p = 0.79$; Fig. 3e). In the discrimination phase, this group showed increased exploration of object C (Student's t test, $t = -2.29$, $p = 0.05$). There was also higher exploration of object C in the discrimination phase than object B in the memorization phase (Student's t test, $t = -2.91$, $p = 0.04$).

The increased dose group (C50A100) did not differ in exploration time in the memorization phase (Student's t test, $t = 0.80$, $p = 0.45$; Fig. 3f), but explored more object C than the object A in the discrimination phase (Student's t test, $t = -4.36$, $p < 0.001$). Fish also explored more object C in the discrimination phase than object B in the memorization phase (Student's t test, $t = -5.01$, $p < 0.001$).

The locomotion parameters differed between the caffeine treated groups. Average swimming speed was higher for C00A00 and C50A50 groups, and lower for C00A50 group in the memorization phase (One Way Anova, $F = 3.50$, $p < 0.001$; Fig. 4a). The caffeine groups did not differ in terms of average speed in the discrimination phase (One Way

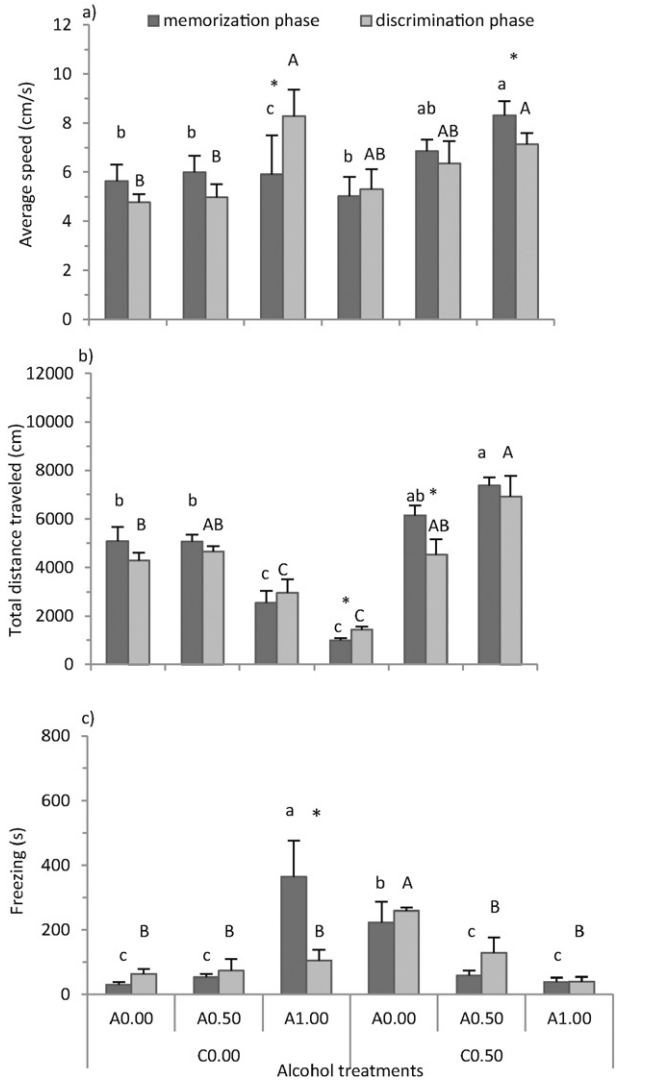

Fig. 2. Locomotor parameters for the alcohol exposure groups. (a) average speed ± SEM, (b) total distance traveled ± SEM and (c) freezing ± SEM. The alcohol treatment conditions are shown on the x-axis. The letter C represents chronic alcohol exposure and the values that follow are the concentrations of alcohol used (0.00% and 0.50%). The letter A represents acute alcohol exposure and the values that follow are the concentrations of alcohol used (0.00%, 0.50% and 1.00%). At least one different letter indicates statistical difference by One Way Anova ($p < 0.05$). Lower case letter signalize the comparison between the groups in the memorization phase and capital letters signalize the comparison between the groups in the discrimination phase. Asterisk indicates statistical differences between memorization and discrimination phases of the same alcohol treatment group.

Anova, $F = 0.68$, $p = 0.64$). Only C50A50 group showed differences in average speed between the memorization and discrimination phases (Student's t test, $t = 3.72$, $p = 0.005$).

For distance traveled, once more C00A50 group showed the lower value while C00A00 and C50A50 groups had the higher values (One Way Anova, memorization phase: $F = 3.32$, $p = 0.01$; discrimination phase: $F = 1.77$, $p = 0.13$; Fig. 4b). There was no significant difference between the memorization and discrimination phases for any of the caffeine groups (Student's t test, $p > 0.05$).

The freezing values were higher for C00A50 and C00A100 than for the other groups in both phases (One Way Anova, memorization phase: $F = 5.51$, $p < 0.001$; discrimination $F = 3.44$, $p < 0.001$; Fig. 4c). The groups did not differ in terms of freezing between the memorization and discrimination phases (Student's t test, $p > 0.05$).

3.3. Alcohol and caffeine treatment

The Fig. 5 shows time of objects exploration in the memorization and discrimination phases for the groups treated with both alcohol and caffeine. The groups exposed to chronic caffeine and acute alcohol, $C_c50A_a0.50$ did not show exploration difference between objects in the memorization phase (Student's t test, $t = -0.71$, $p = 0.49$; Fig. 5a), but explored more object A than C in the discrimination phase (Student's t test, $t = 2.21$, $p = 0.05$). The group $C_c50A_a1.00$ did not show exploratory differences in the memorization phase (Student's t test, $t = -0.71$, $p = 0.49$; Fig. 5b), but explored more object A than object C in the discrimination phase (Student's t test, $t = 2.45$; $p = 0.03$). This last group showed higher exploration of object B in the memorization phase than object C in the discrimination phase (Student's t test, $t = 2.51$, $p = 0.03$).

The groups treated with chronic alcohol and acute caffeine, $C_a0.50A_c50$ did not present exploration differences in the memorization phase (Student's t test, $t = -0.71$, $p = 0.49$; Fig. 5c), while fish explored more object C than object A in the discrimination phase (Student's t test, $t = -2.92$, $p = 0.02$), and explored more object A in the memorization than in the discrimination phase (Student's t test, $t = 5.21$, $p < 0.001$). On the contrary, $C_a0.50A_c100$ did not show difference in objects exploration either in the memorization (Student's t test, $t = 0.69$, $p = 0.51$; Fig. 5d) or in the discrimination phase (Student's t test, $t = 0.78$, $p = 0.45$).

In the memorization phase there were no differences in average speed between groups (Anova One Way, $F = 2.54$, $p = 0.07$; Fig. 6a). In the discrimination phase, average speed was higher for $C_c50A_a1.00$ than for $C_a0.50A_c50$ and $C_a0.50A_c100$ groups (Anova One Way, $F = 7.00$, $p < 0.001$; Fig. 6a). Only $C_a0.50A_c50$ group showed average speed difference between the memorization and discrimination phases (Student's t test, $t = -2.14$, $p = 0.05$).

There were no differences in total distance traveled in the memorization phase (Anova One Way, $F = 2.47$, $p = 0.08$; Fig. 6b) and the discrimination phase (Anova One Way, $F = 1.51$, $p = 0.23$). For total distance traveled, only $C_a0.50A_c50$ group showed difference between the memorization and discrimination phases (Student's t test, $t = 2.06$, $p = 0.01$).

There were no differences between the groups in terms of freezing in the memorization phase (Anova One Way, $F = 1.42$, $p = 0.25$; Fig. 6c). The freezing value $C_a0.50A_c50$ group was the highest in the discrimination phase (Anova One Way, $F = 6.35$, $p = 0.001$). Again, $C_a0.50A_c50$ was the only group that showed difference in freezing between the memorization and discrimination phases (Student's t test, $t = 2.60$, $p = 0.02$).

4. Discussion

In this study we present a view of the effects of alcohol and caffeine on the learning and memory performance of adult zebrafish. Our results provide evidence that acute alcohol exposure as well as withdrawal from both chronic alcohol and caffeine impair discriminative learning. Rather, the natural tendency to explore novelty is expected to motivate the zebrafish to learn about new items in the tank. Not only do our results confirm the ability of zebrafish to discriminate objects, as shown by Oliveira et al. (2015), but we also show that the discriminative performance was dependent upon the alcohol and caffeine dose and exposure regimen.

According to the naturalistic and psychological viewpoints, learning is the ability to change behavior with experience, which provides various benefits to the animal's life. The cognitive ability of discriminative learning involves many areas of the central nervous system (CNS), particularly the hippocampus (Barker and Warburton, 2011; Good et al., 2007; Mumby et al., 2002). This is an important zone also because many drugs affect neurons in this region, for example, alcohol was shown to affect the hippocampus (Norman et al., 2009; Willoughby et al., 2008)

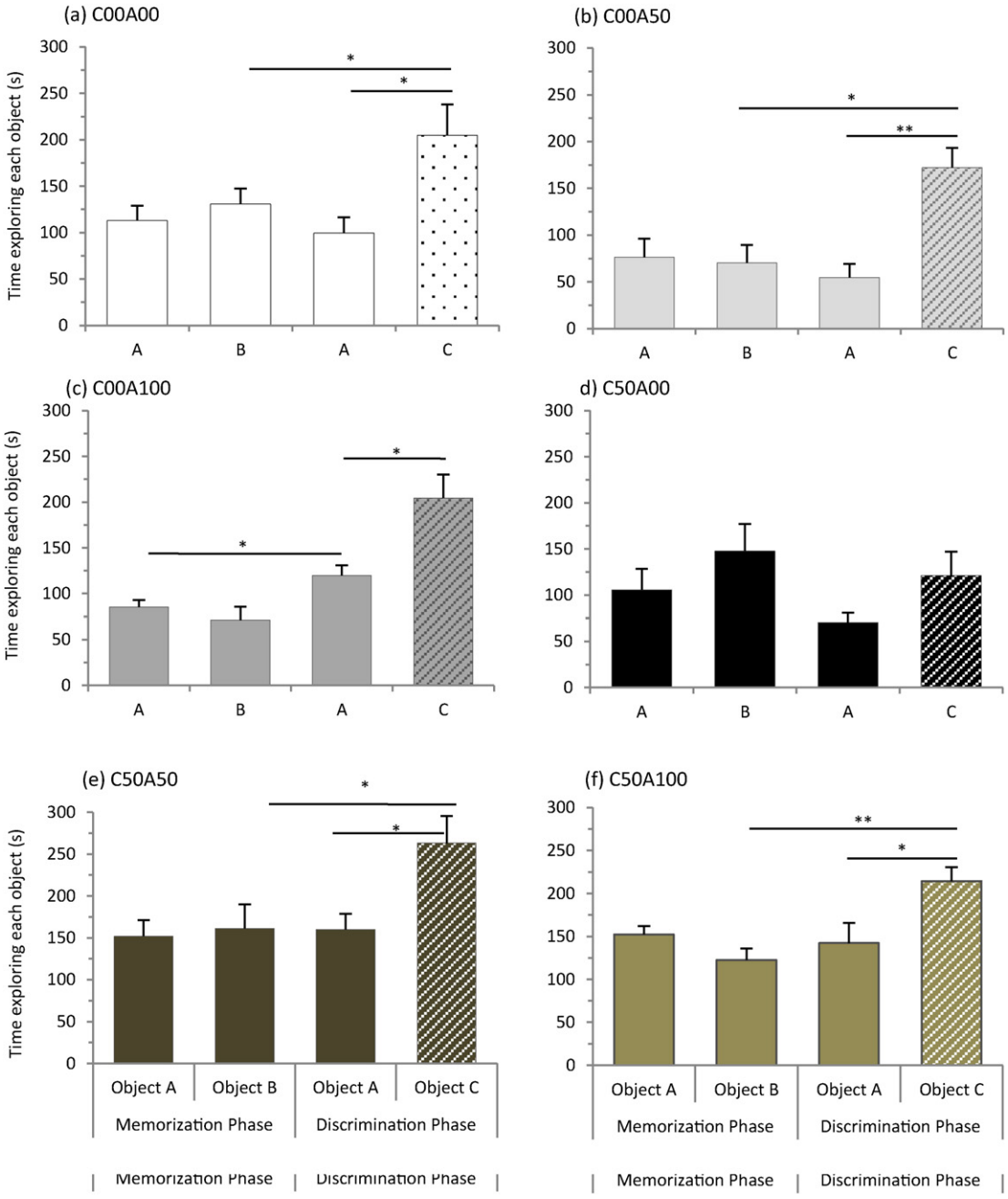

Fig. 3. Zebrafish exploration time for objects A vs. B (memorization phase), or A vs. C (discrimination phase) for the caffeine exposure regimes: (a) C00A00 –Control (n = 17), (b) C00A50 – acute 50 mg/L caffeine (n = 12), (c) C00A100 – acute 100 mg/L caffeine (n = 11), (d) C50A00 – withdrawal (n = 12), (e) C50A50 – chronic (n = 11), and (e) C50A100 – increased dose (n = 8). Bars mean exploration time + SEM in each object, in the memorization and discrimination phases. Fish were observed for 20 min and analyzed using video-tracking software (ZebTrack). Asterisk indicates statistical difference between fish exploration in each pair of objects marked with a bracket (Student t test, $p < 0.05$).

and impair memory (Hamilton et al., 2003; Uecker and Nadel, 1996). In fact, our data corroborates these findings since the use of high dose of alcohol affected fish performance in objects discrimination. However, the exact effects of alcohol in the fish lateral pallium, area that corresponds to the mammalian hippocampus, are still to be confirmed in future studies.

High doses of alcohol have been suggested to promote depression to the CNS (Gerlai, 2013; Quoilin et al., 2013; Roseribloom et al., 2004). In zebrafish, it has been shown that high concentrations of alcohol cause a decrease in activity (Gerlai et al., 2000; Gerlai et al., 2006; Tran and Gerlai, 2013). In this study, we observed this pattern for acute doses of 1.00% alcohol in terms of the objects exploration, total distance traveled and freezing. Although it is not clear if these effects were derived from increased anxiety or sedation, high acute alcohol exposure significantly affected behavior. At first glance, the result of the group under high acute dose of alcohol action seems to indicate their inability to discriminate objects. However, it is possible that the fish did learn but the acute alcohol exposure interfered with their ability to recall memory and/or simply swim properly in the tank. Other studies have also noticed the harmful effects of acute alcohol doses on motion (Gerlai et al., 2000; Tran and Gerlai, 2013), perception, and memory recall (Bartholow et al., 2003; Chacon and Luchiari, 2014). On the other hand, we observed that the moderate acute dose of alcohol (C0.00A0.50) did not alter locomotion patterns but affect objects discrimination, possibly interfering with sustained attention and environmental details perception (Parker et al., 2014; Baiamonte et al., 2015), result that was not observed for fish chronically exposed to alcohol.

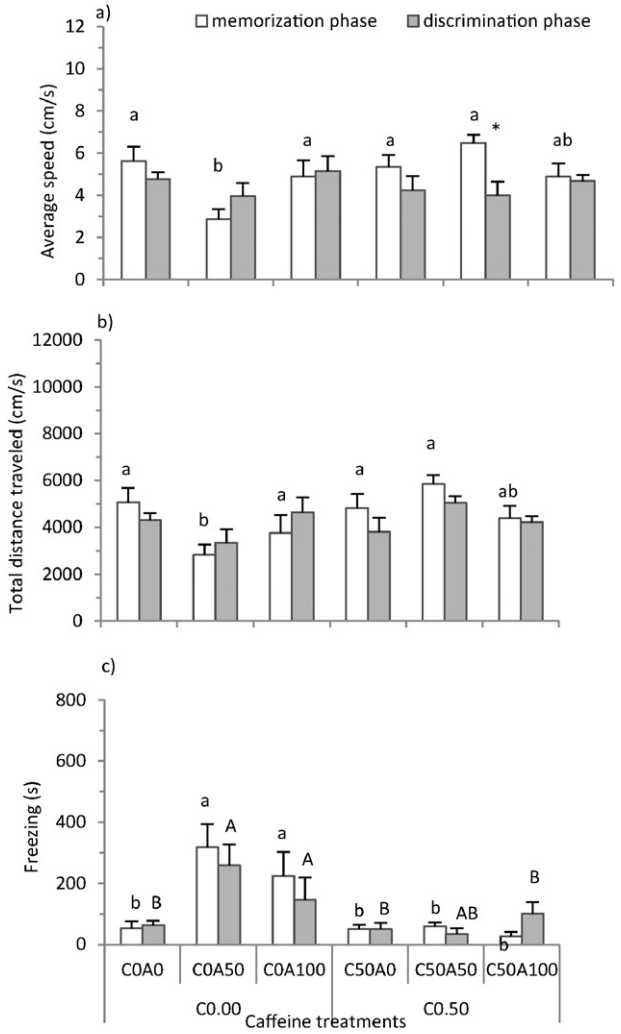

Fig. 4. Locomotor parameters for the caffeine exposure groups. (a) average speed ± SEM, (b) total distance traveled ± SEM and (c) freezing ± SEM. The caffeine treatment conditions are shown on the x-axis. The letter C represents chronic caffeine exposure and the values that follow are the concentrations of caffeine used (00 and 50 mg/L). The letter A represents acute caffeine exposure and the values that follow are the concentrations of caffeine used (00, 50 and 100 mg/L). At least one different letter indicates statistical difference by One Way Anova ($p < 0.05$). Lower case letter signalize the comparison between the groups in the memorization phase and capital letters signalize the comparison between the groups in the discrimination phase. Asterisk indicates statistical differences between memorization and discrimination phases of the same caffeine treatment group.

The main differences on the performance of fish chronically exposed to alcohol appear when we compare withdrawal (C0.50A0.00) against the other groups (C0.50A0.50 and C0.50A1.00). It seems that chronic alcohol exposure promoted tolerance to the drug and decreased the effects of higher acute exposure afterward (acute 1.00%), once these groups have shown performance comparable to the control group in many aspects. This result is in agreement with the study of Boulouard et al. (2002), showing that chronic administration produces tolerance to the adverse effects of acute alcohol exposure in rats, and also in accordance to the observed behavior of zebrafish (Gerlai et al., 2006; Gerlai et al., 2009; Tran and Gerlai, 2013). According to these studies, the tolerance could be partly attributed to altered metabolism of ethanol and to cellular and molecular adaptations of the glutamate and GABA neurotransmissions. In fish, the same glutamatergic and GABAergic systems are present in the telencephalon, and participate in memory formation (Blank et al., 2009; Nam et al., 2004; Xu et al., 2003).

Contrary to the tolerance effect described above, we showed that cessation of alcohol exposure (i.e. withdrawal) leads to a significant decrease in exploration, suggesting higher levels of stress and anxiety in the withdrawal fish. For example, Gerlai et al. (2009) showed that discontinuous alcohol exposure disrupts zebrafish shoaling behavior, Cachat et al. (2010) observed that withdrawal from alcohol causes stress response in terms of increased cortisol release, and Mathur et al. (2011) related the alcohol withdrawal to anxiety-like behavior. In mammals, the clinical symptoms of withdrawal are high anxiety, increased heart rate and perspiration, nausea and headache, increased tremors, and hallucinations (Martinotti et al., 2008; Prat et al., 2009; Wills et al., 2009; Wu et al., 2009). In fish, our results suggest that withdrawal from alcohol can induce inability to properly explore the ambient and difficulty to learn and form memory, behavioral responses associated with the peak of the withdrawal syndrome (Bayard et al., 2004).

On the opposite of the alcohol actions that are considered harmful, caffeine is considered a stimulant psychotropic. Caffeine acts on the CNS through several mechanisms, such as intracellular calcium mobilization, phosphodiesterase inhibition and sodium-potassium pump stimulation (Braga and Alves, 2000), however the most striking effect is the reversible antagonism on adenosine receptors (El Yacoubi et al., 2000; Solinas et al., 2002). Considering that adenosine receptors are found in dopaminergic, glutamatergic and GABAergic neurons (Daly and Fredholm, 1998), by blocking adenosine receptors, caffeine also affects these neurons sensibility.

In our study, withdrawal from caffeine (C50A00) affected discriminative learning. This cognitive impairment was not associated to any locomotor compromising (average speed, total distance traveled and freezing). Comparative studies with rodents during caffeine withdrawal showed poor performance in the aquatic maze task (Bruce, 1989; Khaliq et al., 2012). Cessation of caffeine intake was shown to increase sensitivity to adenosine, and thus provoke decreased alertness, lethargy and drowsiness, altered cerebral blood flow velocity and quantitative EEG (Jones et al., 2000; Rogers et al., 2013).

Animals exposed to acute caffeine (C00A50 and C00A100) showed a slightly decrease in average speed and total distance traveled during the first exposure to the drug, and increased freezing behavior. Similar locomotor effects were reported in rodents and zebrafish (Chen et al., 2008; Marin et al., 2011). These effects may occur due to the caffeine's antagonism on A_1 and A_2 adenosine receptors that affect locomotor activity (Gupta et al., 2014). On the other hand, the acute caffeine exposure did not affect the fish's ability to discriminate the new object from the former ones.

Acute and chronic caffeine exposure allow for cognitive performance in discrimination task comparable to untreated fish (control). Although it was already shown that chronic doses of caffeine leads to tolerance outcome (Satel, 2006), the main effects of prolonged use are related to decrease in fatigue contender and alertness promotion (Striley et al., 2011). The long-term usage of caffeine causes no cognitive impairment (Angelucci et al., 2002; Borota et al., 2014), which is in accordance with our results. Many studies using caffeine support its role as a memory improver (Cunha and Agostinho, 2010). The drug mnemonic effects seems to be related to the non-specific antagonism of adenosine receptors (Cunha and Agostinho, 2010; Fredholm et al., 1999). However, in zebrafish discrimination task, caffeine seems to prevent memory impairment, acting much more as a cognitive normalizer than enhancer.

The consumption of alcohol in combination with drinks containing caffeine has been suggested as responsible for binge drinking and dependence development (Marczinski, 2011). The combination is worldwide reported and its consumption can often cause social problems, interpersonal violence, risky sexual activity and severe intoxication, mainly when indiscriminately used (Naimi et al., 2003). Several beverages such as teas, energetic drinks, soft drinks and others present caffeine as an ingredient (Lozano et al., 2007; Nehlig and Boyet, 2000), and lately alcohol has been added to it. Caffeine does not exert any effect

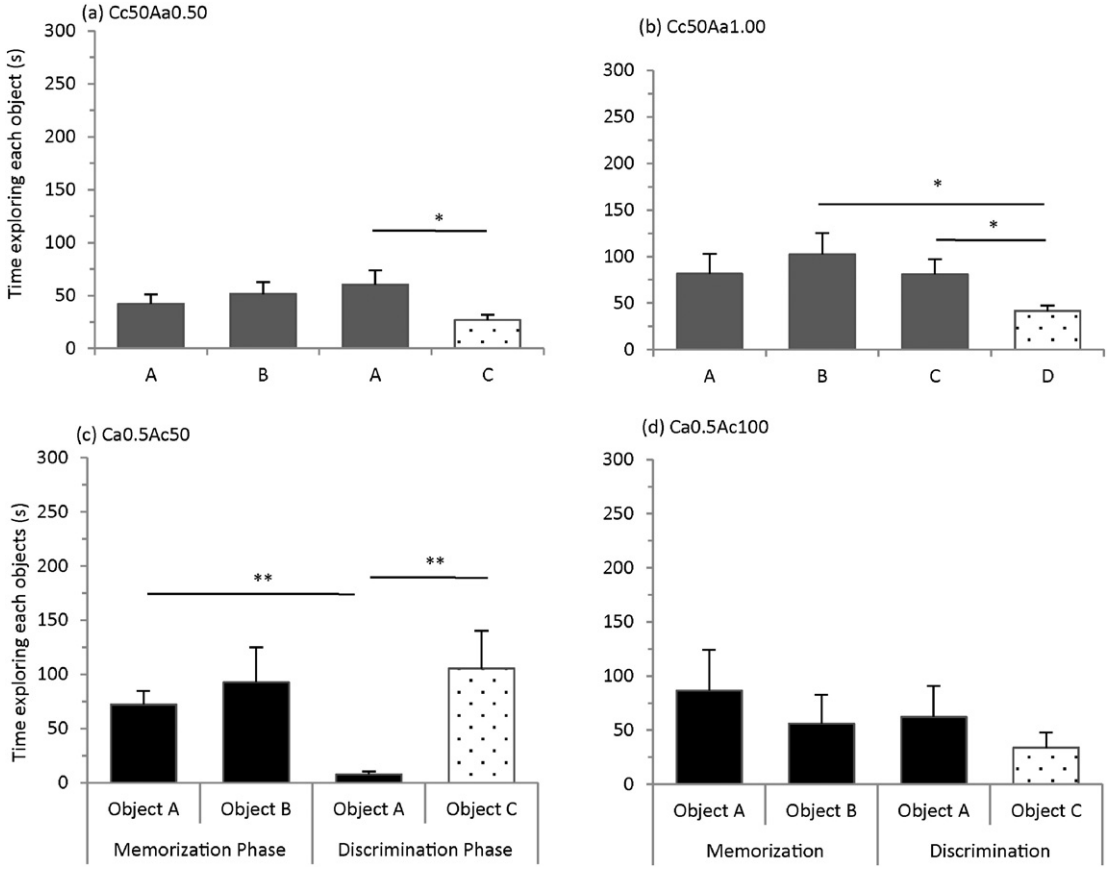

Fig. 5. Zebrafish exploration time for objects A vs. B (memorization phase), or A vs. C (discrimination phase) for the alcohol and caffeine combined treatments. The letter C represents chronic exposure and the letter A represents acute exposure. The letter that follows C represents the alcohol (C_a) or caffeine (C_c) chronic treatment and the values that follow are the concentrations of alcohol (0.50%) or caffeine (50 mg/L) used. The letter that follows A represents the alcohol (A_a) or caffeine (A_c) acute exposure and the values that follow are the concentrations of alcohol (0.50% and 1.00%) or caffeine (50 mg/L and 100 mg/L) used. (a) $C_c50A_a0.50$ (n = 13), (b) $C_c50A_a1.00$ (n = 13), (c) $C_a0.50A_c50$ (n = 9), and (d) $C_a0.50A_c100$ (n = 11). Bars mean exploration time + SEM in each object, in the memorization and discrimination phases. Fish were observed for 20 min and analyzed using video-tracking software (ZebTrack). Asterisk indicates statistical difference between fish exploration in each pair of objects marked with a bracket (Student t Test, $p < 0.05$).

on the alcohol metabolism by the liver and thus, it does not reduce alcohol blood concentrations (Ferreira et al., 2006). Still, when these substances are combined, the caffeine may mask the depressive effects of alcohol (Ferreira et al., 2006), leading to increased and prolonged intake.

In our study, fish chronically exposed to caffeine and acutely exposed to alcohol ($C_c50A_a0.50$ and $C_c50A_a1.00$) did not discriminate a new object. This result seems to be derived from two effects: first, the withdrawal from caffeine that promotes decreased alertness, and then, the acute alcohol effects that is detrimental for learning, results that are also observed in separate. However, the locomotor response observed for these groups seems to reveal the stronger effects of acute alcohol dose for these fish, as average speed, distance traveled and freezing are more related to the alcohol effects than to caffeine withdrawal.

The group exposed to chronic alcohol dose and high acute caffeine dose ($C_a0.50A_c100$) also did not discriminate the objects. For this group, we observed increased mortality during the caffeine exposure, which seems to be related to the high dose of caffeine in combination to alcohol withdrawal. During chronic alcohol exposure, there are both down regulation of GABA receptors and up regulation of glutamate receptors (tolerance related response) (Kang et al., 1998; Piepponen et al., 2002; Tsai et al., 1995). The cessation of the drug intake provoke increase in glutamatergic transmission and, due to the increased number of receptors, the excitatory response is highly increased. When we exposed fish to alcohol withdrawal and high dose of caffeine, the blockage of adenosine receptor may have intensified even more the glutamatergic response, leading to death. Similar response was also observed by Rodriguez et al. (Rodriguez et al., 2014) after high dose of caffeine used for zebrafish. These authors describe curvature body aspect before fish is considered dead.

On the other hand, chronic alcohol exposure followed by moderate acute caffeine dose ($C_a0.50A_c50$) brought about a complete different picture: fish showed objects discrimination and locomotor patterns comparable to the control group, indicating an attenuating effect of the caffeine on the withdrawal from alcohol. Taking into account the same effects of withdrawal described above, the moderate dose of caffeine used afterwards may have slightly blocked adenosine receptors and thus, intensified the sensibility of the glutamatergic and GABAergic neurons in a moderate extent. Therefore, these combined effects seem to have promoted cognitive stimulation, allowing fish to perceive and discriminate objects. There are few studies regarding the effects of this combination, in rodents the adenosine reduces the number of seizures caused by alcohol withdrawal (Malec et al., 1995) and caffeine prevents retrograde amnesia induced by alcohol (Spinetta et al., 2008), and humans report higher tolerance to alcohol when the drug is combined with caffeine (Fillmore, 2003). However, studies approaching signaling pathways and brain areas related to the effects of both alcohol and caffeine are still needed and future studies may contribute to increase the knowledge in this issue.

Although our study is the first to investigate alcohol and caffeine effects on the cognitive performance in zebrafish, many gaps are still open and deserve attention, for instance, the substances effects on long-term memory formation, development of the nervous system and more complex cognitive functions. Also, alcohol and caffeine have dose dependent effect, and other dosages may generate different consequences for the

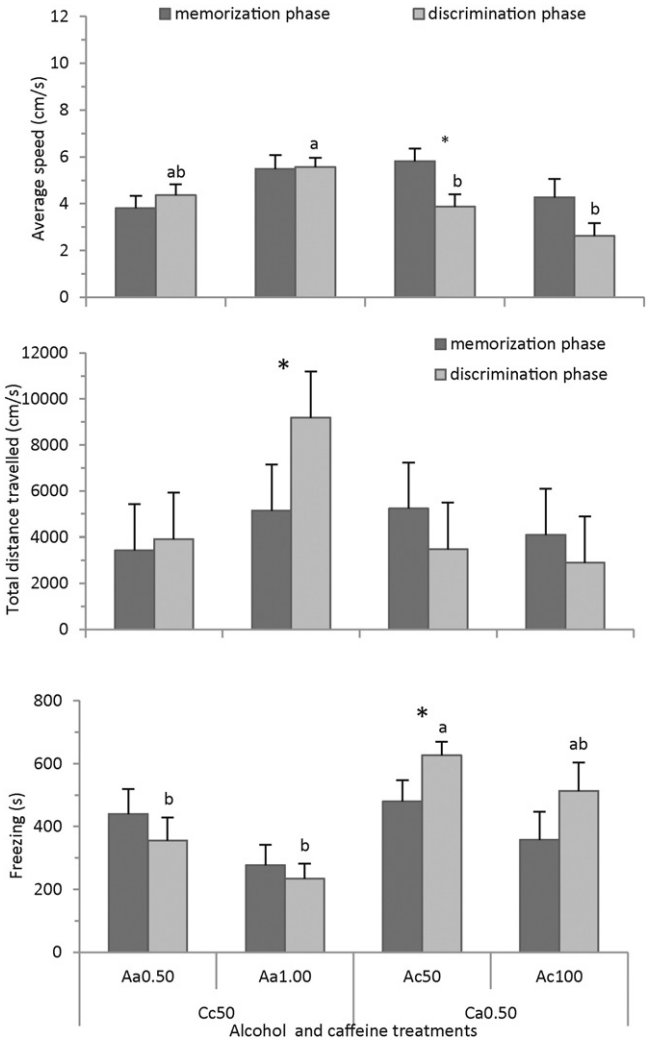

Fig. 6. Locomotor parameters for the alcohol and caffeine combined exposure groups. (a) average speed ± SEM, (b) total distance traveled ± SEM and (c) freezing ± SEM. The letter C represents chronic exposure and the letter A represents acute exposure. The letter that follows C represents the alcohol (C_a) or caffeine (C_c) chronic treatment and the values that follow are the concentrations of alcohol (0.50%) or caffeine (50 mg/L) used. The letter that follows A represents the alcohol (A_a) or caffeine (A_c) acute exposure and the values that follow are the concentrations of alcohol (0.50% and 1.00%) or caffeine (50 mg/L and 100 mg/L) used. (a) $C_c50A_a0.50$ (n = 13), (b) $C_c50A_a1.00$ (n = 13), (c) $C_a0.50A_c50$ (n = 9), and (d) $C_a0.50A_c100$ (n = 11). At least one different letter indicates statistical difference between the groups in the discrimination phase by One Way Anova ($p < 0.05$). Asterisk indicates statistical differences between memorization and discrimination phases of the same alcohol treatment group.

learning process. In conclusion, our study confirms that increased consumption of alcohol is harmful for learning, but caffeine alone does not influence discrimination either in chronic or acute use. However, moderate caffeine exposure after cessation of prolonged alcohol intake seems to reduce the deleterious effects of withdrawal, indicating the caffeine potential as drug that may help to reverse the first effects of alcohol withdrawal. It would be important at this point to invest in techniques that show the effects of alcohol and caffeine in the brain, mainly focusing on areas, neurotransmitters and proteins related to learning.

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