Can Green Tea Alleviate the Effects of Stress Related to Learning and Long-Term Memory in the Great Pond Snail (*Lymnaea stagnalis*)?

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Abstract

Lymnaea stagnalis is a pond snail with a simple nervous system. Recent studies have shown the mechanisms of aging neurons in the *Lymnaea* to be analogous to age-associated diseases of humans and other mammals making *Lymnaea* an excellent model organism for behavioral studies related to the human nervous system. In this experiment, *Lymnaea* was used to explore the relationship between green tea, a mixture known to contain flavonoids; compounds that may have a beneficial effect on human learning and memory, and stress known to negatively impact human memory. The purpose of this experiment was to determine the effects of both green tea and stress on the snails' ability to learn, form, and retain memory. Memory was assessed using an operant conditioning procedure called conditioned taste aversion, which was achieved by exposing the snail to a sweet substance followed by a bitter taste. Thirty-three snails were trained in pond water. After they forgot their training, they were stressed, then retrained and results were compared. The snails were then restressed and exposed to green tea to see if the green tea would alleviate the effects of stress. Results showed that green tea did improve the memory of the snail, while stress harmed the snails' learning and memory. However, the green tea was able to alleviate the effects of the stress-induced memory loss in the snails.

1. Introduction

The Great Pond Snail or *Lymnaea stagnalis* is a freshwater gastropod found in the Holarctic region (Fig. 1, 2). The snail has been used as a model organism in many studies (Audesirk 1984, Dalesman 2006, Hughes 2017) on brain function, memory, stress, etc. Snails were used in these studies because they are easy to train through conditioned learning, have easily observable behaviors linked to memory, and have large neurons. The snail also has a simple nervous system. All of these factors make the snail an excellent model organism.

Dalesman (2013) found that by training snails and observing their behaviors and nervous activity following exposure to stressful situations, the stressful event resulted in some impairment of memory or prevented any memories from forming.

Figure 1. Pond Snail Size Comparison


Figure 2. Labeled Pond Snail

These characteristics make them especially relevant as model organisms with the growing issue of age-associated memory diseases. Scientists have begun testing several theories on what could influence the memory in these organisms (Gottumukkala 2014). For example, a study by Crew (2012) specifically tested the effect of chocolate, known to contain memory-improving flavonoids, on the memory of the snails. The results showed that the snails' exposure to the chocolate demonstrated better memory than those not exposed to chocolate. Dalesman and Sunada (2013) tested how different stressors would alter the snails' ability to learn and retain information. The results from this study illustrated the idea that stress is in fact, very harmful to snails' memory. Audesirk (1984) concluded that the best way to train the snails was by using Conditioned Taste Aversion (CTA). CTA is an operant conditioning procedure achieved by exposing the snail to a conditioned stimulus (sucrose) followed by an unconditional stimulus (potassium chloride).

The significance behind training these snails and observing their learning and memory patterns, is that the results from such studies can be related to human neurology (Orr 2008). This is because the mechanisms of aging neurons in snails have been shown to be analogous to age-associated memory diseases and the aging neurons in other mammals (Ito 1999). In other words, if researchers can understand how to control or alter memory on a molecular level, using the snail, they may be able to obtain a better understanding of memory associated diseases such as Dementia and Alzheimer's in people.

Recent studies (Knezevic 2016) have used pond snails as a model organism to study stress and anxiety. This interest in stress has been motivated by an increase in concern about the impact of stress and anxiety on people. For example, according to a 2017 survey conducted by The National Institute of Mental Health, it was determined that 46.6 million Americans live with or suffer from some type of mental disorder. The most commonly reported mental disorder being Generalized Anxiety Disorder (GAD), with over 40 million Americans diagnosed, ranking GAD at about 85.83% of all mental illness in the United States (The National Institute of Mental Health 2017). Understanding how stress and anxiety affects snails, may help researchers to understand the negative effects stress has on people. There are several factors essential to stressing out a snail enough to harm memory and learning (Dalesman 2013). In one study, scientists exposed the snails to the predatory scent of crayfish. After the exposure, snails were unable to learn and form memories (Hughes 2017). Lukowiak (2014) allowed the snails to live in low calcium pond water for 1 week. The snails need calcium to rebuild and strengthen their shells, so the lack of it was shown to increase their stress. The snails that were stressed were unable to learn and form memories, while the unstressed snails learned and formed memories normally. However, the quickest and easiest way to stress the snails was by overcrowding 15 to 20 of them in 100 mL of pond water for one hour prior to training. As suspected, the snails had lost their ability to learn and remember (Dalesman and Lukowiak 2012).

From the studies on the effect of stress and snail learning and memory, a new question arises: how can the effects of stress on snails be alleviated? To investigate, Crew (2012) tested the effects of several memory-improving flavonoids and other substances to see what could improve a snails' memory. Specifically, a certain flavonoid called epicatechin, which is found in green tea, cocoa, red wine, and blueberries, has been widely tested (Lin 2019). The reason this specific flavonoid was studied was because medical researchers had discovered the many health benefits of green tea in humans such as memory improvement. Green tea has a beneficial impact on memory because the flavonoids and phytochemicals in it improve the brain's cognitive functions (Whiteman 2014). So, when the snails were trained with the exposure to the flavonoid epicatechin, there was a significant increase in their ability to learn and remember (Fruson 2012). Clearly, the ingredients in green tea improve the snails' memory while stress has the opposite effect.

The purpose of this study was to determine if green tea could alleviate the negative effects of stress on the learning and memory in the Great Pond Snail. Knowing that green tea should improve the snail's memory and stress should cause a learning block, the real purpose is to try and reverse the learning block using green tea. I hypothesized that the green tea will be able to reverse the memory/learning block in the snails after they are stressed prior to training. To carry out the study, the snails were trained in pond water as a control trial, then retrained after being stressed. When the stress wore off, they were re-stressed and trained in green tea. Results showed that green tea was able to alleviate the effects of stress on the snails' learning and long-term memory.

2. Methods

2.1 Experimental Overview

The procedure designed to train the snails, with exposure to both stress and green tea was developed using the model CTA procedure created by Lukowiak (2012) (Fig. 3).

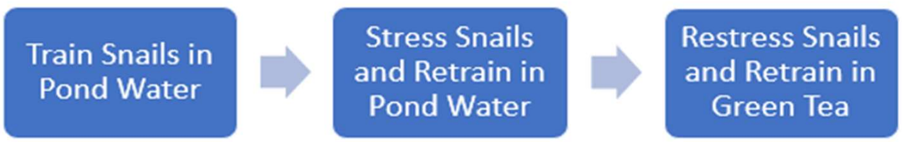

Figure 3. Outline for Procedure

2.2 Care and Maintenance

Snails were kept in a 10 gal tank (Fig. 5). Half of the water was spring water and the other half was pond water. The water was replaced every week and the waste was taken out of the tank every other day. Snails were fed organic romaine lettuce three times a week. Tank temperature remained between 18°C and 22°C. To maintain calcium levels, oyster shells were crushed and added to the water.

Figure 4. Methods Setup

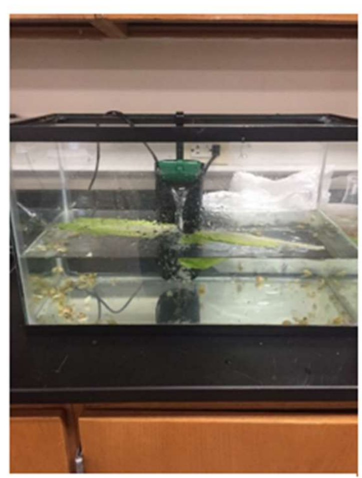

Figure 5. Tank Setup

2.3 Conditioned Taste Aversion (CTA)

Conditioned Taste Aversion (CTA) is an operant conditioning procedure achieved by exposing an organism to a sweet taste, followed by a bitter one. The goal of CTA was to get the organism to associate the two tastes, so when presented with the sweet taste in the future, it will avoid it, remembering its training. This is also known as Taste Avoidance Conditioning.

In this experiment, snails were deprived of food for twenty-four hours prior to training. This was important because during training, the number of bites the snail took was being measured, so food deprivation ensured that the snails were hungry and would take bites.

The first step of CTA is the pre-test. During the pre-test, the snail was placed in a petri dish elevated above a mirror (used to observe the snail bites) (Fig. 6). Next, the snail was exposed to 5 mL of sucrose solution (1.71 g/500 mL). A timer was set for one min and the number of bites of sucrose the snail took was recorded.

Ten min after the pre-test, the snail was exposed to the sucrose solution again for only 15 s. The number of bites was not recorded. After the 15 s, the sucrose was removed from the petri dish using a pipette. Five seconds later, the snail was exposed to a KCl solution (3.725 g/500 mL). The KCl was left in the petri dish for 15 s, then the contents of the dish were emptied, and the pond water was replenished.

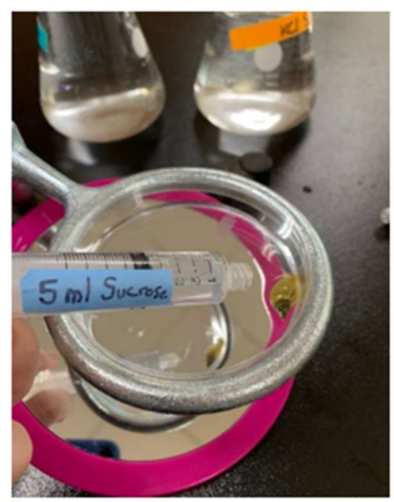

Figure 6. CTA

Ten minutes later, the first post-test was conducted. The procedure of the post-test was identical to that of the pre-test. So, for the 10-min post-test, the snail was exposed to the 5 mL of sucrose solution again. A timer was set for one minute and the number of bites was observed and recorded using a mirror. Two more post-tests were conducted to reach a total of three post-tests per snail. The three post-tests were conducted 10 min, 1 h, and 24 h after conditioning.

2.4 Stressing Procedure

After the snails were conditioned in pond water, all tests were completed, and the snail forgot its training, the snails needed to be stressed. Before stressing, the snails were all given a pre-test. After the pre-test, the snails were stressed instead of being conditioned. To stress the snails, 15 to 20 of them were crowded in 100 mL of spring water. They were crowded and left in the beaker of water for 1 h prior to retraining (Fig. 7). After stressing for one hour, the snails were trained using the CTA procedure, omitting the pre-test, since it was conducted prior to stressing the snails. Once again, 10 min, 1 h, and 24 h after conditioning, the post-tests were conducted.

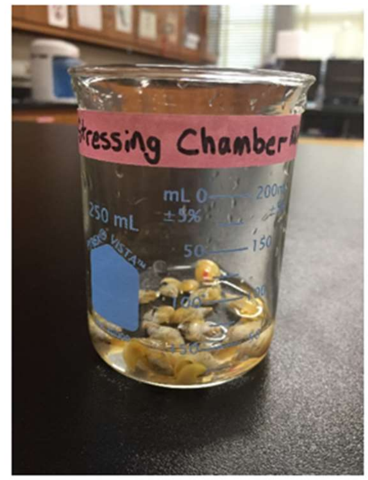

Figure 7. Stressing Chamber

2.5 Stress and Green Tea Procedure

After the snails were no longer stressed and forgot their conditioning once more, they were given another pre-test. This pre-test was crucial since it evaluated if the snails had forgotten their training or not. Ten minutes after the pre-test, the snails were re-stressed using the stressing procedure. After being stressed, the snails were training using the CTA procedure, but instead of being trained in pond water, they were trained in diluted green tea to pond water (1:4). After being trained in the green tea, the normal post-tests followed.

2.6 Statistical Analysis

Data are expressed as the mean number of bites per minute. Statistical significance ($P<0.05$) was determined using a one-way analysis of variance test (ANOVA) to determine if there was a significant difference in the data. Once it was determined that there was a significant difference, a Tukey Pairwise Comparison was used to determine where the differences lie amongst the groups.

3. Results and Discussion

In this experiment, thirty-three snails were used in each group. The first group was pond water only, the second group was stressed, and the third group was stressed and trained in green tea. When the snails were first trained in pond water, most of them displayed good short-term memory, but not long-term, which was expected since the Great Pond Snail does not naturally have good long-term memory (Sunada 2017). After being stressed (group 2), most snails were not able to remember the training at all and took a large number of bites during post-tests. The stressed and green tea snails (Group 3) had improved short, intermediate, and long-term memory, taking very few bites of sucrose in one minute. The mean number of bites for all three groups' tests can be seen below (Figure 8).

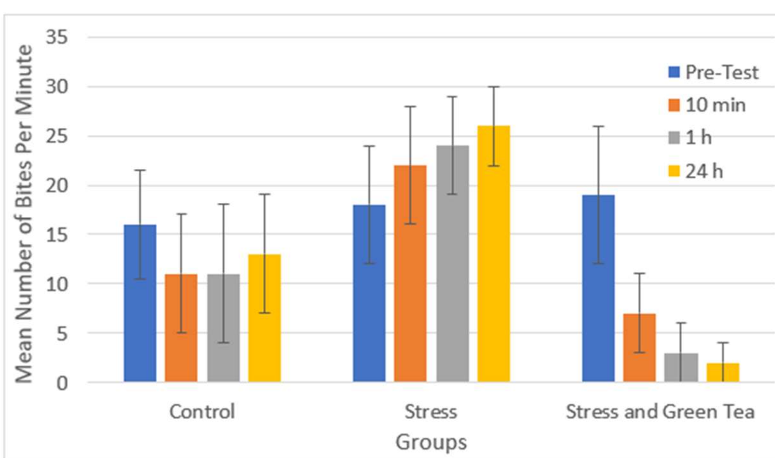

Figure 8. Mean Number of Bites for all Groups. Vertical Bars = standard error

In the pond water trials there was a significant decrease in the number of bites for the 10 min post-test as compared to the pre-test ($P<0.01$, table 1) indicating these snails displayed excellent short-term memory. They retained that memory for the 1 h post-test ($P<0.05$, table 1) but displayed an increase in the number of bites during the 24 h post-test indicating a gradual loss of memory (Figure 8). Seventy-six percent of the snails displayed good short-term (ST) memory, 69% displayed good intermediate (IM) memory, and only 55% displayed good long-term (LT) memory (Figures 9,10,11).

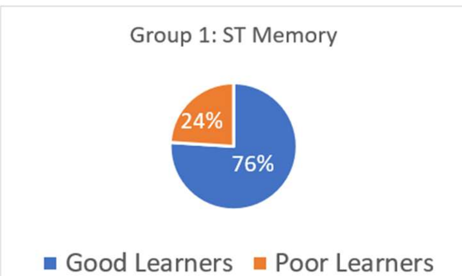

Figure 9. Group 1 ST Memory

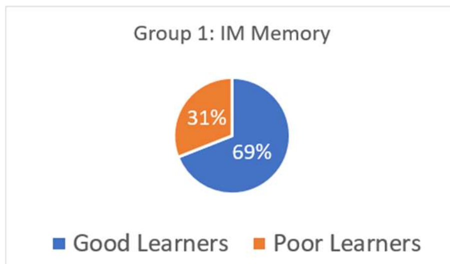

Figure 10. Group 1 IM Memory

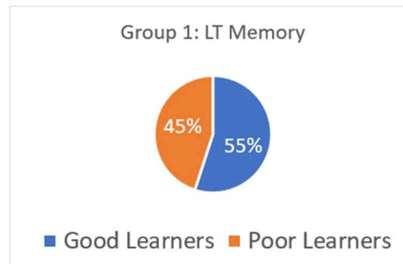

Figure 11. Group 1 LT Memory

In the stress trials there was a significant increase in the number of bites for the 10 min post-test ($P<0.05$, table 1) indicating these snails displayed poor short-term memory. In the 1 h post-test and the 24 h post-test, there was a continuous increase in the number of bites ($P<0.01$, table 1). The stress appeared to have interfered with the snails' ability to learn during training. Eighteen percent of the snails displayed good ST memory, 18% of the snails displayed good IM memory, and only 3% of the snails displayed good LT memory (Figures 12,13,14).

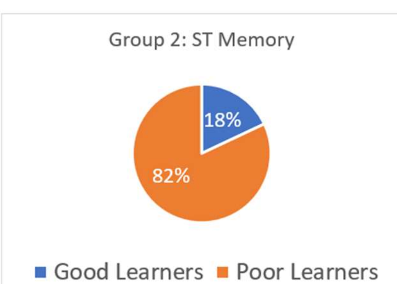

Figure 12. Group 2 ST Memory

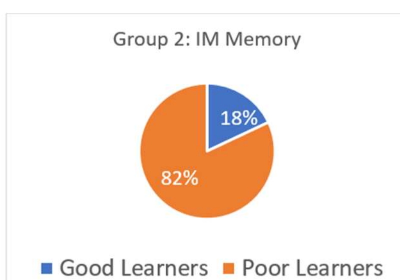

Figure 13. Group 2 IM Memory

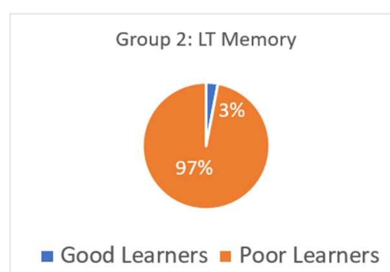

Figure 14. Group 2 LT Memory

In the stress and green tea trials there was a significant decrease in the number of bites for the 10 min post-test ($P<0.01$, table 1) indicating these snails displayed good ST memory. In the 1 h post-test and the 24 h post-test there was a continuous decrease in the number of bites ($P<0.01$, table 1). As time passed, the snails continued to take fewer bites and their memory appears to improve over time. This is the opposite of what happened during the stress trials (Figure 8). Ninety-seven percent of the snails displayed good ST memory, 97% of the snails displayed good IM memory, and 100% of the snails displayed good LT memory (Figures 15,16,17).

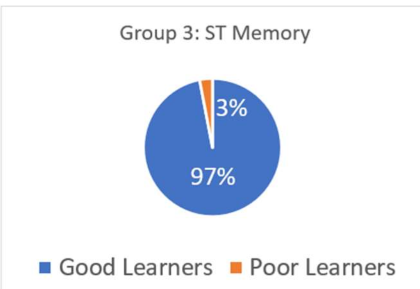

Figure 15. Group 3 ST Memory

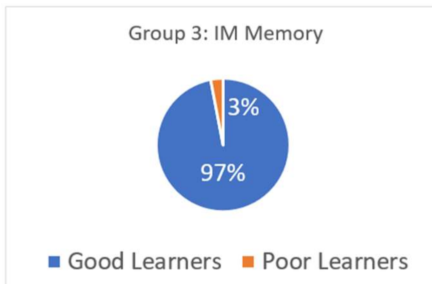

Figure 16. Group 3 IM Memory

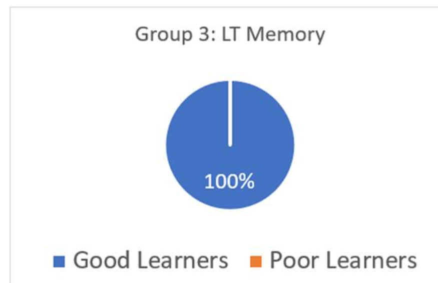

Figure 17. Group 3 LT Memory

The purpose of this study was to confirm that stress impairs learning and memory in the Great Pond Snail and determine if green tea was able to alleviate the effects of stress on learning and memory. The group 1 trails confirmed that the one-trial training was effective, and the snails could be conditioned. The mean number of bites post training had a statistically significant decrease indicating that the training was successful (Figure 8, one-way ANOVA P=4.48E-07). The goal of the group 2 trials was to confirm that stress would affect the snails' ability to learn and remember the CTA. Stress was detrimental to the snails' learning and memory as the mean number of bites post training had a statistically significant increase (Figure 8, one-way ANOVA P=9.50E-07). Green tea was most effective on the long-term memory (group 3). All of the snails were good learners. Green tea was able to alleviate the effects of stress and reverse the imposed learning/memory deficit in the snails. This goes to show that the memory-improving flavonoids are very affective on learning and memory while stress is very harmful. Since the aging neurons in the snails have been shown to be analogous to those in humans, the results from this experiment can be applied to human neuroscience. Stress clearly harms memory, so when students are stressed in school, it will impair their learning and memory. However, daily intakes of green tea could improve any learning or memory deficit caused by being overstressed.

4. Data Analysis

Table 1. Tukey Test: Pairwise Comparisons

Comparison	Pond Water	Stress	Green Tea
Pre-Test vs 10 min	**	*	**
Pre-Test vs 1 h	*	**	**
Pre-Test vs 24 h	ns	**	**
10 min vs 1 h	ns	ns	**
10 min vs 24 h	ns	*	**
1 h vs 24 h	ns	ns	ns

**=Significant P<.01
*=Significant P<.05
ns= nonsignificant

Table 2. P-Values

Group 1	0.004483
Group 2	9.50E-07
Group 3	2.08E-37

To statistically test the data, an Anova test was used followed by a Tukey Test. The Anova test was used to check if the means between the three post-tests were statistically different from each other. P-values were generated, and they displayed that the data was significant (Table 2). The greatest statistical difference was seen in the 24-hour post-test with a p-value of 2.08E-

37. However, all the three p-values were less than 0.01, showing great significance. This means that the results were not just due to chance.

After the Anova test, I wanted to see where the differences lied in the population, so a Tukey Test was used. The Tukey Test generated pairwise comparisons between the pre-test and the different post-tests for each group. It showed whether the differences between each test were significant or not (Table 1). Most of the test were significant within each other, but with some, the number of bites were too close to be significant. For example, in group 3, the 1 h vs 24 h test was nonsignificant (Table 1). This was because in both tests, the snails took very few bites, so when the two tests were compared to each other, there was no difference. However, most tests compared to the pre-test were significant because either the stress or green tea had an influence on the number of bites.

5. Discussion

Since the data was proven to be statistically significant and supported the hypothesis, the hypothesis was accepted. Green tea was able to alleviate the effects of stress on the snails' learning and memory. The greatest improvement, however, was seen in the long-term memory, meaning that the flavonoids contained in green tea have the strongest effects on long-term memory, taking some time to resonate with the snail. Since the mechanisms of aging neurons in the snail have been shown to be analogous to those in humans, the conclusions from my research can be applied to human neurology. In fact, green tea would most likely have the greatest effects on human long-term memory instead of short-term.

Besides the green tea, stress also played a major factor in the findings of this study. Stress caused the snails to take a dramatic increase in the number of bites and a high percentage of group 2 snails were poor learners. Stress clearly caused damage for the snails' learning and memory. This supports the theories that stress is very harmful to human learning and memory. Schools being too stressful for students, will have a direct correlation with lower grades and poor learning and memory. So much so, that it can lead to memory and learning deficits if stress levels are kept high.

To decrease stress levels and reverse the learning/memory deficit, green tea was extremely effective. Green tea is a natural way to reduce stress levels and prevent deficits. The natural way to improve memory has been something scientists have been searching for, so green tea may be the answer.

6. Sources of Error

One of the sources of error in this experiment was the level of stress the snails were under after being taken out of the stressing chamber. Twenty snails had to be in the stressing chamber to induce stress by crowding, but 20 snails could not be trained all at once. Some snails were used as placeholders and stressed several times. Some snails had more time out of the stressing chamber before their training. This could have altered the number of bites they took depending on how stressed they still were.

7. Future Research

I read a study where scientists were able to make a snail remember something that never even happened (Greenwood 2018). Scientists trained snails, then transferred molecules from their brain cells to snails that were never trained. The untrained snails behaved as if they remembered the trained snails' experiences. I found this very fascinating and want to do some similar research in the future. I'd like to find a way to improve memory for people with memory/learning deficits or age-associated memory diseases like Dementia and Alzheimer's.

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Appendix A

Snail	Pre-T	10 min	1 h	24 h
1	8	4	7	10
2	16	6	15	14
3	12	13	13	9
4	9	11	12	9
5	15	14	17	18
6	7	3	19	21
7	21	16	4	2
8	17	9	0	0
9	11	8	10	18
10	13	6	4	17
11	17	0	9	22
12	21	5	7	14
13	23	11	16	24
14	19	0	0	4
15	6	4	12	18
16	14	7	7	16
17	23	11	13	15
18	17	17	11	21
19	9	9	0	0
20	12	12	14	18
21	14	7	7	16
22	18	11	12	13
23	17	12	23	4
24	23	9	18	16
25	15	18	2	14
26	16	7	0	21
27	7	22	24	6
28	21	16	9	11
29	19	12	6	9
30	22	11	15	6
31	13	24	18	17
32	24	17	27	12
33	26	19	22	15
Mean	16	11	11	13
SD	5	6	7	6

Appendix B

Snail	Pre-T	10 min	1 h	24 h
1	14	18	23	22
2	22	29	22	27
3	24	27	26	28
4	18	30	31	21
5	6	11	24	25
6	17	19	29	22
7	11	17	20	19
8	12	9	18	17
9	16	21	21	24
10	27	12	14	28
11	22	26	17	23
12	31	21	23	31
13	8	7	16	29
14	14	22	11	19
15	16	24	26	22
16	17	19	19	30
17	29	29	27	31
18	23	13	16	25
19	16	22	25	25
20	8	19	26	28
21	22	26	29	30
22	21	24	24	26
23	16	28	19	24
24	17	22	27	28
25	14	23	25	25
26	21	28	29	29
27	7	28	29	30
28	24	30	31	31
29	15	29	30	29
30	28	30	30	27
31	22	27	29	29
32	19	28	28	29
33	24	22	24	27
Mean	18	22	24	26
SD	6	6	5	4

Appendix C

Snail	Pre-T	10 min	1 h	24 h
1	16	5	7	6
2	7	2	0	1
3	21	9	5	1
4	11	7	1	2
5	5	0	5	3
6	19	1	0	0
7	14	0	2	2
8	18	3	0	0
9	19	4	4	5
10	27	6	2	1
11	16	9	6	3
12	23	4	3	2
13	18	7	5	4
14	11	3	1	0
15	19	2	1	0
16	15	11	4	2
17	7	7	0	1
18	22	1	0	0
19	26	7	1	1
20	18	12	0	3
21	21	13	0	0
22	20	6	5	5
23	29	8	5	1
24	25	9	6	4
25	16	3	0	0
26	30	7	5	0
27	25	7	0	1
28	17	13	2	2
29	27	15	7	3
30	28	11	8	3
31	27	11	4	0
32	14	8	6	4
33	22	4	2	0
Mean	19	7	3	2
SD	7	4	3	2