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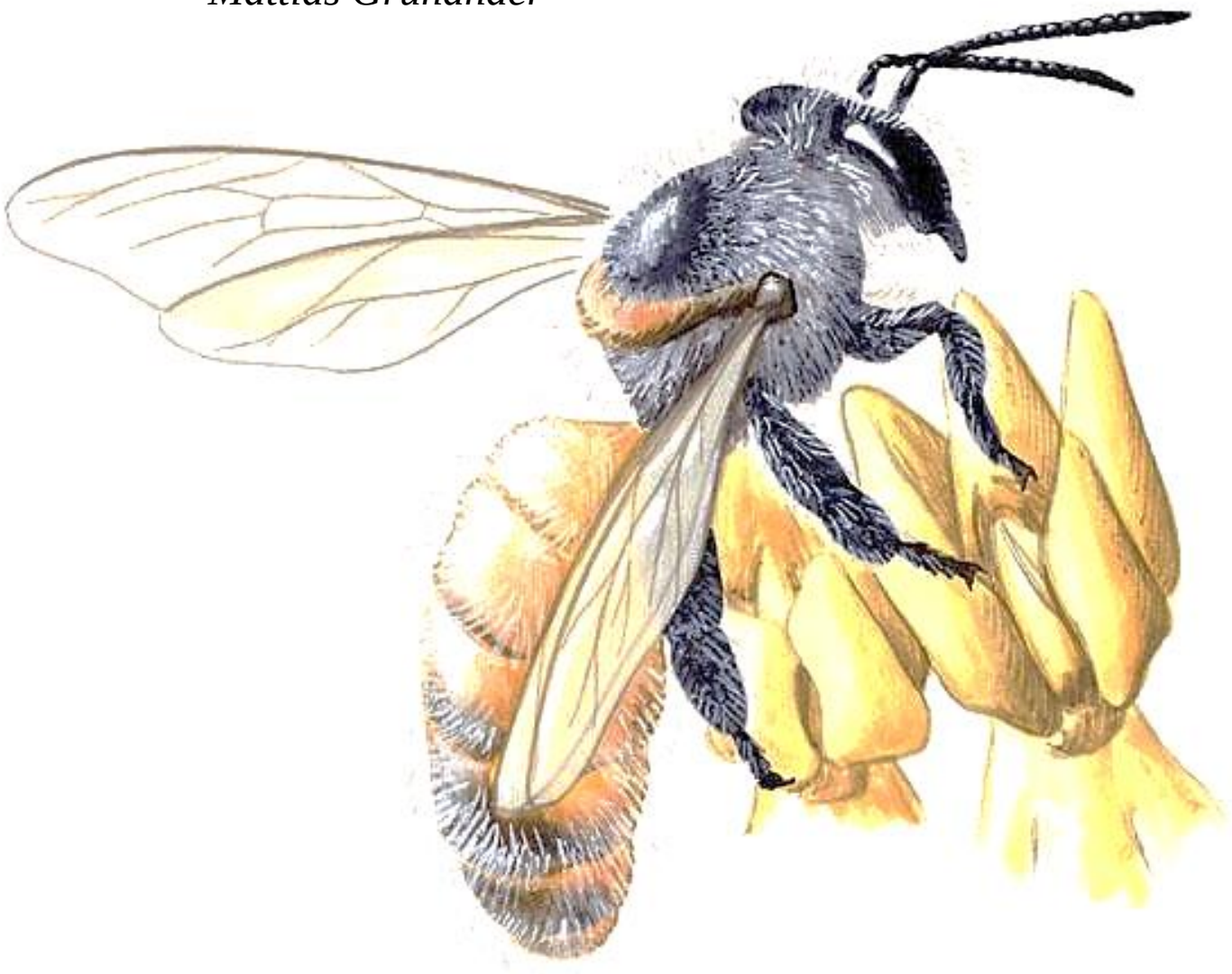
Mattias Grunander



Biology & Environmental Science, Bachelor's Thesis
Uppsala 2015

Independent project/Degree project / SLU, Department of Ecology 2015:16

Induced Defecation in Honeybees
Mattias Grunander

Supervisor:	Olle Terenius, Swedish University of Agricultural Sciences, Department of Ecology
Examiner:	Eva Forsgren, Swedish University of Agricultural Sciences, Department of Ecology

Credits:	15 hec
Level:	G2E
Course title:	Independent Project in Biology – Bachelor's Thesis
Course code:	EX0689
Programme/education:	Biology & Environmental Science
Place of publication:	Uppsala
Year of publication:	2015
Cover picture:	"Michigan Science Art"
Title of series:	Independent project/Degree project / SLU, Department of Ecology
Serial number:	2015:16
Online publication:	http://stud.epsilon.slu.se
Keywords:	Apis mellifera, induced defecation, centrifugation, disease transmission, epidemiological model

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences

Department of Ecology
P.O. Box 7044
SE-750 07 Uppsala, Sweden

Abstract

Growing human populations and technological achievements in later years, have increased interactions between people, and made the world smaller**.** Diseases travel around the globe at an ever increasing speed. There is a growing need to develop an epidemiological model, so that outbreaks and transmission routes can be predicted. Aspects such as genetics, social networks and random contact must be investigated and incorporated, if the model is to fit human populations. Humans would be the preferred test subject, but for practical and ethical reasons, it is not feasible. It is therefore necessary to conduct the experiments on another social animal group.

Honeybees are social insects, living in complex societies. Older foragers collect pollen and nectar from flowers, and younger nurse bees ingest the pollen grains in order to feed the foragers, drones and the queen. There are different levels of genetic relativeness amongst bees, since the queen mate with multiple drones. The bees interact more with their closely related kin than with other bees from the same hive.

Observing disease transmission between bees by analyzing bee feces is a method that has not yet been properly studied. It is in this project assumed that disease transmission of pathogens can be detected, by investigations of the bee feces. To acquire feces from an individual bee is difficult, so means of feces collection from the bees must be developed. Induced defecation by centrifugation is one method in which the bees are centrifuged at a high speed for a short period of time. Centrifuged bees were put in cages, in order to determine whether the centrifuging process affected their long-term survival. The negative side with centrifugation is that it puts the bee in risk of losing its intestines and die. By using the centrifuge, bees suffer from intestine loss at a relatively low speed, 2000 RPM upwards.

The frequency of defecating bees was relatively low, and attempts were made to find "defecation triggers". The bees were exposed to high and low temperature, to smoke, followed by centrifugation. It was concluded that centrifugation does affect bee survival negatively. Smoke combined with centrifugation showed a promising effect on defecation, and should be investigated further.

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Acknowledgments
Special thanks to my supervisor Olle Terenius for coming up with the idea of this project, and whose support, guidance and enthusiasm kept me going. I would also like to thank Louise Nilsson, for her help with the bacteria samples that were collected from the bee-cages. Another thanks to Ingemar Fries, Eva Forsgren and Barbara Locke for helping me understanding the ecology of honeybees, and how to work with bees. Also special thanks to Michigan Science Art, for permitting me to use their illustration on the cover.

1 - Introduction
Pandemic, epidemic, extinction. These words are mentioned more and more in recent years, in newspapers, science reports and popular culture. Tomorrow is hard to predict. But if someone on the street would be asked randomly, if they believe that a larger outbreak is eminent or foreseeably close, the majority of people asked would probably say it is likely.

There have already been several large outbreaks in our time, such as the swine flu in 2009 [1], and the avian flu in 2010 [2].These were smaller outbreaks (regarding the amount of people dying), but they have left an idea of what could have happened or what could possibly be next. Going back in history, outbreaks of diseases like the Spanish flu (1918-1920) led to a large number of deaths in human populations [3]. Problems back then were sanitary conditions, and the lack of knowledge. There are other problems today, the sheer size of the human population is already counting over 6 billion, and is estimated to reach up to 10 billion people as of 2100 [4]. The interaction between people is increasing, connections between and within countries is made easier and faster. It makes the world smaller, and increases physical contact and interaction between people. A severe pandemic/epidemic has potential to spread faster for each technological achievement enabling eased connectivity, and for every child born into this world.

To predict impending outbreaks, it is important to construct a reliable epidemiological model in order to halt, contain and prevent them. In the past, the established models have been mathematical and computer-generated models [5]. Those models are insufficient due to different stochastic variables, as they do not allow random contact or heterogeneity within the population. In other words, they do not allow variety in the population. To create a functional epidemiological model, one could release a disease right into a human population, and observe the effects of social networks and impact of the genetic factors. It is probably the most effective way to construct a model; however, it is not practical to perform trials on human populations for ethical and legal reasons. It is therefore necessary to perform trials on other social animals, honeybees for example.

Honeybees live in complex societies with different levels of genetic kinship between individuals (since the queen mate with multiple drones [6]), and “age polyethism” (chores in the hive based on the bees age). Newly emerged bees start to work when they turn one day old. Their first chores are to tend to the queen and to the eggs, to feed the drones and each other. Up to 13-14 days they are called nurse bees [7]. They will then undergo a series of morphological changes in their bodies, and become foragers. At this stage, they leave the hive in order to go outside to collect pollen, and they do this until they reach an age of approximately 6 weeks. By then their bodies are so worn out that they either die in the hive, or break a wing out on a flying trip.

Bees are a promising modeling group, because ethical approval is not required for invertebrates [8]. Many bees can be tested in a short period of time, and the bees are also relatively easy to handle and to infect with pathogens. The microsporidia *Nosema apis* is an intra-cellular fungi that infects honeybees. It has the ability to create environmental spores, which may infect the bee through infected comb-material, food or water. The spores can possibly also be spread directly between individuals by trophallaxis (the process in which bees feed each other), but that has yet to be

proved. *N. apis* infects the epithelial cells of the gastrointestinal tract of the honeybee, and are void with the feces where it can be detected [9]. By cleaning out the spore-infected feces from the hive, other bees come in contact with the spores and may become infected. Infectious spores can be detected in feces up to a year after being discarded [10].

Throughout evolution honeybees have developed strategies to cope with diseases; the anti-septic propolis is a natural built-in defense mechanism in the hive, protecting against both bacterial and fungal attacks [11]. The honeybees are hygienic; they do not usually defecate in the hive or in confined spaces, since the risk of spreading diseases would increase. There are however ways to induce defecation. By centrifuging the bees, 50 % of the bees could be made defecating, as shown in Lecocq master thesis [12]. Bakker conducted in 2012 a follow-up pilot study of centrifugation of honeybees, with results showing an average induced defecation of 37 % [13]. One can consider if this method of centrifugation could be improved, and more fine-tuned. Centrifugation would be a preferred method, as there are advantages of using a centrifuge; it is relatively cheap, it is fast, it does not take a lot of manpower and the feces is delivered directly into an Eppendorf-tube, ready to be examined. The negative aspects of the centrifugation process are the risks of intestine loss and death. Several bees responded poorly to centrifugation in the follow-up study of Bakker (2012) and lost their intestines.

These are the aims of the project: (1) to achieve 100 % survival of the honeybees. It represents both avoiding intestinal loss, and survival for at least several days post centrifugation. (2) To achieve 100 % defecation in the honeybees.

2 - Material and Methods

2.1 Material

All bees used in the experiments were taken from a colony that was maintained according to standard beekeeping procedures at the Department of Ecology at SLU. The hive used was a Langstroth-type of hive, with replaceable frames in parallel position. The centrifuge used for the experiments was the "Heraeus Biofuge Pico", which is a 24 slot centrifuge, capable of speeds up to 13.000 RPM/min.

2.2 Effects on honeybee long-term survival following centrifugation

2.2.1 – *The long-term survival effects on nurse bees*

Nurse bees were concentrated in the top-box of the hive [14]. They were collected by lifting off the roof, picking up a frame, and shaking them into a jar. Three hundred and twenty nurse bees were collected for centrifugation, and 290 nurse bees were collected as control. The bees were immobilized using CO_2. The immobilized bees were put in small 0.5 ml Eppendorf-tubes with holes in the bottom, the small Eppendorf-tubes were put into larger 1.5 ml Eppendorf-tubes, and centrifuged in a standard centrifuge. The bees were centrifuged for 60 s at a speed of 3000 RPM. The bees that defecated or lost their intestines received unique tags on their backs (notes with different numbers glued on the bee with organic glue). Surviving bees were counted every day, and dead bees were continuously removed. The bees were fed continuously.

2.2.2 – *Comparing nurse bee survival to forager survival*

Two hundred and forty bees in total were collected from the hive, 120 nurse bees and 120 foragers. Foragers were caught outside the hive with a fly net, while entering the hive. In order to facilitate the catching of foragers, a part of the hive entrance was blocked so that the concentration of in-going foragers would be higher. The caught foragers were put in different cages one by one, immediately after they had been caught. They were put in a refrigerator for half an hour, to make them easier to handle. They were then put in the same cage in room temperature for half an hour. This was repeated with both control and centrifuged foragers. Catching 120 foragers on the same occasion would be

physically demanding and time consuming. Twenty foragers were caught first and centrifuged, and another group of 20 foragers were caught soon after (as control). The procedure of collecting foragers was repeated twice the following day. Totally 60 foragers were caught and centrifuged, and 60 foragers were caught as control. Nurse bees were collected by lifting off the roof, picking up a frame and shaking them down into a jar. Sixty nurse bees were collected for centrifugation, and 60 nurse bees were collected for control. Both foragers and nurse bees were immobilized with CO_2, and put into Eppendorf-tubes. The bees were first placed in the small 0.5 ml Eppendorf-tubes with a hole in the bottom), the smaller Eppendorf-tubes was put in larger 1.5 ml Eppendorf-tubes. They were centrifuged for 60 s at 3000 RPM. Defecating bees and bees that lost their intestines received unique tags on their backs (notes with different numbers glued on the bee with organic glue). Surviving bees were counted every day, and dead ones were continuously removed. Bees were supplied with sugar solution all through the experiment.

2.2.3 – *Bacterial and fungal growth in sugar solution*
To investigate if the differences in death between cages could be due to high amounts of bacterial and fungal pathogens, 1 ml of sugar solution was taken from cages which population died very rapidly, or survived and seemed unaffected. The centrifuged nurse bee cages (cages 1-32) were initially given sugar solution on the 12th of June, while the control nurse bee cages (cages A-Ö) were given sugar solution on the 19th of June. One ml samples of the sugar solution from centrifuged and control nurse bee cages were first extracted on June the 24th, and on June the 27th. The 1 ml sugar solution samples collected from the cages were serial diluted in 1:10, 1:100 and 1:500. The diluted samples were spread onto a number of LB-plates. Colonies were counted to measure bacterial and fungal growth.

2.3 RPM-adjustment experiment
The bees were centrifuged at: 1000, 2000, 3000 or 4000 RPM/min in a standard centrifuge. The time intervals were 15, 30, 60 or 120 s. Six hundred and forty nurse bees were extracted from the top-box. They were immobilized with CO_2, put into Eppendorf-tubes and centrifuged. Forty individuals per time-duration and RPM were centrifuged. The relative centrifugal force value (RCF) is calculated from the formula: $11.18\text{^}(n/1000)^2\text{^}r$. Variable "n" is the speed in RPM/min, variable "r" is the centrifuge radius in cm (8.5 cm), and "g" is gravity. RCF(1000): $11.18\text{^}(1000/1000)^2\text{^}8.5 = 95.03$ g, RCF(2000)= 380.12 g, RCF(3000)= 855.27 g, and RCF(4000)= 1520.48 g. A bee centrifuged at 4000 RPM is exposed to a force equivalent to 1520.48 times the gravitation of the earth.

2. 4 Effects on honeybee defecation and intestine-loss due to heating and cooling

2.4.1 – *Heat and cold-exposed bees*
Eighty nurse bees were collected from the hive. They were immobilized with CO_2, and put in Eppendorf-tubes. Forty bees were heated to 37-38 °C in an incubator, and 40 other bees were put in a fridge for 30 min, until they held a temperature of 6-7 °C. They were then instantly centrifuged in groups of 20, at 3000 RPM for 60 s.

2.4.1.1 – *Heat and cold-exposed bees follow-up*
One hundred and eighty honeybees were collected from the hive. They were immobilized with CO_2, put in Eppendorf-tubes, and placed in the fridge. Holding a body temperature of 6-7 °C, they were taken out in groups of 20, and centrifuged at 1000, 2000 or 3000 RPM, in intervals of 15, 30 or 60 s.

2.5 Effects on honeybee defecation and intestine-loss following exposure to smoke

2.5.1 – *Smoke-exposed bees*
Sixty nurse bees were collected from the hive and placed in a fume hood. Smoke (from organic matter such as wood and dried vegetation) was blown onto to the bees for 90 s. They were immobilized by CO_2, put in Eppendorf-tubes and centrifuged at 3000 RPM for 60 s. They were centrifuged in groups of 20 individuals, 10, 15 and 30 min after the initial smoke-exposure.

2.5.2 – *Smoke -exposed hive*
The experiment was conducted by blowing smoke into the hive a few minutes at the time, in intervals of 30 min apart, over a total of 90 min. A total of 120 nurse bees were collected from the hive and centrifuged. The 40 first individuals were collected from the hive, and centrifuged 30 min after the last smoke-exposure. The procedure was repeated 60 and 90 min after smoke-exposure. The bees were centrifuged at 3000 RPM for 60 s.

3 - Results

Honeybee survival post centrifugation

Foragers and nurse bees were both initially tested, in order to find out whether there was any difference in their response to the centrifugation process. The casts were collected in different manners, and similarities between both procedures were few. The nurse bees were easily collected directly from the hive, while the foragers had to be caught by net, one by one. The caught foragers were placed onto the grass in individual cages, and had to wait for the other foragers to be caught (totally 20 individuals before centrifugation), resulting in a longer exposure to external factors for the foragers, in comparison to the nurse bees. Factors such as wind and sun affected the foragers (during collection), and could have dehydrated the foragers, thereby decreasing their chance of surviving the centrifugation process, or their long-term survival in the cages. The foragers, compared to the nurse bees, could also have been more vulnerable to the centrifugation process because of their age (older), and because of the fatigue of being out all day collecting pollen, while the nurse bees stayed in the hive, unexposed to the elements. In the attempt of centrifuging nurse bees, some differences in survival were revealed. The centrifuged nurse bees displayed a high initial mortality, compared to the control nurse bees. Half of the centrifuged nurse bees survived up to 13 days, while half the control nurse bees survived up to 15 days. However, the centrifuged nurse bees managed to outlive the control nurse bees (figure 1). In the experiment of centrifuging foragers, an initial high mortality can be observed with both centrifuged and control foragers. Fifty percent of the centrifuged foragers survived up to 10 days, and 50% of the control foragers survived up to 13 days (figure 2). Similar to the results in the attempt of centrifuging nurse bees, the control foragers also die before the centrifuged foragers.

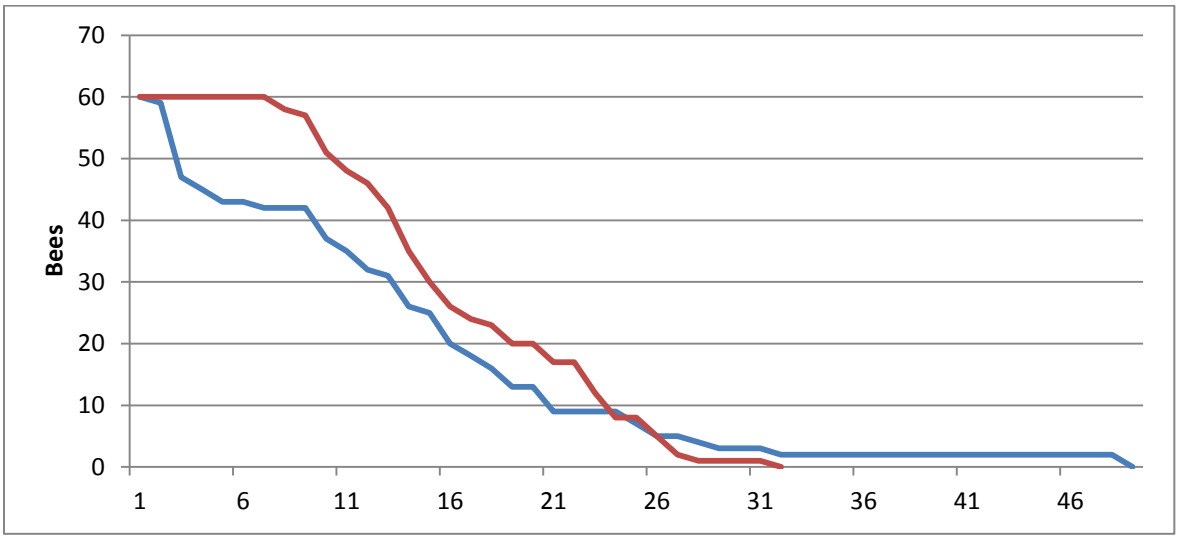


Figure 1. Amount of living nurse bees post 60 s of centrifugation at 3000 RPM.

Centrifuged nurse bee and control nurse bee survival post centrifugation is compared, showing a higher initial mortality for the centrifuged nurse bees than for the control nurse bees. The mortality rate for centrifuged and control bees is relatively similar.
Blue colored line = centrifuged bees, red colored line = control. Day 1 = day of treatment.

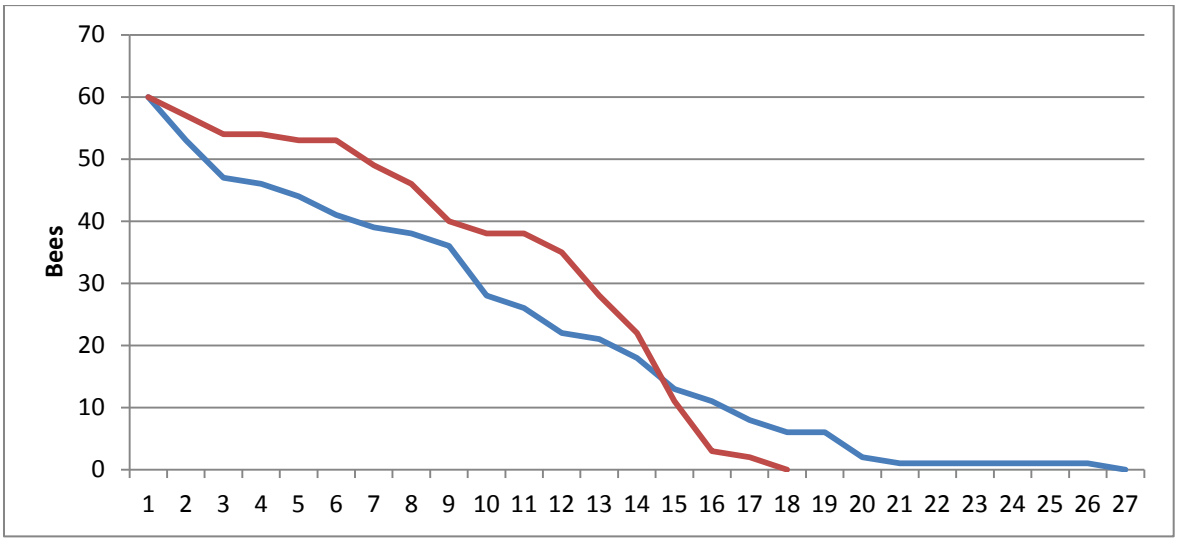


Figure 2. Amount of living foragers post 60 s of centrifugation at 3000 RPM.
Centrifuged forager and control forager survival post centrifugation is compared, showing a higher initial mortality for the centrifuged foragers than for the control foragers. The mortality rate for centrifuged and control is relatively similar.
Centrifuged foragers (blue line) and control foragers (red line). Day 1 = day of treatment.

To analyze the statistic relevance in previous results, the standard deviation between the cages is demonstrated by the use of polynomial standard deviated lines. The idea is to connect survival between cages to unknown random variables, in order to see how large effect those variables have on the survival. By comparing polynomial trend-lines, a small deviation between the centrifuged nurse bees, and control nurse bees was revealed. The deviation seen with the centrifuged nurse bees was relatively low (0.6-0.9 units), except for the initially high deviation, probably related to intestinal loss, caused by the centrifugation treatment. The control nurse bee deviation remains close to 0 during the first 4 days, and stays around a relatively low 0.6-1.1 units throughout the period, giving the centrifuged nurse bees and control nurse bees a relatively similar curve (figure 3). By comparing the polynomial trend-line of the centrifuged foragers to the polynomial trend-line of the control foragers, a few differences can be revealed. Centrifuged foragers have a higher initial deviation in comparison to the control foragers, which is connected to death from intestinal loss. The deviation for the centrifuged forager, although decreasing from an initially high deviation, stays at a relatively high level throughout the period. The control foragers did, as the centrifuged foragers, also have an initial high mortality, leading to a relatively high deviation when compared to the control nurse bees. The deviation did however not decrease after the initial high deviation, but remained steadily at the same level, rising slightly, before finally dropping after 15 days. The forager deviation is overall higher than the nurse bees deviation, around 0.9 – 1.3 unit/day in average for both the centrifuged groups and control groups (figure 4).

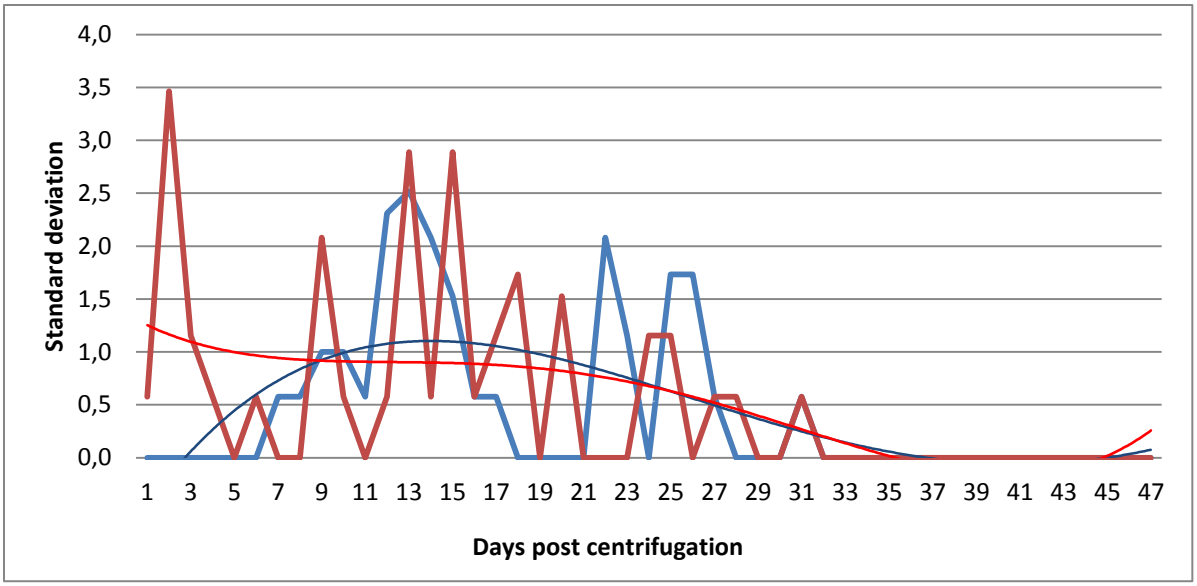


Figure 3. Average deviation in nurse bee mortality following 60 s of centrifugation at 3000 RPM.
Graph comparing the average deviation between centrifuged nurse bees and control nurse bees, showing a relatively high initial deviation for the centrifuged nurse bees compared to the control. The deviation for centrifuged and control is otherwise rather similar throughout the period.
Centrifuged nurse bees deviation is demonstrated by the red thick line and red thin polynomial trend line and control nurse bees deviation is demonstrated by the blue thick line and blue thin polynomial trend line Day 1 = day of treatment.

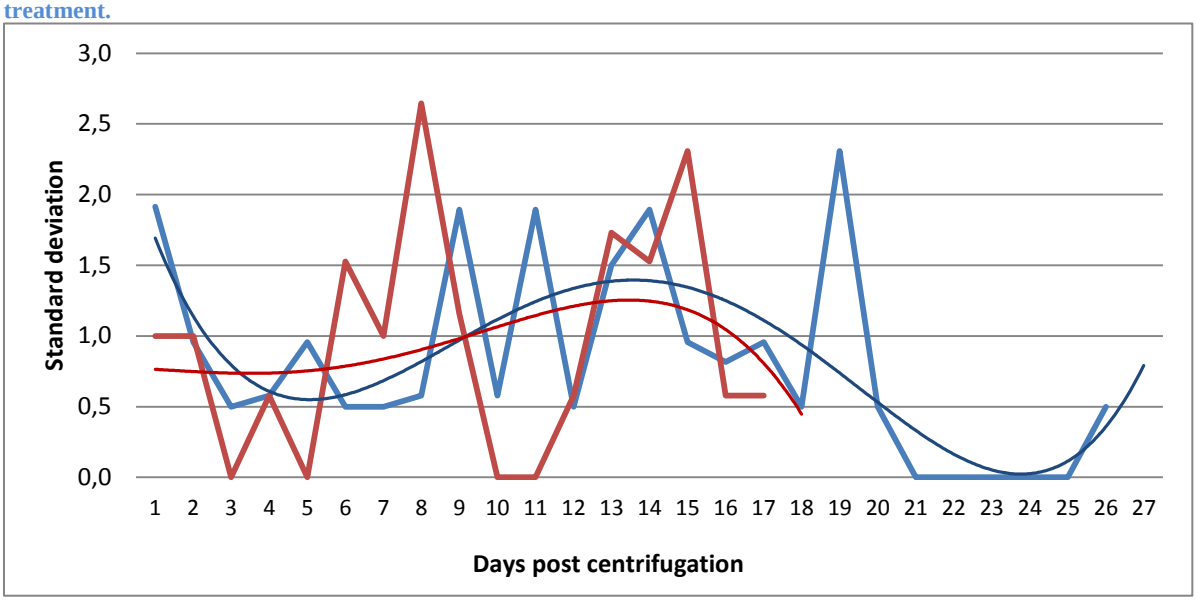


Figure 4. Average deviation in forager mortality following 60 s of centrifugation at 3000 RPM.
Graph comparing the average deviation between centrifuged foragers and control foragers. The centrifuged foragers trend-line show large fluctuation in deviation while the control foragers have a relatively steady deviation throughout the period. Centrifuged forager deviation is demonstrated by the blue thick line plus blue thin polynomial trend line and the control/ non-centrifuged foragers deviation is demonstrated by the red thick line plus red thin polynomial trend line, Day 1 = day of treatment

In order to study centrifuged nurse bees survival more thoroughly, the previous experiment with nurse bees was repeated with a greater number of replicates. Three hundred and twenty nurse bees were collected for centrifugation and 290 nurse bees were collected as a control. They were kept in an incubator after the treatment, in cages of 10 individuals. A significant difference in mortality is shown between centrifuged nurse bees and control nurse bees. An initial high mortality, linked to the centrifugation process can be seen with the centrifuged nurse bees. After the first three days of high mortality, eight days of lower, relatively steady mortality follows. The centrifuged nurse bees then suffers from an increased mortality between days 9 to 11, leaving less than 25 % centrifuged nurse bees alive as of day 12. The control bees, compared to the centrifuged bees, have a steadier

mortality from day one to day twenty, with no sudden dips or fluctuations, and with more than 25 % of the control nurse bees surviving up to day 20. The graph shows a strong correlation between survival and centrifugation, indicating that the nurse bee survival is affected negatively by the centrifugation process (p = 0.0001) (The results are demonstrated by the use of a log-rank test, figure 5).

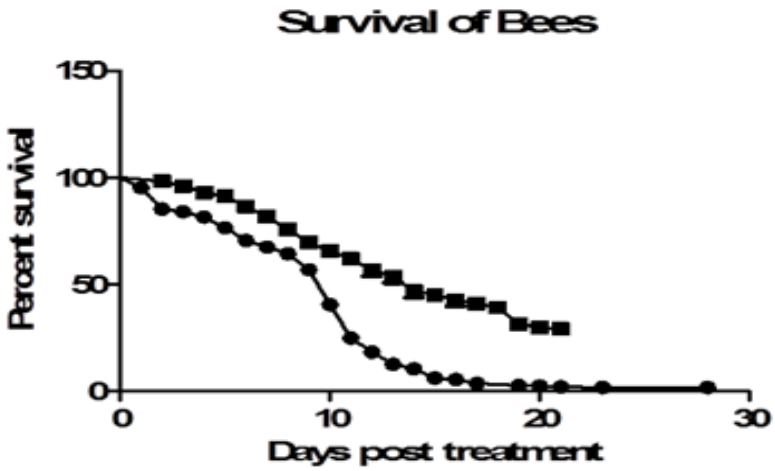
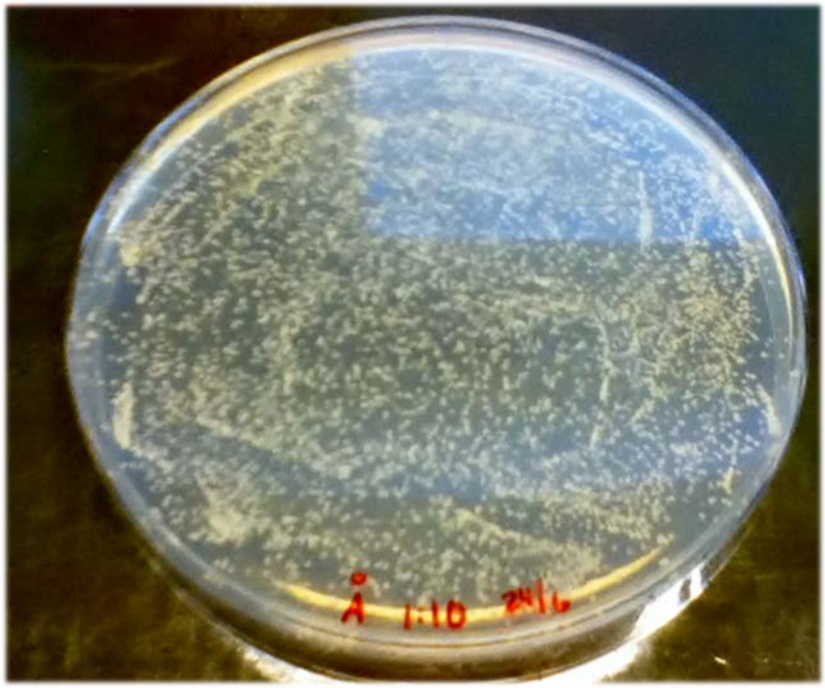


Figure 5. Nurse bee survival curves in percent post 60 s of centrifugation at 3000 RPM.
The graph shows an initial high mortality for the centrifuged nurse bees, followed by 8 days of lower, relatively steady mortality, followed by an increased mortality after day 9, reducing the amount of living centrifuged nurse bees to less than 25 % as of day 12. More than 50 % the control nurse bees were still alive as of day 12, and approximately 30 % as of day 20.
Centrifuged nurse bees are demonstrated by circles and control nurse bees are marked with squares. Day 0 = day of treatment.

Attempt to investigate bacterial growth in the cages.
The previous attempts to determine mortality post centrifugation revealed that the majority of nurse bees survived up to 12-15 days in captivity. The nurse bees should theoretically have been able to survive up to an age of approximately 6 weeks. Additional survival experiments were conducted. One theory was that the survival in the cages was influenced by the amount of bacteria and fungi in the sugar solution they were fed. To analyze the possible contamination of the food as a reason for increased mortality, 1 ml of sugar solution was extracted from selected cages, and was put onto LB agar plates. The sugar solution fed to the cages was tested, in order to confirm that an uncontaminated solution was distributed to the cages. Centrifuged nurse bee cages (cages 1-32) were initially given sugar solution on June the 12th, and the control nurse bee cages (cages A-Ö) were given sugar solution the 19th of June. Samples of sugar solution from both centrifuged and control nurse bee cages were extracted on the 24th of June, and on the 27th of June. Bacterial and fungal colonies were counted to measure growth.



LB agar plate "Å", diluted 1:10, extracted from the cage (Å) on the June the 24.

The samples showing the most rapid growth originated from cages; "5(old)", "3", "25" and cage "R". The highest levels of bacteria were found in cages; "G", "12" and "25" with approximately $8\text{^}10^5$ colonies/ ml sugar solution. The samples from the centrifuged nurse bee cages showed a generally higher amount of bacteria and fungi, in comparison to the control cages (figure 6).

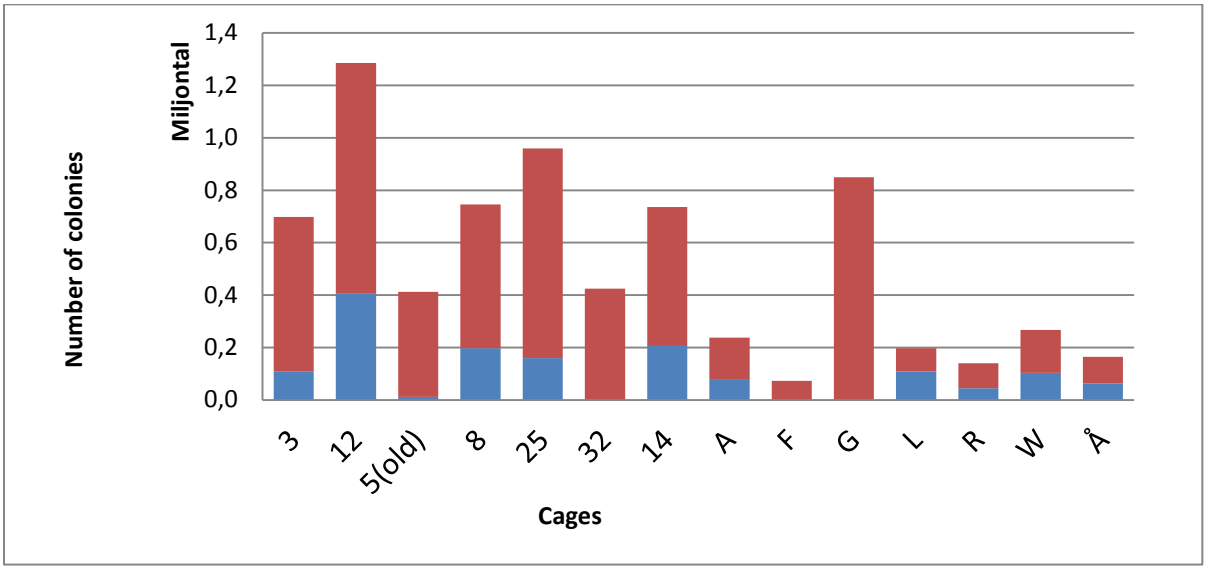


Figure 6. Bacterial growth in sugar solution fed to nurse bees, measured on LB agar plates at two occasions. Cages "5(old)", cage "3", cage "25" and cage "R" showed the most rapid bacterial and fungal growth. Cages with the highest levels of bacteria and fungi were cages; "G", "12" and "25", which all have roughly $8\text{^}10^5$ colonies/ ml. The blue stacks represent the bacterial growth in the sugar solution from selected cages from the 12th of June (for the cages 1-32) and the 19th of June (for the cages A-Ö) to the 24th of June. The red stacks represent the second measurement of bacteria growth in the sugar solution from the same cages, between the dates June 24th to June 27th. All cages started on zero colonies, and the sugar solution from the cages were extracted and measured on two occasions, the 24th of June, and the 27th of June.

Determination of centrifugation speed

In an attempt to reduce intestinal loss and to maximize long term survival, different RPM velocities were tested. If the RPM was set too high, it caused the bees to lose their intestines, if it was set too slow, no defecation was induced. In this experiment, both intestinal loss and induced defecation were measured, combined and separately. The highest amount of combined defecation & intestinal loss occurred when RPM was set at 3000 rpm for 60 s, yielding 58 % defecation and intestinal loss. The second highest amount of combined defecation and intestinal loss was at 4000 rpm/30 s, with 55 % defecation & intestinal loss. The thin black trend-line shows how the combined intestinal loss & defecation increases when the RPM is set higher, and in order to reach 100 % combined defecation & intestinal loss, a RPM velocity set to around 7800-7900 RPM would be sufficient, using only a centrifuge (figure 7). When it came to inducing defecation and avoiding intestinal loss, 2000 RPM was the most effective velocity, in the time-intervals 30 s, 60 s and 120 s. The second most effective RPM overall was 3000 RPM, inducing defecation with 10 % of the bees in average. However, a clear downward trend in induced defecation can be seen the longer bees are centrifuged. The percentage of defecating bees centrifuged at 4000 RPM peaks at 10 %, when the nurse bees are centrifuged for 30 s, but the longer the bees are centrifuged, fewer defecate, giving it a curve similar to the 3000 RPM curve. Thousand RPM is the least effective RPM, resulting in no induced defecation (figure 8). Centrifuging nurse bees at 3000 and 4000 RPM gave a high rate of intestinal loss, at least 25 % for all the time intervals. By centrifuging bees at 2000 RPM, the intestinal loss stayed around a low 5 % in general, except for the 120 s interval where it reached 18 % of intestinal loss. Thousand RPM resulted in no intestinal loss for all time intervals (figure 9). The results presented figures 7, 8 and 9 are all acquired from the same experiment.

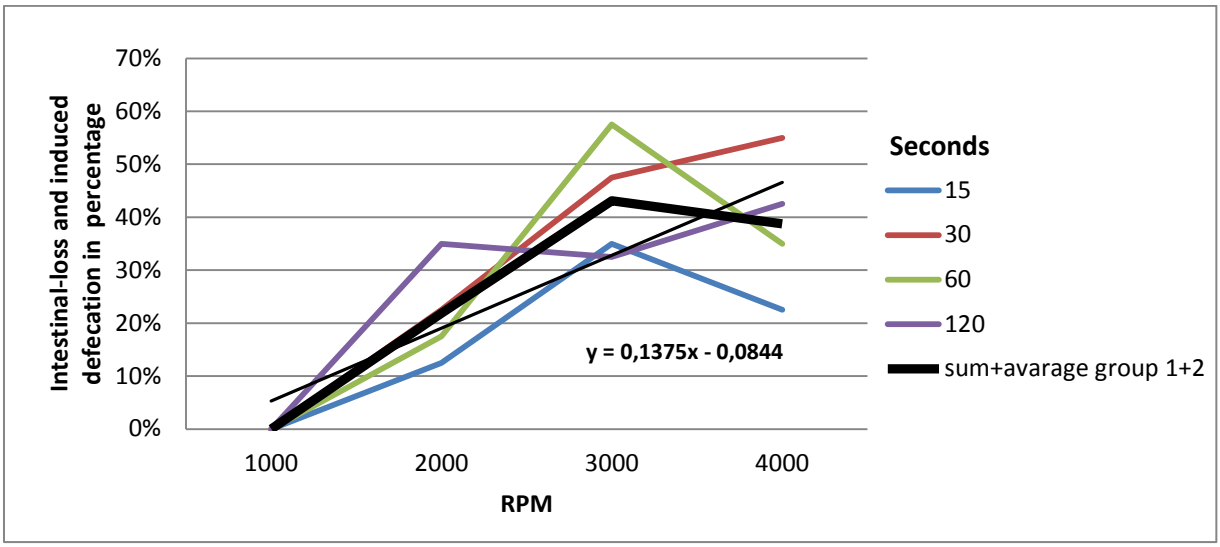


Figure 7. Combined intestinal loss and induced defecation increase/decrease due to RPM adjustments. The highest amount of combined intestinal loss and induced defecation can be seen at 3000 RPM for 60 s. The second highest can be seen at 4000 RPM for 30 s. The black thin trend-line indicates that the increase in combined intestinal loss & induced defecation is connected to the increase of RPM.

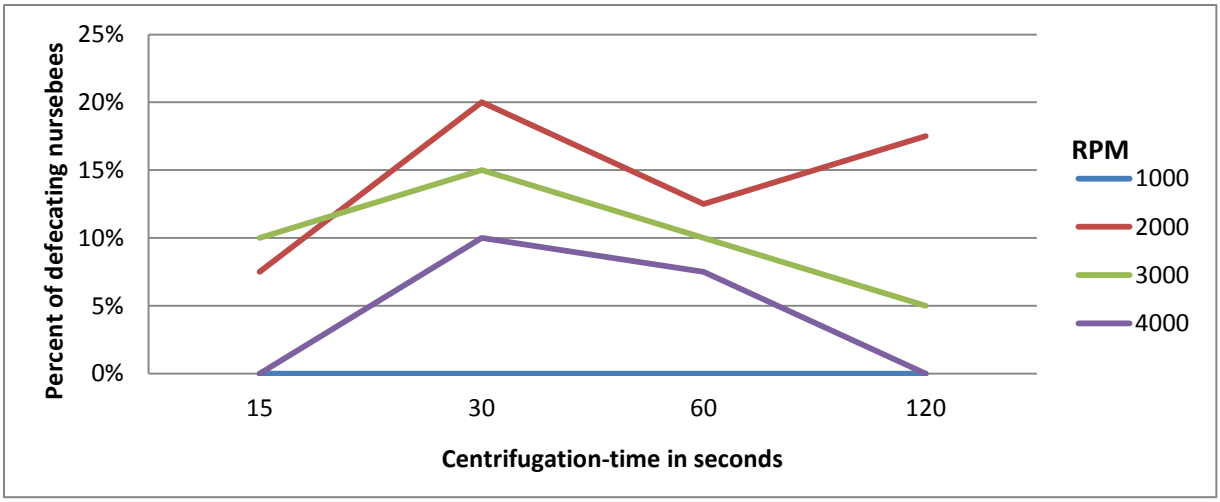


Figure 8. Induced defecation without intestinal loss is presented. The graph shows that the most effective RPM velocity was 2000 RPM in the time-intervals of 30 s, 60 s and 120 s. The second most effective RPM velocity was 3000 RPM, which had a 10 % average in induced defecation.

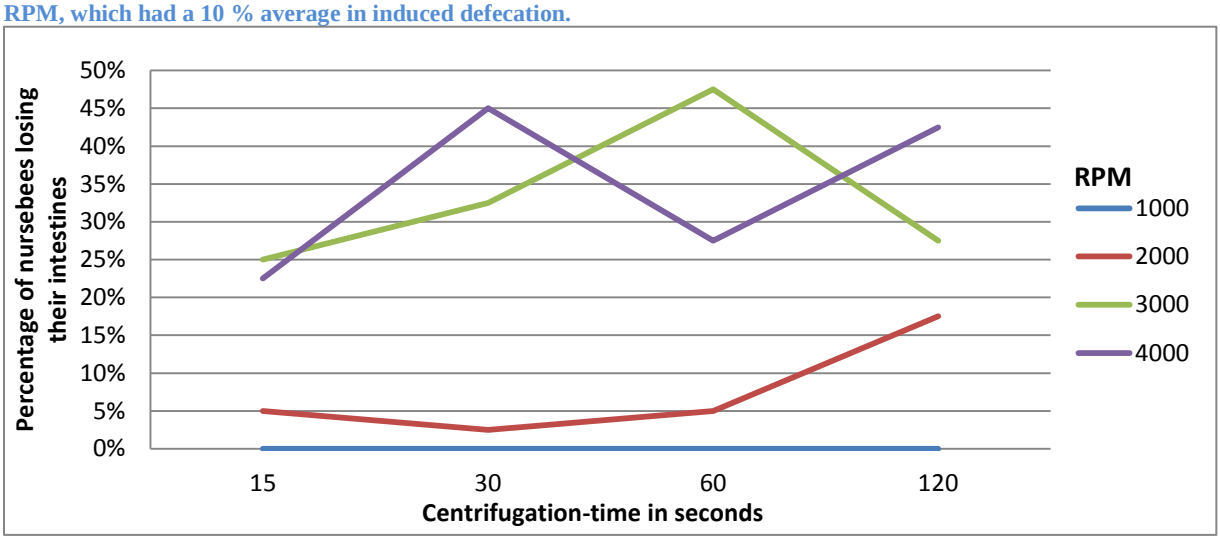


Figure 9. Intestinal loss without induced defecation is displayed. The figure shows that both 3000 and 4000 RPM caused more than 25 % of centrifuged individuals to lose their intestines, for all time-intervals. The figure also shows that 2000 RPM has a relatively low 5 % intestinal loss, in the intervals 15 s, 30 s and 60 s, but a relatively high 18 % intestinal loss when centrifuged for 120 s.

Conducting experimental trials in order to discover defecation triggers

As shown in the previous attempts with centrifugation, the aims of this study could not be achieved by simple adjustments of the RPM speed. There had to be the involvement of another method as well, in order to induce defecation more easily. The effect of short-term temperature decrease/increase on nurse bees was tested in this project. The idea came from the existence of temperature-sensitive enzymes that live in the bee midgut. The experiment was conducted with 80 individuals, 40 individuals were cooled down, and 40 were heated prior to centrifugation. The cold bees showed a significantly higher rate of combined defecation and intestinal loss, compared to the heated bees, both in group 1 and group 2. In group 1 & 2, the amount of combined induced defecation and intestinal loss reached 85 % for the cooled bees, while the heated bees combined defecation and intestinal loss reached 30 % for group 1, and 20 % for group 2 (figure 10). The amount of induced defecation, without intestinal loss, reached 15 % in group 1, both for the cooled bees and the heated bees. In group 2, the induced defecation was 5 % for both cooled bees and heated bees (figure 11). The results presented in figure 10 and 11 are collected from the same experiment.

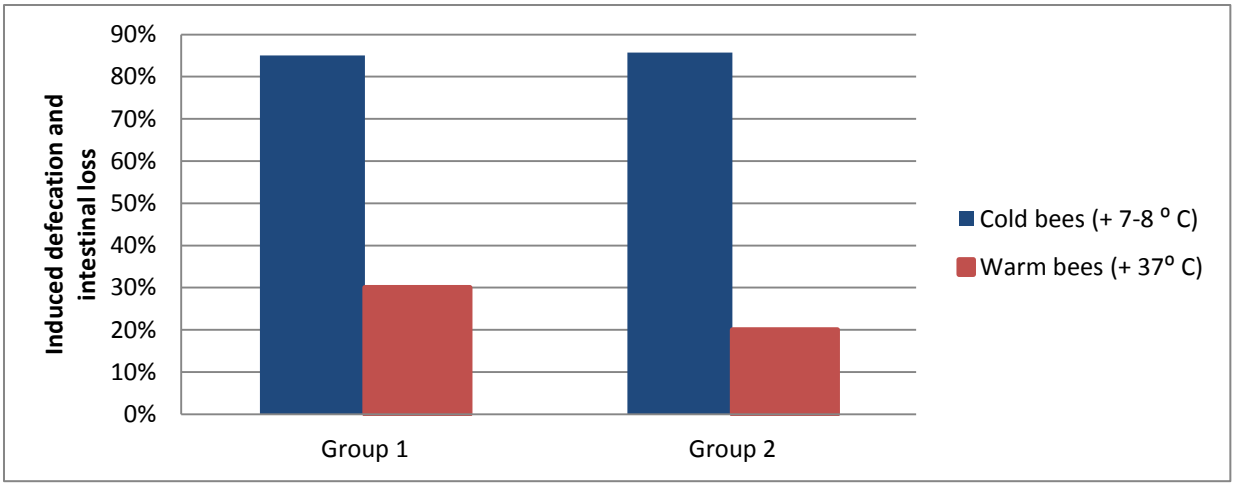


Figure 10. Combined intestinal loss & defecation for two groups of nurse bees following being cooled/ heated and centrifuged at 3000 RPM for 60 s. Combined induced defecation & intestinal loss is measured and reaches over 80 % for both group 1 and 2 with the cooled nurse bees. The heated nurse bees reached 30 % in group 1 and 20 % in group 2.

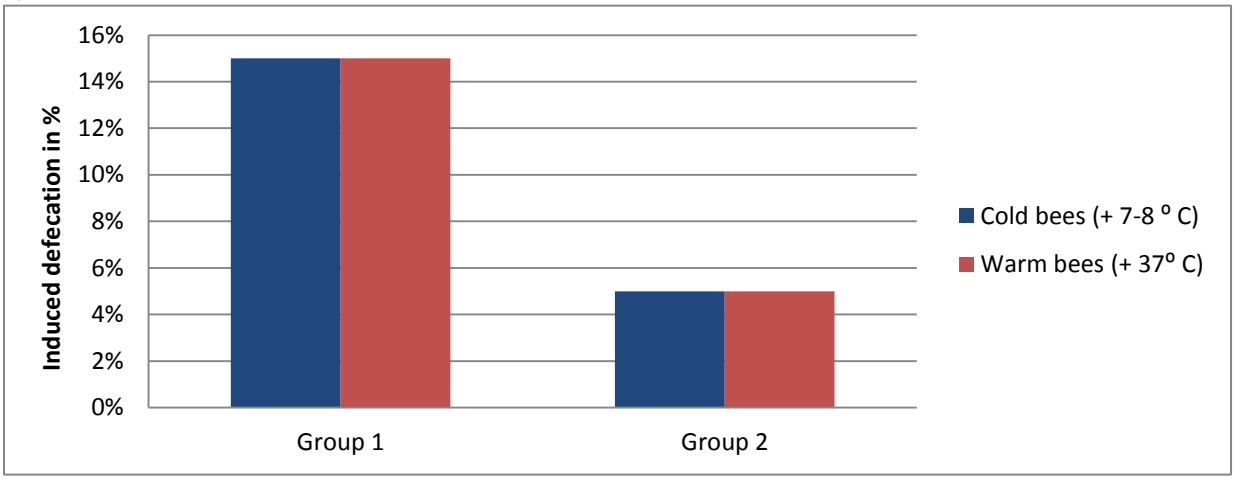


Figure 11. Defecation only in percent after first being cooled or heated, and centrifuged at 3000 RPM for 60 s. Induced defecation is measured without intestinal loss, showing a 15 % induced defecation for group 1, both with the cooled and the heated nurse bees. Group 2 has 5 % induced defecation for both cooled and heated nurse bees.

After conducting the first experiment with heating and cooling, it seemed appropriate to conduct a follow-up experiment, with more replications and more RPM variations, in order to understand the outcomes of the previous trial. It was conducted with 20 individuals per RPM and time-interval, 180 individuals in total. The procedure had similarities to the RPM-approximation experiment (section 2.3, method), except for the pretreatment with cold. Both induced defecation and intestinal loss in percent were measured separately. The quantity of bees losing their intestines was highest when RPM was set at 3000 for 60 s, resulting in a 20 % intestinal loss. Second highest amount of intestinal loss occurred when the RPM was set at 3000 for 15 s, resulting in a 10 % intestinal loss. Ten percent induced defecation occurred in all the tested time-intervals (15 s, 30 s, 60 s), when the RPM was set at 3000 and 2000 RPM (Table 1).

Table 1. Percent of defecation & intestinal loss with nurse bees following cooling treatment and centrifugation at 1000, 2000 or 3000 RPM for 15, 30 or 60s. The highest amount of intestinal loss occurred when RPM was set at 3000 for 60 s, causing 20 % intestinal loss. Second highest intestinal loss occurred when RPM was set at 3000 for 15 s, causing 10 % intestinal loss. The induced defecation was 10 % for all time-intervals when the RPM was set at 2000 and 3000 RPM.

Defecating bees				**Intestinal losing bees**			
Group 1 (nurse bees)				**Group 1 (nurse bees)**			
(RPM/time)	**1000**	**2000**	**3000**	(RPM/time)	**1000**	**2000**	**3000**
15 s	0	10	10	**15 s**	0	0	10
30 s	0	10	10	**30 s**	0	0	0
60 s	0	10	10	**60 s**	0	0	20

Smoke was also used as a potential defecation trigger. Smoke triggers a behavior among bees, alerting them of a nearby fire and potential danger. They move into the hive to gather honey, and to get away from the smoke. This behavior is interesting because it could mean that they would defecate before take-off, since it is extra weight that does not need to be carried. The theory was tested by blowing smoke onto caged nurse bees in a fume hood for a period of 90 s. Totally 60 individuals were immobilized by CO_2, and centrifuged in groups of 20 at 10, 15 and 30 min after the smoke exposure. The first group was centrifuged 10 min after smoke-exposure, resulting in a 10 % loss of intestines and no induced defecation. The second group was immobilized and centrifuged 15 min after exposure, yet again resulting in a 10 % loss of intestines and no induced defecation. The third group was immobilized and centrifuged 30 min after exposure and resulted in no defecation and no intestinal loss. All groups of nurse bees were centrifuged for 60 s at 3000 RPM (Table 2).

Table 2. Induced defecation & intestinal loss measured in percent, after 90 s exposure to smoke and centrifugation at 3000 RPM for 60 s. The groups collected after 10 and 15 min had an intestinal loss of 10 %, and no induced defecation. The bees collected after 30 min had no intestinal loss and no induced defecation.

Time after exposure (min)	**Defecation**	**Intestinal loss**
10 min	0	10
15 min	0	10
30 min	0	0

Because of the poor results achieved in the previous experiment (no induced defecation), an additional experiment with smoke was conducted. The experiment was based on an alternative theory that smoke did not immediately increase the chance of defecation, but kept the bees from defecating. Maybe the smoke triggered a natural defense in the bees, helping them to avoid water deprivation. This is plausible since the bees may fly for a long time to get away from the fire. Smoke was blown into the hive for a few minutes, in intervals of 30 min apart, over a total of 90 min. A total of 120 nurse bees were collected from the hive and centrifuged. The 40 first individuals were collected from the hive and centrifuged 30 min after the last smoke-exposure, resulting in a combined defecation & intestinal loss (not measured separately) of 30 %. The procedure was repeated 60 and 90 min after smoke-exposure, resulting in a 50 % combined induced defecation and intestinal loss with the 40 individuals collected after 60 min, and a 28 % combined intestinal loss and defecation with the 40 individuals collected after 90 min (figure 12).

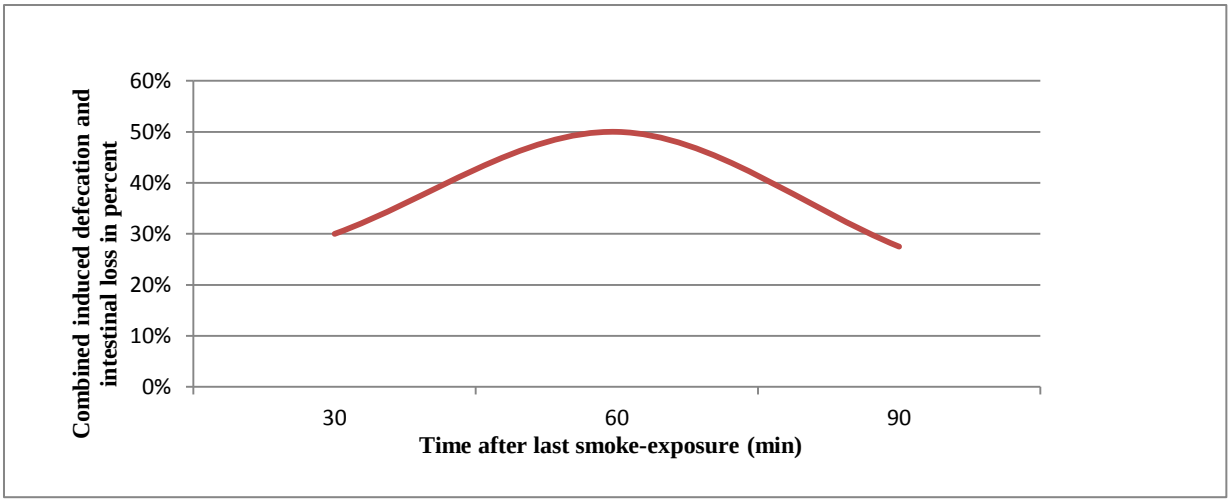


Figure 12. Combined defecation & intestinal loss after smoke-exposure and centrifugation at 3000 RPM for 60 s. Forty nurse bees were collected and centrifuged 30 min after exposure, resulting in a 30 % combined intestinal-loss and induced defecation. The procedure was repeated after 60 and 90 min, resulting in 50 % combined intestinal loss and induced defecation after 60 min, and 28 % after 90 min.
The red line shows theoretical progress of defecation plus intestinal loss over time.

4 - Discussion

Globalization, infrastructural expansion and population density are growing and expanding with an ever-increasing speed. Recent outbreaks of swine flu and avian flu have made it clear that there is a need to study disease transmission, and to create a working epidemiological model for human populations. Honeybees are social insects and are used in the creation of this epidemiological model. One possible way to investigate pathogen transmission between bees is to study the micro-organisms present in their feces. It is therefore necessary to come up with an easy way to induce defecation in the bees. Induced defecation is a small piece in the larger puzzle of studying disease transmission in honeybee populations. In order to make projects like these successful, there is a need for an efficient method to induce defecation.

Defecation only is the preferred outcome of every experiment, but a large amount of intestinal loss is likely an indicator that something about the method (referring to the method of exposing the bees to cold & heat, or to smoke) could have a physical effect on the bees and made them vulnerable to the centrifugation process. For example, the method of heating or cooling bees could have had a dozing effect on the bees, giving them little chance to resist the centrifugal forces, causing them to defecate or lose their intestines. When the effect of the additional method has been determined, the centrifuge can be regulated to spin slower or faster, or for a shorter or longer period of time, in order to reduce the number of bees that lose their intestines, and to optimize the number of defecating bees.

A pilot experiment with nurse bees and foragers was conducted in order to investigate possible differences in their response to being centrifuged. As shown in figure 2, the control foragers died before the centrifuged foragers. This is not an expected result; the centrifuged foragers would be more likely to die before the control bees. Ineffective treatment of the centrifuged foragers affected the results, several foragers had to be immobilized with CO_2 twice, and a few did not wake up again. Those bees were counted as dead on day one. Centrifuged nurse bees compared to the control nurse bees did not produce expected results, with the control bees dying faster than the centrifuged bees (Figure 1). The difference in mortality (between centrifuged and control) was not as high with the nurse bees, as it was with the foragers. The standard deviation was relatively high between the forager cages (Figure 4), in comparison to the nurse bees, indicating that the mortality in the forager cages was influenced to a larger extent by unknown variables.

With foragers left out and with more nurse bees added to a following experiment (Figure 5), a clear difference in survival between the centrifuged and the control groups became visible. It presented an increased mortality among the centrifuged bees, with as much as 75 % of the bees dead as of day eleven. The control groups died at a slower rate, with 50 % dead after 14 days ($p = 0.0001$). In prior studies conducted by Bakker (2012) [13] and Lecocq (2011) [12] the bees were centrifuged with a RPM of 3000 for 30 s. The majority of Lecoqcs twenty centrifuged bees survived up to 8-9 days before death. Bakkers results show a maximum survival of 29 days, with 60 % remaining after 16 days, using 60 bees. One initial goal with this project was that 100 % of the bees would manage to survive several days post centrifugation, but by setting the RPM at 3000 RPM or higher, bee survival is reduced to 75 % as of day 5, and to 50 % as of day 10.

To some extent, the roughness of the centrifugation process can explain why centrifuged bees die at a faster rate than non-centrifuged. With intestinal loss and other possible internal damages from the centrifugation process, it can be expected that the centrifuged bees die at a faster rate than the control bees. But the result that half the control group dies (subjected to no treatment) within two weeks, when they theoretically should live additional 2-3 weeks, is much unexpected (appendix 1 & 2). There must be other factors as well, affecting their survival. One explanation could be the bacterial and fungal growth in the cages (figure 6). In the hive the bees have natural defense mechanisms that protect them (the anti-septic propolis), which is not the case in the cage. It would be fair to say that a large number of bacteria in the cage and in the substrate increase the probability of death for the population residing in the cage. I used the same tweezer (I did not start to sterilize it until late in the project) to remove dead bees from the cages, and there is a chance pathogens have been transferred between cages. The big incubator was not a sterile environment, it was never cleaned or sterilized between experiments and visible amounts of residue consisting of sugar solution, feces and pollen could be spotted on the shelves. Bacteria and fungus could certainly have lingered in the incubator, and contaminated newly arrived cages. The air conditioner helped replacing the air in the incubator, but it could also have eased pathogen transmission.

The idea of using the centrifuge is to induce defecation, without causing serious injuries to the bees. There is therefore a need to find an optimal RPM, where few or no bees get hurt and many bees defecate. Reading prior studies on the subject of induced defecation, nine out of twenty defecating (no intestinal loss) bees seems to be the record to beat [12]. It was done at 3000 RPM for 30 s. The results from the RPM-adjustment experiment pointed to the fact that the combined amount of intestinal loss and induced defecation increased, as the RPM increased (Figure 7). There was also a small but tangible correlation with time and combined intestinal loss & induced defecation as shown in figure 9. However it did not matter much if bees were centrifuged for 15 s or 120 s when the RPM was set as high as 3000-4000, they lost their intestines after 15 s, as well as after 120 s. The result for “only defecating” bees in figure 8, indicates that when bees solely are exposed to centrifugation, the number of defecating bees is the highest somewhere between 2000-3000 RPM. Setting the RPM higher than 3000 RPM, increases the intestinal loss mainly. The RPM experiment gave the first clue of the limit of honeybee physiology, how hard they can be pushed before they suffer from irreversible damage. Both 3000 RPM and 4000 RPM result in a much higher intestinal loss compared to 2000 RPM, shown in figure 9.

Centrifugation alone could not produce the desired level of defecating bees; the centrifugation should therefore be combined with an additional method. A method using natural triggers in bees, combined with centrifugation to maximize defecation. The trials with hot and cold temperatures came from the fact that there is a temperature-sensitive enzyme in the bee midgut, which is involved in food degradation. The enzyme works more efficiently in warmer temperatures [15]. The outcome of the experiment was inconclusive. The first test (Figure 10) showed large differences between the individuals that were tested. The cooled bees showed a significant higher level of combined intestinal loss & induced defecation, compared to the warm bees. Up to 85 % of the cooled bees either defecated or lost their intestines, while around 25 % of the warm bees defecated or lost their intestines. This looked very promising, and it seemed as if the method of cooling had affected the bees. When the experiment was repeated with only cold bees the day after (Table 1), results came out

entirely different, with no defecation and two bees losing their intestines, out of sixty bees in total. They had been treated in exactly the same way as the day before. I cannot provide any explanation to this, except that there must be additional surrounding factors that have not been taken into count.

The outdoor factors such as weather, temperature, humidity and precipitation varied from day to day, and have not been controlled in this study. I was handed data from SLU:s weather station out at Ultuna, but there was not enough time to construct a weather index to match against defecation data. The weather has an effect on the activity of the hive, which in turn regulates the amount of feces produced. High activity in the hive on a sunny day should produce more feces than low activity in a hive on a cold and rainy day. Agitated bees and calm bees could also have produced different results. Some groups of bees were extremely agitated because of frequent visits to the hive the same day. Theoretically, agitated bees should consume more energy and produce more feces than calm bees, and that could have affected the result.

Bees exposed to smoke and hive exposed to smoke produced different results. The first attempt to induce defecation with the bees 10, 15 and 30 min after being smoked proved to be ineffective, two out of sixty bees lost their intestines, and none defecated (Table 2). The theory was that when the honeybees were exposed to smoke, they would crawl back into the hive and gather honey. They would then take to flight to put them away from the threat. In addition to consuming honey and flying off, it also seemed likely that they would get rid of extra weight (such as feces) after takeoff. But the results from the first smoking experiment pointed in the opposite direction, that they did not get rid of excess weight after being smoked. One could speculate if it is due to some kind of defense against water deprivation, since the bees could be flying a great distance in order for them to avoid the fire. My conclusion is that it was more difficult to induce defecation in the honeybees directly after they have been exposed to smoke. The hive was exposed to smoke for a longer period of time in the following experiment. The bees would presumably not defecate during the time of exposure, and there would be a high amount of bees with feces remaining in their guts when they were collected. That is how the second experiment was conducted, smoke was blown into the hive, a few minutes each time, in intervals of 30 min apart over a total of 90 min, and the results came out very different from the first experiment (figure 12). The combined induced defecation and intestinal loss is significantly higher, compared to the first smoking experiment (induced defecation and intestinal loss were not measured separately in this experiment). The combined intestinal loss and induced defecation from bees collected after 60 min was 50%, which is promising. It is however only a pilot, and requires a more extensive study to examine the effects of smoke on honeybees.

Conclusion and further research

It can be concluded from figure 5, that the long-term survival is correlated to centrifugation. How large the effect is, is arguable since fungal and bacterial growth in the cage also play an important role in the survival of the bees. The problem with bacterial growth can be helped by changing the sugar solution every day, and by cleaning the cage at least once a week. This should be done in a future study, so it can be concluded what effect the centrifugation process really has on long-term survival.

Pursuing defecation triggers did not produce reliable results; in fact no result is similar to another. Weather factors shifted from day to day, and were not taken into account in any of the experiments. There were outdoor factors like humidity, wind, warmth and sunshine, which certainly could have affected the results. In further research, an index should be constructed in order to correlate weather factors to defecation. Another idea is to isolate a hive in an indoor environment with constant conditions, in order to try to rule out weather as a factor.

The use of smoke showed promising results, and the method of smoking the whole hive regularly for a longer time period should be investigated further. This ought to be done with shorter time-intervals, like 15 min between smoking sessions for 3 hours, and then collect the bees after 3 hours of smoking, with 15 min intervals.

Additional future research to be investigated is to try to gently squeeze the belly of the honeybee, proposingly with a soft tweezer. A bee that was pinched around its abdomen defecated during an experiment, when it tried to escape from captivity. One could consider if defecation from centrifugation comes as a consequence of the pressure put on the bee's abdomen when gravity pulls the bee backward. This could explain why some bees defecated and some bees did not. All bees were not perfectly positioned inside the Eppendorf-tubes, some bees had their wings reversed and their legs spread in different directions. That might have led to pressure being concentrated on the torso, and not the abdomen, which could be the reason to why some bees did not defecate. To prevent this in further research, the bees should not be allowed to wake up before they are centrifuged. They should not have time to move inside of the Eppendorf-tube, or the chance to brace themselves against the centrifugal forces. In this project, 20 bees were immobilized each time, and that gave the bees plenty of time to regain consciousness. The last 5-6 bees were almost fully awake, resisting quite much, leading to awkward positioning of the bees in the vials, which could have led to a different outcome of results. Ninety five - hundred percent of the immobilized bees gained consciousness in their Eppendorf-tubes before being centrifuged. So if there is a difference between centrifuged conscious bees, and unconscious bees, it remains to be investigated.

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6 - APPENDICES

Appendix 1 – raw-data long-term survival with nurse bees, centrifuged groups.

				1																												
Date/**Cages**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
12-jun	1	0	1	1	1	2	2	1	1	0	0	1	0	0	0	0	1	1	0	0	-	-	-	-	-	-	-	-	-	-	-	-
13-jun	1	0	0	1	0	0	0	0	0	1	2	0	1	1	1	0	1	2	2	2	0	1	0	1	0	0	0	0	0	0	0	0
14-jun	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	2	1	1	1	1	0	3	1	5	1
15-jun	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
16-jun	0	1	1	0	3	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	2
17-jun	0	2	0	2	1	1	0	0	0	0	0	1	0	0	1	0	2	0	1	2	0	2	0	0	0	3	1	0	0	0	2	0
18-jun	0	1	1	0	0	0	1	0	3	0	0	0	0	0	0	1	0	0	1	0	1	0	0	2	1	0	0	1	1	0	0	0
19-jun	0	0	0	1	1	0	0	0	0	0	1	2	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0
20-jun	0	1	0	0	1	1	0	2	0	1	0	0	2	2	3	0	1	0	0	2	0	0	0	0	0	0	0	0	2	0	1	0
21-jun	0	0	1	3	0	0	2	0	2	1	0	3	1	0	0	5	0	1	0	1	1	0	0	0	1	1	1	1	1	2	0	0
22-jun	3	3	1	1	3	1	2	1	1	6	4	0	5	0	3	0	0	6	2	1	3	2	8	4	4	0	2	3	3	0	2	1
23-jun	1	1	0	0	0	0	0	1	1	0	1	0	0	0	0	1	3	0	0	2	1	3	0	0	0	0	0	2	0	1	0	0
24-jun	3	0	3	0	0	2	2	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	2	0	0	3	2	0	1	0	2
25-jun	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	2	0	3	0	0	0	1	1	0	0	0	0	0	0
26-jun	0	0	0	0	0	0	0	3	1	0	0	2	0	3	1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	2
27-jun	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
28-jun	0	0	0	0	0	0	0	0	0	1	0	0	0	0	3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
29-jun	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
30-jun	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
01-jul	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
02-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
03-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
04-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
05-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
06-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
07-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
08-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix 2 – Control Group long-term survival.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	Å	Ä	Ö
19-jun	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-
20-jun	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21-jun	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	3	0	0	0	0	0	0	1	0	1
22-jun	2	0	1	1	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	2	0	0	0	0	0	0	0	0
23-jun	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24-jun	0	0	0	0	2	0	0	1	0	1	0	2	3	1	1	0	0	0	0	0	0	3	0	1	0	0	0	0	0
25-jun	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0
26-jun	0	3	1	0	1	0	3	0	1	0	0	0	2	1	0	1	0	0	0	0	0	1	0	1	0	2	0	1	2
27-jun	0	0	2	0	2	2	2	0	1	0	0	2	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	2	0
28-jun	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	2	1	1	1
29-jun	2	1	0	0	0	0	0	0	0	2	2	0	0	0	0	0	2	0	0	0	1	3	0	0	1	1	0	0	0
30-jun	0	3	1	0	1	1	0	2	0	1	0	0	0	0	2	0	0	1	1	0	1	0	0	1	0	0	0	0	0
01-jul	0	0	0	0	0	1	1	0	1	0	0	0	0	0	3	0	0	0	0	0	0	0	1	1	0	0	0	0	0
02-jul	1	0	0	7	1	0	0	1	0	0	0	0	0	0	2	1	1	3	0	0	0	0	0	1	0	0	1	1	0
03-jul	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0
04-jul	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	0
05-jul	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	3	0	0	0	0	0	0	0	0
06-jul	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
07-jul	0	2	1	0	1	2	0	3	1	3	0	1	2	0	0	1	1	2	0	0	0	2	1	0	0	0	1	0	1
08-jul	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	1	0	0
09-jul	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1