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

The development of tools for network analysis in the honey bee (*Apis mellifera* L.)

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ABSTRACT

The honey bee (Apis mellifera) has accompanied Man for thousands of years, and yet somehow, some aspects of this most studied of insects remain uncertain. To this day, details of the physiology, disease transmission, social organisation and behaviour of this animal are still unclear. The development of technology and computing and the use of tagging and automatic monitoring have already contributed in shedding light on some of the intricacies of sociality amongst insects. In this project, we hoped to develop further tools for the study of disease transmission though social networks in the honeybee, and shed some light on factors which might affect the behaviour of the bees in an experimental setting.

Bees were exposed to Kashmir Bee Virus and *Nosema ceranae* spores, and we attempted to identify the route of transmission of the diseases to sets of uninfected bees. Induced defecation was attempted by exposing honey bee workers to various treatments. Finally, bees were exposed to infra-red and white light treatments in an observation hive in order to determine whether artificial light had an impact on honey bee movement and locomotor activity.

Whilst we would have expected the Kashmir Bee Virus to be transmitted through food exchange, we found no evidence of such pathway. Similarly, bees infected with *Nosema ceranae* spores did not seem to infect other workers through trophallaxis. Induced defecation in worker bees was generally unsuccessful and more work needs to be carried out to identify whether a reliable method can be discovered. Preliminary results of the impact of lights on the activity of the bees in an observation hive support the hypothesis that lights do affect bees for a short period. This body of work should serve as a development of the tools needed for advanced studies of the social networks and exchanges in honey bee colonies.

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Acknowledgments

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1 - INTRODUCTION

Epidemiology, the study of health and disease related events at a population level, has been the subject of much attention in the last few decades. Concerns over growing population densities, urbanization and ease of travel have increased our need for identifying the route of transmission of diseases in order to prevent uncontrollable outbreaks. Furthermore, not only have humans been prone to more rapid transmissions, but animals forced to live in close proximity and large groups have also inherited susceptibility for quick outbreaks leading to large-scale epidemics. Past research with the aim of preventing and containing future outbreaks has focused on the development of large mathematical models, relying on previously collected data and following the movements of already infected people during the course of natural epidemics (Naug, 2010). However, with the development of the network theory, assuming propagation of pathogens via highly structured interaction networks (Otterstatter and Thomson, 2007), replacing previous thoughts of homogeneity amongst individuals in a group, models have become hard to parameterize, leading to unreliable predictions (Naug, 2010). With this in mind, researchers have shifted their attention to more experimental research.

Many animals spend much or all of their time as part of a group. Some groups are simply considered as many individuals with no particular cohesion or interactions; others exhibit mutualistic or semi-social behaviours. On the other hand, some groups are made up of individuals dependent on each other, with a multitude of interactions and even various hierarchical levels. Social insects are known to form such groups where individuals form complex spatial or social networks. Honey bees (*Apis mellifera*) make up exemplary subjects to carry out research into contact network epidemiology.

The eusocial honey bee is found throughout the world. Originally native to western Asia, the Middle East, Africa, and Europe, humans have ensured that this most important of insects followed them wherever they went (Seeley, 2010; Winston, 1987). Our fascination with honey bees goes back thousands of years (Winston, 1987), and many of its features make it a suitable model for study. A healthy colony is densely populated, made up of highly organized and interacting individuals but also segregated depending on their location, age or the task they perform (Naug, 2008). Colonies of honey bees are made up of three castes: workers, queens and drones. Workers carry out most of the tasks necessary for the functioning of the colony, and are all morphologically similar. Some physiological differences arise due to age polyethism, dictating why and which workers do what and when (Winston, 1987). Typically, workers will start with within-colony tasks early in their lives, such as cell cleaning, brood and queen tending, comb building and cleaning. Later on, they tend to carry out tasks on the periphery of the colony, such as ventilating, guarding and finally foraging (Winston, 1987). The colony's social network includes a high number of physical interactions, to disseminate information, exchange food or pheromones or through mutual cleaning. The most relevant interaction in terms of epidemiological significance is that involving trophallaxis, the exchange of nutrients between workers. The trophallactic network in a honey bee colony has been extensively studied. Researchers have spent time mapping the distribution of marked foods throughout the hive, from the periphery to its centre and from the oldest to the youngest individuals (Naug, 2008, 2010). It is widely accepted that trophallaxis has the greatest potential for an orally transmitted pathogen, to spread through the contact network of the colony (Naug, 2008).

The main aim of the project is to identify suitable tools to allow the study of disease transmission through social networks in honey bees. The four main aspects of the work include: (1) the importance of food exchange (trophallaxis) between workers in transmitting pathogens, and the creation of relatedness mediated networks (using trophallaxis to find out if relatedness between workers affects the formation of networks and results in interaction preferences). (2) The potential for faecal analysis, in monitoring disease dynamics whilst keeping the bees alive. (3) The determination of light conditions suitable for studying honey bee behaviour in an experimental in-door setting without disturbance to the subjects. (4) The training of computer software to recognize interactions between, and movements of, individuals and potential instances of disease transmission.

2 - BACKGROUND

2.1 - ***Honey bee diseases***

A multitude of diseases and viruses are present in bee colonies. Many of those are known to exist in individuals and colonies without necessarily causing symptomatic infection. However, some combinations of diseases, the impact of new disease vectors and the effect of managed apiculture have led to increasing concern and could be a major influence regarding the drastic colony mortalities of the last decade (Genersch, 2010). In a bee colony, disease transmission can occur horizontally or vertically. Horizontal transmission occurs within a generation of adult bees, whilst vertical transmission results in infection between adult bees and members of future generations of the colony. The path of transmission of a disease is an important factor in determining its prevalence and virulence. The recent demise of honey bee colonies can be attributed to a wide range of interacting factors and conditions, and there are no doubts that diseases play a part. This study concerns two types of diseases, one a microsporidian pathogen, the other a viral disease. The reasons for this selection include ease of access to the pathogens and their readiness to use, but also the fact that there are still uncertainties regarding their transmission, focusing here on horizontal transmission.

Nosema sp are fungal pathogens belonging to the phylum Microsporidia, which are obligate intracellular fungal parasites. They exist outside the host cell as metabolically inactive spores. Infection occurs when an adult bee ingests the spores present in faeces but also in pollen (Genersch, 2010). These spores then germinate in the midgut, infecting the cells of the midgut epithelium, where they proliferate and produce new environmental spores released into the gut lumen (Fries, 2010). Nosemosis, caused by *Nosema* infection, results in a form of dysentery (Genersch, 2010). For this reason, epidemics, mostly occurring in overwintering bees, are likely the result of a lengthy confinement (L'Arrivée, 1965). It shortens the lifespan of worker bees and results in weak and unprofitable colonies. Diseased bees, unable to fly, usually defecate within the hive, therefore, other bees can get contaminated by cleaning up faecal deposits, and infect others through food-sharing activities (L'Arrivée, 1965). When the queen is infected, a reduction in brood production will result and she is likely to be superseded within one month (L'Arrivée, 1965). *Nosema* is world-wide in its distribution and is usually found wherever bees are maintained (L'Arrivée, 1965).

There are, at present, eighteen known viruses affecting honey bee colonies. The Kashmir Bee Virus (KBV) is part of a complex of closely related viruses from the Family Dicistroviridae,

which also includes acute bee paralysis virus and Israeli acute paralysis virus (de Miranda et al, 2010). First isolated in 1974 in Kashmir and fully described in 1977 (Bailey and Woods, 1977), it now has a worldwide distribution (Hung, 2000). KBV has been detected in a number of organisms other than the *Apis* species of bees such as bumble bees and European wasps (Genersch and Aubert, 2010; Allen and Ball, 1996; Bailey and Woods, 1977).

Many experiments about disease transmission in honey bees result in a large number of individuals being sacrificed for analyses. The potential use of faeces from worker bees could greatly reduce the need for this practice and result in longer lasting experiments and even the potential from tracing the evolution of an infection in a given individual. It has been shown that induced defecation in queen honey bees is possible and often found to occur after a generous feeding (L'Arrivée, 1964).

*2.2 - **Artificial light and honey bee behaviour***

Karl von Frisch, the Austrian ethologist, worked extensively with honey bees and did much research on their vision and perception of colours. He carried out elegantly designed experiments, testing the bees' ability to discriminate between different colours (von Frisch, 1955). These studies, dating back to the middle of the 20th century, showed explicitly that bees did not see red colours but more likely perceived them as a dark shade of grey (Butler, 1976; von Frisch, 1955). Since then, the bee's spectrum of colour has been estimated to be between 0.3 μm to 0.65 μm corresponding from ultra violet to yellow/orange (von Frisch, 1963).

Research has concentrated on establishing the link between day light patterns and honey bee behaviour. For example, it has been shown that bees show strong positive phototaxis (Ben-Shahar et al, 2003 and Erber et al, 2006), but also that foraging bees are more positively phototactic than nurse bees of the same colony (Ben-Shahar et al, 2003). Phototaxis relates to a locomotory movement in response to the stimulus of light. Positive phototaxis occurs if the movement is in the direction of the light source. This makes sense in the way that forager bees, that show strong circadian rhythms, are dependent on light for finding sources of nectar and guiding other bees to them, whereas nurse bees need not rely on daylight cues and typically care for the brood around the clock (Bloch, 2010; Shemesh et al, 2010). Similarly, the impact of weather conditions on the behaviour of different castes has been investigated, foragers were found to be busier on sunny days than on days when light was poor or the

weather was bad, with nurse bees showing little difference in behaviour between day and night (Riessberger and Crailsheim, 1997).

Observation hives provide an experimental setting to study the behaviour of bees. Experiments however usually require manipulation of the subjects during the course of an experiment and such interventions often involve the need for artificial light to correctly identify individual bees. The impact of such light on the behaviour of the bees is still rather unknown in the literature and could interfere with some of the behaviours studied. Furthermore, with the development of more advanced observation techniques such as the use of cameras, lighting is becoming indispensible and yet its impacts are unknown. For example, Meshi and Bloch (2007) used minimal lighting to minimise disruption involving two dim yellow lamps, whereas others have used red light assumed to be invisible to the bees (Kimura T. et al, 2011), but no standards have yet been described. The impact that artificial light might have on normal bee behaviour in an experimental setting is poorly documented. Little work has concentrated on the short term and longer lasting effects that light might have on the locomotor activity in an observation hive.

*2.3 - **Genetic studies of honey bees***

Studies of social behaviour in eusocial insects have been the focus of much research. Attempting to explain and define the evolution of altruism in society has relied on the study of social insects such as bees and ants. Reproduction in a honey bee colony is regulated by the queen. All workers are the offspring of one queen in a colony. However, each queen mates many times, and carries the sperm of between 7 and 20 male drones (Estoup et al, 1994). The result is that workers from the same colony will be more, or less closely related to each other. Therefore, assuming that super-sisters (daughters sharing a father together referred to as a sub-family) have a relatedness of 0.75, whilst the half-sister relatedness (the relationship between two groups of super-sisters) is of 0.25 (Breed et al, 1994), the idea stems from previous research and literature, still debated, hypothesising that (1) members of each subfamily within a colony can recognise each other, and distinguish super-sisters from half-sisters and (2) members of each subfamily use this recognition information and increase the reproductive fitness of their own subfamily at the expense of half-sisters (Oldroyd et al, 1994). The implications of such findings are still widely debated, and whilst some have shown that honey bees can discriminate between super and half-sisters (Oldroyd et al, 1994; Hogendoorn and Velthuis, 1988; Getz and Smith, 1983), others have been quick to question

and limit such discrimination (Underwood et al, 2004; Breed at al, 1994). Kirchner and Arnold (2001) also contradicted the findings of Oldroyd et al. (1991) and found no evidence for sub-family discrimination in the context of the dance communication system. Finally, in their examination of the cuticle hydrocarbon composition of honey bee workers, Arnold et al. (1996) showed that even in a colony headed by a naturally inseminated queen, honeybees possess the discriminatory ability for subfamily recognition in a natural context (Arnold et al, 1996).

More recently, advances in genetics and the full description of the honey bee genome have led to more comprehensive studies into the origin of sociality (Estoup et al, 1994). Extracting DNA from worker bees, combined with selected microsatellite DNA markers has given us a glimpse into the complex system of patriline co-existence and the genetic structure of a colony (Estoup et al, 1994). Multiplex Polymerase Chain Reaction (PCR) is a modified PCR method whereby it is possible to amplify several products in the same reaction (Henegariu et al, 1997). It is used for genetic screening and microsatellite DNA analysis which are nowadays well established techniques in honey bee population genetics (Shaibi et al, 2008). Because of the potential interference of non-specific products with the amplification of specific products, this technique varies somewhat among the research literature and protocols and parameters have to be optimised for each investigation. PCR buffer, primer concentration and annealing temperature are some of the factors that need to be optimised in order to obtain successful amplification and reach the desired yield (Löffert et al, 1999).

2.4 - ***The transmission of food between workers***

The transmission of food between foragers returning to the hive and between workers, drones, and queens inside the hive is an important aspect in the functioning of honey bee society as a eusocial, altruistic super-organism. Indeed, one forager returning to the hive and distributing its bounty amongst numerous members of the colony could be considered the opposite of selfishness and one of the keys to the success of this complex social community (Dietz, 1986; Crailsheim, 1998). Food transmission between two worker bees starts by one of them either begging for or offering food to the other (Free, 1957). A bee asking for food does so by protruding its tongue towards the mandibles of another bee and by directing its antennae towards it (Crailsheim, 1998). A bee, which offers food, opens her mandibles, pushes forward the proximal part of her tongue and regurgitates a drop of fluid between her mouthparts (Free, 1957). Delving into the process of trophallaxis (food exchange) reveals some of its

importance in the functioning of a healthy colony. Indeed, receiving and donating food depends on many factors such as the sex and age of the consumers and donors, food availability and quality, even time of day or weather and season (Crailsheim, 1998). Trophallactic interactions would seem to occur more often between bees of similar age (Naug, 2008). In a honey bee colony, the oldest individuals (mainly foragers) tend to remain at the periphery, whilst the younger ones (newly emerged or nurses) remain at the centre (Winston, 1987). Combined with the flow of food from the outside to the centre of a hive where the stores are, individuals closer in age should interact more with each other (Naug, 2008). Furthermore, more than just resulting in food being transferred from one bee to another, the trophallactic flow of food also provides other information about the quality and quantity of food reserved both inside and outside the hive (Crailsheim, 1998). Finally, it seems evident that such interactions between individuals should be a prime target for the orally transmitted pathogens to spread through a colony (Naug, 2008).

3 - MATERIALS AND METHODS

*3.1 - **The bees***

The bees used in the disease experiments were taken from colonies maintained according to standard beekeeping techniques at the Ecology department of SLU for Kashmir bee disease infections and at Bigården, the bee research facility of Sveriges Lantbruksuniversitet (SLU), Uppsala, Sweden for *Nosema ceranae* infections and induced defecation experiments.

*3.2 - **Disease transmission in cage experiments***

The aim of these experiments is to try to ascertain whether pathogens can be transmitted through trophallaxis, and the role of mouth-to-mouth food transfer in the epidemiology of an infectious disease.

3.2.1 - Kashmir bee virus infection

Groups (1dl, about 100 individuals) of bees were placed in small mesh cages. For controlled infection, each cage was inoculated by feeding it with 1,5 ml of 60 % sucrose solution containing Kashmir Bee Virus in a concentration of $⅓*10^9$ per µl. Once the bees emptied the virus-loaded Eppendorf tube, they were fed a 60% sterile sugar solution. After 24h the cage containing infected bees (cage A) was placed in contact with a cage containing non-infected bees (cage B) with no sucrose solution. After 24h of contact, the two cages were separated.

Cage A with initially infected bees was removed; 50 bees frozen at -80ºC and 50 bees spun in the centrifuge until defecation before being frozen. Cage B bees were fed with a sterile sugar solution and at 24h, 4 days, 8 days and 12 days, after the transmission event, 20 bees were taken from the cage; 10 of them spun and 10 just frozen. Both the tissue and faeces were saved in the freezer at -80ºC.

A qPCR analysis was carried out in order to test the presence and level of virus titers in the various stages of infection and exposure to the virus. We analysed virus levels in control bees of the same colony, the infected bees from cage A, and bees 24 h, 4 days and 8 days after initial contact with infection. The program consisted of incubation at 50ºC and 95ºC for 10 min and 5 min respectively, denaturation for 10 s at 95ºC, 30 s of annealing at 58ºC, and extension at 72ºC for 45 s. The sequence was further duplicated 39 times.

3.2.2 - Nosema ceranae *spores infection*

In this experiment, 1 dl of newly emerged *Nosema*-free bees was fed with a sugar solution containing *Nosema ceranae* spores, obtained from the crushed gut of a heavily infected bee, at a concentration of at least $1*10^7$ spores. The infected bees were kept in a small mesh cage (cage A) with a sugar solution and placed in contact with 1dl of non-infected bees (cage B) with no sugar, for as long as the bees from cage B survived (less than 24 h). Ten days post infection, cage A was placed in contact with another 1dl of nosema-free bees (cage C) with no sugar for 24 hours. Finally, a sample of twenty bees from cage A were placed in another small cage (cage D), separated in two by a mesh. Ten *Nosema*-free bees were placed on the other side of the mesh. Only the 20 bees from cage A received sugar. All the bees stayed in contact for 48 hours, after which the ten bees from cage D were removed and placed in a separate cage with a sugar solution for 10 days. We estimated the number of spores in twenty bees from cage A, the ten bees from cage D, and twenty control bees to check for background levels. The number of spores was estimated under the microscope. The whole abdomen of an individual bee was mashed in 1ml of water. The sample were then diluted 10X to be able to count the number of spores.

*3.3 - **Use of faecal matter from live bees to determine micro-organism levels***

3.3.1 - Pilot study for induced worker bee defecation

Forty bees were subjected to different treatments and placed in individual petri-dishes for 15 minutes. At the end of the recording time, the number of bees that had defecated in its dish

was noted. For each treatment, ten bees were selected at random from a set of 1dL of bees picked from a frame.

Treatments included:

- Control (bees placed in individual petri-dishes)
- Feeding (bees hand-fed with 60% sugar solution and placed in individual petri-dishes)
- CO_2 (bees gazed with CO_2 until immobile (about 20s) and placed in individual petri-dishes)
- Cold (bees placed in the freezer until immobile (about 4mins) and placed in individual petri-dishes)

In another experiment, twenty bees were selected. Ten of the bees were between 24 h and 48 h old, and the other ten were taken from a frame in a hive and therefore of random age. All bees were placed in a 0.5 ml Eppendorf tube with queen candy and fed for 24 h. After this time, all bees were placed in individual petri dishes with the queen candy for another 24 h. Finally, the number of bees that had defecated after 48 h was noted.

3.3.2 - The bee digestive system post-centrifugation

Twenty bees were gazed with CO_2 and immobilised in 0.5 ml Eppendorf tubes, facing up. The tubes were centrifuged at a speed of 3000rpm for 30 seconds. Once spun, the bees were separated into those that defecated and those that had not and frozen at -20ºC. The dead bees were then dissected to determine gut and rectum content.

3.3.3 - The effect of spinning on the survival rate of bees

In a survival experiment, 60 newly emerged bees were separated into 3 treatment groups of 20 bees each. Group A was spun at 1500 rpm for 1 minute. Group B was spun at 3000 rpm for 30 seconds. Group C served as a control. Each group was placed in a separate cage with a 60 % sugar solution. Every day, the number of surviving bees was counted in order to create a survival curve and compare the rate of survival after centrifugation.

3.4 - ***Impact of artificial light on honey bee activity in an experimental setting***

3.4.1 - The bees

The bees used in the experiments were taken from colonies maintained according to standard beekeeping techniques at Bigården, the bee research facility of the Sveriges Lantbruksuniversitet (SLU), Uppsala, Sweden.

We marked the focal bees of random age, taken from a frame within a colony, with numbered tags, of 3 different colours, from 1 to 100. Four hundred bees had white tags with black numbers; one hundred bees had yellow tags with black numbers; and one hundred more with red tags and white numbers. We also added a queen to the hive to recreate a more natural set of behaviours for the workers. The queen was tagged with number 44, and we removed that number from the other sets. This resulted in 595 differently marked bees to work with.

3.4.2 - The observation hive

The observation hive consisted of a wooden box (52.5 x 43.5 x 5.5 cms) with two Plexiglas panels on either side. We placed a honey bee frame, with sufficient capped honey reserves inside the hive. We set up the observation hive in a small room, completely dark with no link to the outside.

3.4.3 - Tracking the bees

One camera was placed 80 centimetres from the hive. The camera was a Basler scout (scA1600-14gm) with a Fujinon HF16HA-1B lens (16mm TV lens). The camera could be controlled using Basler's Pylon driver. The video was recorded using the only available freeware for this purpose, Virtual VCR, Version 2.6.9. Focal bees were tracked using software designed by Cris Luengo at the Centre for Image Analysis, SLU and Uppsala University. The software was originally hoped to be able to detect marked honey bees movements as a series of (X, Y) coordinates across the frame. However, due to time constraints, and hardware malfunctions and low specifications, we used a purpose-built optical flow process, which, disregarding the tags, calculated speed and distance moved by each object (individual bees) for each frame and averaged over 20 s intervals.

3.4.4 - Light treatments

After a 24 h period of habituation to the conditions of the experimental hive and kept in the dark, the bees were exposed to the following light treatments. The bees were filmed for half an hour using infra-red lights. After the first ten minutes, a full bright light (30 $\mu m/m^2.s^{-1}$) was switched on for 10 minutes. The final ten minutes were filmed again using only infra-red

light. This treatment was repeated, at the same time of the day, for 6 days. Subsequently, we used the same protocol for three more days, adding a light of lower intensity (7 $\mu m/m^2.s^{-1}$), resulting in forty minutes films (10 min I.R. / 10 min Low-Intensity / 10 min High-intensity / 10 min I.R.).

*3.5 - **Does variable genetic relatedness affect speed and route of disease transmission?***

3.5.1 - DNA extraction from thoracic flight muscle of honey bees

The sample tissues were transferred to Eppendorf tubes and DNA extraction was carried out using the Chelex 100 protocol (Walsh et al, 1991) regarded as the most suitable for this type of extraction.

3.5.2 - Microsatellite analysis

Using the microsatellite DNA toolkit described by Shaibi et al (2008), multiplex PCR solutions were prepared, containing 10 micro litres of 10-100 ng DNA, 1x PCR-Master-Mix (Promega), and 0.2 μM of each primer (5' –label). Genetic relatedness will be determined using a microsatellite DNA toolkit developed by Shaibi et al. (2008), and consisting of a set of 18 microsatellite DNA markers that can be run in a multiplex PCR. The PCR programme consisted of denaturation for 5 minutes at 95°C, 30 s of annealing at 55°C, extension for 1 min at 72°C, final elongation of 20 min at 72°C in a GeneAmp 9700 thermocycler.

*3.6 - **Statistical analysis***

All statistical work was conducted using Microsoft Excel and Minitab 16. We conducted a Fisher's Exact Test to look for association between defecation and rectum content in worker bees. For comparisons of survival in bees having received different treatments, we use a non-parametric Kruskal-Wallis one-way analysis of variance by rank test. We also performed a Mann-Whitney U test when comparing two treatments.

4 - RESULTS

*4.1 - **Disease transmission in cage experiments***

4.1.1 - Kashmir bee virus infection

The data resulting from the qPCR analysis does not show that we successfully established infection in the original bees. Therefore, All KBV-samples had barely detectable levels of virus:

A dilution series ranging from 8.5 ng/µL to a 10^{-9} dilution of the stock was run along all samples in the qPCR. The samples had higher CT-values (31 or higher) than the -9 standard (CT of 29). Hence, virus levels were at less than 10^{-18} grams per sample.

RP49 (housekeeping gene) CT values were around 14-16 which indicate successful qPCR. The very low levels of virus detected could have been explained either by unsuccessful qPCR reactions, errors in extraction or degraded RNA. However, considering the levels of RP49, these possibilities seemed unlikely. Having reasonable levels of the RP49 also indicated normal transcriptional levels; if the bees were overtly infected or had, for some reason, down-regulated transcription in their cells we would not see the expressions of RP49 that we did.

Some of the results could also have been explained by contamination of the samples. The background bees were RNA-extracted in the virus lab, where the surfaces and equipments could have been contaminated. We had positive results in samples of water extracted along with the RNA-samples. Cleaning the QiaCubes thoroughly could help to avoid traces of viruses or their RNA.

Table 1: Summary of C(t) ranges for all groups of bees used in a qPCR to detect levels of Kashmir bee virus in comparison with the RP49 gene used as a standard level of transcription.

Bee	n	KBV / RP49	C(t) range
Control	5	KBV	32 - 34
Control	5	RP49	14 - 16
Cage A	bulk 10 bees	KBV	36 - 37
Cage A	bulk 10 bees	RP49	17 - 20
24 H	5	KBV	31 - 34
24 H	5	RP49	14 - 27
4 Days	6	KBV	33 - 37
4 Days	6	RP49	14 - 16
8 Days	5	KBV	34 - 37
8 Days	5	RP49	14 - 15
Extrac. Water	1	KBV	31

4.1.2 - Nosema ceranae *spores infection*

The first group of bees placed in contact with the infected bees all died very rapidly, within 24 h. A sample was taken and no spores were found. Ten days post infection; the bees were placed in contact with another set of Nosema-free bees, of similar age. After 24 h all the bees without sugar were dead. It was decided then to use smaller number of bees. Therefore, a sample of 20 infected bees was placed in contact with 10 non-infected bees in one mesh cage separated in two halves. The bees were left in contact for 48 h. Post-exposure, the number of spores in each infected bees was estimated and was in the order of $2*10^7$ to $5*10^7$ spores per

millilitre. Due to time restrictions, the 10 exposed bees were dissected after 5 days and whilst some spores were observed (10^5 in two of the bees), only a very low level of infection was present. This could have been also due to the fact that all the samples were diluted whether we should have checked for infection prior to dilution. Furthermore, three of the ten bees died at day 1, 3 and 4 respectively which would not have allowed for *N. ceranae* spores to develop and propagate.

*4.2 - **Use of faecal matter from live bees to determine micro-organism levels***

4.2.1 - Pilot study for induced worker bee defecation

For all the treatments tried (Control, Feeding, CO_2, Cold), only one of the forty bees defecated after being hand-fed (Feeding treatment) and placed in a petri-dish.

Forty-eight hours post extraction from the hive, only 5 of the 20 bees had defecated in a petri dish. All five were taken directly from the bee hive, therefore none of the bees which were between 24 h and 48 h old had defecated.

4.2.2 - The bee digestive system post-centrifugation

After centrifugation of twenty bees at 3000rpm for 30 seconds, 9 bees defecated. A Fisher's exact test examining the significance of the association between centrifugation, defecation, and the presence of food in the rectum was carried out. We found that there was no significant difference in the presence of food in the rectum between bees that were centrifugated and bees that were not (P=0.15). There was also no significant difference in the presence of food in the rectum between bees that defecated and bees that did not during centrifugation (P=0.19). There could have been a trend that could have been identified if the sample groups had been larger.

Table 2: Counts representing bees' rectum contents in relation to the presence or absence of food before and after centrifugation, with or without defecation.

	Centrifuged Defecated	Did not defecate	**Control**
Food in rectum	7	5	17
No food in rectum	2	6	3

4.2.3 - The effect of spinning on the survival rate of bees

As previously observed, centrifugation of the bees resulted in about 50% success. Nearly half of the bees tested defecated inside the tube. This result whilst not optimal showed that there was some promise in this technique for induced defecation. However, it was also important to test the impact that centrifugation might have had on the physiology of the bees through its effect on lifespan.

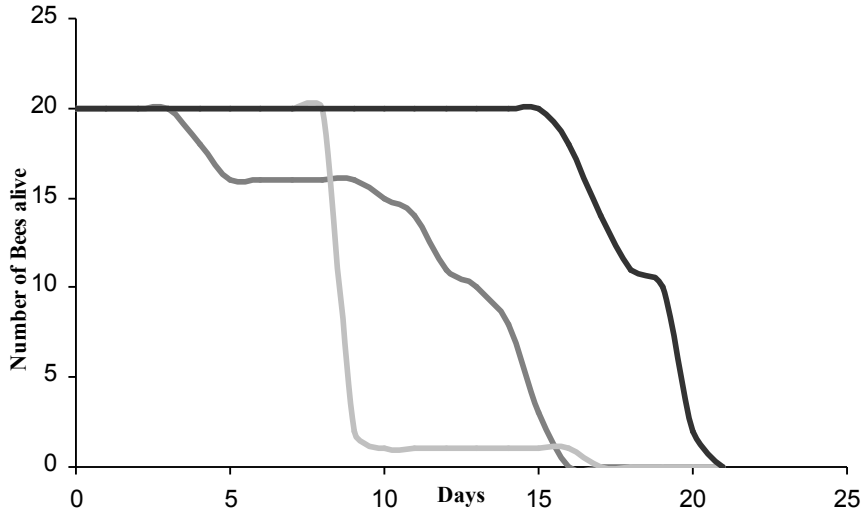

Figure 1: Graph showing the survival curves of 24h old bees after having undergone two different centrifugation treatments. Black curve represents the control bees. Dark grey curve, bees centrifuged at 1500rpm for 1 minute. Light grey curve, bees centrifuged at 3000rpm for 30 seconds.

A Kruskal-Wallis one-way analysis of variance by rank showed that there was a significant difference ($P<0.05$) between the survival rate of bees that have undergone a treatment in the centrifuge and those that did not. Furthermore, a Mann-Whitney U test revealed that there was a significant difference ($P<0.05$) between the survival rate of bees for each treatment and for the control bees, however, there was no significant difference between the two treatments of centrifugation ($P=0.9$).

4.3 - ***Trial video recording***

A short 16 minutes film was successfully recorded consisting of around 50 tagged bees (random tag numbers), one queen, and other untagged workers.

The video was subsequently reduced to a 1 minute film in which bees were manually tracked by Cris Luengo.

http://vimeo.com/25507078

4.4 - ***Impact of artificial lighting on honey bee behaviour in an experimental setting***

Weather over the duration of the experiment was very similar over the days, consisting of mainly large cloud cover, with occasional showers and sunny outbreaks. Preliminary results, showed a significant difference between the first 10 minutes (darkness), the next 10 minutes (bright white light) and the last ten minutes (darkness) (See table 3).

Table 3: Results of a non-parametric Kruskal-Wallis test on the medians of the activity levels over the different treatment periods, for the six days of recording.

Kruskal-Wallis Test on total Activity

Time	N	Median	Ave Rank	Z
0-10	149	115740	196,0	-3,19
10-20	148	116265	209,9	-1,57
20-30	149	139800	264,5	4,76

H = 23,55 DF = 2 P = 0,000

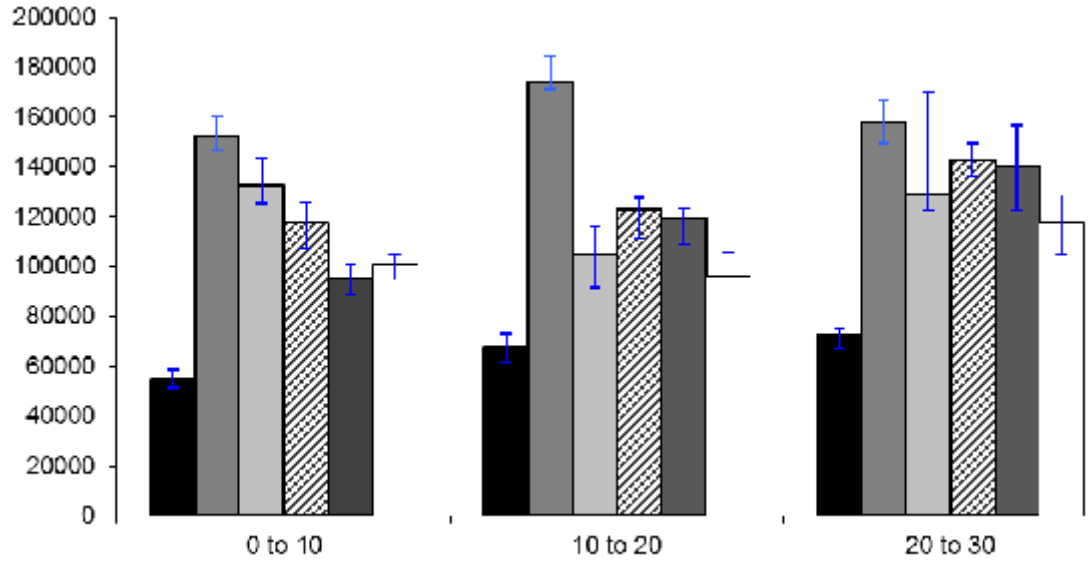

Figure 2: Histogram of the activity levels of the bees (in arbitrary units). 0 to 10 represent the first en minutes of recording in darkness; 10 to 20 represent the next ten minutes, recorded with a bright white light facing the observation hive; 20 to 30 represent the last ten minutes in complete darkness again. Each bar represents the Median for the day at that period of recording. From left to right: day 1 to day 6.

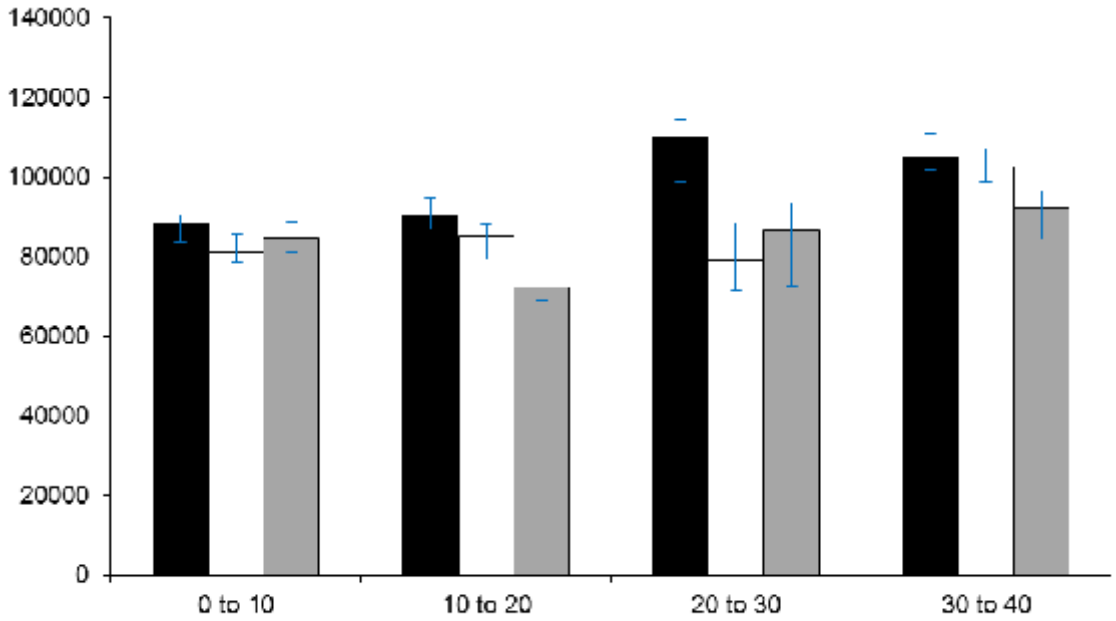

Figure 3: Histogram of the activity levels of the bees (in arbitrary units). 0 to 10 represent the first ten minutes of recording in darkness; 10 to 20 represent the next ten minutes, recorded with a low intensity white light facing the observation hive; 20 to 30 represent ten minutes with high intensity white light; 30 to 40 represent the last ten minutes in complete darkness again. Each bar represents the Median for the day at that period of recording. From left to right: day 7 to day 9.

It seems however, that the bees have been behaving differently over the days. For example, on day 5, there seemed to be nearly constant increase in activity from the start to the end of recording, going through the treatments (Figure 3), and indeed there is a significant difference in activity between the three periods of recording ($P<0.025$) (Table 4). This trend can be observed although to a much lower degree on days 1 and 4. However, on days 2 and 3 seem to behave very differently. For example, on day 2 (Figure 3) we can see that the bees were more active upon the start of the light treatment and resumed to more normal activity levels after the end of the treatment. Once again there is a significant difference. However, in the case of day 2, the difference lies between the period of light treatment and the other two. There was no difference between the first period of darkness and the second period of darkness (see table 5).

Table 4: Results of a non-parametric Kruskal-Wallis test for day 5

Kruskal-Wallis Test on Activity on Day 5

Time	N	Median	Ave Rank	Z
0-10	30	95093	16,2	-7,53
10-20	30	119285	49,9	1,13
20-30	30	140080	70,4	6,40

H = 23,55 DF = 2 P = 0,000

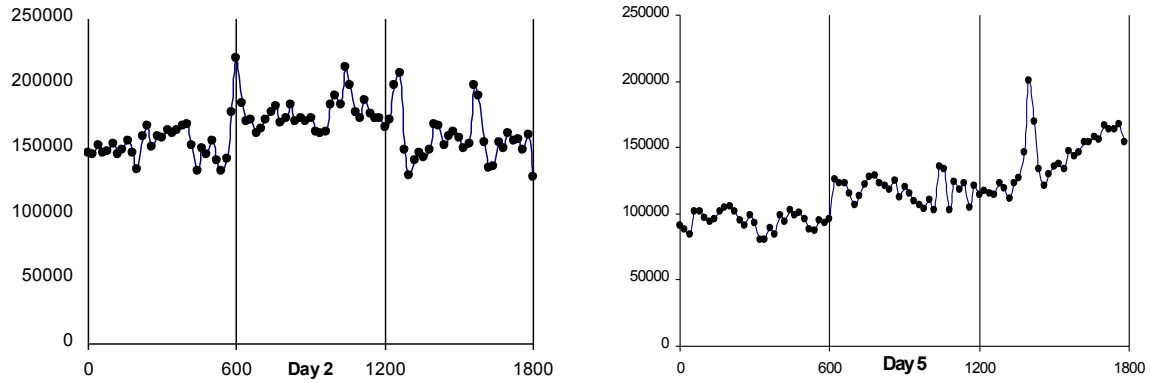

Figure 3 Scatter plots of day 2 and day 5, with added smoothed line and major grid lines highlighting the 3 different treatment periods. Time on the x-axis is in seconds, while the values on the y-axis represent an arbitrary unit of movement and speed. We can clearly see the different patterns emerging over the three periods.

Table 4: Results of a non-parametric Kruskal-Wallis test for day 2

Kruskal-Wallis Test on Activity on Day 5

Time	N	Median	Ave Rank	Z
0-10	30	152185	29,9	-4,07
10-20	30	173870	70,5	6,21
20-30	31	155040	37,8	-2,12
H = 39,87 DF = 2 P = 0,000				

Time	N	Median	Ave Rank	Z
0-10	30	152185	28,5	-1,10
20-30	31	155040	33,5	1,10
H = 1,20 DF = 1 P = 0,273				

5 - DISCUSSION

*5.1 - **Disease transmission***

The current results do not support the hypothesis that viruses and other infections are transmissible through food exchange between worker bees. Previous work showed that Deformed Wing Virus (DWV) was successfully transmitted between infected bees and disease-free bees and that trophallaxis could play a part in the oral transmission of DWV (Möckel et al, 2011; de Miranda and Genersch, 2010). However, in a similar set-up using Kashmir Bee Virus, no instance of the virus could be found in the exposed bees. Previous studied showed that bees injected with purified KBV particles died within six days. Moreover, it seems that if the virus was ingested, the bees remained unaffected (Bailey and Woods, 1977; Genersch and Aubert, 2010). However, some research points to food resources as possible routes of transmission for KBV (Chen et al, 2006) and that the virus can lead to death in bees by feeding at a level of around 10^{11} virus particles per bee (de Miranda et al, 2010). This highlights the importance of the transmission route in the development of overt infections. Finally, it would be uncharacteristic to discuss the transmission of a virus without the mention

of *Varroa destructor*, a mite more and more intimately linked to disease outbreaks in honey bee colonies. The bees in the experimental set-up were taken from a colony which was supposedly mite-free, originally aimed at minimising Deformed Wing Virus infections. A number of studies have successfully linked the presence of *V. destructor*, as both virus activator (Yang and Cox-Foster, 2005), and direct virus vector (Shen et al, 2005; Chen et al, 2004), with the presence of overt infections and transmission of KBV to bee brood (Genersch and Aubert, 2010; Chen et al, 2004), which could explain why the bees did not develop high enough virus titers, or overt and symptomatic infections in the analysis. Whilst the results point to a conclusion that Kashmir Bee Virus is not transmitted through trophallaxis, they could also be explained by the fact that not enough of the virus was fed to the bees. Indeed, most of the bees that were infected through bulk feeding were still alive around 12 to 15 days post infection. With more time, experiments could and should be carried out to confirm or refute the findings.

The experiment to test the transmission of *Nosema ceranae* through trophallaxis was met with some difficulties. The first group of bees was successfully infected with spores, however, the first group of exposed bees, had died within 24 h post exposure. The infected bees were then left on their own for ten days and exposed to another set of bees. Once again, all the exposed bees died within 24 h. This means that it became impossible to test whether Nosema spores had been transmitted through food exchange. Some possible reasons why all the exposed bees died so quickly could be that once the two boxes were attached, the exposed bees did not have enough air, or perhaps the infected bees did not feed the exposed bees that had no sugar. This last point raises more questions, such as why the infected bees did not feed their sugar-less sisters. The Nosema-free bees are bees that had just emerged and had clean guts with no fungal infection. The experimental set-up meant that they were kept without nurses to feed them. They had never been fed by another bee and might have lacked the ability to adequately process the given sugar solution in view of successfully exchanging food with their newly emerged sugar-less fellows. Previous research found that bees kept without nurses did not develop well (Crailsheim, 1998; Crailsheim and Stolberg, 1989). They had reduced levels of amino acids, including minerals and vitamins (Crailsheim, 1998; Crailsheim and Leonhard, 1997). Another possibility, first come across in a study by Naug (2008) is that the infected individuals 'purposefully' stopped interacting, effectively turning themselves into sinks in order to reduce the diffusion of the contagion. In the final experiment, we put in contact twenty infected bees with ten non-infected ones separated by just one mesh. The uninfected bees survived for

48 h with no sugar solution, suggesting that the other bees with sugar had been offering them food. It could be that there is a threshold regarding the number of bees that can be kept alive through trophallaxis, but this raises a further question as to why all the exposed sugar-less bees died rather than a portion of them that were not fed.

5.2 - ***Inducing defecation in a worker bee***

All attempts at inducing the defecation in a worker bee were generally unsuccessful. As the results show, we did not find any reliable, efficient and timely method that allows for the use of faeces for epidemiological purposes. Some of the bees did not defecate for 48 hours of observations. Whilst the two treatments involving anaesthesia (CO_2 and freezing) of the bees gave us poor results, there is some indication that other anaesthetic chemicals could be more successful. For example, in listing all the methods used to put bees to sleep, Hansson (1980) realised that one of the side-effects of nitrous oxide overdose was a tendency to defecate upon waking up in surviving bees. There could be an opportunity for testing various dosages of nitrous oxide to reach a high percentage of defecating bees with no other life impairments. Feeding sugar solutions to the bees or queen candy was very unsuccessful, but perhaps feeding the bees with more proteins such as derivatives of pollen would also result in a better outcome.

Centrifugation originally showed to have the most potential, as the defecation is instant and the faeces already deposited in an Eppendorf tube suitable for analysis. However upon closer examination, some of the drawbacks of using this method emerged. For example, size of the bee, or age of the bee or time since last defecation. Moreover, a survival experiment showed that bees which had been centrifuged lived less long than bees that had not. However, more experimentation could prove fruitful. As we showed, nearly 50% of the bees had defecated and survived for nearly ten days which could be enough to test the evolution of fast-developing infections.The apparent dichotomy between the ease of inducing defecation in a queen honey bee (L'Arrivée, 1965; Czekonska K. and Chuda-Mickiewicz B., 2007) and the difficulties involved in induction in a worker bee serve as an example of the complexities of caste differentiation.

As previously mentioned, the case for finding a reliable way to induce defecation in worker bees stems from the fact that it could allow for a more detailed and thorough analysis of the tracing and evolution of a pathogen (as long as it can be detected in the gut and rectum of the

bee) in one bee, without the need to kill individuals at various stages. With recent advances in technology and microbiological tools, the need to trace diseases in faeces could become redundant as it might be possible to extract micro litres of haemolymph or even faeces using microscopic syringes with no damage to the bees.

*5.3 - **Impact of artificial lighting on honey bee activity in an experimental setting***

The results do at first glance support the hypothesis that lights used in an experimental setting to observe bee behaviour have an impact on the locomotor activity of the bees. The direction and duration of the effect of the lights still need to be looked at more closely, as more than one pattern seems to emerge in terms of the changes in activity levels of the bees. This impact of the light can be observed when using a high intensity light but disappears when using a lower intensity (7 $\mu m/m^2.s^{-1}$). This validates the method used by Meshi and Bloch (2007), who used a light intensity of 1.55 $\mu m/m^2.s^{-1}$ to run their experiments. Furthermore, in this case caution should be taken in interpreting the results as heat produced by the intense white light on the Plexiglas of the observation hive could have had an impact on the behaviour of the bees and their activity levels. This is because the light was directly facing the observation hive rather than being diffuse like in Meshi and Bloch (2007).

This experiment also highlighted some modifications that should be made to optimise successful tracking of all the tagged bees. For example, the bees walking on the Plexiglas cover, with their ventral side facing the camera could not be tracked as their tag was not visible. This could be resolved by either modifying the observation hive, and the space between the frame and the cover, or by marking the underside of the abdomen of the bees, most likely with paint. This also seems to be in accordance with a previous study by Meshi and Bloch (2007) who found that, in their version, tracking efficiency was low when only the thorax of the bees was painted.

CONCLUSION AND FURTHER RESEARCH

The complexities of honey bee physiology and behaviour are highlighted in this research in many ways. Disease transmission has already been the subject of many studies, but in this case there is scope for improving the experimental design in order to statistically and significantly prove or refute transmission through trophallaxis. *Nosema ceranae* infection was straight forward, transmission using cages though proved more ambiguous. In a further experiment, a number of bees could be infected, tagged and segregated in one half of a frame

with a sugar solution and another number of non-infected bees. Another set of *Nosema*-free bees could be placed in the other half of the frame with no sugar solution but contact with the fed bees through a mesh. This way, we could use our knowledge in video tracking and recording to analyse if the tagged bees are offering food to the other bees, and then after a certain time if *Nosema ceranae* transmission has occurred. This experiment could answer the question as to why in our experiment, bees in contact with infected bees seemed to die and also whether *Nosema ceranae* could be transmitted through trophallaxis

There is potential for a method to induce defecation in worker honey bees, therefore bypassing the need to kill individuals for various analyses. A project could be set up to determine what affects defecation: bee age, bee size, time since last feed, centrifugation speed, centrifugation time. While tagging bees, we noticed that bees that had been refrigerated and handled as they were slowly regaining movement often defecated. This success rate could potentially be included in a straight forward experiment.

In terms of the effect of light on honey bee activity, we have succeeded in shedding some light on the impact that high intensity light might have on the activity of an experimental group of bees. Further research should concentrate on types of light such as red lights, often assumed to be invisible to bees. A similar experimental set up could be used for this purpose.
In relation to software development, there is the aim to use the recorded videos to design a tracking software, able to analyse movements but also to determine which bee was in contact with which, for how long, and how many times. Once the recording is completed and the data extracted from the software, the bees could be prepared and DNA extracted from them to test for genetic relatedness and whether this has an effect on the creation of networks of transmission.

Ultimately, while the scope of this research was limited to the development of tools, the envisioned goal remains to be able to introduce social networks and interactions in honey bees into a model, fit for predicting and analysing epidemiological data and disease transmission and spread in human populations.

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