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Improved cognitive functions and behavioural response after exposure to low-level near-infrared laser in snails (*Ariophanta laevipes*)

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Abstract
Terrestrial pulmonates possess photosensitive neurons in their brain that may possibly influence several cognitive functions and therefore can be apt to study the effect of light on cognition and behavioural response. In this paper, we report results from cognition based T-maze studies on pulmonate snail, *Ariophanta laevipes*. Cognitive abilities of the snails were established as, time taken to complete the maze pre and post subjection of the brain to low level near-infrared laser for a period of 5 and 10 days. A significant change in the cognitive abilities and behaviour was observed in snails exposed for 5 days to low level near –infrared laser at a wavelength of 650 $\pm$ 10 nm, in comparison to the control and experimental groups. The results of the studies confirmed that low level light at near infrared wavelengths plays an important role in cognition abilities like memory-formation/retention and learning.

Keywords: Low-level near-infrared laser, photobiomodulation, cognition, snail

Introduction

Low level light therapy or photobiomodulation is a therapy that utilizes light in the far-red to near-infrared region of the spectrum (630 – 1000 nm) as a non invasive treatment procedure for numerous health based disorders [1]. Low level light therapy is known to show a biphasic dose response which is highly dependent on the depth of tissue infiltration by near-infrared light and is proportional to the absorption and scattering coefficients [2]. Defined action spectra's have been constructed for specific cellular light-based responses which confirm that light absorption within the tissue happens through molecular chromophores [3]. In a recent study conducted in mice, it was confirmed that near-infrared light is highly neuroprotective and due to its non toxic effects in the brain it can be applied intracranially to cure certain neuron-based disorders [4].

Cognition is a mental process involved with acquisition, processing retention and use of information, which engages the numerous electrophysiological, neurochemical, neuropsychological and biochemical processes of the neurons in the brain [5]. The brain as we know differs in shape, size and cellular complexity from invertebrates to vertebrates but in relation to behaviour, memory and learning there is always some sort of similarity. Behavioural studies in invertebrates such as molluscs are usually specific to food and mating and therefore there may be a good probability that their cognitive abilities would be dependent on their behaviours [6, 7]. Molluscs have been useful models in basic neuroscience research [8] but studies have been very limited in the field of neurobehavioral science and cognition. Sound and music in the form of meditative chants are already known to augment the cognitive capacity of the brain in snails, which has been demonstrated in earlier studies [9, 10, 11, 12]. A recent study, demonstrates the effect of frequencies generated by tuning forks on the cognitive capabilities of snails [13].

Photosensitive neurons that are highly sensitive to light exist in the central ganglia of the brain of several molluscs, besides the photoacceptor cells in the bilateral eyes [14]. The effect of light on these photo excitable neurons studied in snail, *Helix aspersa*, has shown a direct correlation to physiological functions such as growth and sexual maturation [15]. Long wavelength light is known to stimulate energy metabolism and energy production through photo acceptor molecules present in tissues [16]. Mitochondrial respiration via photoneuromodulation of cytochrome oxidase activity in the brain is a known therapeutic target for neuroprotection and cognitive enhancement [17]. Till date there are several studies on the therapeutic capabilities of low- level near-infrared light but there are very few studies that demonstrate its capabilities in cognition and behaviour [18, 19, 20]. There also seems to be very little information on cognition and behaviour in molluscs and its comparison to vertebrate cognition. The intention of this study was to validate whether low level near-infrared light enhances cognitive abilities and behavioural responses in the brain, which may be non-specific in terms of species with neuronal tissue but may differ in mechanisms from invertebrates to vertebrates.

T-Maze based studies, has helped understand the reasons for cognitive abilities and cognitive dysfunctioning in animals [21] and therefore was chosen as a model in this study, to assess cognitive abilities of the snails. T-maze is a variant form of Y-maze that is widely used to evaluate cognitive and motor capabilities in animals and has helped understand numerous cognitive based behaviours that subsist in animals [22]. The present T-maze study was designed to justify whether there exists a difference in cognitive abilities and behaviours within a known population of snails. The study was also used to assess the exposure of low level near-infrared laser on brain and whether it can enhance cognitive abilities and behaviours in snails. The results of this study would help determine the importance of light in the life of these snails and could also be extrapolated as suggested therapy in veterinary science.

Materials and Methods

Collection and Maintenance

Pre Study 1

Ariophanta laevipes snails were collected from Sanjay Gandhi National Park, Mumbai, India (19.2500° N, 72.9167° E) in the month of July 2016. All studies were carried from 16th July to 25th August 2016 and were carried out in a household setup. The snails were not segregated as per sex but were acclimatized for a period of 2 weeks as a group before Study 1. During acclimatization, snails were placed in well ventilated and hydrated PVC plastic boxes that were maintained on a 12: 12 light: dark schedule (7.00 am: 7.00 pm) at room temperature (30 - 33°C). The snails were fed daily on lettuce leaves *ad libitum* which were procured from the local market and was thoroughly washed and cleaned before providing as feed. The PVC plastic housing was washed and cleaned on a daily basis during which the snails were moved into a similar housing apparatus.

Post Study 1

Based on the results from Study 1, 12 snails were randomly divided into control and experimental groups of 6 snails each. For both groups, the snails were placed in well ventilated and hydrated compartments pre-designed in PVC plastic boxes with each compartment measuring 6 cm x 2.5 cm x 6 cm. The housing was designed in such a way that the barrier between the two compartments was opaque and prevented the snails from interacting with each other. This housing format was uniform for both control and experimental groups. Snails of both the groups were fed daily on lettuce leaves *ad libitum* and the PVC based compartments for each of the snails were washed and cleaned on a daily basis. Snails were acclimatized in this setup for a period of 3 weeks prior to Study 2 and were maintained on a 12:12 light: dark schedule (7.00 am: 7.00 pm) at room temperature (30 – 33 °C).

Study 1, 2, 3 and 4

The T-maze runs were carried out during the day, from 6.00 am – 2.00 pm and during the study the T-maze was kept well hydrated and well ventilated. The studies were carried out in a household setup at room temperature (30 - 33°C) and the maze was thoroughly washed after each snail run in order to reduce the interference of mucus trail-following behaviour in the snails. Mucus trail-following is a known behaviour observed in snails that is used for activities such as homing, grouping and reproduction in snails [23, 24]. Post the runs in Study 2, 3 and 4, the snails were placed in their individual compartments and were fed fresh lettuce *ad libitum*. After completion of all the T-maze based studies, the snails were released at the collection area in the Sanjay Gandhi National Park, Mumbai, India (19.2500° N, 72.9167° E).

Equipment

T-Maze

A self-designed, enclosed, well ventilated and hydrated PVC plastic T-maze, with start and goal arms measuring 6 cm x 3 cm x 3 cm was used to conduct the experiments. The dimensions of all the arms in the T-maze were kept similar for both control and experimental groups (Figure 1). The T-maze was designed based on some of the T-mazes designed to study rat behaviour [25].

Laser Source

The source for the low-level near-infrared laser light was a classic pen-sized laser pointer with a maximum output power of < 5 mW and a wavelength of 630 $\pm$ 10 nm. Lower wavelength lasers of approximately 630 nm have shown therapeutic effects and therefore this wavelength was chosen for the study [20]. The light source for this laser as described by the manufacturer was a 5630smd led, with a luminous flux of 270 - 300. The pen-sized laser pointer was manufactured by Guangzhou Nantian Sources Co., Ltd, China and was procured online from eBay, India. This pen-sized laser was classified under Class IIIa category of lasers and therefore sun glasses were worn as a mode of protection for the eyes during the exposure period for all the studies.

Data Analysis

ANOVA Two factor without replication and Student T test were some of the statistical tests used to determine the significance and variation of the data obtained during the study. Significance was determined and confirmed using the F, F critical and P values with the significance level maintained at $p \leq 0.05$ and F > F critical.

Procedure and Analysis: Study 1

The study was conducted on 40 snails that were acclimatized in a group, for a period of 2 weeks. The snails were starved for a period of 12 hrs before the experiment. 10 snails were randomly selected and placed in 4 groups, and the run was conducted for each of the groups. During each run, the group of 10 snails were placed at the starting arm of the T-maze with fresh lettuce placed in the right-hand side goal arm, as a reward. For each run, the start time was recorded on placing the 10 snails in the starting arm at the starting point and the end time was recorded when the snails would reach their reward. During the run, behaviour for each of the snails were observed and documented. Similar pattern was followed for all the 4 groups and time taken for each snail to reach the food source was documented.

For the snails that completed the run in $\leq$ 25 minutes, the time was noted and they were placed as a group in a PVC plastic box filled with fresh lettuce. 12 snails from this set were randomly chosen and were divided into control and experimental groups with 6 snails each, for the next set of experiments. The two groups of snails were acclimatized for a period of 3 weeks prior to study 2. Snails that did not complete the test within 25 minutes, were stored as a group in well-ventilated PVC plastic box with lettuce *ad libitum* and were later released at the collection area.

Results: Study 1

Run Time Analysis

Of the 40 snails that participated in the run, 37 snails successfully completed the run with a maximum time of 45.6 mins and a minimum time of 6.1 mins (M = 17.05, SEM $\pm$ 1.834) (Figure 2). A threshold time of 25 mins was applied to the run time for all snails, based on which nine snails were eliminated for the next set of experiments. Two snails did cross the threshold time limit, but since they were within the 30 minute time frame, they were considered for the next set of experiments. A total of 30 snails were chosen for the next set of experiments. Based on these results it is evident that the cognitive capacity or intelligence levels of the brain differs between snails within the same species and same population. Since this experiment was based on a single run, it was difficult to confirm the memory-retention/formation of the snails which could have been possible if multiple runs were conducted.

Behaviour Observed

Three snails that did not complete the run, demonstrated behavioural patterns, which comprised of circular movements in the maze and isolation in specific corners of the maze. The snails that took > 30 mins to reach the food source spent most of their time exploring the maze and were unsure about their won moves. The snail which completed the run in the shortest time travelled in one specific direction, without changing its path. Snails that completed the task within the 30 mins time frame spent most of their time exploring the maze before advancing towards the food source. Most of the snails used the lid of the maze to explore and travel towards the food source rather than utilizing the surface. Some snails were also seen interacting with each other during the run and moving in parallel towards the food source.

Procedure and Analysis: Study 2

12 snails that were randomly selected, acclimatized and grouped as control and experimental were used in this study. Two identical T-mazes were used for this study, such that runs for both control and experimental groups could be carried out simultaneously. Just like Study 1, the snails were starved for a period of 12 hrs prior to the study. Fresh lettuce was placed at the right-hand goal arm of the T-maze as a reward. Start time and end time for each snail were recorded similar to Study 1 and the maze was thoroughly washed after each snail run to prevent the mucus trail-following behaviour.

A minimum of 10 runs was carried out for each snail in each group and were done in such a manner that each snail would have sufficient amount of rest time before the next run. Exhaustion in the snails was managed by keeping a gap of approximately 30 mins before the next run for each of the snails. The runs for both control and experimental groups were conducted simultaneously and the start and end time was recorded. Food was only provided once the snail completed its 10^{th} run and the snails were placed in their individual compartments, which were filled with fresh lettuce. During the study, the behaviour observed for each of the snails was recorded. The run time data obtained from this study was labelled as Day 0.

Results: Study 2

Run Time Analysis

No significant difference was observed between the run time for the snails in the control (M = 12.075, SEM $\pm$ 0.283) and experimental group (M = 10.3, SEM $\pm$ 0.329) (ANOVA; F value = 1.748, F critical = 3.325, P = 0.211) (Figure 3, 4 and 6). A difference in time taken to complete the run was observed between snails in each group, but was considered as a confirmatory result to the results observed in Study 1 which showed a remarkable difference between the intelligence and cognitive abilities for each of the snails. In the experimental group, Snail 3 showed a remarkable memory-retention/formation with a constant reduction in time over the 10 run period (Figure 5). Snail 1 and Snail 4 did demonstrate a similar behaviour but was not as significant as observed in Snail 3(Figure 5).

Behaviour Observed

Isolation of the snails in this run did not impact their run time and no behavioural changes were observed within and between the groups. Some snails used the lid to navigate towards the food well rather than using the surface. The time lost during the runs was due to the exploratory behaviour observed in some of the snails. The snails from both control and experimental groups showed a similar pattern of movement in each of the runs, with some of them moving first into the left arm and then moving towards the food source in the right arm or rapidly moving towards the food source in the right arm. A similar pattern of movement in every run conducted was observed in the snails that travelled directly from the start arm to the food source in the right arm.

Procedure and Analysis: Study 3

The 6 snails from the experimental group were individually exposed to low-level near- infrared laser light for a period of 5 days. During exposure time, the laser light was held exactly 1 cm above the brain region of the snail with a continuous exposure time of 1 min 40 sec. The snails were exposed twice a day at 12 hr intervals i.e. 9.00 am and 9.00 pm respectively. After an exposure period of 5 days, the maze runs were conducted on Day 6 wherein the snails of both groups were starved for a period of 12 hrs prior to the run. The maze runs for both control and experimental groups were conducted as per Study 2. Start time and end time for each snail in each group and their behaviour observed was recorded. The run time data obtained from this study was labelled as Day 5.

Results: Study 3

Run Time Analysis

A significant difference in the run time was observed between Day 5 of the experimental group (M = 7.2, SEM $\pm$ 0.130) and Day 0 (T = 3.421, P = 0.006) and Day 5 (T = 4.415, P = 0.001) of the control group (M = 13.105, SEM $\pm$ 0.638) and Day 0 (T = 3.423, P = 0.006) of the experimental group (ANOVA; F = 4.524, F critical = 3.325, P = 0.020) (Figure 3, 4 and 6). No significant difference in run time was observed between Day 5 and Day 0 of the control group (T = 0.414, P = 0.687) and Day 0 (T = 2.107, P = 0.061) of the experimental group (Figure 3, 4 and 6). A significant difference ($p \leq 0.05$) in the Day 5 run time was observed in snail 1 (T = 2.449, P = 0.024), snail 3 (T = 3.125, P = 0.005) and snail 4 (T = 3.168, P = 0.005) in comparison to Day 0 of the experimental group and Day 0 and Day 5 of the control group (Figure 5). No significant difference in run time was observed in snail 2 (T = 1.397, P = 0.179), snail 5 (T = 1.350, P = 0.193) and snail 6

(T = 0.544, P = 0.592) between Day 5 and Day 0 of the experimental group. Snail 1, 3 and 4 showed a linear run time curve on Day 5 in comparison to Day 0 of the experimental group with the start time of Day 5, as the end time of Day 0 run (Figure 5). The linear pattern in the run time period was observed in snail 1, 3 and 4 over the 10 run period suggests an improvement in the learning pattern and memory retention post exposure.

Behaviour Observed

During exposure, the snails were quite comfortable with the laser focused on their brain and did not move away from the laser source, but retracted the eye bearing tentacle whenever the laser would directly fall on it. At the end of the 5 day exposure period, no signs of dullness, restriction in movement or change in feeding behaviour were observed in the snails of the experimental group and control group. Snails of the experimental group were quicker and rapid in behavioural movements on Day 6 as compared to the snails of the control group. Snails 1, 3 and 4 seemed to have a strong memory-formation/retention of the runs from Day 0, which was evident from the start time of Day 5 that remained similar throughout the 10 runs carried out on Day 5 (Figure 5). Similar movement patterns as observed in Study 1 and 2 were observed in this study, with snails choosing their exclusive paths to reach the food source.

Procedure and Analysis: Study 4

Snails of the experimental group were individually exposed to low-level near- infrared laser light for 5 more days and all the procedures were kept similar to Study 3. Start time and end time for each snail in each group was recorded and the behaviour observed was documented. The run time data obtained from this study was labelled as Day 10.

Results: Study 4

Run Time Analysis

No significant difference was observed in the experimental group (M = 7.095, SEM $\pm$ 0.193) after 10 day exposure when compared to the results of Day 5 (ANOVA F = 4.456, F critical = 5.050, P = 0.063) but the results were significantly different when compared to Day 0 (T = 3.015, P = 0.013) and control group results on Day 10 (M = 14.58, SEM $\pm$ 0.359) (Figure 3, 4 and 6). Snail 1 (T = 2.458, P = 0.024), Snail 3 (T = 3.552, P = 0.002) and Snail 4 (T = 3.933, P = 0.0009) showed a significant reduction in run time when compared to Day 0 for each of the snails but was not significant when compared to Day 5 (Figure 3, 4 and 6. No significant difference in run time was observed in Snail 2 (T = 0.695, P = 0.495), Snail 5 (T = 0.951, P = 0.353) and Snail 6 (T = 1.041, P = 0.311) between Day 10 and Day 0 of the experimental group (Figure 3). Snail 1 and Snail 3 showed a linear run time curve on Day 10, similar to Day 5 and in comparison to Day 0 of the experimental group (Figure 5). A similar linear pattern in the run time period as seen on Day 5 was observed in Snail 1 and Snail 3 over the 10 run period for Day 10, which could be the threshold capacity of the cognitive ability of the brain (Figure 5).

Behaviour Observed

During exposure the snails were quite comfortable with the laser focused on their brain and did not move away from the laser source, but retracted the eye bearing tentacle whenever the laser would directly fall on it. At the end of the 10 day exposure period no signs of dullness, restriction in movement and feeding behaviour was observed in the experimental group and control group. Snails from the experimental group were more definite in their moves which were similar to the observations of Day 5. Snail 1 and Snail 3 seemed have a strong memory-formation/retention of the runs from Day 0 and Day 5 which was evident from the start time of Day 10 that was maintained throughout the 10 runs carried out on Day 10(Figure 5).

Discussion

Learning and memory-formation/retention are cognitive abilities associated with the brain and its neural network system, which assist animals in several cognitive based behaviours like, recollecting predators, differentiating between foods, locating food sources, mate selection, etc [26, 27]. Cognitive based studies in invertebrates are limited, but they are known to demonstrate memory-formation/retention and also perform certain learning-based behaviours [28]. In a recent study, learning and formation of long-term memory was demonstrated in pond snail *Lymnaea stagnalis,* which showed a significant variability within the natural occurring populations of these snails [29]. In another study conducted in the same species, it was proved that the mechanisms that control cognitive related differences in these snails are highly conserved and rarely differ from their original type [30]. Food foraging is a dietary based behaviour which helps animals to differentiate and trace their food using cognitive based memory-formation/retention [31]. Snails are known food foragers [32] and therefore food was considered as the major source of attraction to study cognitive abilities in snail, *Ariophanta laevipes*. A self-designed T-maze was used to study the cognitive ability of the snails and to understand the various behavioural patterns of learning and memory-formation/ retention demonstrated by these snails.

This paper is a first time report of cognition and behaviour in snail *Ariophanta laevipes* and comprises of four studies. Based on the results of Study 1, a clear differentiation in the cognitive ability was observed between individual snails of the same population (Figure 2). Intelligence is a mental ability for reasoning, problem solving and learning and is known to highly differ within human populations [33]. From the results of this study, a clear difference in intelligence levels was also observed between the snails within the same population, suggesting a highly conserved mix of behavioural patterns which the snails exploit to perform their day to day activities, especially while foraging for food. Molluscs use their mucus trails for navigational purposes which interim is used by other snails to search and explore [23, 24]. This behaviour was evident in Study 1 and therefore in order to prevent any interference from this behaviour, the mazes were thoroughly washed after each and every run for all studies.

Light supports several physiological and cognitive functions in snails [34]. The molluscan brain which manages learning and memory-formation consists of photosensitive neurons in the central ganglion which utilizes light to trigger several electrophysiological transmissions [15]. In Study 3, a significant enhancement was observed in the cognitive abilities of the snails on exposure of their brains to low level near infrared laser light shown as a significant reduction in run time (Figure 3). The enhanced cognitive abilities were evident with the linear pattern observed in the run profile in the 10 runs conducted on Day 5 and Day 10 in comparison to Day 0 and the runs of the control group (Figure 6). These patterns clearly suggest the enhanced learning and memory-formation/retention abilities which can be correlated to the affirmative effects of exposure to low level near-infrared laser.

Low level near infrared laser transcranial therapy in known to improve cognitive and emotional brain functions in humans [18] and modulates the bioenergetics by absorption of photons by specific molecules in the neurons. Laser exposure at 600 – 1150 nm is shows better tissue penetration because of light scattering at low wavelengths and absorption of higher wavelengths by water present in the tissue [35, 36]. These findings correlate well with the findings of this paper, wherein repeated exposures of low level near-infrared light at 650 $\pm$ 10 nm showed a significant improvement in learning and memory- retention/formation of the snails. The movement patterns recorded during the runs were exclusive to individual snails in the experimental group. Based on the similarity in movement patterns observed between runs, it can be confirmed that snails have utilize their cognitive capacity of their brain to memorize and learn. Patterns once memorized by the brain are stored as memory and is exploited by the snail while carrying out its next move, in this case the run. This behaviour was more linear in the experimental group as compared to the erratic readings observed in the control group (Figure 5) suggesting the enhanced effect due to low level near-infrared laser exposure resulting in an improved response.

The improvements in cognitive abilities post exposure to low level near-infrared lasers are solely related to mitochondrial respiration which has been used as a therapeutic target for neuroprotection and cognitive enhancement in humans [17]. In the present study, exposure of low level near-infrared laser at 650 $\pm$ 10 nm could have enhanced the mitochondrial respiratory functioning in the brain of the snails, resulting in enhanced cognitive abilities observed as improved memory-formation/retention and learning. Further exposure for a period of 10 days did not show a major change in the cognitive abilities of the snails which suggests the limitations of the brain as well as the exposure. Based on the observations in Study 4, it is clear that the brain in snails encompass a limited cognitive capacity (Figure 3) which can be correlated to the limited neuronal connection in the brain or limitations in the excitation of the photo neurons. Over exposure of low level near infrared laser can result in inhibition of cell growth and irreversible changes in the tissue and therefore the dose selection should be done very carefully [37].

In the wild, snails avoid direct exposure to sunlight by moving into shady places and can therefore regulate the excitations of the photosensitive neurons that regulate the cognitive capacity of the brain. Based on the results of this study, light certainly plays an important role in managing cognition and behaviour of snails especially at wavelengths in the near-infrared zone. This study is a first time report of cognitive abilities in snail, *Ariophanta laevipes* and the differences in cognitive abilities between individuals of the same species. It also demonstrates the importance of near-infrared light exposure on the cognitive abilities of these snails. Sunlight exists as a mixture of wavelengths but through a sorting and filtering mechanism gets fragmented and absorbed within tissues providing support to various cellular processes in the brain Understanding these photo-based mechanisms would definitely open up a new area of research in the field of invertebrate cognition. In view of the importance of light in the life of animals, low level near-infrared laser based enhanced animal cognition techniques can be recommended as a therapy in veterinary science.

Ethics statement

Ethical approval is not required for research work with *Ariophanta laevipes*; however every effort was made to restore suffering of animals, ensuring adequate food, clean oxygenated water and sufficient ventilation. The stress treatments used in the study have no long-term effects on the animals beyond the brief exposure periods and therefore the animals were released back into the wild post experiments. No specific permits were required for the described field collections. The Sanjay Gandhi National Park collection site is accessed via a public highway and is not situated on private or protected land. The collection of *A. laevipes* for this study did not involve endangered or protected species.

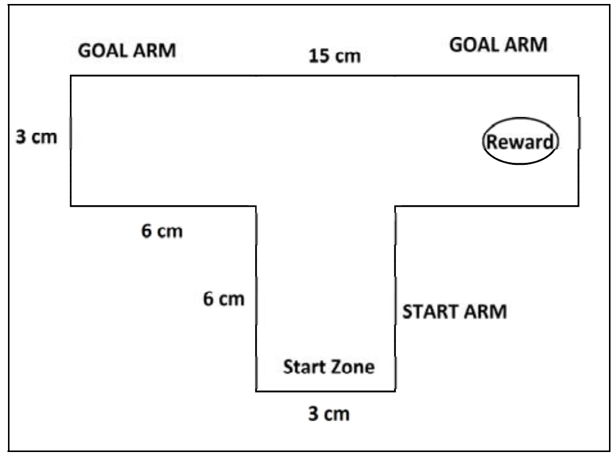

Fig 1: Self-designed T-Maze sketch.

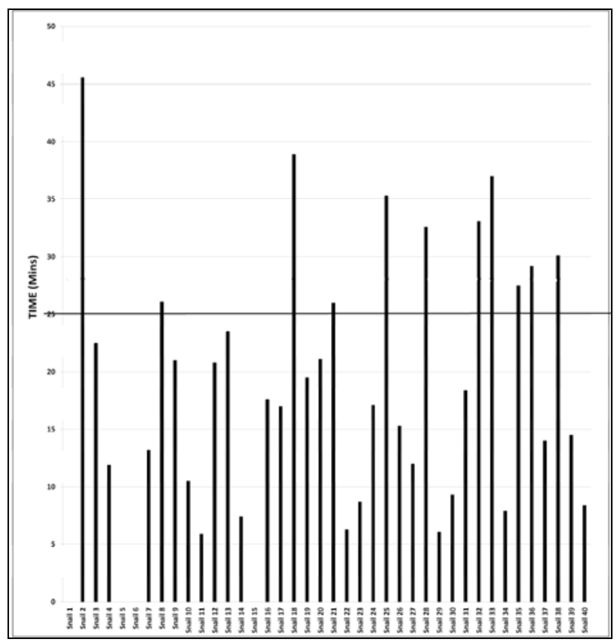

Fig 2: Actual single run time of 40 snails with a threshold level (*horizontal line*) fixed at 28 minutes.

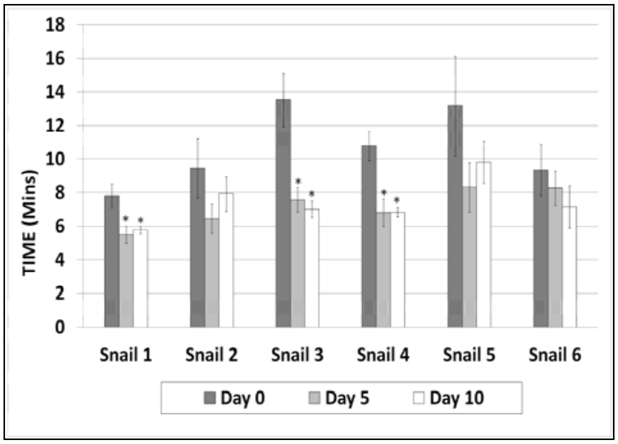

Fig 3: Run time of the experimental group recorded on Day 0, Day 5 and Day 10 where the run time of snails 1, 3, and 5 on Day 5 and Day 10 were significantly reduced in comparison to the run time on Day 0 ($p<0.05$) suggesting the enhanced cognitive effects of low level near–infrared laser exposure on snails. *Asterisk* indicates a significant difference with a value $p<0.05$.

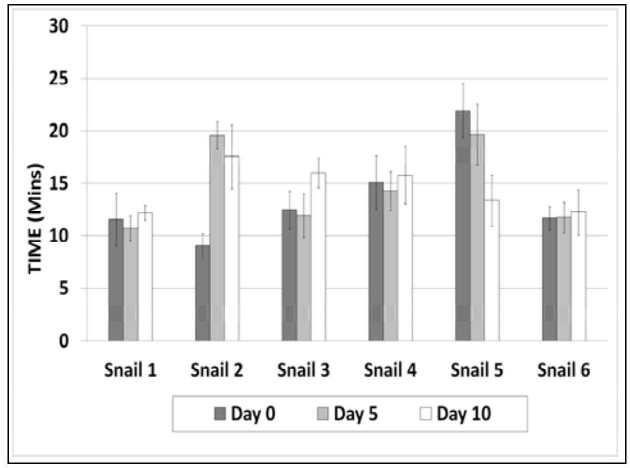

Fig 4: Run time of the control group recorded on Day 0, Day 5 and Day 10 where the run times recorded for all the snails were not significantly different ($p>0.05$) confirming the enhanced cognitive effects of low level near-infrared laser exposure on snails.

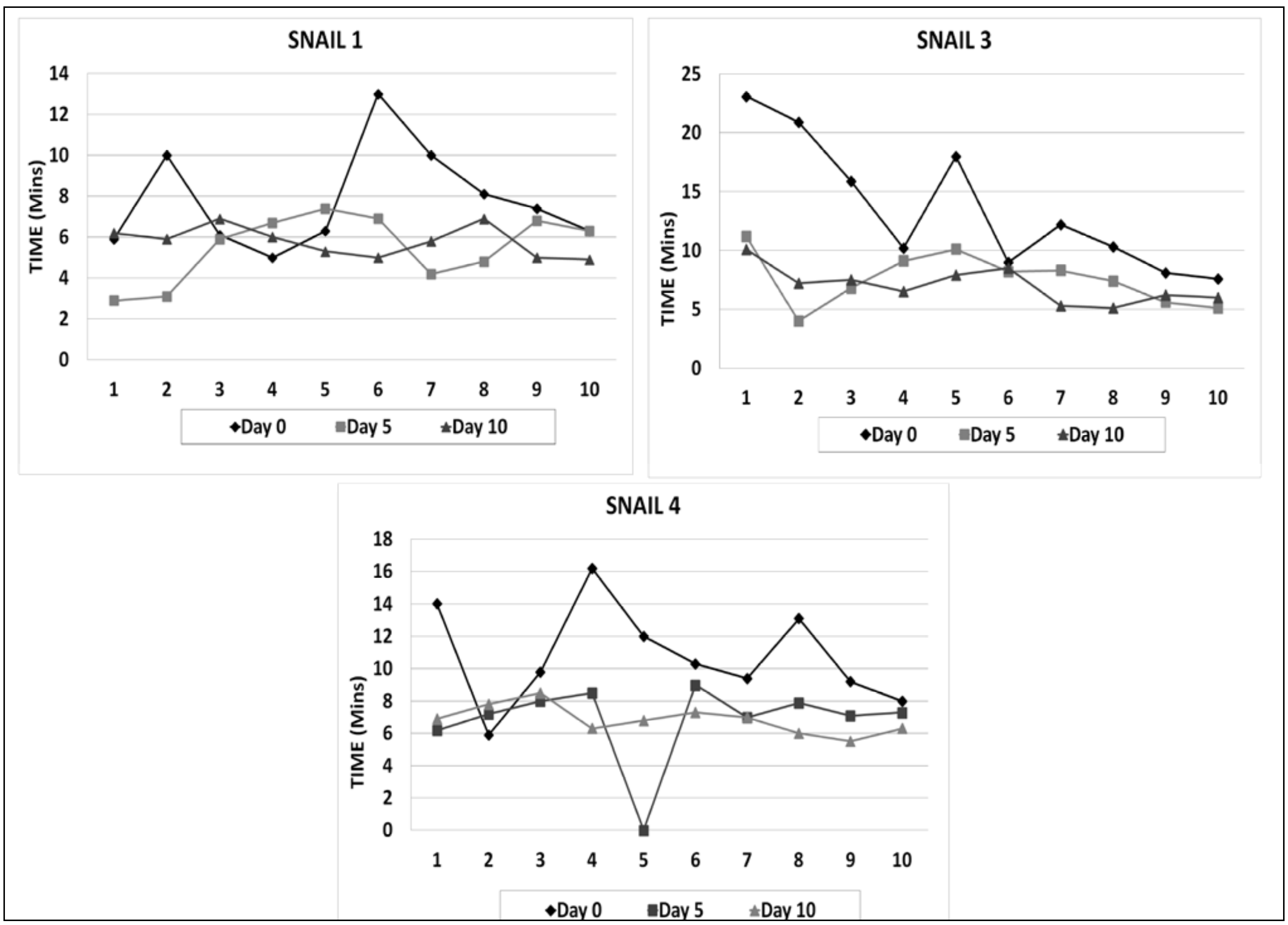

Fig 5: Run time for 10 runs conducted for snails 2, 3 and 4 on Day 0, Day 5 and Day 10 where a significant linear pattern in the run time was observed between Day 0 and Day 5 and 10 ($p<0.05$) suggesting enhanced cognitive abilities in memory-formation and retention due to exposure of low level near-infrared laser exposure on snails.

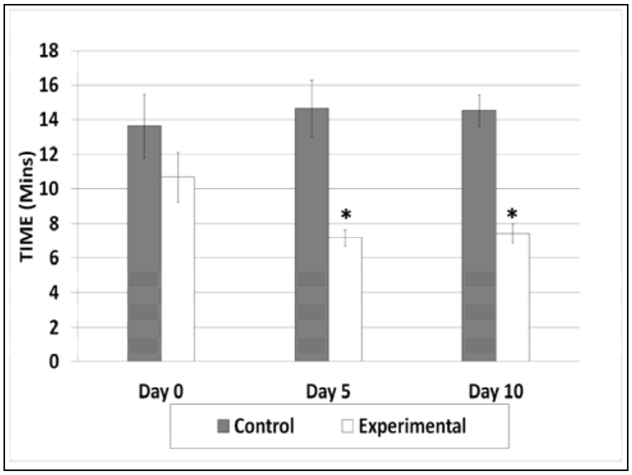

Fig 6: Average run time comparison of control and experimental groups for Day 0, Day 5 and Day 10 where the average run time of snails on Day 5 and Day 10 were significantly reduced as compared to run times recorded on Day 0 ($p<0.05$) confirming the enhanced cognitive abilities in the snails exposed to low level near-infrared laser and a threshold effect observed with higher exposure on comparison of run times between Day 5 and Day 10 ($p>0.05$). *Asterisk* indicates a significant difference with a value $p<0.05$.

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