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Increasing Zebrafish (*Danio rerio*) Numbers in a Limited Tank Space Reduces Night-Time Fish Sleep-Like State and Induces Aggressive Behaviour

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Abstract

Inadequate sleep, besides decreasing consolidation of learning and memory, is also known to be associated with various major mental illnesses such as anxiety, psychotic disorders, mood, distress, anger, and paranoia. The studies of inhabitants of crowded city with limited space also showed similar increase in major mental illness. However, reports on the possibility of inadequate sleep contribution to heightening major mental illness in crowded urban areas with limited space are scarce. Using Zebrafish (*Danio rerio*) as the animal model, this investigation showed that sleep-like state (SLS) duration decreased linearly with increasing population in a limited fish tank space. Increase in fish numbers causes ever increased struggling for preferential sleeping space, resulting in the generation of aggressive and depressive behaviour. Isolating individuals from groups also causes increased anxiety followed by depression. Zebrafish SLS needed maintaining limited distance from one another. However, increasing fish numbers increases invasion of individuals' space leading to shorter average and lower total SLS during night-time. Besides depending on fish numbers, duration of SLS also depends on air quantity, presence of drugs that decrease or stabilize motor-neuron coordination. Drugs such as alcohol decreased SLS while melatonin stabilized it. This investigation, therefore, clearly suggests the applicability of using Zebrafish as an animal model in understanding the increasing mental illness in limited space urban cities.

Keywords: Zebrafish; Sleep; Population; Alcohol; Melatonin; Aggressive

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Introduction

Sleep is a naturally recurring neurological phenomenon characterized by reversible consciousness obtained after progressive reverse of sensory and voluntary muscles excitation. Sleep lowers sensory neuron communication with the surrounding environments. In contrast, wakefulness is a sleep-like state (SLS) but the individual displays alertness and engages in coherent cognitive communication with the external world. SLS is an easily reversible state compared to the state of being comatose [1]. Sleep is an evolutionarily conserved behaviour and its mechanism and functions are not yet fully understood [2]. It is generally accepted that sleep plays an important role in consolidating learning and memory [3]. Lack of proper sleep is shown to impair performance in cognitive tasks, decreased attention and reduced responsiveness to external stimuli as well as altered locomotion [2]. Sleep deprivation could be induced by many factors such as over consumption of caffeine-containing foods, or external factors like noise. The recent explosion of population and increasing settlements in a limited space could also contribute to depriving sleep. In fact, one report showed that frequent inadequate sleep increased in a linear fashion with the number of individuals in the household [4]. There have been reports of association between population growths and increasing risk of major mental illnesses such as anxiety, psychosis, mood, distress, anger, and paranoia [5]. However, the contribution of inadequate sleep manifested behaviour changes in crowded household is not clearly defined. Recently, Zebrafish (*Danio rerio*), has become a valuable animal model for studying sleep related neurological disorders. Zebrafish have been shown to possess characteristics of SLS diurnal circadian rhythm [6]. According to Yokogawa et al. [7] any period of immobility lasting longer than 6 seconds is defined as Zebrafish sleep. Zebrafish clock and sleep-related genes, similar to mammal's hypocretin/orexin system have been cloned and its roles in rhythmic behavioural projection show similarity to that of mammals [8]. Further, drugs that alter mammalian sleep have been shown to have a similar impact on Zebrafish sleep [6]. Therefore, current investigation analysed the pattern of Zebrafish night-time SLS behaviour and how this behaviour is affected by fish numbers in limited

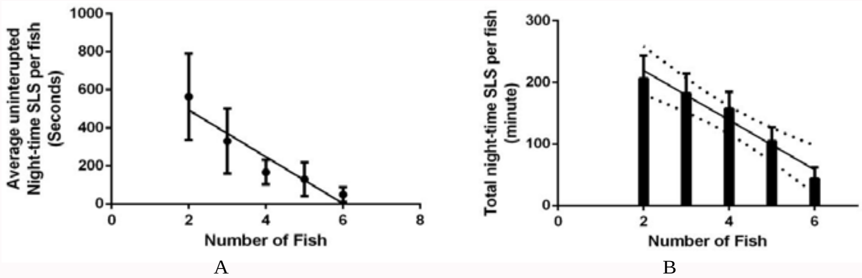

Figure 1: The SLS of Zebrafish negatively impacted by the number of fish in a limited tank space. (A) Average uninterrupted SLS during night-time decreased linearly with increasing fish number (Y=-122.8*X+739.4; at 95% confidence interval, R=0.9079). (B) Total SLS during night-time decreased linearly with increasing fish number ((Y=-40.30*X+300.2; at 95% confidence interval, R square=0.9550). Each point represents means ± SDS of three replicate experiments.

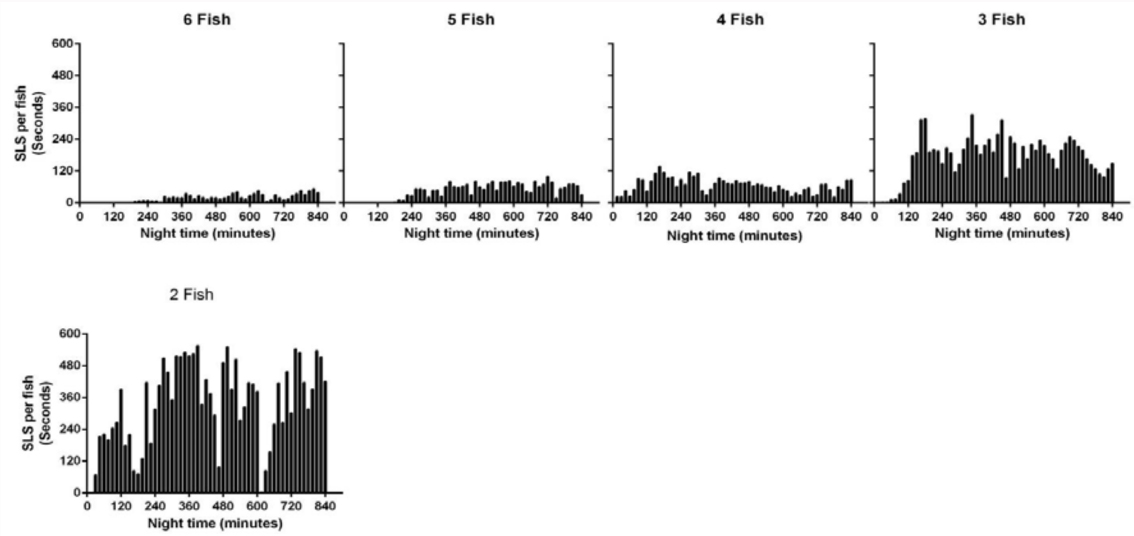

Figure 2: Representative night-time SLS profiles of different fish number in a Novel tank of size 47 cm × 6.5 cm × 30 cm with water to air interface surface of 305.5 cm². Each bar represents the total cumulative SLS (lowest inactivity's for more than 6-second) in 15 minutes of night-time.

water to air surface tank. This observation indicated that average and total night-time SLS duration decrease linearly with increasing fish numbers in the same tank. Proper oxygenation of the fish tank, however, showed slight improvement of total SLS duration. In a populated fish tank, before the onset of sleep, fish showed an intense struggle (aggressive chasing) for occupying the water-air interface of the tanks. These behaviours did not lessen in the consecutive days of observation if provided the same fish numbers in the tank. Fish with dominant character (winner in the chasing) always occupied positions near the water-air interface, while others occupied the bottom half of the tanks. During SLS, the fish always occupied a constant distance from the other fish and the maintenance of this constant distance is critical in fish sleep. This in turn depends on the neurological coordination and stability. Melatonin, the natural endogenous inducer of sleep, stabilized sleep neurological coordination while alcohol reduced it.

Methodology

Healthy adult zebrafish showing normal swimming and feeding were selected and maintained at optimum conditions under a normal light/dark (14/10) cycle. The selected fish were housed 6 fish/6 L in a tank [size 30 cm × 20 cm × 7 cm (water to air interface surface of 600 cm²)]. For recording sleep, all the 6 fish were transfer to the Novel tank [(size 47 cm × 6.5 cm × 30 cm) and water to air interface surface of 305.5 cm²]. The daytime recording started with the help of a CCD camera fitted in front of the tank connected to a desktop computer, using the iSpy software at 10 frame/second for 10 hours from 9 AM to 7 PM. Fish were fed twice, once at 10 AM and again at 5 PM. During the daytime recording, the conditions ensured that fish had less stress. At 6 pm the Novel tank was cleaned to remove the uneaten food particles and 80% of the tank water was replaced with fresh fish water of conductivity of ~1,500 µS/cm, and pH ~7. The oxygen saturation of the recording tank was maintained at 8.0 mg/L with the use of air stone connected to an air pump and the temperature at 28°C. At 7 pm, a night-light (black light) was turned on and daytime white light turned off with the help of an automatic on and off timer regulator. Night-time recording (10 frames/second) was set for 14-hour, which included 2-hour before completely turning on the night-light through progressive dimming of the day-light. Night-time recording also included 2-hour after the night-light was completely turned off through progressive brightening of day-light. All recordings were carried out in a closed room to avoid any external influences and disturbances. The same procedure continued except, one fish was removed before the onset of night-time recording in the consecutive days. The experiments were repeated three times, each time with a different set of six fish. A similar procedure was applied for night-time recording under various conditions, that is, after reducing the air supply from the air pump, or in the presence of 0.5%

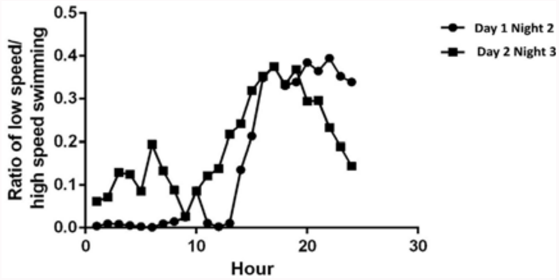

Figure 3: The profiles of 24 hours swimming behaviour in isolated single Zebrafish from its group during two and three consecutive daytimes and night-times. Each value represents the ratio of slow swimming distance (0.3 cm in 6-second) to fast swimming distance (above 0.3 cm in 6-second) in an hour.

(v/v) alcohol or 1 mg/L melatonin (from Nature's Bounty Inc, USA). The analysis of the recorded movies was carried out with NIH Image J software, wrMTrck plugin. For calculating the total SLS duration, all the times in which fish swam uninterrupted at maximum speed of 0.3 cm/6 seconds were computed together. Statistical comparison of significance was carried out using Microsoft Excel Single Factor ANOVA.

Results and Discussion

Zebrafish SLS decreased linearly with increasing fish number in the tank

Since SLS in Zebrafish is defined as any period of minimum swimming distance lasting longer than 6 seconds, the uninterrupted minimum swimming distance (0.3 cm/6 seconds) were tracked and computed together. As can be seen from Figure 1A, Zebrafish uninterrupted SLS became shorter and shorter with the increasing fish number in a Novel tank containing 6L of fish-water with a limited water-air interface surface of 300 cm^2. For 6 fish in the same tank, the average uninterrupted SLS was approximately one minute and the total SLS duration was around 40 minutes during the entire 14-hours of night-time recording (Figure 1B). Analysis of night-time SLS for every 15 minutes from the onset of the night-light, showed a wave rhythmic pattern that became more visible and larger with the decrease in fish number. The frequency of the wave rhythmic pattern was much higher with the increase in fish number (Figure 2). Therefore, the results clearly suggest, with the increase in fish number in a fixed limited space, the frequency of exit from the SLS also increased correspondingly.

Since recording sleep was carried out by removing one fish at a time from the group of 6 every day before the night-light turn-on, it was expected that last fish should have higher SLS average. However, no SLS was visible from the last fish during the first night. One of the probable reasons for this was the generation of anxiety due to loss of shoaling. It has been reported earlier that removal of a single hungry fish from their shoaling fail to show feeding response, it instead showed high-speed escape swimming behaviour [9]. Unlike earlier observation, complete lack of SLS from the lone fish indicated that the night-light induced anxiety instead of stimulating calming effects. Lack of SLS continued in the first daylight of the single fish, but in second night, the fish showed more pronounced SLS with no or negligible night wave rhythmic SLS (Figure 3). In the second daylight however, fish showed smaller wave of SLS with a sharp increase and faster decrease in third night-time SLS. The same behaviour was visible in the third daytime and fourth night-time (data not included) and was reproducible. Available reports suggest that zebrafish sleep is suppressed by light and melatonin hormone, while the hypocretin/orexin system only regulates sleep at night [10]. A second report suggests that isolated shoaling zebrafish undergo depression-like behaviour, in which fish stop swimming and stay still for long periods [11]. Stoppage of swimming for a few seconds to minutes often could be observed in shoaling zebrafish but under the non-aggressive conditions and were experimentally non-reproducible. Increased anxiety during the first night by the last fish left without any shoaling partners, and appearance of SLS in the second and following days were however reproducible. These results therefore strongly agree with the social signal transduction theory of depression, suggesting

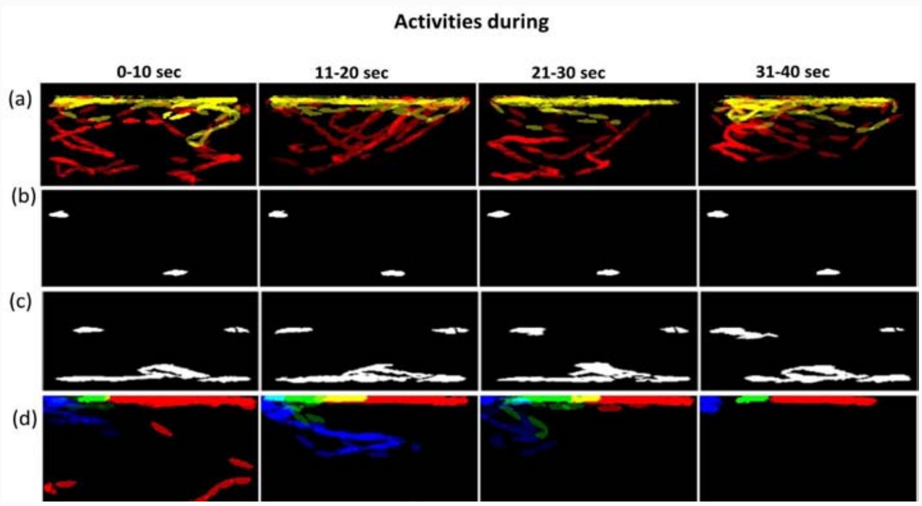

Figure 4: The representative sum image of Zebrafish behaviour (each image represent behaviour in 10-second) during night-time.
a) Struggle for selecting prefer zone in the tanks. Fish at water-air interface (yellow) vigorously defending its space from encroachment from the bottom fish (red).
b) Relatively calm periods with long duration of SLS.
c) Water-air interface fish maintaining theirs SLS while bottom fish showed frequent interruption in their SLS.
d) Migration of bottom fish (red) to the top half of the tank and evade the space of water-air interface fish-green and blue fish. Disturbance of green fish disturbed the blue and started a chain of new struggle for prefer space.

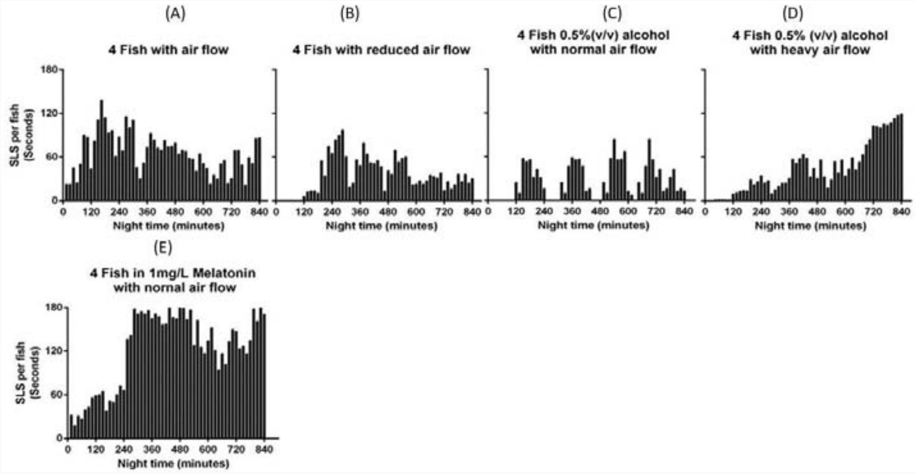

Figure 5: Effect of (A) normal airflow, (B) reduced airflow, (C) 0.5 % (v/v) alcohol at normal airflow, (D) 0.5% (v/v) alcohol at heavy airflow and (E) 1 mg/L melatonin with normal airflow on Zebrafish night-time SLS. Representative night-time SLS profiles of different population of fish in a Novel tank of size 47 cm × 6.5 cm × 30 cm with water to air interface surface area of 305.5 cm^2. Each bar represents the total accumulative SLS (lowest inactivity's for more than 6 second) in 15-minutes of night-time.

anxiety leads to depression disorder [12].

Onset of zebrafish SLS in a group follows sequential repeatable events

The first event was occupying the preferred zone in the tank, usually, the water-air interface. The fish near the water-air interface showed a strong tendency to defend its territory [Figure 4a, yellow fish] against the approaching fish [Figure 4a, red fish], which led to extreme chasing and fighting. The winner (dominant) took the position near the water-air interface whereas the weaker took the position at the bottom half of the tank (Figure 4b). Usually large size fish, either male or female, occupied the water-air interface while smaller sizes occupied bottom half of the tank. The struggle took much longer under minimum air supply from the air-pump into the tank. If the fish were of equal dominant characters, they occupied near the water-air interface but maintained equal distance from each other [Figure 4c-top fish] and they also showed higher average SLS. In contrast, bottom fish have lower average SLS because of their inability to keep distance from one another [Figure 4c-bottom fish]. Furthermore, bottom fish had a high tendency to move to the water-air interface, leading to disturbance in the SLS of water-air interface fish. The whole sequential event repeated many times creating wave-like rhythmic SLS. Before the onset of daylight however, the whole set of events reversed, in which water-air interface fish showed increased tendency to move down to the bottom, and the bottom fish aggressively defends its position. This behaviour continued even 2-hour after the onset of daylight. Use of large size females and small size males in the group resulted into courtship like behaviour. This was because of the increased aggressive nature of small males before the onset of daylight that occupied the bottom half of the tank during the whole night-time with lowered average SLS. Such courtship behaviour has been reported earlier [13]. Dominant female and small sized males however, showed little success in reproductive efficiency unlike dominant male and small size females. These studies therefore agree with the male dominant dependent reproductive success [14,15]. However, contribution of reproductive success by non-dominant male condition by increasing aggressive behaviour could not be ruled out. One report showed even 12 fish/L water did not effectively reduce reproductive efficiency [16]. Our results also clearly agreed with such possibility of translating increasing aggressive behaviour into courting behaviour.

Maintaining SLS longer, depends on many factors

Duration of SLS in the tank with a group of fish not only depends on keeping the distances between the fish but also depends on other factors. When given fresh fish water, but if oxygen saturation level reduced to 7.0 mg/L from 8.0 mg/L, the duration of SLS is also heavily reduced (Figure 5B) when compared with fish in 8.0 mg/L oxygen saturated water (Figure 5A). Close analysis showed that fish at the water-air interface were unaffected. However, bottom dwelling fish became more aggressive and the frequency of their coming to the water-air interface greatly increased, leading to disturbance of water-air interface fish. In the presence of continuous daylight, fish distribution took a bell-shape curve with time [figure not included] at the upper top 25% of the tank when oxygen saturation level were maintained at 7.0 mg/L and 8.0 mg/L respectively. Nearly 90% to 98% of fish occupied at the upper top 25% of the tank and mostly at the water-air interface in both of the oxygen saturated conditions. The activities lasted four 4 hours in the case of 7.0 mg/L oxygen saturated water compared to nearly 2 hours in the case of 8.0 mg/L oxygen saturated water. However its occurrence in both water conditions, corresponded to the timing of 2 hours after switching off of daylight and switching on of nightlight under normal housing conditions. No aggressive behaviour was observable during these periods and no SLS observed. If the daylight switch was off within this 4-hours and night light switched on, the aggressive swimming and chasing activities initiated with slow appearances of SLS. This observation clearly suggests that reduction of water oxygen saturation level increases the fish migration from bottom to the water-air interface of the tank. However, induction of observable SLS took only after switching off of daylight. Delaying the daylight switch off did not reduce the aggressive competition for preferred spaced but reduced total night-time SLS.

Replacing fish water with fish water containing 0.5% (v/v) alcohol greatly reduced SLS at normal air supply (Figure 5C). Treatment of fish with the same alcohol concentration continuously for 9 weeks has been reported earlier without significant reduction of fish health [17].

Treatment of fish with the same concentration of alcohol was also shown to produce significant different in their color preference in T-maize tank [9] and place preference in Novel tank [18]. Similarly, under current investigation alcohol not only affected the bottom fish, but also the water-air interface fish. Alcohol caused short resting type SLS with spike of arousal with sudden high-speed swimming, leading to increased encroaching of each other's space. These observations were contrary to the effect of alcohol on the sleep-awake state in rats, which suggested alcohol has hypnotic (increased sleep-maintaining) effects [19]. In fish water without alcohol, SLS can be visually identified by the dropping tail and longer SLS, associated with slow movement but with minimum displacement from original position. In the case of 0.5% (v/v) alcohol, the swim speeds were slow and SLS appeared like that of resting bouts (define by shorter inactivity duration and longer arousal state) [20]. In contrast, increasing air supply above the normal level, increases the SLS in a time dependent manner with longer SLS 2 to 3 hours before the daylight switch on [Figure 5D]. Alcohol is a known inhibitor of motor-neuron coordination and it is also responsible for aggravated sleep related breathing disorder [21,22]. SLS in Zebrafish therefore appears to not only be controlled by motor-neuron coordination, but also influenced by oxygen saturation level of fish water.

In contrast, melatonin which was reported to suppress Zebrafish sleep in the presence of light [10], showed robust increase and stable SLS when given during the night-time under the night-light (Figure 5E). This study also strongly supports the earlier findings that melatonin causes improvement of homeostatic control of sleep, and sleep consolidation [23,24]. Melatonin, a natural hormone produced by pineal gland, is a major sleep-inducing hormone in zebrafish and is therefore, unlikely to perturb the motor-neuron coordination required during the Zebrafish SLS [24].

Conclusion

Understanding of sleep deprivation in relation to ever increasing mental illness in a crowded urban area with limited space is critically important. Using Zebrafish as the animal model in the current investigation demonstrated that sleep is heavily affected by the number of fish in limited space, air quality, and drugs that influence motor-neuronal coordination and stability. Melatonin, the natural endogenous sleep inducer stabilized sleep through strengthening neurological coordination while alcohol reduced it. Increasing fish number in a limited space caused intense aggressive behaviour for securing preferential space during night-time. Fish that submit to the dominant fish during night-time display reverse and heightened aggressive behaviour during day-time. Isolation of a single fish from their habitual groups led to anxiety followed by depression leading to increased sleep duration both in day and night-time. Therefore, study of sleep using zebrafish as animal model, effectively replicated the findings in human as reported earlier [4,25].

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Author's Contribution

Mohammad Kutub Ali: Research supervisor and coordinator, Protocols designer, experimenter, data analysis and manuscript drafting.

Hasina Lateefah Nicholson: Participated during experimentation and discussion as part of a master's thesis, at the Department of Basic Medical Science, The University of the West Indies, Mona campus, Kingston, Jamaica.

Ethics

This research was conducted after the approval of ethic committee Department of Basic Medical Science, The University of the West Indies Mona campus, Kingston, Jamaica.

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