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# Alarm Test: A Novel Chemical-Free Behavioural Assessment Tool for Zebrafish

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## Abstract

Zebrafish (ZF) is an incredible animal for the study of neurological disorders. Its behaviour is like higher vertebrate animals, which makes it gainful and robust. Understanding the psychological and biological implications of housing settings for ZFs is very crucial in improving the replicability and dependability of ZF behavioural research. Individual housing triggers depression-like symptoms that suggest that housing conditions have negative effects on ZF and can result in the data discrepancy. Based on various behavioural analyses, we have evaluated that the ZFs kept in isolation and the ZFs kept in herd conditions exhibit different behavioural patterns. Interestingly, normal isolated subjects exhibit similar behavioural patterns as Alzheimer disease (AD)-induced subjects; hence, this can have serious implications on any study concerning behaviour of ZFs. Therefore, we have reported a new behavioural test named “Alarm Test”, which effectively discriminates normal isolated subjects from AD subjects. Alarm Test is observed to be better than other tests used for studying fear and anxiety in ZFs as it uses the indigenous compound released by ZFs during fear and makes use of the same for analysis. This can reduce the involvement of chemicals during behavioural analysis as well as sacrifice of ZFs for collection of alarm substance.

**Keywords:** zebrafish, behaviour, alarm test, Alzheimer’s disease, predators

## 1. Background

The behaviour of animal and human is metaphorically related to each other. The study of animal behaviour sheds light on understanding the complex human emotions [1]. In the quest for knowledge on human cognitive abilities and to understand the functioning of the central nervous system, animals are used as a prototype to show different behaviours when subjected to experiments on stress, chemical-induced diseases, cognitive impairment, environmental toxins, thirst, hunger and many more [2]. Zebrafishes (ZFs) are small and proved to be fruitful for many high-throughput applications (**Figure 1**).

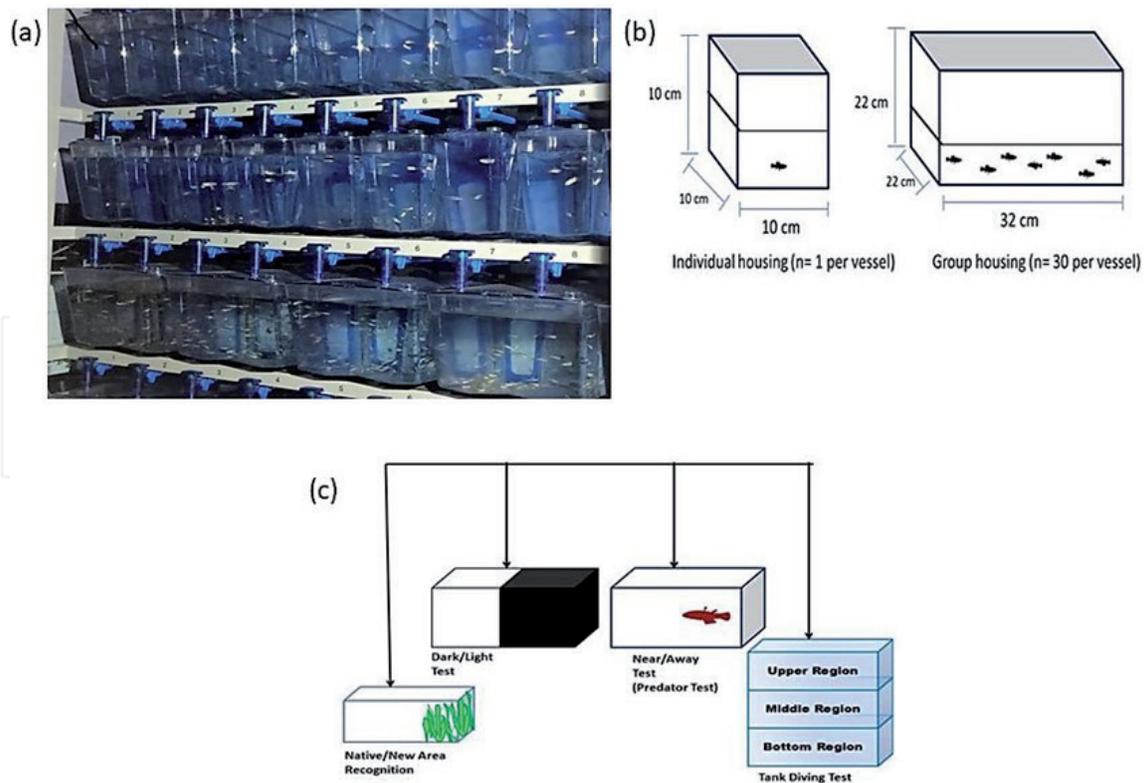
They have the tendency to remain in groups and exhibit social behaviour which is known as shoaling [3]. Thus, ZF has been used for mimicking the social behaviour of humans. Studies have reported that the anxiety level and stress response



**Figure 1.**  
*Utilization of zebrafish in different sciences and domains.*

can be measured in ZFs by evaluating their diving pattern in tank diving test and so forth for another test as well [4]. However, these tests have some limitations which have not been reported anywhere. For example, during our experiments we observed that control behaviour of ZF subjects when introduced in a new vessel is extensive thrashing from wall to wall of that vessel, uninterrupted and vigorous movement for around 2 minutes, heavy breathing and escape actions. This behaviour is replicated by the first few fishes introduced in the same vessel. This is regarded as an ideal control behaviour. As we add more fishes (mostly fourth, fifth and so on), this ideal control behaviour was difficult to attain. The ZFs in the same vessel were now sensing the presence of some substance presumably released by every fish that entered before them in that water, which was hindering with their ideal behaviour. We named this compound as “alarm substance”. The new behaviour exhibited by them was that, as soon as the subject entered the vessel, it was abnormally silent and did not move at all for a long period of time which is mostly 3–4 minutes. Even after little encouragement, the subject moved a little and then settled at the bottom of the vessel. In this chapter, we have tried to report these limitations as well as suggestions to overcome these issues so that correct behaviour of ZFs can be studied.

Additionally, the social isolation disrupts the growth performance and innate immune response of ZFs, and it reflects in their behaviour and temporal-spatial judgements [5]. ZFs exhibit this depression in the form of increased cortisol in their body and show decreased anxiety. Stressful housing conditions, i.e. social isolation, are sometimes inevitable in experiments wherein subjects are exposed to oral doses of chemicals, a precondition in that experiment [6, 7]. Often research communities pay attention to the toxin-treated behaviour, but none has reported



**Figure 2.** (a) Zebrafish facility. (b) Zebrafishes housed individually and in group. (c) Diagrammatic representation of the behavioural test setups, i.e. native/new area, dark/light test, near/away (predator test) and tank diving test.

the differences in behaviour of control ZFs when kept in isolation and herd conditions (**Figure 2a** and **b**). The results obtained from such experiments can be misleading as the subjects already have the depression symptoms, and, therefore, through behavioural assessment of such subjects, conclusive results cannot be obtained.

The alarm substance is not very easily obtained from ZF. Typically, the scientists collect the alarm substance by inducing a stimulus of the presence of a predatory fish and then sacrificing the ZFs by creating cuts in their epidermal layers [8] or by keeping predators with ZFs [9]. Since it is not wise to keep the predator and subject in the same apparatus and also to sacrifice subjects for extraction of the alarm substance, few studies recently have also mentioned the usage of few chemicals like hypoxanthine 3-*N*-oxide which induces the similar effect [10]. This is now trending among scientific fraternity, but this also brings an issue of unforeseen effects of this compound on ZF subjects even when exposed for a short duration of time, i.e. during behavioural assessment. Therefore, in the present chapter, we have proposed a new test named *alarm test* which is chemical free and does not involve sacrificing ZF subjects for the retrieval of alarm substance and can be used for the accurate assessment of fear and anxiety in ZFs.

## 2. Need of behavioural assessments in animal model methods

Although rats and mice are unquestionably used as the most reliable and successful models for studying any neurodegenerative diseases, there is a need to look into some other options which are cost-effective and simple like most importantly an option to replace higher mammals as subjects in research. Zebrafish, a non-mammalian species that has already been established as vertebrate development model, opens new doors for the investigation of brain mechanisms. It is an

emerging animal model for studying psychiatry and neurological behaviour. Both human and zebrafish have similarities in their psychological behaviour, because of which it becomes the most popular model for understanding the complexity of behavioural and phenotypic patterns of human brain disorders. Additionally, this species has some well-characterized features which make it cost-effective and robust. Another hope in this system is the sequence similarity of the vertebrate zebrafish with the humans.

While ZFs can be used for modelling liver cancer [11], diabetes [12, 13], cardiovascular disorders [14, 15] and other metabolic disorders [16, 17], the major contribution of this model is projected in neurodegenerative sciences. This is due to the extensive similarity of its brain structure with that of the humans [18, 19]. The incidences of neurodegenerative diseases in the growing population have increased worldwide, and it is expected that its prevalence will rise in the next few years. In different forms of neurological disorders, the most common is Alzheimer's disease which constitutes around 50–60% of the major public health issues. But still no curative treatment has been found for any of the neurodegenerative problems. For better understanding of the mechanism of action and in order to reproduce the same lesions, symptoms and causes of these pathologies, a series of animal models has been designed and used worldwide. Since the brain anatomy and behaviour of ZF and human are similar and ZFs are readily available in large numbers, therefore ZFs are favoured over other animal models. Their study can shed some light on understanding the complex human pathologies and subsequent behaviour. Since neurodegenerative diseases have a typical behavioural pattern and symptoms, therefore the ZF models are also scrutinized on the basis of their replicability at behavioural levels along with the disease pathology.

Various neurodegenerative models are established on zebrafishes through different routes, i.e. oral and intraperitoneal. Often research communities pay attention to the toxin-treated behaviour, but no one has paid attention to the regular and normal behaviour of the ZF. The present study is novel and reports for the first time the differences in the behaviour of control fishes kept in alone and herd conditions. Our study also raises a question whether these subjects' behaviour is correctly judged or not.

### **3. Existing zebrafish-related behavioural tests**

A total of four different behavioural assessment techniques were generated in our laboratory setup (**Figure 2c**). The inference from the test is also elaborated along with the test write-up. The commonly performed behavioural tests with ZFs are as follows:

#### **3.1 New/native area recognition**

The box was made of non-toxic glass and measured 30 × 22 × 22 cm. The box was divided into two equal chambers: empty area (native area) and area with plants and stones (new area). The box was kept open from above. There were two small circular openings (diameter, 4 cm) in the separator wall between the two chambers which allowed the fish to move between the two areas. A live camera was positioned above the box, which was connected to a computer so that subjects can be observed from a distance. The test ran for 10 minutes and started after the fish was placed into the new area. Activity measures (mean time spent in both areas) were hand-coded by an experimenter blind to the experimental group.

Subjects were also observed for other unusual behavioural patterns like freezing and erratic movements [20].

### 3.2 Dark and light test

The box was made of non-toxic glass and measured 30 × 22 × 22 cm. The box was divided into two equal chambers: a dark chamber and a light chamber. The box was kept open from above at the light chamber. There was a small circular opening (diameter, 4 cm) in the separator wall between the two chambers which allowed the fish to move between the light and dark partitions. A live camera was positioned above the light/dark box, which was connected to a computer so that subjects can be observed from a distance. The test ran for 10 minutes and started after the fish was placed into the light chamber. After completion of the task, the fish was returned to its test facility. Activity measures (mean time spent in both chambers) in the light and dark compartments were hand-coded during the experiment by an experimenter blind to the experimental group. Subjects were also observed for other unusual behavioural patterns [10].

### 3.3 Tank diving test

The protocol for the novel tank diving test used was modified from Egan et al. [21]. Fishes were transferred to the test tank which measured 60 × 30 × 46 cm (length × width × height). As soon as the fish was transferred to the apparatus, the live camera was started, and subject was monitored from a distance. The camera filmed the tank from the front which was already marked for three sections: bottom, middle and upper. Subjects could freely explore the tank for 10 minutes. The test was analysed on the three parameters: time spent in the bottom area; time spent in the middle area; and time spent in the upper area of the tank. Plus, the subject's frequency of shifting in all the three sections was hand-recorded separately [22].

### 3.4 Predator avoidance test

The box was made of non-toxic glass and measured 30 × 22 × 22 cm. The area was filled with water. A live camera was positioned above the chamber, which was connected to a computer so that subjects can be observed from a distance. The test ran for 10 mins and started after the fish was placed into the chamber. After 2 minutes *C. punctatus* (predator) was introduced in front of the subject so close that it can sense its presence. The behavioural activity aroused after the introduction of object, and it was monitored and comprehended. Activity measures (mean time spent near the subject/away from the subject) were hand-coded during the experiment by an experimenter blind to the experimental group. Subjects were also observed for other unusual behavioural patterns [10].

## 4. Anomalies in behavioural assessment setups

### 4.1 Comparison of models and assessment of robustness of test results

For differentiating between models and behavioural tests, 8–12-month-old wild-type ZFs (n = 90) were procured from a certified vendor. All the conditions and procedures were maintained according to the Institutional Animal Ethics Committee. ZFs were acclimatized for 2–3 months in laboratory conditions. Water

temperature was maintained at 28°C. ZFs ranged in weight from 0.3 to 0.5 g and were divided equally into three groups (n = 30) labelled as herd control (HC), isolated control (IC) and Alzheimer's disease (AD) model [23]. In both AD and HC group, ZFs were kept together in the same tank, while in the IC group, ZFs were kept individually in 30 small vessels. Standard conditions of 12 hours of light/dark cycles were maintained, and food was provided twice a day. The fishes were not collected as part of a faunal survey. Also, fishes were not harmed or sacrificed for the current experiment. The experimental conditions did not distress any subject to cause lasting harm. Predator fishes were not used for behavioural analysis neither any chemical was used for induction of fear. Graphs were prepared using GraphPad Prism 6, and statistical analysis was performed with student's t-tests (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001). The error bars represent the standard error mean.

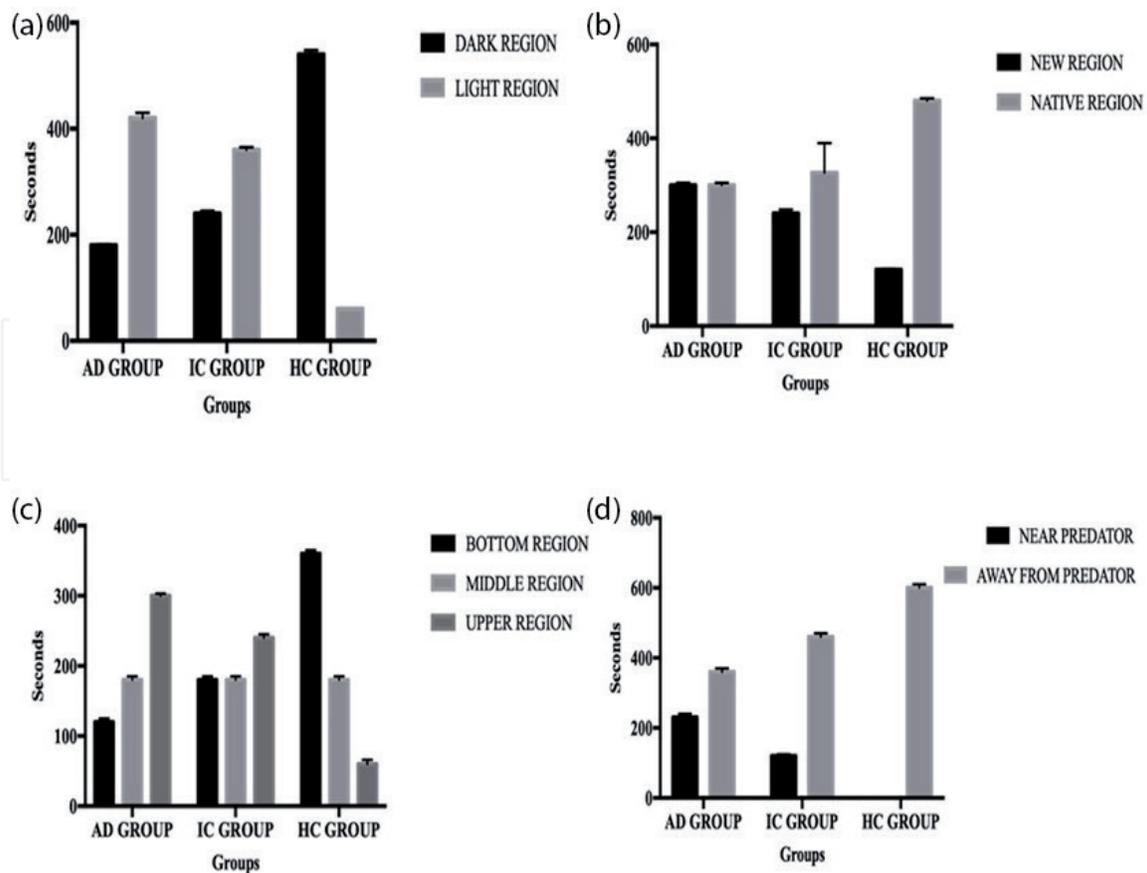
There are various ZF animal model studies that require route-dependent caging. For example, subjects dosed orally in water are kept in separate vessels, and those dosed intraperitoneally are kept together in the same vessels (**Figure 2**). During our experiments, it was observed that the control ZFs which were kept alone (IC group) for more than 3 weeks had some issues in learning and retaining feeding trainings. Their memory and cognition got worse with time, and a period came when it was very difficult to judge the differences between isolated control subjects (IC group) and cognitively impaired subjects (AD group; AlCl<sub>3</sub> induced). Thereafter special attention was given to the behaviour of IC, HC and AD groups of ZFs. The subjects were assessed on nine different parameters [20]. The following parameters were observed for 3 weeks and are discussed in **Table 1**.

It was observed that subjects kept in HC group exhibited ideal normal behaviour. Their anxiety levels were very high and different from those kept in IC group for 3 weeks. Isolated control subjects from IC group displayed less erratic movement, freezing, head-butting, thrashing, etc. During the experiment subjects were also tested for few already established behavioural tests like dark/light, new/native area, predator (near/away) and tank diving test (**Figure 3**).

We observed that isolation was causing depression in socially secluded IC group. The behavioural tests like tank diving test and novel object test gave an indication

Parameters	Isolated control (IC) group			Herd control (HC) group			Alzheimer's disease (AD) group		
	7	14	21	7	14	21	7	14	21
Anxiety	+++	++	+	+	+	+	+++	++	+
Hypo-locomotion	NA	+	++	NA	NA	NA	+	++	+++
Erratic movement	+++	++	+	NA	NA	NA	+++	++	+
Freezing	+++	+	+	NA	NA	NA	+++	++	+
Backward swimming	NA	NA	NA	NA	NA	NA	+	++	+++
Photokinesis	+++	++	+	+++	+++	+++	+++	++	+
Thrashing	+++	+	+	NA	NA	NA	+++	+	+
Adaptation to social environment	+++	++	+	NA	NA	NA	+++	++	+
Head-butting	+++	++	+	NA	NA	NA	+++	++	+

**Table 1.** Analysis of ZF behaviour when put to isolation conditions (IC) and herd conditions (HC) and in Alzheimer's disease (AD) model.



**Figure 3.** Behavioural tests conducted between herd control (HC), isolated control (IC) and Alzheimer’s disease (AD) model group. (a) Dark/light test, (b) native/new area test, (c) tank diving test, (d) predator avoidance test.

that the behaviour of isolated subjects was almost like the AD model ZF’s. For example, the ideal behaviour of ZFs in tank diving test is to swim at the bottom, i.e. away from predator. AD model ZFs are generally found at the upper region of the tank since they do not feel fear as the disease progresses (**Figure 3c**). The similar pattern was observed for isolated subjects of IC group. Likewise, when IC and AD groups were compared using predator avoidance test, both IC and AD groups were very much alike (**Figure 3d**). Similarly, both IC and AD groups were unable to locate their native area in new/native area test (**Figure 3b**). Also, during light and dark test, there was not much difference between IC and AD group (**Figure 3a**). These findings indicate that if someone setups an experiment for assessing the behaviour of neurodegenerative and cognitively impaired ZF subjects and then if controls are also kept in isolation, then it will be difficult to categorize them based on the behavioural results, and the results obtained from such experiments will be misleading.

There was one more critical observation: i.e. usually the test setups published worldwide have a single test apparatus wherein all the fishes are tested for behavioural parameters. Typically, the water is never changed in that apparatus during an experiment. Interestingly, during behavioural assessment it was observed that fishes have a special way of communicating. Every time a new fish from HC group was added to the test apparatus (light/dark, new/native, etc.), their ideal behaviour changed dramatically. The behaviour of the first few fish in all the tests was exhibiting their ideal behaviour, i.e. extreme thrashing and anxiety, while the fishes (from same group: herd control (HC)) added later to the same test were showing different behaviour, i.e. remaining still at one location for 2–3 minutes and did not move much. Although these subjects are from cognitively alert group, i.e. HC

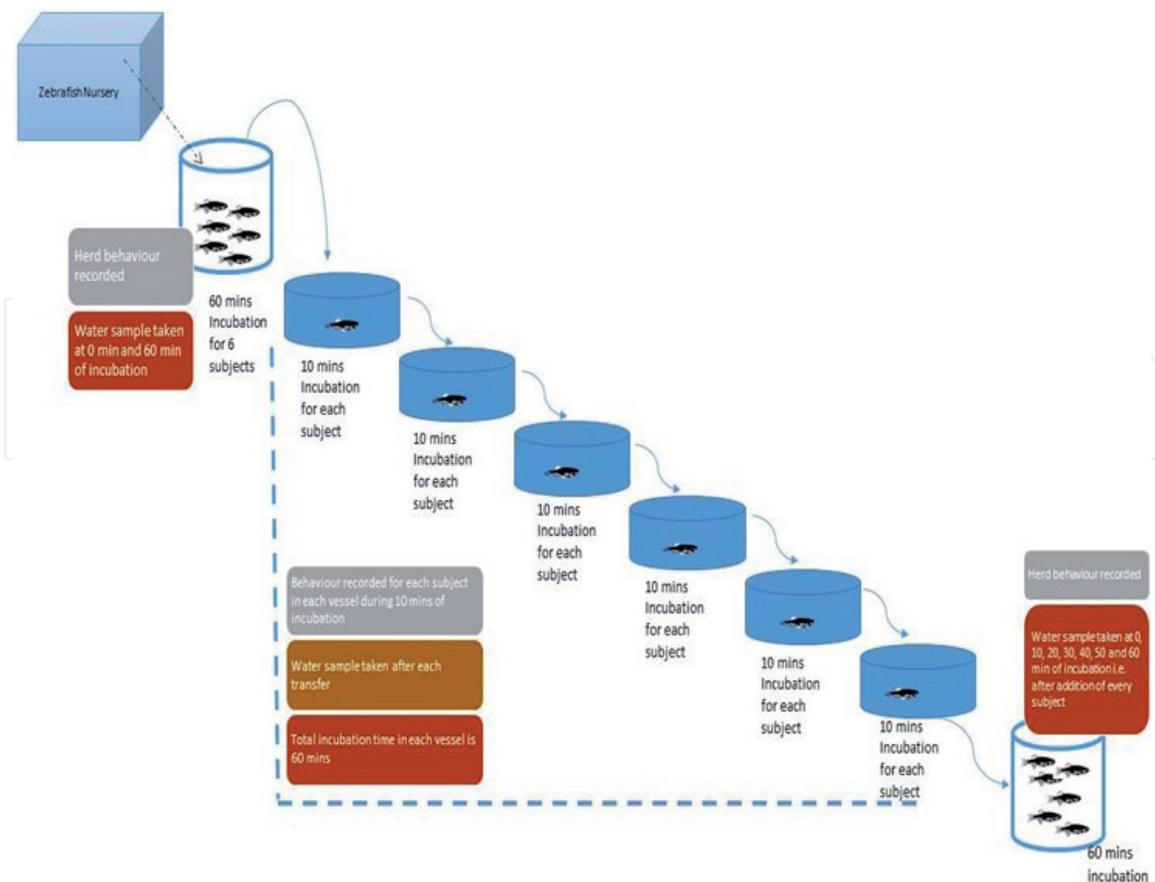
Group, even then, their behaviour was not close to their ideal behaviour. Therefore, an inference was made that the first few fishes released something in the water which was interfering with the behaviour of other fishes. These results prompted our study to formulate a new test for understanding this unusual behaviour.

This new test is called as “alarm test”, and it was utilized to answer few issues invoked during the study. The first issue was to differentiate correctly between cognitively impaired subjects and socially isolated subjects. The second was to evaluate the presence and potency of the alarm substance, and the last was to emphasize on changing the water from behavioural setups after introduction of each fish during behavioural assessment.

## 5. Making of the novel “alarm test”

### 5.1 Collection of alarm substance

A test setup was created (**Figure 4**) for collection of test-water. It was done by adding ZFs one by one in the vessels. It was observed that the first fish to enter in the first vessel exhibited lot of anxiety-provoked thrashing and erratic swimming. But the next fish after the first one displayed lesser anxiety and was more attentive than the first one. The silence in their behaviour grew with each added subject in that vessel. It was concluded that every fish which entered the vessel was leaving some sort of signal in the water that was alarming them for any danger. The silence in their behaviour was not calmness; instead, it was the fear of a probable predator as they displayed extreme stillness and jumping movements simultaneously.



**Figure 4.** Illustrative diagram on devising a protocol for collection of alarm substance in test water from ZFs.

## 5.2 Alarm test setup

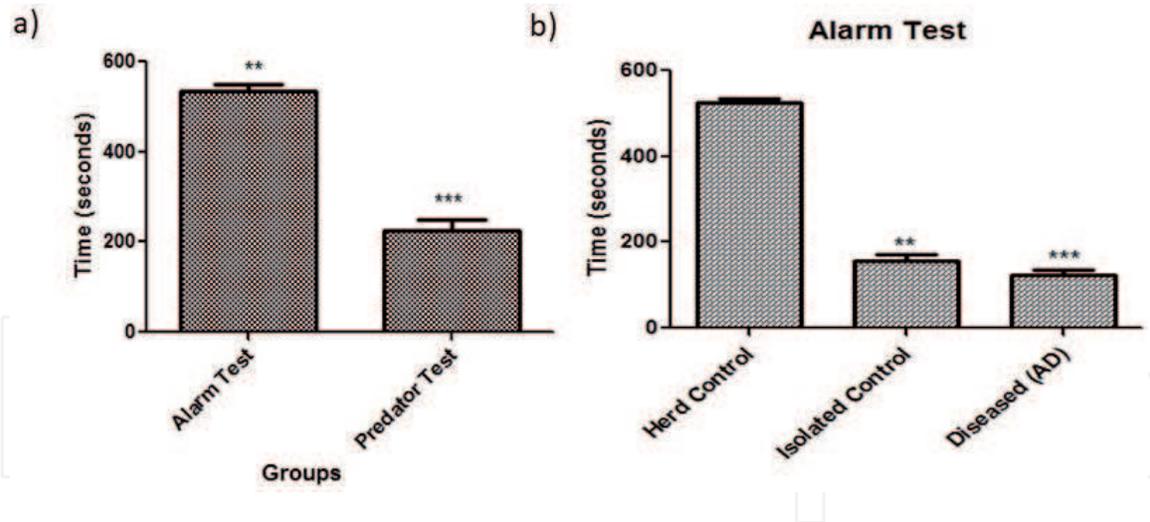
A new test was formulated to understand how zebrafishes communicate fear and anxiety with each other. Their behaviour was extensively studied by setting-up a test protocol which comprised of two big glass vessels (water capacity: 1 litre) (14.5 × 10.5 × 14.5 cm) and six small vessels (water capacity: 100 ml) (10 × 10 × 10 cm). The vessels were kept open from above. Two big vessels were filled with 400 ml water and small vessels had 50 ml water each. Less water was kept for inducing stress to the subject (both space and new area stress) (Figure 4).

## 5.3 Alarm test protocol

The test ran for 130 minutes and started after six fishes were placed into the first big vessel one by one (Figure 4). The fishes were added to the first big vessel in the interval of 10 minutes each, and water sample was collected consecutively. After 60 minutes of incubation, one subject was transferred to the first small vessel and kept there for 10 minutes. Later it was taken out and transferred to the second small vessel and likewise was left in each of the six small vessels for 10 minutes each. Eventually the first subject was transferred to the last big vessel and rested there till all the ZFs reach the last big vessel. Similar steps were followed for rest of the five fishes. Every time a fish was transferred from one vessel to the other, the water sample was collected, and subjects' behaviour was documented. The water sample collected from all the vessels in short intervals of 10 minutes was later stored and was labelled as test-water. The similar protocol was repeated five times. Thereafter to examine the test-water, the new set of ZFs was taken from the nursery and was put to two different conditions. One subject was put in the first vessel with fresh water, and the other was put in the second vessel with test-water. The water in the first vessel was changed for every new fish, while the second vessel with test-water was not changed. Results were replicated with random subjects from the nursery, and it was confirmed that the test-water was able to induce fear-like symptoms in ZFs.

## 6. Advantage of alarm test over other tests

The alarm test was later compared to the predator avoidance test. For that we took two separate vessels. The first vessel was similar to the predator avoidance test, and the second vessel had test-water. The ZFs in the first vessel exhibited thrashing and maintained distance from the predator, but after analysing the subject for a few minutes, it became normal. However in the second vessel, the ZF subject did not show any thrashing and head-butting; instead, the subject displayed increased alertness and fear symptoms, i.e. the subject was extremely silent and was pinned at one location immediately after putting in the water. It continued for approximately 10 minutes, and after that it acclimatized in the environment. The similar test was repeated for HC, IC and AD group. It was observed that the HC group responded to both predator and alarm test very well. The IC group did not respond very well for predator test (*C. punctatus*) but was able to respond equally well for alarm test. On the contrary, the AD group did not respond to either predator (*C. punctatus*) or alarm test. Therefore, this test-water was proven to be useful in detecting cognitively impaired subjects over socially isolated subjects (Figure 5).



**Figure 5.**

(a) Comparison between alarm test and predator test. (b) Differences in sensitivity of alarm test for AD (Alzheimer's disease), IC (isolated control) and HC (herd control) models ( $P < 0.01^{**}$ ) ( $P < 0.001^{***}$ ).

Lastly, it is very important to note that research groups which work on ZF behavioural analysis must change the water after each testing as this might interfere with the ideal behaviour of ZF.

## 7. Conclusion

Behavioural study is one of the best approaches to identify the changes occurring in the body. Expanding the scope of behavioural tests is an imperative process in this area [9, 21]. In addition, ZF is presently developing as a helpful model for studying neurobehavioural changes, including typical and neurotic conditions. It shows anxiety-like behaviour, thrashing, head-butting and changes in its conducts in response to any external stimuli. Since ZF is a social animal, it is always preferred to remain in herd [24]. Our work is in accordance with this information as we are reporting behavioural changes of control ZF in herd and in isolated conditions. The isolated fishes were showing behaviour similar to ZFs from the AD model. So, it is advisable to keep them in herd while establishing any pathological model, as isolation can severely induce depression even to normal fishes used as controls which can give faulty results. Since many at times it is not feasible with every experiment to keep ZFs in herd, we have devised a new test to be called as alarm rest for behavioural analysis. It is a chemical-free test for analysing the ideal behaviour of ZFs. This test was equally effective for the fishes kept in either isolated or in herd conditions.

There are studies which suggest that fishes have the tendency to release cortisol and chondroitin sulphate in stress conditions [5]. One study has reported that chondroitin triggers fear behaviour in ZFs [25]. This substance released by one fish acts as an alarming sign for the rest against any danger. Similarly, another study by Ramsay et al. [26] had used cortisol as a parameter to evaluate the stress response in ZF [27, 28]. They reported that crowded conditions help in optimizing the health, growth and reproduction abilities of ZF. This is optimum for their overall growth. Keeping ZFs in herd conditions is a good thing, but when it comes to behavioural test, the fishes are assessed individually for all parameters. In the present work, we have observed that the substance released by the first few fishes in any behavioural test tanks interferes and alters the behaviour of the next fishes in the row. Therefore, it is advisable to keep changing the water in the vessels during tests (native/new, predator avoidance, etc.) in an experiment. This observation is novel and an intimate

scientific fraternity about the limitations and errors during the behavioural assessment of animal models.

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