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Chapter 3

Zebra Fitness: Learning and Anxiety After Physical Exercise in Zebrafish

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Additional information is available at the end of the chapter

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Abstract

In the recent years, a new branch of physical training has emerged, the high-intensity interval training (HIIT). In contrast to continued exercise regime used in most of the trainings, HIIT proposes a regime of short periods of maximum intensity exercising and brief less intense recovery periods, which are repeated until complete exhaustion. HIIT is calling the attention of those who search for fast escalation in physical performance; however, the stress caused by this type of training may affect other systems functioning, such as cognition. Thus, we investigated the effects of two physical regime protocols, traditional endurance and HIIT on zebrafish learning, memory, and anxiety-like behavior. To that, fish were trained for 30 days and submitted to a latent learning test, objects discrimination test, and novel tank test. Our results showed that HIIT does not affect long lasting memory, evaluated through the latent learning task, but it impairs discriminative learning. On the other hand, both training protocols decrease anxiety-like behavior. This study confirms that zebrafish show good performance in learning tasks and that cognitive performance is dependent upon the regime of physical exercise and cognitive task used.

Keywords: training, latent learning, objects discrimination, novel tank, *Danio rerio*

1. Introduction

It is well known that regular physical activity is an efficient way to improve health in terms of respiratory and cardiovascular functioning [1–3]. In addition to the effects in somatic functioning, studies with humans and animal models show that physical exercise affects cognition,

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improving memory, and learning performance [4, 5]. For instance, positive results are reported in terms of attention [6, 7], executive function [8], and motor skills [9].

The benefits of regular training are remarkable in the elderly population. Aging is accompanied by a decline in cognitive abilities, which in some cases occur faster and in a pathological manner [10]. Physical activity retards the cognitive decline, reduces the risk of dementia and neurodegenerative disorders [11–13]. Continuous exercise through lifespan is important to maintain the protective effects in elderly [14] and is also associated with large gray matter in later life [15].

Besides the protective effects against damages due to aging, physical activity can improve cognitive performance in different stages of life. Young adults who practice at least 8 h of physical activity per week present better results in a sustained attention task when compared to individuals with low levels of activity (less than 2 h per week) [16]. In male adolescents, highly intense exercise is associated with the improvement in working memory and in brain-derived neurotrophic factor (BDNF) levels [17]. In children, physical activity improves performance on verbal and mathematical tests, cognitive flexibility and working memory [18, 19].

The benefits mentioned above seem to be related to structural changes in the brain. Physical activity modifies the structure of the hippocampus [20], the most important brain area for learning and memory, and is associated with an increment in gray matter volume probably through BDNF expression [21]. Moreover, it enhances plasticity [22] and stimulates neurogenesis [21].

Another benefit associated with physical activity is the improvement in psychological health. Emotional disorders are common in modern society and impact several domains of one's life. Physical activity has been postulated as a promising treatment strategy [23]. A large body of literature shows that it reduces anxiety, stress, and depression symptoms as it promotes positive mood and greater well-being [23–25]. These behavioral alterations are associated with physical activity modulation of neurotransmitters such as dopamine, associated of the reward system, and serotonin, which plays a role in well-being [26]. Physical activity also interferes in the stress-regulation axis and may buffer stress effects [24].

Many factors can interfere in the beneficial effects of exercise, including individual characteristics such as age, health status, sedentary level, cognitive abilities, and type of exercise [6, 21, 27–30]. More intense activities such as the high-intensity interval training (HIIT) have gained popularity more recently. HIIT is characterized by short periods of maximum intensity exercising interspersed with brief less intense recovery periods. However, it is not clear how this type of exercise impacts health and cognition. HIIT has been elected as the preferred type of training among those who practice physical activity due to its fast conditioning response. However, the trade-off in maintaining HIIT or adopting the traditional training deserves investigation. In this sense, the present study aims at investigating the cognitive and anxiolytic effects of HIIT in relation to traditional training.

Regarding physical activity, zebrafish swimming ability improves with training [31] and enhances associative learning response [32]; therefore, it offers a good organism model to the study of physical activity on cognition.

2. Materials and methods

2.1. Animals and housing

Zebrafish (*Danio rerio*, 3-month age, both sexes) were acquired from a local farm (Natal, Brazil) and maintained in storage tanks (50 L) for a month prior to the experiments. Tanks formed a system with multistage filtration, containing mechanical, biological, and an activated carbon filter, and UV light sterilizing unit. Temperature was kept at 28 ± 1°C, and pH and dissolved oxygen were measured regularly. Light was maintained in a 12/12-light/dark cycle. Fish were fed twice a day *ad libitum* with commercial food (38% protein, 4% lipid, Nutricom Pet) and *Artemia salina*. All the experiment procedures were performed with the permission of the Ethical Committee for Animal Use of the Federal University of Rio Grande do Norte (CEUA 054–2016).

2.2. Groups and training

From the stock population, 115 fish were randomly assigned to one of the three groups according to the type of physical training: traditional endurance, HIIT, and sedentary. These fish were housed in separate in smaller tanks (40 × 20 × 25 cm, 15 L) and underwent 7 days of acclimation before beginning the training phase (30 days), after which the tests were performed. The experimental phase was developed in the same environment water quality conditions of the stock fish.

Traditional endurance (n = 34): once a day, fish were transferred to a training tube, always in groups of 5. The training tube consisted in transparent tunnel connected to a 180 L/h submerse water pump (Moto Bomba SARLO S300, 220 V, 60 Hz. Sarlobetter Equipaments Ltda., São Caetano do Sul-SP) placed inside a glass tank [32]. Each group was trained for 30 min, during which the first 15 min fish could not exit the tube and was forced to remain swimming against the water current. Then, the tube was open and fish that could not swim was pushed by the current to the tank. After leaving the tube, they were returned to the home tank until the next session.

HIIT (n = 39): the high-intensity training consisted in a progressive increment of water current intensity. For this, we used water pumps with different powers. During the first 10 days, animals were submitted to a forced swim against the water current powered by a pump of 180 L/H (Moto Bomba SARLO S300, 220 V, 60 Hz. Sarlobetter Equipamentos Ltda., São Caetano do Sul-SP). Between days 11 and 20, a pump with 300 L/h (Moto Bomba SARLO S360) was used and in the last 10 days, it was changed to a 520 L/h pump (Moto Bomba SARLO S520). Training sessions lasted 10 min inside the water current followed by 5 min interval, repeated three times (total time of training: 30 min).

Sedentary (n = 42): fish that was not subjected to training were placed once a day in the training tube without any water current for 30 min. After that, they were returned to the home tank until the next session.

2.3. Latent learning

In this paradigm, fish explore a maze for a training phase without any reinforcement until the test day [33, 34]. The maze has two side arms with the same size (left and right) and a central tunnel that connects the start box to a goal box, where a reward was placed only on the test day. Maze walls were transparent allowing fish to see all the compartments. Exploration/training sessions occurred once a day, for 30 min, during the last 15 days of physical training. Fish group was placed in the start box for 30 s to habituate and then, the start box's door was opened, allowing fish to explore the maze. From each training group, some animals were trained only to explore the right tunnel of the maze (traditional endurance n = 7; HIIT n = 6; Sedentary n = 7) and some animals were trained to explore only the left tunnel of the maze (traditional endurance n = 9; HIIT n = 8; Sedentary n = 8). The reward chamber was empty during the 15 days of training.

On the test day (31st day of physical training), a small shoal of five fish was positioned in the goal box as reward stimulus to experimental fish. Animals were put individually in the start box and after 30 s the door was lifted, and fish could explore the maze for 10 min. During the test, the right and the left tunnels of the maze were open, so that fish could choose the patch to the goal box. Fish behavior was recorded using a handy cam (Sony Digital Video Camera Recorder; DCR-SX45) above the maze and the following parameters were analyzed: time spent in each tunnel of the maze and in the goal box, first tunnel chosen; latency to enter the shoal area; duration of time with the shoal.

2.4. Objects discrimination test

The object discrimination test consisted in three phases: habituation, memorization, and discrimination phases [35]. The three phases occurred in a 15 L tanks (40 × 20 × 25 cm) with the walls covered with white paper to avoid any extern interference. Habituation phase lasted 5 days (25th–30th days of physical training), during which fish were transferred to the test tank and allowed to explore it for 15 min to reduce possible isolation stress.

On the memorization phase (31st day), two objects (named of A and A') were introduced in the tank test and positioned in each side of the tank, 30 cm from each other. Objects were equal in size, color, and shape. Fish was individually introduced into the tank and explored the objects for 10 min; after which animal was returned to an individual tank. On the next day (32nd day), the discrimination phase occurred, which object A' was replaced by a new one (named object B) with same shape and size but different color. Fish explore the tank and objects for 10 min. During memorization and discrimination phases, fish behavior was recorded from above using a handy cam (Sony Digital Video Camera Recorder; DCR-SX45). Behavior records were analyzed for the time fish spent around each object during both phases [36].

2.5. Novel tank test

The novel tank test is one of the most common tests to evaluate anxiety in zebrafish. It is based on the innate response of fish to dive and freeze when submitted to a new environment/

situation [37]. The test took place after the end of physical training (day 31). Fish was individually transferred to tank (20 × 12 × 15 cm) and behavior was recorded for 10 min with a handy camera (Sony Digital Video Camera Recorder; DCR-SX45) positioned in front of the tank. It evaluated average swimming velocity, total distance traveled, freezing duration, and distance from the bottom of the tank.

2.6. Statistical analysis

For the latent learning test, one-way analysis of variance (ANOVA) was performed to investigate the main effect of training regime and behavioral parameters during the test trial. When significant effects were identified, post hoc Student Newman Keuls test was conducted to reveal significant ($p \leq 0.05$) group differences.

We compared the objects exploration time in memorization and discrimination phases, and also between the two phases using Two-Way ANOVA followed by Student Newman Keuls when significance was found.

We also calculated an index of how much fish explored each object during both phases. The exploration index for the memorization phase (exploration of memorization = E_m = A1 + A2) and discrimination index for the discrimination phase (discrimination index = D_i = B − A3) were calculated to verify whether there were differences in object exploration time between groups [38]. Correlations between exploration in the memorization and discrimination phases was compared using Simple Linear Regression (Pearson's correlation).

All the locomotor parameters from the novel tank test were statistically compared using One-Way ANOVA. For all tests, we considered the probability level of $p < 0.05$ for statistical significance.

3. Results

3.1. Latent learning

Figure 1 presents the number of correct choice fish made during the test day and the time spent in each tunnel on the test day, when both the right and the left tunnels of the maze were open. One-Way ANOVA showed neither difference in the number of correct choice between the three groups ($F(2,44) = 0.76$, $p = 0.47$; **Figure 1a**) nor in the number of correct choice considering the tunnel where fish was trained before (right trained fish: $F(2,20) = 0.61$, $p = 0.55$; left trained fish: $F(2,24) = 0.32$, $p = 0.72$; **Figure 1b**). Also, time spent in the correct tunnel did not differ between groups ($F(2,44) = 0.04$, $p = 0.96$; **Figure 1c**) and did not differ considering the tunnel where fish was trained before (right trained fish: $F(2,20) = 0.10$, $p = 0.90$; left trained fish: $F(2,24) = 0.31$, $p = 0.74$; **Figure 1d**).

However, during the test day, in which a shoal was presented at the reward area, animals from HIIT group showed lower latency to enter in shoal area compared to the others groups

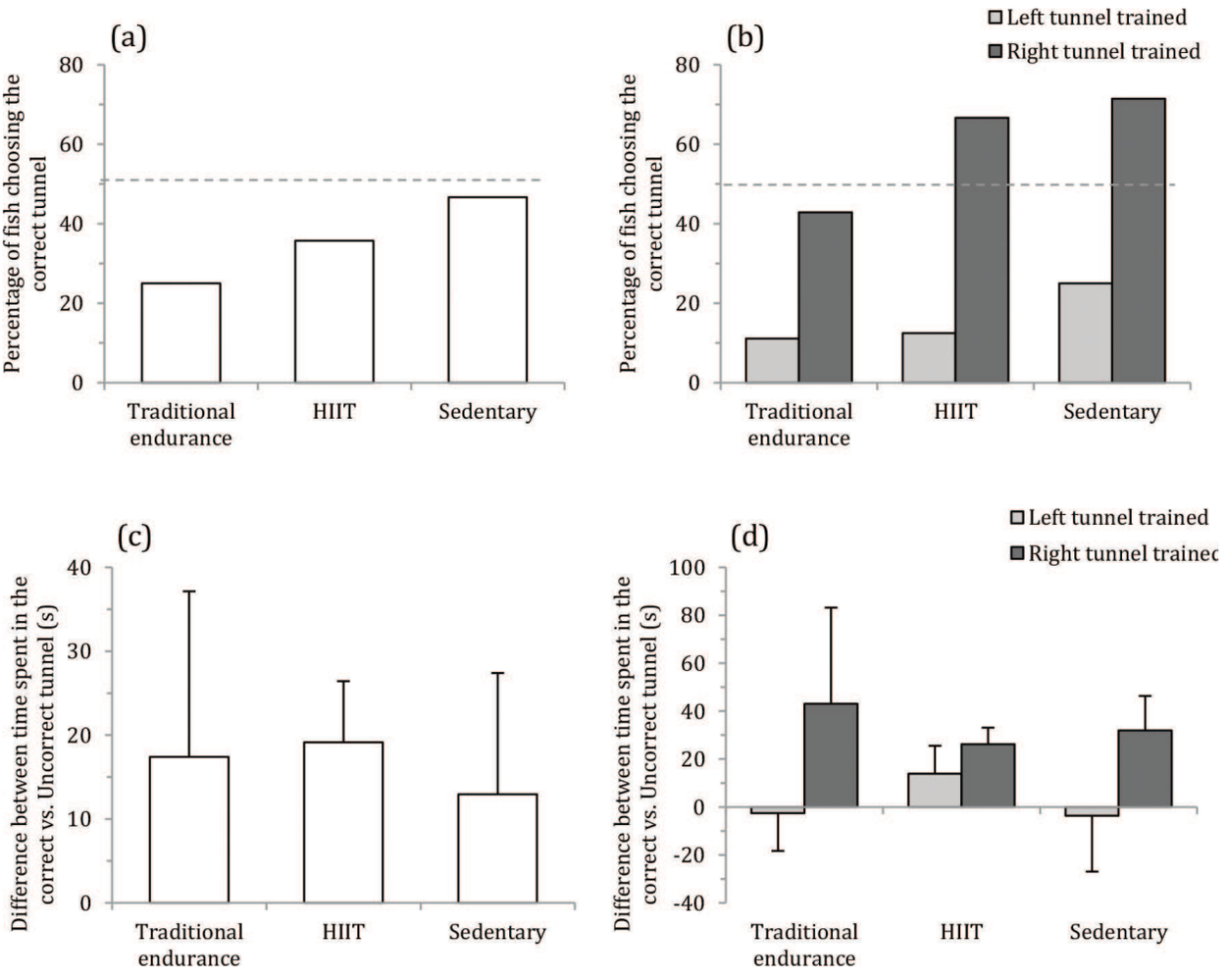


Figure 1. Number of fish that chose the correct tunnel (a, b) and time spent in each tunnel (c, d) after leaving the start box on the probe trial, during which both the right and the left tunnels of the maze were open. The correct choice was the left tunnel for fish that were previously trained with the left tunnel open, and it was the right tunnel for fish that were trained with the right tunnel open. Panel (a) and (c) show the total number of fish that made correct choice and time spent in the correct tunnel irrespective of left or right tunnel training for each treatment. Panel (b) and (d) show the correct choice and time spent in the correct tunnel of each group considering whether the fish were trained with the left or right tunnel: light gray bars represent the fish trained with the left tunnel open and dark bars represent the fish trained with the right tunnel open. Training regimes were: Traditional endurance (n = 16), HIIT (n = 14) and sedentary (n = 15). The physical training conditions are shown on the x-axis: traditional endurance group was trained daily in a current water tube until fish could not swim against it and was pushed to the calm water; high-intensity interval training (HIIT) group was trained to maximum swim against the current three times of 10 min with 5 min interval; sedentary group was put inside the training tube for the same period the other groups trained, but no water current was generated. All panels show that there were no differences between groups. For further details of the results of statistical analysis, see results.

(One-Way ANOVA $F(2,44) = 3.44$, $p = 0.04$), and the same HIIT group spent less time in shoal area (One-Way ANOVA $F(2,44) = 20.70$, $p < 0.001$), as shown in **Figure 2**.

3.2. Objects discrimination

Figure 3 depicts the difference between the time exploring objects A and A′ in the memorization phase and A and B in the discrimination phase for all the training groups. For traditional

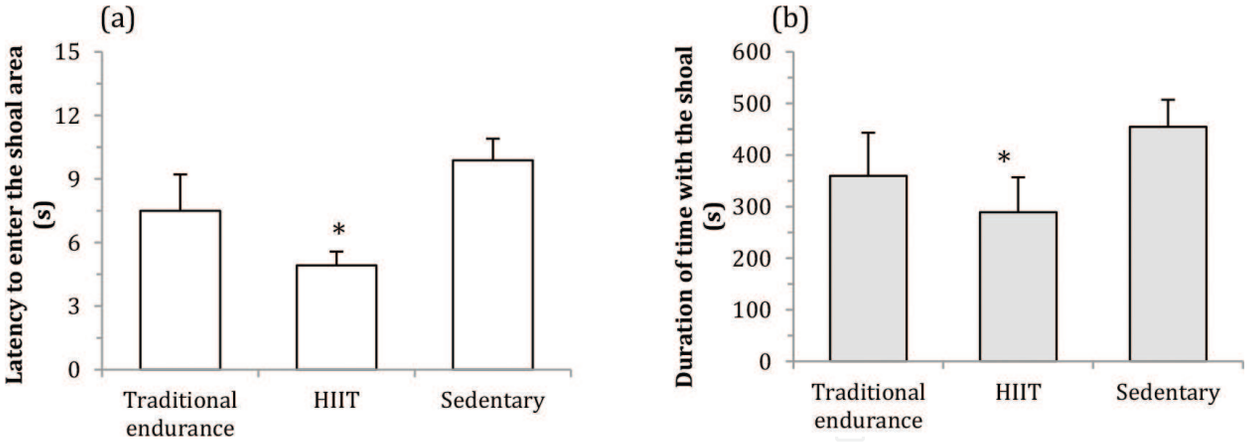


Figure 2. (a) Latency to leave the start box and (b) duration of time zebrafish spent in the goal box during the probe trial. Bars show mean ± SEM; sample size equals 16 for traditional endurance group, 14 for HIIT group, and 15 for sedentary group. The physical training conditions are shown on the x-axis: traditional endurance group was trained daily in a current water tube until fish could not swim against it and was pushed to the calm water; high-intensity interval training (HIIT) group was trained to maximum swim against the current three times of 10 min with 5 min interval; sedentary group was put inside the training tube for the same period the other groups trained, but no water current was generated. During the latent learning training sections, fish were allowed to explore the maze with only one tunnel open. During the probe trial, both tunnels were open and fish were allowed to use the tunnels to reach the goal box, where a conspecific shoal was presented. Asterisk indicates statistical difference between groups (one-way ANOVA, $p < 0.05$).

endurance group, Two-Way ANOVA of this data set revealed a significant effect of the objects ($F(1,56) = 4.02$, $p = 0.04$), no effects of the phase ($F(1,56) = 1.63$, $p = 0.21$), but the objects × phase interaction ($F(1,56) = 9.01$, $p = 0.006$) was also significant. Student Newman Keuls test showed that exploration time of the novel object on the discrimination phase significantly ($p < 0.01$) differed from exploration time of the other objects (**Figure 3a**). For the HIIT group, Two-Way ANOVA showed no significant effect of the objects ($F(1,56) = 0.20$, $p = 0.65$), no effects of the phase ($F(1,56) = 1.85$, $p = 0.18$), and also no effects of interaction ($F(1,56) = 0.50$, $p = 0.48$) (**Figure 3b**). For the sedentary group, Two-Way ANOVA indicates significant effect of the objects ($F(1,56) = 29.93$, $p = 0.001$), no effects of the phase ($F(1,56) = 0.78$, $p = 0.38$), but again significance was observed for the objects × phase interaction ($F(1,56) = 9.78$, $p = 0.003$). Student Newman Keuls test revealed that exploration time of the novel object on the discrimination phase was significant different ($p < 0.01$) from exploration time of the other objects (**Figure 3c**).

Figure 4 shows the correlation between exploration index (E_m; memorization phase) and discrimination index (D_i; discrimination phase) for the training groups. For the traditional endurance group, E_m and D_i were significant (ANOVA, $F(1,14) = 5.67$, $p = 0.03$), showing a directly proportional relationship, as demonstrated by the angular coefficient ($y = 0.66x - 1.90$) and Pearson's correlation coefficient ($r = 0.04$; **Figure 4a**). For HIIT, there was no significant correlation between E_m and D_i (Pearson's correlation coefficient $r = 0.00007$, $p = 0.39$; **Figure 4b**). For the sedentary group, E_m and D_i were significant (ANOVA, $F(1,7) = 3.67$, $p = 0.04$) and showed proportional relationship, as observed from the angular coefficient ($y = 0.42x + 28.81$) and Pearson's correlation coefficient ($r = 0.10$; **Figure 4c**).

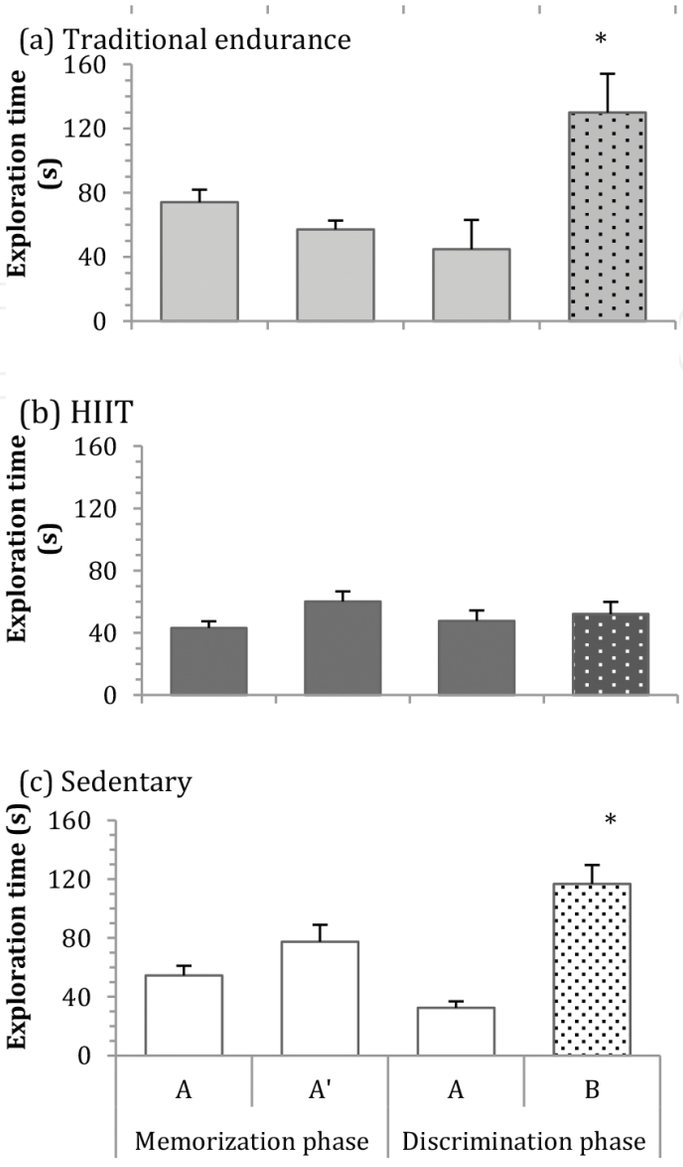


Figure 3. Zebrafish exploration time for objects A vs. A' (memorization phase), or A vs. B (discrimination phase) for the physical training exposure regime groups: (a) traditional endurance, in which fish were trained daily in a current water tube until it could not swim against it and was pushed to the calm water (n = 8), (b) high-intensity interval training (HIIT) group, in which fish were trained to maximum swim against the current 3 times of 10 min with 5 min interval (n = 14), and (c) sedentary group, in which fish were put inside the training tube for the same period the other groups trained, but no water current was generated (n = 15). Bars mean exploration time + SEM in each object, in the memorization phase (two equal objects) and in the discrimination phase (two different objects). Fish were observed for 10 min in each section. Asterisk indicates statistical difference between fish objects exploration (two-way ANOVA, $p < 0.05$).

3.3. Novel tank test

Figure 5 presents difference between the training groups related to average speed, total distance traveled, freezing behavior and distance from the bottom of the tank. One-Way ANOVA showed that training regime did not affect average speed ($F(2,33) = 2.07$, $p = 0.14$; **Figure 5a**), total distance traveled ($F(2,33) = 1.89$, $p = 0.17$; **Figure 5b**), and distance from the bottom of

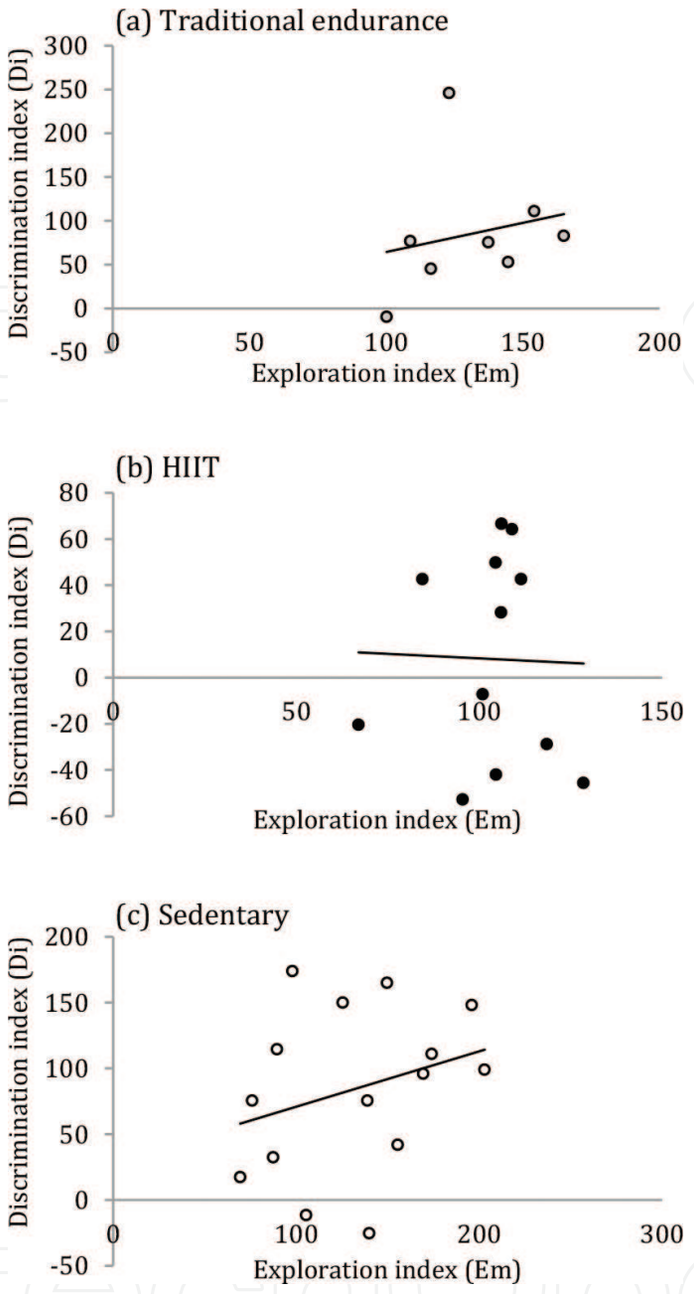


Figure 4. Linear regression between discrimination index (D_i) and exploration index (E_m) in the memorization phase. (a) Traditional endurance, in which fish were trained daily in a current water tube until it could not swim against it and was pushed to the calm water (n = 8), (b) high-intensity interval training (HIIT) group, in which fish were trained to maximum swim against the current three times of 10 min with 5 min interval (n = 14), and (c) sedentary group, in which fish were put inside the training tube for the same period the other groups trained, but no water current was generated (n = 15). For further details of the results of statistical analysis, see results.

the tank ($F(2,33) = 0.08$, $p = 0.92$; **Figure 5d**). However, freezing behavior, a characteristic mainly related to anxiety, was affected by exercising. One-Way ANOVA showed that sedentary group presented higher freezing behavior than the traditional endurance and HIIT groups ($F(2,33) = 9.62$, $p < 0.001$; **Figure 5c**).

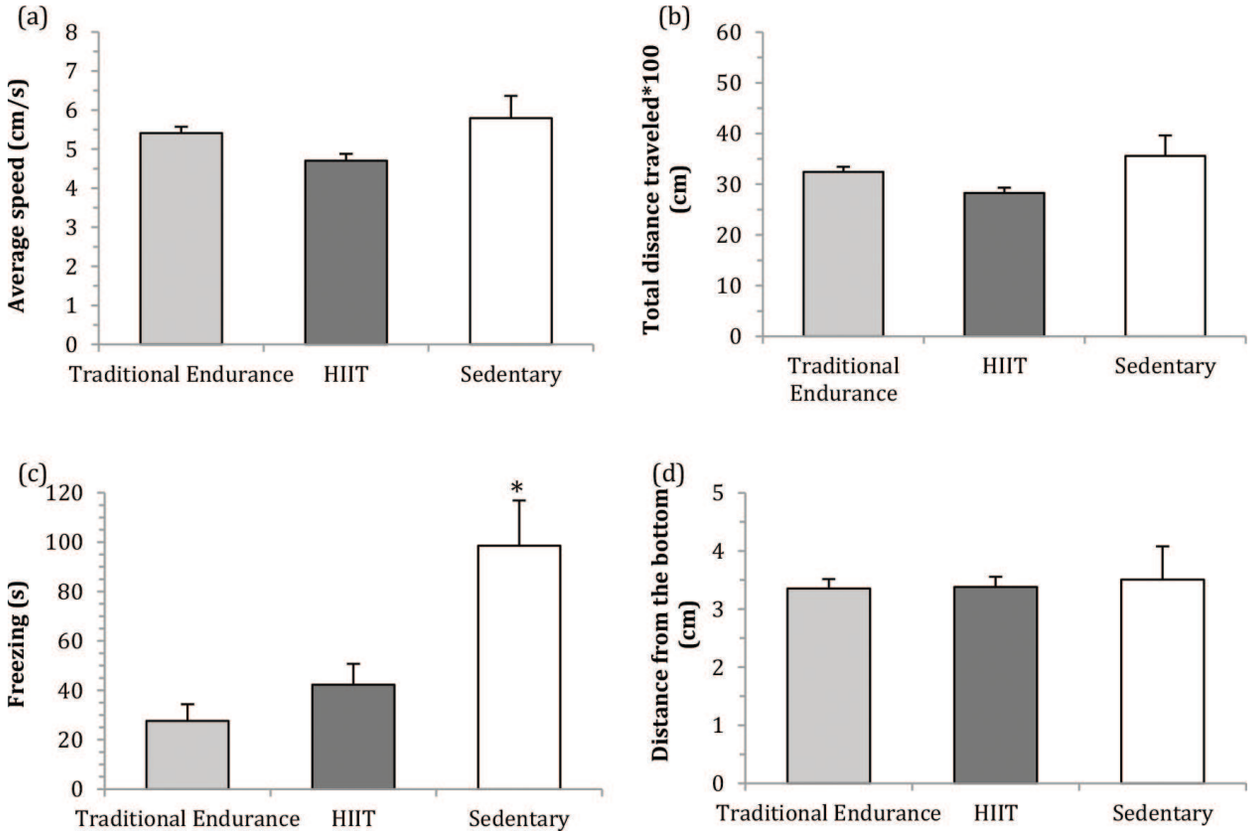


Figure 5. Behavioral analysis in the novel tank test for each treatment: traditional endurance (n = 10), HIIT (n = 11), and sedentary (n = 12). Behavioral tracking applied to compare (a) average speed swimming, (b) total distance traveled by the fish, (c) freezing behavior, and (d) distance from the bottom of the tank between the three groups. Error bars represent standard error. Data corresponds to 15 min of behavioral observation during the test. Asterisk indicates statistically differences (one-way ANOVA, $p < 0.05$).

4. Discussion

In this study, we show the effects of traditional endurance and high-intensity interval training (HIIT) on the learning and anxiety-like behavior in adult zebrafish. Our results evidence that HIIT does not affect long lasting memory, as observed in the latent learning task, but it impairs discriminative learning, as observed in the object discrimination task. We reinforce the zebrafish intrinsic explorative behavior, both directed to novelty and social groups, and showed that physical training affects these responses. However, both HIIT and traditional exercise regime seems to have decreased anxiety-like behavior in zebrafish. This study confirms that zebrafish show good performance in learning tasks and that cognitive performance is dependent upon the regime of physical exercise and cognitive task used.

Our results are in agreement with other studies on learning in zebrafish [33–35, 38, 39]. Latent learning has been tested in zebrafish previously [33, 34] and the results are in line with those reported here. Even the lateralization (right tunnel bias in the first choice) was observed in these other studies using the same protocol we used. Regarding this response (right turn), the

mechanisms that govern this bias are still not understood but a growing body of literature has pointed out lateralization in zebrafish brain [33, 34, 40–42] probably due to asymmetrical biochemical and neurobiological processes. Regarding the objects discrimination using the same protocol we have presented, Oliveira et al. [35], Santos et al. [39], and Pinheiro-da-Silva et al. [38] showed zebrafish ability to recognize the novel object in the discrimination phase of the tests. However, even though the zebrafish is considered a translational model for humans, physical exercise has received little attention, and as far as we know, only two studies approach this issue. One of them relates physical exercise and aging in a protocol that emphasizes swimming performance [31], while the other evaluates the effects of traditional training and associative learning [32]. In the former, the authors have shown that training enhances cognitive performance in zebrafish compared to sedentary fish. However, in the present study, we use both traditional endurance and HIIT to compare how these two different types of training affect cognition. We observed that chronic traditional training (i.e. daily walking, running, or cycling) allowed zebrafish to latent learning and object discrimination, what was not observed in high-intensity training.

Many recent studies have been approaching the high-intensity exercise protocols [2, 8, 28, 43]. It is of particular interest because this mode of training has spread through most of the gyms and young people have been adopting mainly this type of training due to the fast conditioning response it promotes [8]. In the present study, we intent to simulate HIIT protocol by exercising the fish acutely in a high-intensity swimming activity and then letting them rest for a short time before repeating the process. This procedure has negatively affected the fish ability to discriminate objects (**Figures 3** and **4**) but is had no effects on latent learning (**Figure 1**). We also observed that HIIT fish decreased time to reach the shoal in the latent learning test, suggesting it performed the task faster than the other groups. However, HIIT fish showed decreased time with the shoal. Shoaling is a behavioral pattern usually observed in zebrafish due to its high social behavior [44, 45], suggesting the training protocol decreased fish interest in maintaining social interaction. We believe that HIIT may have caused an increased exploratory effect, leading the fish to explore the tank more and shoaling less in the latent learning test. The same effect may explain why fish did not discriminate objects: HIIT may have made the fish to explore the tank of the objects more than focus its attention on the objects itself. These results seem to agree with the decreased anxiety-like behavior (decreased freezing) observed in the novel tank test, which may lead fish to be more explorative than presenting the need for social interaction.

As it is known, many variables may affect exercise training on cognition, such as the cognitive function examined and the level of physical training [2]. Regular physical exercise promotes a number of benefits in the organisms [46, 47] and may have positive effects on cognitive function [32, 48]. In the present study, we confirm that traditional training protocols (continued exercising without high-intensity periods) had beneficial consequences: fish behaves as the control (sedentary) in the latent learning and in the objects discrimination task, but it decreased anxiety-like behavior in the novel tank. Thus, while it seems that traditional endurance cognitive function is comparable to sedentary fish, the anxiety response was significant lower in the fish continuous trained.

Perhaps the most interesting effect, we found in this study was the decreased freezing behavior in the physical training groups. These fish were exposed to training for a prolonged period of time, and tested to the novel tank only at the end of the training period. Different from the cognitive tasks, in which fish were allowed to explore the tank in advance, in the novel tank protocol, fish had only one chance to explore and learn about the tank. Somehow fish used to physical training perceived the novel tank as less threatening, and spent its time exploring the tank instead of showing freezing behavior. In this sense, physical exercise improves one's performance when facing a novel situation, response that is lost when the animal is re-submitted to the tank several times to reduce stress, as we have done on the two cognitive tasks used in the present study.

In fact, our results reinforce the zebrafish as a valuable model organism for throughput screening of behavioral- and cognitive-related training regimes. While physical exercise is highly encouraged and indeed recommended due to several health benefits related to its chronic practice, it seems that the new category of high-intensity training still needs investigations and search for the balance between the benefits of exercising and the damages of acute intense stress. In this sense, our study is far from responding the question: other cognitive protocols should be tested, novel HIIT protocols need to be developed, and several physiological and neural parameters need to be measured, such as O_2 consumption, metabolic rate, catecholamine and corticosteroid release indices, and brain-derived neurotrophic factor levels. Moreover, an alternative form of high-intensity training that also demands attention, coordination, and decision making should also be approached in future studies, such as the game-based activities. Some authors have studied these activities in humans and showed positive cognitive results afterwards [49, 50].

Therefore, while our results are robust in showing there are several effects of the physical training regime on behavior and cognition, we still need to understand how exercising models the body and the brain to cause such effects and what is the extent to which exercising if indeed beneficial. Taken together, these results indicate that high-intensity exercising implies in decreased short-term learning and confirm the zebrafish as a trustful, reliable, and efficient model for basic translational research of the effects of physical activity/training on cognition and behavior.

5. Conclusions

Overall, our results zebrafish is a profitable animal model for basic translational research. Physical exercise in the modern society is medically recommended and rigorously followed in search of increased health and willingness to study/work. However, not all exercising regimes are adequate to anyone and the effects on the body and the brain may exceed the beneficial limit and cause injury, as the decline in short-term learning and spatial navigation observed herein after high-intensity training regime. Therefore, while exercising is important for health, it seems that a trade-off between exercise type and intensity should be taken into account when searching for the enhancement of body and brain. Still, additional studies are needed for a thorough understanding of the effects of physical training and the limits it may impose to our health.

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Conflict of interest

Hereby we confirm the absence of any conflict of interest related to this work.

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