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Study of Stress, Anxiety and Depression in Type 2 Diabetic Model of Zebrafish (*Danio rerio*)

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Abstract:
Type 2 Diabetes (T2D), which causes hyperglycemia, affects the central nervous system, leading to certain neurobehavioral disorders. These days, zebrafish (*Danio rerio*) has emerged as a promising model organism for experimental studies of neurobehavioral disorders like stress, anxiety and depression. The aim of this study was to investigate the effects of high sucrose induced hyperglycemia or T2D on stress, anxiety as well as depression like behavior of zebrafish. Hyperglycemia was induced in adult zebrafish by immersion in 83.25 mM sucrose solution for 14 days after performing the study for survival in sucrose solutions. The animals were divided into 2 groups in replicates: control and sucrose-treated hyperglycemic groups. Afterwards, the behavioral performance was evaluated in both the groups using novel tank diving test and light-dark box test.High-sucrose induced hyperglycemic group produced robust anxiogenic effects like increased latency, reduced exploration in the top of the tank, increased erratic movements and freezing behavior in the novel tank diving test and scototaxis like behavior in the light-dark box test. Overall, our results confirm that an unfamiliar environment of high-sucrose induced hyperglycemia in the zebrafishes can evoke relatively simple, yet robust stress, anxiety and depression-like behavioral responses.

Keywords: Type 2 Diabetes, Hyperglycemia, Zebrafish, Stress, Anxiety, Depression, Novel tank test, Light-dark box test.

INTRODUCTION

Type 2 Diabetes (T2D), mainly recognized by decreased insulin sensitivity and hyperglycemia, is one of the world's prominent causes of death.[1,2] Worldwide, the number of diagnosed diabetes cases reached 366 million in 2011, and is predicted to reach 552 million by 2030[3]. T2D leads to increase in blood glucose levels due to either the loss of production or function of insulin, a hormone that promotes the glucose transport from blood into cells. These days, increase in blood glucose levels, i.e. hyperglycemia, can results in many severe complications, leading to increased susceptibility of neurobehavioral disorders like stress, anxiety and depression even.

Zebrafish are used as an important model organism for biomedical research in the studies of developmental biology and genetics from long years ago in the past.[4] However, in the recent past, its importance has exponentially increased for the neurobehavioral research for the studies of stress, anxiety and depression even. The reason behind this is that neuroendocrine system of zebrafish delivers robust biological response to neurobehavioral disorders, since it contains vertebrate neurotransmitters also.[5-7] Besides these reasons, zebrafish also offers several advantages like its small size, high fecundity, less expensive, requires low maintenance cost and its homology to humans.[8,9]

A number of studies has already been done in regard to the neurobehavioral research for examining behaviors like stress, anxiety, depression, addiction, sleep, social behavior, learning and memory in normal zebrafish larvae and adults[10-17]. However, the effect on these neurobehavioral studies due to hyperglycemia in zebrafish are still lacking in the literature. Therefore, in the present study, we have tried to quantify the neurobehavioral phenotypes of stress, anxiety and depression in hyperglycemic model of adult zebrafish using manual registration of the behavior in the novelty- based paradigms i.e. novel tank diving test and light-dark box paradigm. Potentially anxiogenic experimental manipulations used here included acute and prolonged exposure to water sucrose rich diet.

Novel tank diving test (similar to open-field test) and light-dark box test have already been used in the case of rodents for analyzing anxiety and stress responses. These tests use the innate behavior of zebrafish to protect themselves in unversed environment from predators by diving and residing at the bottom or in the dark areas of the tank, till they feel safe enough to explore the tank.[12,18,19] Several previous studies have already shown the stress and anxiety-like behavior in the zebrafish using different anxiolytic or anxiogenic agents and characterized the anxiety response by increased latency to enter the upper half of the novel tank, less time spent in the top of the tank, as well as increased erratic movements and freezing behavior in the novel tank diving test.[18,20] Similar studies have also characterized the decreased entries and duration in the light part of the light-dark box apparatus, thus showing scototaxis i.e. dark preference.[21-25]

MATERIALS AND METHODS

Animals and Housing

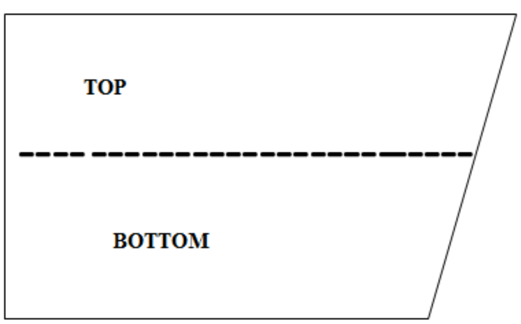
The wild type (short fin) adult zebrafish (*Danio rerio*) strain (6-8 months old) of both the sexes were obtained from a Fish Research Centre, Ranchi. The fish were given at least 1 week to acclimate the constant laboratory conditions in the stock water tanks before all experiments were conducted. Animals were housed in the groups of 8-10 fishes per 10-litre tank. The fishes were maintained in filtered facility water, with both room and water temperatures maintained at approximately 28°C. Illumination was provided by ceiling and wall- mounted tube lights on a 12-12 hr cycle (on: 9:00; off: 21:00). Fish were fed twice daily Tetramin flakes food (3.5 mg/fish/day; Optimum company, Thailand). All fish used in these experiments were experimentally naive and randomly chosen adults of both the sexes from different clutches.

Induction of hyperglycemia

Groups of 10 adult animals were placed in 7-ltr water tank, containing 83.25 mM sucrose solution, which were diluted in water and maintained during 14 days at room temperature for inducing stable hyperglycemia, as described in previous study[26].

Novel Tank Diving Test
For this test, zebrafish were placed individually in a 1.5-litre trapezoidal tank (Dimension: 15.2cm height × 27.9cm top × 22.5cm bottom × 7.1cm width; Figure 1) maximally filled with normal filtered facility water. Novel tanks were allowed to rest on a level, stable surface and were divided into two equal virtual-horizontal parts, marked by a dividing line on the outside wall. Once the setup for novel tank completes, swimming behavior of zebrafish was recorded by two trained observers over a 6-min period. The following endpoint behavioral measures were recorded during this test: latency to reach the upper half of the tank (in sec), total number of transitions (entries) to the upper half of the tank, total time spent in the upper half of the tank (in sec), total number of erratic movements, and total number of freezing bouts. Erratic movement was defined as sharp changes in the direction or velocity and repeated rapid darting behaviors. Freezing was defined as a total absence of movement, except for the gills and eyes, for 1 sec or longer. These behavioral endpoints were selected based on the previous zebrafish studies using the novel tank diving paradigm.[12,18,27]

Light-Dark Box Test
For this test, zebrafish were placed individually in a tank (Dimension: 15cm height × 45cm length × 10cm width; Figure 1 with half black-half white walls and filled with water to a height of 10 cm. The bottom area of the tank should be non-reflective to avoid the tendency of animals to behave in relation to their own reflection. This apparatus also contains sliding central doors, having the same color of the tank side, thereby forming a central compartment, having dimension of 15cm height × 10cm length × 10cm width[25,28,29] (Figure 1). During experiments, the tank must be rotated after each trial to eliminate the orientation effects. The apparatus should be positioned in such way that all the areas of the tank are illuminated by optimal and homogenous lighting, since the results of this test are also sensitive to the light amounts. Once the setup for light-dark box test completes, the fish were transferred to the central compartment, keeping the sliding doors on for 3-5 min for acclimation, then it was removed to allow the fish to explore the apparatus and then light-dark preference of zebrafish was recorded by two trained observers for a period of standard 15-min testing sessions. The following endpoint behavioral measures were recorded during this test: latency to enter white zone of the tank (in sec), total number of transitions (entries) to white zone of the tank, total time spent in white zone of the tank (in sec), and white: total time spent ratios. These behavioral endpoints were selected based on the previous zebrafish studies using the light-dark box paradigm.[25]



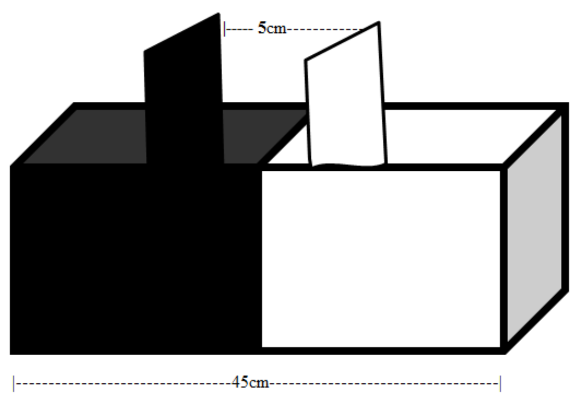


Figure 1: Schematic representation of Novel Tank Diving Test Apparatus and Light-Dark Box Test Apparatus for characterization of zebrafish behavior.

Video- aided tracking and analysis of zebrafish behavior
In the present study, after complete setup of both the tests, manual recording of zebrafish behavior was performed by two trained observers separately. Trials were recorded to a computer for the observation period of 6-min and 15-min using a camera of mobile phone (16.0-Megapixel, Samsung Galaxy J7) for the Novel tank diving test and light-dark box test respectively.

Statistical Analysis
Data were analyzed using Graphpad Prism software (version 5.01). All data were expressed as mean ± standard error of the mean (SEM). Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test for significance between control and experimental groups. $p < 0.05$ was considered to be statistically significant.

RESULTS

Novel Tank Diving Test
Highsucrose diet induced hyperglycemia or type 2 diabetes (T2D) produced robust effects on zebrafish behavior, including a significantly higher latency to explore the upper half of the tank, decreased number of entries and lesser time spent in the top with respect to control group. T2D also significantly increased the frequency of erratic movements, with a trend to increased frequency of freezing behavior, compared to control group (Figure 2). The detailed datasets for the same are provided in the Table 1.

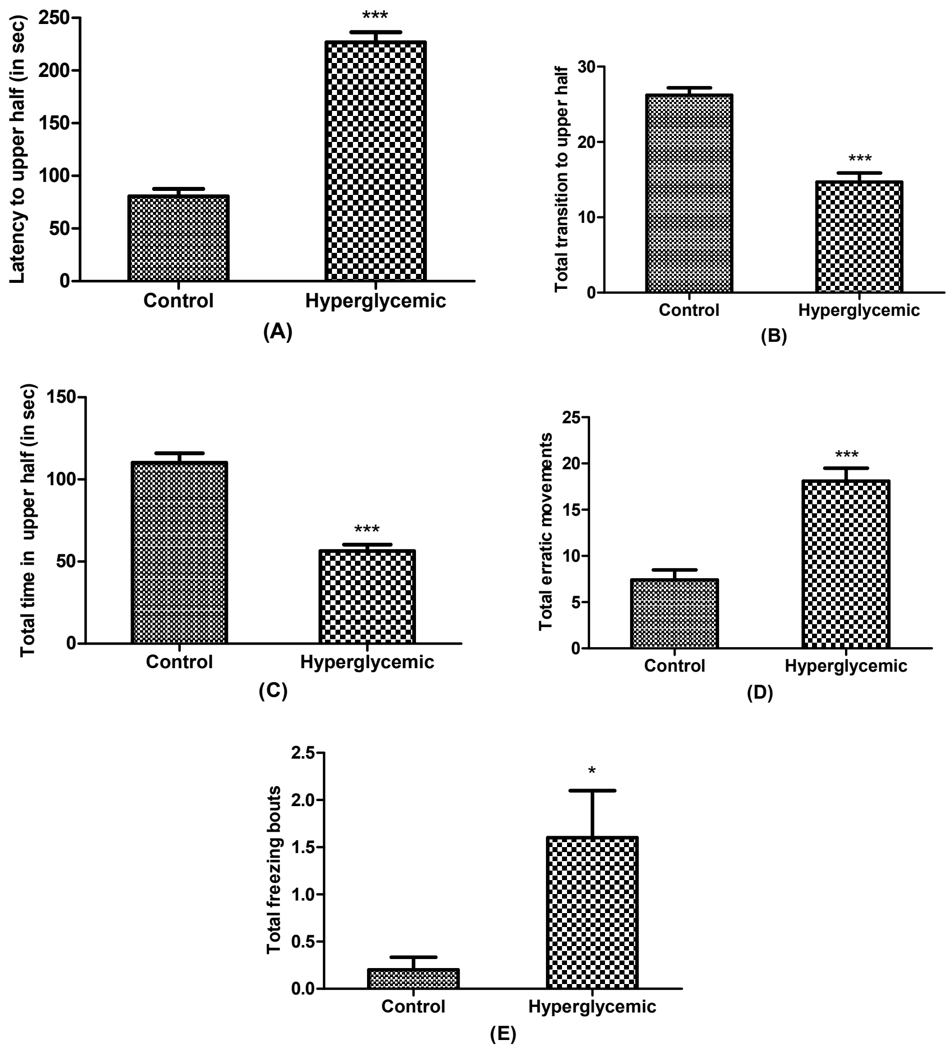


Figure 2: Effects of type 2 diabetes on the different behavioral parameters of zebrafish in the novel tank diving test (n = 10): (A) Latency to upper half; (B) Total transition to upper half; (C) Total time in upper half; (D) Total erratic movements; (E) Total freezing bouts.
Data are presented as mean ± SEM. Data were analyzed by t-test. The *represents a significant difference from the control group. *$p < 0.05$, ***$p < 0.001$.

Table 1: Change in different behavioral parameters of zebrafish due to type 2 diabetes (T2D) in the novel tank diving test.

Behavioral Parameters	Control	Type 2 Diabetes (T2D)
1. Latency to upper half (in sec)	80.6 ± 7.087	226.8 ± 9.581
2. Total transition to upper half	26.20 ± 0.975	14.70 ± 1.193
3. Total time in upper half (in sec)	110.2 ± 5.771	56.40 ± 3.922
4. Total erratic movements	7.40 ± 1.097	18.10 ± 1.394
5. Total freezing bouts	0.20 ± 0.133	1.60 ± 0.498

The change in behavioral parameters in zebrafish recorded after each minute was also found to be significant in this study (Figure 3). For example, number of transitions to upper half after each minute, time spent in upper half after each minute, number of erratic movements after each minute compared were significant in T2D zebrafish in respect to the wild type controls. The detailed datasets for the same are provided in the Table 2.

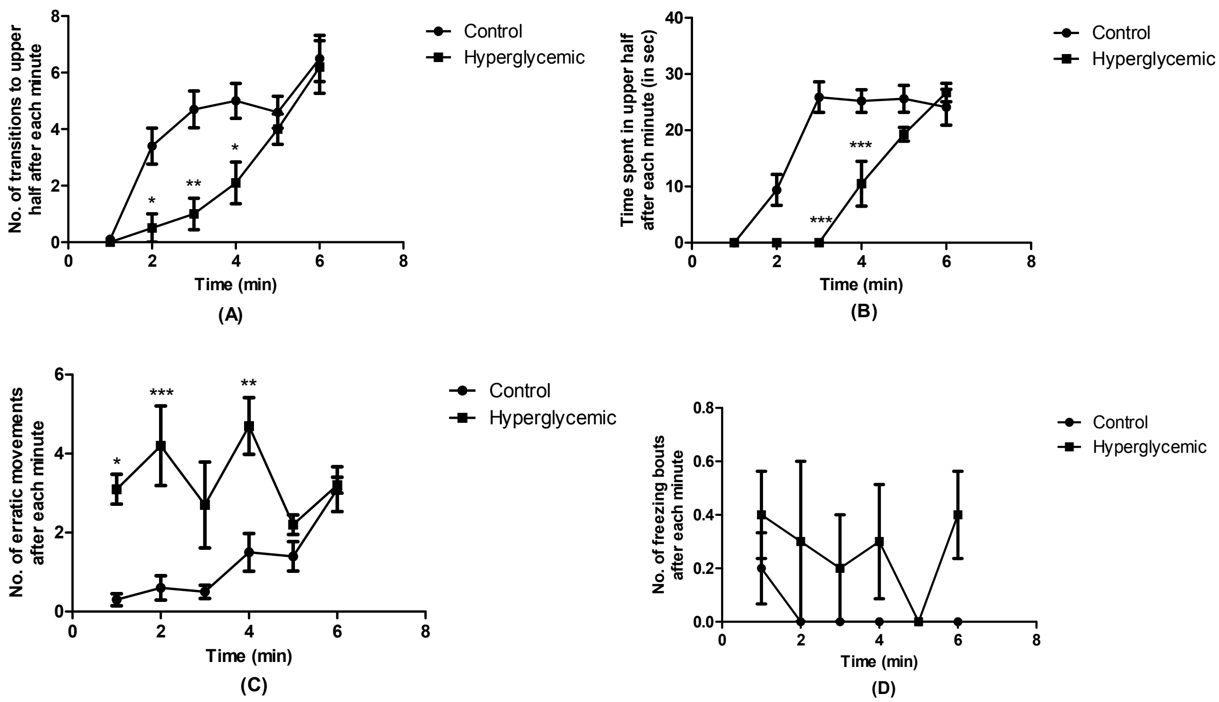


Fig. 3: Change in different behavioral parameters of zebrafish in the novel tank diving test, recorded after each minute (n = 10): (A) Transition to upper half; (B) Time spent in upper half; (C) Erratic movements; (D) Freezing bouts.

Data are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test, used for multiple comparisons. The * represents a significant difference from the control group of each particular day. *p < 0.05, **p < 0.01, ***p < 0.001.

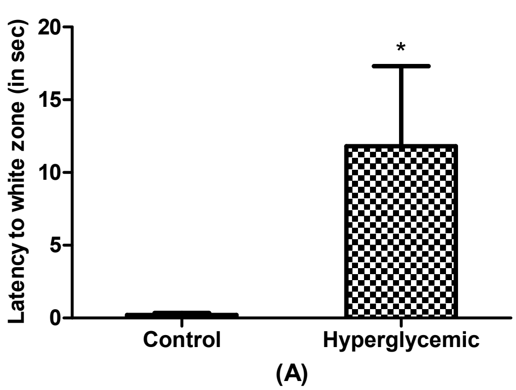
Table 2: Change in different behavioral parameters of T2D zebrafish in the novel tank diving test, recorded after each minute in comparison to control group.

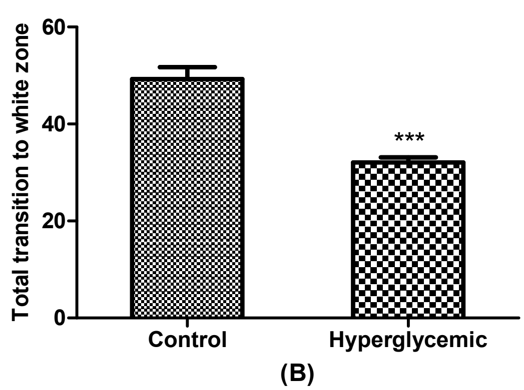
Behavioral Parameters	Control					
	1 min	2 min	3 min	4 min	5 min	6 min
1. Transition to upper half	0.10 ± 0.100	3.40 ± 0.636	4.70 ± 0.650	5.00 ± 0.614	4.60 ± 0.561	6.50 ± 0.819
2. Time spent in upper half (in sec)	0.00 ± 0.000	9.40 ± 2.758	25.90 ± 2.730	25.20 ± 2.015	25.60 ± 2.386	24.10 ± 3.185
3. Erratic movement	0.30 ± 0.152	0.60 ± 0.305	0.50 ± 0.166	1.50 ± 0.477	1.40 ± 0.371	3.10 ± 0.566
4. Freezing bout	0.20 ± 0.133	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000

Behavioral Parameters	Type 2 Diabetes (T2D)					
	1 min	2 min	3 min	4 min	5 min	6 min
1. Transition to upper half	0.00 ± 0.000	0.50 ± 0.500	1.00 ± 0.557	2.10 ± 0.737	4.20 ± 0.489	6.20 ± 0.928
2. Time spent in upper half (in sec)	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	10.50 ± 3.984	19.30 ± 1.230	26.70 ± 1.633
3. Erratic movement	3.10 ± 0.378	4.20 ± 1.009	2.70 ± 1.086	4.70 ± 0.715	2.20 ± 0.249	3.20 ± 0.200
4. Freezing bout	0.40 ± 0.163	0.30 ± 0.300	0.20 ± 0.200	0.30 ± 0.213	0.00 ± 0.000	0.40 ± 0.163

Light-Dark Box Test

The observed behavioral responses and indices of zebrafish anxiety assessed in the light/dark box test should generally be in parallel to those observed in the novel tank test. However, some differences in pharmacological results with variations of these tests have been observed, suggesting that these models may target different aspects or subtypes of anxiety[30]. In line with this, anxiety levels were found to be attenuated with the high sucrose diet induced hyperglycemia or T2D. For example, T2D zebrafishes showed significant increase in latency to cross into white half of the light/dark box apparatus. However, the total number of transitions and total duration in white zone were found to be significantly decreased in the case of T2D, as compared to control zebrafishes (Figure 4).The detailed datasets for the same are provided in the Table 3.





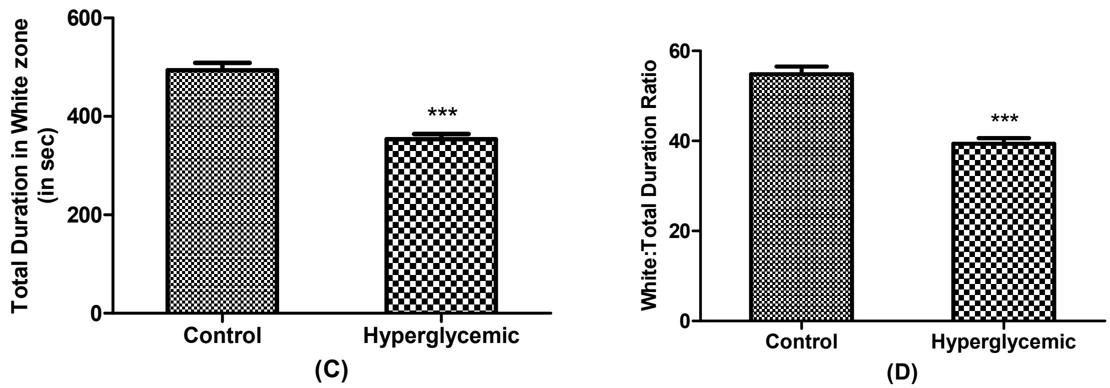


Figure 4: Effects of type 2 diabetes on the different behavioral parameters of zebrafish in the light/dark box test (n = 10): (A) Latency to white zone; (B) Total transition to white zone; (C) Total duration in white zone; (D) White: Total duration ratio.

Data are presented as mean ± SEM. Data were analyzed by t-test. The * represents a significant difference from the control group. *p < 0.05, ***p < 0.001.

Table 3: Change in different behavioral parameters of zebrafish due to type 2 diabetes (T2D) in the light/dark box test.

Behavioral Parameters	Control	Type 2 Diabetes (T2D)
1. Latency to white zone (in sec)	0.20 ± 0.133	11.80 ± 5.515
2. Total transition to white zone	49.30 ± 2.409	32.10 ± 1.038
3. Total duration in white zone (in sec)	493.5 ± 15.36	353.7 ± 10.75
4. White : Total duration ratio	54.83 ± 1.706	39.37 ± 1.238

Moreover, the change in behavioral parameters in zebrafish recorded after each 3 minutes were also found to be significant in this study (Figure 5). For example, number of transitions to white zone after each 3 minutes and time spent in white zone after each 3 minutes were also significant in T2D zebrafish in respect to the wild type controls. The detailed datasets for the same are provided in the Table 4.

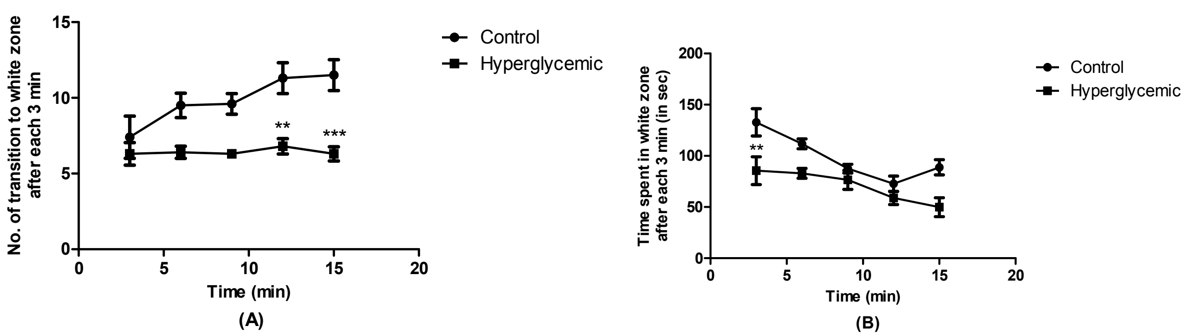


Figure 5: Change in different behavioral parameters of zebrafish in the light/dark box test, recorded after each 3 minutes (n = 10): (A) Transition to white zone; (B) Time spent in white zone.

Data are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey post-hoc test, used for multiple comparisons. The * represents a significant difference from the control group of each particular day. **p < 0.01, ***p < 0.001.

Table 4: Change in different behavioral parameters of T2D zebrafish in the light/dark box test, recorded after each 3 minutes in comparison to control group.

Behavioral Parameters	Control				
	3 min	**6 min**	**9 min**	**12 min**	**15 min**
1. Transition to white zone	7.40 ± 1.392	9.50 ± 0.806	9.60 ± 0.686	11.30 ± 1.023	11.50 ± 1.014
2. Time duration in white zone (in sec)	132.7 ± 13.31	111.8 ± 4.832	87.50 ± 4.169	72.70 ± 7.491	88.80 ± 7.462

Behavioral Parameters	Type 2 Diabetes (T2D)				
	3 min	**6 min**	**9 min**	**12 min**	**15 min**
1. Transition to white zone	6.30 ± 0.746	6.40 ± 0.400	6.30 ± 0.213	6.80 ± 0.512	6.30 ± 0.472
2. Time duration in white zone (in sec)	85.50 ± 13.57	82.90 ± 4.790	76.50 ± 9.195	58.90 ± 6.406	49.90 ± 9.236

DISCUSSION

In the present study, different behavioral parameters of zebrafish were observed and quantified in the novel tank diving test and light-dark box test, in which manual phenotypic measurements were performed. The behavioral parameters measured in the present study (Figures 1–4) proved to be highly sensitive to environmental and pharmacological challenges. The novel tank diving experiments were performed by using manual marking of 2 different zones, as top and bottom variation of the tank. The simplified protocol of the novel tank diving paradigm and light-dark box paradigm has already been used for phenotyping of zebrafish anxiety in the normal zebrafishes.[12,25]

In the Novel Tank Diving test, the significant increase in latency to upper half, decrease in number of entries and time spent in upper half is demonstrating the stress and anxiety-related phenotypes in zebrafish and in the following test i.e. Light-Dark Box test, the zebrafish are showing the similar kind of result (i.e. anxiety-like behavior), as measured by using different parameters. Moreover, increased frequency of freezing bouts has also been observed in the first test, signifying depression like behavior in zebrafish. The result thus suggests that hyperglycemic zebrafishes are showing the behavioral phenotype of stress, anxiety and depression. On the contrary, control group of zebrafishes tend to show better or normal performance due to lack of stress, anxiety and depression. Novel Tank Diving Test and Light-Dark Box Test have already been clearly demonstrated with the good performance in normal zebrafishes.[12,25]

In general, our data confirm that an unfamiliar environment of high-sucrose induced hyperglycemia in the zebrafishes can evoke relatively simple, yet robust anxiety-like behavioral responses, compared to control fish. Zebrafish cohorts acutely exposed to T2D displayed robust anxiety, evidenced by higher latency to initiate top exploration, fewer transitions to the top, more erratic movements and more freezing bouts. The anxiogenic effect seen here in zebrafish is in line with human response to caffeine challenge[31] and rodent data on anxiogenic effects of caffeine.[32,33]

Moreover, another study on chronic treatment with anxiolytic drugs like fluoxetine in zebrafish[12] demonstrates an overall increase in time spent in the top portion, lower latency to top exploration, and higher average top transitions. Behavioral and physiological data from this study agree strongly with that seen in previously published in rodent studies showing that chronic exposure to such kind of anxiolytic drug reduces anxiety[34,35], corticosterone responses in mice[35]and HPA sensitivity in rats.[36,37] Collectively, these findings further validate the zebrafish model of anxiety in its behavioral, pharmacological and endocrine aspects.

On the other hand, the light-dark box test used for this experiment is very good and additional behavioral assay to quantify anxiety-like behavior in adult zebrafish. We have used this test in addition to novel tank diving paradigm, which serves as a useful complement to it because of its unique ability to assess light-dark aversion. However, factors like sex, age, difference in strain, social status, as well as their interactions with different environmental factors (like lighting of the apparatus or testing room) among zebrafishes, can indeed modulate light-dark box behavior of zebrafish. Nevertheless, this experiment is performed keeping all the conditions strictly constant for all the zebrafishes.

In this study, several different domains like locomotion (overall activity), reduction of exploration (anxiety), avoidance behavior, erratic movements (fear- and/or escape-like behavior) and freezing bouts (depression-like behavior) was carefully observed and analyzed for zebrafish behavior, thus proving zebrafish as a good animal model of stress.

Since, in the previous studies, it has been reported that manual observation can measure the zebrafish behavior relatively well, intrinsic inter- and intra-rater variability with the persistence of simple human errors.[12] Therefore, we have tried to obtain the data extraction by using manual observation of videos. Apart from this, we have also tried to reduce the other factors like the presence of experimenters in the testing room, which may also influence zebrafish behaviors. Thus, keeping these factors in mind, we have standardized manual video-tracking method of phenotypic assessment, resulting in more precise results of higher validity.

In summary, we showed robust stress, anxiety and depression related phenotypes in zebrafish following exposure to high-sucrose induced hyperglycemia or T2D. We applied video-tracking tools in both novel tank diving and light-dark box tests for the behavioral analysis of zebrafish, which helped in dissecting anxiety responses from overall activity, thus proving high reliability of this method for the study of zebrafish anxiety. Taken together, these results also confirm zebrafish as a valid, reliable, and efficacious model for basic translational research of stress-related metabolic disorders.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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