Sub-lethal effects of imidacloprid, a neonicotinoid insecticide, on bumblebees (*Bombus terrestris)*

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Abstract

Bees are increasingly facing multiple and interacting threats. One of the threats that has received increased attention lately is neonicotinoids: a group of systemic neuro-active pesticides that disturb the transmission of signals in the insect´s nervous system. Neonicotinoids are the most widely used pesticides in the world, and protect a variety of crops against invertebrate pest. Despite being used in relatively small quantities, several studies have shown sub-lethal effects of neonicotinoids on honeybees (*Apis mellifera*) exposed to neonicotinoids. However, there is still a lack of knowledge on the effects on other important pollinators. A wide range of ecological and physiological traits vary decisively among bee species, indicating that studies on honeybees may not provide satisfactory predictions of the effects on other bee species.

The present study aimed to develop a new experimental method to quantify the sub-lethal effects of imidacloprid on bumblebee colonies (*Bombus terrestris*). More specifically the aim was to determine how dietary exposure to imidacloprid affects learning, and consequently the ability to forage and thus pollinate, in a non-*Apis* species. Bumblebees were exposed to three different dosages of imidacloprid through artificial nectar (sugar water), ranging from realistic field levels (1 μg/L and 10 μg/L) to distinctly higher levels (100 μg/L) in a chronic exposure regime, lasting for eight days. Bumblebees not exposed to imidacloprid were used as control. To assess whether imidacloprid influences learning, the bumblebees were tested systematically in a flying arena containing nectar-filled (rewarding) and water-filled (unrewarding) artificial flowers of two different colors. The bumblebees were tracked by cameras, allowing for analysis of the trajectory of bees. In particular, the learning behavior was quantified (how well bees discriminate between rewarding and non-rewarding flowers) and pollination efficiency (flowers visited during a foraging bout). In addition, the health of the colonies was assessed after exposure to imidacloprid by counting the surviving bumblebees in different developing stages. To assess food intake the number of honeypots were counted, and the amount of nectar consumed during the exposure period were measured.

The overall results were an experimental design that was applicable, and that learning, locomotor activity, survival and food consumption are negatively affected in a dose-dependent manner when bumblebees are exposed to imidacloprid. Moreover, both the behavioral results and the results assessing the health of the colony show that field-realistic doses of imidacloprid have sub-lethal effects on bumblebees.

Abbreviations

ACh	Acetylcholine
AIC	Akaike information criterion
AICc	Corrected Akakie information criterion
AVM	Abdominal ventilation movements
APVMA	Australian Pesticides and Veterinary Medicines Authority
CAS	Chemical Abstract Service
CNS	Central nervous system
Da	Dalton
DMSO	Dimethyl sulfoxide
EC_{50}	Effective concentration where 50% of the maximal effect occurs
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
GLM	Generalized linear model
IUPAC	International Union of Pure and Applied Chemistry
K_{ow}	The octanol/water partition coefficient
LED	Light-emitting diode
LC_{50}	Lethal Concentration where 50% of the organisms are dead
LD_{50}	Lethal Dose where 50% of the organisms are dead
LOEC	Lowest observed effect concentration
Log	Logarithm
mPa	Megapascal
N	Number of observations

nAChR	Nicotinic acetylcholine receptors
NOEC	No observed effect concentration
QAIC	Quasi- Akaike information criterion
Sp	Species
PER	Proboscis extension reflex
PPP	Plant Protecting Products
UiO	University of Oslo
UOH	University of Hertfordshire
UV	Ultra violet

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1 Introduction

1.1 Modern farming

As the world population is growing both in number and in wealth, the need for food is increasing (Kastner et al., 2012). As of today, we are using 38% of the world's ice-free terrestrial surface for agriculture (Foley et al., 2011), and whether the earth is capable of providing enough food to all its inhabitants when exceeding 9 billion people is still an open question (Godfray et al., 2010). An increase in the global population, combined with an increased per capita demand for high-quality food, highlights the importance of efficient use of cropland, i.e. intensive farming, using irrigation, fertilizers, and pesticides. Agricultural intensification has already reduced the area of land used to feed one person by a third in the period 1963 to 2005 (Kastner et al., 2012). Environmental conservation is one key aspect of a successful future food production. Loss of biodiversity, increasing greenhouse gas emissions, deforestation and water degradation must be prevented to reach the food demand (Foley et al., 2011). Included in loss of biodiversity, and an essential aspect of a productive and sustainable agriculture are the pollinators, animals that transfer pollen from the male flower to the female flower, enabling fertilization. Pollinators improve yields for 70% of the 124 crops used directly for human consumption (Klein et al., 2007), and account for 5–9.5% of the economic value of human food (Gallai et al., 2009; IPBES, 2016). Animal-mediated pollination is also essential for wild plant biodiversity as 87.5% of all angiosperms are being pollinated by animals (Ollerton et al., 2011).

Pollinators are under threat from several drivers (Potts et al., 2010), and there are concerns regarding their ability to maintain the ecosystem service they provide to pollinating crops and wild flowering plants (Steffan-Dewenter et al., 2005; Vanbergen et al., 2013). In North America and Hawaii, half of the native bee species are declining (Kopec et al., 2017), and in Europe, nearly half of the bumblebee species have a declining trend (Nieto et al., 2014). Globally, bees are the most important pollinators, and there are concerns about their status (Goulson et al., 2008; Kearns et al., 1998; Vanbergen et al., 2013). The paradox is that one of the major threats to bees is the industry that needs them the most: The agricultural sector (Carvalheiro et al., 2013; Goulson et al., 2005; Kennedy et al., 2013; Potts et al., 2010; Westrich, 1996). Since pollinating insects and plants are linked elements in an ecosystem, they may decline together (Biesmeijer et al., 2006). Moreover, for generalists' pollinators like bumblebees and honeybees, the decline is especially concerning because removing the most-linked pollinators has been

shown to lead to a more rapid decrease in flowering plants than removing the least-linked pollinators (Dunne et al., 2002; Memmott et al., 2004; Solé et al., 2001). The link between decreasing bee species richness and decreasing seed set in insect-pollinated plants has been shown in the Netherlands and the United Kingdom (Biesmeijer et al., 2006).

Several factors affect the decline in abundance and diversity of pollinators, ultimately affecting the ecosystem services they provide (see Potts et al. 2010 for a review). Some well-documented examples from agriculture are fragmentation of landscapes (Cameron et al., 2011; Williams et al., 2009), the lack of floral resources (Goulson et al., 2005), overgrazing by domesticated animals (Xie et al., 2008), pathogens from the commercial breeding of bees (Cameron et al., 2011), competition between native bees and honeybees (Cane et al., 2017; Thomson, 2004), fungicides (Simon-Delso et al., 2014), and insecticides (Whitehorn et al., 2012). These stressors are not new, however many of them have increased in intensity during the last decade. It is therefore essential to understand how current agricultural practices affect bee populations to maintain an efficient agriculture and a healthy terrestrial ecosystem.

1.2 Pesticides

Pesticides are products used to protect plants against harmful organisms or disease. "Pesticide" is a broad term, which includes herbicides, fungicides, insecticides, acaricides, nematodes, molluscicides, rodenticides, growth regulators, repellents and biocides. When referring to protection of plants, the term Plant protecting products (PPPs) is also used (European Commision, n.d). PPPs are important for the yield of cultivated plants, protecting them from pests and competition from weeds. Data from 1988-1990 shows that PPPs protected ~32% of the worlds eight major food crops (Oerke et al., 2004). Pesticide use has increased in the last half-century, and in 2012, ~3 billion kg were produced (EPA, 2017). Of the PPPs, herbicides have the highest market share, followed by insecticides with a market share of 29% (EPA et al., 2017). Insecticides are consisting of five major classes: chlorinated hydrocarbons, organophosphorus compounds, methyl carbamates, pyrethroids and neonicotinoids (Tomizawa et al., 2011)

1.2.1 Neonicotinoids

The newest insecticides are the neonicotinoids, divided into two groups: N-nitroguanidines (contains a nitro-group) and N-cyano amidines (contains a cyano-group). N-nitroguanidines

include imidacloprid, thiamethoxam, clothianidin, dinotefuran and nitenpyram. N-cyano amidines include acetamiprid and thiacloprid (Jeschke et al., 2011). Neonicotinoids were launched in 1991, and since then, they have become the most widely used insecticides in the world (Casida et al., 2013). The discovery was awarded an international price in Research in Agrochemicals by the American Chemical Society (Tomizawa et al., 2011).

In the present study, the following five properties of neonicotinoids are suggested as causes of their success:

1) They are highly soluble, small molecules (250 and 300 Da (Simon-Delso et al., 2015)) with a low octanol/water partition coefficient (K_{ow}) of -0.55 to 1.26 (UOH, 2017). This gives them systemic properties which allow them to enter plant tissue and to translocate into all parts of the plant (primarily via xylem transport) giving the whole plant protection. This also protects the plant indirectly by hindering transmission of viruses that use insects as vectors (Magalhaes et al., 2009; Nauen et al., 2008)

2) When the neonicotinoids were introduced to the market, there was an expanding resistance against one of the main insecticides at the time, organophosphates, and there was a need for a new solution (Brattsten, 1990; Simon-Delso et al., 2015).

3) In general, vertebrates have a lower number of nicotinic receptors in the nervous system with high affinity to neonicotinoids compared to insects (Simon-Delso et al., 2015), and thus neonicotinoids has a lower binding affinity to vertebrates than insects. As a result of the difference, neonicotinoids show higher toxicity to invertebrates than vertebrates (including human) (van der Sluijs et al., 2013).

4) Use of neonicotinoids on seeds or in the soil, rather than spray application, is supposed to make them safer for agricultural workers compared to other insecticides like carbamates and organophosphates. Also, if sprayed they do not have a high vapour pressure, (values of 2.8×10^{-8} and 0.002 mPa at 25 °C), and gaseous forms are temporary (Bonmatin et al., 2015).

5) Their half-life in aerobic soil conditions is long, resulting in long-term crop protection (Bonmatin et al., 2015; Fossen, 2006).

In the mid-2000s, several studies raised awareness of the effect neonicotinoids had on beneficial pollinators, especially honeybees and bumblebees (Wood et al. (2017) and references therein). This lead to the European Safety Authority (EFSA) conducting a risk assessment that ended in a moratorium on three neonicotinoids in 2013 (clothianidin, thiamethoxam, and imidacloprid) (EFSA, 2013a, 2013b, 2013c). The scientific information is currently being evaluated by EFSA, and in the end of 2017 a new risk assessment is to be completed.

While the success of neonicotinoids is proven by their market share, the ecological consequences are starting to visualize. It appears that the success factors of neonicotinoids are the same factors that make them detrimental for wildlife.

1.3 Neonicotinoids: honeybees and other bees

There is an ongoing debate concerning neonicotinoids and their possible harm to bees. On the one side there are arguments about the reliability of the research conducted, and the critique is among others that the doses used in the studies are above the field-realistic concentration, and that bees will avoid food containing neonicotinoids (Carreck et al., 2014). These arguments have recently been refuted, as it has been seen that bees do not recognize the toxicant (Kessler et al., 2015). Bees recognize toxic non-nutrients with gustatory neurons in sensilla on the proboscis (Wright et al., 2010), but this recognition is absent in *A. mellifera* and *B. terrestris* when exposed to neonicotinoids (imidacloprid, thiamethoxam, clothanidin)(Kessler et al., 2015). When neither *A. mellifera* nor *B. terrestris* recognize neonicotinoids, there is not enough knowledge to conclude on whether the same mechanism is present in other bee species or not.

There are approximately 20 000 bee species in the world (Michener, 2007), and they live in different habitats and have different traits. There are solitary bees that live alone, sub social bees that care for their broods (eggs, larvae and pupa), highly eusocial bees that always lives in colonies where the queen cannot live alone, primitively eusocial bees where the queen lives alone for a period of time, semi-social bees where the colony consists of females of the same generation, communal colonies where two or more females use the same nest, and quasisocial bees where a few females cooperate on looking after broods and the nest (Michener, 2007). In addition, there are large differences in adaption to various environments, and there are bees with long tongues or short tongues, parasitic bees and bees who nest in the ground or in threes (Michener, 2007). These variations between the different species in this large group of insects

illustrate that differences in species sensitivity to neonicotinoids most likely exist. The limited research that has been done on the subject suggests that there are indeed differences between bumblebees and honeybees when it comes to sensitivity to neonicotinoids (Alkassab et al., 2017; Cresswell et al., 2012) and between bumblebees and solitary bees (Scott-Dupree et al., 2009). Moreover, there are differences in sensitivity between different species of bumblebees (Baron et al., 2017). It seems that solitary bees are the most susceptible to neonicotinoids, while honeybees are the most resistant, and bumblebees are somewhere in between depending on the bumblebee species. Another consideration when it comes to species difference is that neonicotinoids can be stored in the soil long after application (A. Jones et al., 2014; Woodcock et al., 2017), making the soil a possible exposure route for the bees nesting in the soil. These studies show that the use of honeybees as an indicator species may give results that are not applicable to other bee species, and more research on how different PPPs affect different bee-species is needed.

1.3.1 Sub-lethal effects

Sub-lethal effects can appear at different levels of biological organization within an organism, including the nervous system, the muscular system and the reproductive system (Alkassab et al. (2017) and references therein). Impairment of important processes in the subcellular level can affect organs and functions that are essential for the organism, and as a result this can have negative influence on the whole population. Such impairment has been seen for instance as loss of worker survival, lower reproduction and altered forager behaviour (Mommaerts et al., 2010).

To evaluate the potential risk PPPs have on pollinators, guidelines like EFSAs "Guidance on the risk assessment of plant protection product on bees" are used (EFSA, 2013d). They state in this risk assessment that sub-lethal effects have the potential to affect the development and the survival of the colonies'. However, the test presented in the assessment is only identifying unacceptable harm defined in the specific protection goals outlined by EFSAs panel on plant protection products and their residues (EFSA, 2010). Sub-lethal effects are not assessed as the lack of information hindered EFSA to make quantitative links between sub-lethal effects observed in laboratory studies and effects on colonies (EFSA, 2013d).

Cognitive performance

Cognitive performance is essential for bees, as they forage in a shifting environment with changing flower recourses. The dynamic nature of their environment demands that the bees learn to forage on highly rewarding flowers in an efficient way, and locate different patches of flowers in addition to keeping track of their relative position in the environment (Chittka et al., 1999; Menzel et al., 1996). To learn and memorize the different flower cues (odour, size, pattern, height, color etc.), the bees need to associate these with reward in form of nectar and pollen (Chittka et al., 1999). The recognition of flowers can either be an initial preference for a specific color, (bumblebees have a preference for blue and violet flowers (Chittka et al., 2004; N. E. Raine, Ings, Dornhaus, et al., 2006)), by trial and error (Chittka, 1998) or by learning from others (Dawson et al., 2013).

Learning in bumblebees are connected to the mushroom bodies of the bumblebee brain, and as they learn more, these parts of the brain grow (B. M. Jones et al., 2013). When exposed chronically to field-realistic doses of imidacloprid, the synaptic units in the bees mushroom bodies decrease, and both olfactory and visual learning and memory are impaired (Peng et al., 2016). Neonicotinoid exposure can therefore affect the bees ability to remember the location of floral resources and their flower handling skills, ultimately reducing their amount of food intake (Thompson et al., 2015). Several studies have shown adverse effects of imidacloprid exposure on visual learning (Decourtye et al., 2004; Han et al., 2010), short- and long-term memory (Tan et al., 2015) and navigation (Fischer et al., 2014). In addition the access time of flowers (i.e. the time it takes before a bee find the nectar within the flower) is impaired when a bee has been exposed to imidacloprid (Morandin et al., 2003).

Effects on Reproduction

For bumblebees that rely on cooperation for the survival of the colony, a reduction in reproduction can lead to food-shortage and thus impairment of the colony's reproductive success. Exposure from imidacloprid has been shown to decrease reproduction in bees as the viability of the sperm stored in queen spermatheca decrease with 50% when honeybee queens are exposed to sub-lethal doses (Chaimanee et al., 2016). Moreover, imidacloprid has a downregulating effect on vitellogenin and hexamerin 70b in honeybee queens, suggesting reduced longevity and reproduction (Chaimanee et al., 2016). For bumblebees, exposure to imidacloprid also lead to decreasing feeding rates, affecting brood production (Laycock et al.,

2012; Mommaerts et al., 2010) and the number of surviving broods (Scholer et al., 2014; Tasei et al., 2000).

Effects on food intake and locomotion

The main target for neonicotinoids is the nicotinic acetylcholine receptors (nAChR), but it is shown that they also affect other organs like the midgut and Malpighian tubules by altering the tissue structure, nuclei, mitochondria and endoplastmatic reticulum (Catae et al., 2014). As these organs are responsible for metabolism and excretion, it is likely that the harm caused to these organs will contribute to a decrease in uptake of both nectar and pollen. This decrease has been shown in several studies (Baron et al., 2017; Cresswell et al., 2012; Gill et al., 2012; Laycock et al., 2012). Moreover, reduced feeding on pollen is suspected to have a negative effect on ovary development (Baron et al., 2017) with reduced brood production as a consequence.

Locomotion is essential for a bumblebee colony's ability to forage and care for broods, and a key factor for locomotion is metabolism. In insects, metabolism is linked to respiration (Contreras et al., 2010), and CO_2 can be released in discontinuous ventilation events, or bursts, by abdominal ventilation movements (AVM) (Brattsten, 1990; Kuusik et al., 2002). Imidacloprid is shown to impair AVM burst production and duration (Hatjina et al., 2013) and thus has a negative effect on the $CO_2 - O_2$ exchange and on metabolism.

1.4 Motivation

Since PPPs are essential for the needed growth in agriculture, and thus food-supply for humans, it is important to understand the risks they pose for non-target organisms and especially those that provide ecosystem services to agriculture itself. Several reviews have been written since the EU moratorium, and they highlight the urgent need for more research on sub-lethal effects of neonicotinoids on wild bees, and bumblebees in particular (Alkassab et al., 2017; Goulson, 2013; Wood et al., 2017). A major criticism of previous studies relates to the effects of field-realistic dosages, i.e. exposure levels relevant for what the bees experience in real life situations (Blacquiere et al., 2012). Also, there is a lack of studies investigating effects of imidacloprid on the whole hive, assessing effects on a population level.

Even if EFSA suggests a total ban for imidacloprid, clothianidin, and thiamethoxam and if the European Commission proposes legislation that the European Parliament adopts, the three neonicotinoids currently under the moratorium are still legal in countries outside the EU. The regulatory bodies in the US (US environmental agency, EPA) and in Australia (Australian Pesticides and Veterinary Medicines Authority, APVMA) are not advising to restrict the use of neonicotinoids. Understanding the impacts these neonicotinoids have on pollinators is of particular importance as adverse effects on these organisms will influence agriculture, and thus food production on a global scale.

1.5 Aims and hypotheses

The main aim of the present study has been to test how exposure to sub-lethal concentrations of imidacloprid affects bumblebees by using a new method. To accomplish this, a quantification of the following has been done: 1) behavioural effects - the impairment of ability to learn and to visit flowers (pollinate), 2) reproduction and health of the hive through measurements of the frequency of dead broods and adults, and 3) the amount of food consumed. In particular, the objectives and hypotheses tested were as follows:

Objective 1: Develop an experimental setup that allows the study of behavioural effects of plant protecting products on bumblebees. Extending an established method (N. E. Raine, Ings, Ramos-Rodriguez, et al., 2006) that assesses individual learning by including interaction between bumblebees and by including exposure to a PPP is expected to be feasible.

Objective 2: Identify and quantify the potential behavioural effects of the PPP imidacloprid on a non-*Apis* species, *Bombus terrestris,* in a dose-response manner.

H1: Bumblebee foraging is impaired in a dose-dependent manner when exposed to increasing doses of imidacloprid.

H2: Bumblebees' ability to select rewarding flowers is impaired in a dose-dependent manner when exposed to increasing doses of imidacloprid.

H3: Bumblebee locomotor activity is impaired in a dose-dependent manner when exposed to increasing doses of imidacloprid.

Objective 3: Assess the health of *Bombus terrestris*-hives after chronic exposure to the PPP imidacloprid at increasing doses.

H4: Reproduction is reduced by imidacloprid in a dose-dependent manner.

H5: Survival of offspring is reduced by imidacloprid in a dose-dependent manner.

H6: Survival of bees (worker, males, and queens) is reduced by imidacloprid in a dose-dependent manner.

H7: Storage of nectar is reduced by imidacloprid in a dose-dependent manner.

H8: The amount of pollen and nectar eaten by the bumblebees is reduced by imidacloprid in a dose-dependent manner.

2 Materials and method

2.1 Study species

The species used in this study is *Bombus terrestris dalmatinus* Dalla Torre, 1882 (Figure 1), which is a subspecies of the Buff tailed bumblebee (*Bombus terrestris* L. (1758)) (Rasmont et al., 2008). Bumblebees (tribe Bombini, genus *Bombus)* are in the order Hypenoptera, family Apidae, subfamily Apinae, together with other tribes like the true honey bees (Apini), orchid bees (Euglossini) and Stingless bees (Meliponini) (Michener, 2007). There are approximately 250 bumblebee species in the world, assigned to 15 subgenera (Cnaani et al., 1997). Bumblebees are found from the lowland tropical forest to the Arctic tundra and are most abundant in temperate and cold regions of the Northern Hemisphere and alpine habitats (Williams, 1998)

Bumblebees are holometabolous insects that undergo complete metamorphosis, and each bumblebee goes through four stages: egg, larva, pupa, and adult. They have a haploid system of sex determination: Fertilized eggs develop into females, and unfertilized eggs develop into males. The queen usually mates only once, and with the sperm stored in a spermatheca, she controls the sex of the offspring by either liberating or not liberating the sperm when the eggs pass through the oviduct. The larvae stage consists of four stages, of which the last stage is called prepupa (Cnaani et al., 1997; Michener, 2007).

B. terrestris dalmatinus is the most widely reared subspecies and it has evolved into a subspecies so distinct that it is described as a true species (Rasmont et al., 2008). It originates from the Balkans, the Urals, and Asia (Rasmont et al., 2008). It forms larger colonies and is less aggressive than other subspecies. This attribute makes it easy to breed, and it is a common bumblebee in greenhouses in Europe, North and South America and Asia (Acosta et al., 2016; Dafni, 1998). *B. terrestris* is mass bred both in Norway and in the rest of Europe (Ødegaard et al., 2015). This species is one of the most numerous in Europe and is found from the north in Narvik, Norway to Argentina in the south and east to Kazakhstan and Turkmenistan (Martinet et al., 2015; Schmid-Hempel et al., 2014; Ødegaard et al., 2015). It has also been introduced to Japan, Chile, China, New Zealand, South Africa, Mexico and Argentina (Ødegaard et al., 2015). Due to its importance for agriculture and wildlife, to its availability and to its calm temperament, *B. terrestris* was chosen for this study.

Figure 1: The study species *Bombus terrestris dalmatinus* inside one of the hives.

B. terrestris was first discovered in Norway in 1951, and it is now our most common bumblebee. Wild *B. terrestris* queens are found in Norway from early March to mid-November. They nest on the ground, and the colonies can contain several hundred individuals (Ødegaard et al., 2015).

Like all bumblebees except the cuckoo bumblebees (*Bombus Psithyrus sp,*), Buff-tailed bumblebees are social insects; the hive consists of a monandrous queen and workers (Goulson et al., 2002). The workers are mostly nectar gatherers, followed by pollen and nectar gatherers, while specialist pollen gatherers are rare (Goulson et al., 2002). Worker bumblebees wary in size, and different-size workers have various functions (allotheism). Larger bumblebees are usually foragers, while small bumblebees tend to do within-nest tasks (Dyer, 2002). Also, nectar gatherers are generally larger than pollen gatherers (Goulson et al., 2002).

The Queen initially produces two broods (Michener, 2007). The first brood consists of 3-8 egg cells, with on average two eggs in each. The eggs will develop into larvae, and after some time, each of these larvae will make its own cocoon. On top of the cocoons, new egg cells (6-13) are laid, with each egg cell containing on average five eggs. The Queen then stops laying eggs, and the emergence of the first workers is just in time to assist the queen in the feeding of the second batch of larvae during their last larval stage. After the pupa formation of the second brood, the

next stage in the nest development begins, and the number of egg cells increases linearly in time. In this juncture, there are approximately eight eggs in each egg cell. The mortality of both eggs and larvae is low as long as the hive has not reached competition point, where aggressive interactions and egg laying from workers has started. (Duchateau et al., 1988)

2.2 Study chemical: imidacloprid

Imidacloprid (Figure 2) is one of the neonicotinoids that is currently under a moratorium in the EU. The CAS-name is 1-[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine (C9H10ClN5O29), and the IUPAC-name is (E)-1-(6-chloro-3-pyridyl-methyl)-N-nitroimidazolidin-2-ylideneamine. Imidacloprid, like the rest of the N-nitroguanidines, has shown to be more toxic to insects than N-cyanoamidines (Iwasa et al., 2004; Mommaerts et al., 2010). It is suggested that the higher toxicity observed in N-nitroguanidines is a result of higher metabolizing of the N-cyanomidines and variation between the different nAChRs subunits in bees (Blacquiere et al., 2012).

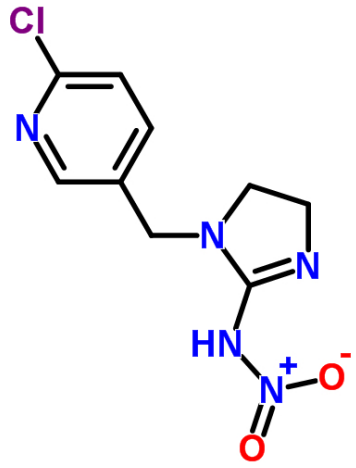

Figure 2: Molecular structure of the neonicotinoid imidacloprid (retrieved from Chemspider)

Like the other neonicotinoids, imidacloprid acts as an agonist on the nAChR receptors of insects, which are placed in the synaptic neuropil regions (Mushroom bodies) of the insects' central nervous system (Jeschke et al., 2013). They induce the same agonistic activation of receptors as the natural neurotransmitter acetylcholine (ACh), by causing an inward current that leads to action potentials being generated (Jeschke et al., 2013).

2.2.1 Pesticide risk assessment

EFSA define the lethal pesticide concentration or dose as the concentration or dose at which half the members of a tested population are dead after a specified test duration (LC_{50} or LD_{50}). In addition to lethal concentrations, imidacloprid can cause sub-lethal effects defined as biochemical, physiological and behaviour endpoints (i.e. cholinesterase activity, survival, development, longevity, locomotion/mobility, navigation/orientation, feeding behaviour and learning performance). To determine the maximum concentration that does not give an effect "No Observed Effect Concentration" (NOEC) is used. To determine the lowest concentration that gives an effect, "Lowest Observed Effect Concentration" (LOEC) is used. LD_{50} and NOEL for honeybees have been summarized by EFSA and are showed in Table 1.

Table 1: LD_{50} and NOEC for acute and chronic oral and contact toxicity given as ng/bee or μg/L in sucrose solution. The concentrations are based on numbers from EFSA (EFSA, 2013b)

Type of exposure	**Response**
Acute: oral toxicity	LD_{50} = 3.7 ng/bee (active substance) LD_{50} = 5.6 ng/bee (formulation)
Acute: contact toxicity	LD_{50} = 81 ng/bee (active substance) LD_{50} = 42 ng/bee (formulation)
Acute: oral toxicity	NOEL = 1.2 ng/bee
Acute: contact Exposure	NOEC= <2.5 ng/bee
Chronic: toxicity	NOEC = 10 μg/L – 48 μg/L sucrose solution
Chronic: Sub-lethal effects	NOEC for foraging behaviour = 20 μg/L sucrose solution
Chronic: Sub-lethal effect	NOEC from olfactory memory = 24 μg/L sucrose solution

EFSAs risk assessment is outlined for honeybees, and there is no specific risk assessment for bumblebees and solitary bees (EFSA, 2013d). Assessment factors are used to make up for the lack of direct risk assessment for wild bees. The assessment factor for extrapolating from a honeybee endpoint to an endpoint for bumblebees and solitary bees is 10, but there are many different bee-species, and it is challenging, most likely impossible, to find a number that fits them all.

The vast amount of studies on bees and neonicotinoids are conducted on honeybees, and while data for other species like bumblebees are growing, the knowledge on how neonicotinoids affect other bees is sparse. A comparison between honeybees and bumblebees shows that dietary imidacloprid in nectar causes decreased feeding rate in bumblebees and negative effects on their locomotor activity, while honeybees do not show any response (Cresswell et al., 2012). This finding indicates that there are differences that need to be addressed. The lack of studies comparing different bee-species under the same conditions makes it difficult to suggest that one species is more sensitive to neonicotinoids than other species.

2.3 Pilot study

Ahead of the present study, a pilot study was conducted to test the experimental setup and to get experience in handling bumblebees. The pilot study was based on published studies done on bumblebees and honeybees (Devillers et al., 2003; L. Evans et al., 2014; N. E Raine et al., 2008; N. E. Raine, Ings, Ramos-Rodriguez, et al., 2006). Different approaches were tested in the laboratory, and unforeseen challenges were dealt with as they appeared. Based on these pilot experiments described in Appendix A, a final experimental setup was designed.

The pilot was conducted during May – July 2015 at the Department of Biosciences, University of Oslo (UiO), with the help of the French student Johann Kieffer that had an internship at the University of Oslo. Three *Bombus terrestris* queens with developing broods were obtained from Pollinering AS in Bryne and placed in wooden nest boxes made in the workshop at the Department of Biosciences (referred to as "in-house"). The bumblebee colonies were bred at UiO, and when the colonies were large enough, the bumblebees were trained in a flying arena containing blue and yellow flowers. Each emerging bumblebee was tagged and placed in a wine cooler for sedation. As a result of lessons learned during the pilot, the final experimental set-up was changed and improved accordingly.

Improvements of experimental setup based on experience from pilot

Limited effect of the sedation of the bumblebees' due to a too high temperature in the wine cooler used resulted in the bumblebees removing the tags when released back into the hive. The time in the wine cooler varied from 10 – 120 min as the temperature in the wine cooler increased when the door was opened. The conclusion was thus that a wine cooler is not ideal for sedation of bumblebees.

It was challenging to remove the bees from the nest box as the bees had to be caught while being away from the other bumblebees to prevent them from crawling onto the device used for trapping bumblebees. This experience resulted in a change in the tracking of the bumblebees from tagging the thorax of the bumblebees with a number-tag to tracking them with cameras making the catching and trapping unnecessary.

In the pilot, a red light was used to eliminate colour preferences in the bumblebees. The red light complicated handling of the bumblebees as it was hard to see them. As the bumblebees only eat nectar and pollen inside a hive covered with a lid, the chance of connecting any colour with reward is small, and in the proceeding experiments, the red light was removed.

The bees did not visit the flowers as often as observed in previous studies e.g. (L. J. Evans et al., 2014a, 2014b; N. E. Raine, Ings, Ramos-Rodriguez, et al., 2006) and they tended to walk on the floor instead of flying. A possible reason for this was the positioning of the entrance too close to the ground of the flying arena. To motivate bees to fly and forage, the entrance was moved further up on the wall, the standard fluorescent lighting was switched to daylight-simulating UV-LED, and the flowers were filled with attracter (fructose/glucose/saccharose solution, 1.27 kg/L; Koppert B.V., Berkel en Rodenrijs, Netherlands).

In the pilot, it was observed that bumblebees had a preference for the yellow flowers over the blue flowers, 79% versus 21%. This observation resulted in a change of the planned rewarding colour from yellow to blue. Yellow was initially planned as rewarding flowers as bumblebees has previously been shown to have an initial preference for blue (Chittka et al., 2004; N. E. Raine, Ings, Dornhaus, et al., 2006). The preference for yellow might be due to the coloration of the artificial flowers; the yellow flowers were slightly green, and bees have an initial preference for green over blue (Giurfa et al., 1995).

To minimize the discrepancy between colonies, healthy bumblebees were considered as a premise for doing this experiment. Due to this premise, it was decided to obtain bumblebees to the main study from a company that does research on pollination and that works closely with universities around the world. As this was not the case for the company that provided the bumblebees in the pilot it was decided to use Koppert, a well-established company that meet all the criteria mentioned above. A change in bumblebee provider made the in-house made hives used in the pilot unnecessary as the bumblebees arrived in standard hive boxes from Koppert. The new hives resulted in a change in the set-up regarding the transfer of bumblebees

from the hives to the flying arena as the hive had a different design from the in-house made hives used in the pilot.

2.4 Experimental setup and design

The main experiments were conducted at Department of Biosciences, University of Oslo in the spring of 2017 (11. January – 25. May). The bumblebee behaviour part was performed in a climate room in the phytotron, and the preparation of imidacloprid in attracter solution and dissection of hives were conducted in the toxicology laboratory.

To assess learning and flower visitation of bumblebees, a custom-made flying arena covered with a transparent lid was used (130 × 100 × 35cm). The arena was made of Plexiglas® and covered with white plastic plates on the inside walls and the floor (Figure 3). To avoid dark spots that could disturb the video recordings of the bumblebee locomotor activity, white tape was used to cover the joints between the floor and the walls and the bolts keeping the flying arena together. The plastic plates were brushed with sandpaper to prevent reflections that would disturb the video analysis. The hive entrance was placed on the upper side of one of the walls so that the bumblebees were forced to fly out of the entrance, instead of walking straight onto the floor. Inside the flying arena, a landing platform was placed below the entrance, and a green coloured pattern made of tape was applied around the entrance to help the returning workers to orientate back to the hive (Figure 3 and Figure 4).

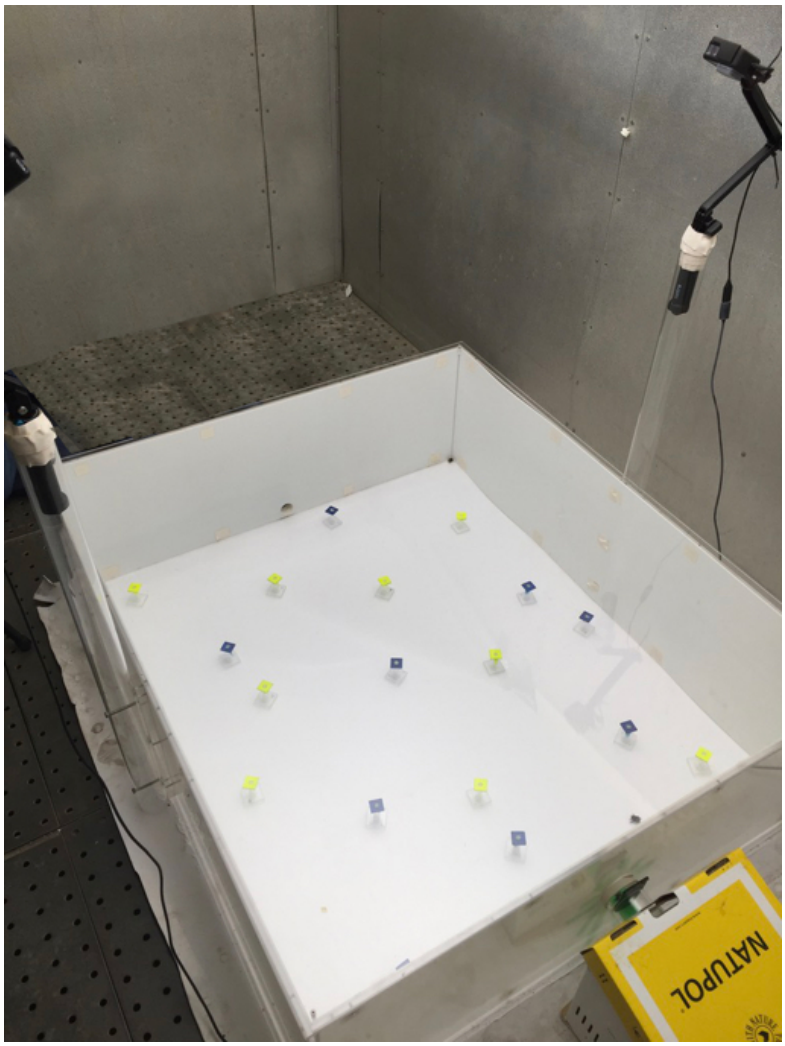

Figure 3: Experimental set-up showing the flying arena with 9 blue (rewarding) and 9 yellow (unrewarding) artificial flowers. The hive is the yellow box in the lower right corner of the image, connected to the flying arena with a tunnel. The camera and the camera mount are visible on the right side of the image.

Two cameras (GoPro Hero 5 Black) were used to film the behaviour in the flying arena; set to the specification ISO 3200, 60 frames per second, 2704 × 1520 pixels resolution and the "linear" lens setting. Each camera had a high-speed memory card (SanDisk Memory Card Secure Digital Micro 64GB SDXC Extreme 90MB/s UHS-1), and were connected to a USB-hub (Sandstrøm USB-hub m. 4 × USB 3.0-porter) for power supply. The videos were transferred to a hard drive (LaCie Porsche Design P9233 3TB USB 3.0) using the software GoPro Studio version 2.5.10. Each camera was connected to a frame (GoPro “The frame”) and mounted on (GoPro 3-Way Mount – Grip) attached to a Plexiglas® cylinder (60 cm) made in-house. The cylinders were bolted on the centre of each long side of the flying arena, allowing the cameras to film the whole flying arena from each side. The start and stop of filming were controlled by a remote control (GoPro Smart Remote) connected to the cameras with Wi-Fi. The remote control allowed for

synchronized filming and subsequent simultaneous analysis of recordings of the hive from different angles. The videos were transferred to an in-house storage facility run by the University (Abel, cod-node) for backup.

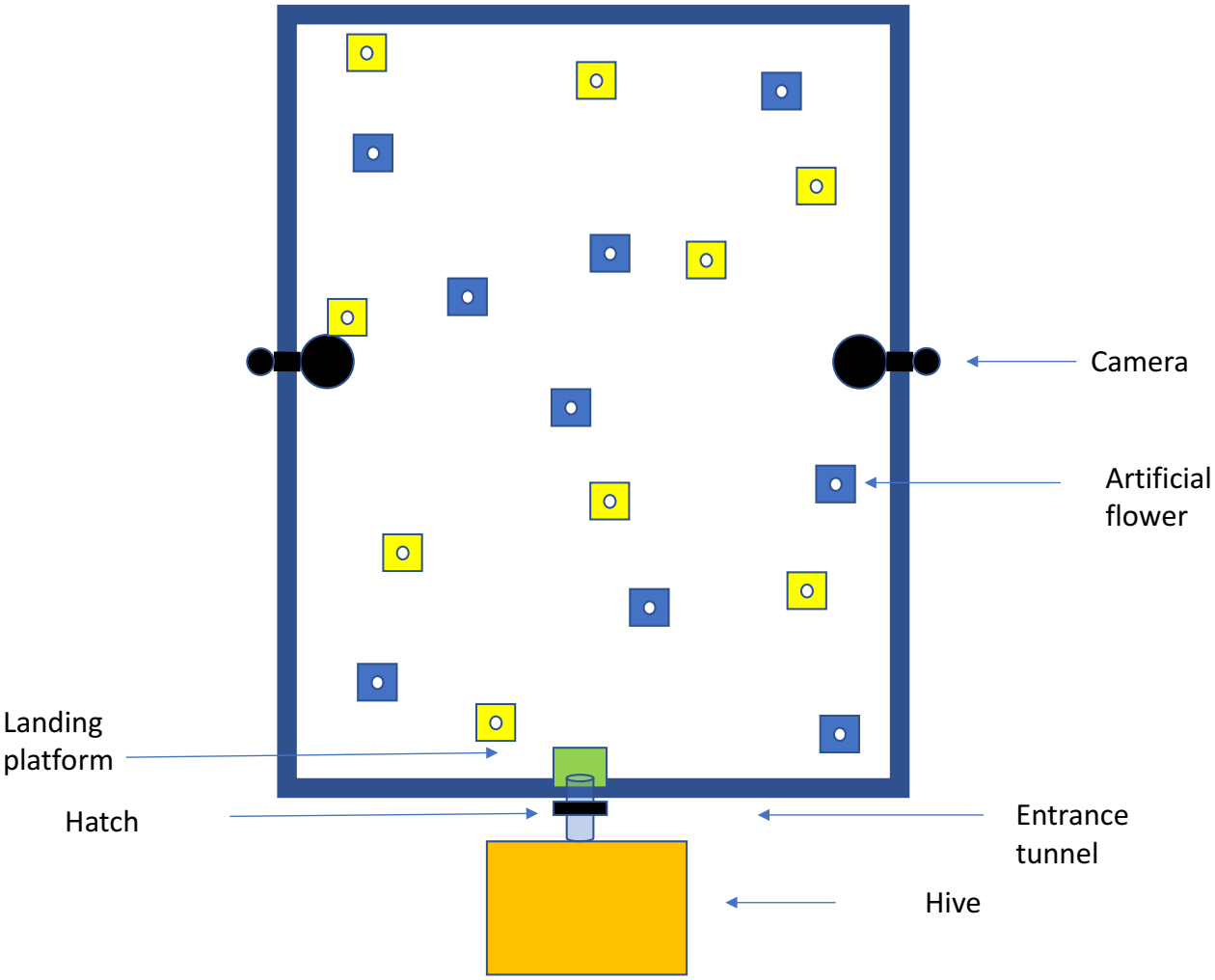

Figure 4: Schematic overview of the flying arena (130 × 100 × 35 cm) used in the experimental setup, showing artificial flowers as squares in blue and yellow. The positions of the cameras are showed on each of the long-sides of the flying arena, and the hive is the yellow box connected to the flying arena with a tunnel. The landing platform is showed as a green square in connection with the hive tunnel entrance.

Portable UV-lights (Valoya C series, NS12) with an array of UV-LEDs were attached to the roof of the climate room, centred above the flying area. The power adapter connected to the UV-LEDs delivered direct current, eliminating the problem of flickering, which is important due to the bumblebees flicker fusion frequency being 200 Hz (Srinivasan et al., 1985), as well as important for avoiding flickering in the video recordings. This particular light resembled natural daylight, including UV-light which has been shown to be useful for bumblebees when

they search for flowers (Peitsch et al., 1992). A diffuser sheet was placed between the light source and the flying arena to spread the light, i.e. to prevent the LEDs in the light array from looking like point sources of light.

To simulate flowers, 72 artificial flowers (24 × 24 mm), made in-house of Plexiglas®, were used: 18 yellow and 18 blue (Figure 5A) and 32 bi-coloured in blue and yellow (Figure 5B). The artificial flowers were attached to a foot (12.5 mm diameter, 40 mm length, with a 24 × 24 mm base) to prevent the flowers from falling over. The base was brushed with sandpaper to prevent reflections. In the centre of each artificial flower, a hole in the size of a 1.5 mL micro centrifuge tube was made. The flowers were spray-painted using Biltema plastic primer and fluorescent sparVAR spray-color RAL 1026 fluorescent yellow and 3107 fluorescent blue.

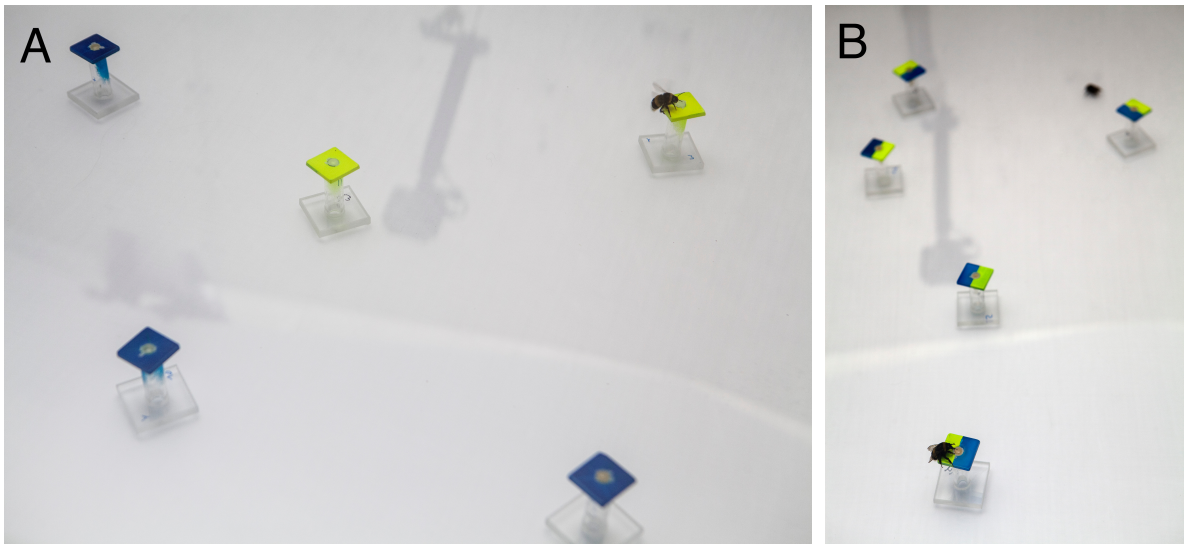

Figure 5: Detail pictures of the flying arena during testing (A) and training (B). The left picture shows two yellow (unrewarding) and three blue (rewarding) artificial flowers. The circular marks in the centre of the flower are containing an Eppendorf tube that allows for a small amount of artificial nectar to be placed in the flower. In the upper right corner a B. terrestris is visiting a yellow (unrewarding) flower. The right picture shows five bi-coloured artificial flowers, one bee on a flower and one bee on the floor. Photo: Simen Kjellin

Before each training or testing, 18 micro centrifuge tubes (1.5 mL) were weighed by placing empty tubes in a micro centrifuge rack on a laboratory balance (Sartorius CP622). The weight was noted before and after adding liquid in the micro centrifuge tubes. For training, all 18 tubes were filled with artificial nectar and placed in the bi-coloured flowers. For testing nine were filled with artificial nectar and placed in blue flowers, while nine were filled with tap water and placed in yellow flowers. To reduce smell trace, the artificial flowers were washed with 30% ethanol in water solution between each training or test in the flying arena, in addition two sets of artificial flowers were used.

20 queenright colonies (presence of a fertile queen) of *Bombus Terrestris,* each containing 80-100 workers and brood at various stages of development was obtained from a continuous mass rearing program (Natupol Beehive; Koppert B.V. Berkel en Rodenris, Netherlands) through the Norwegian company LOG. Import of bumblebees to Norway was applied for, and it was approved by the Norwegian Environment Agency. All colonies were laboratory-raised, meaning that the bumblebees had never seen flowers or colours before the experiments. Two of the colonies were used as test colonies for choosing between two different flying arenas. These were the flying arena which was employed in the pilot study, and a higher flying arena (98 × 100 × 198 cm) made of a steel-frame clothed with mosquito net with a door covering one side providing access to install artificial flowers. The two test colonies were in addition used to investigate the bumblebees' ability to forage on the artificial flowers, for problem-solving regarding capturing of the bumblebees, for weighing of the empty nectar bag, for testing the mixing of fluid in the nectar and for fitting of equipment such as the tunnel between the hive and the flying arena. One colony was sick on arrival, with dead and trembling bumblebees which did not eat, resulting in the exclusion of this hive from the experiments.

The bumblebee colonies were delivered in standard plastic nest-boxes (25.4×22.9×12.7 cm) covered in a cardboard box. The bumblebees remained in these nest boxes for the duration of the study, except when participating in experiments in the flying arena. Under the nest, there was a container with 1000 mL artificial nectar. This nectar was provided to the bumblebees' *ad libidum* through a tube with a sponge from which the bumblebees could access the artificial nectar. The bumblebees were allowed to feed on artificial nectar (Attracter: fructose/glucose/saccharose solution, 1.27 kg/L; Koppert B.V., Berkel en Rodenrijs, Netherlands) at all times, except for the last day before training or testing. The removal of nectar one day before testing was done to encourage bumblebees to forage for nectar in the flying arena. The nests were kept in a controlled environment (28 °C and 55% relative humidity) for the full duration of the study, except during transport from Koppert (~ 1 day).

During exposure, the bumblebees were fed freeze-dried pollen provided by Pollinering AS, Bryne, Norway. The freeze-dried pollen was mixed with 50% sugar water (1.5 dL water per 3 dL dried pollen) using a hand blender under heat until the pollen pellets were dissolved and the mixture was homogenized. The pollen was stored in a fridge (+4 °C). To feed the bumblebees, pollen was pushed through the sprinkles in the inner plastic hive, in the same place each time. The feeding followed a schedule where the colonies were split into two groups. Each group was

fed with 4-5 g of pollen every second day. The schedule was set up such that the colonies were starved for one day before testing in the flying arena.

An optional hatch that allowed bumblebees to enter, but prevented bumblebees from coming out of the hive, was a part of the inner nest box from Koppert. This hatch was removed, and a tunnel (length: 8 cm, outer diameter: 6cm, inner diameter: 5 cm) was placed on the inner hive, allowing bumblebees to enter the tunnel to move between the hive and the flying arena (Figure 6). The tunnel was wide enough to allow bumblebees to pass each other. While mounting the tunnel on the flying arena, a piece of paper was placed in the hole to prevent the bumblebees from escaping. The paper was installed at the same time as the hatch was gently removed, the hatch in the tunnel was closed, and additional escaping routes, e.g. as small holes between the tunnel and the inner hive, were sealed with tape to prevent the bumblebees from escaping.

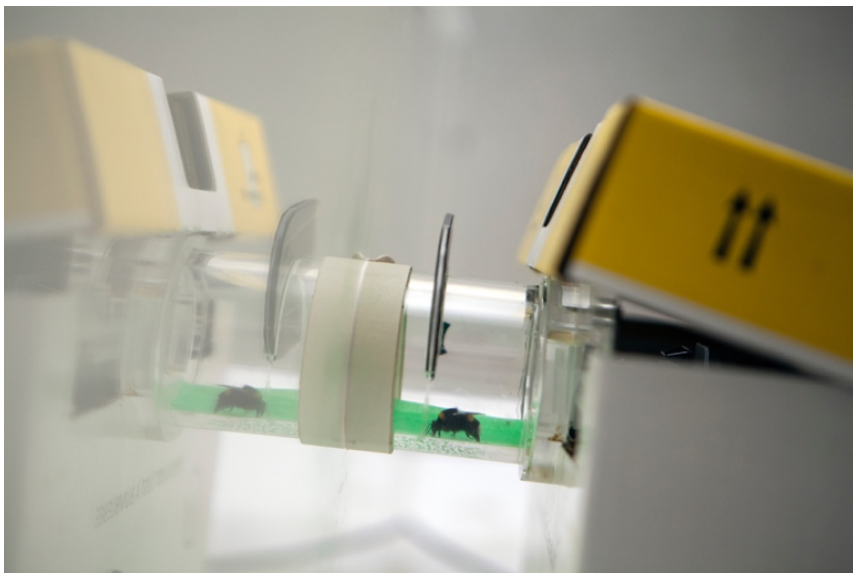

Figure 6: The figure shows the tunnel connecting the hive to the flying arena, and the optional hatch in the tunnel allowing for the control of bumblebees entering the flying arena. Photo: Simen Kjellin.

To estimate the effects of exposure to imidacloprid on the bumblebees, two types of response variables were measured; learning and flowers visitation, and the health of the hive. To assess learning and flower visitation, the bumblebees were first trained on 18 bi-coloured, blue and yellow, artificial flowers in the flying arena. All flowers contained reward in the form of artificial nectar to allow color-naïve bumblebees to associate both blue and yellow color with reward. After eight days of diet exposure to imidacloprid a maximum of 15 bumblebees were allowed to forage on 9 blue and 9 yellow flowers. The blue flowers were rewarding, containing artificial nectar, while the yellow flowers were unrewarding containing tap water. The learning

and flower visitation in exposed, and control, foragers were assessed by filming the bumblebees' exploration of flowers in the flying arena. The recordings were processed using a custom-made computer program (Bumblebee identification software) made by Henrik Anderson Sveinsson, PhD-student in Computational Physics (se Appendix B). The data extracted from the video were the identities of visited flowers, and the time frames during which bumblebees spent time on each flower. To assess the physical condition of the hive, each hive was dissected after the bumblebees had been tested in the flying arena, and living and dead: adults, pupa, big larva, small larva and queens, and empty as well as full honeypots were counted. Food consumption was also assessed by observing whether pollen was eaten or not, and the amount of nectar consumed during the exposure was measured by weighing the nectar bag before and after the exposure period. A timeline of the stages of the experiment, as well as explanatory variable time frames (for statistical analysis) is shown in Figure 7.

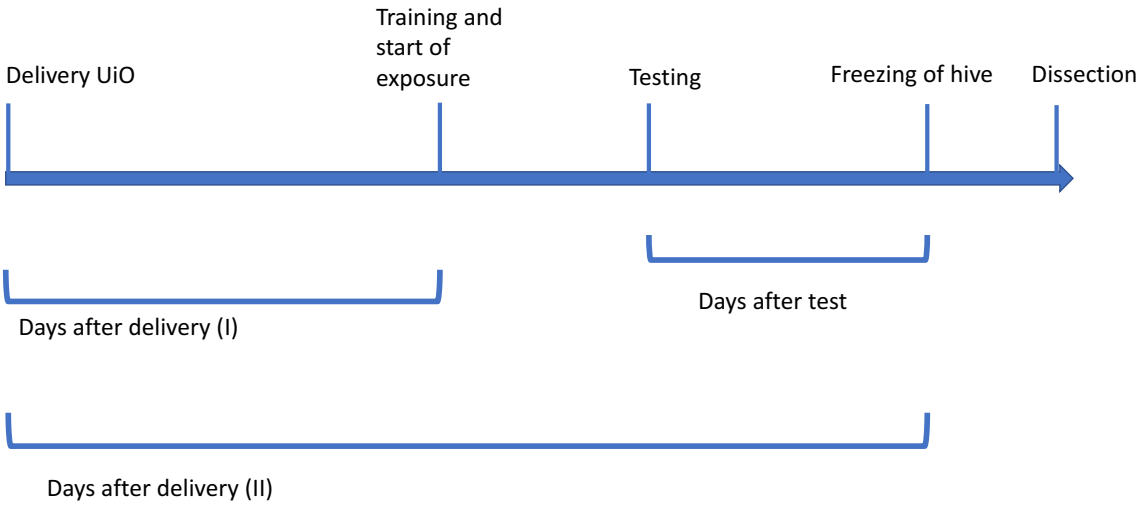

Figure 7: Overview of the timeline of the experiments starting with the arrival of the bumblebees hives at UiO, continuing with the exposure period lasting from the training to the freezing of the hive. The lower half of the figure describes three timelines: the days after delivery to the start of the exposure named "Days after delivery (I)", the days after test to the freezing of the hive, named "Days after test", and the days after delivery to the freezing of the hive, named "Days after delivery (II)".

2.5 Treatment

Colonies were randomly assigned to the different treatment levels and exposed for eight days, starting on the day of training and ending one day before the test. The bumblebees were exposed

orally via drinking artificial nectar *ad libitum* from the nectar bags. The experiment was blinded from the onset of the treatment until the dissection of hives was finished.

Each treatment level of imidacloprid was considered a group, and each hive regarded as a replicate. There were four groups: 0 μg/L (control), 1 μg/L, 10 μg/L and 100 μg/L imidacloprid. Each group consisted of four colonies (out of 16 colonies in total). The dosages used were set from field realistic dosages, based on studies done on residuals of imidacloprid in plants (Table 2). The concentration of 10 μg/L is on the higher end of the field realistic range, while the 100 μg/L was used to assess the effects of imidacloprid at extreme dosage levels. Due to the systemic properties of imidacloprid, this PPP will appear in both nectar and pollen in the field, whereas in this study the bumblebees were exposed only through nectar.

Table 2: Overview of the range of detected concentrations of imidacloprid relative to the exposure pathway in plants. The numbers are retrieved from literature concerning concentrations of imidacloprid in plants.

Residuals	Country	Reference	Plant
3.9 ± 1.0 μg/kg in pollen	Germany	(Schmuck et al., 2001)	Sunflower
31.9 ± 1.0 μg/kg in nectar			
0.5 - 36 μg/kg in pollen	France	(Laurent et al., 2003)	Sunflower
~10 μg/kg in capitule	France	(Bonmatin et al., 2003)	Sunflower
~3 μg/kg in pollen			Maize
2.1 μg/kg in pollen	France	(Charvet et al., 2004)	Maize
6.6 μg/kg in male flowers			
4.1 μg/kg in stems and leaves	France	(Bonmatin et al., 2005)	Maize
6.6 μg/kg in male flowers			
2.1 μg/kg in pollen			
1.1 – 5.7 μg/kg in pollen loads from honeybees	France	(Chauzat et al., 2006)	Pollen loads from honeybees
6.6 μg/kg in nectar	USA	(Krischik et al., 2007)	Buckwheat
10 ± 3 μg/kg in nectar		(Stoner et al., 2012)	Squash
10 ± 8 μg/kg in pollen			
9.58 ±0.8 μg/L in nectar	USA	(Byrne et al., 2014)	Citrus

2.6 Laboratory procedures

Pure imidacloprid (Sigma Aldrich, UK) was dissolved in distilled water and dilution was performed to obtain the right concentration. To avoid using acetone or dimethyl sulfoxide (DMSO) which might affect the results (Cresswell et al., 2012), a dilution scheme that kept the concentration of imidacloprid in the water below 610 mg/L were chosen, as this is the limit for precipitation in imidacloprid (Lewis et al., 2016). To achieve blinding of the doses, the dilutions were done such that the dilution factor of the last dilution (final solution to nectar bag) was constant between doses (1:99), resulting in the amount of final solution to be added to the nectar bag being independent of the desired concentration level of imidacloprid in the nectar bag. This

dilution procedure made it possible to change the amount of solution applied to the nectar as a response to the amount of nectar in the bag, not the concentration of the solution.

Imidacloprid breaks down quickly in water exposed to light at wavelengths between 200 and 300 nm (Zheng et al., 2004). Due to the rapid degradation, the stock preparation was performed in a dim room, and the stock and diluted solutions were kept in containers covered with aluminium foil and stored at +4 °C out of UV-light (the half-life of imidacloprid in aqueous solutions is 1.2 hours at 290 nm radiation). The nectar bag was not exposed to light as it was inside a cardboard box that was placed inside the outer cardboard box of the hive. Degradation of imidacloprid is not observed when it is kept in the dark in an aqueous solution (Moza et al., 1998).

To test for precipitation, 1.5 mL from a solution with a concentration of 10 000 µg/L imidacloprid was transferred using a micropipette into a 500 mL glass bottle, and 200 mL of artificial nectar was added. The bottle was covered with aluminium foil and placed in a fume hood overnight. No precipitation was observed. To test whether the imidacloprid solution would mix in the nectar bag, 50.2 mg of methyl blue powder was weighed on an analytical balance (Mettler Toledo AG204), using a disposable weighing boat. The powder was transferred to a 500 mL bottle; the remaining powder was removed from the weighing boat using distilled water from a 100 mL bottle, this gave a concentration of 502mg/L methyl blue. 9.17 mL of the solution was added to a 1247g nectar bag using a micropipette, and the solution was mixed thoroughly by turning and pressing the bag. This resulted in the blue color spreading throughout the nectar bag.

To calculate the right amount of volume from the stock solution needed to make the intermediate and final solution, the dilution equation below was used:

$$V_1 C_1 = V_2 C_2 \qquad \text{(Equation 1)}$$

Where V_1 is the volume of starting solution required to make a new solution, C_1 is the concentration of the starting solution, V_2 is the final volume of the new solution and C_2 is the concentration of the new solution. In these dilutions, the unknown was the volume of starting solution, V_1,

Stock solution

40,1 mg imidacloprid powder was weighed in a disposable weighing boat on a Mettler Toledo AG204 Analytical Balance. The powder was transferred into a 500 mL bottle, and the remaining powder was flushed from the weighing boat into the 500 mL bottle using distilled water from a container of 100 mL, before adding the rest of that 100 mL of water. Imidacloprid and water were mixed using a magnet agitator and a heater (Fethnika rotamix) at temperature setting 4, and 400 rpm; this gave a temperature of 45 °C in the solution. The resulting stock solution had a concentration of 401 mg/L.

Final solution

Dose 1μg/L: From the stock solution 791 μL was transferred with a micropipette (Thermo finnpipette 200-1000μl) into a 50 mL volumetric flask and water was added, so the volume reached the line of the flask. This gave a concentration of 6.3 mg/L. The solution was stored in a 50 mL centrifuge tube. From the intermediate solution, the above procedure was repeated, and a final solution with the concentration of 100 μg/L was obtained.

Dose 10μg/L: From the stock solution 2,500 mL was taken out with a micropipette (Eppendorf 5000) into a 50 mL volumetric flask and water was added, so the volume reached the line of the flask. This gave a concentration of 20 mg/L. The solution was stored in a 50mL centrifuge tube. From the intermediate solution, the above procedure was repeated, and a final solution with the concentration of 1000 μg/L was obtained.

Dose 100 μg/L: From the stock solution 7,906 mL was taken out with a micropipette (Eppendorf 5000) into a 50 mL volumetric flask and water was added, so the volume reached the line of the flask. This gave a concentration of 63.2 mg/L. The solution was stored in a 50 mL centrifuge tube. From the intermediate solution, the above procedure was repeated, and a final solution with the concentration of 10 000 μg/L was obtained.

An overview of the intermediate and final solutions is found in Table 3.

Addition of final solution to the nectar bag

To prepare for the last step of dilution, nectar was removed from the bag of one of the test hives, and the bag was cleaned and dried in a drying cabinet for 48 h. The empty and dry bag was then

weighed to 36 g, and this weight was assumed for all nectar bags. To measure the right amount of final stock, the following equation was used.

$$V_s = \frac{C_2}{C_1 - C_2} \frac{M_{\text{measured}} - M_{\text{plastic}}}{\rho} \quad \text{(Equation 2)}$$

V_s is the amount of stock to be added, C_1 is the concentration in final solution, C_2 is the concentration of the dose, $M_{measured}$ is the mass of the nectar bag, $M_{plastic}$ is the mass of the nectar bag without nectar and ρ is the mass density of the nectar = 1,321 g/ mL.

The mass density of the nectar was calculated using the relationship:

$$\text{Density} = \frac{\text{Mass}}{\text{Volume}} \quad \text{(Equation 3)}$$

The treatment was applied using a micropipette, and the mean volume of solution inserted in the nectar bag was 6.36 mL.

Table 3: overview of the intermediate and final concentration of the solutions applied to artificial nectar.

Intermediate	**Final**
I_1: 6,30 mg/L	F_1: 100 µg /L
I_2: 20.0 mg/L	F_2: 1000 µg /L
I_3: 63,2 mg/L	F_3: 10 000 µg /L

2.7 Learning and flower visits

The bumblebees in each hive entered the arena two times; first on the day of initiating the exposure (before the start of the exposure), and then nine days later. The first flight in the arena was characterized as *training*, and the last flight was described as *testing*. Training was done to habituate color naïve bumblebees (bumblebees with no preference for colours based on interactions with flowers) to artificial flowers, and to implement a connection between yellow and blue and reward. For one hour, an unlimited number of bumblebees were allowed to forage from 18 bi-coloured, blue and yellow, artificial flowers in the flying arena. All flowers in the training-period contained reward (artificial nectar) to allow color naïve bumblebees to associate both colours with reward. During both training and testing the UV-lights were turned on, and all other light sources in the room were turned off.

After the eight days of exposure (in addition to one day of starving), the bumblebees were tested in the flying arena containing nine yellow and nine blue artificial flowers. The blue flowers were rewarding (each contained ~1,5 mL artificial nectar), and the yellow flowers unrewarding (each containing ~1,5 mL of tap water). Despite blue being the preferred color for bumblebees (Chittka et al., 2004; N. E. Raine, Ings, Dornhaus, et al., 2006), yellow was chosen as the rewarding flower due to yellow being the preferred color in the pilot study. It has also been showed that *B. terrestris dalmatinus* have less preference for blue flowers than other *B. terrestris* sub-species (Ings et al., 2009).

2.7.1 Behaviour analysis

The experiments in the flying arena were recorded with video cameras over a period of two hours. During this period, the bumblebees were not allowed to return to the hive. When 15 bumblebees were present in the flying arena, or if the time had exceeded one hour, the hatch in the tunnel was closed so that new bumblebees were unable to enter the flying arena (Figure 6).

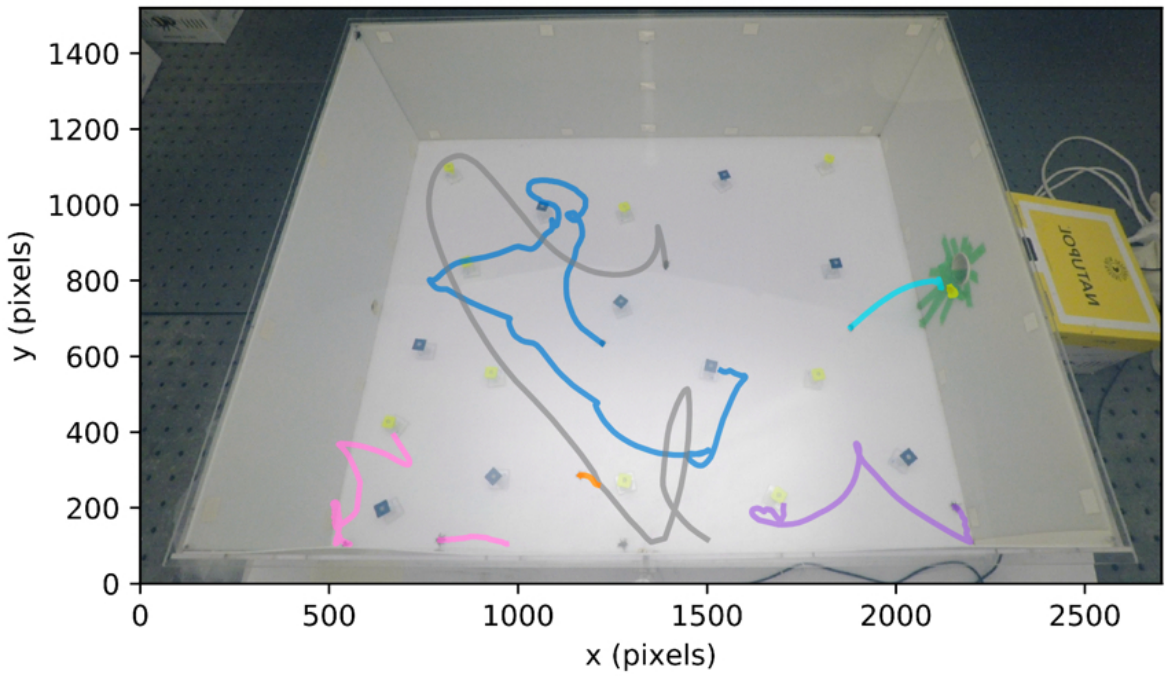

Figure 8: An example of how the tracking of bumblebees can be visualized from the software developed for this experiment. The coloured lines are trajectory fragments that follow each bumblebee as long as the bumblebee doesn't escape the region visible to the camera or the tracking software.

The Bumblebee tracking software was applied to the recordings of bumblebees to assess the learning outcome (Figure 8). The analysis aimed to extract the number of flowers of each colour

that were visited by the hive as a unit. A visit to a rewarding flower (blue) was counted as a success, and a visit to an unrewarding flower (yellow) was counted as a failure. A flower visit was defined as frames where both video streams simultaneously showed the bumblebees centre within the border of a flower, meaning that the bumblebee was standing on a flower. A schematic view of a data set resulting from this procedure is shown in Figure 9. Standing on a flower was regarded as a measurement of selecting a flower because a bumblebee can detect nectar via chemosensors in its feet or via extending the proboscis (de Brito Sanchez et al., 2008). The inclusion of both video streams in the definition of flower visit detection eliminated false visits as false detections never happened simultaneously on both cameras for time intervals long enough to be a problem. To exclude instances where a bumblebee was hovering over a flower instead of landing, the criterion for flower visit was set to be the detection of a bumblebee on more than half of the frames in a two-second time interval (see appendix B for further details about the tracking software).

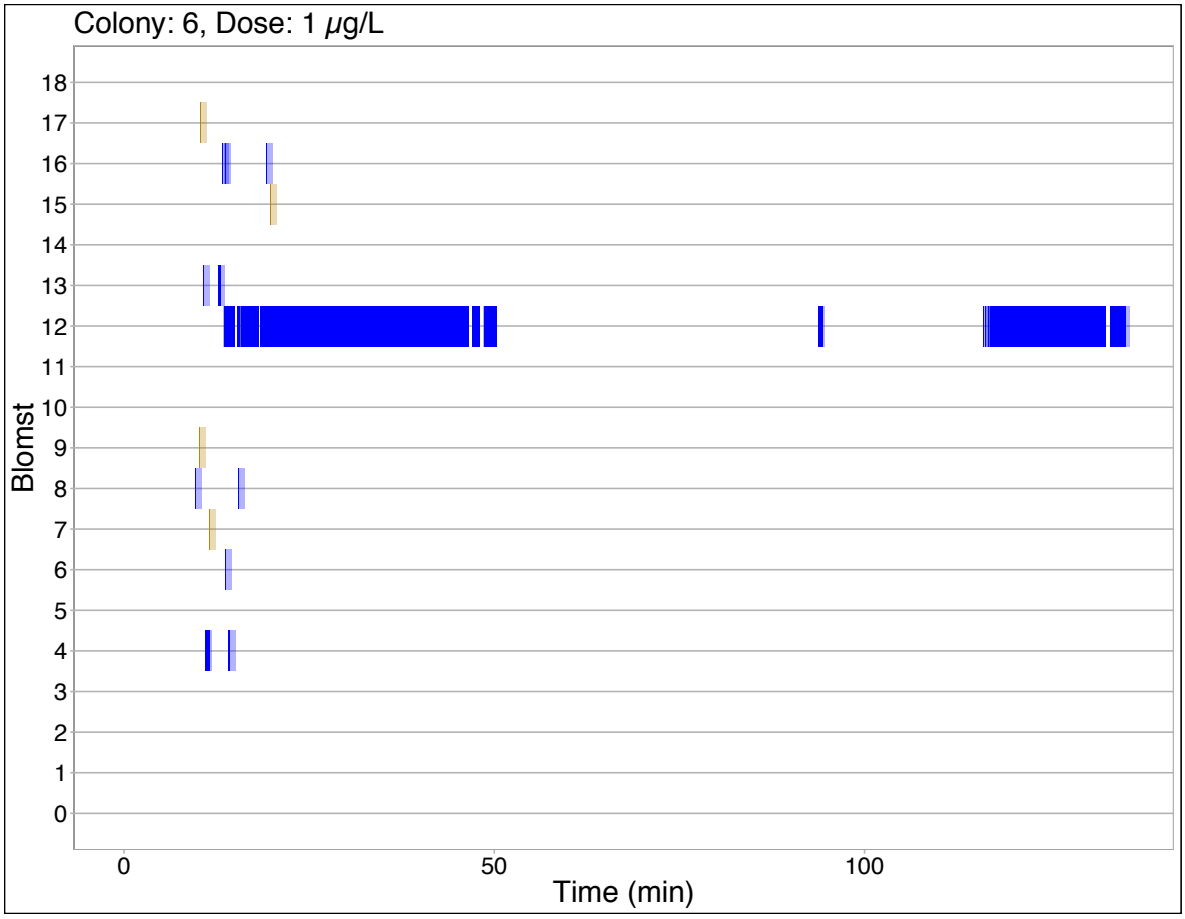

Figure 9: schematic view of the output from one colony during one test in the arena. The x-axis shows the time in minutes, and the y-axis represents the assigned number for each flower. The coloured lines show which flower that were visited and the time length of each visit.

The locomotor activity levels (the proportion of time that animals spend active) were measured, with help from Ph.D.-student Henrik Andersen Sveinsson, by constructing trajectories using the Python package Trackpy 0.3.3 (Allan et al., 2014). From the trajectories, all the speeds of the bees were extracted and presented as a histogram over the speeds (Figure 10A). As expected from previous studies on animal locomotor activity and trajectory data (Edelhoff et al., 2016), it was observed a clear difference between fast-moving animals and slow-moving animals, thus it was decided to use a threshold value on the speed distribution to distinguish slow and fast movement. The threshold value was chosen as the speed at which the histogram plateaued, and was set by visual inspection of the data. The same threshold value of 3 pixels per 1/60 second was chosen for all experiments, since all plateau onsets fell approximately on the same value. The proportion of the speed measurements at speeds below this threshold value were considered *passive*, and the speeds above the threshold were considered *active*. The *activity level* (locomotor activity) of the colony was then defined as the proportion of the speed measurements being above the threshold, and can be read off the cumulative distribution of bumblebee speeds (Figure 10B). It can also be seen from the cumulative distribution that the relative measured activity levels between hives is not particularly sensitive to the choice of threshold speed value.

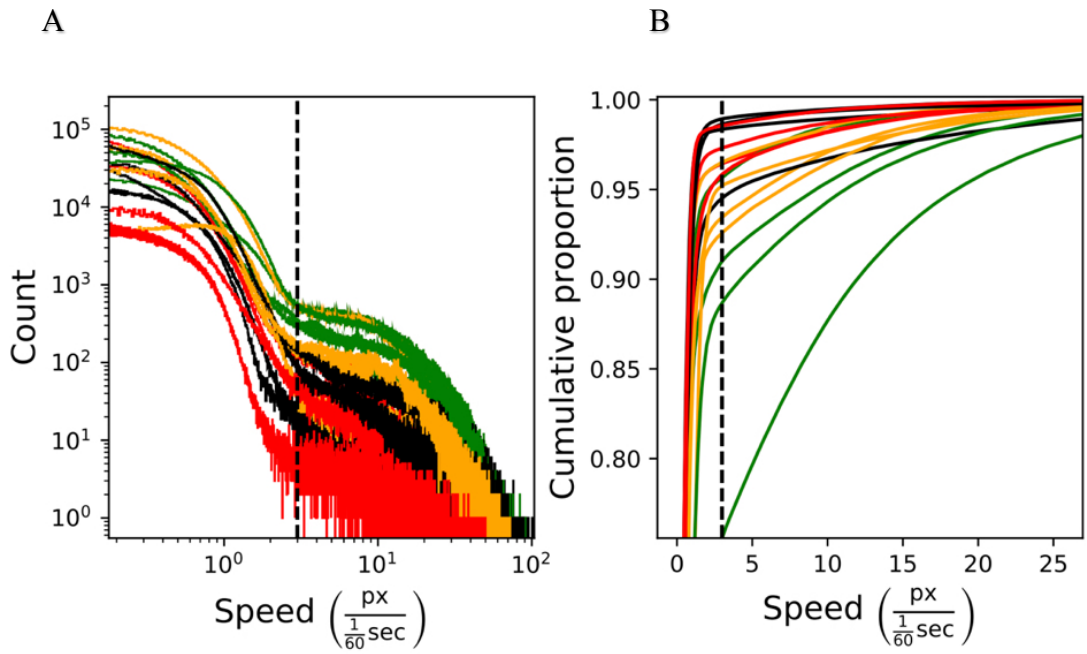

Figure 10: Panel A) shows histograms of the calculated speeds for different colonies with speed on a logarithmic x-axis and number speed measurement on a logarithmic y-axis. The vertical dashed line represents the threshold value above which the bees are considered active. Panel B) shows the cumulative speed distributions resulting from integrating and normalizing the data in panel A), with speed on the x-axis and cumulative proportion of speed measurements on the y-axis. For each colony, the proportion of speed measurements being below a given speed can be read off this plot. Again, the vertical dashed line represents the threshold speed. For both panels, colours represent different concentrations of imidacloprid: Control (green), 1 µg/L (orange), 10 µg/L (red) and 100 µg/L (black)

2.8 Dissection of hives

At the end of the exposure period, the bumblebees remaining in the hives were killed by freezing them for a minimum of 48 hours in a -20°C freezer. During dissection, the hives were randomized, thus the imidacloprid treatment levels (dose) were not known.

During dissection, the following units were counted: number of eggs, pupa, big larva, small larva, adults, honeypots. In addition, it was noted whether the queen was alive or not, whether pollen was present or not, and the total amount of nectar consumed was measured.

Brood production was assessed by counting all egg cells containing eggs, larvae (in two different stages), and pupa. The larvae were split into two categories as bumblebees first form an egg-cell containing several eggs that develop to larva before each larva develop a separate bulge and become separated from the other larvae (Michener, 2007). The larvae in the cells with several larvae were defined as small larvae, and larvae that had developed a separate cell

were classified as big larvae. The broods and the adult bumblebees were divided into living or dead, depending on the color and physical shape (Table 4). Food stores were measured as the number of honeypots containing nectar compared to the honeypots not containing nectar. The nectar bag was weighed at the start of the exposure period, and at the end of the exposure period to assess the food consumption. The amount of imidacloprid dilution added for exposure was added to the starting weight, and the amount consumed nectar was calculated by subtracting the end-weight from the start-weight.

Table 4: Characteristics used to classify living or dead broods and adults in the hive during dissection of the hives.

	Eggs	**Larvae**	**Pupa**	**Adult**
Dead	Color: grey Other: not containing solid substance	Color: grey / black / orange Other: bloated	Color: grey / black Other: shrunken and dry	Other: found on the side of the inner nest box, shrunken to a size beyond normal shrinking due to freezing
Alive	Color: white Other: containing solid substance	Color: white / light yellow Other: normal shape	Color: white / light grey Other: moist	Other: found in the inner nest box, normal shrinking due to freezing

2.9 Statistical analyses

Statistical analysis was performed using R statistical software (R Core Team, 2017). An overview of packages and the usage of these packages is shown in Table 5. For a total overview of the data from the hive, see Appendix C.

Table 5: Overview of the packages applied in R to do the statistical analysis of the data from the hives and the behaviour. The overview does not include the default packages that comes with R.

R-package	Citation	Use
car	(Fox et al., 2011)	Find the best probability distribution for the dataset
Ggplot2	(Wickham, 2009)	plots
GGally	(Schloerke et al., 2017)	Check for correlation between explanatory variables in each dataset
plyr	(Wickham, 2011)	Reordering dataset
tidyr	(Wickham et al., 2017)	Reordering dataset
multcomp	(Hothorn et al., 2008)	Dunnett´s test
MuMIn	(Bartoń, 2016)	AICc and QAICc
MASS	(Brian et al., 2002)	LC_{50} and EC_{50}

For the analysis, each hive was treated as the unit of replication. All model fits were checked using Residual vs. Fitted-, QQ-, Scale-location- and Residual vs. Leverage-plots. Correlations between explanatory parameters in each dataset where checked using pairwise scatterplot, correlations coefficients and variance inflation factors. Shapiros Wilk´s test with additional histograms were used to test for normality in each dose for each response variable.

Generalized Linear Models (GLM) were fitted to address how the different parameters counted or measured varied with imidacloprid concentration. Doses were log10-transformed to allow for the fitting of the data to a sigmoid dose-response curve. Two additional explanatory variables were added to control for the number of days that had passed since that hive was delivered to UiO and the days that had passed between the day of the testing and the day of the freezing (Table 6 and Figure 7).

Table 6: Overview of the three explanatory variables used in the statistical analyses.

Explanatory variable	**Behaviour data**	**Data from hives**
Dose	A numeric variable with four values: 0, 1, 10, 100	A numeric variable with four values: 0, 1, 10, 100
Days after test		A numeric variable describing days between day one of receiving the hives to the day the hive was put in the freezer.
Days after delivery	A numeric variable describing days between day one of receiving the hives to the last day of test	A numeric variable describing days between the day the hive was tested to the day the hive was put in the freezer.

For data that were either successes or failure (dead adult bumblebees, dead pupa, dead big larvae, dead small larvae, empty honeypots, locomotor activity (active/passive) and unique or total flowers visits to rewarding or unrewarding flowers) the probability for the response based on the explanatory variables were calculated to find whether the bumblebees were impaired as a response to an increase in dose. This was done using GLM with family quasibinomial and link function logit (not probit due to non-normality). The link function transforms the shape of the curve to (hopefully) a straight line for a linear fit. Due to overdispersion, meaning that the sample variance exceeds the theoretical data, the quasibinomial family was chosen rather than the binomial. The relative response in each response variable was the number of responding units in each group divided by the total number of units in the group. To account for a minimum response level above 0, Schneider-Orelli's formula was used to correct for the minimum level:

$$\text{Schneider} - \text{Orelli´s formula} = \frac{b-k}{1-k} \qquad \text{(Equation 3)}$$

Where b is the relative response (mortality, flowers visited, empty honeypots, locomotor activity) in each treatment and k is the relative response in control. Since the binomial family only accepts values between 0 and 1, the lowest observed response in a data set was used as the minimum response level. This relation was only used to test positive relationships between dose and response, since subtracting a minimum value on a negative relationship would mean setting the highest-dose response to 0.

In mortality and number of honeypots analysis, the total number of units was used as weights in the model fit. For locomotor activity, all weights were set to 1, letting the quasibinomial dispersion parameter handle the dispersion. The number of speeds measured could in principle have been used as weights, but speed measures resulting from trajectories are highly correlated, leaving the actual number of "trials" uncertain.

As the data in total visits were counts, without a success or failure, a Poisson family distribution with an identity link function was used. Due to overdispersion being detected in the data when applied to a Poisson GLM, a quasi-Poisson distribution was used to not violate the assumption for doing Poisson regressions (Reitan et al., 2016).

To find the p-value, the default test in GLM, Wald Chi-squared test, was used. The comparison between each treatment group and the control was obtained by using a GLM with dose as factor in a multiple comparison test: Dunnett's test. The level of significance was set to a p-value of 0.05 or lower. If neither of the exposure groups were on, or above this number in the Dunnett's test, the results from this test is not mentioned in the results.

LC_{50} was calculated for adults and broods to find the concentration where 50% of the individuals in each dose was dead. ED_{50} was calculated for the amount of nectar consumed to find the concentration where 50% of the maximal effects was observed. Both of these calculations were done on the fitted model (GLM) using the MASS package in R.

2.9.1 Data from hives

For the hive analysis, the proportion of dead small larva, dead big larva, dead pupa, dead adults, and empty honeypots, in addition to amount of nectar consumed, consumption of pollen or not and queen (dead or alive) were set as individual response variables. One response variable (eggs), was removed from the final analysis due to missing data points that would make the subsets less robust for further analysis.

To fit the data from the consumed nectar to a sigmoid curve their values were divided by the maximum value in the dataset. In figures, both the data and the fit is transformed back to its original range of values to better show the content of the actual data. The GLMs used on data from the hives contained three explanatory variables: Dose, days after test and days after delivery.

2.9.2 Behaviour data

The response variables were split into two groups: one group was the total number of visits to all flowers, counting e.g. two visits to one flower as two visits, the other group was unique flowers visited, not considering whether the flower was visited several times. This distinction was made to examine whether the flower choice was a result of an individual choice or whether the bumblebees made a choice as a result of a bumblebee already sitting on a flower. For both groups, the total number of visits (either total visits or unique visits) were the response variable to examine whether the total number of flower visits was affected by the exposure level. To measure the bumblebees' preference, the proportion of rewarding flower visits (either total visits, or unique flowers) was the response variable. Unrewarding flowers were used as a response variable to measure whether exposed bumblebees followed their initial preference for yellow flowers.

The response variable for locomotor activity was the *activity level* (proportion of time in fast motion). The GLMs used on behaviour data contained two explanatory variables: Dose and days after delivery.

2.9.3 Model selection

Model selection was applied to derive the best-fitted model for each data set. Model selection included the most complex model, containing all parameters relevant for the model and simpler models (Table 6 and Appendix D). Akaike´s Information Criterion (AIC) was used, corrected for overdispersion by using quasi AIC (QAIC) that includes the variance inflation factor (c) and balance over- and underfitting when overdispersion is present. AIC was also adjusted for small sample sizes by using QAICc that gives more penalty for extra parameters (Kim et al., 2014; Richards, 2008). AIC with the corrections find the most parsimonious model as a balance between how well the model fits the data and the complexity (number of parameters) of the model. Akaike´s weight is the weight of evidence for each model, and was used as a measure

of model selection uncertainty, thus low model selection uncertainty was assumed if all or most of the weight was in one model. To compare models, $\Delta QAICc_i = QAICc_i - min(QAICc)$, where i refers to a model, and min refers to the model with the lowest QAICc value, was used. If a model had $\Delta QAICc < 2$ it was checked whether this variable changed the deviance, and such a model was selected solely if it had a ΔQAICc-value less than the ΔQAICc -values of all of the simpler models, since the loss of degrees of freedom when one variable is added results in a weaker model. Also, the importance value of the explanatory variables was calculated as a measure of what variable explained the most in the models.

GLM does not provide an R^2, but a calculation of explained deviance (pseudo-R^2) was calculated for each model (Equation 4).

$$\text{Pseudo} - R^2 = 100 \times \frac{\text{null deviance} - \text{residual deviance}}{\text{null deviance}} \quad \text{(Equation 4)}$$

Pseudo-R^2 is not directly comparable to the standard R^2, and cannot be interpreted as the explained variance in the dataset. The measures from pseudo-R^2 are relative measures between similar models, indicating how well the model explains the data relative to the other models. In the present study, the McKelvey-Zavoina pseudo-R^2 was used, as this is argued to be the best to mimic the ordinary least squared – R^2 (Veall et al., 1994).

3 Results

3.1 Learning and pollination by bumblebees

Below only the results from the final (best) model from the model selection are presented. For a complete overview of the results from the model selection procedure, see Appendix D.

Total number of visits by bumblebees to flowers

There was no relationship between dose and the total number of flowers visited or number of unique flowers visited (Figure 11A and B). The best models describing the variation in both total flower visits and unique number of flower visited was a flat model without any explanatory variables. Dose was not significant ($p > 0.05$) in the models with dose as an explanatory model, and had importance values of ~0.25. There was, however, a direct difference between control group and the 10 μg/L in the unique number of flowers visited (Dunnett's test p-value = 0.0090).

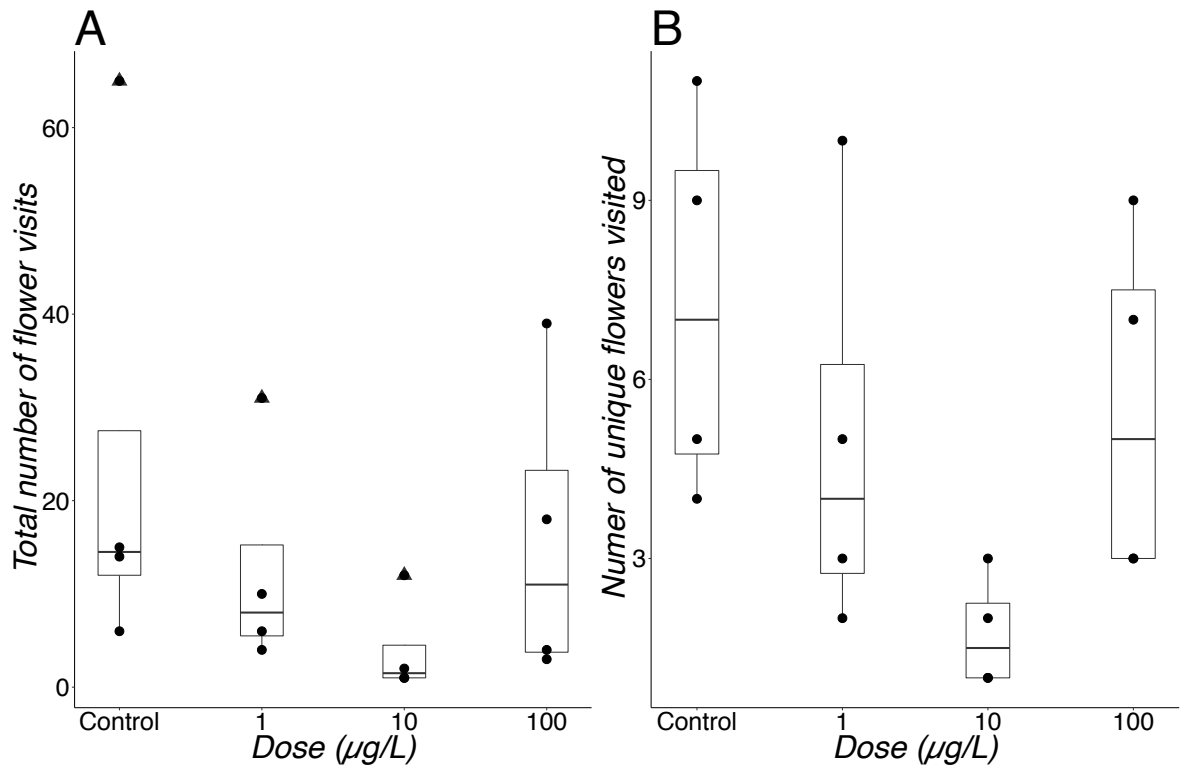

Figure 11: Plot A) shows the relationship between dose on the x-axis and total visits to all flowers on the y-axis (n = 231 flower visits). Plot B) shows the relationship between dose on the x-axis and total unique flowers visited on the y-axis (n = 78 unique flowers visited). The boxes show the variation in the dataset with the horizontal line being the median, the lower and upper bar the first and third quartiles, respectively, and the whiskers show the min/max- values in the dataset (except in the presence of outliers). The black triangles are extreme values beyond 1.5 times the interquartile range.

Proportion of visits by bumblebees to rewarding flowers

There was a negative relationship between increasing dose and proportion of visits to rewarding flowers (Figure 12A and B). Dose was included in the best model describing the variation in both frequency of visits to rewarding flowers (blue) and frequency of unique rewarding flowers visited. For total visits to rewarding flowers, dose had a Wald p-value of 0.041, and an importance value of 0.76. For number of unique rewarding flowers visited the dose had a Wald p-value of 0.030 and an importance value of 0.80.

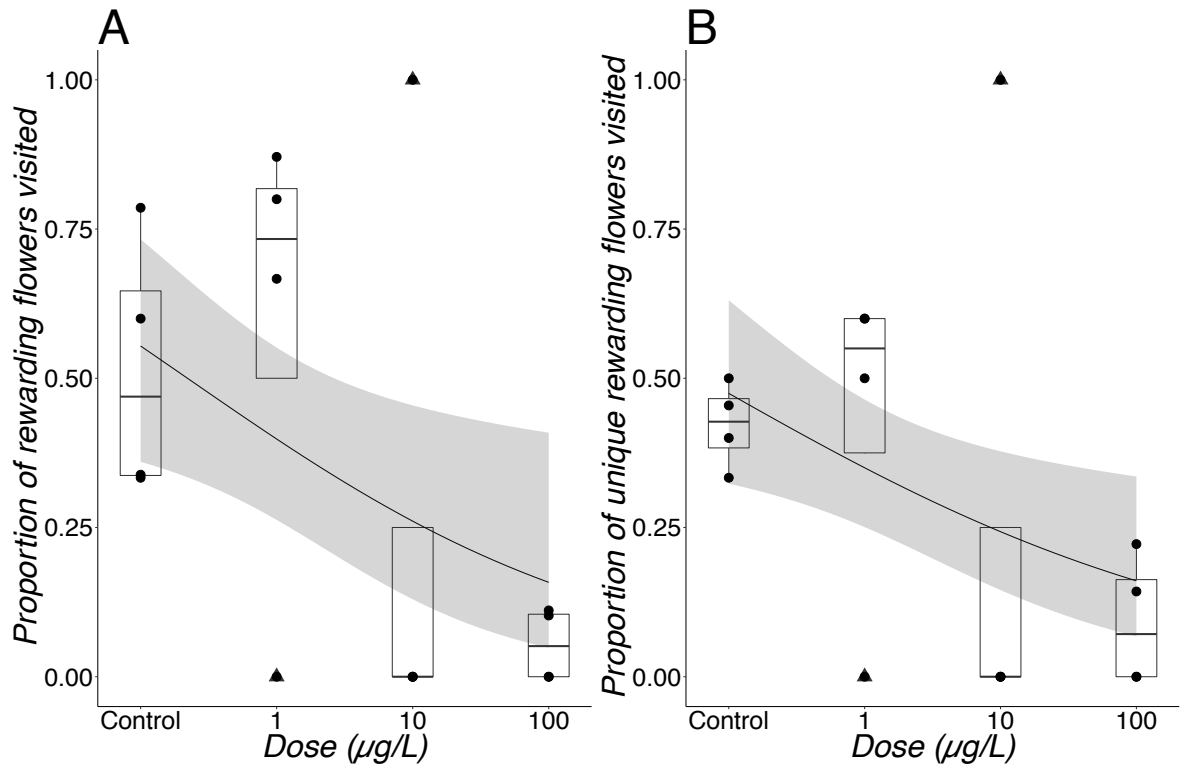

Figure 12: Plot A) shows the relationship between dose on the x-axis and the proportion of rewarding flower visits on the y-axis (n = 90 rewarding flower visits). Plot B) shows the relationship between dose on the x-axis and proportion of unique rewarding flowers visited on the y-axis (n = 26 unique rewarding flowers visited). The boxes show the variation in the data with the horizontal line being the median, the lower and upper bar the first and third quartiles, respectively, and the whiskers show the min/max-values in the dataset (except in the presence of outliers). The black triangles are extreme values beyond 1.5 times the interquartile range. The shaded area shows the 95% confidence bands.

Bumblebee locomotor activity in the flying arena

There was a negative relationship between increasing dose and the relative amount of time spent moving at a speed higher than the threshold value (Figure 13). The locomotor activity level has been normalized by the maximum level of locomotor activity among all flight tests. The best model contained dose with a Wald p-value of 0.0048 and an importance value of 0.33. There was also a direct difference between the control group and the group exposed to 10 µg/L (Dunnett's test p-value = 0.014) and between the control group and the group exposed to 100 µg/L (Dunnett's' test p-value = 0.0091).

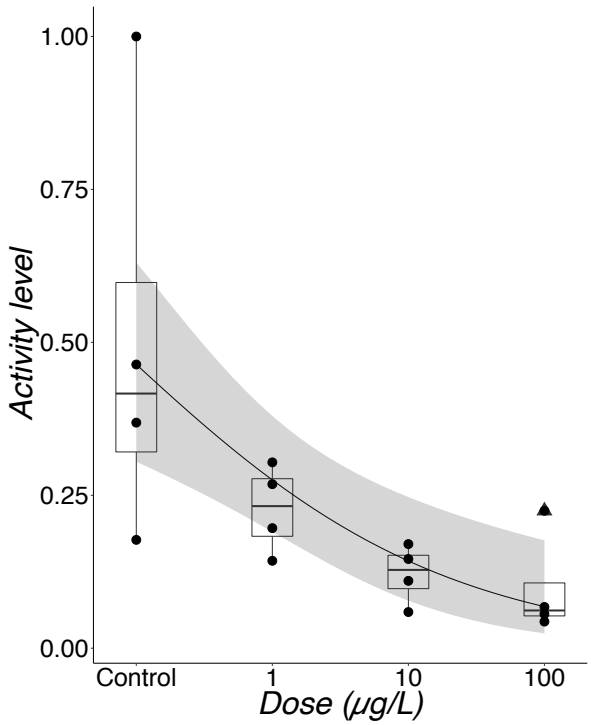

Figure 13: The relationship between dose on the x-axis and the proportion of locomotor activity above the threshold speed-value on the y-axis. The boxes show the variation in the data with the horizontal line being the median, the lower and upper bar the first and third quartiles, respectively, and the whiskers show the min/max-values in the dataset (except in the presence of outliers). The black triangle is an extreme value beyond 1.5 times the interquartile range. The shading shows the 95% confidence bands.

3.2 Reproduction and colony health

The overall variance in the number of dead and living bumblebees in different developmental stages within the colonies was high, as shown by the standard deviation values listed in Table 7, with three significant numbers. The control group had a smaller combined population (eggs, larvae, pupa and adults (mean = ~147)), than the groups exposed to imidacloprid (mean=~193, ~177 and ~182), at dose 1 µg/L, 10 µg/L and 100 µg/L, respectively (Figure 14 A). The control group also had the lowest proportion of produced broods compared to all the imidacloprid exposed groups (Figure 14 B). In two of the colonies (one in the control group and one in the 100 µg/L) the count of dead adult bumblebees is missing. The best model explaining the variation in total broods in the colonies contained days after delivery (Wald test p-value = 10.06, importance value = 0.99). The best model containing dose (Wald test p-value = 0.045, importance value = 0.59) also contained days after delivery. In this model, there was a positive

relationship between dose and number of broods. For the number of adult bumblebees in the hive the results are exactly the same as for broods, but with a negative sign, (individuals are either adults or broods) the proportion of adult bumblebees in the colony decreased with increasing dose.

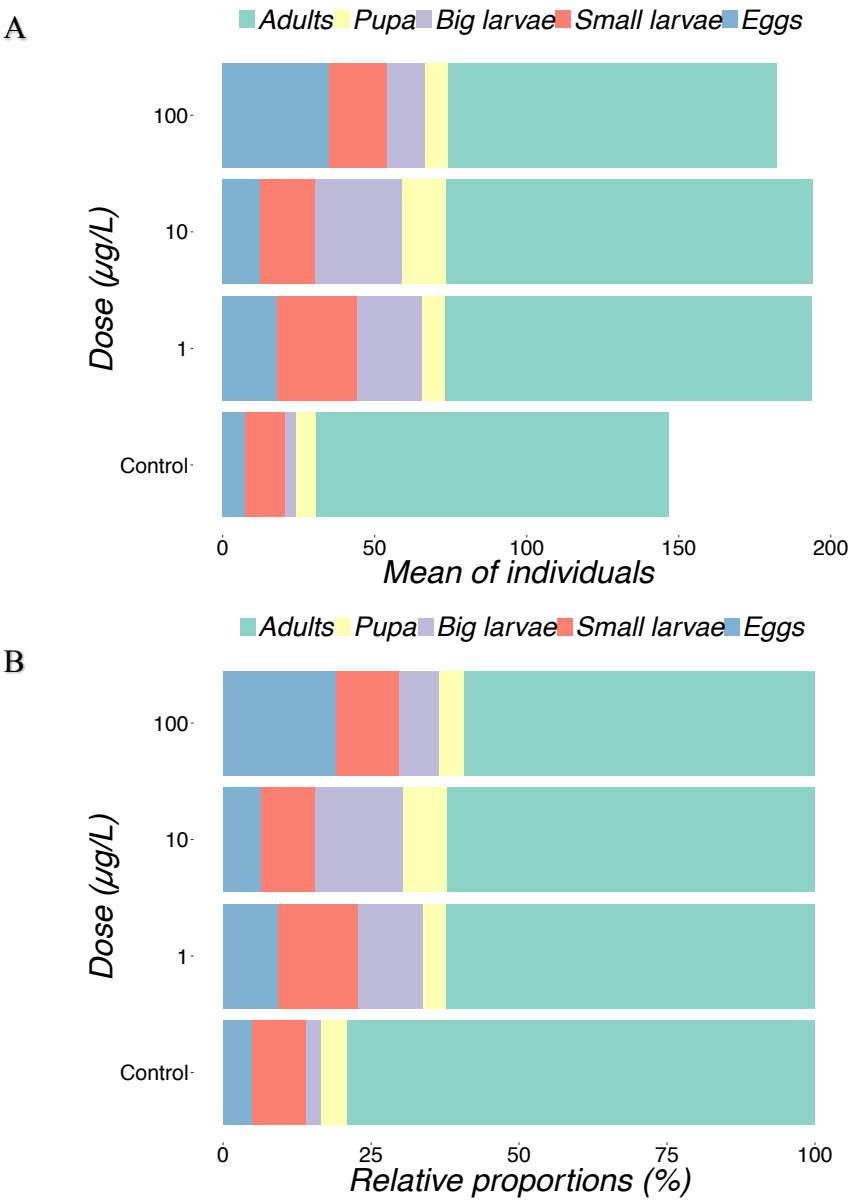

Figure 14: The developmental stages of bumblebees in the hives, divided in the four exposure-groups. A) present the total number of individuals (dead and alive), and B) present the relative proportion of bumblebees in different developing stages: eggs (n=579), small larvae (n=615), big larvae (n=528), pupa (n=291), adult (n=3364).

Table 7: Different developmental stages of bumblebees (Bombus terrestris) categorized as living or dead. Data is presented as minimum and maximum levels, mean ± standard deviation

	Control			1 µg/L		
	Min - Max	**Median**	**Mean ± SD**	**Min - Max**	**Median**	**Mean ± SD**
Living adults	66 – 147	126	116 ± 36.3	37 – 120	94	86.2 ± 41
Dead adults	105 – 124	117	115 ± 9.6	115 – 188	158	155 ± 34.2
Living pupa	0 – 14	5.5	6.25 ± 6.84	0 – 19	4	6.75 ± 8.99
Dead pupa	0 – 17	4.5	6.5 ± 8.02	2 – 17	7.5	8.5 ± 6.35
Living big larva	0 – 23	2	6.75 ± 11	0 – 57	4	16.2 ± 28.5
Dead big larva	0 – 3	0	0.75 ± 1.5	0 – 69	17	26.5 ± 33.2
Living small larva	0 – 62	18.5	24.8 ± 29.9	0 – 120	21	40.5 ± 56.6
Dead small larva	0 – 5	1.5	2 ± 2.44	0 – 40	0	29 ± 24.5
Living eggs	0 – 36	11	14.5 ± 15.8	0 – 65	28.5	30.5 ± 35.4
Dead eggs	0 – 0	0	0 ± 0	0 – 21	0	5.25 ± 10.5
Nectar	240 – 306	325	312 ± 54.6	234 – 392	295	304 ± 78.5
Full honeypots	52 – 107	86	82.8 ± 22.8	30 – 68	63	56 ± 17.6
Empty honeypots	13 – 34	31.5	27.5 ± 9.81	46 – 109	51.5	64.5 ± 29.9

Table 7 continued: Different developmental stages of bumblebees (Bombus terrestris) categorized as living or dead. Data is presented as minimum and maximum levels, mean ± standard deviation

	10 µg/L			100 µg/L		
	Min - Max	**Median**	**Mean ± SD**	**Min - Max**	**Median**	**Mean ± SD**
Living adults	41 – 117	71	75 ± 32.4	46 – 71	51.5	55 ± 11
Dead adults	99 – 162	136	133 ± 26	115 – 211	170	179 ± 29
Living pupa	0 – 21	10.5	10.5 ± 11	0 – 7	4	3.75 ± 3.3
Dead pupa	3 – 33	19	18.5 ± 14.7	1 – 27	10	12 ± 13
Living big larva	0 – 41	12	16.5 ± 19.5	0 – 13	1	4 ± 11.3
Dead big larva	11 – 61	45.5	40.8 ± 17.9	0 – 41	20.5	20.5 ± 30.6
Living small larva	5 – 68	15	25.8 ± 28.6	0 – 86	0	21.5 ± 15.1
Dead small larva	0 – 29	6	10.2 ± 13.4	0 – 35	16	16.8 ± 15.1
Living eggs	0 – 43	13.5	17.5 ± 18.7	0 – 125	4	33.2 ± 61.3
Dead eggs	0 – 20	0	7.25 ± 14.5	0 – 130	8	36.5 ± 62.8
Nectar	102 – 268	123	154 ± 76.9	37.4 – 128	101	92.2 ± 40
Full honeypots	23 – 64	45	44.2 ± 17.5	26 – 93	45	46.8 ± 31.4
Empty honeypots	53 – 111	53.5	67.8 ± 28.8	15 – 109	98	80 ± 43.9

3.2.1 Survival of bumblebee-broods and adults

The best model explaining the variation in overall mortality proportion contained both dose and days after test (Dose: Wald p-value=0.023, importance value=0.75, Days after test: Wald test p-value=0.0025, importance value=0.99). In this model, there was a positive relationship between dose and number of dead individuals. In the control group the proportion of dead eggs, small larva, and big larva were 0%, 7%, and 10%, respectively. There was an increasing trend of dead bees from the control group to the highest dose, in which the proportion of dead individuals in the same three developmental stages was 52%, 44% and 84% (Figure 15).

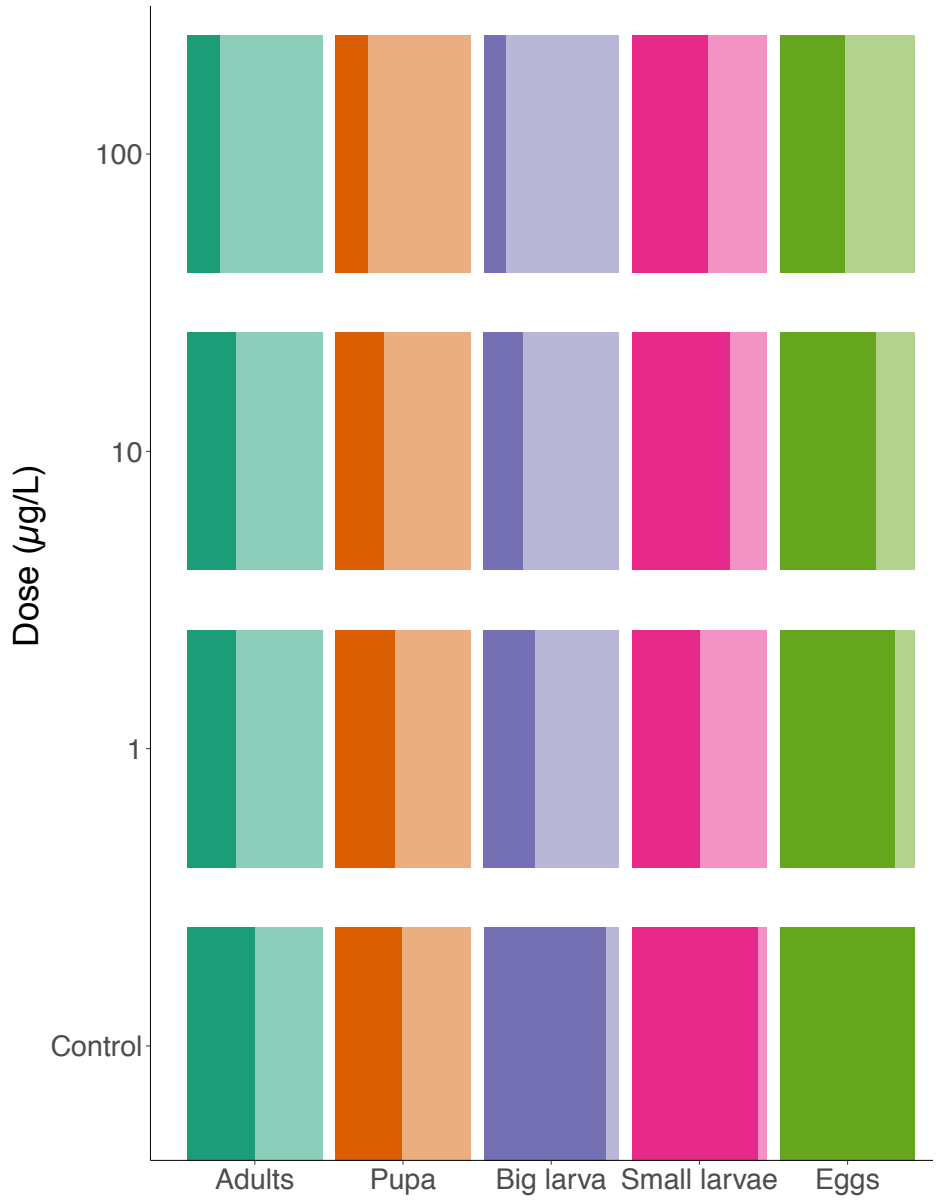

Figure 15: Proportion of living (dark color) and dead individuals (light color) in different development stages, grouped by the level of exposure.

Proportion of dead broods in bumblebee colonies

There was a positive relationship between proportion of dead broods and increasing dose (Figure 16). The best model explaining the variation contained dose and days after test (Dose: Wald test p-value= 0.0077, importance value = 0.91, Days after test: Wald test p-value = 0.00093, importance value = 0.99). LC_{50} is 19 µg/L with a 95% confidence interval from 2 µg/L to 153 µg/L. There is a difference between control and the highest dose (Dunnett's test p -value = 0.0047).

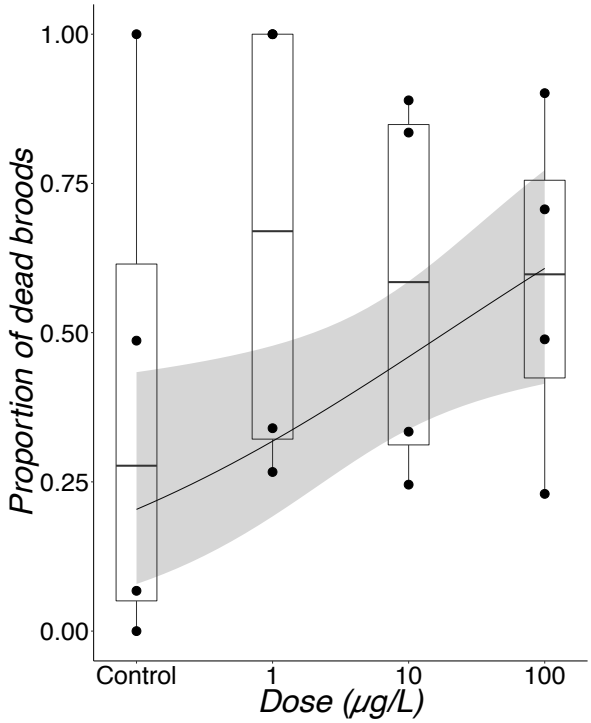

Figure 16: The relationship between the dose on the x-axis and the proportion of dead broods on the y-axis (n =897 dead broods, of total n = 2013). The boxes show the variation in the data with the horizontal line being the median, the lower and upper bar the first and third quartiles, respectively, and the whiskers show the min/max-values in the dataset. The shading shows 95% confidence bands.

Proportion of dead adult bumblebees in the colonies

There was a positive relationship between the proportion of dead adult bumblebees in the colony and increasing dose (Figure 17). The best model contained dose with a Wald test p-

value of 0.039, and an importance value of 0.72. There was also a direct difference between the control group and the highest dose (Dunnett's test p-value = 0.032). LC_{50} is 33 µg/L with a 95% confidence interval from 9 µg/L to 125 µg/L.

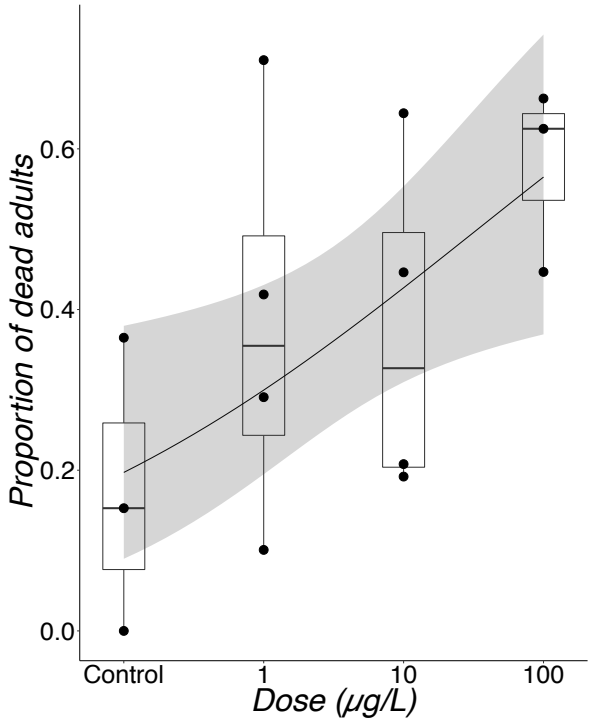

Figure 17: The relationship between dose on the x-axis and the proportion of dead adults on the y-axis (n = 2033 dead adults of total n = 3364). The boxes show the variation in the dataset with the horizontal line being the median, the lower and upper bar the first and third quartiles, respectively. The whiskers show the min/max-values and the shading shows 95% confidence bands.

Bumblebee queens in the colonies

The best model contained days after test with a Wald test p-value of 0.056, and an importance value of 0.81. Dose was not significant (Wald test p-value = 0.41).

Number of dead pupa in the colonies

There was a positive relationship between all explanatory variables (increasing days after test, dose and days after delivery) and proportion of dead pupa (Figure 18). The best model contained all three variables with significant Wald p-values (days after test: 0.000011, dose: 0.011, days after delivery: 0.00075) and high importance values (days after test: 1.0, dose: 0.90, days after delivery: 1.0). 17 days after test, all of the pupa in all the colonies were dead.

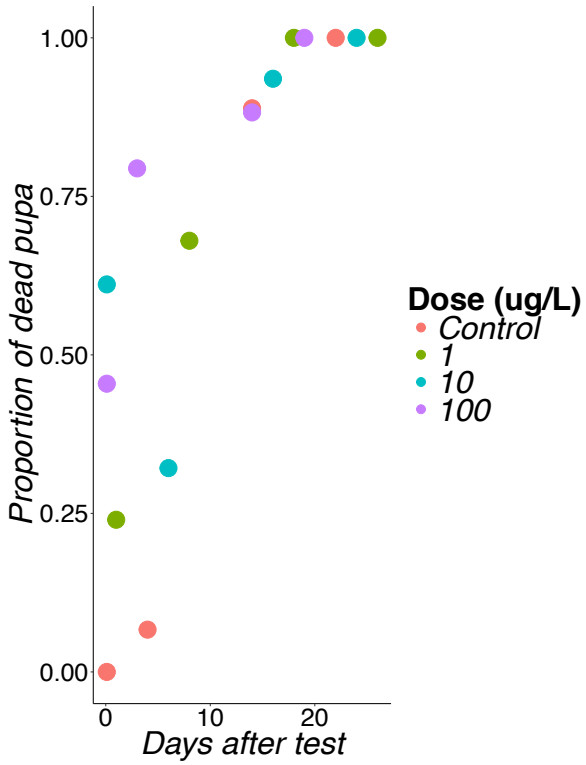

Figure 18: The relationship between days after test on the x-axis and the proportion of dead pupa on the y-axis (n = 182 dead pupa of total = 291). The points represent the hives, and the dose is represented by the colours.

Proportion of dead big larvae in the colonies

There was no relationship between increasing dose and proportion of dead big larvae (Figure 19). The best model contained days after test with a high importance value (0.98), but this was not significant (Wald test p-value = 0.084).

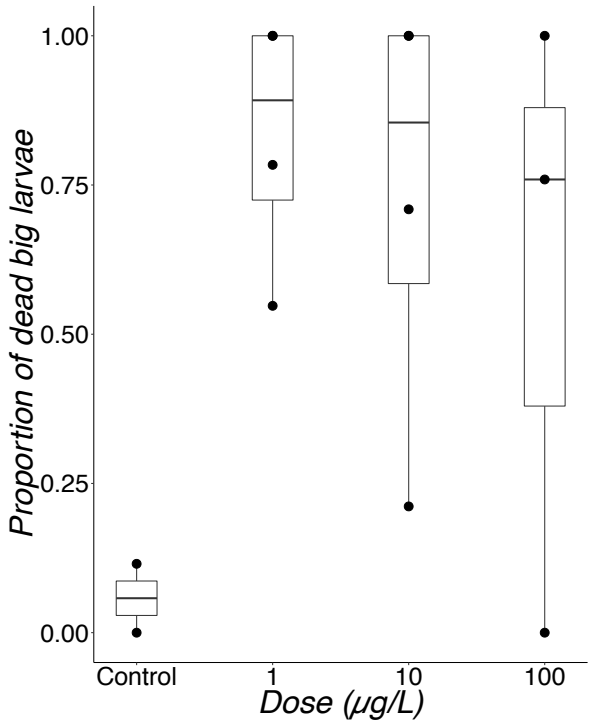

Figure 19: The relationship between dose on the x-axis and the proportion of dead big larvae on the y-axis (n = 354 dead big larvae of total n = 528). The boxes show the variation in the dataset with the horizontal line being the median, the lower and upper bar the first and third quartiles, respectively. The whiskers show the min/max-values in the dataset.

Proportion of dead small larvae in the colonies

There was no relationship between increasing dose and proportion of dead small larvae (Figure 20). The best model did not include any explanatory variables. For models containing variables, both days after delivery and dose were equally good in two different models, but they did not have a relationship with mortality (Walt test p-value ~ 0.15).

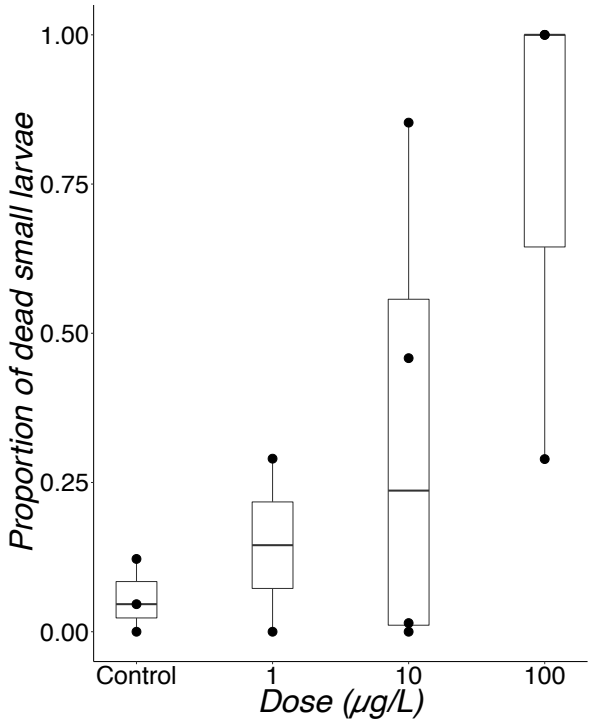

Figure 20: The relationship between dose on the x-axis and the proportion of dead small larvae on the y-axis (n= 165 dead small larvae of total n = 615). The boxes show the variation in the dataset with the horizontal line being the median and the lower and upper bar the first and third quartiles, respectively. The whiskers show the min/max-values in the dataset, and the x-axis displays a proportion between 0 and 1.

3.2.2 Food intake

Proportion of empty Honeypots in the hives

There was a positive relationship between increasing dose and proportion of empty honeypots in the hive (Figure 21). The best model contained dose with a Wald test p-value of 0.023 and an importance value of 0.77. There was also a direct difference between the control group and the 10 µg/L group (Dunnett's test p-value = 0.037) and between the control group and the 100 µg/L group (Dunnett's test p-value = 0.019).

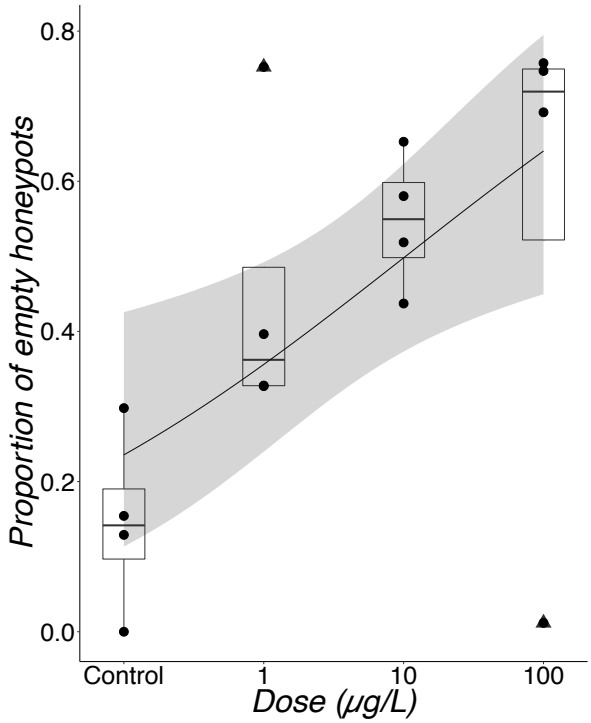

Figure 21: The relationship between dose on the x-axis and the proportion of empty honeypots on the y-axis (n=959 empty honeypots of total n = 1878). The boxes show the variance in the dataset with the horizontal line being the median and the lower and upper bar the first and third quartiles, respectively. The whiskers show the min/max-values (except in the presence of outliers), the black triangles are extreme values beyond 1.5 times the interquartile range, and the shading is 95% confidence bands.

Pollen consumed by the bumblebees

There was no relationship between dose and whether pollen was eaten or not. The best model contained dose with a Wald test p-value = 0.12, and an importance value of 0.80.

Amount of nectar consumed by the bumblebees

There was a negative relationship between increasing dose and amount of nectar consumed (Figure 22). The best model contained dose with a Wald test p-value of 0.00038 and an importance value of 0.97. In the model fitting, the amount of nectar was normalized by the maximum amount of nectar consumed among all hives, but for Figure 22, the values and the model fits have been transformed back to the range of the raw data. There was also a direct difference between the control group and the 10 µg/L group (Dunnett´s test p-value = 0.0085) and between the control group and the highest dose 100 µg/L (Dunnett´s test p-value < 0.001). EC_{50} was 6 µg/L with a 95% confidence interval from 2 µg/L to 16 µg/L.

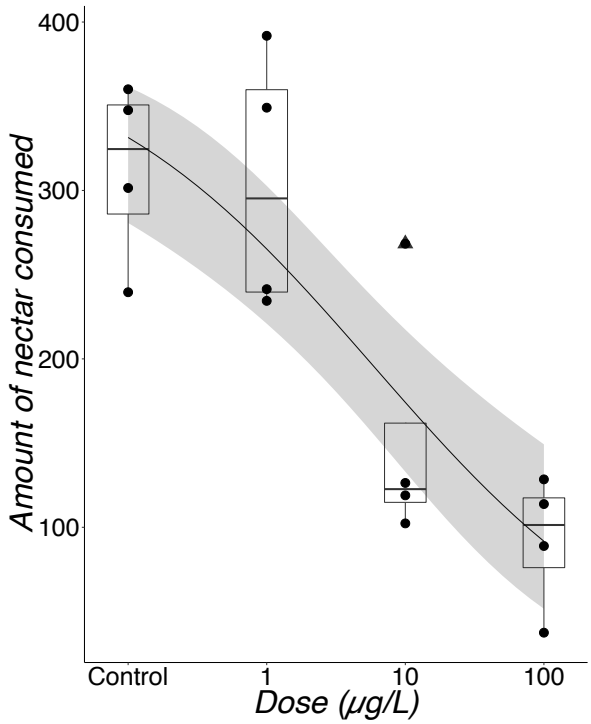

Figure 22: The relationship between dose on the x-axis and the volume of nectar consumed on the y-axis. The boxes show the variation in the dataset with the horizontal line being the median, the lower and upper bar the first and third quartiles, respectively. The whiskers show the min/max-values (except in the presence of outliers). The black triangle is an extreme value beyond 1.5 times the interquartile range, and the shading the 95% confidence bands.

4 Discussion

The present study aimed to develop a new method for assessing learning in bumblebees chronically exposed to a field realistic concentration of the neonicotinoid imidacloprid. This was done by using rewarding and non-rewarding flowers in a set-up that allowed for several bumblebees to forage at the same time. The study also aimed at simplifying the observation of foraging by using video-recordings and a new software to track the bumblebees. There are to the author's knowledge no previous ecotoxicological studies that evaluate learning and flower visitation using this method. Additionally, the study assessed whether imidacloprid affected the reproduction, survival and food consumption of the bumblebee colony. Both the behavioural results and the results assessing the health of the colony show that field realistic doses of imidacloprid have negative sub-lethal effects on bumblebees.

4.1 Experimental design

The present study was modified from two research areas within bee-ecology, one concerning behaviour in bees and one concerning sub-lethal effects on bees. The studies concerning behaviour used methods that assess learning in bumblebees (L. J. Evans et al., 2014b; N. E Raine et al., 2008; N. E. Raine, Ings, Ramos-Rodriguez, et al., 2006). The basis of these studies is that artificial flowers in two contrasting colours are placed in a flying arena, and a single bee is allowed to forage. The central modifications in the present study compared to other studies are that several bees were allowed to forage simultaneously, the addition of a PPP stressor and video recording of the behaviour and analysis of the recordings instead of manually observing. In the previous studies, the principal aim was to assess learning in bumblebees in general, using each bee as a replicate and scoring errors and observing the learning progression. The studies concerning sub-lethal effects are studies assessing impairment of bumblebee learning when exposed to different doses of PPPs. The main change from these studies is the test setup. Former studies have used Proboscis extension reflex (PER) methods (Decourtye et al., 2004) or T-maze methods (Tan et al., 2015). The present study used the methods from the behaviour studies, i.e. artificial flowers, combined with the exposure to a PPP. Also, the present study focused on field realistic doses (and a positive control of 100 μg/L). The modifications were done to make the experiment as field realistic as possible, with several bumblebees foraging on artificial flowers and field realistic doses, and to make tracking of bumblebees more efficient.

Based on the experiences from the pilot, several improvements were made to the main experiment. Also, modifications were done after the arrival of the bumblebees to UiO. These included, e.g. change and improvements of the flying arena, installing daylight-simulating UV-light, troubleshooting regarding the bumblebees' reluctance to return to the hive, installation of cameras to the new flying arena, modifications to the exposure procedure and other major and minor details. This resulted in a setup sufficiently sensitive to measure sub-lethal effects of imidacloprid on bumblebees. The Bumblebee video tracker was developed in parallel with and after the experiments, as data from experiments were necessary to develop the software.

4.2 Learning and pollination

This study aimed to mimic a field realistic situation by having flowers in two different colours, one rewarding and one unrewarding. The flower set-up can be seen as a realistic situation as bumblebees have to choose the most rewarding flowers in the natural environment. The overall results from the learning and locomotor activity experiment show that there is a negative relationship between learning and locomotor activity when the PPP dose increases.

4.2.1 Overall foraging by bumblebees

Contrary to the expectations (H1), foraging was not impaired by increasing doses of imidacloprid. Impairment was expected as bumblebees have been shown to increase their access time (the time it takes before the bumblebee find the nectar in the flower) when exposed to imidacloprid (Morandin et al., 2003). Since the behaviour-test of the bumblebees was over a limited amount of time (2h), the expectation was that the number of flowers visited would decrease in a dose-dependent manner. However, the bumblebees in the control group and the group exposed to the highest concentration of imidacloprid (100 μg/L) visited approximately the same number of flowers. The same number of flowers visited can indicate that they did learn to forage on artificial flowers during the training period and that the long-term memory was not affected by exposure to imidacloprid, as information stored in long-term memory is hard to erase (Chittka, 1998). An interesting feature in the dataset is that there is no difference between the control group and the highest dose (100 μg/L) in the unique number of flowers visited, but a difference between the medium dose (10 μg/L) and the control. This difference indicates that the bees in the 10 μg/L –group foraged poorer than all other groups. While this result is statistically significant there is high uncertainty in the dataset, and the finding is

contrary to other studies where no effect has been found with the same dosing exposure (Feltham et al., 2014; Schmuck et al., 2001; Stanley et al., 2015). Other studies have also shown that bumblebees exposed to higher doses of imidacloprid use longer time to access flowers (Morandin et al., 2003; Ramirez-Romero et al., 2005). The increase in access time is contrary to the results presented in the current study as the bees had two hours to forage and the group in the highest dose did not access fewer flowers than the any of the other groups.

Since pollination is the moving of pollen from one flower to another flower, the ability to access flowers is important for wild plants and crops. The results from overall foraging by bumblebees in the present study show that this ability is maintained even at a high dose of imidacloprid. The model selection showed that none of the explanatory variables, meaning dose, days after delivery and days after test, explained the variance in flower visitation frequency, and thus suggesting that imidacloprid did not affect the bumblebees' ability to transfer pollen from one flower to another. An aspect that amplifies the suggestion that pollination is not affected by imidacloprid is that the bumblebees visited new flowers, as seen in unique flowers visited. If the bumblebees only had visited the same flowers in the highest dose, the transfer of pollen between different flowers would have been severely impaired. However, it is necessary to investigate if the pollen transfer efficiency is affected, as the present study focussed on the frequency of flower visits, not the absolute transfer of pollen.

4.2.2 Learning

In line with the expectations (H2), the bumblebees' learning was impaired in a dose-depended manner when exposed to increasing doses of imidacloprid. This impairment was seen by decreasing visits to blue (rewarding) flowers when the dose increased. The effect was most visible when the dose increased to 10 μg/L, and there was no effect of the lowest dose 1 μg/L. The bumblebees in the control group and the 1 μg/L group also visited more unique blue (rewarding) flowers indicating that they actively chose not to visit yellow flowers to see if they had a reward, or that they learned to visit blue flowers from other bees visiting blue flowers. The results from the combination of total flower visits and total visits to unique flowers suggest that the bumblebees in the control group and the lowest dose (1 μg/L) learned that the blue flowers were rewarding while the two groups exposed to the highest doses (10 μg/L and 100 μg/L) did not learn the system. The results from yellow flower visits show that the groups exposed to the two highest doses followed their initial preference for yellow flowers. These

results are consistent with earlier studies done on olfactory learning using PER and thiamethoxam on bumblebees with the same concentration (~10 μg/L) (Decourtye et al., 2003; Stanley et al., 2015). The underlying mechanism of the impairment of learning can be explained by disturbances in the learning centre (mushroom bodies) in the bee's brain (Peng et al., 2016) as this is connected to learning centre (B. M. Jones et al., 2013). The mushroom bodies are affected by imidacloprid due to the location of nAChRs being mainly in this part of the bee-brain (Kreissl et al., 1989). The density of neuropilar concentrations (nerve fibres with numerous synapses found in concentrations of nervous fibre) in the mushroom bodies that are necessary for both olfactory and visual learning decreases when bees are exposed to imidacloprid (Peng et al., 2016).

The mechanism provoking learning and memory in the bumblebees' brain is essential for the survival of bumblebees' due to the many tasks that require these features. These tasks are diverse and ranging from handling flowers, remembering where the most rewarding flower patches are, and to the bumblebees ability to navigate and finding their way back to the hive (Fischer et al., 2014). Impairment of these skills can cause harm to the individual, compromising the ability to find rewarding flowers, as shown in the current study, or the bumblebees homing ability (Fischer et al., 2014). Moreover, the results from the overall visitation to flowers combined with the learning results did not show a reduction of visits, rather a switch from choosing the rewarding flowers to choosing flowers with the colour initially preferred (the unrewarding flowers). Indicating that the bumblebees use more energy on finding flowers than actually foraging from flowers.

Impairment of learning and memory can cause harm at the population level as it is likely that as the individual foraging decreases so will the collective food gathering that is essential for social insects (N. E Raine et al., 2008). For bumblebees, learning is vital as they are generalists and different flowers can be rewarding at various times of the day and throughout the season. As generalists are necessary for maintaining many plant communities, a reduction in bumblebee populations due to imidacloprid can reduce the seed set in insect pollinated plants (Biesmeijer et al., 2006) and thus cause a decline in populations of plant species depending on insect pollination for seed set.

4.3 Reproduction and colony health

4.3.1 Reproduction

A reduction in reproduction was expected as the imidacloprid dose increased, due to several severe effects of imidacloprid on reproduction showed in earlier studies. These effects include a downregulating effect on vitellogenin and hexamerin 70b in honeybee queens, that can lead to reduced longevity and reproduction. (Chaimanee et al., 2016). Also a reduction in food intake was expected, as this correlates with a reduction in reproduction (Laycock et al., 2012). Contrary to the expectations (H4), reproduction was not reduced in imidacloprid exposed colonies, it was rather the contrary; reproduction in the colony was positively correlated with increasing dose. This relationship was only visible if corrected for days after the test, which had an adverse effect on the number of broods in the hive. A cause for the results can be that with increasing dose the number of broods increases as a response to fewer broods developing into adults' due to the positive relationship between increasing dose and brood mortality. This is coherent with the results that show an increase in broods with increasing dose; a decrease of adult bumblebees with increasing dose and an increase in brood-mortality with increasing dose. Another explanation can be an increase in worker-production as is seen in bumblebee colonies exposed to imidacloprid (Gill et al., 2014), as more foragers are produced to compensate for the decrease in foraging bouts. Following this line of thought, it is possible that the increase in brood production was to make up for less food in the hive because this can simulate a colony with fewer foragers. Also, the increase in reproduction can be explained as to maintain the colony as more of the worker bumblebees died in the colonies exposed to the higher dose of imidacloprid.

However, these results can also be explained by other factors than the exposure; the bumblebees' were exposed for a short amount of time relative to the time it takes for a bumblebee to develop new broods (the time from egg to a worker is 22 days (Michener, 2007)). Thus, the variation in the number of offspring in the colonies can be explained by factors like the size of the colony, the age of the hive, the reproduction rate of each colony, and condition of the colony prior to the exposure. Other studies have shown that the reproduction rate decreases when exposed to field realistic doses of imidacloprid (Gill et al., 2012; Laycock et al., 2012). Other studies have also found that reduced feeding on nectar and pollen is correlated with lower brood production (Laycock et al., 2012). These results are not consistent with the

present study, which shows a reduction on feeding, while the amount of broods produced is higher in the exposed groups.

4.3.2 Mortality

In line with the expectations (H5), the overall mortality of broods increased when the dose increased. While this relationship was found for the overall brood-mortality, it was not found in small or big larvae. The lack of a relationship for dose and mortality in big larvae can be a result of high variation in the dataset, as the mean mortality of big larvae was low in the lowest dose (10%) and high in the highest dose (84%), but with individual hives in the highest dose having 0% dead larvae. The same trend in the datasets are seen in small larvae. For pupa, the results are consistent with the overall mortality when both days after test and days after delivery are included in the model. The results from pupa mortality show that the proportion of dead pupa in the control-group was small, while the mortality in the highest doses were high even when the colonies had been out for few days after the test. This result may indicate that imidacloprid can have an adverse effect after exposure over a longer time-period. The results showing increased mortality in broods when exposed to higher doses of imidacloprid are coherent with other studies done with field realistic doses (Gill et al., 2012; Laycock et al., 2013).

In line with the expectations (H6), the mortality of adult bumblebees showed a positive relationship with dose. The LC_{50} was estimated at ~33 μg/L, but due to the low number of replicates and the large variation in the dataset, the 95%-confidence interval is too wide to conclude (8.7 μg/L to 126 μg/L). The same was seen in total brood mortality with an LC_{50} of 19 μg/L and a 95%-confidence interval ranging from 2.3 μg/L to 153 μg/L. While the LC_{50} has a wide confidence interval, it reflects the information provided by previous studies, where mortality have been found to vary depending on the experimental setup. In one study LC_{50} ranged from 39 μg/L to 59 μg/L based only on whether the bumblebees were foraging or not (Mommaerts et al., 2010) . Other studies have found no effect of field realistic doses (Gill et al., 2012; Tasei et al., 2000). This variation in concentrations that cause 50% mortality are consistent with studies done on honeybees, where no effects have been found when honeybees have been exposed to field realistic concentrations (Faucon et al., 2005; Schmuck, 2004). In comparison doses far beyond the field realistic range have been found to cause 50% mortality

(ranging from 50μg/kg in nectar (Suchail et al., 2000) to 600 μg/kg in nectar (Suchail et al., 2001).

Contrary to expectations (H6), the queen mortality did not respond to increasing doses of imidacloprid. Queen mortality in bumblebees have divisive results, showing both increased mortality (Scholer et al., 2014) and no effect (Gill et al., 2012). While this result may be interesting, it is highly uncertain as this study only included 16 queens.

Overall, the results of the present study show that mortality appears in a dose-dependent manner in both broods and adults. A loss of either worker or adults poses a great treat to the colonies ability to maintain the most important function of surviving: Feeding. However, the results suggest that a field realistic dose is not sufficient to cause elevated mortality as a significant difference between the control and the other concentrations was only observed between the highest concentration and the control for both broods and adults.

4.3.3 Food intake and locomotor activity

In line with the expectations (H3) the locomotor activity of bumblebees in the flying arena decreased as the dose increased. This decreasing trend was visible at the 10 μg/L-dose, indicating that imidacloprid has an effect on locomotor activity level when consumed in field realistic doses. Moreover, the ED_{50} was calculated to 6 μg/L with a 95% confidence interval from 2 μg/L to 16 μg/L. These result diverges from earlier results where locomotor activity of bumblebees is not affected even when exposed to 100 μg/L of imidacloprid (Baron et al., 2017). That study analysed the locomotor activity by measuring the mean speed of bees inside the hive during an average time span of 45 seconds. The analysis method is different in the present study, where the 2-hour recording of each colony was analysed according to a threshold speed value. The method used in the present study is more robust due to the length of the analysed recordings and the recordings of bees in a flying arena where they were able to move more freely than in a hive.

It is likely that several factors contribute to the reduced locomotor activity. One of them can be the impairment of gas exchange between CO_2 and O_2 (Hatjina et al., 2013). Ventilation in insects is generated from a nerve cord in the CNS (Bustami et al., 2000) which is dependent on functioning nAChRs (Buckingham et al., 1997). As ventilation is connected with metabolizing in insects, the depolarisation of postsynaptic neurons caused by imidacloprid acting on the

nAChRs (Buckingham et al., 1997) can lead to lower metabolizing (Contreras et al., 2010; Hatjina et al., 2013), and thus inhibiting the need for food. Another factor is the response from other organs than the brain. Even if the brain is the active site for imidacloprid, other organs can be affected. An organ that can be affected is the midgut of bees, where most of the biotransformation of chemicals takes place (Catae et al., 2014). The midgut suffers structural changes in the first three days of exposure, but seems to recover after this period (Catae et al., 2014). The Malpighian tubules, responsible for the excretion, are also impaired by exposure to neonicotinoids, by structural changes that can reduce uptake of nutrients, and prevent excretion (Catae et al., 2014).

Whether imidacloprid affects feeding is still unclear. In some studies exposure to imidacloprid had no effect on food intake (Tasei et al., 2000), while other studies show that both consumption and storing of food decreases when bumblebees are exposed to imidacloprid in field realistic doses (Cresswell et al., 2012; Gill et al., 2012; Laycock et al., 2012; Scholer et al., 2014). These last results are consistent with the present study, where, in line with expectations (H7, H8), both the amount of nectar consumed and the storage of food were negatively affected by increasing doses of imidacloprid. However, the decreasing trend is likely not due to an aversion against imidacloprid as bumblebees prefer sugar solution spiked with imidacloprid over regular sugar water (Baron et al., 2017; Kessler et al., 2015). Nor is the decrease likely due to inhibition of the PER-reflex or relaxation of the proboscis (Kessler et al., 2015).

Another explanation for the reduced feeding could be the lack of navigation due to exposure from neonicotinoids (Fischer et al., 2014), that can lead to the bumblebees being unable to locate the food. A challenge with applying this hypothesis to the current study is that the bumblebees had artificial nectar available in both the feeder and in the honeypots at all times. Also, the overall number of visits to artificial flowers was not impaired, which indicates that long-term memory was not affected, and it is reasonable to assume that the location of the honeypots and feeder was stored in the long-term memory. Another explanation can be that imidacloprid suppresses the need to feed. However, the underlying toxicological reasons for the detrimental effects are unknown. It is suggested that there is a detoxification system that is overwhelmed when it reaches a certain threshold (Cresswell et al., 2012). The detoxification hypothesis is based on a twice inflicted dose-response curve (or the “Eagle effect” after the biologist Harry Eagle that first described it (Eagle et al., 1948)), where the feeding rates are lower when exposed to small doses, before the feeding increases when the dose increases. This

is when the detoxification is believed to be induced (Cresswell et al., 2012). When the process reaches a threshold, and the bees are no longer capable of removing the toxicant, the system collapses and the feeding rate decreases. However, a twice inflicted dose-response curve is not seen in the results from the present study, where the feeding rate is as normal for the group exposed to 1 μg/L before it has a dose-dependent decrease in the two highest doses. To address this issue, it would be necessary to include several treatment doses closer in concentration than in the present study.

The results from the present study are not sufficient to conclude that the low activity level of the exposed bumblebees was a direct consequence of reduced feeding, but there is evidence for a strong correlation based on the information on the biological processes. Nevertheless, the sub-lethal effects on locomotor activity and food intake can have serious consequences on bumblebee colonies even if they are not correlated. Reduced locomotor activity leading to smaller distances covered when searching for rewarding flowers and a decreased feeding rate is detrimental for the individual and thus negative for the survival of the colony.

4.4 Context

Understanding the effects imidacloprid has on important pollinators like bumblebees is essential for making evidence based decisions. This is especially true for neonicotinoids as they are the most widely used insecticides in the world (Casida et al., 2013). For a rapidly expanding population, efficient agriculture is the foundation of a functioning society. As both the ecosystem service of pollination and the use of pesticides are vital for efficient agriculture, it necessary to investigate whether the different pesticides are compatible with the ecosystem service of pollination. Earlier studies have been criticized for not using field realistic doses (Blacquiere et al., 2012), and the need for studies on sub-lethal effects of neonicotinoids on non-*Apis* species have been highlighted (Alkassab et al., 2017; Goulson, 2013; Wood et al., 2017) together with studies investigating effects of imidacloprid on the whole hive, assessing effects on the population level not only the individual level. The results from the present study give insight on how the overall colony fitness of a non *Apis*-species is affected by a field realistic dose of imidacloprid. Using the knowledge from studies that have assessed the effects on the cellular level, the organ level and the individual level combined with the present study gives a better understanding of the negative effects field realistic doses of imidacloprid have on a population.

EFSAs suggestions on the further use of the three neonicotinoids currently under moratorium are supposed to be due in November 2017. The call for papers that are making the foundation of the decision making regarding the three neonicotinoids is closed, hence these results and this method will not be considered. However, Europe is a small provider of food compared to other continents (Ramankutty et al., 2008) and other regulatory bodies like EPA and APVMA is not considering a restriction in use of neonicotinoids. The uncertainty connected to the effects of neonicotinoids on pollinators emphasize the importance of understanding realistic exposure of these PPPs.

5 Conclusions

The overall goal of this study was to test how exposure to a sub-lethal concentration of imidacloprid affects bumblebees. The study specifically aimed to develop an experimental setup that allowed the study of bumblebee learning at the colony-level in a field realistic laboratory setting. Based on the results, this was successfully accomplished. However, there are still improvements that can strengthen the experimental setup (see future directions). Nevertheless, the experimental setup is functional.

The other goals of this study were to identify and quantify the potential behavioural effects of the PPP imidacloprid on a non-*Apis* species, *Bombus terrestris* in a dose-response manner and to assess the characteristics of *Bombus terrestris*-hives after chronic exposure to imidacloprid at field realistic and higher doses. The colonies in the present study showed an overall dose-dependent impairment of learning ability, locomotor activity, food consumption as well as an increase in both brood and adult bumblebees. The combined impact of imidacloprid is severe, and it seems that bumblebees are sensitive to the dietary levels of imidacloprid used in the present study. Moreover, they respond in a dose-dependent manner, suggesting that the higher the dose, the more severe is the impact of imidacloprid. This result is not coherent with the detoxification hypothesis, but the doses in the current study cover a wide range with low resolution, possibly too low to resolve a detoxification hypothesis.

While these results show that imidacloprid is harmful to bumblebees it is worth noting that this is a laboratory study and that a laboratory is substantially different from the environment. This type of study methods allows for the control of several factors, but by doing this, the magnitude of the uncontrolled factors remain unknown. It is challenging to extrapolate from single foraging bouts on artificial flowers in the flying arena to an intricate landscape with complex flowers in the field. Moreover, the other stressors like diseases, parasites, monoculture (unbalanced diet) or the presence of other pesticides or chemicals are absent in the laboratory. Nevertheless, there is a link between learning, memory, locomotor activity, food and the success of the colony regardless of the environment.

6 Improvements and future studies

To make the experimental setup easier to use and less prone to errors, several improvements can be made. Regarding the automatization of the analysis of the behaviour, it might be possible to extend the bumblebee tracking program to track individual bumblebees (Perez-Escudero et al., 2014), and thus assess the number of unrewarding flowers probed by a single bumblebee before probing a rewarding flower. Moreover, to gain more knowledge on learning, the time before visiting the first flower, and the time before visiting a rewarding flower is an interesting parameter to assess. The tracking and analysing of individual bumblebees should be done with several bumblebees in the flying arena simultaneously, as in the present study. The inclusion of several bumblebees in the colonies makes the experiment more field realistic, as bumblebees often forage in the presence of other bumblebees. Identification of each bumblebee can also be used when assessing learning from other bumblebees' by observing if it is an increase in the number of rewarding flowers choices after the first bumblebee has landed on a flower. To make that type of analysis, it is necessary to have cameras of higher quality that can capture more pixels in one frame, and with less distortion. The floor in the laboratory used in the present study was uneven, and it bounced when walking. To make the analysis possible with individual bumblebees, it is favourable with an even and stabilized floor.

While the results of the present study contribute to the knowledge about neonicotinoids, the study has limitations that makes it less environmentally relevant. Since the bumblebees did not forage during the exposure period, the metabolizing was not realistic, moreover the pollen was not dosed, and this is an essential nutrient for queens and broods. Another aspect not taken into consideration in the present study is the number of foragers compared to nursing bees, nor the number or quality of sexuals, thus the survival of the next generation is therefore not assessed when proposing the risk imidacloprid poses to bumblebees. The additional stressors that bumblebees experience, e.g., several plant protection products, parasites and habitat fragmentation are beyond the scope of the present study. An additional stressor is climatic differences, which is an interesting aspects as winter honeybees have been found to be more sensitive to imidacloprid than summer honeybees (Decourtye et al., 2003). In addition, the bumblebees used are reared bumblebees, and there is uncertainty in the difference in susceptibility for reared bumblebees and wild bumblebees. Moreover, if the method developed in the present study is to be more environmentally relevant, it should cover a whole flowering period and include an assessment of the homing ability of the bumblebees.

Bumblebees foraging performance improves with experience while foraging performance in bumblebees exposed to imidacloprid is reduced (Gill et al., 2014). An experiment lasting longer with the bumblebees foraging each day can provide knowledge on how the learning in bumblebees' progress when exposed to different doses of imidacloprid. Additionally, there is a need for more knowledge on how imidacloprid and other neonicotinoids affect different bee-species.

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Appendices

Appendix A: Pilot

A pilot study was conducted to test the experimental setup and to get experience in handling bumblebees. The pilot study was based on studies done on bumblebees and honeybees: (Devillers et al., 2003; L. Evans et al., 2014; N. E Raine et al., 2008; N. E. Raine, Ings, Ramos-Rodriguez, et al., 2006). Different approaches were tested in a laboratory, and unforeseen challenges were dealt with as they appeared. Based on these pilot experiments described below, a final experimental setup was designed.

The pilot was conducted during May – July 2015, at Department of Biosciences, University of Oslo, with the help of the French student Johann Kieffer that had his internship at the University of Oslo. Three *Bombus terrestris* queens with developing broods were bought from the company Pollinering AS in Stavanger, Norway, early May 2015. To make the bumblebees associate the flowers in the experiments with reward and for not having them associate one color more than the other color with reward the bumblebees were color naïve, meaning that they did not associate any color with reward. The queens were placed in wooden nest boxes (28 cm x 10 cm x 11 cm) with an air vent hole covered with wire netting, made in the workshop at Department of Biosciences, the University of Oslo (referred to as "in-house") (Figure 23). The entrance to the nest box was on one of the short sides (2,5 cm in diameter). The nest box had two lids, one made of wood to cover the hive when the light was one, and one made of Plexiglas® to make it possible to observe the hive; both had a hole to attach a syringe, an air vent hole and a circular hatch (4 cm in diameter). The queens and emerging workers were fed ad libitum with sugar water (50% sugar) through a syringe with the tip cut off, placed upside down from the lid of the nest box. Feeders were initially filled with sugar water every 2-3 days, and after the colony developed every 1-2 days depending on the amount of sugar water eaten. Defrosted honeybee-collected pollen imported from Latvia by Pollinering AS was mixed with 50% sugar water (Dan sugar) using a hand blender under low heat until the pollen-pellets dissolved and the mixture homogenized. Pollen was formed into small balls and placed in a small feeder (2,5 cm in diameter) inside the nest box; pollen was refilled and changed every second day.

Figure 23 Overview of the experimental set-up in the pilot study.

As workers need approximately 22 days to develop from larvae, we maintained colonies for 14 days in a controlled environment (28°C, 55% relative humidity) (Duchateau et al., 1988). To ensure that the bumblebees were color naïve we kept the bumblebees in a dark room. The only light source was an infrared light bulb (Osram theratherm infrarot 220-230 V, 250W) connected to a light fixture as bumblebees have low sensitivity to red (Peitsch et al., 1992).

To obtain information on earlier training, age and to individually trace the bumblebees, each emerging bumblebee was uniquely marked with a queen tag (Opalith number plates, Honningcentralen SA) attached with glue or marking paint from 1-100, one color for each hive (yellow, red and blue). Before tagging, the bumblebees were trapped using a marking cage or a queen trapper (both from Honningcentralen SA) before they were placed in a wine cooler (+6-+15°C) to sedate them. After a period in the wine cooler (this varied from 20 min to 120 min) the bumblebee was sedated, and the tag was glued on the thorax of the bumblebee. The marking was done in infrared light so that the bumblebee would not see colors. As the glued tags often detached from the bumblebees, the individuals that had lost their tags were retagged with a new tag.

To have flowers that were easy to handle, that had the same appearance, and that was not limited by the season, 72 artificial flowers were made in-house of Plexiglas® to be used in the flying arena. The artificial flowers were squared (24 x 24 mm) placed on a vertical plastic cylinder (12,5 mm diameter, 40 mm length) to raise them from the floor, and a squared foot attached (24 x 24 mm) to prevent them from tipping over. Of these, 32 flowers were bi-colored in blue and yellow (each color 12 x24 mm), 18 flowers were painted yellow, and 18 flowers were painted blue (24 x 24 mm) using Biltema plastic primer spray and fluorescent sparVAR spray-color RAL 1026 Fluorescent yellow and 3107 blue Fluorescent. In the center of each artificial flower was a hole in the size of a 2 ml Eppendorf tube (7 mm diameter) to hold an Eppendorf tube containing sugar water.

To accustom the color-naive bumblebees to artificial flowers, and for the bumblebees to make the connection between specific colors and reward (sugar water, 50% water, 50% Dan sugar), the bumblebees were trained in pairs to forage from 18 bi-colored, blue and yellow, artificial flowers. The training was performed in a custom-made flying arena (130 x 100 x35cm) made of Plexiglas®, covered with a transparent lid and clothed with dark plastic plates on the sides and a white plate on the bottom to avoid and reduce disturbance to the bumblebees. During training, all flowers contained a reward of sugar water in the 2 ml Eppendorf tube placed in the hole in the center of the flowers. Rewarding flowers of both colors allowed color-naïve bumblebees to associate both blue and yellow flower with reward. The bumblebees were able to forage for 15 min each.

The day after a training bout on the bicolored flowers, the testing face started. After one training bout in the flying arena, the bumblebees were tested in pairs in the flying arena

containing nine yellow and nine blue artificial flowers. To test the experimental set-up both flowers contained 2 ml sugar water (reward), and the strength of color preference was measured by calculating the proportion blue or yellow flowers they landed on. Before each testing, the flowers were weighed to record the amount of liquid ingested by bees. By direct visual inspection and registration, the number of flowers visits, both blue and yellow, the time before probing a flower, time resting, the time walking and the time flying were recorded for each bee. Between each test, the flowers were cleaned and replaced by the other pair of blue and yellow flowers, rearranged and placed randomly in the flying arena to prevent scent marks or flowers positions as reward predictions. Under the testing, the light was on to allow registration of the flight behavior. As a result of lessons learned during the pilot, the final experimental set-up was changed and improved accordingly.

Appendix B: Automatic flower visit detection

Henrik Andersen Sveinsson

Development of the bumblebee identification software

Development of the bumblebee identification software was done alongside experiments. It could be done in no other way due to time restrictions and tight regulations regarding the time of year when bumblebees can be imported to Norway.

Libraries

The analysis program was developed using Qt creator and the OpenCV computer vision library. This allowed for computationally efficient bumblebee detection due to the Graphics processing unit (GPU) functions in OpenCV.

Per-video stream detection procedure

Each video stream was analysed using the following steps (in order): A region of interest crop using manual input, a colour filtration to detect flowers and distinguish blue flowers from bumblebees, a difference of Gaussians filter to make bumblebees appear as light spots, a threshold operation to make a binary picture of bees and background and finally a blob detection on the binary image to detect bumblebees from the binary picture. Most of the computations were done on the GPU. Only the blob detection and subsequent analyses of the data were done on the CPU.

The final result of this procedure is a set of pixel coordinates for each identified blob (bee) in each frame. A 2-hour video contains around 450000 frames at 60 fps, which means this is a computationally demanding procedure.

All videos were analysed using the same input parameters, which are given below in the step-by-step description of the detection procedure.

Bee detection procedure and parameters

Below is an ordered list of the steps in the bee detection scheme that was applied. Parameters are either given in units of pixels (for spatial quantities) or Lab colour scale values mapped onto the interval [0, 255] for all parameters L, a, b in the Lab colour space.

Detect flowers

(this is done on a frame not containing bumblebees):

1. Apply a gentle blur using a Gaussian filer of with width parameter 32 pixels but only acting on domains of 3x3 pixels. This is to remove noise.
2. Cut the region of interest to avoid detecting objects outside the flying arena.
3. Convert frame from BGR to RGB to Lab colour representation
4. Find blue and yellow pixels by the following criteria on the L, a and b values in the Lab representation of the frame (in logical notation): Blue if ((L > 140 and b < 125) or (L < 140 and b < 122)), yellow if ((L>150 and a > 105 and b >140)).
5. Detect canny edges of these detected regions. OpenCV call: createCannyEdgeDetector(128, 200, 5)
6. Find contours using the canny edges. These are the borders of each flower. OpenCV settings on the findContours function: CV_RETR_EXTERNAL, CV_CHAIN_APPROX_SIMPLE.
7. Approximate the border with its minimum bounding rectangle (for subsequent computationally efficient checking of whether a point is inside or outside the flower border.)
8. Using the centers of these rectangles from frames corresponding to the two different cameras, find the homography relating the representation of the physical plane containing the flowers seen from the two different cameras. OpenCV: findHomography with the RANSAC method.
9. Sort the flowers seen from one of the cameras by starting with a random flower and then always finding the nearest not yet sorted flower to that flower. Now every flower has a number. Match flowers seen from one camera to the ones seen from the other camera using the homography found in the latter point. Now, corresponding flowers seen from the two cameras have the same number.

Detect bees:

1. Read frame from video file.
2. Cut the region of interest to avoid detecting objects outside the flying arena.
3. Convert frame from BGR to RGB to Lab colour representation
4. Apply a gentle blur using a Gaussian filer of with width parameter 32 pixels but only acting on domains of 3x3 pixels. This is to remove noise.
5. Colour filtration. The goal is to obtain an image where bumblebees are the only dark spots. Therefore, coloured regions corresponding to the flowers and the hive entrance has to be identified. The criterion is the following (on the Lab values, in logical notation):
   ((L>140 and b < 124) or (L<140 and b<121)) and a < 112.
6. Apply Gaussian filter on L-channel on pixels satisfying the colour filtration criterion. Apply a Gaussian filter of width 3 pixels in x and y direction on domains of size 32x32 pixels (32x32 is the limit of the GPU implementation of OpenCV). Due to the 32 pixels limit of OpenCV on GPU, we apply this filter 8 times to achieve sufficient smearing of the L value.
7. Apply a difference of Gaussians filter on the L channel: lower width parameter: 11 pixels upper width parameter: 25 pixels
8. Bees will now appear as light spots. Choose pixels above a threshold value of 100 on the L channel.
9. Use the OpenCV SimpleBlobDetector with a minimum area of 100 pixels and a maximum area (not used in practice) of 2000 pixels.
10. Take the blob centres as the bee positions.
11. For each detected blob (bee), check whether it is inside the bounding rectangle of a flower.

The parameters chosen here do not origin from any fundamental property of bees or mathematics. They are simply a result of qualified trial and error and visual inspection of the quality of the detections. The parameters are specific for the experimental setup they were applied to, but the program can be used for other experimental setups if satisfactory parameters can be found. Implementing filters for other colours is a matter of changing the logical expressions related to the colour filtration.

Output

The output of the bee detection software is the following:

The position of all detected blobs in each frame on each video stream. These blobs are mostly actual bumblebees, but false detections do occur, especially on blue flowers with a low colour saturation. In practice, this means flowers that are behind a highly reflecting part of the top lid (due to setup geometry).

The colour of all flowers, and a flag indicating, for each video stream, whether the flower is visited by a bumblebee. The number of bumblebees on the flower is unknown.

The further analysis of this data to investigate the effect of imidacloprid exposure on learning was explained in the main text.

Flower visit criteria

Bumblebees were regarded as having no identity, i.e. they were indistinguishable. Thus, when designing the criteria for flower visits, the focus was on the flowers rather than the bees, and the detected property was whether a given flower was visited by a bumblebee in a given time frame. A visit was defined as a blob (a bee) having its centre within the border of the flower. This generally means that the bee is standing on the flower. In can also mean that the bee is hovering over the flower at a very low altitude.

A flower visit was defined as an event where a flower was detected to be visited by a bee from both video streams simultaneously. This mitigated the problem of false bee visit detections on blue flowers, as these false detections never (to our knowledge) happen simultaneously for sufficiently long stretches of time to be a problem. To make the method even more robust, the flower visit data were smoothed over 2 second intervals, thus eliminating problems due to short-time (in practice flickering) false detections. The final flower visit criterion was that a flower was considered visited at a point in time if a bee visit was detected on more than half of the frames in the 2 second interval surrounding that point in time.

False visit detections occur in individual frames on individual video streams, but after smoothing of data, and combining data from both streams, no false positives have been seen. Of around 20 random checks of detections, all were real flower visits.

Trajectory reconstruction

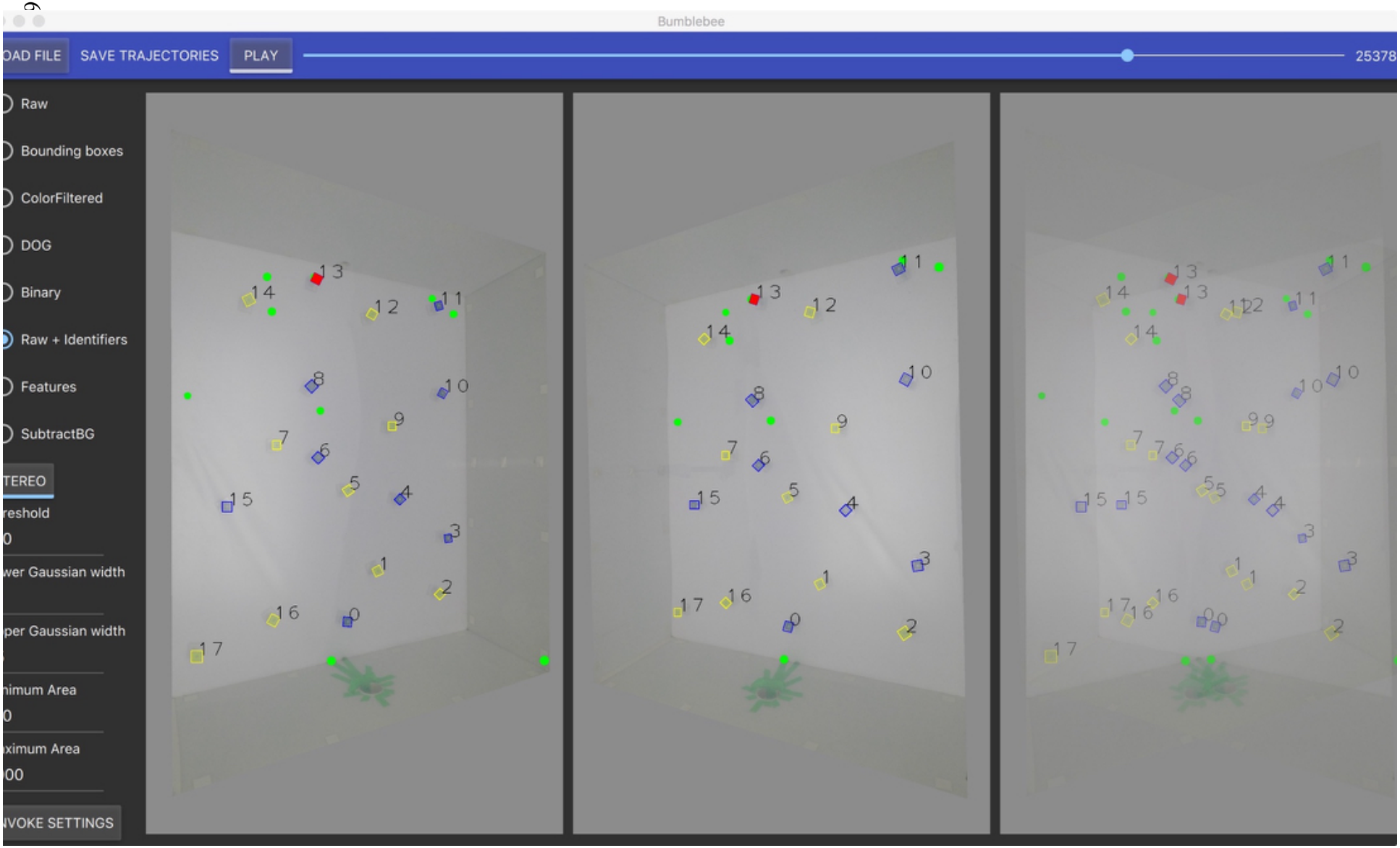
Trajectory fragment reconstruction was done using the trackpy python module version 0.3.3. Fragments means that only partial trajectories are obtained. Bees sometimes cross paths or disappear below flowers, in such a way that trackpy is unable to make *one* single path for each bumblebee. This also means that the identity of the bee belonging to each path is unknown. The settings allowed for a bee to disappear from the scene for up to 10 frames (1/6 second) and still be considered for belonging to an existing trajectory. Positions of bees in subsequent frames were predicted using the velocities from the last two frames. The maximum number of pixels the bees were allowed to move between frames was set to 40. These settings are rather arbitrary, but are confirmed to reasonable by overlaying the calculated trajectories on a movie of an experiment and verifying that the constructed trajectories follow the bumblebees.

Computer power considerations

The procedure of identifying the bumblebees is computationally heavy. For running the program, we had access to a high-end desktop computer with an Intel i7-6950X Extreme Edition with 10 computing cores. Additionally, the computer had an NVIDIA P100 graphics card, which is NVIDIAs best computing graphics card. The graphics card was necessary due to the heavy use of filtering-like operations on the video stream, and the CPU was necessary for the blob detection. On this type of computer, all 16 videos, totalling at around 60 hours of video, could be analysed in 5-6 days. Essential to the video analysis was a graphics card with a lot of memory on it. Running the program took around 2 GB of graphics memory, and to finish the analysis in the 5-6 days timeframe, 4 analyses had to be run simultaneously, requiring at least 8 GB of graphics memory, which is substantially more that on regular desktop computers.

The trajectory reconstruction procedure was computationally much cheaper, and could be done in around 1 day on a new MacBook Pro with 4 cores.

Figure 1: Screenshot of the bumblebee detection software. On the left panel, one can choose what property of the analysis to look at. In this screenshot, it is set to the "Raw + Identifiers" mode, which means the raw video stream is shown overlaid with the detected bees as green dots, the flowers indicated with rectangles and numbers, and the flowers being visited by a bumblebee indicated in red. The first two (from the left) images show the arena seen from camera A and camera B, respectively, and the last frame shows the two first images laid on top of each other in order to verify that the identified flower numbers are consistent between the cameras.



Appendix C: Data from hives

Table 1 overview of the different development stages of bumblebees in each hive

Dose (µg/L)	Number of hive	Bumble bees	Dead adult bumble bees	Big larvae	Big dead larvae	Larvae	Dead larvae	Eggs	Dead eggs	Pupa	Dead pupa	Queens yes (1) no (0)
0	1	147	NA	4	0	62	3	36	0	10	0	1
0	16	138	105	23	3	36	5	16	0	14	1	0
0	5	115	124	0	0	1	0	6	0	1	8	0
0	8	66	117	0	0	0	0	0	0	0	17	0
1	2	68	138	0	5	0	0	0	0	0	9	0
1	6	37	188	0	3	0	0	0	0	0	2	0
1	17	120	178	57	69	120	49	65	21	19	6	1
1	15	120	115	8	29	42	0	57	0	8	17	0
10	7	117	138	0	54	13	11	0	0	0	3	0
10	12	41	162	0	37	5	29	8	29	2	29	0
10	13	81	99	41	11	17	0	43	0	21	33	1
10	14	61	133	25	61	68	1	19	0	19	9	1
100	3	53	NA	0	0	0	10	0	0	6	5	0
100	9	71	155	13	41	86	35	125	130	7	27	1
100	11	50	211	3	0	0	0	0	0	0	1	0
100	10	46	170	0	41	0	22	8	16	2	15	1

Table 2 Overview of the different explanatory variables and food intake and storing of each colony

Dose (µg/L)	Number of hive	Weight of bag before exposure (g)	Weight of bag after exposure (g)	Difference in amount of nectar eaten (g)	Pollen	Days after delivery	Days after test
0	1	1083,05	735,37	347,68	1	49	0
0	16	735,11	433,64	301,47	1	58	4
0	5	987,20	627,11	360,09	1	68	14
0	8	910,16	670,58	239,58	1	54	22
1	2	1045,28	696,12	349,16	0	57	26
1	6	890,67	498,83	391,84	1	66	18
1	17	627,02	392,56	234,46	1	61	1
1	15	610,61	369,20	241,41	1	67	8
10	7	921,62	653,23	268,39	1	52	24
10	12	890,67	764,33	126,34	1	59	16
10	13	575,34	473,01	102,33	1	76	0
10	14	763,55	644,58	118,97	1	58	6
100	3	1038,52	1001,08	37,44	0	53	0
100	9	663,68	574,76	88,92	1	72	3
100	11	1171,84	1058,03	113,81	0	59	19
100	10	1035,13	906,66	128,47	0	62	14

Appendix D: Model selection

This appendix was generated from the R-script used for model selection

Honeypots

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm3	-0.59257	0.58463	NA	NA	2	-237.73	23.371	0.0000	0.53988	1.0000	31.37100
modsel.glm8	-0.24782	NA	NA	NA	1	-328.64	26.173	2.8016	0.13302	4.0585	0.00000
modsel.glm4	-2.66195	0.55738	NA	0.03406	3	-226.97	26.312	2.9410	0.12407	4.3514	35.06898
modsel.glm2	-0.66219	0.58832	0.00617	NA	3	-237.05	26.964	3.5925	0.08958	6.0271	31.57372
modsel.glm7	-3.07603	NA	NA	0.04623	2	-306.16	27.797	4.4252	0.05907	9.1396	7.73665
modsel.glm6	-0.25951	NA	0.00108	NA	2	-328.61	29.248	5.8763	0.02859	18.8805	0.00687
modsel.glm1	-3.14816	0.56490	0.01480	0.03924	4	-223.73	30.467	7.0952	0.01554	34.7305	36.10957
modsel.glm5	-3.41747	NA	0.01103	0.04984	3	-304.14	31.302	7.9310	0.01024	52.7461	8.37629

Importance

	importance
dose	0.76908
days_after_delivery	0.20892
days_after_test	0.14395

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.59257	0.28635	-2.0694	0.05749
dose	0.58463	0.22833	2.5604	0.02265

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.24782	0.26723	-0.92739	0.3684

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-2.66195	2.28949	-1.1627	0.26586
dose	0.55738	0.23073	2.4158	0.03115
days_after_delivery	0.03406	0.03729	0.9134	0.37766

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.59257	0.28635	-2.0694	0.05749
dose	0.58463	0.22833	2.5604	0.02265

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	1.0136	0.44728

Dead adults

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm3	-0.84879	0.55485	NA	NA	2	-303.26	22.629	0.0000	0.45746	1.0000	30.6694
modsel.glm2	-1.41328	0.56711	0.04360	NA	3	-257.24	24.514	1.8850	0.17825	2.5664	42.6715
modsel.glm8	-0.52981	NA	NA	NA	1	-420.48	24.820	2.1910	0.15296	2.9907	0.0000
modsel.glm6	-1.05423	NA	0.04113	NA	2	-376.85	26.082	3.4526	0.08140	5.6198	11.3757
modsel.glm4	0.32305	0.57015	NA	-0.01908	3	-298.03	26.428	3.7988	0.06846	6.6819	32.0214
modsel.glm7	-0.08177	NA	NA	-0.00724	2	-419.67	28.091	5.4620	0.02980	15.3485	0.2053
modsel.glm1	-3.45610	0.55520	0.05879	0.02985	4	-250.11	29.235	6.6062	0.01682	27.1974	44.5568
modsel.glm5	-3.66780	NA	0.05884	0.03853	3	-363.18	29.485	6.8559	0.01485	30.8137	14.9716

Importance

	importance
dose	0.72099
days_after_test	0.29131
days_after_delivery	0.12993

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.84879	0.29044	-2.9224	0.01278
dose	0.55485	0.24020	2.3099	0.03948

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.41328	0.46835	-3.0175	0.01171
dose	0.56711	0.22996	2.4662	0.03133
days_after_test	0.04360	0.02783	1.5666	0.14550

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.52981	0.26486	-2.0004	0.0668

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.84879	0.29044	-2.9224	0.01278
dose	0.55485	0.24020	2.3099	0.03948

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	1.5298	0.58353

Dead pupa

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm1	-8.12204	0.59452	0.24538	0.10397	4	-22.467	52.916	0.0000	0.90219	1.0000e+00	88.6032
modsel.glm5	-9.01548	NA	0.23758	0.12507	3	-27.830	57.364	4.4473	0.09763	9.2409e+00	80.2352
modsel.glm2	-1.10177	0.75171	0.18970	NA	3	-35.509	69.983	17.0662	0.00018	5.0802e+03	68.2506
modsel.glm6	-0.43288	NA	0.15945	NA	2	-48.447	87.604	34.6880	0.00000	3.4073e+07	48.0610

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm3	0.29185	0.35959	NA	NA	2	-74.860	131.004	78.0875	0.00000	9.0466e+16	6.8432
modsel.glm4	-0.71869	0.30824	NA	0.01636	3	-74.375	133.843	80.9266	0.00000	3.7410e+17	7.6004
modsel.glm7	-1.46502	NA	NA	0.03108	2	-77.131	134.735	81.8186	0.00000	5.8437e+17	3.2996
modsel.glm8	0.51266	NA	NA	NA	1	-79.245	135.132	82.2160	0.00000	7.1280e+17	0.0000

Importance

	importance
days_after_test	1.00000
days_after_delivery	0.99982
dose	0.90237

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-8.12204	1.61820	-5.0192	0.00030
dose	0.59452	0.19838	2.9969	0.01113
days_after_test	0.24538	0.03398	7.2215	0.00001
days_after_delivery	0.10397	0.02322	4.4784	0.00075

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-9.01548	2.35574	-3.8270	0.00210
days_after_test	0.23758	0.04904	4.8444	0.00032
days_after_delivery	0.12507	0.03344	3.7399	0.00247

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.10177	0.41088	-2.6815	0.01885
dose	0.75171	0.27262	2.7574	0.01631
days_after_test	0.18970	0.04703	4.0339	0.00142

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.29185	0.37649	0.77519	0.45112
dose	0.35959	0.32605	1.10286	0.28869

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	-0.81164	1.551

Dead small larvae

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm8	-1.0033	NA	NA	NA	1	-132.633	18.543	0.0000	0.42736	1.0000	0.00000
modsel.glm6	-1.5248	NA	0.09911	NA	2	-110.675	20.023	1.4798	0.20392	2.0957	18.33061
modsel.glm3	-1.4260	0.59913	NA	NA	2	-110.780	20.033	1.4902	0.20286	2.1067	18.24300
modsel.glm7	-1.1042	NA	NA	0.00164	2	-132.624	22.209	3.6658	0.06836	6.2520	0.00721
modsel.glm4	3.6451	0.99659	NA	-0.08664	3	-97.169	23.392	4.8488	0.03783	11.2956	29.60642

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm2	-1.7920	0.50334	0.07988	NA	3	-97.413	23.416	4.8731	0.03738	11.4336	29.40284
modsel.glm5	-2.7583	NA	0.10387	0.01948	3	-109.673	24.637	6.0943	0.02030	21.0549	19.16709
modsel.glm1	1.6376	0.77855	0.05255	-0.05612	4	-93.171	29.280	10.7364	0.00199	214.4780	32.94358

Importance

	importance
dose	0.28006
days_after_test	0.26359
days_after_delivery	0.12848

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.0033	0.40424	-2.482	0.03047

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.52478	0.52019	-2.9312	0.01501
days_after_test	0.09911	0.06284	1.5774	0.14579

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.42597	0.49635	-2.8729	0.01659
dose	0.59913	0.37894	1.5811	0.14495

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.42597	0.49635	-2.8729	0.01659
dose	0.59913	0.37894	1.5811	0.14495

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	2.3801	1.1579

Dead big larvae

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm6	-0.44048	NA	0.22594	NA	2	-56.008	22.826	0.0000	0.71468	1.0000	60.616
modsel.glm2	-0.62448	0.43167	0.20588	NA	3	-50.207	25.692	2.8668	0.17045	4.1928	65.851
modsel.glm5	-1.02080	NA	0.23098	0.00878	3	-55.864	27.122	4.2968	0.08338	8.5711	60.747
modsel.glm1	2.24472	0.64556	0.17421	-0.04483	4	-47.963	30.697	7.8710	0.01396	51.1877	67.876
modsel.glm4	7.58308	1.14175	NA	-0.12100	3	-70.422	30.803	7.9772	0.01324	53.9806	47.610
modsel.glm3	0.17701	0.80454	NA	NA	2	-100.221	34.003	11.1771	0.00267	267.3484	20.721
modsel.glm8	0.71024	NA	NA	NA	1	-123.185	36.341	13.5157	0.00083	860.7888	0.000
modsel.glm7	5.04518	NA	NA	-0.06905	2	-110.030	36.483	13.6569	0.00077	923.7692	11.870

Importance

	importance
days_after_test	0.98248

	importance
dose	0.20033
days_after_delivery	0.11136

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.44048	0.60460	-0.72856	0.48149
days_after_test	0.22594	0.11913	1.89660	0.08443

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.62448	0.74462	-0.83865	0.42126
dose	0.43167	0.62248	0.69346	0.50381
days_after_test	0.20588	0.13765	1.49570	0.16561

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.02080	4.89052	-0.20873	0.83885
days_after_test	0.23098	0.13400	1.72371	0.11548
days_after_delivery	0.00878	0.07336	0.11974	0.90706

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.17701	0.45910	0.38557	0.70717
dose	0.80454	0.47322	1.70015	0.11717

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	-0.22002	0.65714

Blue flowers

	(Intercept)	dose	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm2	-0.41287	-0.62987	NA	2	-50.448	27.240	0.000	0.58357	1.0000	27.6642
modsel.glm1	1.67963	-0.65140	-0.04344	3	-47.115	29.605	2.365	0.17887	3.2626	34.1081
modsel.glm4	-0.44895	NA	NA	1	-64.759	29.621	2.381	0.17745	3.2887	0.0000
modsel.glm3	1.07433	NA	-0.03166	2	-62.368	31.786	4.546	0.06011	9.7084	4.6223

Importance

	importance
dose	0.76244
days_after_delivery	0.23898

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.41287	0.31588	-1.3071	0.21225
dose	-0.62987	0.27978	-2.2513	0.04095

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.67963	1.93786	0.86674	0.40180
dose	-0.65140	0.28345	-2.29809	0.03880
days_after_delivery	-0.04344	0.04008	-1.08401	0.29806

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.44895	0.32973	-1.3616	0.19344

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.41287	0.31588	-1.3071	0.21225
dose	-0.62987	0.27978	-2.2513	0.04095

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	-0.65549	0.5584

Yellow flowers

	(Intercept)	dose	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm2	0.41287	0.62987	NA	2	-50.448	27.240	0.000	0.58357	1.0000	27.6642
modsel.glm1	-1.67963	0.65140	0.04344	3	-47.115	29.605	2.365	0.17887	3.2626	34.1081
modsel.glm4	0.44895	NA	NA	1	-64.759	29.621	2.381	0.17745	3.2887	0.0000
modsel.glm3	-1.07433	NA	0.03166	2	-62.368	31.786	4.546	0.06011	9.7084	4.6223

Importance

	importance
dose	0.76244
days_after_delivery	0.23898

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.41287	0.31588	1.3071	0.21225
dose	0.62987	0.27978	2.2513	0.04095

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.67963	1.93786	-0.86674	0.40180
dose	0.65140	0.28345	2.29809	0.03880
days_after_delivery	0.04344	0.04008	1.08401	0.29806

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.44895	0.32973	1.3616	0.19344

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.41287	0.31588	1.3071	0.21225
dose	0.62987	0.27978	2.2513	0.04095

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	-0.65549	0.5584

Locomotion activity

	(Intercept)	dose	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm4	-1.16720	NA	NA	1	-5.5027	13.291	0.0000	0.52341	1.0000	0.00000
modsel.glm2	-0.96932	-0.82456	NA	2	-4.8216	14.566	1.2751	0.27666	1.8919	45.72041
modsel.glm3	-0.93347	NA	-0.00472	2	-5.5018	15.927	2.6354	0.14014	3.7348	0.23262
modsel.glm1	-1.23157	-0.83371	0.00533	3	-4.8151	17.630	4.3389	0.05979	8.7536	45.95449

Importance

	importance
dose	0.33645
days_after_delivery	0.19993

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.1672	0.32489	-3.5926	0.00267

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.96932	0.24424	-3.9688	0.00140
dose	-0.82456	0.24606	-3.3510	0.00475

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.93347	1.35078	-0.69106	0.50083
days_after_delivery	-0.00472	0.02651	-0.17794	0.86132

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.96932	0.24424	-3.9688	0.00140
dose	-0.82456	0.24606	-3.3510	0.00475

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	-1.1756	0.45922

Nectar amount

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm3	0.73987	-0.96280	NA	NA	2	-5.8709	16.665	0.0000	0.59502	1.0000	62.5185
modsel.glm2	0.21178	-0.96623	0.04830	NA	3	-5.3622	18.724	2.0596	0.21247	2.8004	71.9049
modsel.glm4	1.10363	-0.95791	NA	-0.00601	3	-5.8304	19.661	2.9960	0.13304	4.4726	62.6158
modsel.glm1	-0.69053	-0.98609	0.05182	0.01441	4	-5.3941	22.425	5.7597	0.03341	17.8116	72.3795
modsel.glm8	0.20206	NA	NA	NA	1	-10.9698	24.225	7.5604	0.01358	43.8258	0.0000
modsel.glm6	-0.28564	NA	0.04536	NA	2	-10.4493	25.822	9.1568	0.00611	97.3569	10.5655
modsel.glm7	1.74679	NA	NA	-0.02543	2	-10.6755	26.274	9.6093	0.00487	122.0753	2.2495

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm5	0.60492	NA	0.04299	-0.01423	3	-10.3132	28.626	11.9614	0.00150	395.7249	11.2220

Importance

	importance
dose	0.97393
days_after_test	0.25349
days_after_delivery	0.17282

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.73987	0.24661	3.0001	0.00955
dose	-0.96280	0.20757	-4.6385	0.00038

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.21178	0.35126	0.6029	0.55695
dose	-0.96623	0.20793	-4.6470	0.00046
days_after_test	0.04830	0.02444	1.9763	0.06973

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.10363	1.87081	0.58992	0.56536
dose	-0.95791	0.21674	-4.41953	0.00069
days_after_delivery	-0.00601	0.03058	-0.19640	0.84733

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.73987	0.24661	3.0001	0.00955
dose	-0.96280	0.20757	-4.6385	0.00038

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	0.76846	0.22199

Pollen present or not

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm3	2.4221	-1.4599	NA	NA	2	-6.5355	17.994	0.00000	0.31797	1.0000	27.3618
modsel.glm2	4.7800	-2.0476	-0.13722	NA	3	-5.3502	18.700	0.70631	0.22337	1.4236	40.5357
modsel.glm4	-7.1919	-1.7423	NA	0.16517	3	-5.4666	18.933	0.93917	0.19882	1.5993	39.2417
modsel.glm8	1.0986	NA	NA	NA	1	-8.9974	20.280	2.28631	0.10137	3.1366	0.0000
modsel.glm1	-3.1219	-2.0421	-0.10740	0.12632	4	-4.8920	21.420	3.42618	0.05733	5.5461	45.6288
modsel.glm6	1.9009	NA	-0.06558	NA	2	-8.5053	21.934	3.93946	0.04436	7.1687	5.4695
modsel.glm7	-3.9814	NA	NA	0.08523	2	-8.5231	21.969	3.97514	0.04357	7.2978	5.2712
modsel.glm5	-2.9470	NA	-0.05435	0.07911	3	-8.1784	24.357	6.36269	0.01321	24.0792	9.1021

Importance

	importance
dose	0.79749
days_after_test	0.33826
days_after_delivery	0.31293

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.4221	1.34048	1.8069	0.09231
dose	-1.4599	0.90578	-1.6117	0.12933

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.78003	2.16171	2.2112	0.04555
dose	-2.04761	0.99295	-2.0621	0.05977
days_after_test	-0.13722	0.08506	-1.6132	0.13070

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-7.19194	7.69737	-0.93434	0.36716
dose	-1.74229	0.96728	-1.80124	0.09490
days_after_delivery	0.16517	0.13561	1.21795	0.24489

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.4221	1.34048	1.8069	0.09231
dose	-1.4599	0.90578	-1.6117	0.12933

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	1.6592	0.57829

Queen live or dead

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm6	1.23499	NA	-0.19645	NA	2	-7.1873	21.306	0.0000	0.53529	1.0000	32.0994
modsel.glm2	1.04451	0.63737	-0.21570	NA	3	-6.6370	23.923	2.6177	0.14460	3.7019	37.2978
modsel.glm5	-3.13513	NA	-0.20755	0.07246	3	-6.8841	24.381	3.0751	0.11504	4.6533	34.9635
modsel.glm8	-0.51083	NA	NA	NA	1	-10.5850	24.519	3.2132	0.10736	4.9858	0.0000
modsel.glm7	-4.94487	NA	NA	0.07259	2	-10.0899	26.679	5.3736	0.03645	14.6845	4.6772
modsel.glm3	-0.76167	0.44128	NA	NA	2	-10.1512	26.793	5.4870	0.03444	15.5410	4.0986
modsel.glm1	-2.45937	0.54617	-0.21959	0.05898	4	-6.4820	28.000	6.6944	0.01883	28.4232	38.7621
modsel.glm4	-4.79797	0.39715	NA	0.06645	3	-9.7672	29.718	8.4126	0.00798	67.1067	7.7257

Importance

	importance
days_after_test	0.81376
dose	0.20585
days_after_delivery	0.17830

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.23499	0.91377	1.3515	0.19796
days after test	-0.19645	0.09407	-2.0882	0.05553

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.04451	1.10170	0.94809	0.36038
dose	0.63737	0.70963	0.89816	0.38543
days_after_test	-0.21570	0.11980	-1.80040	0.09503

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-3.13513	5.65599	-0.55430	0.58878
days after test	-0.20755	0.10443	-1.98746	0.06836
days_after_delivery	0.07246	0.09501	0.76263	0.45930

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.76167	0.65677	-1.15973	0.26556
dose	0.44128	0.51961	0.84925	0.41003

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	1.7261	1.8172

Unique blue flowers

	(Intercept)	dose	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm2	-0.61889	-0.51755	NA	2	-18.055	42.630	0.0000	0.51914	1.0000	27.6072
modsel.glm1	1.55015	-0.54139	-0.04380	3	-16.810	43.877	1.2478	0.27818	1.8662	38.8437
modsel.glm4	-0.69315	NA	NA	1	-21.115	45.421	2.7915	0.12856	4.0381	0.0000
modsel.glm3	1.08246	NA	-0.03586	2	-20.085	46.522	3.8927	0.07413	7.0031	9.2947

Importance

	importance
dose	0.79731
days after delivery	0.35231

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.61889	0.24298	-2.5471	0.02325
dose	-0.51755	0.21552	-2.4014	0.03079

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.55015	1.35655	1.1427	0.27376
dose	-0.54139	0.21016	-2.5761	0.02303
days_after_delivery	-0.04380	0.02727	-1.6062	0.13223

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.69315	0.25959	-2.6702	0.01747

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.61889	0.24298	-2.5471	0.02325
dose	-0.51755	0.21552	-2.4014	0.03079

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	-1.1958	0.68413

Unique yellow flowers

	(Intercept)	dose	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm2	0.61889	0.51755	NA	2	-18.055	42.630	0.0000	0.51914	1.0000	27.6072
modsel.glm1	-1.55015	0.54139	0.04380	3	-16.810	43.877	1.2478	0.27818	1.8662	38.8437
modsel.glm4	0.69315	NA	NA	1	-21.115	45.421	2.7915	0.12856	4.0381	0.0000
modsel.glm3	-1.08246	NA	0.03586	2	-20.085	46.522	3.8927	0.07413	7.0031	9.2947

Importance

	importance
dose	0.79731
days_after_delivery	0.35231

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.61889	0.24298	2.5471	0.02325
dose	0.51755	0.21552	2.4014	0.03079

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.55015	1.35655	-1.1427	0.27376
dose	0.54139	0.21016	2.5761	0.02303
days_after_delivery	0.04380	0.02727	1.6062	0.13223

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.69315	0.25959	2.6702	0.01747

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.61889	0.24298	2.5471	0.02325
dose	0.51755	0.21552	2.4014	0.03079

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	-1.1958	0.68413

Unique flowers

	(Intercept)	dose	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm4	4.8750	NA	NA	1	-42.481	40.804	0.0000	0.61273	1.0000	0.0000
modsel.glm2	5.2118	-0.67356	NA	2	-41.293	42.878	2.0739	0.21723	2.8206	7.1501
modsel.glm3	4.0032	NA	0.01752	2	-42.431	43.840	3.0353	0.13433	4.5615	0.2967
modsel.glm1	4.5630	-0.66701	0.01297	3	-41.263	46.489	5.6851	0.03571	17.1596	7.3293

Importance

	importance
dose	0.25294
days_after_delivery	0.17003

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.875	0.82601	5.9019	3e-05

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	5.21178	0.95468	5.45917	0.00008
dose	-0.67356	0.73330	-0.91852	0.37390

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.00321	3.42470	1.16892	0.26195
days_after_delivery	0.01752	0.06744	0.25984	0.79877

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	5.21178	0.95468	5.45917	0.00008
dose	-0.67356	0.73330	-0.91852	0.37390

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	6.9954	6.9865

Total flowers

	(Intercept)	dose	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm4	14.438	NA	NA	1	-154.91	22.455	0.0000	0.62182	1.0000	0.0000
modsel.glm2	15.757	-2.6399	NA	2	-148.27	24.780	2.3245	0.19449	3.1972	5.3765
modsel.glm3	22.954	NA	-0.17119	2	-153.09	25.325	2.8700	0.14806	4.1997	1.4784
modsel.glm1	24.059	-2.7752	-0.16551	3	-146.13	28.175	5.7194	0.03562	17.4559	7.1026

Importance

	importance
dose	0.23011
days_after_delivery	0.18369

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	14.438	4.3205	3.3417	0.00446

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	15.7575	5.0214	3.13804	0.00726
dose	-2.6399	3.7747	-0.69938	0.49577

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	22.95412	17.89943	1.28239	0.22053
days_after_delivery	-0.17119	0.33538	-0.51043	0.61771

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	15.7575	5.0214	3.13804	0.00726
dose	-2.6399	3.7747	-0.69938	0.49577

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	5.7795	7.3853

Total broods

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm2	0.17745	0.36309	-0.10588	NA	3	-204.88	26.637	0.00000	0.51063	1.0000	71.983
modsel.glm6	0.41180	NA	-0.10416	NA	2	-273.77	27.365	0.72771	0.35489	1.4389	60.360
modsel.glm1	2.89828	0.46575	-0.12593	-0.04145	4	-183.18	30.189	3.55249	0.08644	5.9076	75.644
modsel.glm5	0.81535	NA	-0.10672	-0.00600	3	-273.17	31.367	4.73018	0.04797	10.6450	60.462
modsel.glm7	-4.28303	NA	NA	0.05948	2	-539.82	45.794	19.15718	0.00004	14452.0177	15.473
modsel.glm3	-0.75567	0.34518	NA	NA	2	-559.97	47.190	20.55323	0.00002	29045.4271	12.073
modsel.glm4	-3.76980	0.25816	NA	0.04863	3	-503.46	47.320	20.68295	0.00002	30991.7167	21.608
modsel.glm8	-0.52216	NA	NA	NA	1	-631.53	48.838	22.20101	0.00001	66204.6846	0.000

Importance

	importance
days after test	0.99992
dose	0.59710
days after delivery	0.13446

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.17745	0.25918	0.68464	0.50774
dose	0.36309	0.16137	2.25002	0.04589
days_after_test	-0.10588	0.02245	-4.71670	0.00063

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.41180	0.27365	1.5049	0.15822

	Estimate	Std. Error	t value	Pr(>|t|)
days_after_test	-0.10416	0.02558	-4.0725	0.00155

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.89828	2.08283	1.3915	0.19425
dose	0.46575	0.17866	2.6069	0.02618
days_after_test	-0.12593	0.02699	-4.6657	0.00089
days_after_delivery	-0.04145	0.03152	-1.3151	0.21782

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.75567	0.3142	-2.4051	0.03321
dose	0.34518	0.2532	1.3633	0.19782

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	2.1892	1.3051

Dead broods

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm2	-1.52232	0.57109	0.13726	NA	3	-110.15	27.588	0.0000	0.82395	1.0000e+00	73.28195
modsel.glm1	-1.05400	0.60069	0.13393	-0.00736	4	-109.85	31.909	4.3209	0.09498	8.6751e+00	73.38451
modsel.glm6	-1.06868	NA	0.14277	NA	2	-174.77	33.311	5.7231	0.04711	1.7489e+01	52.44914
modsel.glm5	-4.09312	NA	0.16116	0.04548	3	-154.42	33.999	6.4114	0.03340	2.4672e+01	58.95693
modsel.glm4	3.53862	0.91512	NA	-0.07158	3	-213.99	42.626	15.0384	0.00045	1.8431e+03	40.44366
modsel.glm3	-0.76225	0.59983	NA	NA	2	-257.79	45.333	17.7453	0.00012	7.1341e+03	26.39328
modsel.glm8	-0.27878	NA	NA	NA	1	-339.84	54.138	26.5504	0.00000	5.8258e+05	0.00000
modsel.glm7	-0.14174	NA	NA	-0.00216	2	-339.77	57.205	29.6173	0.00000	2.6997e+06	0.02121

Importance

	importance
days_after_test	0.99944
dose	0.91949
days_after_delivery	0.12882

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.52232	0.29693	-5.1269	0.00019
dose	0.57109	0.18141	3.1481	0.00770
days_after_test	0.13726	0.03222	4.2597	0.00093

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.05400	2.14047	-0.49242	0.63131
dose	0.60069	0.23079	2.60279	0.02311
days_after_test	0.13393	0.03666	3.65341	0.00331

	Estimate	Std. Error	t value	Pr(>|t|)
days after delivery	-0.00736	0.03332	-0.22082	0.82895

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.06868	0.28678	-3.7264	0.00226
days after test	0.14277	0.03921	3.6416	0.00267

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.76225	0.34405	-2.2156	0.04380
dose	0.59983	0.26755	2.2419	0.04168

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	1.2708	0.46649

Total mortality

	(Intercept)	dose	days_after_test	days_after_delivery	df	logLik	QAICc	delta	weight	evidence_ratio	pseudo_r2
modsel.glm2	-1.98026	0.54187	0.09752	NA	3	-278.17	24.335	0.0000	0.68230	1.0000	65.7091
modsel.glm6	-1.51204	NA	0.08954	NA	2	-429.65	26.766	2.4305	0.20239	3.3711	44.0329
modsel.glm1	-3.52630	0.52003	0.10943	0.02265	4	-272.38	29.143	4.8081	0.06165	11.0679	66.5785
modsel.glm5	-4.35418	NA	0.10994	0.04169	3	-406.35	29.814	5.4790	0.04408	15.4793	47.4415
modsel.glm4	3.28402	0.58211	NA	-0.06762	3	-518.00	34.587	10.2518	0.00405	168.3284	31.3156
modsel.glm3	-0.86110	0.44682	NA	NA	2	-621.48	34.966	10.6309	0.00335	203.4532	16.6504
modsel.glm8	-0.55129	NA	NA	NA	1	-737.83	36.630	12.2950	0.00146	467.5424	0.0000
modsel.glm7	2.06614	NA	NA	-0.04177	2	-693.53	38.046	13.7108	0.00072	948.9772	6.2489

Importance

	importance
days_after_test	0.99041
dose	0.75135
days after delivery	0.11050

Summary of the highest ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.98026	0.40552	-4.8832	0.00048
dose	0.54187	0.20546	2.6373	0.02310
days after test	0.09752	0.02508	3.8888	0.00252

Summary of the second ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.51204	0.40023	-3.7779	0.00263
days after test	0.08954	0.02864	3.1267	0.00875

Summary of the third ranked model.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-3.52630	2.97495	-1.18533	0.26329

	Estimate	Std. Error	t value	Pr(>|t|)
dose	0.52003	0.21599	2.40762	0.03683
days_after_test	0.10943	0.03486	3.13896	0.01053
days_after_delivery	0.02265	0.04291	0.52782	0.60913

Summary of the dose only model

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.86110	0.36047	-2.3888	0.03421
dose	0.44682	0.28800	1.5515	0.14675

EC/LC50 estimate from the dose only model

	log10dose	SE
p = 0.5:	1.9272	0.99122