Effects of Neonatal Oxidative Stress on the Social Affinity and Survival in Larval Zebrafish (Danio rerio)

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Research

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Abstract

Background

Exposure to oxidative stress (OS) incurs various consequences in animals' life from molecular to organismic level. Survival and social affinity are the two representative fitness components of animals that can be impacted by early exposure to OS during the developmental process. However, the effect of early OS exposure on survival and social affinity has not been well addressed.

Results

In this study, we treated 0, 1, and 5 mM of hydrogen peroxide solution to four-days post fertilization (dpf) larval zebrafish (*Danio rerio*) for 30 mins, and examined their survival until 19 dpf and social affinity at 28 dpf. There were no significant differences in survival among the treatment groups. On the other hand, social affinity was reduced in the individuals exposed to 5 mM hydrogen peroxide solution. All the groups demonstrated a similar level of locomotion.

Conclusions

These findings indicate that mild neonatal OS exposure did not affect the survival but partially hampered social affinity. Whether and how partially hampered social affinity in larval zebrafish persists to adulthood need to be investigated.

Background

Reactive oxygen species (ROS) is a general term of molecules containing oxygen radicals. They are highly reactive and they bind to and disrupt many vital biomolecules, such as proteins, lipids or mediators of inflammatory responses [1]. ROS can be derived from not only energy metabolism [2], but also environmental factors such as pollution, radiation, and nutritional status [3, 4].

Since ROS can mediate damage to cell structure and cellular processes of living organisms, their effects have long been studied in various aspects of biological processes. In response to ROS, living organisms go through the state of oxidative stress (OS), which generally refers to a state of imbalance between the manifestation of ROS and the ability to detoxify the reactive intermediates [5]. Although a certain amount of OS is required for normal developmental processes [6, 7], OS is usually regarded to have a negative impact on normal cellular processes, such as cell signaling [8] and organismal performance such as survivorship [9, 10], reproduction [10, 11], locomotion [12], cognitive function [13], and social affinity [14, 15].

However, in contrast to the negative effect of OS in cellular level, the effect of OS on organisms in systemic level is not completely understood. So far the studies on the association between OS and

organismic performance yielded conflicting results. For instance, Grover et al. [16] observed that adult *Drosophila* showed decreased survival when fed with high-dose of hydrogen peroxide but survival did not decrease with low-dose hydrogen peroxide. However, when *Drosophila* larvae were exposed to low-dose oxidants, tert-butyl hydroperoxide known to increase OS, their life span was extended up to 30% via microbiome remodeling [17]. Thus, the organismic effect of OS seems to depend on various factors including the severity of OS and the developmental stage of the organism. However, further studies are required to investigate how different levels of OS induced at early developmental stage can change organismal performance such as survival and social behaviors.

In this study, we aimed to elucidate the effect of neonatal OS on the survival and social affinity of zebrafish. Zebrafish is a low-cost model organism with short generation time and high fecundity [18, 19]. Since zebrafish exhibit social behaviors such as shoaling and schooling after certain age [20], it is an appropriate model organism to examine the development of social behavior from larval stage. Using this advantage, we induced OS shortly after hatching and observed the survival and social behavior of larval zebrafish.

Methods

Maintenance and mating

Adult zebrafish were maintained on a circulating system (ZebTech) under 12L : 12 D cycle at 26 to 30 ℃, pH between 6 to 8, and conductivity between 400 to 600 μS. Adults were fed with Tetrabits (Tetra) twice a day with occasional provisioning of Daphnia and Artemia. Mating pairs were randomly selected and mating was conducted with the sex ratio of 1:2 or 2:2 (male : female). After 2-3 hrs, eggs were retrieved and placed in petri dishes using transfer pipettes and maintained at 28 ℃ and 12L : 12 D cycle until 4 dpf (days-post fertilization). Fresh fish water was refilled every day. After full consumption of the yolk (which occurred around 7 dpf), the larvae were moved to 1L breeding tanks and fed with ground Tetrabits twice a day. Larval fish from this mating were used for the experiment.

Induction of oxidative stress

We used hydrogen peroxide to directly induce OS. As a member of ROS, hydrogen peroxide is often used for induction of oxidative stress in many studies, and production and accumulation of cellular hydrogen peroxide are known to result in lipid peroxidation and alteration of cellular redox status [21, 22]. At 4 dpf, individual larvae were randomly assigned to four treatment groups : No treatment, 0 mM, 1 mM, and 5 mM hydrogen peroxide solution. The concentration of hydrogen peroxide solution was determined from a previous study that treated 5 mM hydrogen peroxide solution to zebrafish embryos to induce OS for the examination of survival [23]. Individuals in *No treatment* group did not experience any of the treatment processes. Those in *0 mM*, *1 mM* and *5 mM* groups were first immersed in fish water, 1mM and 5mM hydrogen peroxide solution respectively for 30 minutes, washed with Hank's Balanced Salt Solution, and transferred to breeding tanks. Individuals of the same treatment group were kept together in the breeding tank throughout the experimental period.

Survival

The survival of larval zebrafish was monitored every day until 19 dpf. We counted the number of survivors and the survival rate was calculated by the number of survivors over the initial number. The difference in the survivorship of four treatment groups was investigated with Kaplan-Meier survival curves and pairwise log-rank tests. Since the survivorship can be different depending on the developmental stages, we also examined early (1-10 dpf) and late (11-19 dpf) survival separately.

Social affinity

At 28 dpf, we conducted the behavioral test to examine whether the four treatment groups differed in the social affinity towards conspecifics. We used the experimental scheme used by Dreosti et al. [20]. U-shaped acrylic water tanks with partitioned ends were used. A focal individual was placed in the bottom part of U-tank and the behavior was recorded. This is defined as *Alone* condition, in which the behavior was monitored in non-social context. After 15 minutes of recording the behavior in *Alone* condition, three individuals from the same treatment group were randomly placed in either end of U-tank. This is defined as *With others* condition, and any change in the behavior of the focal fish between *Alone* and *With others* conditions corresponds to social affinity.

We analyzed the behavior of the focal fish using EthoVision XT 11.5. The frames in which the focal fish stayed in social zone (i.e. near the compartment where other individuals were placed) were defined as social cue frames (SC frames) and the frames in which the focal fish stayed opposite to the social zone were defined as non-social cue frames (Non-SC frames). With these results, we calculated the social preference index (SPI = (SC frames – Non- SC frames)/total frames)[20]. In order to compare SPI values between *Alone* and *With others* conditions for each treatment condition, we conducted paired t-tests. Since the numbers of SC or non-SC frames can be influenced by the activity level of the focal individual, we additionally analyzed average velocity.

Results

The survival of all the treatment groups is shown in Figure 2. Early survival (during 1-10 dpf) of *0 mM* and *1 mM* tended to be higher than that of other groups, but late survival (during 11-19 dpf) seemed higher in *5 mM* than that of other groups. However, these differences were not statistically significant and we found no difference in the survival in the pairs of treatment groups (Table 1).

Social affinity was detected from all groups. Heatmaps showed a clear indication of the focal individuals spending more time in the social zone (Figure 3A). Two lines of statistical results verified this notion. Firstly, one-sample t-tests show that SPI values of the larval zebrafish in *With others* condition significantly deviate from zero in *No treatment* ($t=12.402$, $P<0.001$), *0 mM* ($t=2.50$, $P=0.012$) and *1 mM* ($t=2.102$, $P=0.051$) but not in *5 mMWith others* condition ($t=0.972$, $P>0.05$) and *Alone condition* in each treatment groups (for all $P>0.233$: Figure 3B). Secondly, paired t-tests show a significant increase in SPI in *With others* in comparison to *Alone* conditions in *No treatment*, *0 mM* and *1 mM* (Figure 3B: $t=4.384$,

P<0.001; t=2.728, P=0.016; t=2.196, P=0.042, respectively). For *5 mM*, the difference was marginally not significant (t=1.871, P=0.081). Notably, the increase in SPI in *With others* condition was greater in the individuals that were not exposed to OS (i.e. *No treatment* and *0 mM*; P<0.01) than those that were exposed to mild levels of OS (i.e. *1 mM* and *5 mM*; P>0.04). On the other hand, the movement velocity of the focal zebrafish did not change with the presence of social cues (Figure 3C: paired t-tests: t=0.483, P=0.636 for *No treatment*; t=0.403, P=0.692 for *0 mM*; t=0.677, P=0.508 for *1 mM*; and t=0.705, P=0.492 for *5 mM*). Similarly, the total distance travelled during 10 mins of observation did not change with the presence of social cues (Figure 3D: paired t-test: t=0.171, P=0.867 for *No treatment*; t=0.071, P=0.944 for *0 mM*; t=0.919, P=0.371 for *1 mM*; and t=0.835, P=0.417 for *5 mM*). These indicate that the increase in SPI values in *With others* conditions was not due to the systematic difference in activity levels of the focal zebrafish.

Discussion

In this study, we show that neonatal OS, which was induced by 1 mM and 5 mM hydrogen peroxide solution at 4 dpf, does not affect the survivorship of the larval zebrafish up to 19 dpf. On the other hand, social affinity was slightly reduced with OS induced by 5 mM hydrogen peroxide. Since there were no significant changes in the activity levels among the treatment groups and the presence of social cues, we concluded that the slight disruption in social affinity was indeed mediated by OS-induced damage.

Previously, there were several reports for the association between oxidative stress and the social behavior of zebrafish. Müller et al. [24] showed that repeated ethanol exposure enhanced shoal cohesion even with the oxidative damage measured by the increased level of lipid peroxidation in the brain tissue. Strungaru et al. [25] showed that acute exposure to gold caused antisocial behavior of zebrafish, but only temporarily. Oxidative damage was also detected from the lipid peroxidation marker and the expression of various antioxidant enzymes.

Even though both studies found the alteration of social behavior when the chemical stressors were provided, the results were conflicting. Our results, using hydrogen peroxide solution to induce OS, seem more consistent with the findings of the latter study, although in our case the effect of OS, albeit weakly, appeared to last longer than what the latter study suggested. Considering that the treatment of hydrogen peroxide solution did not alter the survival of the larvae and slightly reduced social affinity in our study, it is possible that the effect of the treatment may be totally undetectable when the larval zebrafish become adults, which may imply that the treated concentration was too low to alter organismal performance in long term. How neonatal OS can affect the performance of mature fish will be an interesting future question to answer.

Our results suggest that neonatal OS, even if it is mild, may reduce the development of social affinity and there are several possible explanations for this. One possibility is that neonatal OS might disrupt the locomotory ability of the individuals that generally affects any performance based on behavior. A previous study described that OS disrupted the locomotory ability of mosquitofish such as traveling

distance and swimming speed [12]. As the social affinity measure that we used in our study is calculated based on the number of frames at the location of the focal individual, reduced locomotion in the individuals that were exposed to greater OS would result in lower velocity and smaller traveling distance values regardless of the presence of the social cues. However, in our experiment, the traveling distance and movement velocity of the focal individuals did not differ among the treatment groups. Thus, we can rule out the possibility that the slight disruption of social affinity that we observed is based on OS causing low level of locomotion.

The other possibility is that the neonatal OS hampers kin recognition process. Kin recognition in zebrafish is known to involve olfactory imprinting process that occurs on 6 dpf [26]. In our experiment, the treatment groups were maintained as social groups since 4 dpf. Thus, the larval zebrafish that we studied may have perceived the social mates as kin and exhibited strong social affinity towards them. OS, induced on 4 dpf in our study, can negatively influence the development of central nervous system and olfactory epithelium that are responsible for the olfactory imprinting process [27], and thus reduce the strength of social affinity measured on 28 dpf in the groups that were exposed to greater OS.

OS can also damage specific neural pathways. It has been well known that OS can degenerate dopaminergic neurons and dopamine autooxidation due to OS can lead to various neurodegenerative diseases such as schizophrenia and Parkinson's Disease in humans [28-31]. In zebrafish, the correlation between dopaminergic system function and shoaling behavior has been reported [32]. Considering these results, dopamine may work as a key factor that connects OS and development of social affinity in zebrafish.

The focus of our study lies on revealing the effect of OS on the fitness of larval zebrafish. In that sense, our results are not sufficient to address the proximate mechanisms for the effect of OS. Future studies could examine the association between neonatal OS and social behavior in the zebrafish more thoroughly by narrowing down the time window of measuring the social behavior and additionally measuring the activities of antioxidation enzymes. Also, any physiological signature of neonatal OS exposure could be examined using biochemical quantification of OS and neural imaging. This would enable the understanding of the proximate mechanisms of the effect of neonatal OS on the fitness of social animals.

Declarations

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Authors' contributions

DS Lim, SY Kim, SY Jun, EJ Choi, SW Ko and S-I Lee conceived and designed the study. DS Lim, SY Kim, SY Jun, EJ Choi, and SW Ko carried out the experiments. DS Lim, SY Kim, SY Jun, EJ Choi, and S-I Lee drafted the first manuscript. ES Seo helped maintenance and controlling fish populations and aided experimental procedure.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1. Comparison of survivorship. Chi-square (above diagonal) and P values (below diagonal) from pairwise log-rank tests between two different concentration groups for whole period during which the survival of larvae was monitored (A), and for early (B) and late (C) stages separately.

(A) Whole period (1-19 dpf)	No treatment	0mM	1mM	5mM
No treatment	-	0.078	1.846	2.273
0 mM	0.221	-	1.842	2.268
1 mM	0.826	0.825	-	4.036
5 mM	0.868	0.868	0.955	-
(B) Early stage (1-10 dpf)	No treatment	0mM	1mM	5mM
No treatment	-	0.078	1.846	2.273
0 mM	0.996	-	1.842	2.268
1 mM	0.718	0.984	-	4.036
5 mM	0.991	0.987	0.890	-
(C) Late stage (11-19 dpf)	No treatment	0mM	1mM	5mM
No treatment	-	0.078	1.846	2.273
0 mM	0.918	-	1.842	2.268
1 mM	0.984	0.983	-	4.036
5 mM	0.991	0.990	0.998	-

Figures

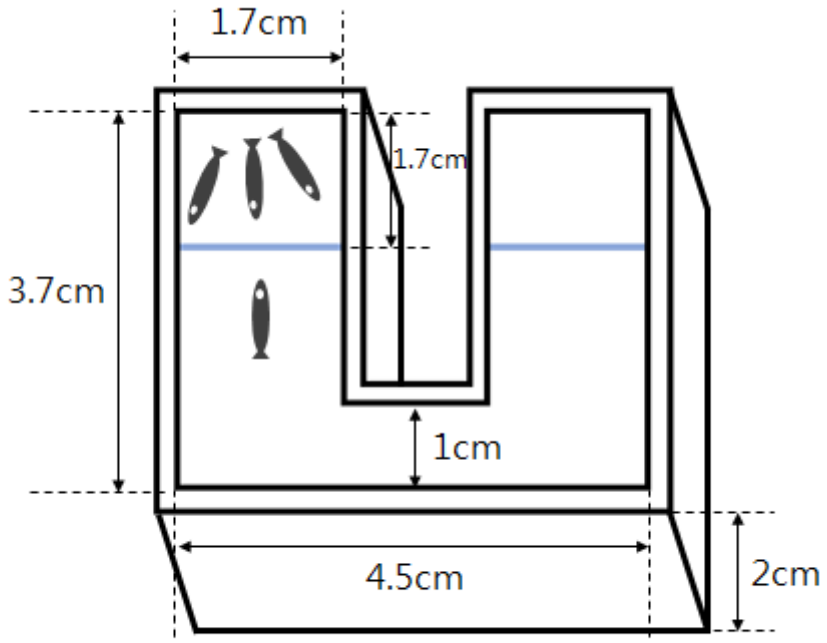

Figure 1

U-shaped tank for social affinity test.

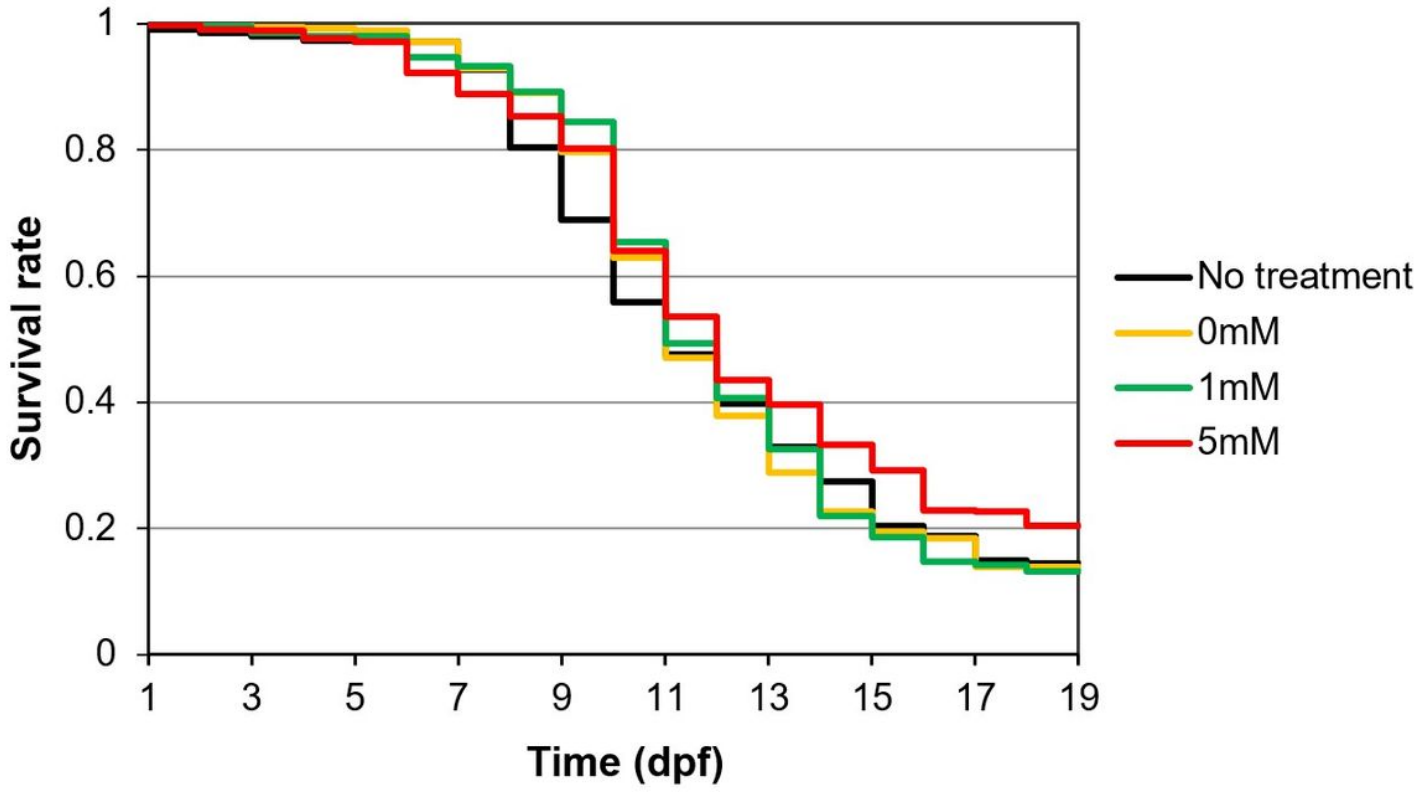

Figure 2

Survival curves of the larval zebrafish exposed to different concentrations of hydrogen peroxide. Four treatment groups of No treatment, 0 mM, 1 mM, and 5 mM showed similar survivorship.

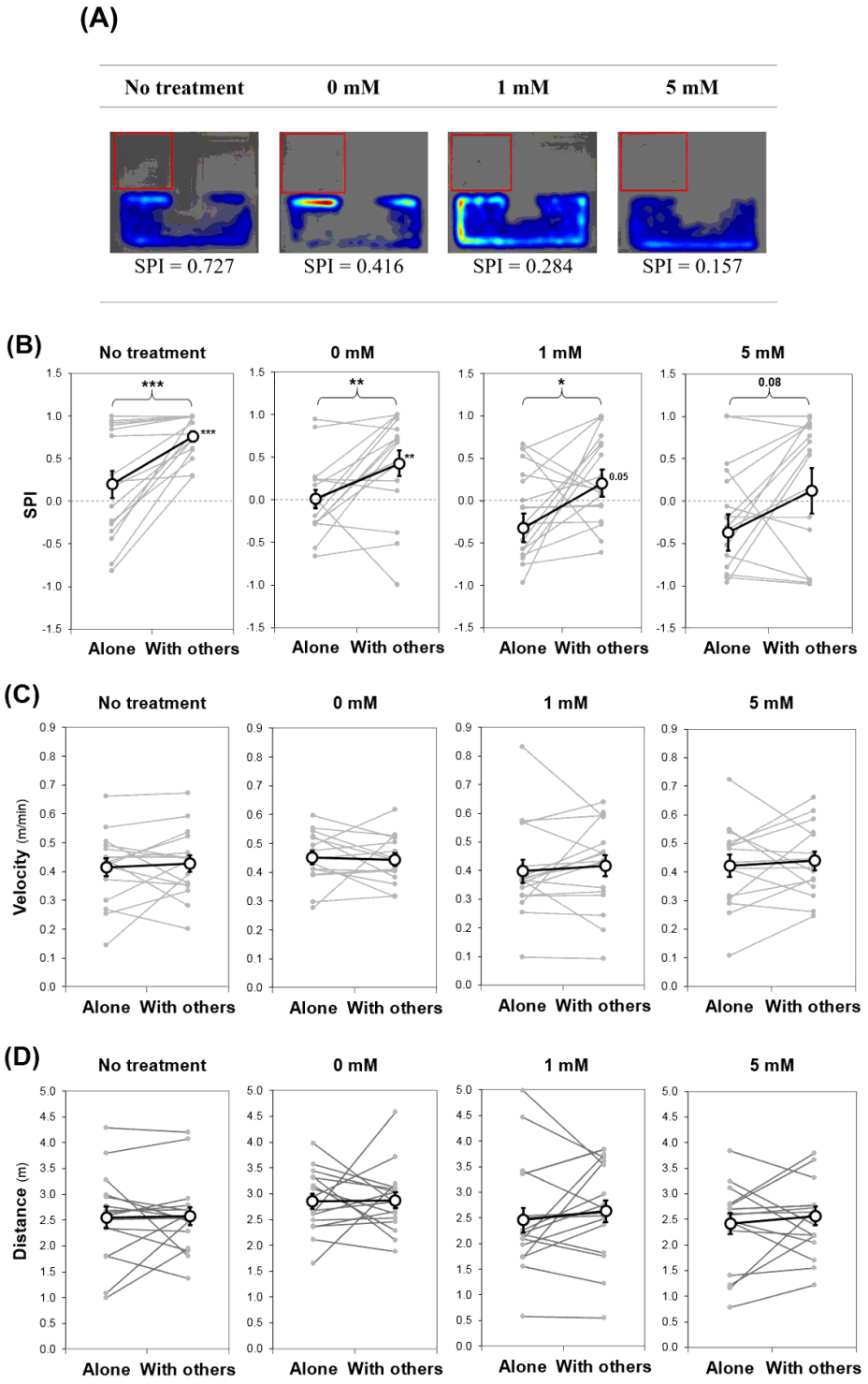

Figure 3

Results of social affinity tests. (A) Representative heatmaps of the location of focal zebrafish during the behavior test. Red box marks the location of the social zone (i.e. location of other individuals). Red color of the map represents longer time and blue color represents less time spent on that spot. For every group,

we chose representative heatmaps whose SPI (social preference index) value is most similar to the average SPI value. (B) Change in SPI of focal individuals between Alone and With others conditions (gray circles connected with lines). Average SPI values and their standard errors for each condition are shown as open circles with error bars. Significance levels of paired t-tests that compare the chances in SPI values between Alone and With others are given above brackets and the symbols '***', '**', and '*' denote $P < 0.001$, $0.001 \leq P < 0.01$, and $0.01 \leq P < 0.05$ respectively. For marginally non-significant pair (i.e. 5 mM), the actual P value was given. Significance levels of one sample t-tests for each condition (see text for details) are given next to the open circles and the notation for symbols are the same as paired t-tests. (C), (D) Change in the velocity of movement (C) and total distance travelled during 10 mins (D) of focal individuals between Alone and With others conditions. All individuals did not differ in the velocity or distance between Alone and With others conditions. For (B) - (D), the sample sizes were 32, 32, 36 and 32 respectively for the treatment groups. Equal numbers of fish were used for Alone and With others conditions.