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The Zebrafish (Danio rerio) as a Model for Studying Voluntary Physical Exercise and its Effects on Behavior and Metabolism

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HIGHLIGHTS

- The proposed voluntary physical exercise model does not cause stress in zebrafish.
- The zebrafish in the light period voluntarily chooses to frequent areas with faster water.
- The animals in the VPE group showed a higher maximum acceleration and speed during the locomotion test.

Abstract: The objective of this study was to develop a viable and low-cost model of voluntary physical exercise that could be applied to studies on metabolism and behavior. 40 male zebrafish (Danio rerio) were studied, divided into two groups: control and voluntary physical exercise. The model consisted of two aquariums connected by a translucent tube and a video camera on the side to measure physical exercise parameters of the animals. The animals showed higher acceleration and maximum speed and had a higher frequency of activity in the light period. In this model of voluntary physical exercise, we observed better performance in locomotor assessment tests, which was not accompanied by increased anxiety or changes in biochemical parameters related to lipid metabolism. Zebrafish responded positively to voluntary physical exercise and this model appears to be a good option for further studies.

Keywords: fish; novel tank test; locomotor activity.

INTRODUCTION

Zebrafish (Danio rerio) is a well-established animal model for several research fields, such as genetics, developmental biology, human diseases, and ecotoxicology [1-5]. It has a low maintenance cost, high reproduction rate, short generation time (approximately 3 months), and large number of eggs per spawning, allowing many animals to be studied at once [6]. Because zebrafish has high genetic, anatomical, and physiological homology with mammals, it has great potential for the development of exercise-related models.

Any type of exercise is beneficial to health [7-13], but given the need to evaluate physical performance and the effects of exercise on diseases, it was necessary to develop models of aerobic [14, 15] and sprint exercise training [16]. A voluntary physical exercise (VPE) model for zebrafish has not yet been described, although such model is already widespread in rodents [17-21]. Using zebrafish as a model of VPE would offer all the advantages that the model has while allowing the study of VPE-stimulating drugs and their possible application to metabolic diseases. Thus, the objective of the present study was to evaluate the potential of zebrafish as a viable and low-cost VPE model that could be applied to studies on metabolism and behavior.

MATERIAL AND METHODS

Ethics statement

All experimental procedures were performed at the Central Animal Facility of Federal University of Lavras (Lavras, Minas Gerais, Brazil) and were approved by the animal research ethics committee of Federal University of Lavras (protocol 042/2019).

Experimental animals

Forty male zebrafish (Danio rerio) with an aged 12 months, mean weight of 0.562 ± 0.135 g, kept under a 14 h light:10 h dark photoperiod, were used. The water quality parameters (temperature, pH, and ammonia concentration) were monitored daily and kept within the ranges recommended for the species. The animals were randomly divided into two groups: VPE (n = 20) and control (CT) (n = 20).

Voluntary physical exercise system

The VPE system was adapted from McDonald and coauthors (2007) [22]. The system aimed to measure the amount of VPE using video monitoring equipment, where the maximum number of animals per hour in the tank with water flow was quantified. It consisted of two translucent tanks (22.5 cm height × 33 cm length × 23 cm width) connected by a translucent plastic tube (60 cm length × 5cm) centered at 8 cm from the bottom of the tanks. This connecting tube was adjusted to the same height so that no current was produced inside the tube. The cross-sectional area of the tube was large enough to allow the zebrafish to move from one tank to the other.

The animals in the VPE group were acclimated to the system for 5 days before starting the experiment, during which the water flow simulator remained off. On the first day of the experiment, all animals were allocated to tank A, where there was no water flow-generating mechanism. Then the water flow simulator in tank B was connected to the system where the animals of the VPE group were located. Thus, the fish that opted for VPE passed through the tube to reach tank B. The water flow in tank B was generated with a pump (flow rate 2500 L/h). The water flow was restricted to this tank, as there was no water flow in the tube or in tank A. The time of day when the zebrafish preferred to perform physical activity was recorded using a high-definition infrared monitoring system (Giga Security, Brazil).

The animals in the CT group were kept in an identical system, but the water flow was kept off throughout the experiment; thus, in the system where the CT animals were kept, both tank A and tank B had the same characteristics (Figure 1).

The zebrafish were fed commercial feed (45% crude protein; Alcon, Brazil). The fish were fed three times a day to apparent satiety.

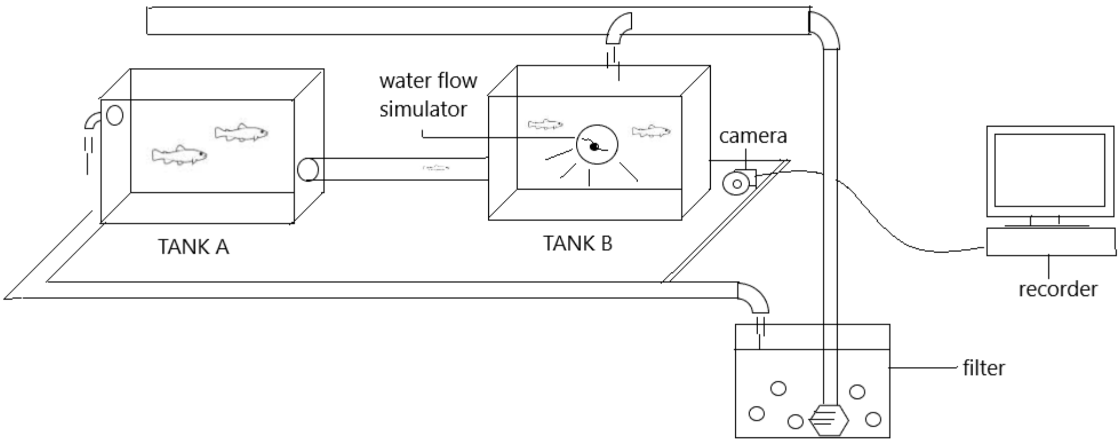

Figure 1. Model of voluntary physical exercise.

Locomotion in a regular tank

The locomotion protocol in still water was adapted from Blazina and coauthors (2013) [23] consisting of, different fish with the same characteristics were placed in a rectangular tank (27.5 x 14 x 10.5 cm, length x height x width) with 2 L of treated water. Fish locomotion was video recorded for 6 min and the Maximum speed (cm/s), Maximum acceleration and (cm/s), were automatically analyzed by the EthoVision XT® software (Noldus).

Euthanasia and biochemical parameters

At the end of the experiment, the animals were anesthetized with benzocaine (250 mg L-1) [24] and euthanized. Blood was collected according to Carneiro and coauthors (2020) [25], and blood glucose levels were immediately measured using a portable glucometer (Accu-Check, Roche Diagnostics, Rotkreuz, Switzerland). Total cholesterol (TCHO), triglycerides (TG), and lactate dehydrogenase (LDH) were analyzed according to the protocol described by Sancho and coauthors (2009) [26] using the following kits: Bioclin total cholesterol (Ref. K083), LDH Bioclin (Ref. K014-2), and BioTecnica triglycerides (Ref. 10.010.00), respectively.

Analysis of cortisol

Cortisol was analyzed in five fish from each group using the extraction protocol proposed by Canavello and coauthors (2011) [27] and quantification by enzyme-linked immunosorbent assay (ELISA) (Monobind Inc., USA).

Histological analysis

Five fish were fixed whole in 10% formaldehyde aqueous solution for at least 48 h. Standard histological processing was then performed [28], and the fish were embedded in paraffin to cut 4-μm-thick sections in a manual microtome (Lupetec, MRP2015, Brazil). The slides were then stained with hematoxylin–eosin. The images were obtained using a light microscope (Motic, USA) coupled to an image capture system (Moticam 3+, USA). The visceral adipose tissue was identified, and from 15 nonsequential fields, the area of the adipocytes was measured, as described in Virote and coauthors (2020) [28].

Novel tank test

At the end of the experimental period, the novel tank test was performed with all animals, according to the protocol of Cachat and coauthors (2011) [29]. The parameters time spent on bottom (s), time spent on top (s), distance traveled at the top (cm), distance traveled on the bottom (cm), frequency of top entries from bottom, frequency of bottom entries from top, stillness duration (s) were analyzed using EthoVision XT® software (Noldus).

Statistical analysis

The data are presented through descriptive statistics (mean, median, and standard deviation). The normality and homogeneity of variances were evaluated by the Shapiro-Wilk and Levene tests. Statistical comparisons between two means were performed by Student's t-test and the Mann-Whitney U test. A p-value <0.05 was considered statistically significant (Prism 7.04, GraphPad Software, La Jolla, CA, USA)..

RESULTS AND DISCUSSION

In recent decades, zebrafish has been used in diverse areas of biomedical research, so it is important to develop new models involving this species, as well as to understand its responses to new methods. To date, this is the first study to propose a model of VPE specific to zebrafish.

In rodents, the preference for the activity is linked to VPE being highly rewarding [30], and according to Palstra and coauthors (2010) [15], that could be extrapolated the zebrafish. But the results of the present study showed that zebrafish tended to remain in the tank without water flow (Figure 2A). This can be explained by the zebrafish take a prefer still or slow moving water [31]. The time the fish stayed in the tank with water flow was higher in the light period (Figure 2B), which result can be explained by the higher activity of zebrafish in the daytime [32].

The animals in the VPE group showed a higher maximum acceleration and speed

during the test locomotion in a regular tank (Figures 2C and 2D). Similar results were found by Gilbert and coauthors (2013) [33], who evaluated the effect of intermittent exercise on zebrafish performance using the critical velocity method and found that it improved the maximum endurance and sprint swimming speeds in young and middle-aged fish. McDonald and coauthors (2007) [22] evaluated the performance of rainbow trout (Oncorhynchus mykiss) with the critical velocity method after the animals had passed through the VPE system and reported an improvement in sprint performance and fatigue threshold. The results observed may be related to the improvement in aerobic capacity, causing an increase in mitochondrial and vascular density, increased ventilation and blood ejection volume, and better recruitment of oxidative muscle fibers [34-36].

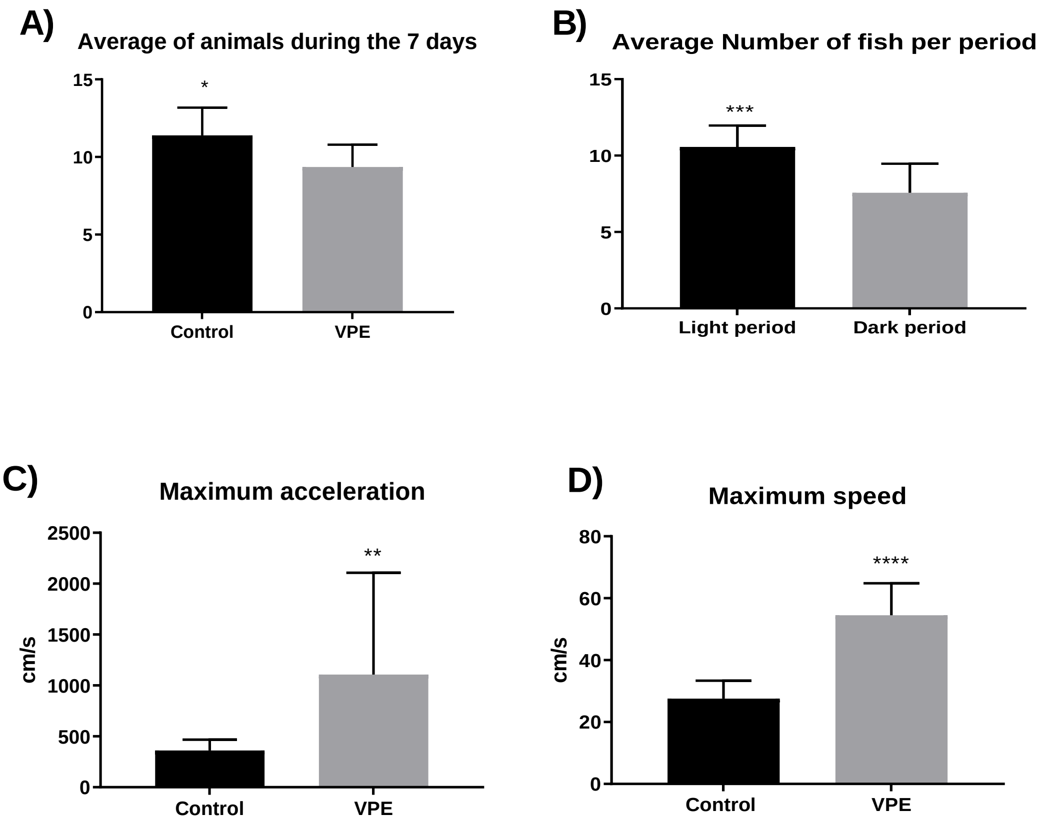

Figure 2. (A) Preference for voluntary physical exercise over time (p= 0.0359*). (B) Preference for voluntary physical exercise throughout the photoperiod (p= 0.0005***). (C) maximum acceleration comparison (p= 0.0095**). (D) Maximum speed comparison (p<0.0001****). Data are mean ± SD.

We wanted to determine whether the model was of VPE and not stress, since in mammals, VPE levels are measured individually, not in groups as done in the present study. The responses to stress can be divided into three categories [37, 38]. The primary response corresponds to increased levels of plasma corticosteroids, specifically cortisol; to be considered a stressful level, it should be above 30-40 ng/ml [37]. We evaluated the primary responses and found that the animals were not in a state of stress, but there was an increase in the cortisol levels in the VPE group (Table 1). This variation may be related to non-stressors [37], such as increased cortisol for greater stimulation of gluconeogenesis to meet the energy demands of the new activity and to increase protein synthesis [39]. Secondary stress responses include numerous metabolic, hematological, hydromineral, and structural variables [37]. We evaluated two parameters, blood glucose and LDH (Table 1), but these did not change with VPE. The secondary stress response does not depend on the primary stress response, as Pickering and coauthors (1982) [40] found that cortisol and LDH in brown trout subjected to 2 min of handling returned to their resting levels within 4 h, but the peak blood glucose occurred at 4 h. Tertiary stress responses are related to the fish as a whole, i.e., to its growth rate, metabolic rate, thermal tolerance, reproductive capacity, and others [37].

Table 1. Behavioral effects after 7 days of exposure to a voluntary physical exercise system. Measures 3, 7 and 8 are expressed as mean ± SD, and measures 1, 2, 4, 5, and 6 are expressed as median.

Measures	Control	VPE	P-value
1. Time spent on bottom (s)	350.2	352.9	0.911
2. Time spent on top (s)	0.000	0.2338	0.603
3. Distance traveled on the top (cm)	3.928 ± 0.13	3.565 ± 0.22	0.176
4. Distance traveled on the bottom (cm)	0.0000	0.0003	0.665
5. Frequency of top entries for bottom	0	0	0.869
6. Frequency of bottom entries for top	0	1	0.898
7. Stillness duration (s)	182.2 ± 30.6	175 ± 29.31	0.865
8. Total distance traveled (cm)	1154 ± 259.4	1168 ± 242.4	0.969

To determine the anxiety levels of the animals, we used the novel tank test because it is a well-established test [29]. We did not observe significant differences in variables related to anxiety behavior between the groups. The combined evaluation of primary, secondary, and tertiary stress responses indicates that the proposed model does not cause stress in zebrafish (Table 2).

Last, we evaluated whether exposure to the VPE system would alter factors related to lipid metabolism, as occurs in rodents [41, 42], but no differences were found in TCHO, TG, or adipocyte area (Table 1). The nonobservance of effects in the VPE group may be because the animals did not have any previous metabolic alteration. Studies of rodents typically use longer periods and animals with some pathology, such as obesity, or some metabolic syndrome–related comorbidity [43-47].

Table 2. Effects of 7 days of exposure to a voluntary physical exercise system on biochemical, hormonal, and visceral adipocyte area variables.

Variables	Control (Mean ± SD)	VPE (Mean ± SD)	P-value
TCHO (mg/dL)	50.084 ± 1.213	53.034 ± 3.706	0.129
TG (mg/dL)	129.392 ± 9.947	126.675 ± 6.263	0.619
LDH (U/L)	19.942 ± 6.499	17.535 ± 5.981	0.605
Blood glucose (mg/dL)	54.428 ± 17.643	45.125 ± 17.422	0.323
Cortisol (ng/g body weight)	3,765 ± 0,4725	13,64 ± 1,921	0.001**
Adipocyte area (µm²)	1674.368 ± 935.015	2278.844 ± 442.505	0.227

CONCLUSION

Zebrafish responded positively to voluntary physical exercise and this model appears to be a good option for further studies.

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