Investigating the effects of the neonicotinoid pesticide clothianidin on bumblebee foraging using an automated monitoring system

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Abstract

The aim of this project was to design a monitoring system for tracking foraging bouts of individual bumblebees and use this system to test how different sub-lethal doses of the neonicotinoid pesticide clothianidin affected foraging behaviour on individual bumblebees. The system consisted of a nest box connected to a dedicated camera box operating as an entrance/exit. Each worker bumblebee was equipped with a 2mm x 2mm data matrix on its back, enabling me to identify every individual. A low-cost web camera was installed in the entrance/exit box and connected to a standard desktop computer. A motion detection software controlled the recording system, taking a series of pictures each time it detected motion, i.e. when a bumblebee left or entered the hive. I used a tailored software (bTools) to scan each picture for bCodes. The software returns a text string containing the ID of the bumblebee(s) found in the picture and the exact time the picture was taken. By using these timestamps, I was able to generate data on activity patterns, i.e. number and lengths of foraging bouts, on an individual level.

In total 36 bumblebee (*Bombus terrestris*) colonies were exposed to field-realistic concentrations of clothianidin though nectar, at 2 different concentrations (6.8 µg/L and 13 µg/L) and a negative control (0 µg/L) for 9 days. After the exposure period, 30 individuals from each colony were marked with bCodes and the colony released for observations for 7 days. At the end of the release period, all colonies were dissected to assess the colony condition.

The exposure to the clothianidin did not affect the colony growth rate or the average number of foraging bouts. However, clothianidin affected the foraging behaviour in an interaction with temperature. Foraging bout length of bumblebees exposed to the high concentration of clothianidin (13 µg/L) significantly increased with temperature, while those exposed to low dose (6.8 µg/L) and the control were not affected by temperature. I conclude that the effect of clothianidin exposure on bumblebee foraging behaviour is temperature sensitive and that local climatic conditions and future climate change scenarios should be considered in risk assessments of clothianidin and other insecticides.

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1 Introduction

1.1 Modern agriculture and use of pesticides

The increasing demand for plant-based food products has forever changed agriculture. Modernization and the establishment of large-scale cultivations require a transition from extensive to intensive cultivation of crops, leading to increasingly more sophisticated techniques and machinery – mechanization, GMO, regular watering and use of artificial fertilizers and pesticides on a massive scale. Different methods are used for pest control in agriculture, including both preventative (quarantine and varied cultivation practices) and direct control (mechanical, biological and chemicals) measures. The most used pest control method is chemical, due to the ease of application, relatively low cost, compared to other methods, and high effectiveness. Various poisonous substances used are collectively named pesticides. These substances include herbicides, fungicides, insecticides, acaricides, nematodes, molluscicides, rodenticides, growth regulators, repellents and biocides. All pesticides contain an active substance, which determines its activity, i.e. targeted organism group. While providing benefits in the form of increased yield, reduced workload and other resources needed, the usage of pesticides can have huge environmental disadvantages. The impact the pesticides have on the environment is not limited to the area of application as they can easily spread to the environment through air, soils and runoff water. Only about 25% of the total amount of the pesticide used settles on the sprayed crops, while the rest evaporates, is blown away by wind or is absorbed into soil (Owens and Feldman 2004 and references therein) . Pimentel (1995) estimated that only ~ 0.1% of pesticides are absorbed by the target organism. Due to the need for long term crop protections, most pesticides are persistent in the environment, though their half-lives vary with their structure, complexity and environmental conditions (Briggs et al. 1990).

Even though pesticides are designed with a specific target organism, they can cause sublethal or lethal effects in non-target organism groups. Insecticides used in agriculture against parasites and herbivores can negatively affect beneficial insects, including bees and other

pollinators (Kiljanek et al. 2016; Basu and Chakrabarti 2015; Kumar et al. 2018). The number of insects in the world is dropping at an alarming rate; Sánchez-Bayo and Wyckhuys (2019) suggest that as much as 40% of all insect species are threatened with extinction and may disappear in the next few decades. A study done by Nieto et al. (2014) showed that 46% of European bumblebee species are in recession. The insect population decline is well documented, with habitat loss and fragmentation due agricultural intensification as the main driver, climate change and pesticides as additional causes (Potts et al. 2010; Vanbergen and Initiative 2013; Potts et al. 2016; Sánchez-Bayo and Wyckhuys 2019). A decrease in insect pollinator populations can have severe consequences for plant communities as 87.5% of all angiosperms depend on pollination services delivered by pollinating insects (Ollerton et al. 2011) and for the agricultural production as pollinators increase the yields from 35% of plants used for human consumption (Klein et al. 2007).

Constantly growing demand for plant-based products requires intensification of agriculture and usage of plant protection products (PPPs). Although modern PPPs are much safer than older compounds (Toxicology Education Foundation 2018), no product has been yet produced to be completely harmless to non-target organisms. Therefore, it is crucial to understand how pesticide usage affects the ecosystem and its components. Expanding this knowledge will help to maintain productive agriculture and conserve the ecosystem.

1.2 Neonicotinoids

Neonicotinoids is the collective name for a group of synthetic chemicals used as insecticides. The development of this group of insecticides began in 1980s by the Shell company and continued in the 1990s by Bayer (Kollmeyer et al. 1999). Among the neonicotinoids, the most commonly used compounds include imidacloprid, thiamethoxam and clothianidin (Claydon 2017). Neonicotinoids are related to nicotine, which was used as an insecticide from 17th century (Tomizawa and Casida 2005). Neonicotinoids are classified based on the active substance: N-nitroguanidines, N-cyanoamidines and nitromethylenes (Tomizawa and Casida 2005; Elbert et al. 2008). The differences in the chemical structure between these groups neonicotinoids affect their susceptibility to degradation, application methods and toxicity (Jeschke et al. 2013). These differences are also reflected in the level of toxicity to non-target

organisms like bees. N-nitroguanidines group which includes imidacloprid, thiamethoxam and clothianidin are highly toxic to bees, while neonicotinoids in N-cyanoamidines group (thiacloprid and acetamiprid) have lesser toxic effects (Manjon et al. 2018).

Neonicotinoids interact with nicotine-stimulated receptors, located in the central nervous system of insects, functioning as an antagonist of acetylcholine (Brown et al. 2006). Acetylcholine is a neurotransmitter – a substance that enables the transfer of nerve signals. Neonicotinoids bind to the acetylcholine receptors (AChR) and in larger doses inhibit them. The acetylcholine specific enzyme (AChE) is not able to break down neonicotinoids. As a result, they accumulate in the synaptic cleft and cause continuous generation of nerve impulses in the postsynaptic neuron, resulting in convulsions, paralysis and ultimately the death of the insect (Nakagawa 2001; Brown et al. 2006; Simon-Delso et al. 2015).

The advantage of neonicotinoids is lower efficiency of binding to receptors in vertebrates compared to invertebrates. Neonicotinoids are less acutely toxic to mammals and other vertebrates, especially those living in freshwater ecosystems and coastal areas exposed to pesticides through rainwater and/or agricultural runoff. Neonicotinoids are highly persistent. Calculating the exact half-life and persistence of neonicotinoids is difficult due to their sensitivity to environmental factors. For example, depending on the soil and humidity in the soil, the half-life of clothianidin varies from 148 to 1155 days (Bonmatin et al. 2015a; EPA 2003a.), imidacloprid from 40 days to 997 days (Bonmatin et al. 2015a; Gervais et al. 2010) and for thiamethoxam between 47-301 days (Bonmatin et al. 2015a; Gupta et al. 2008). The other very important advantage of neonicotinoids is the versatility in application methods (Ensley 2018). The most common application method is as seed dressings and integration with soil as granules (Jeschke et al. 2011). Other methods include foliar spraying, mixing with irrigation water, drenching of flower bulbs and roots or injections into tree trunks (Simon-Delso et al. 2015).

Despite multiple benefits, neonicotinoids have been a source of controversy for some time due to suspected negative effects on non-target organisms, especially bees, and other pollinators. Neonicotinoids are systematic insecticides, which means that once taken up by a plant, it will be distributed to all plant tissues, including pollen and nectar (Krupke et al. 2012; Stoner and Eitzer 2012), which makes it relatively easy for a non-target organisms to come in

contact with the insecticide. One of the main differences to older pesticides is the water solubility of the neonicotinoids (Wood and Goulson 2017). This means that the knowledge of the properties of old pesticides like persistence, adsorption, volatility, and degradation in the environment of fat-soluble insecticides cannot be directly extrapolated to the neonicotinoids.

The neonicotinoid used in this study was clothianidin. Clothianidin belongs to the nitroguanidine subgroup of neonicotinoids. The IUPAC chemical name is (E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine. The molecular formula is $C_6H_8ClN_5O_2S$. Clothianidin is an odour- and colourless powder and is classified as Toxicity Category III (Slightly toxic and Slightly irritating) and “not likely” a human carcinogen (EPA 2003). Short term oral exposure in mammals might cause moderately toxic effects, while long term exposure may lead to reproductive and developmental effects (EPA 2003).

1.3 Effects of neonicotinoids on Bees

Most studies done so far have investigated the effects of imidacloprid, which is the most widely used neonicotinoid, on honey bees, in particular effects on foraging behaviour. Although honeybees and bumblebees belong to the same family (Apidae) they differ in many aspects like physiology, foraging behaviours, colony size and life cycle (Michener 1990). Many studies have suggested, bumblebees (and solitary bees) might be more susceptible to neonicotinoid exposure than honeybees (Osterman et al. 2019; Rundlöf et al. 2015; Wintermantel et al. 2018). It has therefore become evident that studies of negative effects of neonicotinoids on honeybees cannot directly be extrapolated to wild bees, including bumblebees.

Non-target organisms can come in contact with insecticides though multiple pathways. Pollen and/or nectar contaminated with neonicotinoids can be collected by foragers and brought back to the colony (Goulson 2013). This means that bees can be exposed to neonicotinoids not only as adults but also in the larval stage (as larvae feed on contaminated pollen). Mitchel et al. (2017) found traces of neonicotinoids in 75% of honey samples from around the world. The concentration of neonicotinoids exceeded 0.10 ng/g in 48% of the honey samples. This

concentration is considered safe for human consumption per EU and US regulations but would most likely have negative effects on bees as it corresponds to the lowest concertation at which harmful effects have been observed (Mitchell et al. 2017).

Learning and memory in bees is connected to the mushroom bodies in their brain (Jones et al. 2013). Exposure to sub-lethal doses of neonicotinoids leads to impairment of the function of synapses in these regions of the brain, thus leading to reduced visual and olfactory learning and memory (Decourtye et al. 2004; Han et al. 2010; Williamson and Wright 2013). Neonicotinoids also interfere with the basic motor skills of bees - walking, flying or grooming, which can impair the normal functioning of these insects (Williamson et al 2014). Uncoordinated movements, hyperactivity and convulsions have been observed in exposed bees (Blacquière et al. 2012). Long-term exposure to neonicotinoids interferes with food consumption and foraging (Decourtye et al. 2004) and has a negative effect on homing (Fischer et al. 2014; Yang et al. 2008). A decrease in foraging effectiveness can lead to food shortage and have a detrimental effect on colony survival and reproduction.

Bumblebee colonies exposed to sub-lethal doses of neonicotinoids experience lower queen production and survival rate (Spivak 2017; Baron et al. 2017). As bumblebee hives have a yearly cycle, this can lead to reduced number and health of colonies the following year (Baron et al. 2017). Several studies have shown that neonicotinoids negatively affect the larval development of bees (Abbott et al. 2008; Decourtye et al. 2005; Gregorc and Ellis 2011; Tasei et al. 2001; Tasei et al. 2000). Decourtye and Devillers (2010) showed that larvae fed with food contaminated with imidacloprid experienced prolonged larval period and that a decrease in reproduction can have much more acute effects on a bee colony than the loss of older foragers.

Neonicotinoids also negatively affect the bee's immune system making the insects more susceptible to parasites and pathogens (Brandt et al. 2016). Contradictory, Pettis et al. (2013) showed that exposure to neonicotinoids can decrease the possibility of *Nosema ceranae* infection. However, Alaux et al. (2010) suggest that there is a synergistic interaction between *Nosema* infection and exposure to imidacloprid, resulting in higher susceptibility to pathogens.

Based on numerous studies showing a plethora of negative effect of neonicotinoids on a variety of bees the European Union decided to prohibit usage of the three most common neonicotinoids in 2013. Numerous studies have since confirmed the key role of these insecticides in pollinator declines. A new rapport from European Food Safety Authority (EFSA 2019) concludes that neonicotinoids are indeed harmful to bees. However, these pesticides are still widely used in other parts of the world. Expanding the current knowledge about the effects these pesticides have on pollinating insects as well as developing new methods for documenting such effects is therefore crucial for both bee conservation and global food production.

1.4 Aims of the study

The aim of this study has been to assess how exposure to sub-lethal concentrations of clothianidin affects bumblebee foraging and colony development. To achieve this, I developed an automated hive monitoring system to assess individual foraging bout lengths. In addition, I assessed within-colony mortality rates by counting dead workers and larvae within the colonies.

The objectives and hypotheses were as follows:

Objective 1 – Develop a monitoring system that record bumblebee foraging bouts in a natural setting.

Objective 2 – Assess the condition of *B. terrestris* colonies after exposure to clothianidin at increasing doses.

H1: There is a significant reduction in colony growth rate with increasing clothianidin exposure.

Objective 3 – Quantify behavioural effects of field-realistic doses of the insecticide clothianidin on bumblebee (*Bombus terrestris*) individuals.

H2: The length of foraging bouts increases when exposed to increasing doses of clothianidin

H3: There is an interaction between abiotic factors (weather conditions) and the level of exposure to clothianidin.

2 Materials and Methods

2.1 Study species – Bumblebees

Bumblebees are a part of Apidae family, which belongs to order Hymenoptera. Other members of Apidae- family include honeybees (tribe Apini), stingless bees (tribe Meliponini), carpenter bees (tribe Xylocopini), orchid bees (tribe Euglossini), cuckoo bees (subfamily Nomadinae), and several other less known groups. There are over 300 bumblebee species around the world. They can be found in a variety of climates, from north of the arctic circle (e.g. *B. polaris* and *B. alpinus)* (Heinrich 2004) to warm and humid areas like Indonesia and South America (e.g. *B. transversalis)* (Dornhaus and Cameron 2003).

***Bombus terrestris* – Buff-tailed bumblebee**

The species used in this study was *Bombus terrestris*, in english called Buff-tailed bumblebee or Large earth bumblebee. *B. terrestris* are medium-sized bumblebees. The queen can reach 20-23 mm length, males range from 14-16 mm and workers vary between 11-17 mm (Staverløkk et al. 2012). The body of *B. terrestris* is covered in black hair with 2 yellow/orange bands – the first band at the front of the torso, and the second one on the second segment of the abdomen (Figure 1). The last 2 segments of the abdomen have white hair. *B.terrestris* prefers open and agricultural environments, where it builds nests underground, most often in abandoned rodent burrows (Staverløkk et al. 2012). The nest has a random structure - the wax cells are chaotically arranged. The queen is fertilized in autumn and the queen overwinters below ground. In the following spring, the queen locates a suitable nest site and establishes a new colony. Number of individuals in a single colony can vary from several dozen to couple of hundreds (Duchateau and Velthuis 1988; Goulson et al. 2002).

B. terrestris is a generalist and visits flowers of a wide spectrum of plant species. On plants with deep flower corona (e.g. red clover and vetches), *B.terrestris* can often be observed as a pollen and nectar robber because of its relatively short tongue length (Koeman-Kwak 1973). In Southern Norway, *B. terrestris* queens can be observed as early as March, as one of the first

emerging bumble bee species emerging in spring, and as late as October (Staverløkk et al. 2012).

Figure 1 Bombus terrestris queen foraging on a Tropaeolum minus *flower in autumn before overwintering. Photo: Pawel Jan Kolano. Date: 20 September 2019*

B. terrestris is one of the more common bumblebee species in Europe. It has naturally spread across Europe and eastwards to Kazakhstan and Turkmenistan. It has been introduced to i.e. Japan, New Zealand, Chile and Tasmania where it has established sustainable population (Torretta et al. 2006; Schmid-Hempel et al. 2014; Semmens et al. 1993). In many places, *B. terrestris* is considered an invasive species (Schmid-Hempel et al. 2014; Naeem et al. 2018) and its introduction has been suggested to be the main reason for declines in native bumblebees in several areas (Naeem et al. 2018; Matsumura et al. 2003; Geslin and Morales 2015). The main reason for the introduction of *B. terrestris* is that it can easily be bred commercially and used as a pollinator for greenhouse crops, mainly tomatoes and strawberries. Thanks to the use of bumblebees in agriculture, farmers are able to avoid hand-pollination of e.g. tomato flowers, which is very time consuming, or mechanical pollination which is expensive (Hayo et al. 2006).

B. terrestris was chosen because of their robustness and ease of handling as well as the availability of commercially bred bumblebees in Norway. The colonies were purchased from the company Bombus natur AS, located near Bryne, South-Western Norway.

2.2 Experimental setup and design

I conducted the experiment in spring 2019, from May 8th- July 15th at Kristine Bonnevies Hus, the University of Oslo. The handling of bumblebees took place in a climate room in the basement of the Phytotron, while the behaviour experiment was done outside of the Animal Facility (IBV-ANIMAL). The preparations of nectar-clothianidin solutions I did in the toxicology lab.

The experiment involved 36 colonies divided into 6 replicates. Each replicate contained 6 hives of which 2 received control dose (not exposed), 2 received low dose (6.8 µg/L) and 2 received the high dose (13 µg/L) of clothianidin. The concentrations used were within field-realistic concentrations, based on studies measuring residuals of clothianidin in leaves, nectar and pollen (Table 1)

Table 1 Overview of field-realistic clothianidin concentrations detected in plants, pollen loads, and nectar found in returning foragers.

Concetration in ng/g	Plant/Origin	Tissue	Reference
1.8±1.7	Maize	Pollen	Xu et al. 2015
0.58 ± 0.64	Oilseed Rape	Nectar	Xu et al. 2015
13.5 mean ± SE)	Sunflower	Leaf	Mogren et al. 2016
4 (mean ± SE)	Buckwheat	Leaf	Mogren et al. 2016
4 (mean ± SE)	Phacelia	Leaf	Mogren et al. 2016
3.5 (mean ± SE)	Garden tickseed	Leaf	Mogren et al. 2016
1 (mean ± SE)	Mustard	Leaf	Mogren et al. 2016
0.4 (mean ± SE)	Partridge pea	Leaf	Mogren et al. 2016
0.4 (mean ± SE)	Safflower	Leaf	Mogren et al. 2016

Table 1 Continued

1.6 (mean ± SE)	Mustard	Pollen	Mogren et al. 2016
1.2 (mean ± SE)	Buckwheat	Pollen	Mogren et al. 2016
0.5 (mean ± SE)	Scorpionweed	Pollen	Mogren et al. 2016
2.27 ± 3.52 (mean ± SD)	Oilseed rape	Pollen	Botías et al. 2016
2.18 ± 3.99 (mean ± SD)	Oilseed rape	Nectar	Botías et al. 2016
5.4	Oilseed rape	Nectar (from bumblebees)	Rundlöf et al. 2015
10.3 ± 1.8	Oilseed rape	Nectar (from honeybees)	Rundlöf et al. 2015

The experiment was blinded from the start of the exposure until the end of the dissection. Concentrations were assigned to the colonies randomly by a third party, not related to the project. The dilution series was designed in such a way that the volume of the solution (15 ml) added to the nectar tank in the last step of the dilution series was the same for all treatment levels. The 6 flasks containing the 3 different doses were delabeled and a number from 1 to 6 was assigned to each flask to enable me to link each colony to a particular treatment level when all data was recorded.

Figure 2 Overview of the experiment timeline, containing 2 of 6 replicates. Starting with the delivery of the bumblebees at UiO, continuing with exposure including the transfer of the colonies to the new colony boxes, release of the colony and finally the dissection. The numbers below the timeline indicate the length of each section. The placement of the 2 timelines shows the overlap between the replicates.

Each replicate followed the same procedure from the start to the end of the experiment. Figure 2 shows the timeline of two replicates. The day after receiving the colonies from the producer, I exposed the colonies to the clothianidin (or control) for 9 days through artificial

nectar. I used the nectar supplied in the colony boxes from Bombus Natur. However, this nectar had a concentration of 50% sugar, while bumblebees prefer sugar concentration of approximately 30% (Willmer 2011). I, therefore, lowered the sugar content in the nectar solution by adding 585 mL distilled water to 900 ml nectar. The 30% nectar was then transferred back to a clean nectar tank, together with 15 ml of the clothianidin (or control) solution from the assigned flask. A detailed laboratory protocol is included in Appendix 1.

The nectar supplied by the producer resided in a plastic nectar tank, placed below the colony box. The nectar was delivered into the colony box by a plastic tube and a sponge placed inside of the colony box. The colonies had unrestricted access to the nectar during the exposure period. During the exposure, the colonies were kept indoors at temperatures ranging from 17.5 to 18.5 °C. On the fifth day of the exposure, I transferred the whole colony (all individuals and the nest structure) from the original plastic container to a custom-made colony box made of wood. The new colony boxes used the same nectar tanks and supplied the nectar to the colony in the same way as the original boxes (Figure 3). I had 2 sets of colony boxes (12 boxes in total), so that replicates 1,3 and 5 used one box set and replicates 2,4 and 6 used another. For the rest of the exposure period, the colonies were left undisturbed to acclimatize to the new colony boxes (Figure 3). I weighed each nectar tank at the start and at the end of the exposure period to assess the volume of consumed nectar during the exposure.

During the whole exposure period, the colonies were fed pollen substitute in form of a patty - KVIKKPOLL. Pollen is the main source for proteins and lipids (Vaudo et al. 2016) and is necessary for the development of the hive. Bombus Natur provided pollen for the duration of the transit. New pollen was provided on the transfer day (15 grams) and at the marking day (6 grams).

Figure 3 Six bumblebee colonies during the exposure period after transfer to the custom-made colony boxes.

I conducted the behaviour experiments on queenright colonies only, i.e. colonies with a single queen. Bumblebees are social insects with a social order based on dominance. Having multiple queens or no queen might promote aggressiveness between workers and unwanted behaviour changes which could influence the results of this study (Sibbald and Plowright 2013; Free 1955). To reduce the variation in colony condition, all colonies without (5) or with

multiple queens (2), colonies with fewer than 30 workers (2) and colonies with more than 200 workers (2) the end of the exposure period were excluded from the experiment.

2.3 Marking

To be able to identify individual bumblebees I used a set of software tools called bTools designed by Tim Gernat (Gernat et al. 2018). By use of this software, I generated a set of 2048 unique bCodes. A bCode is a two-dimensional data-matrix and works by the same principle as a QR code and a barcode (Gernat et al. 2018). Each bCode contains a different value which, when scanned by software provided with bTools, distinguish the codes from one another. See Appendix 2 for the settings and measurements of the bCodes. The bCodes were printed on weatherproof paper (Rite in the rain All-weather Printing Paper) using a standard laser printer and then cut out using a scalpel. To optimize readability and the process of applying the codes to the bumblebee backs, I tested different sizes for the bCodes. Codes of 2.2 mm x 2.2 mm (length x width) were ultimately chosen as they provided the largest bCodes without disturbing the bumblebees. I attached the bCodes to the bumblebees back, between the wings, using a small drop of Loctite Super Glue. See Appendix 3 for step by step marking procedure. I conducted the marking procedure under a red lamp (wave length of approx. 650 nm) as bumblebees cannot see the light above 580 nm (Riddle 2016) and therefore will not fly under these light conditions.

2.4 The monitoring system

To be able to monitor individual bumblebees leaving and entering the colony boxes I constructed an automated detection system using web-cameras to record the bCodes tagged to each individual bumblebee, as they left and entered the colony. I designed colony- and monitoring boxes as follows:

2.4.1 The colony box

I used a ready-made storage container as the base for the colony box (Figure 4). The container measured 30 cm x 20 cm x 15 cm (length x width x height) and was made of untreated

pinewood, as wood preservatives might repel bumblebees and/or be toxic (Kalnins and Detroy 1984). I divided the container into an outer and an inner chamber by a fibreboard with a connecting hole in the middle. The outer-chamber provided more room for the bumblebees, a place for applying feeding stations with pollen and nectar, and functions as an entrance to the colony. I lined the floor in both chambers with plastic foil and corrugated cardboard to prevent any moisture, waste and rot from contaminating the floor of the chamber. In addition, I lined the inner chamber with water-repellent cotton to provide building materials and isolation for the colony. Ventilation holes were drilled in the sidewalls of the outer chamber and were secured with plastic insect netting to prevent any escape attempts. The lid for the colony box was made from 2 separate layers – 1 sheet of transparent Plexiglas and 1 sheet of fibreboard. The transparent lid enabled inspection of the hive without opening it, while the fibreboard was used as cover to block sunlight (as the Buff-tailed bumblebees normally nest underground). I attached the Plexiglas lid to the colony box with glue pads (UHU patafix) and secured it with Gaffer tape.

Figure 4 Custom made colony box. The box was made of pinewood, measured 30 cm x 20 cm x 15 cm. The box was divided into two chambers. The chamber on the left side was used for the nest, while the right chamber was used as an entrance, a place for feeding station. The lid for the colony box was made of Plexiglass.

2.4.2 The monitoring box

The monitoring box housed the camera and the entrance/exit tunnels. The box was custom made to specific dimensions - 16 cm x 10 cm x 16 cm (length x width x height) made of untreated pinewood, similar to the colony boxes. At the bottom of the box, I applied another Plexiglass lid under which I mounted two tunnels, to separate the bumblebees leaving the hive from the ones entering the hive. To dictate the movement direction, I installed a pair of one-way doors in both ends of each tunnel. The doors were made of a thin sheet of transparent plastic mounted on a simple hinge in a 45-degree angle. To reduce the speed of the bumblebees as they left or entered the monitoring box, I installed a "chokepoint" in each tunnel. This to enable the cameras to take sharp enough pictures for the bCodes to be recognizable. The chokepoints were made of a small plastic cable duct attached to the Plexiglass lid (Figure 5) and I used fibreboard to fill the empty room below the ducting.

Figure 5 A close up of the camera and the entrance/exit tunnels. The direction in each tunnel is dictated by a set of 1-way doors. The smaller white tunnels, which are placed directly under the camera, are used to reduce the speed of the bumblebees.

Placed directly above the entrance/exit tunnels was a camera (Logitech HD PRO WEBCAM C920) selected because of its mounting system, with build-in height and tilt adjustments and a tripod mounting bracket, performance (i.e. resolution, frame rate and shutter speed) and low price. To provide constant lighting I mounted a strip of white LED lights in the top corner of the box. I used LED lights because they do not produce any heat, they are power efficient, easy to mount and cheap. This placement of the LED strip was carefully chosen to avoid any reflections and possible shadow spots. Each camera had to be set up separately as the boxes differed slightly from each other. The general placement and adjustment of camera position were done using the mounting system, while smaller, more precise adjustments were done in the camera software iSpy 7.2.0.0. For exact settings for each camera, see Appendix 4.

2.4.3 The computer and software

A standard desktop computer (OS: 64-bit Windows 7. Processor: Intel Core i5-2500. Memory: 8GB RAM. Graphics Card: Intel® HD Graphics 2000. Hard Drive: 500 GB) controlled the cameras through the software iSpy 7.2.0.0 I chose this particular software due to it being open-source, easy to use, of its numerous functions and its control over the manual camera settings. The cameras were configured to two-frames motion detection, recording and comparing the current frame with the previous. If the difference between two frames was bigger than a given threshold, the software started taking pictures. The constant lighting inside of the camera box enabled me to set a 98 % sensitivity trigger range i.e. 2% difference between the frames was enough to trigger the camera.

The hardware set up allowed me to control up to 3 cameras at the same time at a resolution of 960 x 720 pixels, 30 frames per second. While more than 3 cameras are possible, it greatly reduces the overall performance of the system and increases the possibility of a system crash (due to excessive number of devices sharing the same Windows-driver). Because of this limitation, 2 separate computers were used (with identical hardware and software specifications) to control the six cameras operating at the same time.

2.5 Release and observations

The colonies were divided randomly into 2 groups (left and right) and connected to one of the two monitoring stations (PC with monitoring boxes connected) accordingly. The randomization disabled me from assigning the boxes in a systematic order with respect to exposure level, so in theory, both colonies exposed to the same concentration could be mounted to the same station. Both monitoring stations were placed under a roof, so the computers and colony boxes were sheltered from the weather (Figure 6). The distance between the stations was 5.5 meters. Due to limited space, colonies were placed on a shelf unit. The colonies were separated vertically from each other by placing each colony on a different shelf (top, middle and bottom) and horizontally by placing them on either right or left side of the shelf (Figure 6). Colony entrances were painted with different colours to reduce drifting between the colonies.

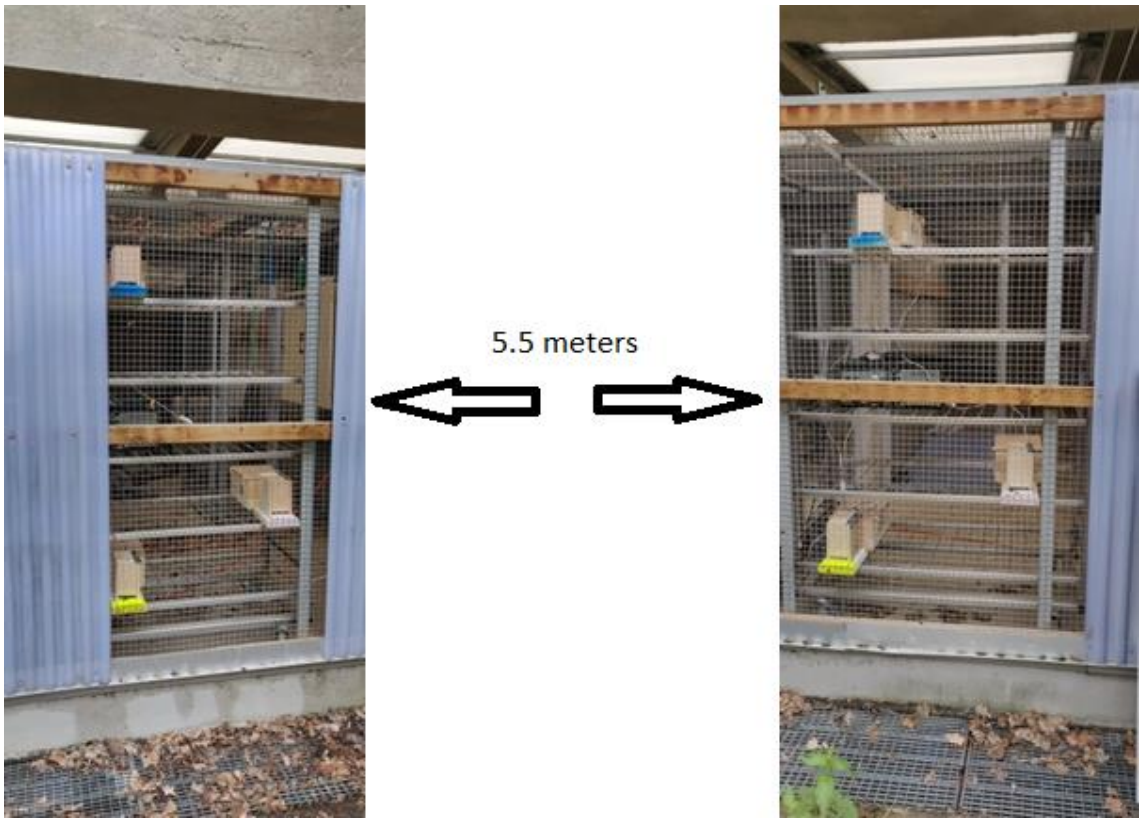

Figure 6 The monitoring stations. The placement of the monitoring boxes was the same in both stations. The distance between the blue - yellow and blue-white hives was 1.3 meters, while the distance between yellow-white hives was 1 meter. The distance between the stations was 5.5 meters.

Figure 7 Bumblebee worker marked with bCode foraging on Anthriscus sylvestris *flowers in Urtehagen at University of Oslo (approx. 50 meters from the monitoring stations and colony boxes). Photo: Malin Røyset Aarønes. Date 29 May 2019*

The release and observation part of the experiment lasted for 7 days for each replicate, with the monitoring stations operating 24 hours/day. The colonies were left undisturbed during this phase. During the release period, the nectar feeders were removed, and no pollen provided, to encourage foraging (Figure 7). On the last day of the release period, I closed the exit on the hive boxes to collect all the bumblebees. To ensure that most of the bumblebees had returned to the hive, I closed the exit between 01:00 and 02:00. The following morning, all colonies were taken inside and dissected. I copied all pictures taken onto a portable hard drive (WD My Passport 1TB) for further processing on a separate computer.

2.6 Dissection of the hives

To evaluate the condition of the colony after the experiment, I assessed the following:

- number of alive individuals, separating marked and non-marked individuals
- number of dead individuals, separating marked and non-marked individuals
- number of larval pods with alive larvae
- main queen dead/alive
- number of new queens

After the evaluation of the colony condition, the bumblebees from the same hive were collected into a glass jar, marked with the date and colony number and then killed by keeping the jars at -21 °C.

2.7 Picture processing

I did all post-experiment picture processing on a separate computer (OS: 64-bit Windows 10. Processor: Intel Core i7-6700k. Memory: 16GB RAM. Graphics Card: Nvidia GTX 980. Hard Drive: M.2 Solid State Drive). While the computers used in monitoring station were capable of picture processing, using a machine with higher processing power drastically reduces the processing time. To process the pictures, I used a selection of software. To sort and gather all the pictures as well as creating data registries for bTools I used simple Windows Command Prompt. Using the bCode detection software included with bTools, I was able to scan each picture for bCodes and extract the bCode data. I used R x64 3.5.2 and FME Workbench 2019.1.3 for sorting through bCode data. I created the final dataset using Microsoft Excel 2016. The detailed step by step description of the picture processing, including workflow and software used, can be found in Appendix 5.

2.8 Statistical analyses

All Individuals that made at least one foraging bout during the release period were included in the analyses. I excluded the lower and upper extremes for the foraging bout length, i.e. foraging bouts shorter than 3 minutes and longer than 180 minutes, (Figure 8). This because it is unlikely that a foraging bout is shorter than 3 minutes and longer than 180 minutes. There is also a possibility of the system not registering a returning bumblebee, resulting in inflated foraging bout length. The decision to limit the foraging bout length was based on studies where the mean foraging bout duration varied from 55 to 65 minutes (Minahan and Brunet 2018; Gill and Raine 2014). However, Gill and Raine (2014) observed an increase in foraging bout length during the experiment. Due to these facts, I chose the upper limit for foraging bout length to 180 minutes. In total, 964 out of the total 6908 foraging bouts were removed.

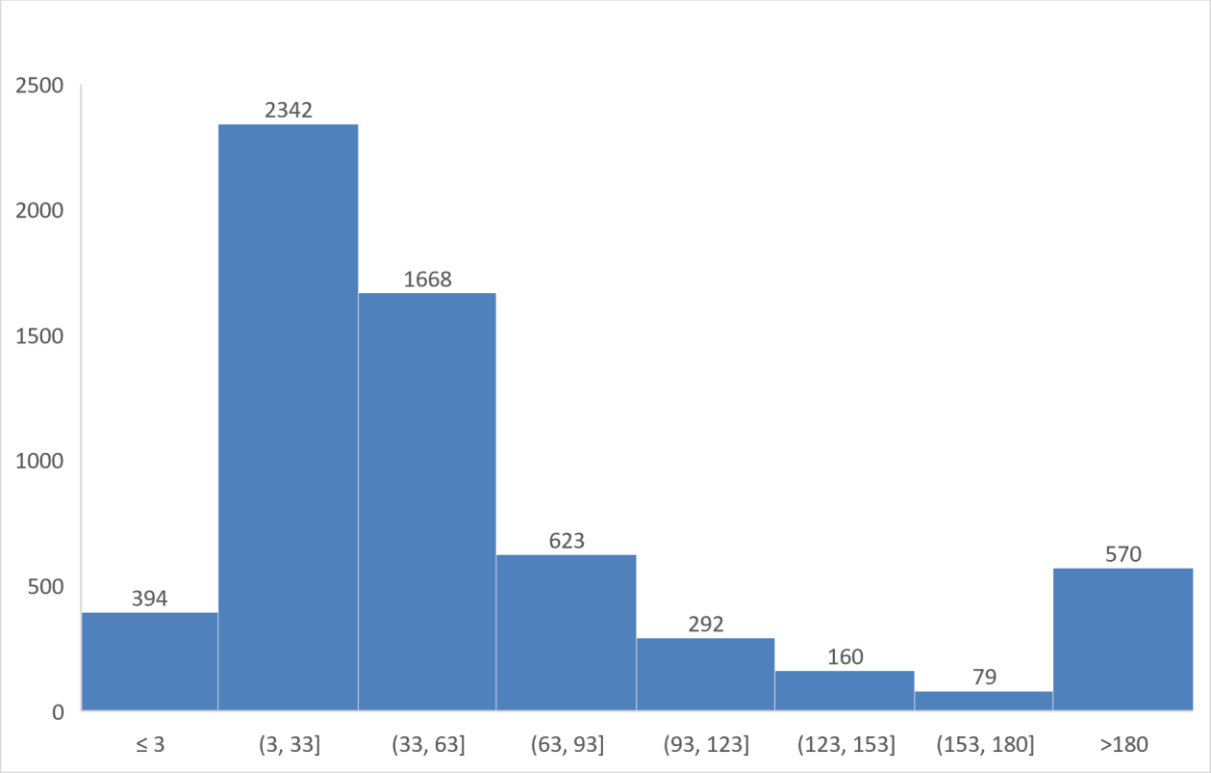

Figure 8 Foraging bout length frequency chart. The y-axis represents the number of foraging bouts, while the x-axis is the foraging bouts grouped by duration into bins which do not overlap. The graph includes all foraging bouts. 9.9 % of all registered foraging bouts were longer than 180 minutes.

Model selection for Foraging bout length

All statistical analysis was done using R 3.5.2. To assess what factors affected foraging bout lengths, I used a generalized linear mixed model (GLMM) approach in the lme4 package (Bates et al. 2015). I made a full GLMM model (i.e. including all potential explanatory variables shown in Table 2) and used two different model selection procedures "model.sel" from MuMIn package (Bartoń 2013) and "drop1" included in the base RStudio package to double-check that the selection procedures revealed the same best model. I selected the best model based on the Akaike information criterion corrected for small sample sizes (AICc) (Brewer et al. 2016).

Colony-level data

I used the Shapiro-Wilk test to assess the distribution of the colony level data (i.e. colony growth and average number of foraging bouts per day) and checked homoschedacity using Levene's test. I used One-way ANOVAs to test for significant difference between the treatment levels.

Table 2 Overview of the explanatory variables used in statistical analyses for the foraging bout length.

Explanatory variable		**Definition**
Treatment		Factor variable with 3 different levels of exposure: Control, Low and High
DailyTemp		Numeric variable with the mean daily temperature at Norwegian Meteorological Institute (MET), 530 meters from the study site.
Time of day	sin1 + cos1	Cyclic time-of-day variable. sin1+cos1 used as time-of-day covariate. sin1 + cos1 + sin2 + cos2 is higher order of the time-of-day variable.
	sin2 + cos2	
DailyPrecipitation		Numeric variable with the mean daily precipitation
DateID		Numeric variable with the number of days into release period ranging from 1 to 7.
MeanWind		Numeric variable with the daily mean wind speed at MET (Norwegian Meteorological Institute), 530 meters from the study site.
(1 | Week/HiveID/BeeID)		Random variable. Used to describe variance between weeks (numeric variable Week), hives (factor variable HiveID) and individual workers within the hives (numeric variable BeeID)

3 Results

Eleven of the 36 colonies involved in the experiment had to be excluded due to issues with their condition. The foraging behaviour observation part of the experiment was conducted on 25 colonies - control group (not exposed - 9 colonies), low concentration group (6.8 µg/L - 9 colonies) and high concentration group (13 µg/L - 7 colonies). During the release and observation period (in total 35 days, 7 days per replicate) the system registered 6908 unique foraging bouts – control group (2519), low concentration group (2425) and high concentration group (1965).

3.1 Colony growth rate

There was no significant difference in the population growth rate between the control, low and high concentration groups ($F_{2,22}$=0.391, p = 0.681). The growth rate in the control group varied between -0.68 and 1.17 (mean = 0.05, SD = 0.58). The growth in the low concentration group varied between -0.66 and 1.18 (mean = -0.07, SD = 0.66) whilst the growth rates in the high concentration group varied between -0.47 and 1.89 (mean = 0.25, SD = 0.97) (Figure 9).

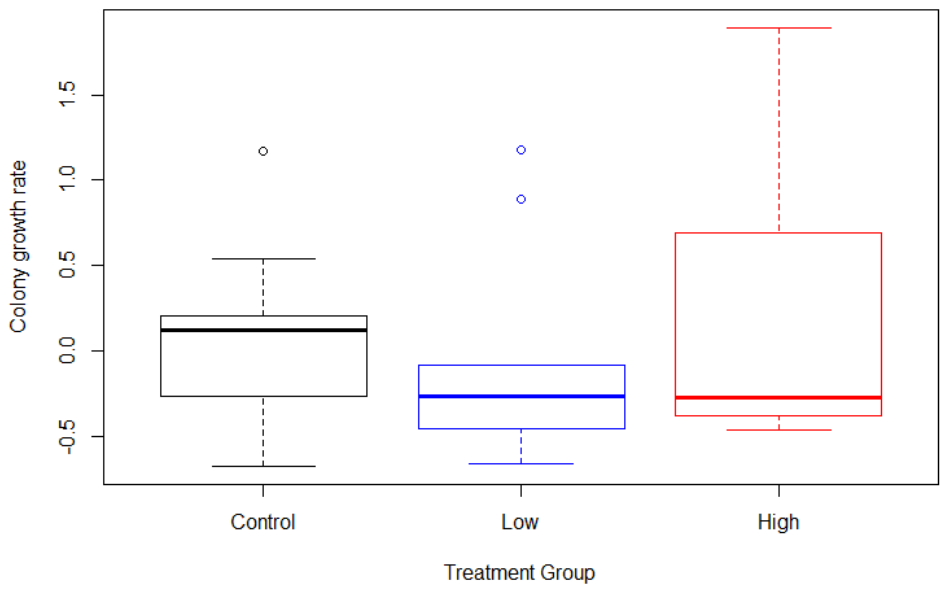

Figure 9 Colony growth rate in the colonies based on the treatment group. Growth rate above 0 indicates positive growth, whilst the growth rate below 0 represents decline in the colony. The solid line represents the median, the outline the 25th and 75 percentiles and the whiskers 1.5 times the interquartile range. Any data points outside that range are indicated by dots

3.2 Average number of foraging bouts

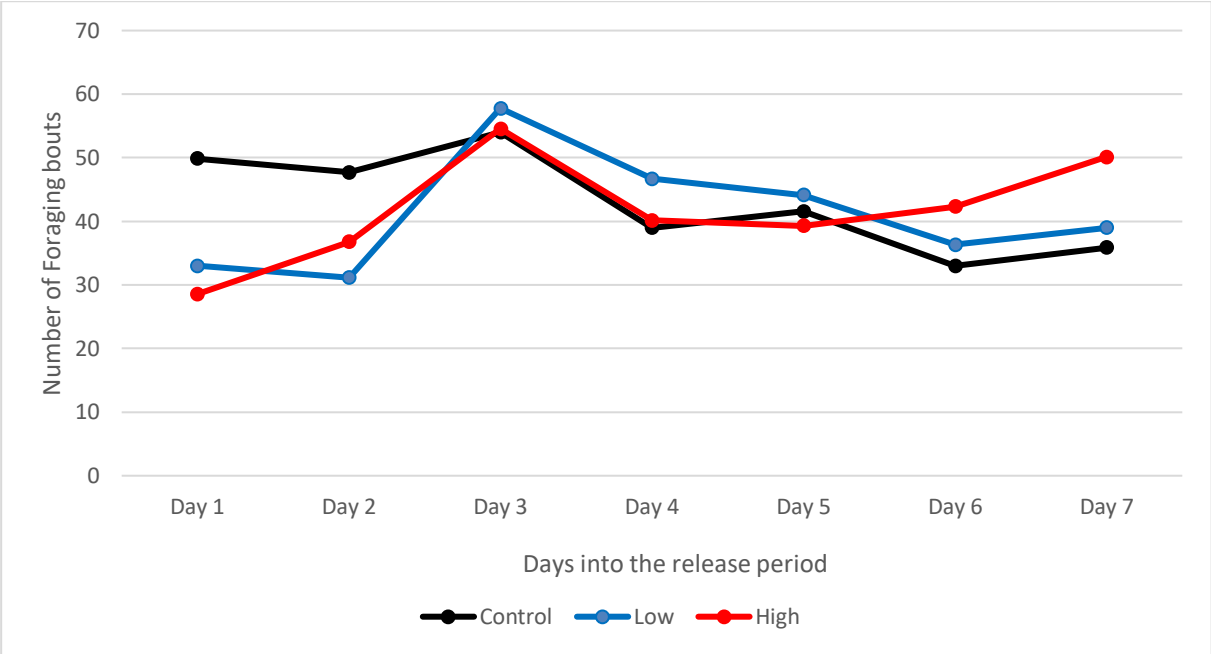

Figure 10 The average number of foraging bouts per day per treatment level. There was no significant difference between any of the treatments.

The One-way ANOVA comparing the average number of foraging bouts per day (Figure 10) taken by marked individuals (30 individuals in each hive) exposed to the control group (mean = 43.4, SD = 8.49) and the two different concentrations of clothianidin – low (mean = 41.2, SD = 9.22) and high (mean= 43.1, SD = 6.65) were not significantly different ($F_{2,18}$= 0.159, p = 0.854).

3.3 Foraging bout length

The GLMM model best describing the variation in foraging bout length included an interaction between treatment and average daily temperature, average daily precipitation, time of day and the number of days into the release period.

Table 3 Linear regression fit for the best fitting GLMM.

Fixed effects:		Estimate	SE	df	t value	P
(Intercept)		31.29092	6.78883	81	4.609	< 0.001
High Concentration		-18.20288	8.64096	91	-2.107	0.04
Low Concentration		0.18785	8.82355	126	0.021	0.98
Average daily temperature		0.38877	0.33324	5369	1.167	0.24
Time of day	Sin1	-12.99691	1.5158	5500	-8.574	< 0.001
	Cos1	-8.68262	1.01022	5496	-8.595	< 0.001
	Sin2	-5.13552	0.90211	5473	-5.693	< 0.001
	Cos2	3.21853	0.80652	5463	3.991	< 0.001
Average daily precipitation		-0.1713	0.06633	5608	-2.582	< 0.01
Days into the release period		1.97578	0.25175	5881	7.848	< 0.001
Interaction High Concentration:Temperature		1.50514	0.45134	5283	3.335	< 0.001
Interaction Low Concentration:Temperature		0.31142	0.4827	4759	0.645	0.52

Overall, the foraging bout length was shorter in bumblebees exposed to high concentrations of clothianidin (p=0.04) (Table 3). The exposure to lower concentration of clothianidin did not have a significant effect on foraging bout length, as compared to the control (p=0.98) (Table 3). The mean daily temperature alone did not have a significant effect on foraging bout length (p = 0.24) (Table 3) but was included in the best model in interaction with treatment. For all treatment levels the foraging bout length increased with temperature, although the increase was significantly stronger under high exposure (p = 0.001) (Table 3). The low concentration group had consequently longer foraging bouts compared to the control, while the high concentration group had the shortest foraging bout lengths under low temperature and the longest foraging bout length under high temperature. Figure 10 shows the daily mean temperature dependency for the different treatment levels.

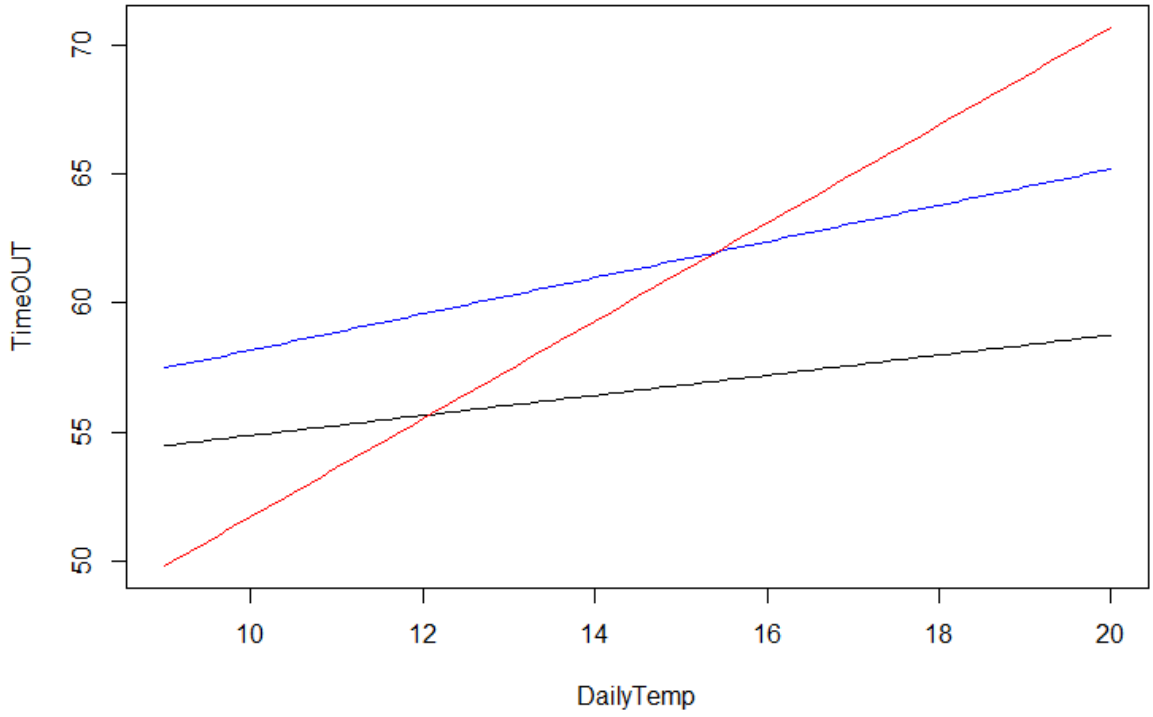

Figure 11 Mean daily temperature dependency for the different treatment levels given that all other fixed effects are kept on their average values. Y-axis represents the foraging bout length in minutes, and x-axis the mean daily temperature in C. Black line shows the control, blue line the low dose (6.8 ug/L) and the red line represents the high dose (13 ug/L)

Mean daily precipitation had a significant negative effect on the foraging bout length; increase in daily precipitation decreased foraging bout length for all treatment groups (p= 0.01) and the length of the foraging bouts significantly increased during the release period (p = < 0.001) (Table 3).

The length of the foraging bouts varied significantly through the day following a complex harmonic function (Table 3). The foraging bouts taken during the night were significantly shorter than foraging bouts taken during the day (Figure 12). The shortest foraging bouts were taken between 03:00 and 04:00, whilst the longest foraging bouts between 10:00 and 20:00 (Figure 12). Bumblebees in all treatment groups were most active between 08:30 and 23:00 (Figure 13). There was no difference between the treatment groups.

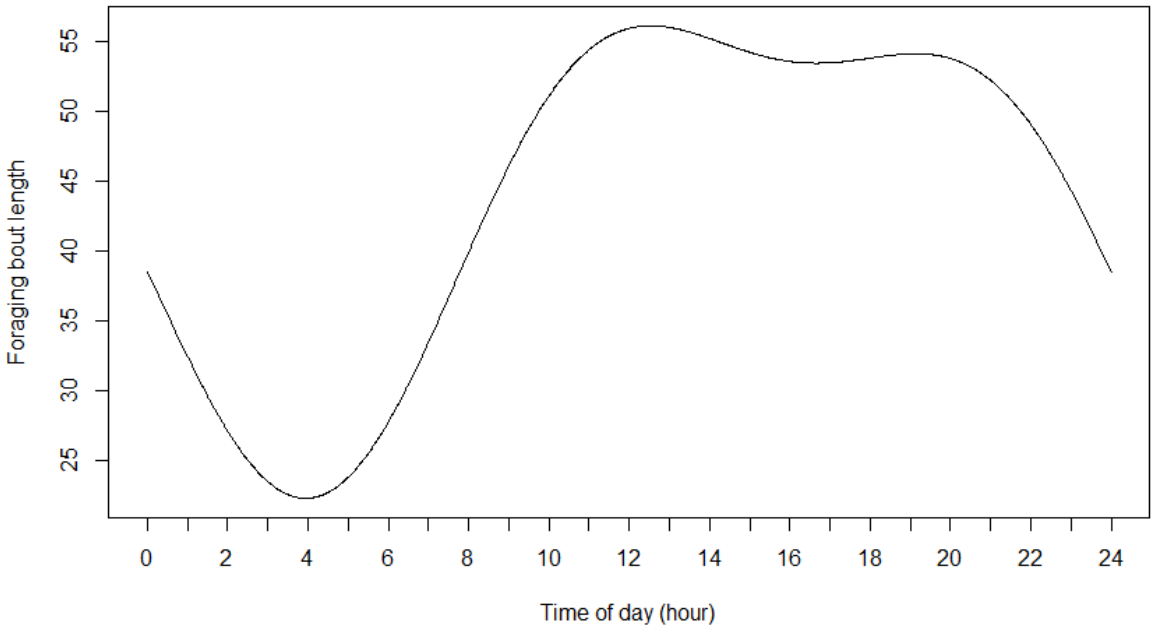

Figure 12 Changes in foraging bout length during the day for the control group.

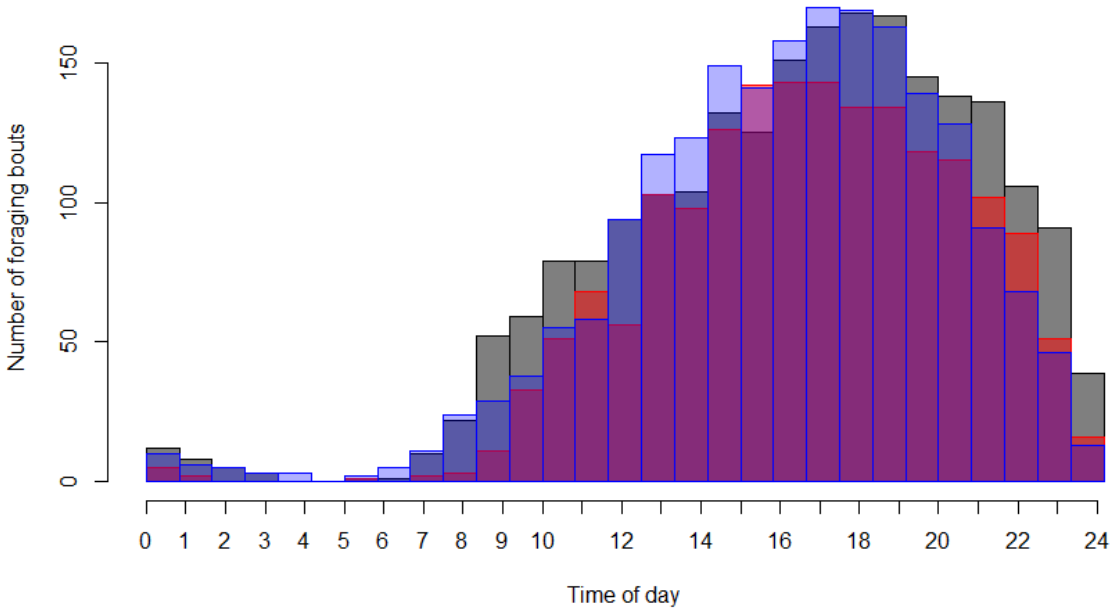

Figure 13 Daily activity of the bumblebees. The grey histogram shows the activity of the control group, the blue represents the low concentration group, whilst the red shows the high concentration group. There is no significant difference between the treatments. The main foraging activity window was between 8:30 and 23:00.

3.4 The performance of the monitoring system

In the course of the whole experiment, the system worked for 35 days. During that time, 5,613,031 pictures were taken. The size of a single picture averaged on 65.5 kb and all pictures together occupied 368 GB of hard drive space.

A detection rate, as defined as the number of successful detections, i.e. the bCode is readable, divided by the total number of detections of bCodes (including also those where the identity of the code could not be defined) was 81.8 %. The detection rate varied from 75.7% - 85.1% among the monitoring boxes.

4 Discussion

4.1 Colony growth rate

There was no difference in colony growth rate between the 3 treatment groups giving support to my hypothesis (H1: There is no significant difference in colony growth rate in the different treatment groups).

The treatment levels used in this study have previously been shown to be sub-lethal (Table 1 p.9)(Stanley et al. 2015; Scott-Dupree et al. 2009). There is contradicting evidence for the effect of neonicotinoids on colony growth rate. Multiple studies report a negative effect on the colony growth (Gradish et al. 2009; Scott-Dupree et al. 2009; Bryden et al. 2013), while others found no effect (Stanley and Raine 2017; Laycock et al. 2013). In the present study, 15 out of 25 hives experienced a decrease in colony size. The colonies delivered from Bombus Natur were in different stages of development, which could have affected their overall condition and consequently their mortality rates (Matheson 1996). Queens in early colony development stages produce more workers. As the colony enters late development stages, the queen stops producing workers, and focuses on laying unfertilized eggs, which will develop into males, and producing new queens (Matheson 1996). Once this process starts, the queen loses her dominance over the hive, and this can lead to aggressive behaviour between the workers and the new queens, resulting in increased mortality (Sibbald and Plowright 2013; Free 1955). Due to the variation in colony condition and differences in development stage among the colonies used, the results of this study cannot be used to confirm whether the exposure to neonicotinoids negatively affects the colony growth or not.

4.2 Average number of foraging bouts per day

There was no difference in the average number of foraging bouts per day carried out by workers under the different exposure levels. The results are comparable to a study done by Gill and Raine (2014), in which there was no significant difference across the treatment groups in the daily number of foraging bouts during the first week of the experiment.

All 3 treatment groups followed a similar trend – the highest average number of foraging bouts per day increased from day 1, through day 2 and peaked at day 3 of the release period. After the 3rd day the number of foraging bouts declined until the very end of the release period when the average started to increase again. The low concentration group did on average more foraging bouts than control and high concentration groups on the last day of the observation period, though the difference was not statistically significant.

It is worth noting that the average number of foraging bouts per day for high concentration group started increasing earlier than for the control and low concentration groups (Day 5 for high concentration groups and Day 7 for control and low concentration group). A study by Williamson et al. (2014) showed that honeybees were able to metabolize clothianidin at concentrations between 1.8 – 2.4 µg/L within 24 hours (Williamson et al. 2014). In my study, bumblebees were exposed to higher doses of clothianidin over a longer period, and they had access to contaminated nectar stored within the hive after the exposure period. Therefore, I assume that the detoxification period, i.e. the time it takes to remove all traces of clothianidin from the organism, for the bumblebees in this study was longer than 24 hours. Thus, the increased foraging activity of the two exposed groups at the end of the observation period might be due to the stimulating effect of neonicotinoids on bee activity at low doses (Matsuda et al. 2001)

4.3 Foraging bout length

For all treatment levels, the foraging bout length increased with temperature. The control group and the low concentration group followed a similar trend although the foraging bout length for the control group was consequently shorter. The effect of temperature on foraging bout length was significantly stronger for the high concentration group, suggesting that the effect of clothianidin exposure depends on local weather conditions. Therefore, the H2 hypothesis (H2: The length of foraging bouts increases when exposed to increasing doses of clothianidin) is partially supported, as only the high concentration group was significantly affected by temperature.

Bumblebees exposed to the high concentration made longer foraging bouts than the control bumblebees above 12°C but shorter foraging bouts below 12°C. This indicates that the bumblebees were more vulnerable to changes in temperature when exposed to high concentrations of clothianidin. Bumblebees cannot take off until the temperature of their flight muscles is above 30 ^{o}C and the temperature of the thorax during flight must be kept between 30 ^{o}C to 40^{o}C (Sanborn 2005; Heinrich 1981). Neonicotinoids have been shown to alter the thermoregulation of honey bees. Tosi et al. (2016) showed that honeybees exposed to thiamethoxam at three different doses (0.2, 1, 2ng/bee), the higher doses resulted in a decrease of the thorax temperature, while the lowest dose increased the thorax temperature. Results of a study done by Potts et al. (2017) show similar results for bumblebees. Paus-Knudsen (2017) showed that exposure to field-realistic doses of imidacloprid (10 µg/L) significantly reduced the locomotor activity (i.e. flight speed) of the bumblebees. In the same study, the bumblebees' learning was impaired at 10 µg/L, resulting in fewer visits to rewarding flowers. Peat and Goulson (2005) suggest that bumblebee foraging preferences might be affected by the weather; Bumblebees preferably collected pollen in warmer, low humidity conditions and switched to collecting nectar in high humidity conditions. Due to the aforementioned negative effects of neonicotinoids on thermogenesis, bumblebees exposed to neonicotinoids might struggle to adapt to these changes or incorrect interpret temperature stimuli, resulting in longer foraging bouts. We have an incomplete understanding of the interaction between neonicotinoid exposure and temperature on bumblebee foraging behaviour. To close the knowledge gap, future studies on effects of neonicotinoids should include temperature interaction.

The foraging bout length increased significantly with days into the release period. As forager bumblebees age, their foraging efficiency might decrease (Tobback et al. 2011). The increase of the foraging bout length might also be a result of chronic effects of exposure to clothianidin (Gill and Raine 2014), so the colonies most likely utilized stored nectar gathered while under exposure.

Although bumblebees are quite robust and able to withstand rain and wind, the foraging bout length decreased with increasing precipitation, which is an expected result. Precipitation might affect the availability of food sources as many flowers close their coronas during rain

(Bynum and Smith 2001). Rain can dilute and/or remove nectar and pollen from open flowers (Lawson and Rands 2019). Flying in the rain requires more energy and decreased visibility might disrupt/weaken sensory signals, increasing the possibility of a bumblebee forager using more resources than it was able to gather (Lawson and Rands 2019). Thus, wet conditions might discourage foragers from continuing the foraging bout and prompt them to return to the colony.

The duration of foraging bouts varied throughout the day. Only 62 of the total number foraging bouts were made between 00:00 and 05:00. The main activity period for bumblebees in this study was between 09:00 and 22:00. Between 10:00 and 20:00 bumblebees made longest foraging bouts (> 50 minutes) and shortest foraging bouts between 03:00 and 04:00 (<20 minutes). During the observation periods, the sunrise in Oslo was between 03:30 and 04:00 which might explain the elevated activity (Lundberg 2006). In a study done by Stelzer and Chittka (2010), *B.terrestris* workers showed a strong diurnal rhythm, with main activity between 08:00 and 23:00 with only low or close to no activity during the night. The activity patterns showed in their study are comparable to the activity I report here.

The H3 hypothesis (H3: There is an interaction between abiotic factors (weather conditions) and level of exposure to clothianidin.) is supported, as the interaction between the treatment level and temperature was significant. However, no interaction between the treatment level and other abiotic factors (average daily precipitation and average wind speed) was included in the best model.

4.4 The performance of the monitoring system

To my knowledge, this is the first attempt to use a camera-based bumblebee colony monitoring system to monitor bumblebee colonies in a semi-natural experiment. Other behaviour experiments have used Radio Frequency Identification (RFID), a system that uses radio waves transmitted from an RFID tag attached to a bee to an RFID reader to identify the individual. This means that the RFID reader does not rely on visual clues to recognize the different tags. This simplifies the monitoring system, as the reader might be placed in multiple locations, unlike in a camera-based system where the camera must be placed above

the passing bumblebees. RFID completely removes the possibility of data loss due to visual contamination/degradation of the tags.

The main advantage of the system used in this study over RFID based solutions are the costs. My system was based on computer hardware from 2013 (see 2.4.3) and open-source software, making it extremely cost-efficient. The most expensive part of this system were the cameras (purchased for ~€70 each) and computers capable of handling the software can be purchased for ~€50 each. That said, RFID readers are not the only costly part of an RFID system. While an RFID reader can be built for around 300-400€, the price of a single RFID tag can be as much as $1.5. In the present study, 750 bumblebees were marked with bCodes. If an RFID based system was used instead, the cost of RFID tags would be 1125$, which is double the cost of entire (i.e. 6 camera boxes and 2 computers) system used in this study.

One of the main issues with any monitoring system is misreading, i.e. when an object, in this case a bumblebee, is not detected or is incorrectly detected when passing through the monitoring checkpoint. My system had an average detection failure rate of 18.2%. Less advanced RFID systems have a similar detection failure rate, ranging from 10 % (De Souza et al. 2018 and references therein) to 49% (Tenczar et al. 2014) depending on the system used. More advanced system, which uses multiple readers, have close to 0% detection failure rate but such systems are even more expensive than the simple ones described above.

The system used in this study is still in an early stage of development and many improvements can be made. Earlier designs of the system relied heavily on high-resolution pictures. However, as the design of the system improved, the reliance on the quality of the camera became less important. A single-board computer, like Raspberry Pi, equipped with a camera module, could be adapted to work with this system, making it even more affordable, and portable, without compromising its performance.

The system used in the present study is based around an image sensor and thus the main sources of misreadings in this system are caused by poor readability of the bCode in the pictures. In the current set up, at least 85% of the bCode pattern must be recognizable by the detection software to correctly identify the identity of the bee. If a bCode is not recognizable the software will automatically assign a BeeID value of “-1”. Due to fact that

the camera boxes were handmade, the tolerances varied slightly between them. As a result, settings for each camera had to be manually adjusted. Automatization of the manufacturing process can lower the variance between boxes and thus help to improve the detectability. As 3D printing becomes more and more accessible and cost-effective, it would be a possible solution.

In the present study, the bCode degradation was not a problem, as the number of failed detections did not differ significantly between 1st and 7th day of the release period. However, over longer periods, bCodes might become unreadable due to discolouration, water damage and/or pollen and nectar obstructing the bCode, even if waterproof paper was used. One possible solution to this problem is laminating the printed bCodes with 1.5 mils (0.038 mm) laminating film. This solution could possibly extend the longevity of a bCode but would also add another step to bCode production.

5 Conclusions

The overall goal of this study was to assess the effects of field-realistic concentrations of clothianidin on bumblebee foraging behaviour, specifically the length of the foraging bouts. To achieve this, I developed a monitoring system capable of tracking foraging bouts of individual bumblebees in semi-natural settings, which would be a budget-friendly alternative to currently available monitoring systems. The overall performance of the monitoring system is promising; The number of successful detections averaged 81.8% which is comparable to less advanced RFID systems. However, there are still improvements that can increase the performance of this system and make it an even more attractive alternative to other monitoring systems. Nonetheless, the monitoring system is usable.

I did not find any effect of clothianidin exposure on colony growth rate showing that the selected exposure levels were in the sub-lethal range. Despite the fact that the average number of foraging bouts per day did not differ between the clothianidin exposure levels, I have shown that clothianidin exposure increased the foraging bout length. Moreover, I found that the effect of clothianidin is temperature dependent, as the increase in foraging bout length was strongest for the highest exposure level.

The results of this study underline the need for semi-natural experiments where bumblebees are exposed to several stressors at the same time. Detecting and understanding the interactions between several stressors is of utmost importance to be able to correctly manage and help both the pollinators and their environment. Although I have not monitored the behavioural change occurring during the foraging bouts and therefore cannot identify the exact mechanism for the observed changes in foraging bout length, I have shown that the effects of clothianidin exposure are context-dependent. This finding suggests that risk assessments of pesticide cannot be directly extrapolated to new environments and other climatic conditions. Furthermore, the foraging bouts under high exposure were longest at higher temperatures, suggesting that the negative effects of clothianidin, and maybe also other pesticides, will be stronger under future global warming.

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Appendix

1. Exposure

All containers have been been treated with 0.1 M hydrochloric acid for 24 hours to remove /lower the risk of contamination and thereafter washed 6 times with distilled water to remove the residual acid from the flasks. All dilutions and mixing have been done in a fume hood with lights turned off and all containers were wrapped with aluminium foil as clothianidin is prone to photodegradation – measured aqueous photolysis half-life was <1 day (Federoff and Barrett 2005). No significant degradation of clothianidin occurs in the dark (Federoff and Barrett 2005). All solutions were stored in a refrigerator at 4°C in containers marked with content, concentration, name of the student and date.

Stock and Intermediate solutions

Stock solution – In preparing of the stock solution, 0.02 g clothianidin was weighted in a disposable weighing boat and then flushed into a 200 ml glass bottle with 100 ml distilled water and mixed on a magnetic stirrer Rotamix 560 MMH at 500-600 RPM, temperature setting 3. The solution was allowed to cool before proceeding to the next dilution step. The concentration of the stock solution was 200 mg/L.

Intermediate solution 5 mg/L –1250 µL of Stock solution was pipetted out and mixed with 48.75 ml distilled water in a 50 mL flask. The resulting concentration was 5 mg/L. The solution was transferred to a clean 100 ml glass bottle.

Intermediate solution 684 µg/l - 6840 µL of Intermediate 5 mg/l was pipetted out and mixed with 43.16 ml distilled water in a 50 mL flask. The resulting concentration was 684 µg/l. The solution was transferred to a clean 100 ml glass bottle.

Intermediate solution 1300 µg/l - 13,000 µL of Intermediate 5 mg/l was pipetted out and mixed with 38 ml distilled water in a 50 mL flask. The resulting concentration was 1300 µg/l. The solution was transferred to a clean 100 ml glass bottle.

Distilled water solution – 50 ml of distilled water was measured using a measuring cylinder and transferred to a clean 100 ml glass bottle.

Adding the final solution to nectar tanks

The contents of the nectar tanks were emptied into a 1500 mL Erlenmeyer flask. Each nectar tank was then cleaned both inside and outside using distilled water and allowed to dry out. Nectar solution was diluted from 50% concentration to 30% concentration using distilled water. To obtain 30% concentration, 900 ml of 50% nectar was measured using a measuring cylinder and mixed with 585 ml distilled water in 1500 mL Erlenmeyer flask.

For the concentration of 0 µg/l**,** 15 ml of **distilled water** was added to the nectar using a 5000 µL micropipette (Eppendorf research) and mixed thoroughly using a magnetic stirrer Rotamix 560 MMH at 200-250 RPM until the solution becomes homogenous (around 3-4 minutes). The solution was then transferred to a nectar tank and marked with the hive designation.

For the concentration of 6.9 µg/l, 15 ml of **Intermediate solution 684 µg/l** was added to the nectar using a 5000 µL micropipette (Eppendorf research) and mixed thoroughly using a magnetic stirrer Rotamix 560 MMH at 200-250 RPM until the solution becomes homogenous (around 3-4 minutes). The solution was then transferred to a nectar tank and marked with the hive designation.

For the concentration of 13 µg/l, 15 ml of **Intermediate solution 1300 µg/l** was added to the nectar using a 5000 µL micropipette (Eppendorf research) and mixed thoroughly using a magnetic stirrer Rotamix 560 MMH at 200-250 RPM until the solution becomes homogenous (around 3-4 minutes). The solution was then transferred to a nectar tank and marked with the hive designation.

2. bCode generation

bTools is a java-based program pack. The detailed description of the program functionality and all required files (bcode_detector.jar, converter.jar and bcode_maked.jar) can be found at http://www.beemonitoring.igb.illinois.edu/ and at https://github.com/gernat/btools.

The bTools require Java to be installed (https://www.java.com/en/download/) on the computer and it is operated using Command Prompt in Windows. Step by step process is described below. Steps marked with ***[CMD]*** sign at the beginning are Command Prompt commands and the ***[CMD]*** must not be included in the command.

1. Download the bcode_marker.jar
2. ***[CMD]*** **cd C:\Users\PawelJK\Desktop\Bcode\Maker**
3. **Change the working directory to the directory containing the bcode_maker.jar**
4. ***[CMD]*** **java -jar bcode_maker.jar square.side.length=8 padding=2 bcode.file=bcodes.png**
5. This command is used to determine the size of the bCodes. The bCode size formula is defined as (square.side.length * 12 + padding * 2) / printer_resolution * 25.4 (Gernat et al. 2018). Using values specified above and printer DPI of 1200, results in bCodes with a side length of 2.12 mm. The command outputs the bcodes.png file which includes all 2048 bCodes. The bCodes are segregated into 8 groups with 256 bCodes each. It is recommended to save the bcodes.png as a PDF file. This enables better

manipulation of the image e.g. cropping, resizing etc. as well as printing the image as PNG file or using Windows Photo Viewer to print, may cause degradation in print quality.

3. Bumblebee marking

For the marking of the bumblebees a simple marking station was made (Figure 12) To mark the bumblebees the following procedure was used:

1. Replace the colony box lid with one with an access hole.
2. Using long tweezers, grab a bumblebee and transfer to the marking cage (Figure 13)
3. Using the plunger, carefully move the bumblebee into the correct position in the marking cage (Figure 13)
4. Apply a small amount of glue to the back of the thorax between the wings.
5. Using a toothpick or small tweezers place the bCode directly on the glue.
6. Allow the glue to dry for around 15-20 seconds and release the bumblebee back in the colony box.

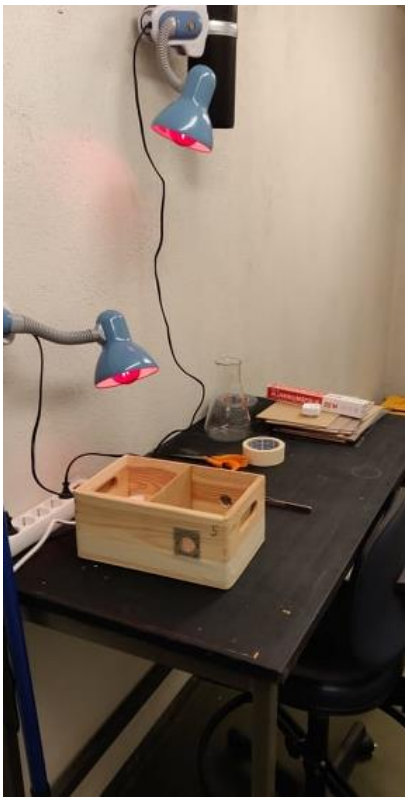

Figure 6 An overview of the marking station. 2 lamps with red LED bulbs were used to calm down the bumblebees.

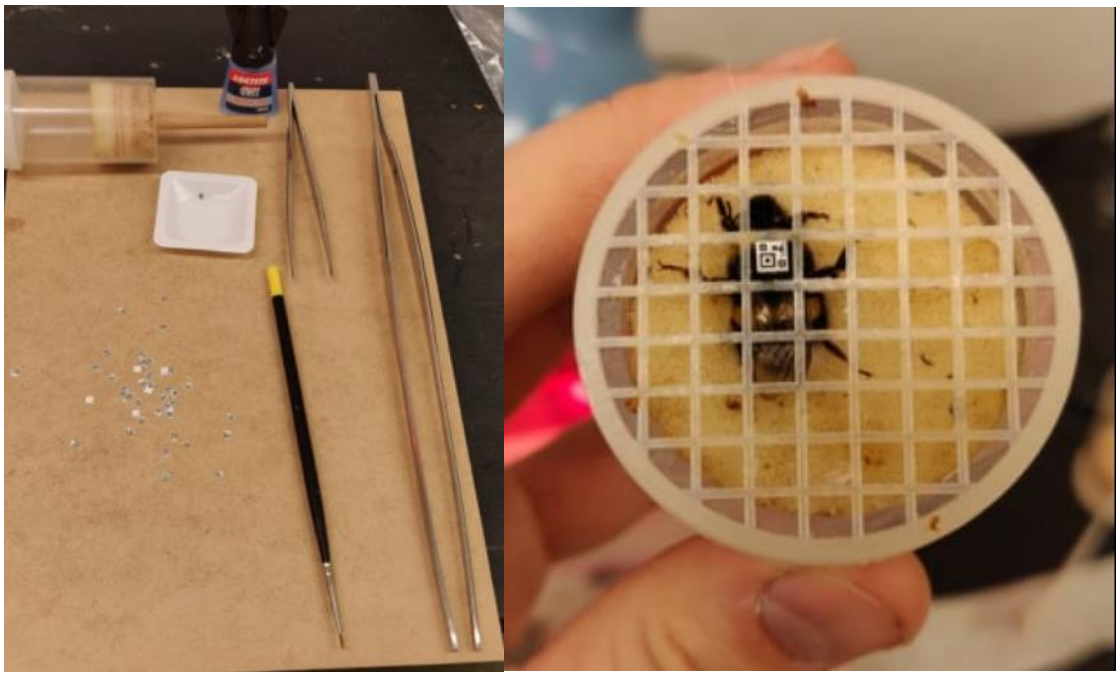

Figure 7 Left picture - The equipment used to mark the bumblebees. From the top left - queen cage used to hold the bumblebee in the correct position. Loctite Super Glue and a small plastic container for the droplets of glue, fine brush to precisely apply small amount of the glue.

4. Setting up cameras and iSpy software.

iSpy is an open-source video surveillance software. The software can be downloaded from https://www.ispyconnect.com/. Any other camera software capable of digital motion detection can be used instead of iSpy.

1. Download and install the iSpy software.
2. Add a camera using the Add button in the top left corner. A new window will pop up. The software will automatically choose the camera. The video resolution settings will depend on the camera itself, the computer specifications and the number of cameras. In this experiment, the video resolution was set to 960 x 720 (24 bit up to 30 fps).
3. Once the camera is added, a new window called “Edit Camera” will appear (Figure 14), containing all settings for the current camera. Two important settings are the name of the camera and the framerate at which the camera operates. The framerate should be set to 30 for both “maximum framerate” and “When Recording”

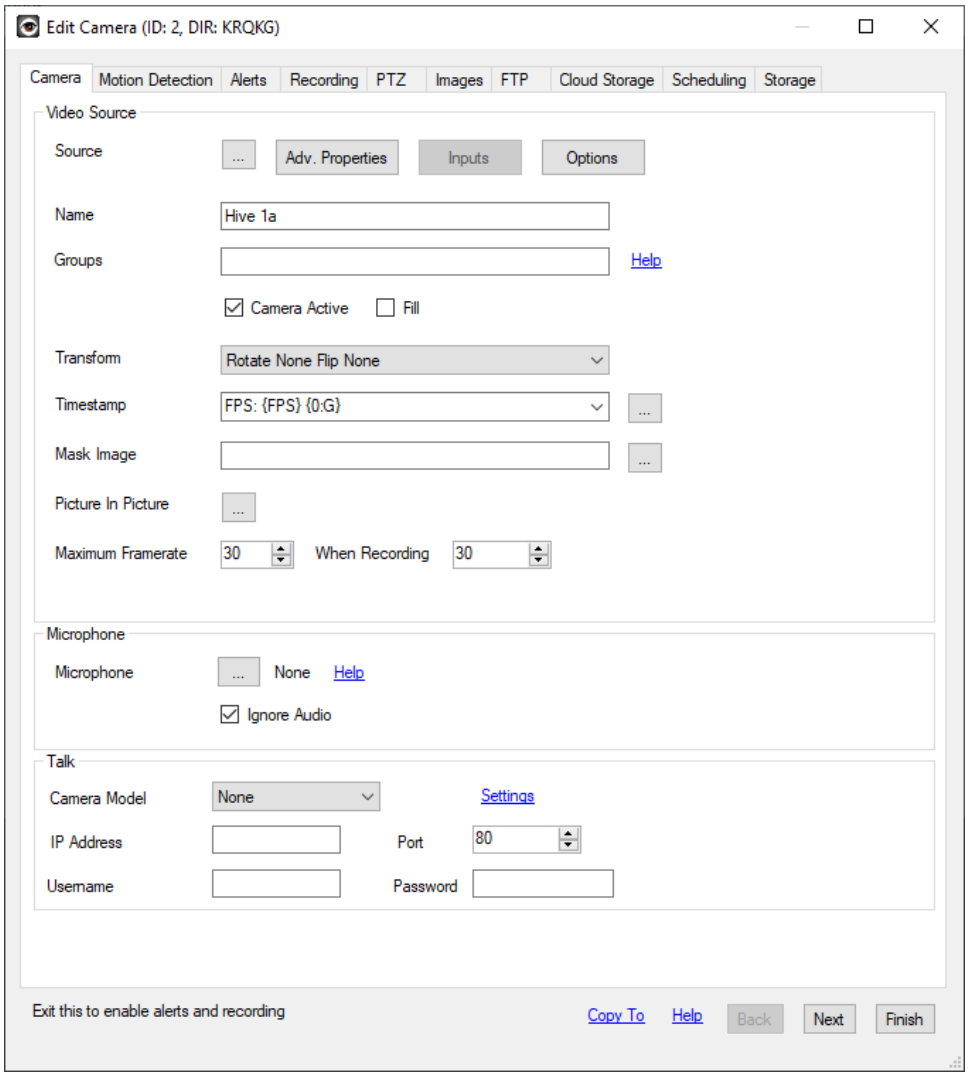

Figure 8 General camera settings. The two important settings are the name of the camera and the frame rate at which the camera operates.

4. The “Adv. Properties” tab contains settings for the image quality and camera control. These settings depend on the camera used and the way it is mounted inside the camera box. Settings used in this experiment are as follows:

   Settings that are the same for all cameras (default if not specified)

   Brightness: 130

   Contrast :130

   Saturation: 130

   Sharpness: 150

   White balance: Auto

Other settings differ based on the position of the camera inside of the camera-box. Each camera has to be set up separately. Zoom and focus settings depend on the distance between the camera lens and the tunnel inside of the camera-box. The camera should be set up in a way that both tunnels are visible. The zoom levels varied from 150 to 160 and the focus between 100-110 depending on the camera.

The exposure slider can be set to value of -5 or left on Auto as the lightning inside of the camera-box is constant. The Pan and Tilt sliders can be used to fine-adjust the camera position (in x and y-axis) above the tunnels.

In the "Options" tab is it important that the "No resize" box is checked. If left unchecked, the software will resize the photos, independent of other settings.

iSpy is capable of recording videos triggered by motion detection. This function has to be disabled in the "Recording" tab (Figure 15). Disabling the recording function will force the software to only take motion triggered pictures. Motion detection is enabled by default and no settings have to be changed.

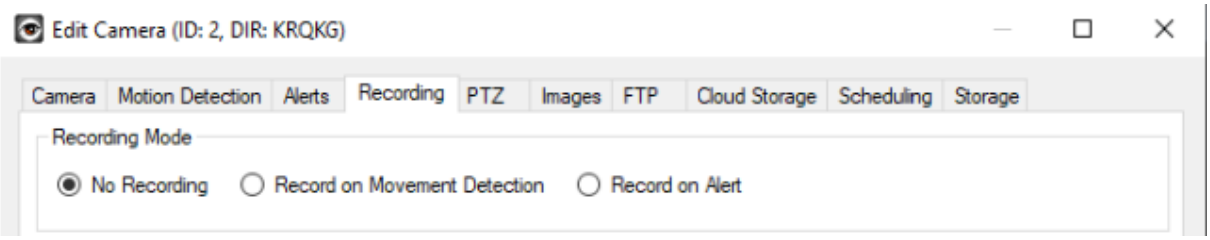

Figure 9 Disabling the motion-triggered recording. This will force the software to only take pictures.

5. In the "Images" tab (Figure 16), Local Saving Enabled has to be turned on. In the "Filename" field the name format has to be set to {yyyy-MM-dd-HH-mm-ss-fff}.jpg. This is very important as the bTools require the images to be named in this format, otherwise it will not function. The quality slider has to be set to 100% to avoid any image compression. The overlay text can be left blank or the camera name can be inserted. The Counter Max setting can be either set to 0 or to maximal value, as it doesn't affect anything.

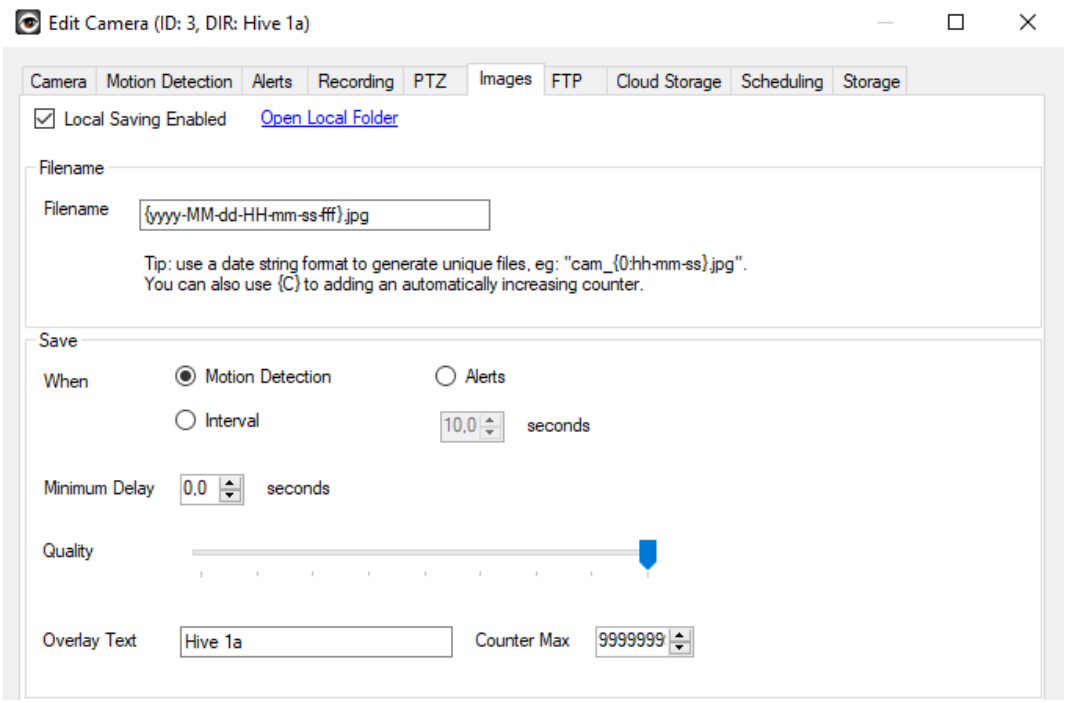

Figure 10 The setting for images. Most important settings here is the Filename as it has to be set to a specific format.

6. A restart of the iSpy might be required. All settings will be automatically applied on start-up and the software is ready to use. Additional cameras have to be set up in the exact same way.

5. Picture processing

Loading and converting pictures in bTools.

Hives must be processed separately. Pictures from each hive have to be grouped in the same folder.

1. ***[CMD]* cd C:\Users\PawelJK\Desktop\Bcode\Hive1a**
   The "cd" command is used to change the current working directory. Specify the folder where the pictures are saved.

2. ***[CMD]* dir /s /b *jpg >jpgList.txt**

   The command "dir" creates a list of all items in the current working directory. The dir command switches are used to specify the command functionality. The /s /b switches are used to list every file and its path, *jpg specifies the file format and >jpgList.txt creates a new TXT file jpgList.txt with the results.

3. Copy bcode_detector.jar into the working directory.

4. ***[CMD]*** **java -jar bcode_detector.jar min.intensity.threshold=40 max.intensity.threshold=110 intensity.step.size=10 min.template.conservation=0.85 conserve.margin=0 image.list.filename=jpgList.txt**

   This command is used to scan through every single picture based on the jpgList.txt.

   The command will output 1 TXT file per picture. This process is highly CPU intensive and the processing times will vary based on the number of pictures and the specifications of the computer. Processing 250,000 pictures will take approximately 2 hours (computer specification listed in Ch. 2.8). The detailed description of each parameter used in the command can be found on http://www.beemonitoring.igb.illinois.edu/.

5. ***[CMD]*** **delete "jpgList.txt"**

   The file jpgList.txt must be deleted either using the command above or manually.

6. ***[CMD]*** **copy *txt merged_detections.txt**

   Used to merge all TXT files created by bcode_detector.jar into one TXT file.

7. Copy converter.jar into the working directory.

8. ***[CMD]*** **java -jar converter.jar raw.bCode.file=merged_detections.txt human.readable.file=converted_bcode_detections.txt**

   Used to convert all detection into human-readable format. The converted bCode detection will be stored as comma-separated TXT files without a header. Each line describes one detection and provides the following information:

   image timestamp, x-coordinate, y-coordinate, orientation vector x-component, orientation vector y-component, ID of bee (Gernat et al. 2018). The column "Timestamp" shows the time when the picture was taken (in UNIX format), both x and y coordinates display the placement of the bCode in the picture. The X-coordinate allows to determine in which tunnel the bumblebee is, and thus the movement direction (either exiting or entering the hive). The columns "orientation vector x-component" and "orientation vector y-component" are not used in this experiment. The ID of bee is used to distinguish between the individual bumblebees.

Data handling and basic sorting in RStudio

```
#Disabling scientific notation and increasing max print limit

options(scipen = 999)

options(max.print=10000)

#Loading the dataset.

df <- read.table("converted_bcode_detections.txt", header=FALSE)

#Adding header

colnames(df) <- c("Timestamp", "XCoord", "YCoord", "Xvector", "Yvector", "BeeID")

#Removing all faulty detections (categorised by a value of -1) and 0 (low entropy detection) based on BeeID column and -1 BeeID

df <- df[ !grepl("-1", df$BeeID) , ]

df <- df[df$BeeID != "0",]

#Converts the x-coordinate values below 350 (centre) to OUT and values above 350 to IN, showing the movement direction of the bumblebee.

df$XCoord = ifelse(df$XCoord < 350, "OUT", "IN")

#Unix timestamp conversion

df$Timestamp <- as.POSIXct(as.integer(as.numeric(as.character(df$Timestamp)) / 1000.0), origin='1970-01-01', tz="CET")

#######SORTING#######

#Sorting data by date/time and BeeID

df.sorted <- df[order(df$BeeID, df$Timestamp), ]; df.sorted

#Removing unused columns

df.col_removed <- df.sorted[,c(1,2,6)]; df.col_removed

#######EXCEL#######

#Saving the new dataset to .csv file

write.csv2(df.col_removed,file = "Hive1a.csv", row.names = FALSE)
```

Removing the duplicate detections using FME Workbench

FME Workbench is a visual data editor which includes many different ready-made transformation tools. FME Workbench can be downloaded from https://www.safe.com/fme/. It is worth noting that the same analysis can be done in RStudio. I chose FME Workbench due to being more familiar with it than I am with R and RStudio.

The system will continuously take pictures if movement is detected and thus a bumblebee can be photographed twice or more times when leaving and entering the colony box. This creates a problem of duplicate detections. A simple sorting system was created using two transformers (Figure 17). DateTimeConverter (Figure 18) transforms the Timestamp column into YYYY-MM-DD HH-MM format, while the DuplicateFilter (Figure 19) detects duplicates based on Timestamp, BeeID and XCoord columns and removes them only keeping unique detections. Finally, the script outputs a new CSV file. Majority of the duplicate detections are removed using this system. However, some detections have to be removed manually using Microsoft Excel.

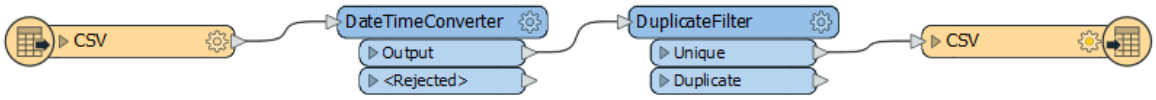

Figure 11 Overview of the duplication removing script. First, the CSV file is loaded into the software, then date/time format is transformed using DateTimeConverter. DuplicateFilter transformer removes any duplicates, only keeping the unique detection. Lastly, the newly created dataset is saved as CSV file.

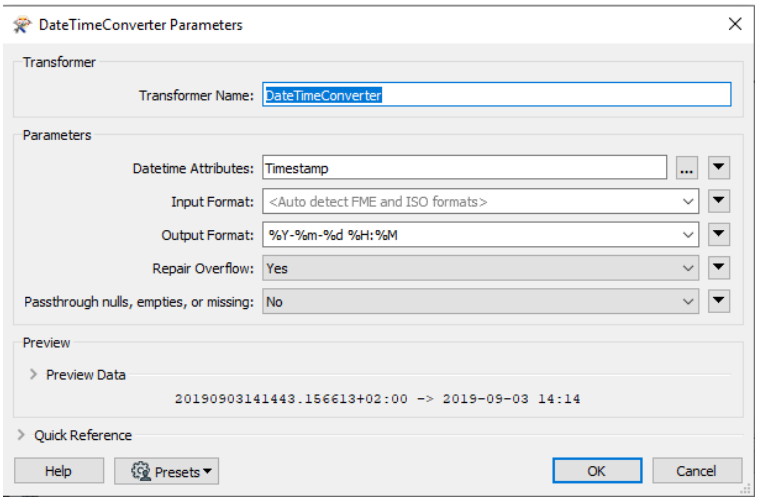

Figure 12 The settings for the DateTimeConverter. It automatically detects the date/time format and transforms it into a specified output date/time format.

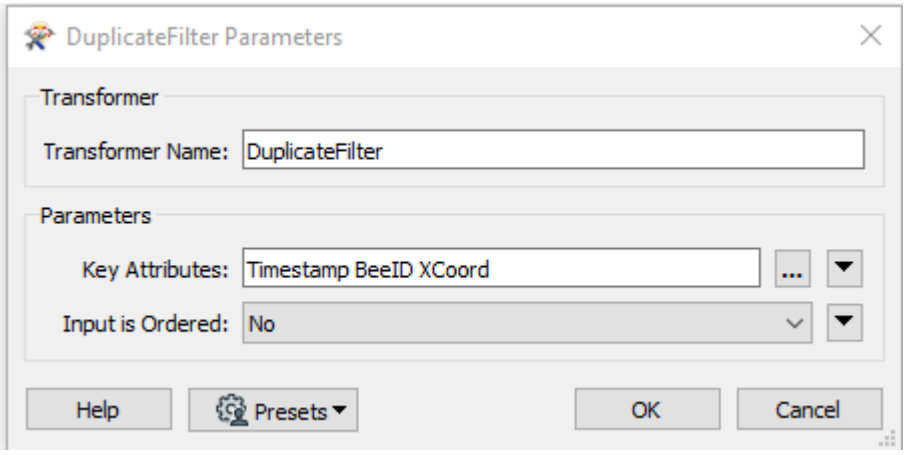

Figure 13 The settings for the DuplicateFilter. The duplicate detection is based on 3 separate parameters: Timestamp, BeeID and XCoord.