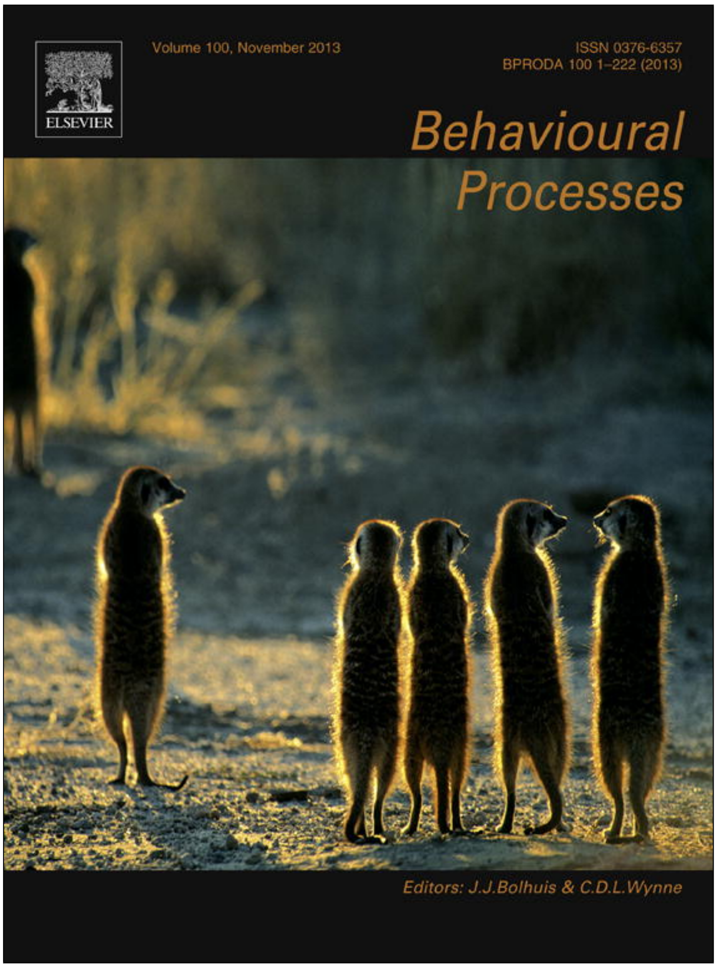
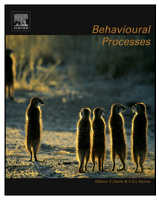

ELSEVIER
Volume 100, November 2013
ISSN 0376-6357
BPRODA 100 1–222 (2013)
Behavioural Processes
Editors: J.J.Bolhuis & C.D.L.Wynne

Behavioural Processes 100 (2013) 44–47

Contents lists available at ScienceDirect

Behavioural Processes

journal homepage: www.elsevier.com/locate/behavproc



Short report

Physical exercise improves learning in zebrafish, Danio rerio

Ana Carolina Luchiari*, Diana Marques Martins Chacon

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

A R T I C L E   I N F O

Article history:
Received 1 February 2013
Received in revised form 18 July 2013
Accepted 19 July 2013

Keywords:
Activity
Behavior
Conditioning
Fish

A B S T R A C T

Zebrafish is an ideal vertebrate model for neuroscience studies focusing on learning and memory. Although genetic manipulation of zebrafish is available, behavioral protocols are often lacking. In this study we tested whether physical activity can facilitate zebrafish's learning process in an associative conditioning task. Learning was inferred by the approach of the feeding area just after the conditioned stimulus (light). Unexercised zebrafish showed conditioning response from the 5th testing day while fish previously submitted to swim against the water current showed learning by the 3rd day of testing. It seems that physical activity may accelerate associative learning response in zebrafish, indicating the benefits of exercise for cognitive processes. We suggest that this preliminary work could be useful for high throughput screening.

1. Introduction

Learning refers to acquiring new information from experiences, made viable by the nervous system plasticity (Kolb and Whishaw, 1998). It is widely observed in animals; both intrinsic (age, gender, nutritional state) and extrinsic factors (temperature, light) may affect it (Coble et al., 1985). The most important neural area involved in learning is the hippocampus, whose vulnerability to degenerative processes leads to neurological diseases such as Alzheimer's (Flood and Coleman, 1990; Bird et al., 2010; Venkateshappa et al., 2012).

Many neuroscience and psychobiology studies (primarily on mammals) focus on behavioral features related to learning and memory. However, research using phylogenically distant animals may shed a light on the mechanism of brain route formation for learning and memory. Due to their strong genetics, their ability to perform cognitive tasks and their simpler neural pathways and brain structures zebrafish represent an adequate model for learning studies (Gerlai et al., 2000; Gerlai, 2002; Pather and Gerlai, 2009; Souza and Tropepe, 2011; Karnik and Gerlai, 2012).

In this study, we aimed to stimulate associative learning in the zebrafish through increased physical activity. Moderate exercise seems to decrease stroke risk (Fujimoto et al., 2012), increase prolactin and endorphin release (Paez et al., 2007), decrease distress (George et al., 2012) and promote hippocampus neurogenesis (Mustroph et al., 2012). Since neurophysiologic systems in zebrafish share many organizational and functional characteristics with other vertebrates, including mammals, this study has potential translational relevance, even to humans. Understanding associative learning may, therefore ultimately help prevent and reverse clinical effects of neurodegeneration and open possibilities to deeply understanding behavioral processes in the functions of the brain. The simplicity of the proposed associative task allowed us to first, quantify the reaction of the experimental zebrafish and second to develop a simple conditioning learning paradigm.

2. Materials and methods

A group of zebrafish (*Danio rerio*) from a local pet store (Natal, Brazil) were maintained in glass aquarium with aerated and filtered water at 28 ± 1 °C, and on a 12/12-light/dark cycle for 1 month prior to the experiments. Fish were fed twice a day ad libitum with commercial food (38% protein, 4% lipid, Nutricom Pet). All animal procedures were performed with the permission of the Ethical Committee for Animal Use of the Universidade Federal do Rio Grande do Norte (CEUA 014/2011).

The experimental strategy was the evaluation of the behavioral effects of physical activity on an associative learning task. Twelve zebrafish were randomly chosen from the stock aquarium to exercise for 20 consecutive days. Each group of 4 fish was submitted to an intense water current, so that the fish would swim against the water flow. The water current was generated by a submersible pump connected to a transparent plastic tube, inside an aquarium (40 cm × 25 cm × 20 cm, 15 L; Fig. 1a). Each fish group was kept in the water current until they were exhausted, which was when the fish were pushed by the water through the tube to the aquarium. The mean duration time inside the tube progressively increased as the days went on. For instance, mean duration time was 5.4 ± 0.6 min on day 1 and reached 18.9 ± 1.0 min by day 10. Due to the increased swimming capacity in the course of the days,

* Corresponding author. Tel.: +55 84 32153409; fax: +55 84 32119206.
E-mail address: analuchiari@yahoo.com.br (A.C. Luchiari).

0376-6357/$ – see front matter
http://dx.doi.org/10.1016/j.beproc.2013.07.020

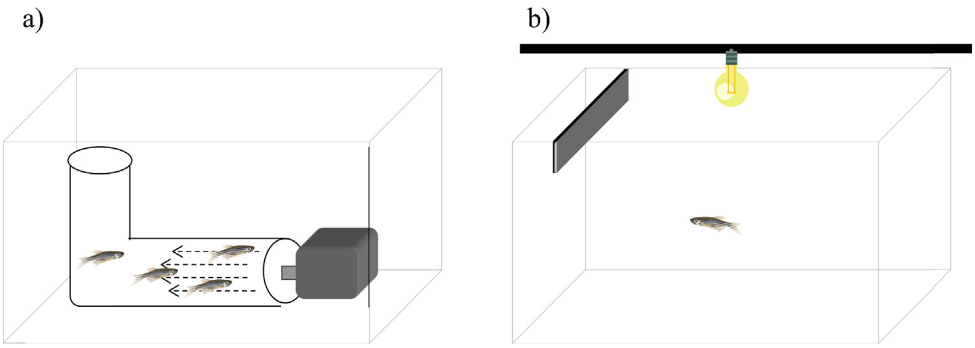


Fig. 1. Schematic draw of (a) physical activity aquarium, containing a submersible pump connected to a transparent tube where groups of 4 fish were placed to swim against the water current until exhaustion, when the water pushed them to the aquarium area, and (b) conditioning task aquarium, with the feeding area at the upper left region and the lamp (unconditioned stimulus) above.

different submersible water pumps were used. For the first 10 days a 280 L/h pump (Moto Bomba SARLO S300, 220V, 60 Hz. Sarlobetter Equipamentos Ltda., São Caetano do Sul-SP) was used, and for the last 10 days it was changed to a 520 L/h pump (Moto Bomba SARLO S520). The mean duration time inside the swimming tube was 7.4 ± 0.6 min on day 10 and increased to 36.5 ± 1.9 min by day 20.

The sessions were conducted once a day for 20 days. All fish performed the same amount of exercise. At the end of these sessions, 8 zebrafish were randomly chosen for the conditioning test. Fish for the control group were kept in one glass aquarium (40 cm × 25 cm × 20 cm, 15 L) without any manipulation for 20 days and received food twice a day ad libitum.

For the conditioning tests, the exercised (3.53 ± 0.05 cm and 0.55 ± 0.08 g) and naïve zebrafish (3.48 ± 0.03 cm, 0.52 ± 0.05 g) were chemically, acoustically and visually isolated from each other in glass aquarium (40 cm × 25 cm × 20 cm, 15 L), where there was a small opaque divide at the upper left region delimitating the feeding area (25 cm × 2 cm) and a lamp on the central area of the aquarium (Fig. 1b). Illumination (910 ± 2 lux) and feeding were used as conditioned and unconditioned stimuli, respectively. Temperature and photoperiod were set the same as in holding conditions; ambient light was 47.5 ± 0.1 lux.

All aquaria were placed behind an opaque curtain so that the researcher could handle the light (on and off) and feed them through small holes in the curtain. It prevented fish from associating the researcher with unconditioned stimuli. Food was offered once a day using a manual feeder (a small recipient connected to a long tube), always delivered at the feeding area just after the light stimulus.

Fish behavior was recorded daily using a digital video camera during a 2 min and 10 s period between 11 and 12:00 h for 8 consecutive days. The records included 1 min before conditioned stimulus and 1 min after the end of the stimulus. Light (conditioned stimulus) was offered for 10 s, followed by food delivery.

The fish dispersion in the aquarium and distance from the feeding area was analyzed. The dispersion area occupied during 1 min before and 1 min after light stimulus was used to estimate the fish activity. The fish distance from the feeding area was registered every 10 s and then the mean distance was calculated. The difference between the distances of feeding area after and before the conditioned stimulus expressed the approach index [AI = (distance from the feeding area after light stimulus) − (distance from the feeding area before light stimulus)]. To validate the index used, we compared the position of the fish prior to the light stimulus on each condition. Learning was inferred from this index: the more negative the values, the more the fish approached the feeding area. The mean approach index from the 8 recorded days of conditioning was grouped into a 2-day period for analysis.

The Friedman test was used to compare the position of the fish before the light stimulus, aiming to validate the approaching index. Repeated-measures ANOVA were used to analyze dispersion and approach index of the exercised and control groups. The post hoc test used was Student Newman Keuls. Independent Student's *t*-test was used to compare the index between exercised and control groups in the same session. A probability level of $p < 0.05$ was used as an index of statistical significance.

The generalized linear analysis (GLM) was conducted to compare the performance of the control and exercised groups along the 8 experimental days. The GLM was used to adjust the response variable (*Y*-approach index) to the explicative variable (*X*-days). The probability distribution model used $f(Y)$ was normal and the link function used was identity ($\delta(\mu) = \mu$). The proposed model showed the following structure: approach index $\sim\beta$ + days, where β is the intercept. Analysis of covariance (ANCOVA) was performed to investigate differences between the control and exercise models (slope parameter).

3. Results

The position of the fish prior to the light stimulus did not differ between control and exercised groups (Friedman: $H = 20.09$, $p = 0.13$). The mean distance of the control group was 5.25 ± 1.78 cm and the exercise group was 5.80 ± 2.38 cm.

Fish dispersion decreased after the light signal over the days; both the control group (unexercised) and the exercised group decreased dispersion immediately after the light stimulus in the second block of days (RM ANOVA control: $F = 3.75$ $p = 0.01$; exercise: $F = 3.11$ $p = 0.03$). There were no significant differences in dispersion between the control group and the exercise group in any block of days (Student's *t*-test, 1st block: $t = 0.09$ $p = 0.92$; 2nd block: $t = -1.21$ $p = 0.23$; 3rd block: $t = 0.30$ $p = 0.76$; 4th block: $t = 0.55$ $p = 0.58$).

The control group showed significant approaching of the feeding area from the third block of days (days 5 and 6) onward and maintained this until the end of the test (RM ANOVA $F = 3.82$ $p = 0.014$). However, the exercised group approached the feeding area after the light signal already on the second block of days (days 3 and 4), and the approaching response increased on the last block of days (7 and 8) (RM ANOVA $F = 4.64$ $p = 0.007$, Fig. 2). The comparison between the two groups indicates significant difference in the approachment of food placement on the second block of days (Student's *t* test $t = 3.09$ $p = 0.004$) and on the fourth block of days (Student's *t* test $t = 3.28$ $p = 0.002$). On the first 2 days there was no difference between the groups (Student's *t* test $t = 0.35$ $p = 0.72$), nor on the third block (Student's *t* test $t = -0.01$ $p = 0.98$, Fig. 2).

The angular coefficient (control: −0.110 and exercise: −0.34002) analysis from the generalized linear model (GLM)

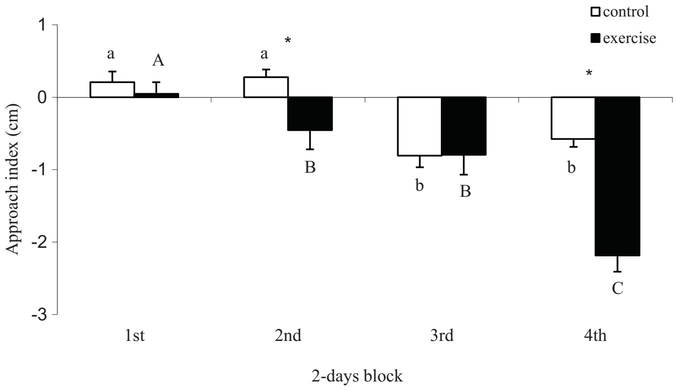


Fig. 2. Approach index of the feeding area (mean ± SEM) of the zebrafish during 8 days of the conditioning test using food as reinforcement. The approach index was calculated by the distance of the feeding area on the 2nd min minus the distance on the 1st min. The more negative the values, the shorter the distance from the feeding area after the light signal. Days 1 and 2 composed the 1st block, days 3 and 4 composed the 2nd block, days 5 and 6 composed the 3rd block and days 7 and 8 composed the 4th block of days. Control group is represented by white bars ($n = 9$). Exercised group (fish that experienced forced swimming for 20 days before the test) is represented by black bars ($n = 8$). Data from 8 days of test was grouped in blocks of 2 days for analysis. Different lowercase letters indicate statistical difference among white bars (control group: RM ANOVA, $p < 0.05$). Different capital letters indicate statistical difference among black bars (exercise group: RM ANOVA, $p < 0.05$). *Indicates difference between control group and exercise group on the same block of days (2nd and 4th blocks: Student's t, $p < 0.05$).

indicated that the exercise group approached the feeding area faster than the control group throughout the experimental days. The linear equation was $y = -0.110x + 0.4635$ for the control group and $y = -0.34002x + 0.68332$ for the exercised group. All data used passed on the normality (control: 0.857 and exercise: 0.138), constant variance (control: 0.262 and exercise: 0.443) and power of performed test (alpha 0.05; control: 0.893 and exercise: 0.231). The ANCOVA showed that control group slope is statistically different from the exercise group slope ($F = 5.457$ $p = 0.021$, Fig. 3).

4. Discussion

We found that fish undergoing physical exercise treatment improve the ability to associate stimulus in the conditioning test. Although significant approaching of the feeding site over time indicates that both the control (days 5–6) and the exercised (days 3–4) groups learn to associate the light stimulus (conditioned) to food (unconditioned stimulus), exercised fish respond faster than controlled fish (Figs. 2 and 3). Our data suggests that physical exercise may have accelerated the process of association and learning. This is the first time exercise is tested for fish in a simple behavioral protocol.

It is possible that these results are due to differences in motor performance, leading to immobility or increased activity levels, which could induce differential learning performance. However, our observation of the dispersion revealed no modifications in motor patterns due to previous exercises. Both groups appear to move normally. Also, the analysis of the distance of the feeding area before the light stimulus did not confirm differences between the groups. The control group showed learning by days 5–6 (Fig. 2). These results reinforce the idea that zebrafish are able to achieve good performance in associative learning tasks, corroborating studies of Bilotta et al. (2005), Pather and Gerlai (2009) and Sison and Gerlai (2010).

Growing number of studies in humans and animals have suggested that exercise may influence brain functions, including cognition, learning and memory. In humans, robust effects of exercise have been linked, especially in the elderly population, to the improvement of memory and decrease of mental illness risk such as senile dementia and Alzheimer's disease (Colcombe and Kramer, 2003; Weuve et al., 2004; Heyn et al., 2004). In agreement with these studies, researches using rodents indicate that physical activity facilitates memory retention by the activation of hippocampal areas in tests utilizing a water maze (van Praag et al., 2005), a radial maze (Schweitzer et al., 2006), aversive tests (Radak et al., 2006) and object recognition (O'Callaghan et al., 2007). In recent years, knowledge about the biological mechanisms that underlie exercise benefits has increased, but what molecular and cellular pathways are involved in the cognition improvement need to be confirmed.

In teleost fish, intense neurogenesis continuously occurs during adult life and several brain cells increase with age and body size (Zupanc and Horschke, 1995; Zupanc, 2006). Studies approaching cellular proliferation in the lateral pallium of the fish brain - considered the evolutionary precursor of the mammalian hippocampus (Friedrich et al., 2010)- linked this phenomenon to environmental

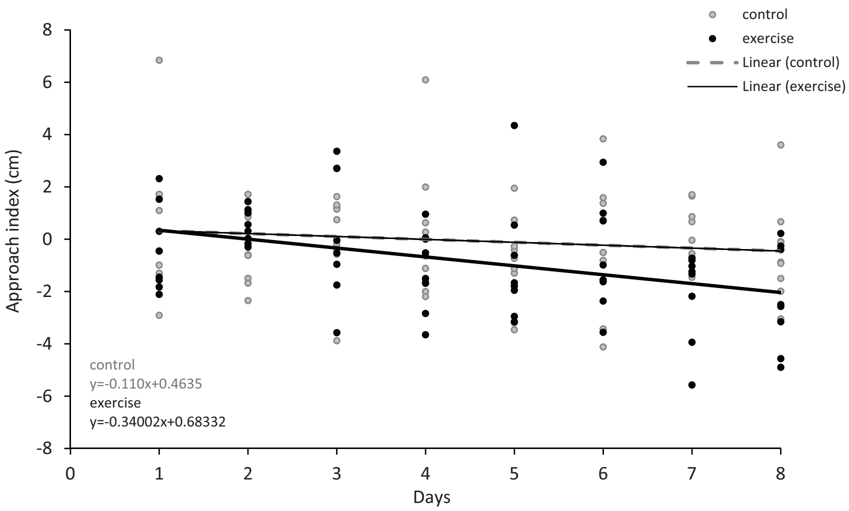


Fig. 3. Linear regression of the zebrafish approach of the feeding area during 8 days of the conditioning test using food as reinforcement. The approach index was calculated by the distance of the feeding area on the 2nd min minus the distance on the 1st min. The more negative the values, the shorter the distance from the feeding area after the light signal. Control group is represented by gray dots ($n = 9$) and exercised group is represented by black dots ($n = 8$). Dashed gray line is the linear regression for the control group, with angular coefficient of −0.110 and solid black line is the linear regression for the exercise group, with angular coefficient of −0.34002. The comparison between groups by ANCOVA test showed statistical difference with $p = 0.02$.

challenges, such as enrichment (von Krogh et al., 2010) and stressors (Gould et al., 1997; Mirescu and Gould, 2006). Physical exercise challenges the body which then signals to the brain centers that may have affected the learning process. This idea corroborates our data of better learning performance in exercised zebrafish. However, the systemic and brain alterations due to exercise as well as its effects on memory retention require investigation and must be addressed in future studies of our group.

Our findings establish zebrafish as a model to study how physical may lead to improved brain processes. The performance parameter measured (distance from an area) is easy to quantify, what suggests this paradigm for high throughput screening, as addiction drugs (Klee et al., 2012) or appetite regulating drugs (Shimada et al., 2012). Although some questions will have to be explored, our results demonstrate significant learning acquisition after the exercise of the zebrafish. It is a key area for future research on neural plasticity and cognitive ability mainly relevant to the neural processes of evolution understanding.

5. Conclusion

Zebrafish is gaining popularity in behavioral brain research but high throughput paradigms are lacking. Our study used a novel paradigm and showed that physical exercise accelerates the zebrafish process of learning in an associative task. The simplicity of the model will make it useful for high throughput screening.

Acknowledgements

The authors thank very much Mrs. Ludmila Schenflede and Mr. Michael Copeland for English review and Ms. Marilia Teixeira for technical assistance.

References

Bilotta, J., Risner, M.L., Davis, E.C., Haggbloom, S.J., 2005. Assessing appetitive choice discrimination learning in zebrafish. Zebrafish 2, 259–268.
Bird, C.M., Chan, D., Hartley, T., Pijnenburg, Y.A., Rossor, M.N., Burgess, N., 2010. Topographical short-term memory differentiates Alzheimer's disease from frontotemporal lobar degeneration. Hippocampus 20, 1154–1169.
Coble, D.W., Farabee, G.B., Anderson, R.O., 1985. Comparative learning-ability of selected fishes. Canadian Journal of Fisheries and Aquatic Sciences 42, 791–796.
Colcombe, S., Kramer, A.F., 2003. Fitness effects on the cognitive function of older adults: a meta-analytic study. Psychological Science 14, 125–130.
Flood, D.G., Coleman, P.D., 1990. Hippocampal plasticity in normal aging and decreased plasticity in Alzheimer's disease. Progress in Brain Research 83, 435–443.
Friedrich, R.W., Jacobson, G.A., Zhju, P., 2010. Circuit neuroscience in zebrafish. Current Biology 20, R371–R381.
Fujimoto, N., Prasad, A., Hastings, J.L., Bhella, P.S., Shibata, S., Palmer, D., Levine, B.D., 2012. Cardiovascular effects of 1 year of progressive endurance exercise training in patients with heart failure with preserved ejection fraction. American Heart Journal 164, 869–877.
George, E.S., Jorm, L., Kolt, G.S., Bambrick, H., Lujic, S., 2012. Physical activity and psychological distress in older men: findings from the New South Wales 45 and up study. Journal of Aging and Physical Activity 20, 300–316.
Gerlai, R., 2002. Phenomics: fiction or the future? Trends in Neurosciences 25, 506–509.
Gerlai, R., Lahav, S., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacology Biochemistry and Behavior 67, 773–782.
Gould, E., McEwen, B.S., Tanapat, P., Galea, L.A.M., Fuchs, E., 1997. Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. Journal of Neuroscience 17, 2492–2498.
Heyn, P., Abreu, B.C., Ottenbacher, K.J., 2004. The effects of exercise training on elderly persons with cognitive impairment and dementia: a meta-analysis. Archives of Physical Medicine and Rehabilitation 85, 1694–1704.
Karnik, I., Gerlai, R., 2012. Can zebrafish learn spatial tasks? An empirical analysis of place and single CS–US associative learning. Behavioural Brain Research 233, 415–421.
Klee, E.W., Schneider, H., Clark, K.J., Cousin, M.A., Ebbert, J.O., Hooten, W.M., Karpyak, V.M., Warner, D.O., Ekker, S.C., 2012. Zebrafish: a model for the study of addiction genetics. Human Genetics 131, SI977–SI1008.
Kolb, B., Whishaw, I.Q., 1998. Brain plasticity and behavior. Annual Review of Psychology 49, 43–64.
Mirescu, C., Gould, E., 2006. Stress and adult neurogenesis. Hippocampus 16, 233–238.
Mustroph, M.L., Chen, S., Desai, S.C., Cay, E.B., Deyoung, E.K., Rhodes, J.S., 2012. Aerobic exercise is the critical variable in an enriched environment that increases hippocampal neurogenesis and water maze learning in male c57bl/6j mice. Neuroscience 219, 62–71.
O'Callaghan, R.M., Ohle, R., Kelly, A.M., 2007. The effects of forced exercise on hippocampal plasticity in the rat: a comparison of LTP spatial- and non-spatial learning. Behavioural Brain Research 176, 362–366.
Paez, L.C., Luque, G.T., Gutierrez, C.V., Oltras, C.M., 2007. Prolactin responses to stress induced by a competitive swimming effort. Biology of Sports 24, 311–323.
Pather, S., Gerlai, R., 2009. Shuttle box learning in zebrafish. Behavioural Brain Research 196, 323–327.
Radak, Z., Toldy, A., Szabo, Z., Siamilis, S., Nyakas, C., Silye, G., Jakus, J., Goto, S., 2006. The effects of training and detraining on memory neurotrophins and oxidative stress markers in rat brain. Neurochemistry International 49, 387–392.
Schweitzer, N.B., Alessio, H.M., Berry, S.D., Roeske, K., Hagerman, A., 2006. Exercise-induced changes in cardiac gene expression and its relation to spatial maze performance. Neurochemistry International 48, 9–16.
Shimada, Y., Hirano, M., Nishimura, Y., Tanaka, T., 2012. A high-throughput fluorescence-based assay system for appetite-regulating gene and drug screening. PLoS ONE 7 (12), e52549.
Sison, M., Gerlai, R., 2010. Associative learning in zebrafish (*Danio rerio*) in the plus maze. Behavioural Brain Research 207, 99–104.
Souza, B.R., Tropepe, V., 2011. The role of dopaminergic signalling during larval zebrafish brain development: a tool for investigating the developmental basis of neuropsychiatric disorders. Review of Neuroscience 22, 107–119.
van Praag, H., Christie, B.R., Sejnowski, T.J., Gage, F.H., 2005. Exercise enhances learning and hippocampal neurogenesis in aged mice. Journal of Neuroscience 25, 8680–8685.
Venkateshappa, C., Harish, G., Mahadevan, A., Bharath, M.M.S., Shankar, S.K., 2012. Elevated oxidative stress and decreased antioxidant function in the human hippocampus and frontal cortex with increasing age: implications for neurodegeneration in Alzheimer's disease. Neurochemical Research 37, 1601–1614.
von Krogh, K., Sorensen, C., Nilsson, G.E., Overli, O., 2010. Forebrain cell proliferation, behavior, and physiology of zebrafish Danio rerio, kept in enriched or barren environments. Physiology & Behavior 101, 32–39.
Weuve, J., Kang, J.H., Manson, J.E., Breteler, M.M.B., Ware, J.H., Grodstein, F., 2004. Physical activity including walking, and cognitive function in older women. JAMA – Journal of the American Medical Informatics Association 292, 1454–1461.
Zupanc, G.K.H., 2006. Neurogenesis and neuronal regeneration in the adult fish brain. Journal of Comparative Physiology 192, 649–670.
Zupanc, G.K.H., Horschke, I., 1995. Proliferation zones in the brain of adult gymnotiformfish—a quantitative mapping study. Journal of Comparative Neurology 353, 213–233.