Behavioural Brain Research 336 (2018) 85–92

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Sex and the housing: Effects on behavior, cortisol levels and weight in zebrafish

Gustavo Kellermann Reolon[a,*], Gabriela Madalena de Melo[a], João Gabriel dos Santos da Rosa[b], Leonardo José Gil Barcellos[b,c,d], Carla Denise Bonan[a]

[a] Pontifícia Universidade Católica do Rio Grande do Sul, Faculdade de Biociências, Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia, Porto Alegre, RS, Brazil
[b] Universidade Federal de Santa Maria, Programa de Pós-Graduação em Farmacologia, Santa Maria, RS, Brazil
[c] Universidade de Passo Fundo, Curso de Medicina Veterinária, Passo Fundo, RS, Brazil
[d] Universidade de Passo Fundo, Programa de Pós-Graduação em Bioexperimentação, Passo Fundo, RS, Brazil

ARTICLE INFO

Keywords:
Sex
Housing
Zebrafish
Behavior
Cortisol
Aversive memory

ABSTRACT

Studies with zebrafish use acclimatizing periods of at least one week immediately before the experiments. During this time, animals can be housed in sexually segregated conditions (only females or males in the tank) or in mixed-sex conditions (both sexes in the tank). The influence of sex and housing conditions regarding the presence of one or two sexes is largely unknown in zebrafish. Our aim was to evaluate the influence that sex and housing regarding the sex of animals had in the open tank task, in the inhibitory avoidance memory test, in cortisol levels and weight in zebrafish. Four groups of animals were used: 1) segregated housed females (only females were kept in the tank); 2) segregated housed males (only males were kept in the tank); 3) mixed-sex housed females (only females were analyzed from a tank containing 50% ratio of each sex); 4) mixed-sex housed males (only males were analyzed from a tank containing 50% ratio of each sex). Males showed higher total distance travelled and mean speed when compared to females. In the inhibitory avoidance memory, sexually segregated animals had higher latencies than their mixed-sex counterparts in the 1 day test and sexually segregated females presented a memory that persisted longer and was able to be reinstated. Whole-body cortisol levels were higher in mixed-sex animals while weight was lower in these fish. To the best of our knowledge, this is the first time that effects of sex and housing regarding sex were investigated in behavior and physiology of zebrafish.

1. Introduction

Zebrafish advantages as a model organism have promoted its use in several research fields. Neurochemistry, behavior and memory, development, toxicology and genetics are among some of them [1,2]. As a relatively new model, the rise in methodologies developed for or adapted to zebrafish can boost even further its use.

Zebrafish is a shoaling species with complex social behaviors. In nature, the shoals appear to have 50% proportion for each sex [3], with animals most likely spending their lives exposed to both sexes. Evidence indicate that exposure or deprivation of contact with the opposite sex could affect behavior and physiology in zebrafish. Female sexual pheromone can induce male courtship behavior [4] whereas prolonged deprivation from male contact can impact females ability to lay eggs [3].

A common practice for experiments with adult zebrafish is to move animals from a growing facility to house them in an experimental facility at least one week before beginning of experiments (acclimation period). In this process, fish can be sorted by sex and housed in sexually segregated housing conditions (only males or females in the tank) or not sorted and housed in mixed-sex housing conditions (both sexes in the same tank). Sorting is usually done to avoid sex as a possible experimental confounder as sex is vastly known to influence from brain neurochemistry to cognition [5,6]. In zebrafish, studies that evaluated sex differences observed some conflicting results. Lower cortisol levels, higher boldness [7] and higher locomotion [8] were found in females. However, locomotion was also found to be higher in males [9] and cortisol was unaffected by sex [8].

Despite the literature evaluating sexual distinctions in zebrafish, the influence of sex *per se* needs to be further investigated. Moreover, if the

* Corresponding author at: Pontifícia Universidade Católica do Rio Grande do Sul, Faculdade de Biociências, Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia, Porto Alegre, RS, Brazil.
E-mail address: gkreolon@gmail.com (G.K. Reolon).

http://dx.doi.org/10.1016/j.bbr.2017.08.006
Received 18 April 2017; Received in revised form 24 July 2017; Accepted 5 August 2017
Available online 16 August 2017

housing conditions regarding the sexes can indeed affect animals, it is an experimental confounder mostly ignored. Several researchers do not mention these housing conditions in the methodological description, even those whose focus is to describe sex differences. Understanding the effects that sex and housing conditions may have in routinely evaluated outcomes for zebrafish, such as behavior and cortisol levels, has the potential to increase experimental sensitivity and reproducibility as well as decrease the necessary sample size.

Taking into consideration that the housing conditions regarding the presence or absence of the opposite sex might influence the behavior and physiology of zebrafish and sexual segregation is frequent for at least one week immediately before experiments, our aim is to evaluate the influence that sex and housing regarding the sex of animals have in the open tank task, in the inhibitory avoidance (IA) memory test, in cortisol levels and weight in zebrafish. To the best of our knowledge, this is the first time that housing conditions regarding the sex is used to evaluate these behaviors and cortisol measurement in this teleost.

2. Materials and methods

2.1. Animals and growing conditions

Adult male and female wild-type zebrafish (6–12 months old) were obtained from our breeding facility. Animals were grown in 50 L tanks filled with water previously treated with AquaSafe® (Tetra, VA, USA) and kept under filtration at a temperature of 26 ± 2 °C and pH at 7.0–8.0 with a maximum density of 5 animals per liter with females/males ratio of ~50%. Fish were subjected to a day/night cycle of 14:10 h (lights on at 7:00 am) and were fed thrice per day with commercial flake food and supplemented once a day with live brine shrimp. All animals used in this study were experimentally naive, healthy and free of any signs of disease. All protocols were approved by the Animal Care Committee from Pontifícia Universidade Católica do Rio Grande do Sul (14/00412-CEUA-PUCRS).

2.2. Experimental groups and housing

Animals were first sorted by sex and weight in the growing facility and then transferred to new tanks. In each experiment, animals of all groups had the same age and sorting was done to avoid weight differences between groups. Four groups of animals were used: 1) segregated housed females (only females were kept in the tank); 2) segregated housed males (only males were kept in the tank); 3) mixed-sex housed females (only females were analyzed from a tank containing 50% ratio of each sex); 4) mixed-sex housed males (only males were analyzed from a tank containing 50% ratio of each sex). The 50% ratio for each sex in the mixed groups was chosen as it is the observed proportion found in nature [3] and also used by several researchers.

During the experiments, tank water, pH, temperature, filtration, light cycle and feeding was the same as in the growing facility. Animals were kept at a density of 1.6 fish per liter and this was maintained the same among the groups. To maximize isolation of sexes, animals were deprived from visual stimulus of other animals, besides those in the home tank, by surrounding each tank with a plastic board at a minimum distance of 5 cm from the tank outer wall.

2.3. Experimental conditions

The four groups were maintained as described above for one week immediately before the experiments. This interval was chosen as it usually is the minimum period found in the literature for acclimatizing zebrafish. Each animal was submitted to either the open tank test followed by weight measurement, inhibitory avoidance task or whole-body cortisol levels measurements. Therefore, different animals were used in each evaluation.

Behavioral tests occurred between 10:00 am and 4:30 pm with environmental distractions kept to a minimum. Fish euthanasia for cortisol measurement was done between 12:00 pm and 1:00 pm. Water from behavioral apparatuses were adjusted to home tank conditions. To prevent that pheromones or other compounds in the water from one group of animals affected the behavior of another group, between assessments of animals from different home tanks, the open tanks and the inhibitory avoidance apparatus along with its electrodes were thoroughly cleaned and water was changed. Moreover, any material in contact with water or the animals of one tank, e.g. a net, was cleaned before being used with animals from another tank.

2.4. Open tank

Open tank was performed similar to previously described [10,11]. The apparatus consisted of a rectangular glass tank of 23.5 cm by 7.5 cm by 20 cm (WxDxH) filled up to 16 cm with water. A Logitech® c525 HD webcam (Logitech®, CA, USA) was placed in front of the open tanks and recorded the experiment. To enhance the contrast between fish and background, the back and lateral outer walls of the tank contained an opaque plastic self-adhesive film, resulting in a homogeneous white background.

Animals were carefully removed from their respective housing tank and placed in the open tank. Immediately after that, recording was started and lasted 15 min. Each test recorded four subjects individually placed in four open tanks, the apparatuses were visually isolated from each other. After the test, animals were weighed in a BG 400 scale (Gehaka, SP, BR). ANY-Maze® software (Stoelting Co, IL, USA) was used to analyze behavior. The open tanks were virtually divided in the ANY-Maze® software into 3 equally sized horizontal zones (bottom, middle, and top) and 4 equally sized vertical zones (column A, B, C and D, from left to right). Line crossings were evaluated counting the crossings from both vertical and horizontal zones. The number of animals used for the open tank test and weight measurement were: 19 for segregated housed females, 23 for segregated housed males, 22 for mixed-sex housed females and 22 for mixed-sex housed males.

2.5. Inhibitory avoidance

Inhibitory avoidance (IA) was performed similar to Blank *et al.* [12]. The apparatus consisted of 18 cm by 9 cm and 7 cm (length x width x height) glass tank separated by a sliding guillotine-type partition in two equally sized compartments. Compartments were externally covered in opaque plastic self-adhesive film. One compartment was covered in the color white and the other in black, designated hereon as white and dark, respectively. The guillotine-type partition was always raised 1 cm above the tank floor to allow zebrafish to swim freely from one side of the tank to the other. The apparatus had two electrodes placed on each far side of the opposing side walls of the dark compartment. The electrodes extend through the wall height and parallel along the tank floor of the dark compartment. They were attached to an 8 V stimulator and administered a final 3,6 ± 0,4 V AC shock when manually activated.

During training, testing and reminder sessions, zebrafish were individually and gently placed in the white compartment of the IA apparatus with the partition between compartments closed. After 1 min of familiarization to the white compartment, the partition was raised, allowing animals to cross to the dark side of the tank. After the fish entered the dark area with their entire body, the sliding partition was closed and, on the training sessions, a pulsed electric shock was administered for 5 s; on the test sessions, no electric shock was administered; and on the reminder sessions, a 2 s shock was administered. After that, in all sessions, all fish were removed from the IA apparatus and placed in a dedicated tank for temporary housing, in which determination of sex was visually done by at least 2 experienced observers.

After sex determination, animals returned to their home tank. Fish were tested on the 1st, 2nd, 7th and 8thday after training (day zero). In the 7th day test, after the animal entered the dark compartment it

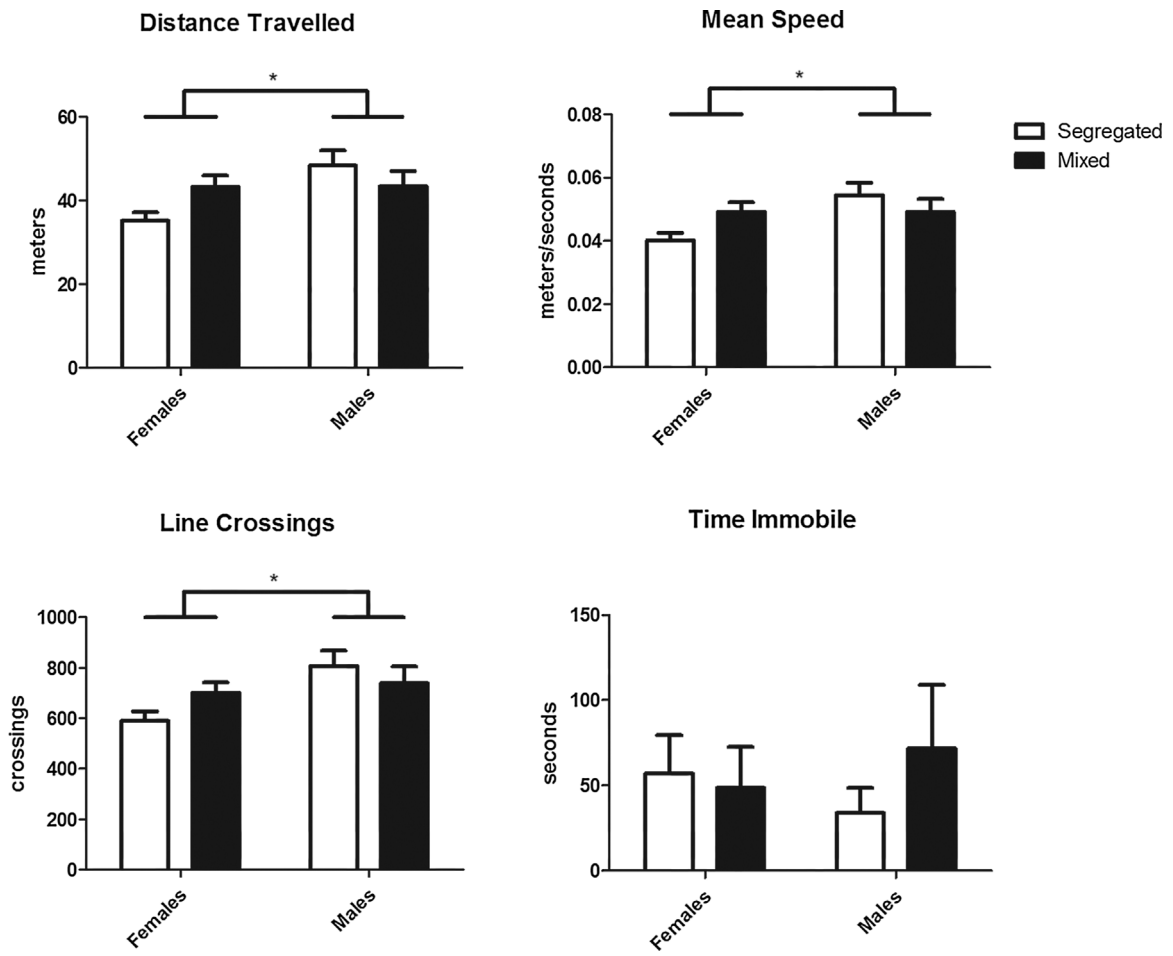

Fig. 1. Effect of sex and housing in open tank locomotor parameters. The data are presented as mean + S.E.M. Two-way ANOVA. n = 19–23. * indicates statistical significance at $p < 0.05$.

received a reminder (reminder session). One day after the reminder session, animals were tested again (8th day). The latency to enter the dark compartment, after raising the sliding partition, was measured in all sessions and the test latencies were used as a measure of retention. Animals had up to 180 s to cross to the dark compartment during training sessions. If it did not cross during this period, it was excluded from the experiment. The number of animals used for the IA task were: 16 for segregated housed females; 14 for segregated housed males, 15 for mixed-sex housed females and 13 for mixed-sex housed males.

To ensure that the increased latency seen in females after the reminder session is indeed due to training effect and not non-specific effects (such as hypersensitivity to shock), a new group of naive separated females (NSF) were not submitted to training, 1st day test and 2nd day test and was submitted to the equivalent of the 7th day reminder session (receiving a pulsed electric shock of 2 s after completely entering the dark area) and were tested 24 h later (equivalent of the 8th day test). The number of animals used in the NSF group was 16.

2.6. Cortisol extraction and analysis

The determination of whole-body cortisol was performed through enzyme-linked immune sorbent assay commercial kit (EIAgen CORTISOL test, Bio ChemImmuno Systems) [13,14]. Fish were captured and immersed on cold water (1–4 °C) and frozen in liquid nitrogen. The cortisol extraction and kit validation was performed according to Sink et al. [15]. Briefly, the tissues were homogenized and suspended in 3 ml of PBS buffer. An aliquot of 1 ml was placed in a new test tube and the sample was suspended again in ethylic ether, followed by freezing in liquid nitrogen. After freezing, the supernatant were placed in a test tube and left overnight until complete ether evaporation. The extract was then suspended in 200 µl of PBS buffer and stored at −20 °C until ELISA analysis. A pool of 3 animals was used per sample. The number of samples used were: 5 for segregated housed females; 5 for segregated housed males, 6 for mixed-sex housed females and 4 for mixed-sex housed males.

2.7. Sex confirmation

After the end of the experiments, animals were euthanized by stunning followed by decapitation for the behavioral experiments and hypothermia followed by liquid nitrogen freezing for the cortisol assay. Sex was confirmed as described in Gupta and Mullins [16]. Briefly, zebrafish were pinned to a dissection mat, gonads were dissected and examined in an Olympus zoom stereomicroscope model SP-ILK (Olympus, Tokyo, JP). Animals were included in the analysis only after the correct gonads confirmation from all fish that shared the same tank. Therefore, the sexual segregation was assured in the segregated groups and the correct proportion of females to males in the mixed groups.

2.8. Statistics

All data are expressed as mean and standard error of mean (SEM). Data was analyzed using a two-way ANOVA to identify the main effects of sex and housing, as well as their interactions, with the exception of IA data, weight and habituation. A cut-off ceiling was imposed during training and testing in the IA task, therefore we analyzed it with the

Table 1
Results of two-way analysis of variance (ANOVA) for effects of sex and housing in behavior and cortisol levels. The degrees of freedom for whole-body cortisol ANOVA are: 1,16. The degrees of freedom for all other dependent variables are 1,82.* represents a statistical difference.

Dependent variable	Effects	F-value	p-Value
Distance travelled in the open tank	Sex	4.332	0.041*
	Housing	0.230	0.633
	Sex x Housing	4.259	0.042*
Mean speed in the open tank	Sex	4.334	0.040*
	Housing	0.242	0.624
	Sex x Housing	4.227	0.043*
Line crossings in the open tank	Sex	5.324	0.024*
	Housing	0.137	0.712
	Sex x Housing	2.674	0.106
Time immobile in the open tank	Sex	< 0.01	0.999
	Housing	0.320	0.573
	Sex x Housing	0.777	0.381
Time in the botton zone in the open tank	Sex	0.319	0.573
	Housing	0.257	0.614
	Sex x Housing	0.057	0.812
Time in the middle zone in the open tank	Sex	0.122	0.728
	Housing	0.296	0.588
	Sex x Housing	1.967	0.165
Time in the top zone in the open tank	Sex	0.865	0.355
	Housing	0.931	0.337
	Sex x Housing	0.0235	0.629
Time in the column A in the open tank	Sex	10.809	0.001*
	Housing	0.032	0.858
	Sex x Housing	0.449	0.505
Time in the column B in the open tank	Sex	1.981	0.163
	Housing	0.019	0.891
	Sex x Housing	0.021	0.884
Time in column C in the open tank	Sex	9.946	0.002*
	Housing	0.619	0.434
	Sex x Housing	0.916	0.341
Time in column D in the open tank	Sex	3.238	0.076
	Housing	0.014	0.908
	Sex x Housing	0.065	0.800
Whole-Body Cortisol	Sex	0.6587	0.428
	Housing	8.149	0.011*
	Sex x Housing	0.094	0.762

nonparametric test of Kruskal-Wallis followed by Dunn's Multiple Comparisons between more than 2 groups or Mann Whitney test for 2 groups. Weight was analyzed using a one-way ANOVA. Habituation between the first and 7th minute of the same group was analyzed using a paired *t*-test. In all comparisons, $p < 0.05$ was considered to indicate statistical significance. SPSS (SPSS Inc., Chicago, IL, USA) and GraphPad (GraphPad, La Jolla, CA, USA) softwares were used.

3. Results

3.1. Open tank locomotor parameters are influenced by sex with a sex and housing effect

Fig. 1 shows locomotor parameters measured in the open tank and Table 1 summarizes the two-way ANOVA analysis. Total distance travelled and mean speed were influenced by sex, with males having a higher activity, but not by housing. There was also a sex and housing interaction. Line crossings were found to be influenced by sex but not by housing and did not interact. Time immobile was not influenced by sex, housing and did not interact with each other. Supplementary Fig. 1 shows distance travelled along time.

3.2. Time spent in vertical zones of the open tank and habituation are not affected by sex and housing

Fig. 2 shows time spent in zones divided vertically and Table 1 summarizes the two-way ANOVA analysis. Time in the bottom, middle or top zone was not influenced by sex and housing, nor did they interact. Sex and housing had no effect on the time spent in the vertical zones. Supplementary Fig. 2 shows time in the top zone along time. Supplementary Fig. 3 shows time and number of entries in the top zone in the first and 7th minute and all groups display habituation as seen in the increase in both parameters.

3.3. Open tank time spent in vertical zones is affected by sex but not housing

Fig. 3 shows time spent in zones divided vertically and Table 1 summarizes the two-way ANOVA analysis. Time spent in column A and C were highly influenced by sex. Females spending more time than males in column A, and males spending more time than females in column C. Time spent in column B and D was not affected by sex. Time spent in all columns was not influenced by housing nor did it interact with sex.

3.4. Memory for inhibitory avoidance is distinct in the groups

There was no difference between groups in training (Kruskal-Wallis test, Fig. 4). On the first day test, the groups with separated females and males had a higher latency than their mixed housing counterparts (Kruskal-Wallis test followed by Dunńs Multiple Comparison test, with both $p < 0.01$). Moreover, the latency to cross to the dark compartment is higher in separated females and males ($p < 0.01$, $p < 0.01$, respectively), but not in the mixed females and males when comparing the respective test and training latencies of each group. These parameters indicate that sexually segregated animals showed memory while mixed animals did not.

In the 2nd day test, separated females had a higher latency than separated males ($p < 0.05$), and no group presented a higher latency when compared with their training. No difference among groups or comparing test and training latency was found in the 7th day test. In the 8th day test, separated females had a higher latency than mixed females ($p < 0.05$) and separated females was the only group to have a higher latency compared to training ($p < 0.05$). These results suggest that isolated females had a memory that persisted longer and was able to be reinstated one week after training.

No statistical difference was observed between the latencies of the reminder session and the subsequent test for the new group of naive separated females (Fig. 5) (Mann Whitney test), indicating that no performance effects in the separated females group is responsible for the 24 h after reminder test increase in latency shown in Fig. 4.

3.5. Whole-body cortisol levels are lower in sexually segregated animals

Fig. 6 shows cortisol levels and Table 1 summarizes the two-way ANOVA analysis. Cortisol levels were influenced by housing but not by sex and there was no interaction.

3.6. Weight is higher in sexually segregated animals

Fig. 7 shows weight levels in the beginning and end of the experiment. The initial and final mean weight of groups was (respectively): 0.189 and 0.288 g for segregated females, 0.203 and 0.287 g for segregated males, 0.185 and 0.248 g for mixed females and 0.200 and 0.256 g for mixed males. The mean increase in groups was: 0.099 g for segregated females, 0.084 g for segregated males, 0.063 g for mixed females and 0.057 g for mixed males.

4. Discussion

Here we show distinct influences of sex and housing (regarding sex segregation or not) in fish behavior, cortisol profiles and body weight. Effects from housing conditions are expected to be more pronounced in social species with complex pheromone communication intra and inter-sex, such as zebrafish [17].

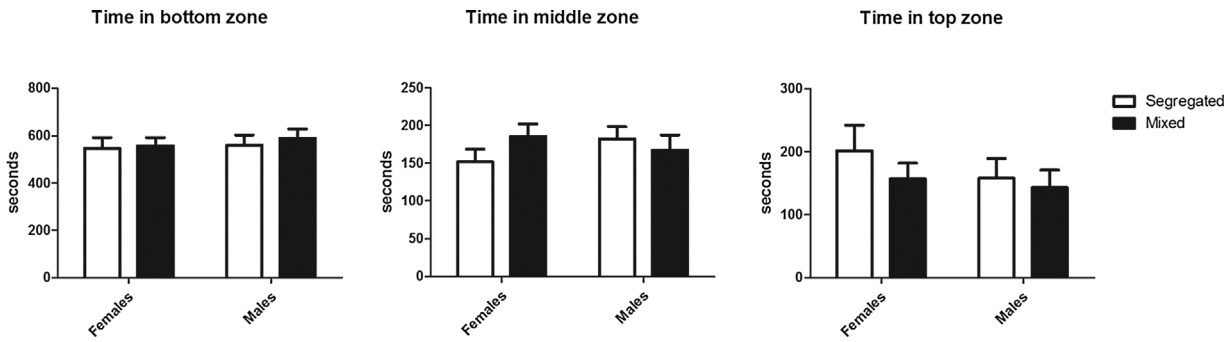

Fig. 2. Effect of sex and housing in time exploring vertical zones in the open tank. The data are presented as mean + S.E.M. Two-way ANOVA. n = 19–23.

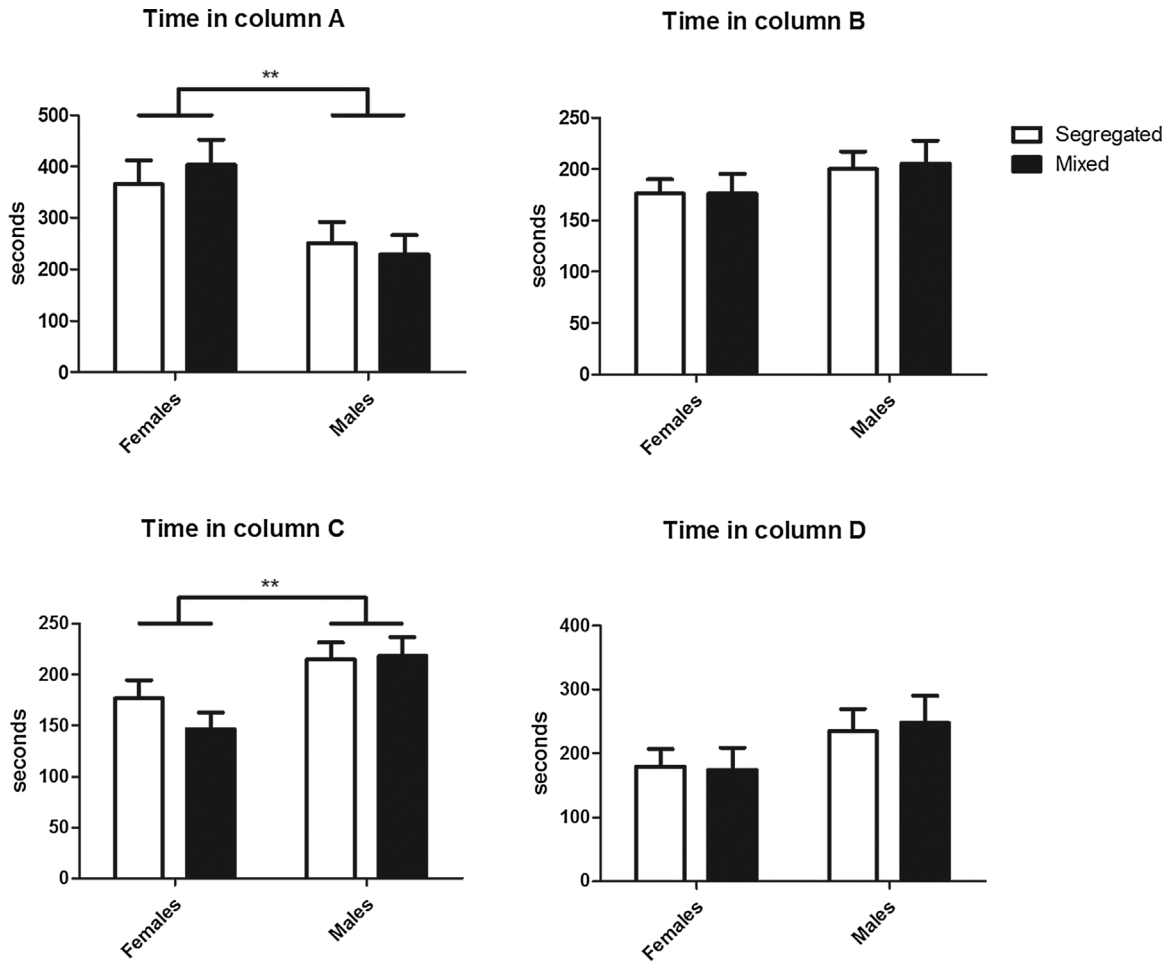

Fig. 3. Effect of sex and housing in time exploring horizontal zones in the open tank. The data are presented as mean + S.E.M. Two-way ANOVA. n = 19–23. ** indicates statistical significance at $p < 0.01$.

We found that locomotor parameters in the open tank were influenced by sex but not housing. We hypothesized that sex influences baseline activity in zebrafish. Previous findings with this teleost found that locomotor parameters were not altered [18,19], dependent of the animaĺs age [20], increased in males [9] or increased in females [8]. Swimming time in the spinning task was not affected by sex [21].

Moreover, we observed that segregated females decreased their locomotion. This result may be due to lack of inter-sex pheromone communication, leading to female behavior alteration. An example of an effect that segregation from male contact causes in female zebrafish is the “eggbound” phenomenon, with impaired egg lay and increased animaĺs size due to necrotic clumped eggs when segregation occurs for 3 weeks [3]. We segregated females for 1 week and this short period did not form necrotic eggs (verified while dissecting animals under the stereoscope). In agreement with our data showing that segregation decreases female activity, Ariyomo and Watt [9] housed animals in a sex-segregated way and observed higher male locomotion, while Moretz et al. [18] used mixed-sex housing and did not find differences between sexes. In this way, our results can help explain the discrepancies of previous findings.

Time immobile and time exploring horizontal zones were not influenced neither by sex nor housing in our study. Tran and Gerlai [22] observed that average distance from bottom was not different between sexes along a 7 day test. Time near surface was also found to be the same between males and females [23]. Nevertheless, Ampatzis *et al.* [19] observed that females spent more time in the upper half of the

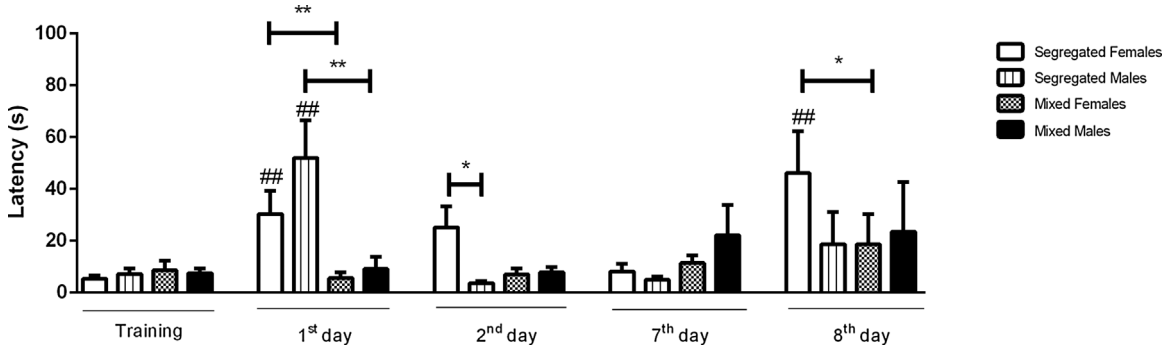

Fig. 4. Effect of sex and housing in the inhibitory avoidance task. The data are presented as the mean + S.E.M. Two-way ANOVA. n = 13–16. * indicates statistical significance at $p < 0.05$ between groups; ** indicates statistical significance at $p < 0.01$ between groups; ## indicates statistical significance at $p < 0.01$ between the test and training of same group.

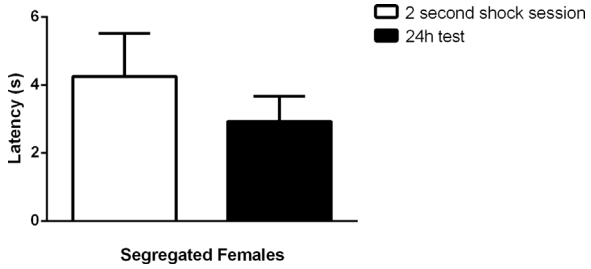

Fig. 5. Effect of sex and housing in the equivalent of the 7th day test and 24 h test. The data are presented as mean + S.E.M. Mann Whitney. n = 16.

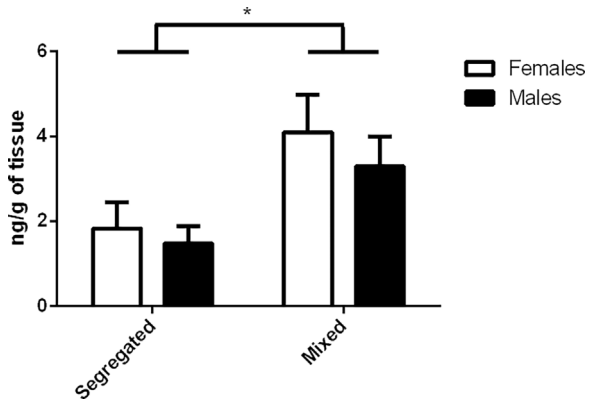

Fig. 6. Effect of sex and housing on cortisol levels. The data are presented as mean + S.E.M. Two-way ANOVA. n = 4-6. * indicates statistical significance at $p < 0.05$ between groups.

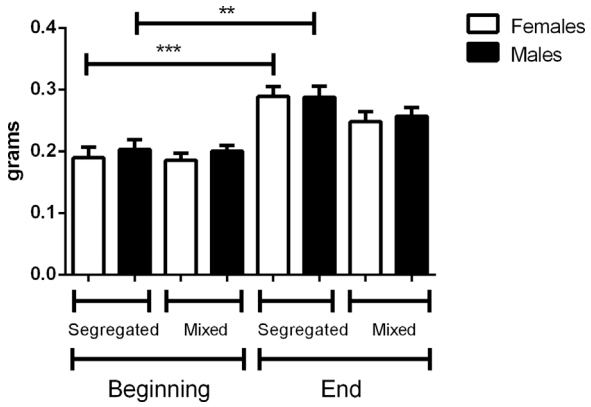

Fig. 7. Effect of sex and housing on weight. The data are presented as mean + S.E.M. One-way ANOVA. n = 19–23. ** indicates statistical significance at $p < 0.01$ and ** indicates statistical significance at $p < 0.001$ between groups.

tank. Habituation, a simple form of spatial memory, is observed in zebrafish exposed to the open tank by increase in time and entries in the upper part of the tank along time [24]. All of our groups displayed habituation, indicating lack of impairment in spatial working memory.

We verified that time exploring vertical zones was influenced by sex but not housing. This teleost has homebase behavior (frequent return to and spend more time in a specific location considered “safe” during trial) [25] when exploring an open tank [26,27]. It is possible that homebase behavior is influenced by sex. As far as we found, only one article explored this matter in a similar way, where sex influence over the time spent in the center of the open tank was found to be near the significant value [28]. Our findings indicate that sex is highly influent over vertical exploration and is relevant to spatial exploration such as homebase behavior. Moreover, the fact that all groups showed habituation in the open tank indicates that the distinct patterns of vertical exploration are not due to unspecific spatial working memory impairment.

Although unexpected, we observed that only the segregated-sex groups showed a 1st day memory. An interesting finding was that memory in the segregated females group persisted for a longer period and was reinstated after one week of training and this result was not due to unspecific effects. Whole-body cortisol levels were measured in different animals from those submitted to the IA task, therefore, a direct causal relationship between aversive memory and cortisol levels is speculative. However, as cortisol levels were measured in animals submitted to the same conditions and at the same time of housing (1 week) as those from animals that were trained in IA, it is possible that cortisol levels on both groups of animals is similar. If so, cortisol at lower levels enhances memory, however, after a threshold it has an impairment effect [29], which might explain our findings that mixed-sex groups with higher cortisol levels showed no 1st day memory. We observed that cortisol levels were independent of sex but influenced by housing. It is possible that mixed-sex housing elicited a higher dispute for mates, resulting in increased cortisol levels. In fact, zebrafish display aggressive behavior to establish hierarchies, dispute territory and mates [2,30] and increases whole-body cortisol after social challenge [31]. Also, in mammals, aggression is increased in male mice when exposed to females [32]. Moreover, pheromones are known to alter behavior and physiology in vertebrates (for a review see [33]). In humans, smelling sweat from the opposite sex increased salivary cortisol levels [34,35]. In salamanders (*Plethodon shermani*), male exposure to female pherormones increases plasma corticosterone [36]. Therefore, the lower cortisol levels seen in sexually segregated animals may be a consequence of lower aggressive behavior and/or absence of the opposite sex pherormones.

One week of sexual segregation is enough for animals to have a higher weight gain than mixed-sex fish. We verified a statistical difference only in segregated groups between the initial and final weight, indicating that housing conditions are affecting the weight of both sexes. We hypothesize that mixed-sex housing alters the energetic

balance possibly by eliciting behaviors that increase physical exercise, such as mate dispute and/or distract animals from feeding. Moreover, the lower cortisol levels observed in segregated-sex animals may also influence their higher weight, as cortisol has catabolic effects in muscles [37].

Moreover, an interesting work from Kurtzman et al. [38] verified an increase in fecundity and egg viability in sexually segregated zebrafish when compared to mixed-sex housed animals. Authors argue that segregated-sex housing allows fish to increase mature gametes reserves and that continuous breeding from mixed-sex housing causes metabolic strain in females. The metabolic strain may increase cortisol levels while decreasing body mass seen in mixed-sex housing animals, which could also be influencing our results.

Zebrafish forms social hierarchies and most information from the effect of those hierarchies arises from the paired paradigm (only 2 fishes in one aquaria, usually for 5 days). This paired paradigm enhances the development of dominance hierarchies that may be more subtle in larger groups of animals, such as in our experimental design, where more members of the shoal facilitate escape and avoidance of repetitive dominant behavior [39]. We did not evaluate how social hierarchies influence the behavioral and physiological parameters regarding sex and housing conditions. Therefore, it is not possible to exclude their impact on the evaluated outcomes. Further studies are required to better understand this topic.

Our results strongly suggest that sex and housing regarding the presence of 1 or 2 sexes in the tank should be taken into consideration. An interesting perspective from this work is to investigate the neurobiological basis for the observed effects. Here it is described for the first time a dissociation between sex and housing regarding sex for commonly investigated outcomes used by researchers working with zebrafish. An interesting point is that all these effects were observed with 1 week of housing, the usual minimal period in which zebrafish may be sexually segregated during acclimation. Our findings might help to understand conflicting results found in the literature, increase inter-study reproducibility and decrease the necessary sample size. It is plausible to suggest that publications using zebrafish should explicit not only the sex of individuals, but also the housing conditions regarding the sexes of animals in their study.

Conflict of interest

Authors declare no conflict of interests.

Acknowledgements

Authors would like to thank Darlan Gusso and Fabiano Peres Menezes for the help in sex determination and Mariana Rieck for proof reading the article. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS). G.K.R. is a CNPq fellowship recipient.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2017.08.006.

References

[1] E.P. Rico, D.B. Rosemberg, K.J. Seibt, K.M. Capiotti, R.S. Da Silva, C.D. Bonan, Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets, Neurotoxicol. Teratol. 33 (2011) 608–617, http://dx.doi.org/10.1016/j.ntt.2011.07.007.

[2] W. Norton, L. Bally-Cuif, Adult zebrafish as a model organism for behavioural genetics, BMC Neurosci. 11 (2010) 90, http://dx.doi.org/10.1186/1471-2202-11-90.

[3] R. Spence, G. Gerlach, C. Lawrence, C. Smith, The behaviour and ecology of the zebrafish, *Danio rerio*, Biol. Rev. 83 (2008) 13–34, http://dx.doi.org/10.1111/j.1469-185X.2007.00030x.

[4] Y. Yabuki, T. Koide, N. Miyasaka, N. Wakisaka, M. Masuda, M. Ohkura, J. Nakai, K. Tsuge, S. Tsuchiya, Y. Sugimoto, Y. Yoshihara, Olfactory receptor for prostaglandin F2α mediates male fish courtship behavior, Nat. Neurosci. 19 (2016) 897–904, http://dx.doi.org/10.1038/nn.4314.

[5] J.M. Andreano, L. Cahill, Sex influences on the neurobiology of learning and memory, Learn. Mem. 16 (2009) 248–266, http://dx.doi.org/10.1101/lm.918309.

[6] L. Cahill, Why sex matters for neuroscience, Nat. Rev. Neurosci. 7 (2006) 477–484, http://dx.doi.org/10.1038/nrn1909.

[7] M.E. Oswald, R.E. Drew, M. Racine, G.K. Murdoch, B.D. Robison, Is behavioral variation along the bold-Shy continuum associated with variation in the stress axis in zebrafish? Physiol. Biochem. Zool. 85 (2012) 718–728, http://dx.doi.org/10.1086/668203.

[8] C.L. Rambo, R. Mocelin, M. Marcon, D. Villanova, G. Koakoski, M.S. de Abreu, T.A. Oliveira, L.J.G. Barcellos, A.L. Piato, C.D. Bonan, Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress, Physiol. Behav. 171 (2017) 50–54, http://dx.doi.org/10.1016/j.physbeh.2016.12.032.

[9] T.O. Ariyomo, P.J. Watt, Effect of hunger level and time of day on boldness and aggression in the zebrafish *Danio rerio*, J. Fish Biol. 86 (2015) 1852–1859, http://dx.doi.org/10.1111/jfb.12674.

[10] F.P. Menezes, L.W. Kist, M.R. Bogo, C.D. Bonan, R.S. Da Silva, Evaluation of age-dependent response to NMDA receptor antagonism in zebrafish, Zebrafish 12 (2015) 137–143, http://dx.doi.org/10.1089/zeb.2014.1018.

[11] J.W. Bortolotto, G.M. de Melo, G. de P. Cognato, M.R.M. Vianna, C.D. Bonan, Modulation of adenosine signaling prevents scopolamine-induced cognitive impairment in zebrafish, Neurobiol. Learn. Mem. 118 (2015) 113–119, http://dx.doi.org/10.1016/j.nlm.2014.11.016.

[12] M. Blank, L.D. Guerim, R.F. Cordeiro, M.R.M. Vianna, A one-trial inhibitory avoidance task to zebrafish: rapid acquisition of an NMDA-dependent long-term memory, Neurobiol. Learn. Mem. 92 (2009) 529–534, http://dx.doi.org/10.1016/j.nlm.2009.07.001.

[13] T.A. Oliveira, G. Koakoski, L.C. Kreutz, D. Ferreira, J.G.S. da Rosa, M.S. de Abreu, A.C.V. Giacomini, R.P. Oliveira, M. Fagundes, A.L. Piato, R.E. Barreto, L.J.G. Barcellos, Alcohol impairs predation risk response and communication in zebrafish, PLoS One 8 (2013) e75780, http://dx.doi.org/10.1371/journal.pone.0075780.

[14] J.G.S. da Rosa, G. Koakoski, A.L. Piato, M.R. Bogo, C.D. Bonan, L.J.G. Barcellos, Impaired brain StAR and HSP70 gene expression in zebrafish exposed to Methyl-Parathion based insecticide, J. Toxicol. Environ. Health A 79 (2016) 1–7, http://dx.doi.org/10.1080/15287394.2015.1099483.

[15] T.D. Sink, S. Kumaran, R.T. Lochmann, Development of a whole-body cortisol extraction procedure for determination of stress in golden shiners, *Notemigonus crysoleucas*, Fish Physiol. Biochem 33 (2007) 189–193, http://dx.doi.org/10.1007/s10695-007-9130-0.

[16] T. Gupta, M.C. Mullins, Dissection of organs from the adult zebrafish, J. Vis. Exp. 37 (2010) e1717, http://dx.doi.org/10.3791/1717.

[17] H.D. Bloom, A. Perlmutter, A sexual aggregating pheromone system in the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan), J. Exp. Zool. 199 (1977) 215–226, http://dx.doi.org/10.1002/jez.1401990206.

[18] J.A. Moretz, E.P. Martins, B.D. Robison, Behavioral syndromes and the evolution of correlated behavior in zebrafish, Behav. Ecol. 18 (2007) 556–562, http://dx.doi.org/10.1093/beheco/arm011.

[19] K. Ampatzis, C.R. Dermon, Sexual dimorphisms in swimming behavior, cerebral metabolic activity and adrenoceptors in adult zebrafish (*Danio rerio*), Behav. Brain Res. 312 (2016) 385–393, http://dx.doi.org/10.1016/j.bbr.2016.06.047.

[20] C. Philpott, C.J. Donack, M.A. Cousin, C. Pierret, Reducing the noise in behavioral assays: sex and age in adult zebrafish locomotion, Zebrafish 9 (2012) 191–194, http://dx.doi.org/10.1089/zeb.2012.0764.

[21] A.R. Blazina, M.R. Vianna, D.R. Lara, The spinning task: a new protocol to easily assess motor coordination and resistance in zebrafish, Zebrafish 10 (2013) 480–485, http://dx.doi.org/10.1089/zeb.2012.0860.

[22] S. Tran, R. Gerlai, Individual differences in activity levels in zebrafish (*Danio rerio*), Behav. Brain Res. 257 (2013) 224–229, http://dx.doi.org/10.1016/j.bbr.2013.09.040.

[23] G.P. Way, A.L. Kiesel, N. Ruhl, J.L. Snekser, S.P. McRobert, Sex differences in a shoaling-boldness behavioral syndrome, but no link with aggression, Behav. Processes 113 (2015) 7–12, http://dx.doi.org/10.1016/j.beproc.2014.12.014.

[24] K. Wong, M. Elegante, B. Bartels, S. Elkhayat, D. Tien, S. Roy, J. Goodspeed, C. Suciu, J. Tan, C. Grimes, A. Chung, M. Rosenberg, S. Gaikwad, A. Denmark, A. Jackson, F. Kadri, K.M. Chung, A. Stewart, T. Gilder, E. Beeson, I. Zapolsky, N. Wu, J. Cachat, A.V. Kalueff, Analyzing habituation responses to novelty in zebrafish (*Danio rerio*), Behav. Brain Res. 208 (2010) 450–457, http://dx.doi.org/10.1016/j.bbr.2009.12.023.

[25] D. Eilam, I. Golani, Home base behavior of rats (*Rattus norvegicus*) exploring a novel environment, Behav. Brain Res. 34 (1989) 199–211, http://dx.doi.org/10.1016/S0166-4328(89)80102-0.

[26] A. Stewart, J. Cachat, K. Wong, S. Gaikwad, T. Gilder, J. DiLeo, K. Chang, E. Utterback, A.V. Kalueff, Homebase behavior of zebrafish in novelty-based paradigms, Behav. Processes 85 (2010) 198–203, http://dx.doi.org/10.1016/j.beproc.2010.07.009.

[27] D.B. Rosemberg, E.P. Rico, B.H.M. Mussulini, A.L. Piato, M.E. Calcagnotto, C.D. Bonan, R.D. Dias, R.E. Blaser, D.O. Souza, D.L. de Oliveira, Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments, PLoS One 6 (2011) e19397, http://dx.doi.org/10.1371/journal.pone.0019397.

[28] S.J. Dahlbom, D. Lagman, K. Lundstedt-Enkel, L.F. Sundström, S. Winberg, Boldness predicts social status in zebrafish (*Danio rerio*), PLoS One 6 (2011) e23565, http://dx.doi.org/10.1371/journal.pone.0023565.
[29] J.L. McGaugh, Memory — a century of consolidation, Science 287 (2000) 248–251, http://dx.doi.org/10.1126/science.287.5451.248.
[30] F.F. Zimmermann, K.V. Gaspary, A.M. Siebel, C.D. Bonan, Oxytocin reversed MK-801-induced social interaction and aggression deficits in zebrafish, Behav. Brain Res. 311 (2016) 368–374, http://dx.doi.org/10.1016/j.bbr.2016.05.059.
[31] M.C. Teles, R.F. Oliveira, Androgen response to social competition in a shoaling fish, Horm. Behav. 78 (2016) 8–12, http://dx.doi.org/10.1016/j.yhbeh.2015.10.009.
[32] V. O'Donnell, R.J. Blanchard, D.C. Blanchard, Mouse aggression increases after 24 hours of isolation or housing with females, Behav. Neural Biol. 32 (1981) 89–103, http://dx.doi.org/10.1016/S0163-1047(81)90317-4.
[33] P.A. Brennan, F. Zufall, Pheromonal communication in vertebrates, Nature 444 (2006) 308–315, http://dx.doi.org/10.1038/nature05404.
[34] C. Wyart, W.W. Webster, J.H. Chen, S.R. Wilson, A. McClary, R.M. Khan, N. Sobel, Smelling a single component of male sweat alters levels of cortisol in women, J. Neurosci. (2007), http://dx.doi.org/10.1523/JNEUROSCI.4430-06.2007.
[35] A.L. Cerda-Molina, L. Hernández-López, C.E. de la O, R. Chavira-Ramírez, R. Mondragón-Ceballos, Changes in men's salivary testosterone and cortisol levels, and in sexual desire after smelling female axillary and vulvar scents, Front. Endocrinol. (Lausanne) 4 (2013) 159, http://dx.doi.org/10.3389/fendo.2013.00159.
[36] S.N. Schubert, C.L. Wack, L.D. Houck, P.W. Feldhoff, R.C. Feldhoff, S.K. Woodley, Exposure to pheromones increases plasma corticosterone concentrations in a terrestrial salamander, Gen. Comp. Endocrinol. 161 (2009) 271–275, http://dx.doi.org/10.1016/j.ygcen.2009.01.013.
[37] J.R. Florini, Hormonal control of muscle growth, Muscle Nerve. 10 (1987) 577–598, http://dx.doi.org/10.1002/mus.880100702.
[38] M.S. Kurtzman, M.P. Craig, B.K. Grizzle, J.R. Hove, Sexually segregated housing results in improved early larval survival in zebrafish, Lab. Anim. (NY) 39 (2010) 183–189, http://dx.doi.org/10.1038/laban0610-183.
[39] E.T. Larson, D.M. O'Malley, R.H. Melloni, Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish, Behav. Brain Res. 167 (2006) 94–102, http://dx.doi.org/10.1016/j.bbr.2005.08.020.