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Effect of hypothermia on cognitive capabilities in snail (*Achatina fulica*) and their recovery post exposure to the Buddhist meditative chant "Om Mani Padme Hum"

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Abstract
In humans, music therapy is a proven relaxation technique that helps manage stress and acts as a form of secondary treatment for several neural based disorders. The basis of this study was to understand whether the "Om Mani Padme Hum" meditative chant, can enhance brain related activities mainly cognitive behaviour in snails post exposure to an induced stress, thus hypothesizing the interaction and recovery to be mediated at a cellular level. Cognition is an important attribute of every living organism which although varies considerably from species to species, helps facilitate their day to day activities. Hypothermia stress and recovery in snail *Achatina fulica* has shown no long-term effects of hypothermia and therefore has been proposed as a model of studying consciousness in a previous study (Pereira 2016). The intention of this study was to validate whether the vibrations generated by the "Om Mani Padme Hum" chant can enhance and improve cognitive abilities and behavioural responses in snails which have been imperilled to hypothermic stress. In this study, a significant effect of hypothermia induced stress was observed as reduced cognitive capabilities of snail *Achatina fulica* despite the quick recovery from the hypothermic induced stressed state. Significant improvement was observed in cognitive abilities of the snails subjected to hypothermic stress, when exposed to the Om Mani Padme Hum chant confirming a therapeutic effect that can be directly correlated to the frequencies associated this chant. The results of these studies confirm that the "Om Mani Padme Hum" meditative chant generates vibrations that can augments cognitive capacity of the brain and simultaneously demonstrate significant therapeutic effects. The significant effect of hypothermia induced stress on cognitive abilities and its recovery from this state is a first time report for this snail species.

Keywords: Hypothermia, Stress, Music, Resonance, Vibrations, Cognition, Snail, Behaviour

Introduction
Music has always been linked to meditation or meditative religious or non-religious techniques which may involve singing in a choir or chanting. Brain regions associated with attention and sensory processing were found to be thicker in persons who would meditate daily in comparison to persons who would not, and the thickness of these areas increased with increasing years of mediation practice (Lazar *et al* 2005) [14]. Meditation is a process that self-regulates the body and mind and maybe associated with psychological and neurophysiological alterations. Meditation studies have been linked to an increased activity in the prefrontal cortex of the brain which is associated with several cognitive based functions (Previc 2006) [25]. EEG recordings of skilled Buddhist monks with years of training have shown a significant rise in gamma wave activity in the 80–120 Hz range while this effect was lower in new meditators. For these Buddhist monks, the purpose of meditation is to free oneself from suffering and gain spiritual liberation which is the same reason for meditative practice in other religions (Davidson and Lutz 2008) [9]. The potential therapeutic effects of music listening reduces stress and modulates arousal and has also shown reduced anxiety in humans (Thoma *et al* 2013) [28].

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 (Majovski and Jacques 1982) [16]. Stress can alter cognitive capabilities and affect the performance of an individual (Staal 2004) [26] and therefore there are several therapeutic techniques that are been developed to overcome stress (Varvogli and Darviri 2011) [29]. Hypothermia is a well know

technique used to slow the metabolic rate which may induce a state of unconsciousness with longer exposure time and lower freezing temperatures in animals. In invertebrates, hypothermia has been used as a technique to immobilize and anesthetize them for various invasive procedures (Cooper 2011) [6]. Land snail *Helix aspersa* demonstrated freezing tolerance based on body size which increased with increase in body size (Ansart and Vernon 2004) [2]. Freeze tolerance or tolerance to hypothermia may help invertebrates survive, but the level of tolerance may differ from species to species, which was evaluated for the species *Achatina fulica* in a previous study (Pereira 2016) [22].

Resonance occurs without regard to distance or time separations and without physical communications. Experiments have provided a confirmation that resonant based experiences are not illusionary or imaginary, but occur through a form of interconnectedness created by resonating frequencies inside and outside the living system (Cambray 2009) [5]. Meditational music is known to generate frequencies and resonant vibrations which augment the cognitive capacity of the brain in snails *Achatina fulica* and at reduced variation in frequencies e.g. Om Mani Padme Hum, can enhance learning ability with an increase in short-term memory gain (Pereira 2015a) [23]. The intention of this present study was to validate whether music generated resonant vibrations can enhance and improve cognitive abilities and behavioural responses in snails that have been subjected to hypothermic stress. The effect may be non-specific in terms of molluscs that do not have auditory features and therefore the effect of these resonant vibrations in this study may be considered as an indirect sensory effect of vibrations at a cellular level.

Collection and Maintenance

Pre study

Achatina fulica (Bowdich 1822), snails (4 – 6 cms) were collected from St. Peter's Catholic Cemetery, Worli, Mumbai, India (18°15'29.5''N, 72.49'19.5''E). 30 snails were collected and acclimatized as a group for a period of 2 weeks. The snails were placed in a ventilated and hydrated PVC plastic box measuring 50 cm x 30 cm x 30 cm and maintained on a 12: 12 light: dark schedule (7.00 am: 7.00 pm) at room temperature (30 - 33°C). The snails were fed on lettuce leaves *ad libitum* which 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.

Study 1 and 2

Post the 2 week acclimatization period, 24 snails were randomly chosen from the batch of 30 snails and were sub-divided by random picking into 4 groups of 6 snails each and were numbered on their shells using a water-proof marker. Each group were placed in individual ventilated and hydrated PVC plastic boxes measuring 30 cm x 20 cm x 20 cm and were acclimatized to this setup for a period of 2 weeks. The snails were maintained on a 12: 12 light: dark schedule (7.00 am: 7.00 pm) at room temperature (33 - 35°C) and were fed on lettuce leaves *ad libitum.* The PVC plastic housings were washed and cleaned on a daily basis during which the snails were moved into a similar housing apparatus. For both studies, post exposures the snails were placed in their individual PVC plastic boxes and their behaviour was observed for a period of 3 hours daily.

In study 1 hypothermic stress was induced for a period of 15 mins for 5 days and for study 2, music exposure was provided for a period of 15 mins for 5 days. Both these exposure times were considered based on earlier studies conducted on the same snail species (Pereira 2016, Pereira 2015a) [22, 23]. Hypothermic exposure was provided during the day, between 8 am to 9 am. Reflex actions of the snails for all groups post hypothermic exposure were checked by observing withdrawal effects after nudging them with a wooden stick. After the three hour observation period, the housing was filled with fresh lettuce *ad libitum.* The T-maze runs were conducted during the day, from 9.00 am to 12.00 pm and during the runs was kept hydrated and ventilated. All maze studies and behaviour-based observations were carried out at room temperature (33 - 35°C). The maze was thoroughly washed after each snail run, inorder 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 (Ng *et al* 2013, Patel *et al* 2014) [19, 21]. Both studies were completed within a period of 12 days post the 2 week acclimatization period. After completion of both studies, the snails were released back at the collection area.

Equipment

T-Maze

A self-designed, enclosed, well ventilated and hydrated PVC plastic T-maze, with start and goal arms measuring 12 cm x 10 cm x 10 cm was used to conduct the experiments. The dimensions of all the arms in the T-maze were kept similar for all three groups (Figure 1). The T-maze was designed based on T-mazes designed to study rat behaviour (Deacon and Rawlins 2006) [10].

Hypothermia Induced Stress

Hypothermia was induced by placing the snails as a group in their individual boxes in a frost-free freezer of a double-door refrigerator at a temperature of -16°C $\pm$ -2°C. The LG GL-D292JSFL double-door refrigerator is equipped with a LG Dura Chill option that maintains optimum cooling inside the refrigerator for up to 10 hrs.

Music Source

In Study 2, snails were exposed to a Tibetan meditational chant from Tibetan Incantations (Nascente) - "Om Mani Padme Hum" with a similar bit rate of 128 kbps. Frequency analysis of this soundtrack was done using the Wave Pad NCH software Version 6.18, which uses a FFT analytical tool to determine the actual frequency recordings of the soundtrack. The highest frequency recorded for the Tibetan meditational hymn was 21371 Hz (21096 Hz + 274.8 Hz) with a range varying from 236 Hz – 21371 Hz and a decibel gain range of – 23 db to -130 db. The nearest sound note recorded for this hymn was E (21096.2 Hz). The soundtrack was played on an I-ball Tarang 2.1 music system with one sub-woofer (20 watts RMS max) and two satellite speakers (10 watts RMS max each) and a total output of 40 watts RMS max with the frequency ranges for - woofer as 20Hz -200Hz and satellites as 100Hz-20kHz. The decibel output range for the Tibetan hymn was 75 – 80 db and frequency range was 260 – 280 Hz. These output values were recorded by means of an Android based Spectral Audio Analyzer Application from RadonSoft. "Om Mani Padme Hum" is a meditational hymn, and is known to generate positive energies within the body through mystical vibrations that are generated while chanting (Misra and Shastri, 2014) [18]. Exposure to this chant has also shown enhanced cognitive effects in various snail species (Pereira

2016, Pereira 2015a, Pereira 2015b) [22, 23, 24] and therefore was chosen for this study.

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 $p<0.05$ and F > F critical.

Procedure and Analysis: Study 1

Study 1 was conducted on 24 snails that were subdivided into 4 groups of 6 snails each, acclimatized for a period of 2 weeks. The snails in each group were numbered and were placed in individual PVC plastic boxes that were labelled as control, hypothermia, music treated and hypothermia + music treated. The control and music treated group were not exposed during study 1 and were fed with lettuce *ad libitum*. Hypothermia was induced to snails of the hypothermia and hypothermia + music treated groups for 15 minutes daily for a period of 5 days and the exposure was conducted during the day between 8 to 9 am. Post hypothermic exposures, the snails belonging to each of the groups were placed at room temperature (33 - 35°C) and revival from the stressed state was observed as the reflex actions of the snails after nudging them with a wooden stick for 15 mins. The exposure and recovery time considered for this study, has been tested earlier for the same species and therefore was considered ideal for this study (Pereira 2016) [22]. Post hypothermic exposure the snails from both the groups were observed for their behaviour for a period of 3 hours. The snails were fed with lettuce *ad libitum* after the 3 hour observation period.

After the 5 day exposure, snails for all groups were starved for a period of 12 hours and the T-maze runs were conducted on Day 6. On the run day, the snails from each group were randomly selected and placed at the starting arm of the T-maze with fresh lettuce placed in the both the arms, as a reward. For each run, the start time was recorded 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. A similar pattern was followed for snails from all the groups and time taken for each snail to reach the food source was documented as the run-time. Fresh lettuce was only provided to the snails after completion of the runs.

Results: Study 1

Behaviour Observed Post Hypothermic Exposure

For the hypothermia and hypothermia + music treated groups, post exposure, the snails did not retreat into their shells and were in a complete knocked out or comatose state with no movement. Recovery from this state was observed within 2 – 3 mins beginning with a first jerk in the head region followed with a slight swirling of the head and movement. Complete recovery was observed within 3 - 5 mins of the 3 hour observation period for both groups. Similar results were obtained in an earlier hypothermic stress induced study for the same species (Pereira 2016) [22]. Over the 5 day exposure period, no significant altered changes in eating habits, crawling patterns, body movements and reflex actions were observed confirming complete recovery after every single hypothermic exposure for both the hypothermia and hypothermia + music treated groups. No behavioural changes were observed in the control groups on day 0 and day 6 or day 0 of the hypothermia and hypothermia + music treated groups.

Run Time Analysis

A significant increase in run time was observed for snails between the control (M = 14.14, SEM $\pm$ 1.879) and the hypothermia (M = 25.82, SEM $\pm$ 1.696) (ANOVA; F value = 28.19, F critical = 6.607, $P<0.05$) (T=4.237, $P<0.000862$) and hypothermia + music treated groups (M = 26.52, SEM $\pm$ 1.815) (ANOVA; F value = 19.962, F critical = 6.607, $P<0.05$) (T=3.845, $P<0.00161$) on day 6 (Figure 2). No significant difference was observed between the hypothermia and hypothermia + music treated groups on day 6 (Figure 2). Run time for the music treated and the control group were similar as the group was not treated.

Behaviour Observed during the T-maze runs

Snails from the control groups used the lid to navigate towards the food well rather than using the surface in each of the runs, with some of them moving into the left arm and then moving towards the food source in the right arm. Snails from the hypothermia and hypothermia + music treated group showed a restrictive movement pattern and seemed indecisive about their movements and therefore took a longer time to reach their reward. The movement patterns observed in the control group were focussed in comparison to hypothermia and hypothermia + music treated groups. Besides, restrictive movements no significant behavioural differences in the maze were observed between the hypothermia and hypothermia + music treated groups during the run.

Procedure and Analysis: Study 2

Study 2 was a continuation of study 1 and was conducted on all 4 groups; control, hypothermia, music treated and hypothermia + music treated. Music treated and hypothermia + music treated groups were exposed to the meditational soundtrack " Om Mani Padme Hum" for 15 minutes daily for a period of 5 days. The music exposure time was considered based on the results obtained in a previous study, where the snails were exposed to the same meditative sound track and demonstrated higher cognitive abilities (Pereira 2015a, Pereira 2015 b) [23, 24]. Post exposures the snails were fed with lettuce *ad libitum*. After the 5 day exposure period, snails for all groups were starved for a period of 12 hours and the T-maze runs were conducted on Day 12. On the run day, the snails from each group were randomly selected and placed at the starting arm of the T-maze with fresh lettuce placed in the both the arms, as a reward. For each run, the start time was recorded 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. A similar pattern was followed for snails from all the groups and time taken for each snail to reach the food source was documented as the run-time. Fresh lettuce was only provided to the snails after completion of the runs.

Results: Study 2

Behaviour Observed post exposure to the Meditative Chant

Post the exposure period, no significant altered changes in eating habits, crawling patterns, body movements and reflex actions were observed in the music and hypothermia + music treated groups.

Run Time Analysis

A significant decrease in run time was observed for snails between control (M = 10.055, SEM ± 0.580) and music treated group (M = 7.19, SEM ± 0.459) (ANOVA; F value = 10.623, F critical = 6.607, $P < 0.05$) (T=4.2953, $P<0.000786$) (Figure 3) on day 12. A significant decrease in run time was also observed between the hypothermia treated (M = 24.23, SEM ± 1.761) and hypothermia + music treated group (M = 9.09, SEM ± 1.133) (ANOVA; F value = 53.097, F critical = 3.287, $P < 0.05$) (T=7.2895, $P<0.000013$) (Figure 4) on day 12. The run time for the hypothermia group (M = 24.23, SEM ± 1.761) was significantly high when compared to the controls (M = 10.055, SEM ± 0.580) (T=7.6828, $P<0.00001$) (Figure 4) on day 12. No significant difference was observed between run time of day 6 and day 12 of the hypothermia treated groups (Figure 4).

Behaviour Observed during the T-maze runs

The snails from the control group showed a similar pattern of movement in each of the runs, with some of them moving into the left arm and then moving towards the food source in the right arm. Snails from the music treated group were quick and would directly move towards the reward in comparison to the control group. Hypothermia treated group showed a perplexed movement pattern with restricted movements as observed in study 1. The movement patterns observed in the hypothermia + music treated group were comparatively smoother and focussed as compared to the hypothermia treated group and control groups but was not as significant when compared to the music treated group.

Discussion

Music enhances cognitive functions of the brain which is dependent on dopaminergic neurotransmission and release of opioid peptides within the brain (Sutoo and Akiyama 2004) [27]. Pulsed transcranial ultrasound has been shown to improve memory functioning in Alzheimer's mice by the break-down of amyloid plaques which may help to boost the weakened cemi-field within the neurons or the microtubules within the neurons (Craddock *et al* 2012) [7]. Music has been shown to enhance cognitive abilities in humans and can interfere with complex cognitive processes in the brain (Wang 2013) [30]. Cognitive based studies in invertebrates known to demonstrate memory-formation/retention and also perform certain learning-based behaviours (Haszprunar and Wanninger 2012) [12]. 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 (Dalesman *et al* 2011) [8].

Snails are ectothermic and do have the ability to survive in cold and freezing temperatures, but as a form of adaptation they can increase their glycerol and proline reserves in the body and alter their carbohydrate metabolism to adapt to the changing temperature (Nowakowska 2011) [20], but longer exposures leads to hypothermia which when beyond a certain threshold limit can result in knockout or unconsciousness/sentience with reversible and irreversible effects (Pereira 2016) [22]. Hypothermic anaesthesia has been considered non-reliable as it may sensitize some species to painful stimuli based on the effects observed in higher animals and therefore is minimally used (Martin 1995, Diaz and Becker 2010, Cooper 2011) [17, 11, 6]. The present study did not show any signs of distress or irreversible effects in the treated snails during the 5 day exposure period, which is evident that the hypothermic stresses which leads to unconsciousness/insentience is reversible for this species of snails confirming the rigidity and sturdiness of this species. The study also confirmed a significant effect of the hypothermic induced stress as an increase in cognitive capabilities of the snails (Figure 2) despite of a quick recovery from the hypothermic induced stressed state.

Music articulates our life, evoking emotions from joyous to sadness and regulating moods. It can be stimulating but depends on structural features such as tempo, pitch, frequency patterns, etc. which can be broadly categorized as pleasant or unpleasant by the listener (Brandt *et al*. 2012) [3]. The potential therapeutic effects of music listening reduces stress and modulates arousal and has also shown reduced anxiety in humans (Thoma *et al.* 2013) [28]. The emotional effects observed after exposure to music is due to the mechanosensory hair cells present in the ear that transmit the sound generated mechanical vibrations via neural impulses to the brain (Bryant *et al* 2002) [4]. Resonant vibrations generated through music are known to affect mood and emotions, which has been mainly focussed on the brain and its cells and not on cellular metabolism, which may be the case in organisms with non-auditory apparatus. Cultured human breast cancer cell line MCF7 showed an alteration in cellular morpho-functional parameters such as cell size and cell granularity when exposed to music generated resonant vibrations conforming to the direct interference of these vibrations with hormonal binding processes that could modulate physiological and pathophysiological processes within these cells (Lestard *et al* 2013) [15]. Yeast cells demonstrated a 12% increase in growth rate and 14% reduction in biomass production with a significant difference in the metabolite profiles on exposure to different sound frequencies, confirming the enhancing effect of these vibrations at a cellular level (Aggio *et al* 2012) [1].

"Om Mani Padme Hum" is a meditational hymn, and is known to generate positive energies within the body through mystical vibrations that are generated while chanting (Misra and Shastri, 2014) [18] and has shown significant increase in cognitive capacity of the brain in snails *Achatina fulica* with enhanced learning ability and an increase in short-term memory gain (Pereira 2015a) [23]. In Study 2, a significant improvement was observed in cognitive abilities of hypothermia induced snails when exposed to this meditative chant when compared to the snails that were not exposed to the meditative chant. This therapeutic effect can be directly correlated to the affirmative effects of the variations in frequencies of this chant. Significant effect of this chant was also observed in the music treated group which showed better focussed maze runs and reduced run time (Figure 3) and therefore confirms the results of some of the earlier studies carried out on these snails (Pereira 2015a) [23]. Meditation is a process that self-regulates the body and mind and maybe associated with psychological and neurophysiological alterations. Meditation studies have been linked to an increased activity in the prefrontal cortex of the brain which is associated several cognitive based functions (Previc 2006) [25].

Music is not perceived by snails as they do not have auditory features but based on this study and previous studies, music does cause an effect, the mechanism for which needs to be evaluated and is beyond the scope of this paper. The mechanisms behind the enhanced cognitive effects post exposure hypothermic stress is unknown but is surely related to vibrations created at different frequencies which have direct impact on the biochemical processes at a cellular level within the neural tissues. Qigong masters have enhanced or reduced biochemical rates during plant growth through their resonating

meditative practices which involves determining the position and velocity of the trajectory of an object that needs to targeted via techniques that involve vibrations and resonance (Jahnke *et al.*, 2010) [13]. Resonance occurs when an object is vibrated at its natural frequency or naturally occurring frequencies. Resonance has been used as a medium to transfer power into all kinds of waves ranging from lasers to microwave ovens and musical instruments. The brain works on electrical activity which exists in the form of brainwaves ranging from high amplitude, lower frequency delta waves to low amplitude, higher frequency beta waves (Zhuang *et al.*, 2009) [31].

In the present study, exposure of the frequencies generated by the chant could have ensued resonating effects inducing biochemical changes in the cells of these snails, resulting in an enhanced therapeutic effect on stressed snails. The mechanisms involved in the effect of these vibrations and frequencies generated by this chant needs to be evaluated in future for which this species of snails could be a recommended model.

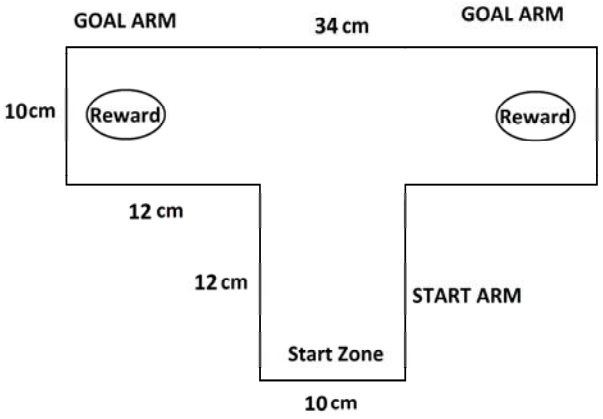

Fig 1: Self-designed T-Maze sketch.

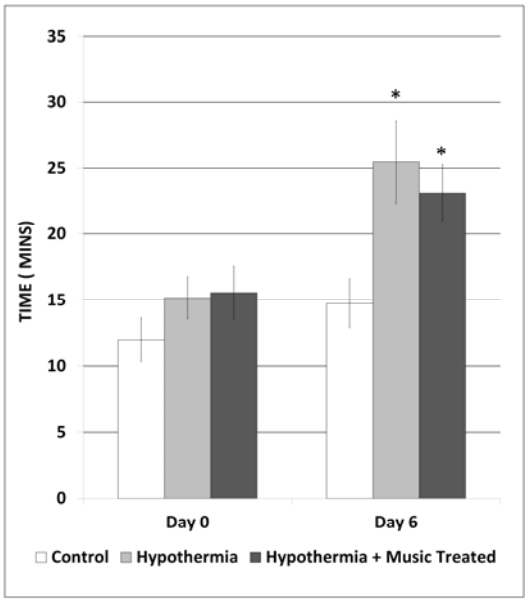

Fig 2: Average run time comparison of control, hypothermia and hypothermia + music treated group on day 0 and day 6, where the average run time of snails subjected to hypothermic stress were significantly increased as compared to control group ($p<0.05$) confirming the reduced cognitive abilities in the snails subjected to stress. *Asterisk* indicates a significant difference between the hypothermia and hypothermia + music treated groups and control group with a value $p<0.05$.

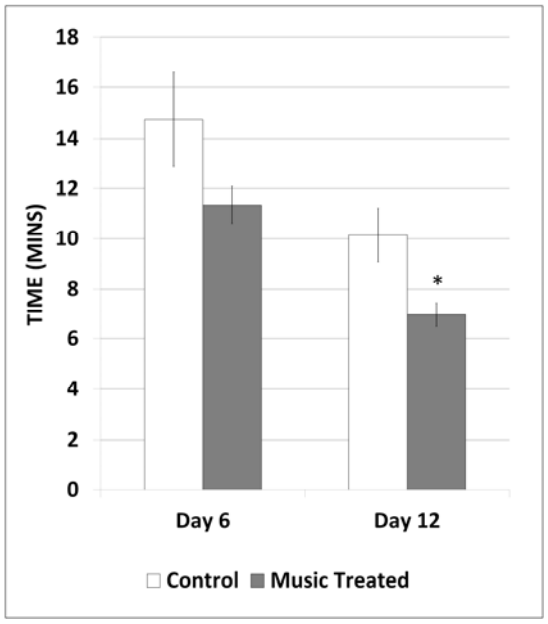

Fig 3: Average run time comparison of control and music treated group on day 6 and day 12, where the average run time of snails exposed to music were significantly reduced as compared to control group ($p<0.05$) confirming the enhanced cognitive abilities in the snails exposed to music. *Asterisk* indicates a significant difference between the control and music treated groups with a value $p<0.05$.

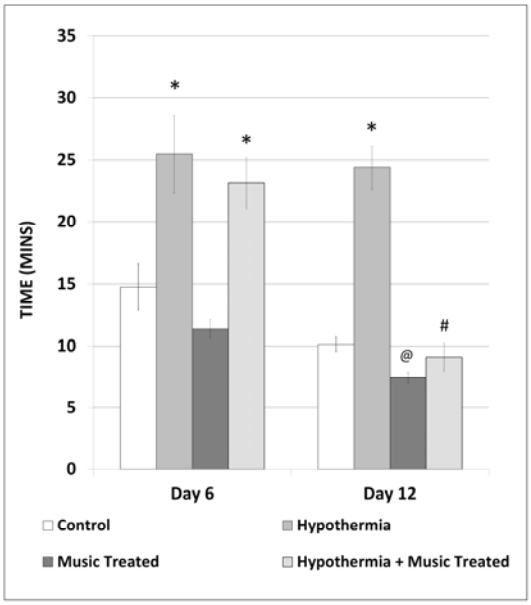

Fig 4: Average run time comparison of control, hypothermia treated, music treated and hypothermia + music treated group on day 6 and day 12, where the average run time of snails that were subjected to hypothermic stress was significantly decreased as compared to the hypothermia treated group ($p<0.05$) confirming the therapeutic effect of the music on the snails subjected to hypothermic stress. *Pound sign* indicates a significant difference between the hypothermia and hypothermia + music treated groups with a value $p<0.05$. *Asterisk* indicates a significant difference between the control and hypothermia treated groups on day 6 and day 12 with a value $p<0.05$. *At sign* indicates a significant difference between the control, hypothermia and hypothermia + music treated groups on day 6 and day 12 with a value $p<0.05$.

Ethics statement

Ethical approval is not required for research work with *Achatina fulica*; 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 collection of *A. fulica* for this study did not involve endangered or protected species.

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