association of long-term effects of caffeine consumption on lifespan, behavior and learning performances in mature honeybees (apis mellifera)

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Master Thesis 30 credits 2012

Abstract

Caffeine is probably the most consumed pharmacologically active substance in the world. It is found in common beverages (coffee, tea, soft drinks), in products containing cocoa or chocolate, and in medications. Honey bee (Apis mellifera) serves as an invertebrate model to understand the complexly organized brains, such as those find in mammals. Caffeine affects learning and memory in several different species, including the honey bee.

The purpose of this research was to (1) test the long term effects of caffeine on the life span (2) ask if the long term consumption of caffeine could enhance the learning performances in the honey bees.
The experiment is divided in a mortality count part and the other part is the olfactory conditioning. The first part is to see if the caffeine has an effect on the lifespan of honeybees, and I expect that it will increase, due to previous studies done on yeast that has shown it has an effect. In our case we will use two different concentrations of caffeine, because we want to see which one will in a way decrease the mortality. Gustatory Response Score (GRS) was used to measure how sensitive bees were to different concentrations of sucrose in water. Measuring was done by monitoring the extension of tongue (proboscis) as a response to the sucrose concentrations between 0- 30 % in water solution. For the learning test it was used an odor of carnation oil with 30% sucrose reward if the bees responded by extension of the tongue. It was done 6 contiguous trials and they got scores for response.

The survival analyses shows that the comparison between controls and the low caffeine concentration is significant, and you can say that the difference between the controls and the high concentration is strongly significant. From the learning test it showed no significant differences between the control and caffeine group of the long term consumption of caffeine. There are many studies on the acute effects on caffeine, and it should continue to be explored, since this treatment condition is more similar to the way that humans consume caffeine.

Sammendrag

Koffein er sannsynligvis den mest brukt farmakologisk aktive stoffet i verden. Den finnes i vanlige drikker (kaffe, te, brus), i produkter som inneholder kakao eller sjokolade, og i medisiner. Honningbier (Apis mellifera) fungerer som virvelløse modelldyr for å forstå den komplekse organiserte hjernen, slik som det finnes hos pattedyr. Koffein påvirker læring og hukommelse i flere ulike arter, deriblant honningbie.

Hensikten med denne forskningen var å (1) teste de langsiktige effektene av koffein på levetiden (2) deretter å spørre om langvarig inntak av koffein kan øke læringsutbyttet hos honningbier.
Eksperimentet er delt i en ”dødelighet teller” del og den andre delen er læringsevne. Den første delen er å se om koffein har en effekt på levetiden hos honningbier, og jeg forventer den vil øke, på grunn av tidligere studier gjort på gjærceller som har vist denne effekten. I vårt tilfelle vil vi bruke to ulike konsentrasjoner av koffein, fordi vi ønsker å se hvilke konsentrasjon som kommer best ut. Gustatory Response Score (GRS) ble brukt for å måle hvor sensitive bier er til ulike konsentrasjoner av sukrose i vann. Måling ble gjort ved å overvåke forlengelsen av tungen (proboscis), som en reaksjon på de ulike sukrose konsentrasjoner mellom 0 - 30% i vann løsning. For å utføre testen ble det brukt en lukt av nellik olje med 30% sukrose belønning hvis biene svarte med forlengelse av tungen. Det ble gjort 6 sammenhengende prøver, og de fikk poeng for hver respons.

Analysene fra overlevelses dataene viser at sammenligningen mellom kontroll og lav koffein konsentrasjonen er betydelig, og du kan si at forskjellen mellom kontroll og den høye konsentrasjonen er sterkt signifikant. Fra lærings testen viste det ingen signifikant forskjell mellom kontroll og koffein gruppe på langvarig inntak av koffein. Det er mange studier på akutte effekter av koffein, og det bør fortsette å bli utforsket mer på det, da denne behandlingenstilstanden er mer lik den måten mennesker konsumerer koffein på.

List of symbols and abbreviations

CS – conditioned stimulus

US – unconditionated stimulus

PER – proboscis extension response

GRS – gustatory response score

LS – learning score

MWU – Man- Whitney U test

ANOVA – analysis of variance

AD – Alzheimer's disease

CR – caloric restriction

LD_{50} – median lethal dose

CHD – coronary heart disease

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1. Introduction

Coffee is the most popular beverage in the world that is consumed every day, especially in the western world. Coffee contains caffeine, which is a stimulant, and therefore coffee drinking is not generally considered as a healthy lifestyle. Although, it does contain high sources of antioxidants and other bioactive compounds (1).

The natural alkaloid found in coffee beans, tea leaves, cocoa beans, cola nuts and other plants is caffeine (1,3,7-trimethylxanthine) (2) (3). The extensive use of caffeine in beverages, food, and many pharmaceutical preparations, muscle relaxants, decongestants and allergy medications, has generated much attention to illuminate the variety of effects and mechanisms of action of this active substance of everyday life. Caffeine acts as an antagonist of adenosine A_1 and A_{2A} receptors in mammals (4), which lead to a cascade of event in activation or inhibition of adenylyl cyclase and cAMP (5). The release of norepinephrine, dopamine and serotonin in the brain and the increase of circulating catecholamines, consistent with reversal of the inhibitory effect of adenosine, are caused by caffeine (2).

The nutritionists are more interested in whether caffeine effects on the energy expenditure (EE) and as a pharmalogical tool to clarify the mechanisms of thermogenesis, due to the increasing evidence to a thermogenetic deficiency as the causative to the etiology of obesity (6).

The increased dopaminergic and glutamatergic transmission in differentstriatal subcompartments is also associated to its activation and enhancing effects of caffeine (8). In humans it has showed that caffeine produces subjective and behavioral effects that are similar to those of typically psychomotor stimulant drugs (e.g., amphetamine and cocaine), and are known to be mediated by dopamine receptors (8).

Caffeine has been associated with Alzheimer's disease (AD) by significantly lowering the risk, while recently caffeine intake was found also to be positively associated by lowering the risk of another neurodegenerative disorder, Parkinson's disease (9). In addition, a study demonstrated that any amount of consistently consumed caffeinated coffee decreased by 15% to 25% risk of dying from cardiovascular disease (CD) (10). The risk of dying from any causes has indicated to be decreased by 10% by daily consumption of caffeine. Another 14 – years of prospective observational study on men and women older than 70 years, indicated that dying prematurely was decreased by 4% when daily consumed a cup of coffee (1).

Furthermore, a study showed significant opposite associations of coffee consumption with deaths from all causes and specifically with deaths due to heart disease, respiratory disease, stroke, injuries and accidents, diabetes, and infections. And therefore, coffee drinking might affect the health; this study was assessed at a single time point, and may not reflect the long term effects of consumption (1).

The effects of ageing on brain and cognition are extensive and have several causes. There are abundant signs of natural aging as we grow older (11). The free radical theory of ageing says that ageing can be seen as a progressive, non-stoppable process partially associated with collections of oxidative damages by biomolecules (12). The most visible signs of aging on human body are grey hair and wrinkles, while the hidden signs are changes to the brain size, aged vasculature, decline in bone and muscle strength, reduced vision and hearing and cognition are common (11). There is a difference in cognitive impairment between individuals. While some have an early start of cognitive deficits, others maintain a very high cognitive function at much later age (13). This heterogeneity, which shows the differences in cognitive deficits, is not well understood, but it is a combination of genetic and environmental factors that appear to contribute to this diversity in the population (14). It is shown that regularly exercise, moderate intake of alcohol, and a healthy diet have a positive result on slowing the aging of brain. Otherwise, a high education or professionally achievement also seems to have a protective effect (11).

The different effects of caffeine found by a large number of studies suggest that consumption of caffeine leads to increased alertness. It has been questioned whether there is the caffeine in coffee which lies behind the behavioral changes or a combination of other compounds. Recent research points to caffeine as the main determining factor of the behavioral effects of caffeine-containing beverages (15).

Honey bees (Apis mellifera) are known to be social organisms with high sophisticated community structure that live in colonies with up to 50 000 individuals. The vast majority of the colony is sterile female workers, a few hundred drones (males) and a single reproductive queen (16) (17) (18) (19). The workers are relatively short lived compared to the queen that additionally lives about 2-3 years (20) (21). Worker bees provide as a model for ageing research because of their flexible ageing that appears to switch tasks within the colony (22).

The starting tasks of a worker bee is as a “nurse”, which implies inside the nest performing larval care, cleaning and building work (22). The worker bees changes from nursing to “forager”, and collects nectar, pollen, propolis and water for the colony. In general the bee will forage until she dies, which could be in between 7-8 days after she began to foraging (23). The nurse bees age slowly compared to the fast ageing in the foragers, but they can show variability in aging when the workers delay or hasten the transition from nursing to foraging, or return from foraging to nursing duties (20).

For the direction of their flight the forager bees uses landmarks, memories of previously visited flowers, and learn to associate to floral odors which rewarded with food (24). Therefore, learning and memory are important abilities for a forager bee to secure a safe return to their nest (25). The honey bees communicate with each other about the direction and distances, with a ritualized body movement. In a way they have an abstract way to communicate about food sources (19). The honey bees are well-established invertebrate models for learning and memory, and are extensively used as a model for age related functional changes in the brain (18). Among the insects, the honey bees are represented at an individual level as one of the most advanced restraint models of learning and memory (18). In the laboratory the associative (Pavlovian) learning can be measured, as the brain function matures during foraging period (26). Learning in honey bees can be tested by behavioral tests of learning in the laboratory. This involves the olfactory conditioning assay, the proboscis extension reflex (PER) (18), which can be used to study the acquisition for an association and period of the memory for the association. Bees are exposed to an odor (The conditioned stimulus with reward; CS+, without reward; CS-), followed by a drop of sucrose as a reward (The unconditioned stimulus, US) delivered to the antennae, and elicits the extension of the proboscis (18) (24).

The accumulating oxidative damage to proteins and lipids in honey bees are associated with the decline of learning and memory (27). From earlier studies of aging, the honey bees showed that behavioral aging in mammals can be modeled in insects, and that they shows functional decline patterns during aging when compared to the findings in mammals (28). This may possibly make them as a key model for age related diseases.

It has been shown that caffeine increase lifespan on yeast cells (29), and it also increases the learning ability in younger honeybees (30). This positive effect of caffeine established my two

main hypotheses in this study. To start with I am interested to see the long-term effects of daily caffeine consumption and hypothesized that the long term effect will increase the life span in honey bees. Secondly, I will measure the daily consumption effects of caffeine on learning ability of matured honeybees, and ask if the long term effect of caffeine could enhance the ability of learning performances in matured honey bees. The experiment is divided in two parts. The first part conducts the long term effects of caffeine with two caffeine concentrations, and the second part is the olfactory conditioning by the long term consumption of caffeine. To avoid having any hive specific outcomes, I have used honey bees from two different hives (Hive 1 and hive 2).

Most studies of caffeine consumption have studied the acute effects by a single dose, while very little is known about the long term effect as a regular consumption of caffeine. However, some suggest that the high consumers express better performance, especially when challenged with non-consumers of caffeine. Yet, there are exceptions which demonstrate that high users show reduced performance, even though the effects are restricted to specific tasks of performance (15).

2. Materials and Methods

The experiments were performed during the spring of 2012 at the Norwegian University of Life Sciences (UMB) in Ås, Norway.

This experiment was divided in two parts. The first part was the Mortality Counts and the second part was the Learning Score test. Both parts were conducted separately and the results from part one gave information about the caffeine concentration to proceed with. See figure 1 for overview.

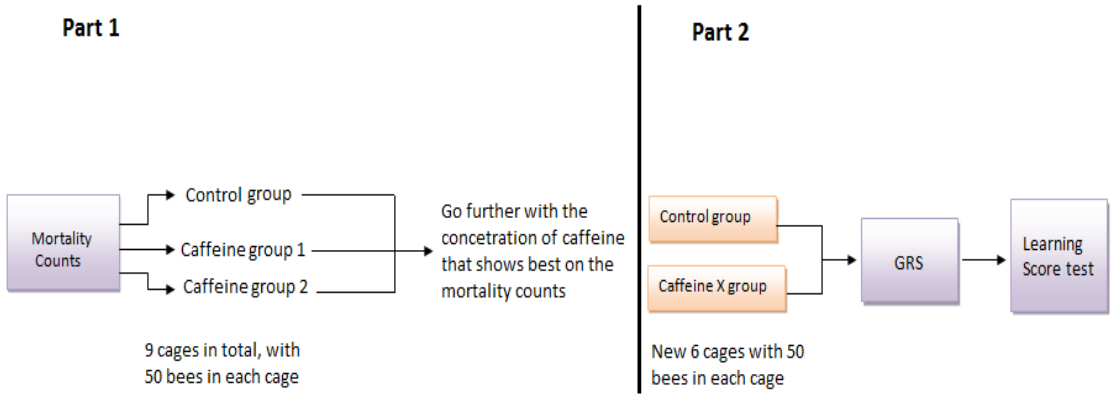

Figure 1: Overview of the experiment setup, divided in two parts.

2.1 Honeybee source

In this experiment we used newly emerged honeybees from two different hives. The two different colonies were selected to look at whether or not there were any hive specific effects on the results.

The newly emerged bees were marked with two different colors depending on which hive they were born from. When the marking was done we distributed the honeybees and placed them back to their hives. Half of the honeybees that were born in hive 1 were placed to grow up in the hive 2 and same for the honeybees born in hive 2. 5 days was to ensure that they got normally growth (conditions) before entering the cages.

The same procedure was done for the second part (Part II).
For the first part, there were marked approximately 900 bees in total from both hives, which was double amount of what needed for the experiment. The bees from hive 1 got a yellow mark on the thorax (about 530 bees), while bees from hive 2 got a red mark (about 112). This was done to distinguish between the two hives. The double amount of bees where marked, as it was expected to get less back from hives when collecting them after 5 days. Because these bees have been out of the hive, when they return, there a chances they may not get accepted back into the original hive (ref ??? boka?).

2.2 Materials

In a 100% food stock solution, caffeine (Sigma) was dissolved in 50% BIFOR (Nordic Sugar), 47% dH_2O, 1% Lipid mix (Sigma), 2% Amino acid mix (Sigma) and administrated in a volume of 1, 25 mg/ml and 0,125 mg/ml concentration.

2.3 Part I: Mortality Counts

The first part was to see if the caffeine has an effect on the lifespan of honeybees. In this experiment, it was used two different concentrations of caffeine to go ahead with the concentration that reduces mortality in bees. The experiment started with marking several bees and put them into 9 different cages. There were approximately 50 bees in each cage, where three boxes were the control groups. Three boxes were fed with caffeine concentration 1,0
and three for caffeine concentration 2.
The control group got high carbohydrate diet with no caffeine: 50% biefor (…), 1% Lipid mix (Sigma) 2% Amino acid mix (Sigma) and dH_2O.

Caffeine groups got the same diet with addition of two different caffeine concentrations: 1. 1, 25 mg/ ml of caffeine and 2. 0,125 mg/ml of caffeine.
When the food stock was made for each group they were added to a 10 ml tube and frozen until use, and left some in the fridge (4°C) for the next day. Before using the tubes there were made four holes so the honeybees easily could absorb the food solution with the proboscis (tongue) extending. In addition to food, bees had access to water (dH_2O) in a 10ml tube which was replaced with new water every day. The tubes were changed and noted down the

consumption each day, so they received new food every day. At first when all of the bees were collected into the cages, they were fed with sucrose solution one day before giving the caffeine.

The bees were kept in an incubator for 30°C with high humidity. The bees were checked twice a day with 12 hours gap. The dead bees were removed, noted down deaths, consumption in mL in a form, and changed the tubes with the food every day (Se the form for mortality counts and consumption data in Appendix 1).
The experiment was done till there were no bees alive, and the data could be analyzed.

2.4 Part II: Olfactory conditioning

Based on the results from Part I, I decided to go ahead with caffeine concentration 2 (0, 125 mg/ml). This time there were prepared 6 cages to add new honeybees in it. The bees were marked and collected the same way as for the Mortality test and were checked every day for deaths as for Part 1. From Part I, it was observed that the mortality curve slightly went downwards after approximately 10-12 days for the control group (about 30% of bees were dead at the time). Therefore, I decided to stop the experiment after 30% of mortality in control group and start the tests. There was tested on about 50 bees in total each day (distributed the 6 cages in 3 days), where 25 from the caffeine group and 25 from the control group tested simultaneously.
The food stock was made with the same diet as for Part 1 with the addition of caffeine for the low caffeine group.

The honeybees were kept on ice for some seconds so they were immobilized and then it was easy to strap them into a small plastic holder with a tape placed around the head and one on the back body. Only the mouthpart and antennas were able to move while strapped. The holders were put into plastic tubes that were numbered. The tube was randomly numbered to make sure that the examiner did not know which group and hive each bee was originally from.
The bees were then force-fed without touching the antenna with 2 µl of 30% sucrose solution to lower the mortality rate. This was done by adding the 2 µl drop on a flat side of forceps and gently placing it under the tongue. The honeybee automatically extended the tongue and

sucked the sucrose solution. After 3 hours, the tests were conducted (See Appendix 2 for the fill out form).

2.4.1 Gustatory Response Score (GRS)

First test to perform on the honeybees after starving was the gustatory response score (GRS). GRS was measured to notice how sensitive bees were to different concentrations of sucrose in water solution. This was done by monitoring the extension of tongue (proboscis) as a response to the sucrose concentrations between 0-30 % in water solution. They got 7 points for responding to all concentrations, and for those that didn't respond to any sucrose concentrations got zero.

Started with 2 µL zero concentration (dH_2O) to 30% sucrose (0, 0.1, 0.3, 1, 3, 10 and 30%) and gently touched it right over the head and led the pipette back and forth between the antennas, without feeding. Each bee got 5 seconds to respond with extending of the tongue. The one that responded got 1 point, while zero to no response, and from this it was measured each bee's subjective sensitivity value for sucrose solutions.

2.4.2 Learning Score (LS)

For the learning test it was used an odor of carnation oil to train the bees to associate the odor with 30% sucrose reward by extending the tongue. This training regarding associative learning is the differential learning of PER (31). It was done 6 contiguous trials and they got scores for responding to the odor. The odor was first presented for 3 seconds (CS+) and then paired with sucrose reward (US) for other 2 seconds. In total each bees got 5 seconds, with 3 seconds to respond to the carnation oil. There were done six trials per individual, and the learning score was noted down on a form (see appendix 2).

The trial started with placing the bee in front of an exhaust fan for about 10 seconds, so the bee could adjust to the airflow before being exposed to the odor (CS+) and US. A test of odor (cineole, CS-) was delivered without the US, before the main odor trials.

For the preparations for two different odors there was made a 10 mL syringe contained with 2 µL of pure odorant on a paper. The odor was delivered by manually pushing the syringe towards the bees for 3 seconds, and after 3 seconds the US was applied (see figure 2). The sucrose solution, the reward, was given by gently touching the antenna and mouthparts. Only the bees that extended proboscis within 3 seconds got a score 1 and were fed (approximately 1

µL), while non respond gave score 0. It was about 10 minute intervals between each conditioning trials to ensure correct memory formation (32).

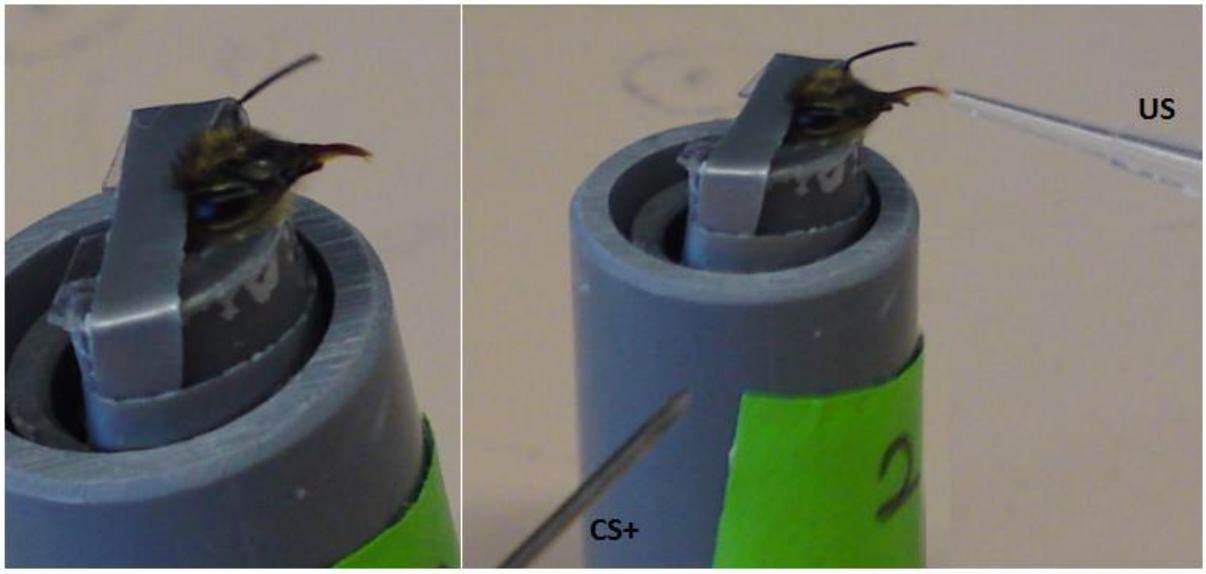

Figure 2: The conditioning trial. Picture on the left showes a honeybee that has learned to associate with the odor by PER. Picture on the right shows a bee feeding with sucrose solution (US), while still getting airflow of the odor (CS+).

2.5 Statistical analyses

The total number of individuals used in the first part, the Mortality counts was 443, with 151 individual from control group (n_c= 151), 145 from 1, 25 mg/ml caffeine concentration ($n_{1,25}$ = 145) and 147 from 0,125 mg/ml group ($n_{0,125}$ = 147). To get an objective assessment of the data collected from mortality counts, the Survival Analysis was done. This analysis shows whether the treatment groups, which in our case were the two caffeine concentrations (0,125 and 1, 25 mg/ml) and the control group. We are interested in getting to know whether there are any differences of the surviving of honeybees in the various treatments they went through (Cox F-test). ANOVA test was used to analyze the effects of multiple categorical independent variables (factors; birth hive and treatment effect).

To analyze the consumption data conducted from the mortality counts the data was first checked to be reliable or not. The correlation analyze was done to test if the "increasing consumption" problem could be removed when there were few bees in the cages. The testes were done by removing cages with less than 9 bees (Total observation, N = 148). A mean plot of the effects was added, ANOVA and the post hoc test were done on the treatment effects. .

The data from learning performance test and gustatory responsiveness was not normally distributed, and the non-parametric tests were used to compare the median scores. Mann Whitney U (MWU) test and Chi-square test were used to compare and assess effects on treatment groups for GRS and LS.

Analyses were conducted using Statistica 6.0 (StatSoft), by a significance level of 5% ($p<0,05$).

3. Results

The result section is divided into two main parts. The first part covers the long term daily effects of caffeine on life span. While the second part covers about the long term effects of caffeine in learning performances.

3.1 Part I: Long term effects of caffeine on survival analysis

The result on the overall analysis tells us that there are effects in the data (Chi-square = 26.3327, df = 2, $p < 0.0001$).

There was performed Cox's F- Test separately for comparing the two treatments of caffeine against the control group to find out what was causing overall statistical influence.
Results from the control; n_C = 148/294 and Caffeine 1; 1.25 mg/ml caffeine, n_1 = 145/294, showed a strong significant differences between survival of bees, with $F_{298, 290}$ = 1,558407 and p= 0.00008.

Then the control was tested with the 0,125 mg/ml caffeine low concentration group: Control; n_c = 149 and 0,125 mg/ml caffeine concentration; $n_{0,125}$ =146.
The results shows a significant difference between the caffeine F(292, 298) = 1,250198, p = 0,02774.

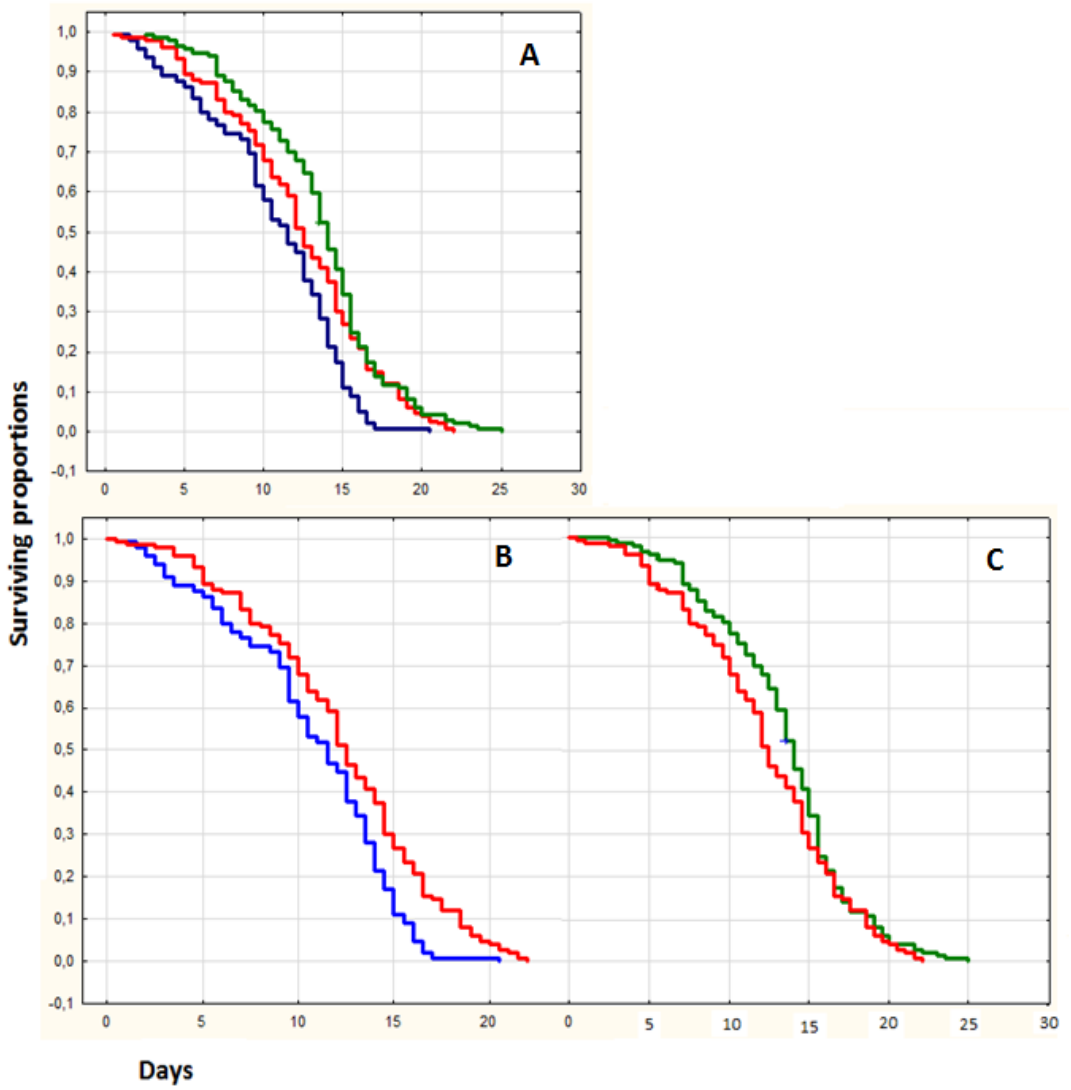

Figure 3: Survival analyses between two caffeine treatments and a control group. Control group is color red, high caffeine concentration blue and low caffeine concentration is green. (**A**) Shows an overview of the surviving proportions per days, and shows significant differences between treatments. (**B**) Shows the high caffeine treatment compared to control group (with a strong significance of p = 0,0008) (**C**) Shows the low caffeine treatment compared with control group (Significance with p = 0,02774).

3.1.1 Other factors

Since I have used bees from two hives, I was interested in finding out whether there were differences in the lifespan due to which hive the bees were born from (independent of treatment groups). Birth hive has a strong significant differences in mortality between hive 1 and hive 2 ($F_{234, 646}$ and p = 0.00004).

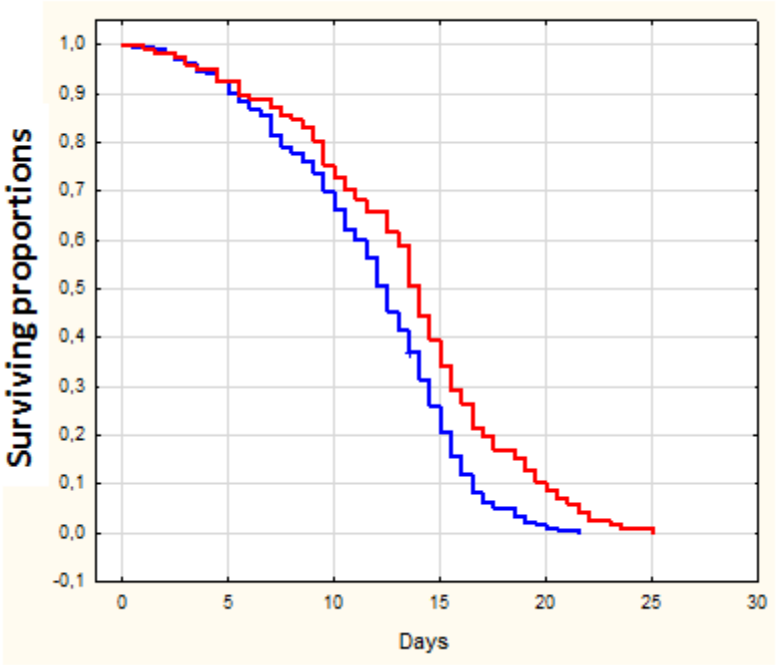

Figure 4: Birth hive effects on the mortality rate between two hives. Total nr of valid observations= 441, Gr 1 (Hive 1, blue) = 323(324), Gr 2 (Hive 2, red) = 117 (Cox- F test).

3.1.2 Food consumption data from mortality counts

A normal consumption for a bee in a day is about 0.015 – 0.04 mL. From the scatter plot on figure 5 the number of bees is placed against consumption in mL per bee. The red rectangle in the figure indicates the unreliable data about the increasing high consumption value with decreasing bees alive in each cage.

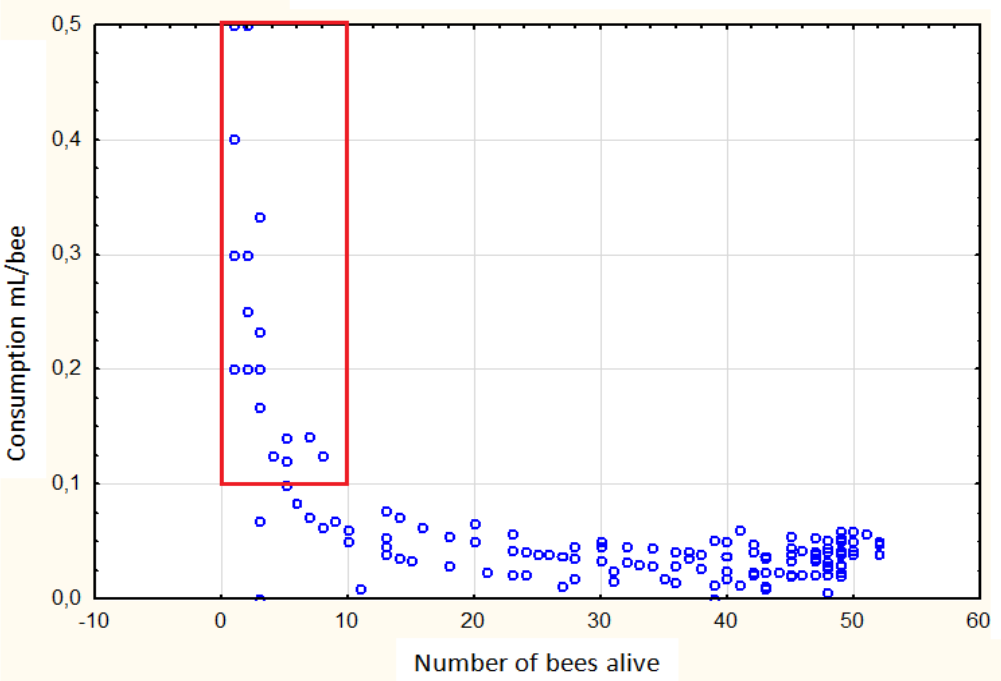

Figure 5: Scatter plot with consumption ml/bee per nr of bees alive. The red rectangle indicates the unreliable consumption data for number of bees alive in each cage.

There were made correlation analysis between consumption and day of the experiment when removed the unreliable data (removed the unreliable data). Total observation was then, N = 148.

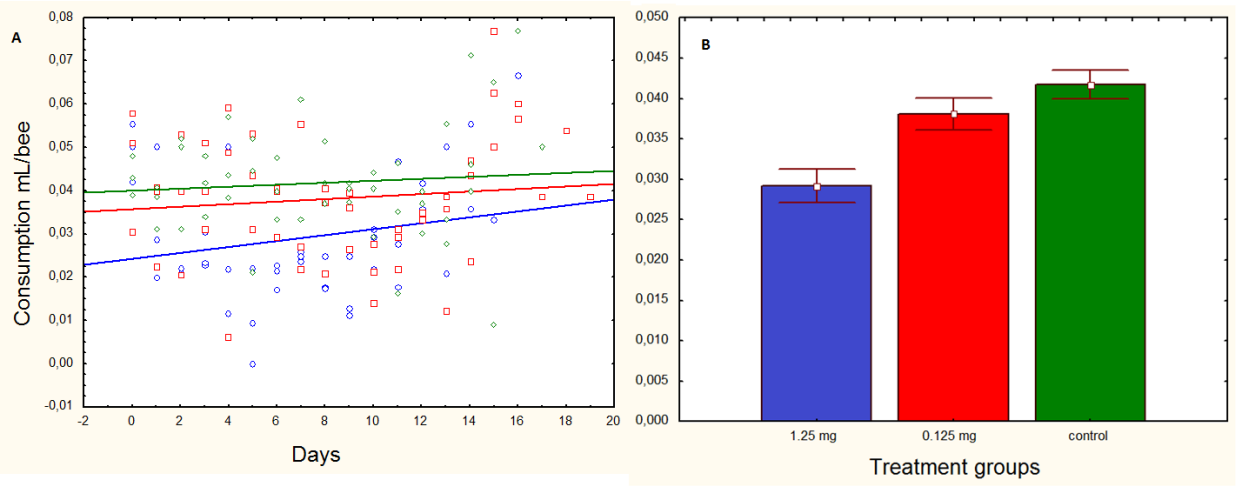

Figure 6: (A) Distribution mL/bee after removing observations of cages with less than 9 bees alive. Scatter plot that shows the corrected data with regression lines for all treatments (Control- green, low caffeine concentration- red and high caffeine concentration- blue). The lines are close to flat and suggest that the problem is resolve, and tells the average consumption in ml per bee with regression lines for each treatment group. **(B) Mean plot of the average consumption for all treatments.** The mean value for consumption mL/bee of the control group is higher compared to the high caffeine concentration group, while the low caffeine group is in between the two other.

ANOVA: Univariate tests of significance for ml/bee.

The three different treatments show a significant effect of consumption (F= 10.9, df = 2 and p = 0.00005).

The post-hoc test shows that the control and the low caffeine treatment group are similar (p = 0,107), while the high concentration of caffeine is different from the other two groups with p = 0,000001 with control group, and p = 0,0002 with the low caffeine concentration group.

3.2 Part II: Long term effects of caffeine in behavior and learning performances

To test the potential long term effects of caffeine treatment on behavior and learning, the bees were fed with a low caffeine diet (0,125 mg/ml) until 30% of the bees were dead and compared with a control group and then tested for GRS and LS.

3.2.1 Effects of caffeine on gustatory responsiveness

Our results do not show a significant effect of caffeine on gustatory responsiveness ($Z = 0.835$, $p = 0.4$; Mann- Whitney U test (MW U)).

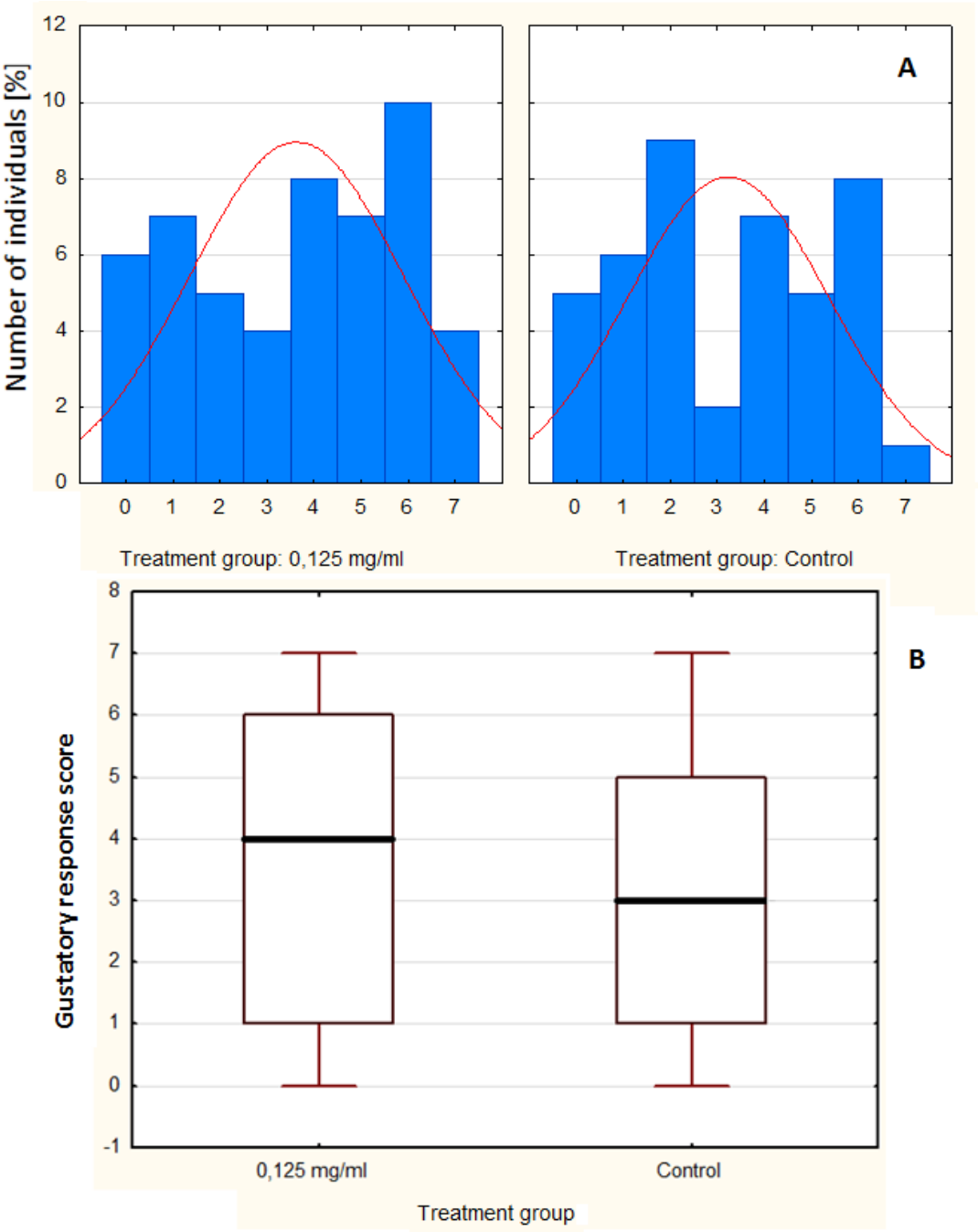

Figure 7: Caffeine treatment and control group do not show differences in the gustatory responsiveness. (**A**) Categorized histogram of GRS values. There is a similar distribution of both control and the caffeine group and no significant difference in between were detected. (**B**) The graph shows medians and interquartile ranges with n = 43/51 for control and caffeine group, respectively.

3.2. 2 Effects of caffeine on Learning score test

We did not detect significant differences of long term caffeine expenditure on learning test when compared to the control group (Z = 0.6, p = 0.5; Mann – Whitney U test).

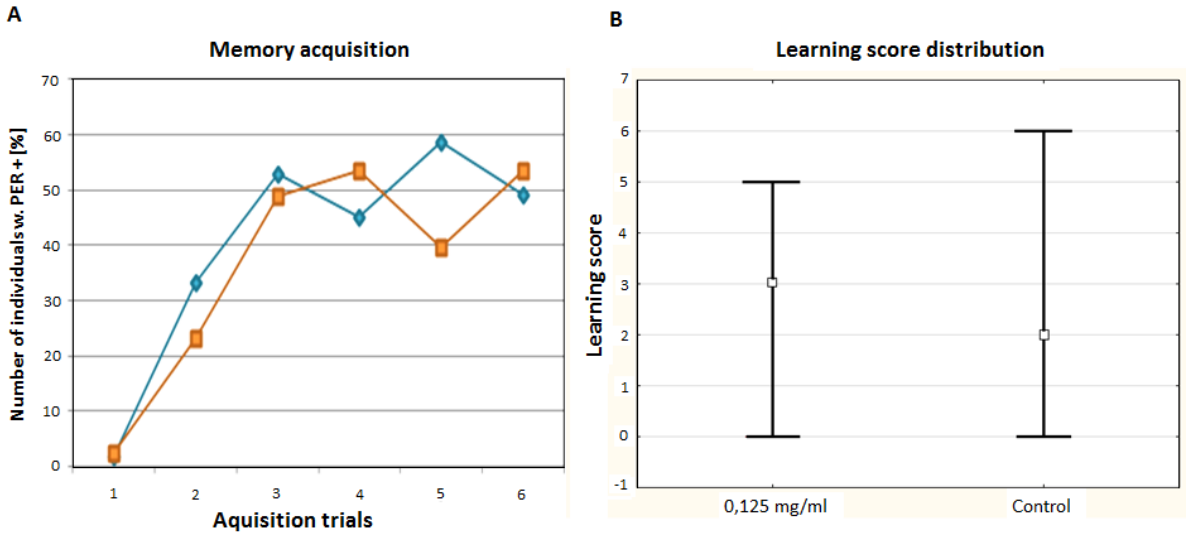

Figure 8: Acquisition trials and median graph for caffeine treatment and control group. (**A**) Shows the acquisition trials (6 CS-US pairings for LS) with the number of PER+, color orange is control group, and blue 0,125 mg/ml caffeine group. (**B**) The graph shows medians and interquartile ranges for both groups, (p = 0.5; MW U).

4. Discussion

The main implications of this study concerns the long term effects of potential use of daily caffeine consumption on the lifespan and compare the learning ability of matured honeybees. Learning performance will also consider how individuals will cope with the representative caffeine concentration of interest. This was done by separating the experiment into two sections, first to conduct mortality counts by measuring the lifespan, and then measure the learning performances. Therefore, these two main sections will be discussed independently in their respective order.

4.1 Long term effects of caffeine on survival

This part was fulfilled over several days, starting with new emerged bees, picked the same bees 5 days later and placed them in a cage-box and then into the incubator. To get high resolution data, I checked the bees and counted them twice a day, changed the food supply and water (dH_2O) on a daily basis. This was to make sure the bees got fresh food every day. Fortunately I didn't experience any abnormal mortality patterns with bees in the boxes when I placed them into the incubator.
The initial mortality was low in almost all boxes to start with, accordingly they were safe in the more or less flat mortality phase, which is typical for young individuals who have not developed stress symptoms yet (23).

4.1.1 Mortality rate between the three groups

The analyses show that there is a significant difference between the three groups. The various treatments turn out to have an impact on mortality and life of honey bees.

4.1.1.1 Effect of high concentration of caffeine compared with control

The analyses showed that the high concentration of caffeine (1, 25 mg/ml) influenced the early mortality compared to the control group. The effect was found for both the higher mortality rate and early decreasing life span in honey bees. We saw a clear pattern when observed the bees in the incubator, and similar pattern from the statistical results as strong significant evidence between these two groups.

This concentration was not immediately toxic to the honey bees, but it showed a clear tendency of reducing the lifespan when compared to the control group.

It is not common that excessive use of caffeine can be lethal. In adult humans the estimated lethal dose of acute caffeine is to be 10 g/person, while it has been reported death by ingesting 6.5 g caffeine, and at the same time a patient survived from 24 g caffeine (2). The median lethal dose (LD_{50}) per kilogram albino rats is 192 ± 18 mg per kg body weight, while in humans LD_{50} is estimated between 150 – 200 mg/kg (ref 1 og 2 se printet ut draft).
It is estimated that a bee drinks about 20 µl sugar solution every day (33). Then the high caffeine group corresponds to a dose of 1, 25 µg/ml x 20 µl, which is about 25 µg caffeine per bee, per day. A worker bee is about 150 mg (23). In other words, the daily dose per bee is 0,025 mg per 0, 00015 kg, which would be the same as about 166,7 mg caffeine per kg bee. The dose is exactly at the lethal dose of humans, while the low caffeine concentration group is 10x lower dose of that, i.e. 0, 125 mg/ml.

Coffee drinking has been associated with coronary heart disease (CDH), but it does not increase the risk of CDH or deaths in humans. Some studies suggest that those that are heavy coffee drinkers (<4-9 cups per day) can increase the risk of getting CDH (34), as in our case the early mortality in high caffeine group.

4.1.1.2 Low concentration of caffeine compared with control

The analysis of the low concentration (0,125 mg/ml) of caffeine and the control group is significant. The less mortality rate in this low concentration of caffeine group indicates as hypothesized that caffeine will increase the lifespan, hence supporting the hypothesis. This results is an important confirmation that reasonable amounts of daily intake of caffeine is related to and affects mortality by increasing the life span of honeybees.

Consequently, caffeine has shown to extend lifespan in yeast cells by implicated several nutrient-sensitive kinases (including the target of rapamycin complex; TORC1). The kinase cascade is shown to be evolutionary conserved and suggesting caffeine as a lifespan extending effects in other eukaryotes as well, including humans (29).
A study in mice suggested that a moderate daily intake of caffeine may delay or in some cases reduce the risk of getting Alzheimer disease (AD) (9).

4.1.2 Hive effects on mortality rate

I tested for replicate effects of source colony (hive birth) and found that the colony origin, the birth place for bees, had an effect on surviving. I detected a significant variation between the two hives that I used for my experiment. The 2 hive showed to have less mortality and higher survival than hive 1.

This can be explained by the fact of individuals belonging to different hives behave differently (23), as in my case where hive 1 responded differently by having a lower survival.

4.1.3 Consumption data

As mentioned above, I noted down the consumption of food in mL every day for all boxes. At the end it gave me a spreadsheet with all the data's for each treatment, and how many bees there were alive at the time point (Days). After collecting all the data I could calculate the mL per bee consumption and do the various analyses. An average food intake for a bee in a day is about 0,015-0,04ml, while the scatter plot on figure 5 shows in the red rectangle the unreliable data that was removed while undertaking the statistics. These data's are not correct value for a bee to consume in mL, and therefore are a source of error. One explanation of the error could be that in the end of the experiment I was too quick with noting down the numbers, and therefore not accurate.

The analyses showed that the treatment had a significant effect on consumption. The control group and the low caffeine treatment group are similar, while the high caffeine treatment is different from the other two groups.
The same average consumption per bee in the low caffeine and control group shows an endurance prolongation of the life with the low caffeine group of bees. Several studies suggest that animals that receive as much food as they want live shorter lives than those that eat somewhat less, this is called "Caloric restriction". Calorie restriction (CR) has shown to extend life span and age-related diseases in rats, mice, fish, flies, worms, yeast, and in variety of species. The mechanisms of CR are unclear, but it says to reduce metabolic rate and oxidative stress. At the same time the aging process are regularly affected by environmental factors and the CR for extending lifespan in various animal models (35). In our case the longevity effect is not caused by the low caffeine group bees eating less, but is further

supported by the reduced survival of the high caffeine group of bees, which actually did eat less than the low caffeine and control group.

The high caffeine causes bees to consume less. This may be due to an effect of the caffeine on the bees, or this food must be less palatable. In addition to sugar the nectar can contain very small amount of other substances. I did not find any literature about bees tasting caffeine.

The effects of caffeine on appetite are unknown in humans, but if it is assumed that there is not any compensatory increase in food intake, the increase of 5% in 24 hours of EE after consumed caffeine can represent an energy deficit. In animals it is demonstrated that caffeine at high doses has a reducing effect of body fat and body weight (6).

4.2 Part II: GRS and LS

The major goal of current research on aging is to define the neural basis of age-related cognitive dysfunctioning. Evidence from studies done on model organisms and humans indicates that aging does not undoubtedly lead to cognitive decline (36). Therefore, I want to see if the long term of caffeine consumption may affect the learning performances in mature honey bees.

The same procedures with new bees were collected as for the part 1 for preparation on this part. From the mortality counts the low concentration came out good and I decided to go further with olfactory learning tests. The bees were kept inside the incubator and fed for approximately 10-12 days with the given diet.

4.2.1 GRS

The gustatory responsiveness test (GRS), was done to measure motivation and sensitivity of each bees to different sucrose concentrations.

The analyses show that both the control group and caffeine group had no significant differences on the GRS results. That means that caffeine does not alter sensitivity for sucrose concentration when compared to the control group.

4.2.2 LS

A previous study of honey bees shows that caffeine improves motivation and cognitive learning (30).

Caffeine showed no differences in learning performances, and this tells us that the long term effects of caffeine does not affect the learning ability of honey bees. At the same time I could find no report in the literature showing the long term effects of caffeine in model organisms, but I did find the acute effects of caffeine when administrated before a session.
The literature shows that even in low doses caffeine may increase alertness, while high doses can lead to anxiety in some individuals. At the same time there is little evidence suggesting impairments following consumption of caffeine (15)

5. Conclusion and future work

My study shows that the long term consumption of caffeine has an increasing effect on the life span, while the high concentration of caffeine showed the opposite. The high concentration of caffeine does not increase the life span of honey bees. The early mortality in the high caffeine group can have a relation with premature death, because coronary heart disease (CHD) is associated with chronic consumption of three or more cups of caffeinated coffee daily. Furthermore, reliable prospective cohort studies have consistently found no association between any amount of coffee consumption and CHD (10). There should be further research regarding the effects of regular daily caffeine consumption effects on human health**.**

I examined the long term effect of caffeine, while most of the studies focused on the acute effects. The results from the caffeine group did not show any effects on the learning ability in matured bees. As I expected the aging brain of the honey bee will have associative effect on regular intake of caffeine. More research is needed on the effect of usual levels of caffeine consumption on performance efficiency. Although, there is some evidence that high consumption is associated with better performance, especially among the elderly (15). There are many studies on the acute effects on caffeine, and it should continue to be explored, as this treatment condition is more similar to the way that humans consume caffeine.

Acknowledgements

First of all, I thank everyone who gave me the possibility to complete this thesis. My deepest appreciation goes to Professor Gro Amdam for giving me the opportunity to do this interesting study, and for her incredible guidance, advice, motivation, for assisting me and offering any insight on how to approach my research.

I also want to thank Dr. Daniel Münch for listening when I had a question and offering me with helpful suggestions, guidance and support in the laboratory, and in the writing process.

Thank you, Claus Kreibich for teaching me about beekeeping and for getting bees when I needed them, and for making sure I had all the chemicals I needed to perform my experiments.

Thank you, Anbjørg Rangberg for helping me with the statistical analyses.
I also want to thank the entire group of "Integrative neuroscience and sociogenomics" at IKBM for a great support.

Finally, I would like to thank my family and friends for their enormous support. To my wonderful fiancée, Waqqas, thank you for listening and for the encouragement to pursue my Master's degree. My sister Maria and my brother Shahnawaz to borrow their cars for the drive from Oslo to Ås, Thank you so much. You saved me a lot of time!
I want to thank my mum and my in-laws for listening to me talk about my research, even though they didn't always know what I was doing and for continually supporting me. I also want to thank my good friend Sumaira for a great companion to Ås every day, and special thanks to my best friend Samin for proofreading this thesis.

To all my friends, thank you for asking about my research and being interested in what I was working on.
Miss you, Dad!

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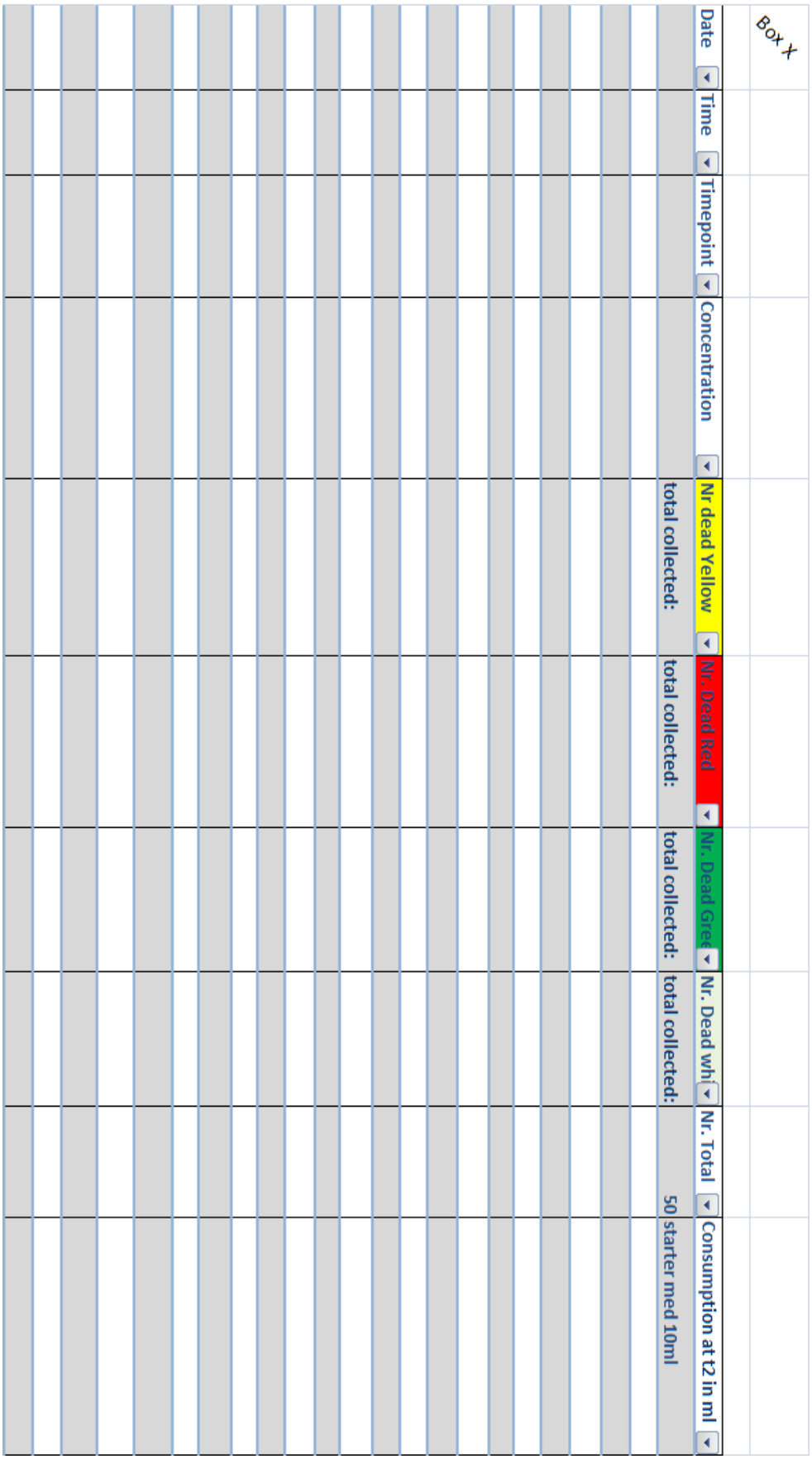
Appendix 1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Time
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Concentration
Nr dead Yellow
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Consumption at t2 in ml
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50
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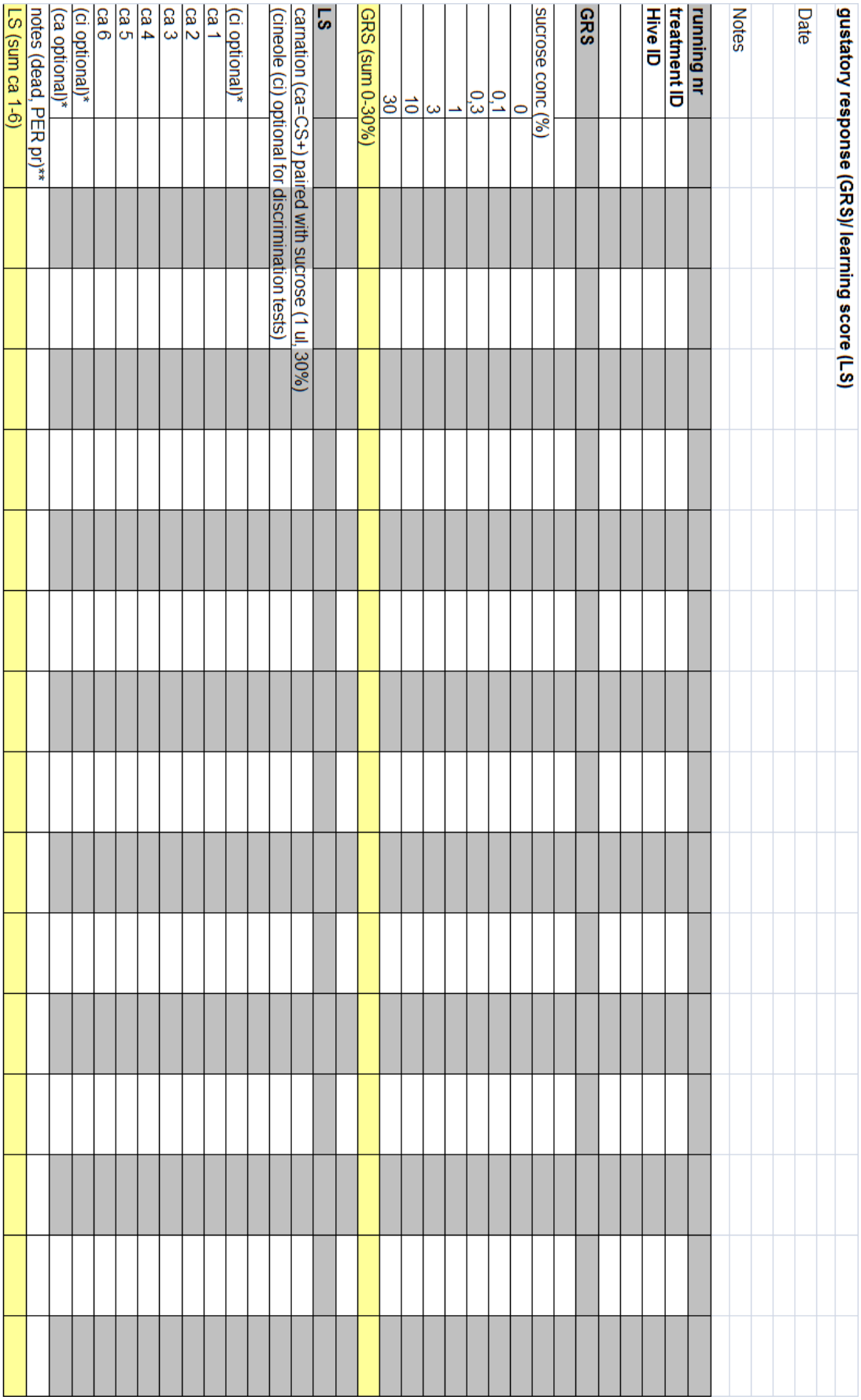
Appendix 2


gustatory response (GRS)/ learning score (LS)
Date
Notes
running nr
treatment ID
Hive ID
GRS
sucrose conc (%)
0
0,1
0,3
1
3
10
30
GRS (sum 0-30%)
LS
carnation (ca=CS+) paired with sucrose (1 ul, 30%)
(cineole (ci) optional for discrimination tests)
(ci optional)*
ca 1
ca 2
ca 3
ca 4
ca 5
ca 6
(ci optional)*
(ca optional)*
notes (dead, PER pr)**
LS (sum ca 1-6)