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ORIGINAL ARTICLE

Impact of sex and environmental conditions on the responses to pain in zebrafish

Impacto do sexo e das condições ambientais nas respostas do peixe-zebra à dor

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ABSTRACT

BACKGROUND AND OBJECTIVES: Adult zebrafish (*Danio rerio*) has been proposed as a low-cost and simple alternative to the use of rodents in laboratory research on novel compounds with antinociceptive potential. This study aimed to assess whether there is an influence of animal sex and the test environment on the orofacial nociceptive behavior of the adult zebrafish.
METHODS: First, cinnamaldehyde, menthol, capsaicin, acidic saline, or glutamate was applied into the lips of the adult male or female zebrafish. Naive groups were included as control. The orofacial nociception was quantified in terms of locomotor activity. In other series of experiments, it was evaluated whether the apparatus, acclimatization, period of test, temperature of the water and color of the open field would alter the nociceptive response to cinnamaldehyde.
RESULTS: The nociceptive behavior did not depend on the sex of the animal, apparatus, time the test was performed or the color of the open field. However, acclimatization promoted nociceptive behavior in naive animals and did not alter the nociceptive response to cinnamaldehyde ($p<0.01$ vs acclimatized naive). The nociception behavior was presented only when the test was performed at a temperature of 26ºC ($p<0.01$ vs naive).
CONCLUSION: The results suggest the need to control the environment and water temperature as an environmental source of variation during the nociceptive behavior test of the adult zebrafish.

Keywords: Environment, Pain, Nociception, Zebrafish.

RESUMO

JUSTIFICATIVA E OBJETIVOS: O peixe-zebra adulto (*Danio rerio)* tem sido proposto como uma alternativa simples e de baixo custo ao uso de roedores em pesquisas laboratoriais de novos compostos com potencial antinociceptivo. Este estudo teve como objetivo avaliar se há influência do sexo do animal e do ambiente de teste no comportamento nociceptivo orofacial do peixe-zebra adulto.
MÉTODOS: Inicialmente, cinamaldeído, mentol, capsaicina, solução salina ácida ou glutamato foi aplicada nos lábios do peixe-zebra adulto masculino ou feminino. Grupos naive foram incluídos como controle. A nocicepção orofacial foi quantificada em termos de atividade locomotora. Em outra série de experimentos, foi avaliado se o aparato, aclimatação, período de teste, temperatura da água e cor do campo aberto alterariam a resposta nociceptiva ao cinamaldeído.
RESULTADOS: O comportamento nociceptivo não dependeu do sexo do animal, do equipamento de teste, do horário em que o teste foi realizado ou da cor do campo aberto. No entanto, a aclimatação promoveu comportamento nociceptivo em animais naive e não alterou a resposta nociceptiva ao cinamaldeído ($p<0,01$ vs naive aclimatado). O comportamento nociceptivo foi verificado apenas quando o teste foi executado a uma temperatura de 26ºC ($p<0,01$ vs naive).
CONCLUSÃO: Os resultados sugerem a necessidade de controlar o ambiente e a temperatura da água como fonte de variação ambiental durante o teste de comportamento nociceptivo do peixe-zebra adulto.

Descritores: Dor, Meio ambiente, Nocicepção, Peixe-Zebra.

INTRODUCTION

Pain is considered a negative affective state associated with tissue damage and it is important to alleviate pain to improve both human condition and animal welfare[1,2]. Although rodent models are widely used in translational pain research[3,4], other experimental models help to evaluate evolutionarily conserved mechanisms underlying nociception and its associated behavioral phenotypes[5,6].
It has been already shown that the adult zebrafish (*Danio rerio*) is a viable alternative to more traditional laboratory models used

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in the search for new compounds with antinociceptive potential[7] with the advantages of significant homology to the human genome and relatively lower cost compared with rodents[7,8].

However, as far as is known, there are no studies that assess the relationship between the test environment and nociceptive behavior of the zebrafish, as well as whether there is interference of the animal's sex or not. It's known, for example, that the water temperature alters the convulsive response of zebrafish to pentylenetetrazole[9]. Study[10] demonstrated that the locomotor activity of zebrafish larvae is sensitive to the time of day and light conditions.

According to other study[11] in zebrafish neurobehavioral, sex is often not reported and the use of both rodent and novel (zebrafish) models may contribute to the understanding of the mechanisms linking sex to brain and behavior. It has been already shown that the corneal nociceptive response induced by hypertonic saline in adult zebrafish is independent of the animal's sex[12]. Authors[13] also reported that there was no interference of sex in the nociceptive response to acetic acid administered on the lips of zebrafish. However, both hypertonic saline and acetic acid promote nociceptive behavior through non-specific mechanisms.

Therefore, the aim of this study was to assess if there is an influence of animal sex and the test environment on the orofacial nociceptive behavior of the adult zebrafish.

METHODS

Adult wild zebrafish (*Danio rerio*) of both sexes, short-fin phenotype, aged 60–90 days, similar size (3.5±0.5cm) and weight (0.3±0.2g), obtained from *Agroquímica: Comércio de Produtos Veterinários LTDA* (Fortaleza, Ceará, Brazil). Groups of 50 fish were acclimated for 24 hours in a 10-L glass tank (30x15x-20cm) containing dechlorinated tap water (ProtecPlus®) and air pump with submerged filter at 25°C and pH 7.0, under near-normal circadian rhythm (14:10h of light/dark). The fish received *ad libitum* feed 24h prior to the experiments.

EVALUATION OF THE INFLUENCE OF SEX ON NOCICEPTIVE OROFACIAL BEHAVIOR IN ADULT ZEBRAFISH

Cinnamaldehyde-induced orofacial nociceptive behavior

Orofacial nociception was induced with cinnamaldehyde (TRPA1 - transient receptor potential cation channel subfamily A member 1 - specific agonist; 0.66μg/mL; 5.0μL) injected into the lips of the animals (male and female; n=8/group), 30 min after pre-treatment with saline (20.0μL). Naive groups (male and female, n=8/each) were included as control. The animals were then placed in a glass Petri dish (10 x 15 cm) divided into quadrants and the nociceptive response was quantified in terms of locomotor activity (number of crossing lines) performed during 0 - 5 min.

Menthol-induced orofacial nociceptive behavior

Orofacial nociception was induced with menthol (TRPM8 - transient receptor potential cation channel subfamily M member 8 - agonist; 1.2mM; 5.0μL) injected into the lips of the animals (male and female; n=8/group) 30 min after pre-treatment with saline (20.0μL). Naive groups (male and female, n=8/each) were included as control. The nociceptive behavior was quantified in terms of locomotor activity (number of crossing lines) performed during 0 - 10 min.

Capsaicin-induced orofacial nociceptive behavior

Orofacial nociception was induced with capsaicin (TRPV1 - transient receptor potential cation channel subfamily V member 1 - agonist; 40,93μM; 5.0μL), dissolved in ethanol, PBS (phosphate buffered saline) and distilled water (1:1:8) injected into the lips of the animals (male and female; n=8/group) 30 min after pre-treatment with saline (20.0μL). Naive groups (male and female, n=8/each) were included as control. The nociceptive behavior was quantified in terms of locomotor activity (number of crossing lines) performed during 10 - 20 min.

Acidic saline-induced orofacial nociceptive behavior

Orofacial nociception was induced with acidic saline (ASIC - acid-sensing ion channels - agonist; 0.1%; 5.0μL) injected into the lips of the animals (male and female; n=8/group) 30 min after pre-treatment with saline (20.0μL). Naive groups (male and female, n=8/each) were included as control. The nociceptive behavior was quantified in terms of locomotor activity (number of crossing lines) performed during 10 - 20 min.

Glutamate-induced orofacial nociceptive behavior

Orofacial nociception was induced with glutamate (NMDA - N-methyl-D-aspartate receptor - agonist; 12.5 mM; 5.0μL) injected into the lips of the animals (male and female; n=8/group) 30 min after pre-treatment with saline (20.0 μL). Naive groups (male and female, n=8/each) were included as control. The nociceptive behavior was quantified in terms of locomotor activity (number of crossing lines) performed during 0 - 15 min.

EVALUATION OF THE INFLUENCE OF THE TEST ENVIRONMENT ON NOCICEPTIVE OROFACIAL BEHAVIOR IN ADULT ZEBRAFISH

Cinnamaldehyde-induced orofacial nociceptive behavior

Orofacial nociception was induced with cinnamaldehyde (see above) injected into the lips of the animals (n=8/group), 30min after pre-treatment saline (20.0μL). Naive groups (n=8/each) were included as control. The nociceptive behavior was analyzed as described above during 0-5 min in the following conditions:

- Test performed in Petri dish (10x15 cm) or beaker (250mL); in this test, groups (n=8/each);
- Test performed in Petri dish with animals acclimated or not 24 hours before the experiments;
- Test performed in Petri dish with different schedule of the experiment (morning or afternoon);

• Test performed in Petri dish with different temperatures of the water: 22, 26 or 30 ºC;
• Test performed in Petri dish with light or dark floors.

All experimental procedure was approved by the Ethics Committee on Animal Research of the State University of Ceará (CEUA-UECE; #7210149/2016).

Statistical analysis

Values are expressed as mean±standard error of the mean (S.E.M). The data obtained were evaluated through the one-way analysis of variance (ANOVA), followed by Tukey's test. In all cases, differences were considered significant if $p<0.05$. All statistical analyses were carried out using the GraphPad Prism 6.0 software (GraphPad Prism Software Inc., San Diego, CA, USA).

RESULTS

Cinnamaldehyde- ($p<0.01$ - $p<0.001$ vs naive), menthol- ($p<0.05$ vs naive), capsaicin- ($p<0.001$ - $p<0.0001$ vs naive), acidic saline- ($p<0.0001$ vs naive) and glutamate- ($p<0.001$ - $p<0.0001$ vs naive) induced nociceptive behavior in adult zebrafish and this effect was not sex-dependent (Figure 1).

Influence of the test environment on nociceptive orofacial behavior of adult zebrafish

Cinnamaldehyde induced nociceptive orofacial behaviour when the animals were tested in Petri dish ($p<0.05$ vs naive) and beaker ($p<0.01$ *vs* naive) as described in figure 2. There was nociceptive response when the test was performed in the

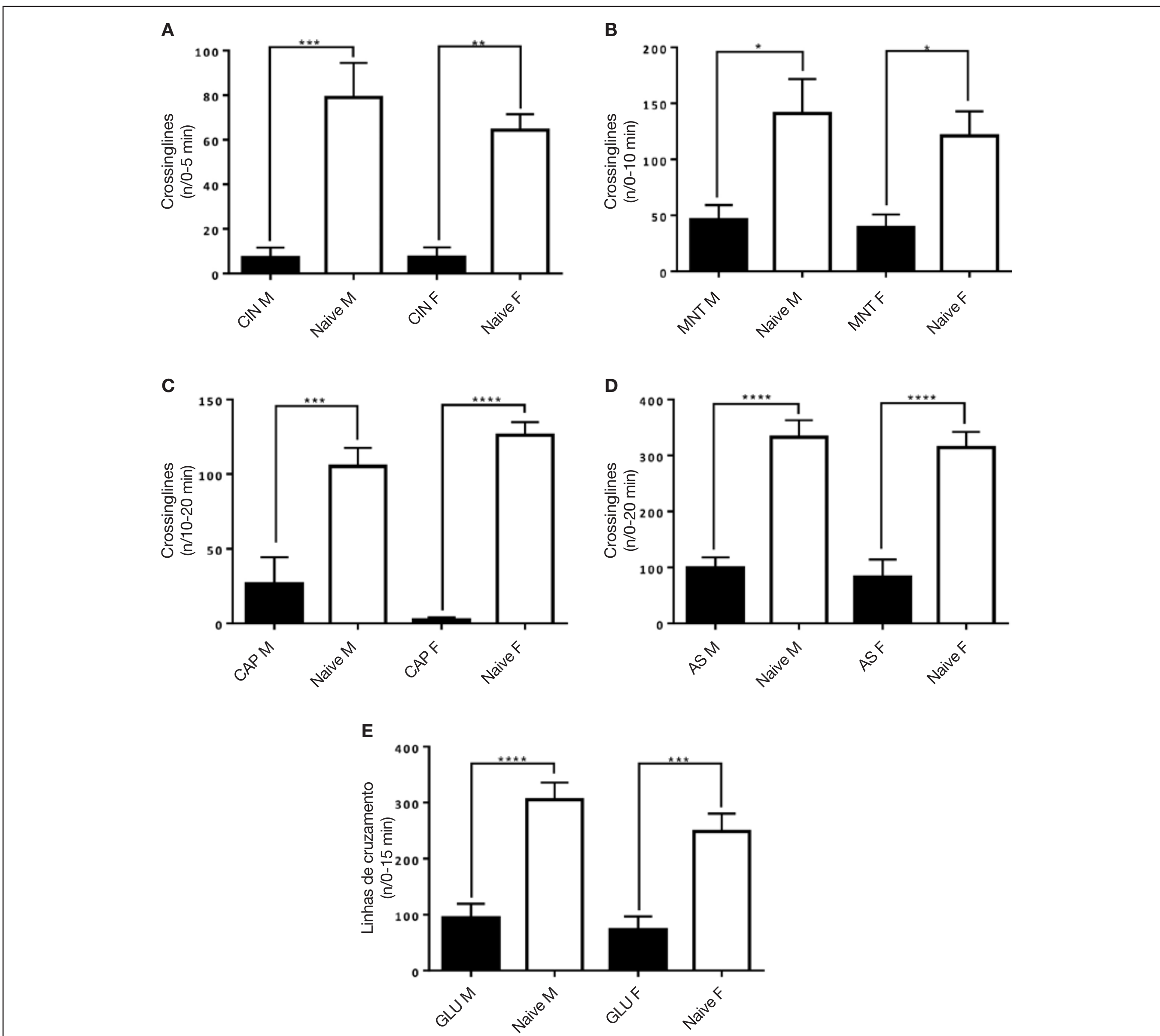

Figure 1. Orofacial nociceptive behavior induced by cinnamaldehyde (CIN - A), menthol (MNT - B), capsaicin (CAP - C), acidic saline (AS - D) or glutamate (GLU - E) in adult male (M) and female (F) adult zebrafish.
The results are expressed as mean values ± standard error. ANOVA followed by Tukey. **p<0.01, ***p<0.001 and ****p<0.0001 vs the respective Naive group.

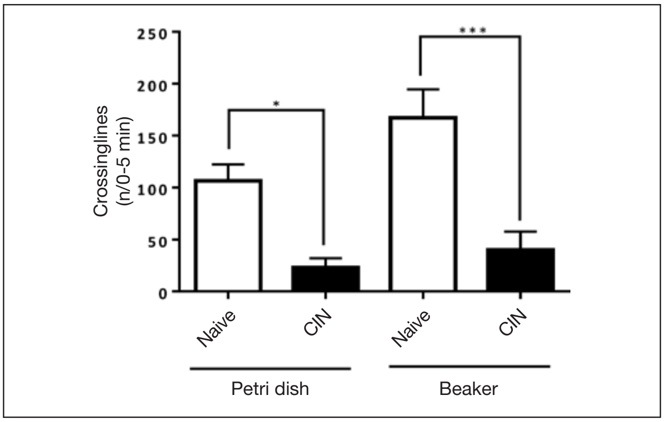

Figure 2. Nociceptive behavior induced by cinnamaldehyde (CIN) in adult male and female zebrafish for test performed in the Petri dish (A) or beaker (B).
The results are expressed as mean values ± standard error. ANOVA followed by Tukey. **p<0.01, ***p<0.001 and ****p<0.0001 vs the respective Naive group.

morning (p<0.01 vs naive) or afternoon (p<0.05 vs naive) as shown in figure 3B.

Whether or not the animals were acclimatized 24h before the test interfered with the nociceptive response, since acclimated naive animals showed nociceptive behavior (p<0.05) like that presented by fish treated with cinnamaldehyde (p<0.01) in comparison with non-acclimatized naive animals (Figure 3A).

The water temperature in which it was possible to verify nociceptive behavior was 26ºC (p<0.0001 vs naive – Figure 4A). There was no difference in response when the test was performed on Petri dishes with a white (p<0.001 vs naive) or black (p<0.01 vs naive) background (Figure 4B).

DISCUSSION

The use of adult zebrafish as an animal model of nociception has proven to be efficient as a tool for the study of new pharmacological targets[14-19]. The decrease in locomotor activity is

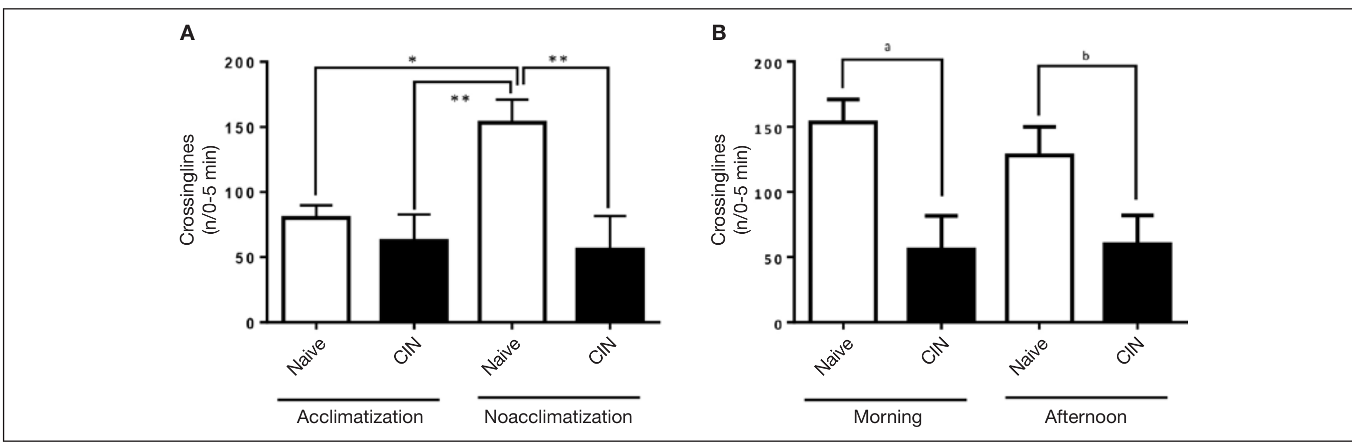

Figure 3. Evaluation of the interference of acclimatization (A) and period of the test (B) on the nociceptive behavior induced by cinnamaldehyde (CIN) in adult male and female zebrafish.
The results are expressed as mean values ± standard error. ANOVA followed by Tukey. **p<0.01, ***p<0.001 and ****p<0.0001 vs the respective Naive group.

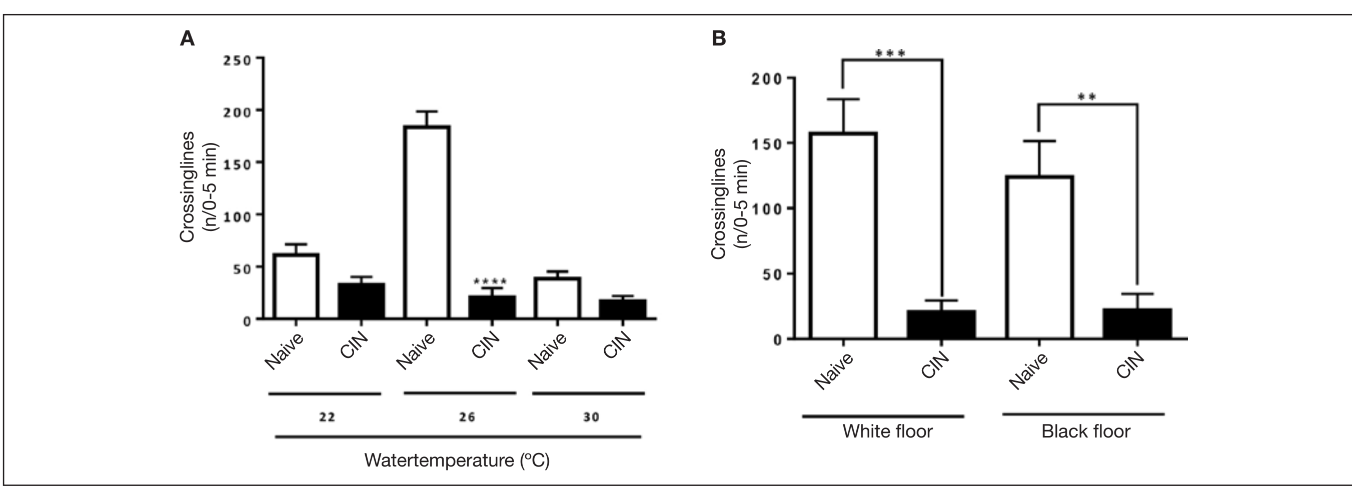

Figure 4. Evaluation of the interference of water temperature (A) and color of the open field (B) on the nociceptive behavior induced by cinnamaldehyde (CIN) in adult male and female zebrafish.
The results are expressed as mean values ± standard error. ANOVA followed by Tukey. **p<0.01, ***p<0.001 and ****p<0.0001 vs the respective Naive group.

one of the parameters of nociception in zebrafish[5] and was used here. To promote accuracy in the translation of research findings using zebrafish, several particularities of the zebrafish biology should be considered[9]. Here, the effect of sex and changes in the test environment on orofacial nociceptive behavior of adult zebrafish was evaluated.

There are few confounding factors in the nociceptive behavior of the adult zebrafish, since acclimatization 24h before the test and water temperature were the only parameters that altered the animals' response. Thus, as in adult zebrafish, the temperature of the test environment is a key factor in the variation of the nociceptive response of rodents[20,21].

It has been reported that there is a difference between the locomotor activity of male and female zebrafish[22,23]. Since the nociception parameter used is locomotor activity, it was investigated if there would be interference of the animal's sex on nociceptive behavior.

The influence of sex on nociceptive behavior in animal models has been widely demonstrated and it appears that females have hyperresponsiveness, although there is no difference between sexes in some rodent strains[24]. Here, a relationship between the animal's sex and the nociceptive response, as previously reported, was no verified[12,13,25].

The behavioral tests using zebrafish are based on confronting the animal with a new environment, experiencing the conflict between the urge to explore the unknown area/object, curiosity and the motivation to avoid potential dangers[8,25]. The nociceptive behavior was not altered by the size of the apparatus, time or color of the Petri dish on which the test was performed.

Study[26] compared the locomotor activity of adult zebrafish in two open fields of different sizes and found that locomotor activity was greater in the larger arena. The animals tested in the beaker were expected to show greater locomotor activity than the animals tested in the Petri dish. Both the animals tested in the beaker and the animals tested in the Petri dish showed nociceptive behavior. Although the nociceptive behavior was more significant in the beaker, there was no difference between the behaviors presented in the beaker and in the Petri dish.

Several studies have analysed whether the locomotor activity of embryos and zebrafish larvae is altered at different times of the day. Authors[27] demonstrated that activity of embryos and larvae was more variable during these periods, while activity was higher and less variable in the afternoon. Study[10] reported higher movement of larvae in the morning. However, other study[28] conclude that time of day did not change the activity of larvae. Although adult zebrafish is known to be more active in the morning[29], no difference was found between the nociceptive response of adult zebrafish tested at morning and at afternoon.

The color of other equipment used in neurobehavioral research, including nets and treatment beakers, is rarely reported in the literature and may play a role[30]. Zebrafish have color vision[31] and their visual physiology is generally like that of mammals[32], making zebrafish a useful tool to study the impact of colors on behaviour[30]. Different colors may affect subjective pain perception[33], but here, using the open field color parameter, there was no difference in nociceptive response of zebrafish when they were tested in both black and white open field.

It was previously showed that adult zebrafish readily (within 5 min) display habituation learning in the open field test[34]. The acclimatization induced nociceptive behavior in naïve zebrafish like animals receiving cinnamaldehyde. Non-acclimatized fish presented high locomotor activity, suggesting the existence of adaptive mechanisms in zebrafish that inhibit nociception in situations of threat and release it in situations of novelty or expectancy[35].

The nociceptive effect is observed at the water temperature of 26º C, while when the animals are tested at water temperature of 22º and 30º C there was no nociceptive behavior. This result agrees with previous findings[9], confirming the need to maintain water temperature as a determining factor for handling zebrafish, since variations in environmental temperature strongly affect fish biology influencing not only their growth, but also reproduction, spontaneous activity and metabolism[36].

CONCLUSION

In summary, the results suggest the need to control the environment and water temperature during the nociceptive behavior test of the adult zebrafish.

AUTHORS' CONTRIBUTIONS

Gerlânia Oliveira Leite
Data Collection, Research, Writing – Preparation of the original
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Data Collection
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Funding Acquisition, Resource Management, Supervision

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