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Pesticide Effects on Bumble-Bees

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Preface and acknowledgement

The present report is the result of a project mainly funded by the Danish EPA (MST-667-00138). The project has been followed by an expert group consisting of Jens Erik Ørum (Department of Food and Resource Economics (IFRO), University of Copenhagen), Jens Erik Jensen (SEGES), Ghita Cordsen Nielsen (SEGES), Nis Schmidt (Dow AgroSciences Denmark A/S), Thomas Secher Jensen (Natural History Museum, Aarhus), Signe Anthon (Environmental Economics, Danish EPA), Ilse Ankjær Rasmussen (International Centre for Research in Organic Food Systems), Niels Holst (Department of Agroecology, Aarhus University), Lise Nistrup Jørgensen (Department of Agroecology, Aarhus University), Johannes Michelsen (Department of Political Science and Public Management, University of Southern Denmark), Rune Vesterager Asmussen (Danish Christmas Tree Association), Helga Hjort (Danish EPA) and Henrik Frølich Brødsgaard (Danish EPA).

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Dansk sammendrag

Baggrund
I den nuværende risikovurdering af pesticider vurderes risikoen for uønskede effekter på honningbier ved at udføre kortvarige (48 timer) tests på disse, hvor alene effekten på voksne individers overlevelse måles. Det er imidlertid usikkert, om en risikovurdering baseret på sådanne simple tests i tilstrækkelig grad beskytter honningbierne mod utilsigtede virkninger af pesticider, da nogle undersøgelser peger på, at ikke-dødelige, men stadig alvorlige effekter på fx reproduktion og orienteringsevne kan opstå ved langt lavere doser. For at undersøge dette er det nødvendigt med undersøgelser, der anvender lavere doser, foregår over en længere periode og måler på andet end dødelighed.

Forholdene i laboratoriet er desuden generelt optimale for testdyrene, mens det i naturen er sjældent, at sprøjtemidlerne er den eneste påvirkning af bierne. Ofte vil der være perioder med for lavt udbud af føde (nektar og pollen), og bier kan også være inficeret af skadelige mikroorganismer (patogener). Det synes derfor oplagt at undersøge, om bier, der i forvejen er stressede af fx sult eller patogener, er mere følsomme over for pesticider end ustressede bier.

Endvidere er honningbien jo ikke det eneste bestøvende insekt, som kan blive påvirket af pesticider. Det er ukendt, hvor godt effektstudier på honningbien stemmer overens med følsomheden af fx humlebier, enlige bier, svirrefluer og sommerfugle. Vi har derfor i dette projekt valgt at fokusere på en anden almindeligt forekommende bestøver, jordhumlen *Bombus terrestris*, som desuden anvendes til bestøvning i bl.a. væksthuse, og som derfor kan købes året rundt. Vi har undersøgt effekten af tre insekticider, som alle anvendes i blomstrende afgrøder, hvor bl.a. humlebier søger føde, og derfor kan forventes at påvirke humlebierne, enten fordi de får stofferne på sig, når de kravler rundt, eller fordi de optager det sammen med pollen og nektar.

Formål
Hovedformålet med projektet var at undersøge

1) hvor godt resultater af tests af pesticider på humlebier udført på forskellige niveauer (varighed, doser, effektmål) stemmer overens, altså med hvor stor sikkerhed kan man ekstrapolere fra fx simple laboratorietests til pesticideffekter i felten
2) effekten af tre udvalgte insekticider på humlebier, når bierne blev udsat for pesticiderne alene eller i kombination med en insektpatogen svamp eller sult

Undersøgelser
Vi har undersøgt effekten af tre insekticider:

- Thiacloprid (Biscaya), et systemisk virkende neonicotinoid som bruges til at bekæmpe skadegørende insekter i rapsmarker
- Pyrethroidet alpha-cypermethrin (Fastac), som i Danmark bruges i kornmarker, rapsmarker og grøntsagsafgrøder
- Lambda-cyhalothrin (Karate), som i Danmark bruges i kornmarker, rapsmarker og grøntsagsafgrøder (inkl. ærter og kartofler), frugtplantager og marker med frøproduktion (inkl. kløver). Mange af disse afgrøder er bibestøvede.

Fire forskellige typer tests blev udført:

1. Korttidsundersøgelser, hvor humlebierne testes på same måde som honningbierne standardmæssigt testes. I vore forsøg blev pesticiderne tilført bierne ved at afsætte en dråbe på thorax (”nakken”). Resultatet af disse tests er dødeligheden efter 48 timer.
2. Længerevarende laboratorieundersøgelser, hvor man ud over dødeligheden kan vurdere effekter på reproduktive mål såsom antal æg og larver. Her brugte vi mikrokolonier uden dronning, som er nemme at overskue. Bierne fik tilført pesticiderne via sukkervand, og effekten blev målt 14 dage senere.
3. Semifelttests, hvor bierne i mikrokolonierne ikke bare levede i en lille boks, men havde adgang til et større bur, hvor de kunne bevæge sig rundt og finde føde i kunstige blomster. Her fik humlebierne pesticiderne i sukkervand, og effekterne blev målt på samme måde som i de længerevarende laboratorieforsøg. Desuden målte vi effekten på biernes adfærd vha. såkaldte RFID-målinger, hvor en slags stregkode limes på bierne, hvorefter deres færden ind og ud af den boks, som de bor i, kan følges vha. en læser.
4. Feltundersøgelser, hvor hele boer af humlebier med dronning blev udsat for pesticider via sukkervand, hvorefter de blev sat ud i områder uden pesticidpåvirkning. Bifamiliernes udvikling blev fulgt ved at veje rederne ugentligt i 8 uger.

I forsøgene i laboratoriet og i semifelttestene undersøgte vi ud over effekten af pesticiderne alene, om humlebierne blev mere følsomme over for pesticiderne, hvis de i forvejen enten var inficeret med en insektpatogen svamp eller var blevet sultet. I felttestene blev alene effekten af pesticiderne undersøgt.

Konklusioner

Hovedkonklusionerne fra projektet er:

1. Vore undersøgelser tyder ikke på, at effekter på humlebiernes reproduktion (målt som ED_{50} for antallet af æg og larver, dvs. den dosis, der forårsager 50% reduktion), forekommer ved lavere pesticiddoser end dem, som humlebierne dør af (målt som LD_{50}, den dosis der forårsager en dødelighed på 50%). Derimod er der indikationer på, at humlebiernes aktivitetsniveau kan være påvirket ved meget lave doser af Biscaya. Dette bør undersøges nærmere.
2. På grund af svingende succes med at måle de forskellige typer effekter er det mest oplagt at sammenligne de forskellige typer tests ved at sammenligne effekten på dødelighed (LD_{50}). Her var tendensen, at humlebierne i de mere komplicerede og langvarige tests var mere følsomme over for pesticiderne, dvs. LD_{50}-værdierne faldt, jo mere realistisk testdesignet var i forhold til humlebier i naturen. Dette kan hænge sammen med, at de mere realistiske tests kører over længere tid og ved mindre optimale betingelser for humlebierne. Desuden betyder det noget for effekten af pesticiderne, om humlebierne udsættes for dem gennem føden eller gennem huden.
3. Resultaterne tyder på, at humlebier ikke er mere følsomme end honningbier over for de undersøgte insekticider. Imidlertid er det værd at tage i betragtning, at der er langt færre bier i et humlebibo end i et honningbistade, og derfor kan fx en halvering af antallet af arbejdere have en langt større effekt på humlebiboets samlede trivsel og overlevelse. Dette gælder ikke mindst først på sæsonen, hvor der kun er dronningen og ganske få arbejdere i boet; da vil en påvirkning af et enkelt individ kunne være afgørende for koloniens overlevelse.
4. Når vi udsatte humlebierne for insektpatogen svamp eller sult forud for pesticidforsøgene, gjorde det overordnet set ikke humlebierne mere følsomme over for pesticiderne. Da sådanne forsøg kan udføres på mange forskellige måder og med andre patogener, bør interaktioner mellem pesticider og andre stresspåvirkninger undersøges yderligere.

5. De anvendte metoder til undersøgelser af pesticideffekter på humlebier i laboratoriet og ude i felten fungerede tilfredsstillende, mens det set-up, vi anvendte til undersøgeler under semi-feltbetingelser i væksthuset, ikke var optimalt. Til sådanne undersøgelser vil det formentlig være bedre at anvende hele humlebikolonier med en dronning, der udsender feromoner og styrer samarbejdet mellem arbejderbierne. Også RFID-teknikken til at følge biernes adfærd skal videreudvikles, før den kan anvendes i større skala og med bedre sikkerhed – fx faldt for mange af mærkerne af under forsøgene.

Summary

Background

In the current standard risk assessment of pesticides, the risk of adverse effects on honeybees is assessed by short-term (48 hours) tests, in which only the effect on adult survival is measured. However, some studies indicate that non-lethal, long-term effects on e.g. reproduction and orientation while foraging may occur at much lower dosages. Hence, a risk assessment based on such simple tests may not detect adverse effects of pesticides on bees and, consequently, regulations based on these risk assessments may not protect these beneficial insects sufficiently. In order to investigate sub-lethal effects of pesticides, studies using lower dosages are required over a longer period.

Furthermore, in laboratory tests conditions are generally optimal for the test animals, while in nature pesticides are seldom the only stressor of bees. In most landscapes in Northern Europe, periods of food (nectar and pollen) scarcity occur, and bees are exposed to a range of different pathogens and parasites. Thus, an interesting aspect to investigate is if bees exposed to other stressors, including nutritional stress or pathogens, are more sensitive to pesticides than non-stressed bees.

Honeybees are not the only pollinating insects affected by pesticides. However, it is unknown whether the sensitivity of wild pollinators, including bumble-bees, solitary bees, hover flies and butterflies, differs from honeybees, which have a very different life history. Therefore, this project focusses on another common pollinator, the buff-tailed bumble-bee, *Bombus terrestris*, which is also used for pollination, e.g. in greenhouses, and therefore can be purchased year round. We have investigated the impact of three insecticides, all of which are used in flowering crops that attract flower-visiting insects. Hence, foraging bumble-bees are expected to be exposed to pesticides by direct contact when handling sprayed flowers or by ingesting them with pollen and nectar.

Objectives

The main objective of the project was to assess

1) to which extent results from different types of tests of pesticide effects on bumble-bees (differing in duration, dosages, end-point) are consistent. Hence, we assess the level of uncertainty when extrapolating results e.g. from simple laboratory tests to pesticide effects in the field

2) the effect of three different insecticides on bumble-bees, when the bees are exposed to the pesticides alone or in combination with an insect pathogenic fungus or starvation

Studies

We have studied the effect of three insecticides:

- Thiacloprid (Biscaya), a systematically acting neonicotinoid used to control harmful insects in oilseed rape fields
- The pyrethroid alpha-cypermethrin (Fastac), used in Denmark in cereal fields, rape fields and vegetable crops
- Lambda-cyhalothrin (Karate), used in Denmark in cereal fields, rape fields, vegetable crops (incl. peas and potatoes), orchards and fields with seed production (incl. clover). Many of these crops are pollinated by bees.

Four types of tests were carried out:

1. Short-term laboratory tests, in which bumble-bees are tested as in the honeybee acute tests. In our study, pesticides were applied topically to the bees by depositing a drop of pesticide on the thorax. Results from these tests show mortality after 48 hours.
2. Long-term laboratory studies that, in addition to mortality, assess effects on reproduction, such as numbers of eggs and larvae. In these studies, we used queen-less micro-colonies, which are easy to manage. Bees were exposed orally by feeding them sugar solution containing pesticide, and the effect was measured during a period of 14 days.
3. Semi-field tests, in which the bees in the micro-colonies did not only live in the small box, but had access to a larger cage in which they could move around and forage on artificial flowers. In these studies, bumble-bees were fed a sugar solution containing pesticide, and the effects were measured in the same manner as in the long-term laboratory tests. Additionally, we monitored behavioural effects using RFID technology, in which bees were marked individually with passive tags, and their movements in and out of a small box placed at the entrance hole of the micro-colony nest box was tracked by a reader.
4. Field studies, in which hives with queen-right colonies of bumble-bees were exposed by feeding them sugar solution containing pesticide and thereafter released into landscapes with a low pesticide load. The development of the bee families was measured by weighing the nests on a weekly basis for eight weeks.

In the laboratory and semi field tests, in addition to studying the effect of pesticides alone, we tested whether the sensitivity of bumble-bees to effects of pesticides increased when the bees were infected by an insect-pathogenic fungus or were starved prior to the experiments. In the field studies, the combined effects of stressors were not studied.

Conclusions

The main conclusions from the project are:

1. Our studies do not indicate that the effects on bumble-bee reproduction (measured as ED_{50} for the number of eggs and larvae, i.e. the dosage that causes a 50% reduction) occur at pesticide dosages below those that cause mortality in bumble-bees (defined as LD_{50}, the dosage that causes a 50% mortality). However, there are indications that the activity level of bumble-bees may be affected by very low dosages of Biscaya. This should be investigated further, as Biscaya is increasingly used in flowering crops.
2. Due to varying success in measuring pesticide effects on non-lethal end-points, results from the different tests are best compared by the parameter LD_{50}. An important finding was that bumble-bees tend to be more sensitive in the more complex and long-term tests, i.e. the LD_{50}-values decreased, the closer the test design mimicked natural conditions. These more field-realistic tests are run over a longer period of time and with less optimal conditions for the bumble-bees. Additionally, it makes a difference to the effect of pesticides whether bees are exposed through food or by direct surface contact.
3. The results indicate that bumble-bees are no more sensitive towards the examined insecticides than honeybees. However, it should be considered that bumble-bee colonies are much smaller (<400 individuals) than honeybee hives (<60000 individuals) and, therefore, e.g. decreasing the number of worker bees may have a far greater impact on the functioning and survival of the bumble-bee hive. This is particularly important early in the season during colony foundation, when the colony only contains the queen and a small number of workers; then the impact on even a single individual may be detrimental to the survival of the colony.
4. In the experiments where bumble-bees were pre-exposed to insect-pathogenic fungus or starvation, bumble-bees were generally not more sensitive to the pesticides. However, since such tests can be conducted under various experimental designs, including exposure to other pathogens, interactive effects of pesticides and other stressors should be investigated further.

5. The methods applied for studying pesticide effects on bumble-bees in the laboratory and in the field worked well, while the set-up we used for tests under semi-field conditions in the greenhouse was less than optimal. For such tests, it is probably better to use entire bumble-bee colonies with a queen that emits pheromones and controls the cooperation between worker bees. The RFID-technique for investigating bee behaviour should also be developed further prior to being used on a large scale and with more certainty – for instance, too many labels fell off during testing.

1. Background

Wild pollinators, including bumble-bees, enhance the yield of many crops, independently of the abundance of commercial honeybees (Garibaldi et al. 2013). In addition, the wild flora depends on bumble-bees and other wild bees. In Europe and North America, bumble-bees have been declining since the 1950s and 1960s, at the onset of industrialization and agricultural intensification (Benton, 2006; Biesmeijer et al., 2006; Colla and Packer, 2008; Goulson et al., 2008; Grixti et al., 2009; Murray et al., 2009). Declines are ascribed to historical changes in land use and landscape composition, leading to loss of nesting sites (Skovgaard, 1943; Kells and Goulson, 2003; Osborne et al., 2008b), decline of forage plants due to change in crops (fewer legumes) and fewer flower-rich hay meadows (Rasmont and Mersch, 1988; Goulson and Hanley, 2005), pathogen spill over and competition from commercial bees (Otterstatter and Thomson, 2008; Wermuth and Dupont, 2010), and pesticide use (Marletto et al., 2003; Woodcock et al., 2016).

In 2012, an expert panel in EFSA (European Food Safety Authority) summarised existing knowledge about the effects of pesticides on bees at the time (EFSA PPR 2012). This report identified several knowledge gaps concerning pesticide effects on bees. In particular, it was concluded that knowledge of pesticide effects was lacking for wild bees (bumble-bees and solitary bees), and effects on end-points (i.e. the parameters used to measure toxic effects) apart from direct mortality, including reproductive, developmental and behavioural end-points. Such sub-lethal end-points should be addressed because low pesticide dosages, which do not lead to increased mortality, may have serious effects at colony and population levels. Furthermore, only few studies have addressed effects of pesticides on bees under field or field-like conditions. Although complex, these studies are necessary to assess the results of short-term laboratory tests that only measure effects on mortality and their relevance in the field to ultimately assess the risk of pesticide use in agricultural crops. EFSA also pointed out the need for more studies of the relation between tests at different tiers, i.e. how results from controlled laboratory conditions can be linked to effects under semi-field conditions and to patterns observed at the complex field scale. Finally, the EFSA report concluded that little is known about effects of repeated pesticide exposure or mixtures of different pesticides. Another EFSA report (EFSA 2014) pointed out the lacking knowledge about interaction effects between pesticides and other stressors, including food shortage, diseases and parasites, especially for non-Apis bees.

1.1 Sub-lethal effects of pesticides on bees

In a pilot study of pesticide effects on bees, we reported that bumble-bees are exposed to sublethal dosages of pesticides in Danish agricultural landscapes (Bruus et al., 2013). However, various studies of honeybees and wild bees indicate that non-lethal effects of pesticides include a broad range of symptoms, including reduction in growth, delayed development, reduced reproduction and change in behaviour (EFSA 2012 and references therein, for neonicotinoids: (Godfray et al., 2014; Godfray et al., 2015) and references therein). Behavioural changes of adult bees include loss of homing ability (Henry et al., 2012) and decrease in foraging activity (Morandin et al., 2005; Mommaerts et al., 2010; Gill and Raine, 2014). Mortality due to homing failure in honeybees exposed to sub-lethal levels of thiamethoxam is at a level which may ultimately lead to colony collapse and, hence, pesticide exposure may be an important causal factor in the bee disease CCD (Colony Collapse Disorder) (Henry et al., 2012). A decline in foraging efficiency (e.g. amount of pollen collected per foraging bout) will lead to a decrease in nest provision, which, in turn, may affect offspring fitness negatively. In bumble-bee colonies exposed to sublethal dosages of the neonicotinoid imidacloprid, colony growth

was impaired, pollen provisioning by the workers decreased, and fewer new queens were produced (Gill et al., 2012; Whitehorn et al., 2012). In conclusion, several studies have documented that sub-lethal exposure to pesticides may have serious detrimental consequences for colony survival and reproduction. In addition, the pollination services provided by these bees are expected to be impaired. Thus, it is a major concern that more subtle effects than acute toxicity of pesticides (including other types than neonicotinoids) may severely affect pollinator populations and biodiversity.

1.2 Bumble-bee biology

Colonies of all nest-building species of bumble-bees, including the study species *Bombus terrestris* L. (Hymenoptera: Apidae), are annual. They are initiated by a queen, which has been mated the previous year. Queens emerge from hibernation in spring, collect pollen and nectar, and provision their nests. Nests are mostly below ground, often in old mouse nests (Goulson, 2003). The first batch of eggs (5-10) hatch into worker bees (sterile females), which help the queen collect forage, clean the nest, and take care of subsequent offspring. As more and more workers are produced, the colony grows and may reach up to 400 workers in *B. terrestris*. At climax, sexual offspring (new queens and males) are produced. Towards the end of colony life, the sexuals leave the nest and mate, and eventually the new mated queens hibernate, completing the annual cycle, as the old colony degenerates (Goulson, 2003).

Several bumble-bee species, including *B. terrestris*, can be found in the field from early spring to autumn. However, the cycle of a single colony ranges from a few weeks to a couple of months, depending on the species (Dupont and Madsen, 2010). Furthermore, within species emergence time may vary by several months. Queens of *B. terrestris* emerge from hibernation as early as February/March and as late as May/June in Denmark (Dupont and Madsen, 2010). Flowers of oilseed rape are an important floral resource for bumble-bees in spring. If oilseed rape fields are sprayed with pesticides, foraging bumble-bee queens and workers may be exposed directly. However, foraging bees provisioning the nest may also bring back poisonous nectar and pollen to the young, when feeding on oilseed rape that has been treated with systemic pesticides.

For the purpose of testing, smaller units, called micro colonies, can be used. These consist of e.g. five bumble-bee workers from the same colony, which are transferred to a smaller nesting box and provided with pollen and sugar water. In the absence of a queen (and her pheromones, which suppress reproduction of the workers), one of the workers will start producing unfertilized eggs, often within a week. All bees of the micro colony help building new brood cells and honey pots; eggs hatch into larvae, which pupate and hatch into adult bees (Regali & Rasmont 1995, Tasei et al. 2000, Mommaerts et al. 2006). Hence, micro colonies function much like full colonies, except that offspring is always male because workers only lay unfertilized eggs. In the micro-colonies, the number of egg cells, larvae and adult males can be monitored non-destructively, in particular if the nesting box is made of transparent material.

1.3 Methods for testing pesticide toxicity on bumble-bees

Although the two above-mentioned EFSA reports give recommendations for future research, their main long-term objective is to establish a scientifically sound and yet cheap system for risk assessment of pesticides on bees, including bumble-bees and solitary bees. Although higher tier testing is in principle included in the present procedure for approval of new pesticides, the only obligatory bee test included is a standard honeybee test (OECD 1998a,b), in which mortality is measured as LD_{50} within 48 hours, i.e. the dosage causing a 50% mortality. The sub-lethal, long-term effects of pesticide exposure on commercial and wild bees summarised in the EFSA report (EFSA PPR Panel 2012) raised the concern that acute standard tests do not adequately address the risk of pesticide use in the field and provided suggestions for improving the risk assessment procedure. Among the recommendations was the suggestion that sub-lethal endpoints should be included in the first tier laboratory tests by implementing

the test system with micro-colonies described by e.g. Mommaerts & Smagghe (2011), and that the methods for semi-field and field testing should be improved. A couple of years later, EFSA (2014) gave extended recommendations about the research needed to fill the knowledge gaps concerning risk assessment of multiple stressors on bees. Since then, the number of studies investigating pesticides effects, in particular neonicotinoids, have sky-rocketed (Godfray et al., 2014; Godfray et al., 2015). However, bee health and pesticide effects are still high on the agenda, e.g. in the EU call Horizon 2020.

Relatively few higher tier studies have been conducted, in particular field studies (but see (Budge et al., 2015; Rundlof et al., 2015; Woodcock et al., 2016). Semi-field experiments are useful because they are relatively small systems in which several physical variables can be controlled in contrast to studies at field scale. In semi-field studies, the foraging of bees is restricted to a small area of sprayed flowers. Hence, they do not have the choice of visiting sprayed versus non-sprayed flowers (EFSA PPR 2012). On the other hand, results of full scale field studies can be difficult to interpret due to a variety of interfering factors, including weather variables, different landscape types (Rundlöf et al., 2008), temporal and spatial distributions in floral resources (Osborne et al., 2008a) etc. Notwithstanding these challenges, studies at large spatial scales using standard hives (entire colonies of bees) may reveal pesticide effects, which cannot be adequately tested under laboratory or semi-field conditions. In particular, these include studies concerning homing and navigation ability at landscape scale.

1.4 Interactions between pesticides and other stressors: Pathogens

As for other livings organisms, the longevity and fitness of bumble-bees is influenced by a variety of biotic and abiotic stressors in the environment. Among these stressors are parasites, which may challenge the immune system of the host and, in the case of parasitic microorganisms acting as pathogens, lead to disease. Infected bumble-bees may also encounter chemical pesticides in the environment when present as residues in pollen, nectar or on treated vegetation. The combination of pathogen infection and pesticides may result in synergistic interactions, where the adverse effect of the combination of stressors is significantly higher than would be expected, based on simple addition of the effect caused by the individual stressor. This has been studied widely in the last decades in relation to more environmentally friendly pest control, and in many cases a combination of a pathogen, e.g. the entomopathogenic fungus *Beauveria bassiana*, and a low dosage of an insecticide has been found to increase the efficacy of the fungus treatment (e.g. Furlong & Groden, 2001; Farenhorst et al. 2010).

Populations of bumble-bees are known to harbour a large number of parasitic organisms of different taxonomic groups, some of which have significant impacts on colony fitness (MacFarlane et al. 1995, Schmid-Hempel 1998, Schmid-Hempel 2001). The microsporidian fungus *Nosema bombi* and other pathogens such as the trypanosome *Critidia bombi* and the neogragarine *Apicystis bombi* are very common in bumble-bees and frequently can be found in high proportions of the populations (e.g., Rutrecht & Brown 2008, Gillespie 2010, Goulson et al. 2012). These pathogens have also been recorded from wild populations of Danish bumble-bees (Steenberg, unpubl. data).

Parasites, including species acting as pathogens, are thought to be one of several causes of population decline in bumble-bees (Cameron et al. 2011), and even when not killing the host, the activation of the immune system is likely to be costly in terms of energy or nutrients. Therefore, the effect of pesticide exposure may increase if bees are also infected, as infected bees may become more susceptible to pesticides compared to non-infected bees. Pioneering laboratory studies on such interactions of stressors in honeybees (Apis mellifera) focused on the gut parasite Nosema ceranae and neonicotinoid pesticides and indicated synergistic effects between the two stressors (Alaux et al. 2010, Vidau et al. 2011). However, recent field studies

were not able to document such effects in honeybees (Retschnig et al. 2015). Similar studies in bumble-bees are still very few and only recently published and used another gut parasite, *Crithidia bombi* in combination with a neonicotinoid and a pyrethroid pesticide, respectively (Fauser-Misslin et al. 2013, Baron et al. 2014). Only Fauser-Misslin et al. 2013 were able to show a synergistic effect of the combination treatment and only on queens.

In contrast to *Nosema sp*., which is the pathogen given the main focus in previous interaction studies involving bees, pathogens and pesticides, *B. bassiana* can be propagated in vitro. It also has the advantage of enabling easy topical application, which is simple, precise and less time consuming than working with pathogens that need to be ingested. Topical application further allows combination with pesticides administered orally without the risk of adverse effects of pesticides directly on fungus propagules. These factors, combined with the fact that *B. bassiana* is known to be virulent to bumble-bees (Mommaerts et al. 2009, Kapongo et al. 2008a) and that it has been reported to cause synergistic effects when combined with pesticides applied against non-pollinators, form the rationale behind choosing *B. bassiana* as a model for a bumble-bee pathogen.

The fungus *B. bassiana* has not previously been included in pathogen-pesticide interaction studies in bees, as it is not a bee pathogen per se, i.e. as a generalist species it is not among those pathogens causing disease in bees, which are afflicted by a range of specialist pathogens with limited host ranges. This ubiquitous entomopathogenic fungus has a wide host range including several hundred species of arthropods and has also been recorded – albeit rarely - from wild populations of honeybees and bumble-bees (see references in Goettel et al. 1990, MacFarlane et al. 1995). Importantly, it has never been reported to cause epizootics in social insects, including bees, under natural conditions. While the scarcity of records from bees could imply a low susceptibility in bumble-bees to infection by this fungus, work on *B. bassiana* used commercially for pest control includes several studies on non-target effects of the *B. bassiana* product BotaniGard® (strain GHA) showing that it is, indeed, virulent to two bumble-bee species used in horticulture for pollination and for vectoring a range of biocontrol products (B. terrestris: Mommaerts et al. 2009, B. impatiens: Kapongo et al. 2008a). While both species are susceptible to infection, the virulence of the fungus seems to depend on formulation and dosage (Al-mazra'awi et al. 2006; Kapongo et al. 2008b; Ramaneidu & Cutler 2013).

B. bassiana has a mode of action very different from that of the array of pathogens occurring in field populations of bumble-bees. These all need to be ingested before starting to multiply in the alimentary tract and other internal organs and tissues. In contrast, *B. bassiana conidia* germinate on the surface of the insect and then penetrate the cuticle and epidermis. This generally takes 1-2 days, depending on isolate, temperature and humidity, and host species. The fungus eventually proliferates in the haemocoel, where it initially grows in a yeast-like manner as blastospores and/or as hyphal bodies and secretes an array of enzymes and mycotoxins. After the death of the host, the fungus switches to hyphal growth and finally emerges from the cadaver, provided the humidity is sufficiently high. This notably takes place through the intersegmental membranes of the dead host (Figure 2). Here it produces dry conidia, which can then be transmitted to new hosts via contact, air currents or rain. Time to kill depends on several factors, including host species, fungus isolate, dosage and temperature, and may vary from a few days to several weeks.

1.5 Interactions between pesticides and other stressors: Food shortage

The agricultural intensification from the 1950-60s and onwards has resulted in a major change of the landscape. Extensively managed grasslands, the number of hedgerows and unmown field margins have decreased, and in the cropped area, fewer insect-pollinated crops are grown. These changes all affect the availability of forage for the bees negatively, leading to a

significant historical decline in food resources for bumble-bees and other insect pollinators in agricultural areas (Rasmont and Mersch, 1988; Goulson and Hanley, 2005). Furthermore, the widespread use of herbicides has a negative impact on flowering of many plants, including important food plants of bees (Bruus et al., 2013; Boutin et al., 2014). In the UK, a significant historical decline of bumble-bee forage plants has been documented (Goulson, 2005; Carvell et al., 2006), and a similar trend must be expected in Denmark. Boutin et al. (2014) includes a study of hedgerow ground vegetation. They found fewer insect pollinated plants in hedgerows exposed to pesticides and showed that these plants also flowered for a shorter period than in organic hedgerows. It has been shown that bumble-bee workers are smaller, indicating food scarcity in homogenous landscapes which are more depauperate in flowers (Persson and Smith, 2011). Periods of starvation have also been detected for honeybees (Kryger et al., 2011). Thus, it is expected that food shortage is a common stressor of bumble-bee populations in Danish agricultural areas, and we assume that starved bumble-bees will spend more time on searching for food than well-fed bees. Thereby, they may visit more flowers and/or fly for longer distances and, consequently, they may in some cases experience a higher degree of pesticide exposure. It has been documented that the physiological status of honeybees strongly influenced their sensitivity to pesticides. For instance, bees fed with abundant, high quality pollen were less sensitive to pesticides (Wahl and Ulm, 1983).

1.6 Aims of the current study

This project has pursued two main aims: 1) To study the relations between test results obtained from different tiers, i.e. tests performed at different levels of realism and complexity; 2) to study the effect of three selected insecticides alone as well as in combination with a pathogen and in combination with starvation.

In the current study, we follow a three-tier approach by measuring a collection of end-points of *B. terrestris* exposed to the same pesticides in experiments at different levels of complexity (tiers). We compare results from experiments carried out under controlled laboratory conditions with results from experiments in semi-field to a large-scale field experiment in order to assess how well responses of lab tests reflect the risk of using pesticides in crop fields. We use *B. terrestris* as a representative of wild bees, firstly because it is one of the most common bumble-bee species in Denmark, and secondly because it is commercially available.

In recent years and in particular since a moratorium was imposed by the EU on the use of three neonicotinoid pesticides in 2013, a range of different studies on the effect of these pesticides on bees has been published. However, we still have limited knowledge on sub-lethal effects of bees exposed to other pesticide types, including the widely used pyrethroids and the cyano-type neonicotinoids. We here assess sub-lethal effects of two pyrethroids (Karate and Fastac) and one cyano-type neonicotinoid (Biscaya) on *B. terrestris*. We investigate the effects of pesticide exposure when bees are exposed to low (sub-lethal) dosages of each of the three pesticides, with and without other stressors (food shortage or pathogens).

Specifically, we address the following hypotheses:

(1) Exposure to sub-lethal dosages of pesticides will result in significantly decreased reproduction and population growth in bumble-bees. This hypothesis is tested using several end-points including egg production, larval development, adult behaviour and activity level.
(2) Tests of pesticide effects at different tiers will result in different effect levels for the same end-point (e.g. LD_{50} and ED_{50}) because the level of complexity during testing will affect the response. Due to overlapping end-points, results from different tiers may be compared directly.
(3) Bumble-bees subject to other stressors are more sensitive to pesticide exposure:

a. Colonies of bumble-bees that are stressed by food shortage are more sensitive to pesticide exposure compared to well-fed bees.
b. Colonies of bumble-bees that are stressed by infection with pathogens are more sensitive to pesticide exposure compared to bees without pathogens.

These hypotheses were tested in a series of bioassays:

The overall approach was to test three insecticides under conditions ranging from those equal to standard honeybee tests to field experiments. In addition, the effects of starvation and pathogen infection on sensitivity to insecticides were studied.

Insecticide selection was based on the likelihood of exposure and knowledge of their effects on honeybees:

- Thiacloprid, a systemic neonicotinoid used to regulate insect pests in oil seed rape fields by spraying (product name Biscaya)
- The pyrethroid alpha-cypermethrin (Fastac), which in Denmark is used in cereals, oil seed rape and vegetable fields, and which has been shown not to repel honeybees (Karise et al. 2007)
- Lambda-cyhalothrin (Karate), which in Denmark is used in cereals, oil seed rape and vegetable fields (incl. peas and potatoes), orchards and fields with seed production (incl. clover). Many of these crops are pollinated by bees. An unpublished preliminary study found decreased pollination activity in a red clover field treated with Karate

Four types of tests were performed (overview in Table 1)

1. Short-term laboratory tests based on the protocol for honeybees (OECD 1998a,b), in which bumble-bee workers were exposed topically to the pesticide, and mortality was estimated after 48 hours.
2. Long-term laboratory tests, in which bees in micro-colonies were exposed orally to a pesticide-containing sugar solution for 6 hours, the tests ran for 14 days, and mortality as well as sub-lethal effects on reproduction were measured as end-points.
3. Semi-field tests in which the micro-colony boxes were placed in a larger cage in which the bees could move around and forage on artificial flowers. The bees were exposed orally to pesticides, and after 14 days, mortality and sub-lethal endpoints as well as effects on bumble-bee activity were estimated by RFID techniques.
4. Field tests, where queen-right colonies (i.e. full colonies where a queen lays the eggs) were exposed to pesticide-contaminated sugar solutions for 24 hours (Karate and Fastac) or one week (Biscaya, because this is a systemic pesticide designed to stay in the plants for a longer period of time). The cages were placed for eight weeks at four different sites, all reckoned to have a low pesticide load from the surrounding landscape. End-points were weekly estimates of colony growth as well as the number and biomass of bees present after 8 weeks, the presence of new queens and the number of larval cells. Bumble-bee activity in relation to pesticide exposure was also assess by RFID measurements.

TABLE 1. Overview of the tests performed in the project, the form of exposure used and the end-points assesses

Test type	Exposure	Mortality	No. eggs	No. larval cells	No. honey pots	Activity
48 h lab	Topical	X				
14 d lab	Oral	X	X	X	X	
14 d semi-field	Oral	X	X	X	X	X
8 week field	Oral			(X)		X

2. Short-term laboratory tests – topical exposure

The aim of the short-term tests was to test the acute toxicity of the insecticides. The outcome can readily be compared with standard toxicity data for honeybees available in international databases.

This kind of test was performed for the three pesticides in combination with either a pathogen or starvation, but also gave results for pesticide alone. Pesticide exposure was established by topical application with pipette on the thorax of the bees.

2.1 Methods

The general approach of the short-term acute tests was to establish micro-colonies of newly hatched workers, expose the bumble-bees to either starvation or fungus for a week, expose the bees to pesticide, and then estimate the acute effect 48 h later.

2.1.1 Bumble-bees

All bumble-bees were bought from EWH BioProduction ApS in Tappernøje, Denmark, as queen-right colonies. These were kept in a room at 27 ˚C, with a light-darkness cycle of 16/8 hours and an approximate air humidity of 60%. When needed, the built-in container for sugar solution was replenished with Api-Invert from Swienty.dk. This product contains 30% sucrose, 31% dextrose and 39% fructose. Before feeding it to the bumble-bees, the solution was diluted 1:1 with tap water. In addition, the bumble-bees were supplied with ample amounts of honey-bee-collected pollen bought from EWH BioProduction, who guaranteed that the pollen was not polluted with pesticides. Large batched of pollen were bought in order to prevent effects caused by differences in pollen quality.

Micro-colonies

In all laboratory and semi-field tests, synchronized micro-colonies were obtained by removing all workers from queen-right colonies upon anaesthetizing them with CO2, marking 10-20 workers with honeybee marker dye (UNI Posca pen), returning the marked workers to the colony and collecting the emerging, unmarked workers one week later. In this way, workers aged 0-7 days were obtained. The concept and design are greatly inspired by the methods presented by those described in e.g. Tasei et al. (2000) and Mommaerts & Smagghe (2011). However, we decided to run the micro-colony set-up in a one- chamber design, where the bees lived and were fed in the same box, in order to save space. Furthermore, we kept the bees at a light-darkness cycle of 16/8 hours in order to facilitate the observation of the bees. Before running the tests, we checked that the bees could live and reproduce in this set-up. The micro-colonies were established by placing five of these workers in a transparent polyeth-ylene box (17 cm x 14 cm x 13 cm) with ventilation holes in lid and sides (see Figure 1). The bees were supplied with sugar solution (same as described above) and pollen grains. The sugar solution was served in a closed plastic tube with a small drilled hole, allowing the bees to feed on the solution, but almost preventing evaporation of water. Thereby, the consumption of sugar solution could be estimated by weighing the tube. Within one week at 27 ˚C, one of the workers started to produce infertile eggs as described by Mommaerts & Smagghe (2011), and hence the production of eggs, larval cells and honey pots may be used as indicators of bumble-bee fitness. At first, a shelter (plastic flower pot with cut-out entrance) was offered to the bees (see Figure 1), but since they did not seem to prefer that, it was given up, as the

observation of reproductive end-points was obstructed by the flower pot, not least because the bees tended to “glue” the flower pot to the surrounding box with wax.

Workers were harvested several times from each queen-right colony. In order to avoid drones, spot checks of five bees were made prior to each “harvest” and the number of antennal segments was counted (drones have 13 segments, workers and queens 12). In case we found drones in a colony, it was no longer used.

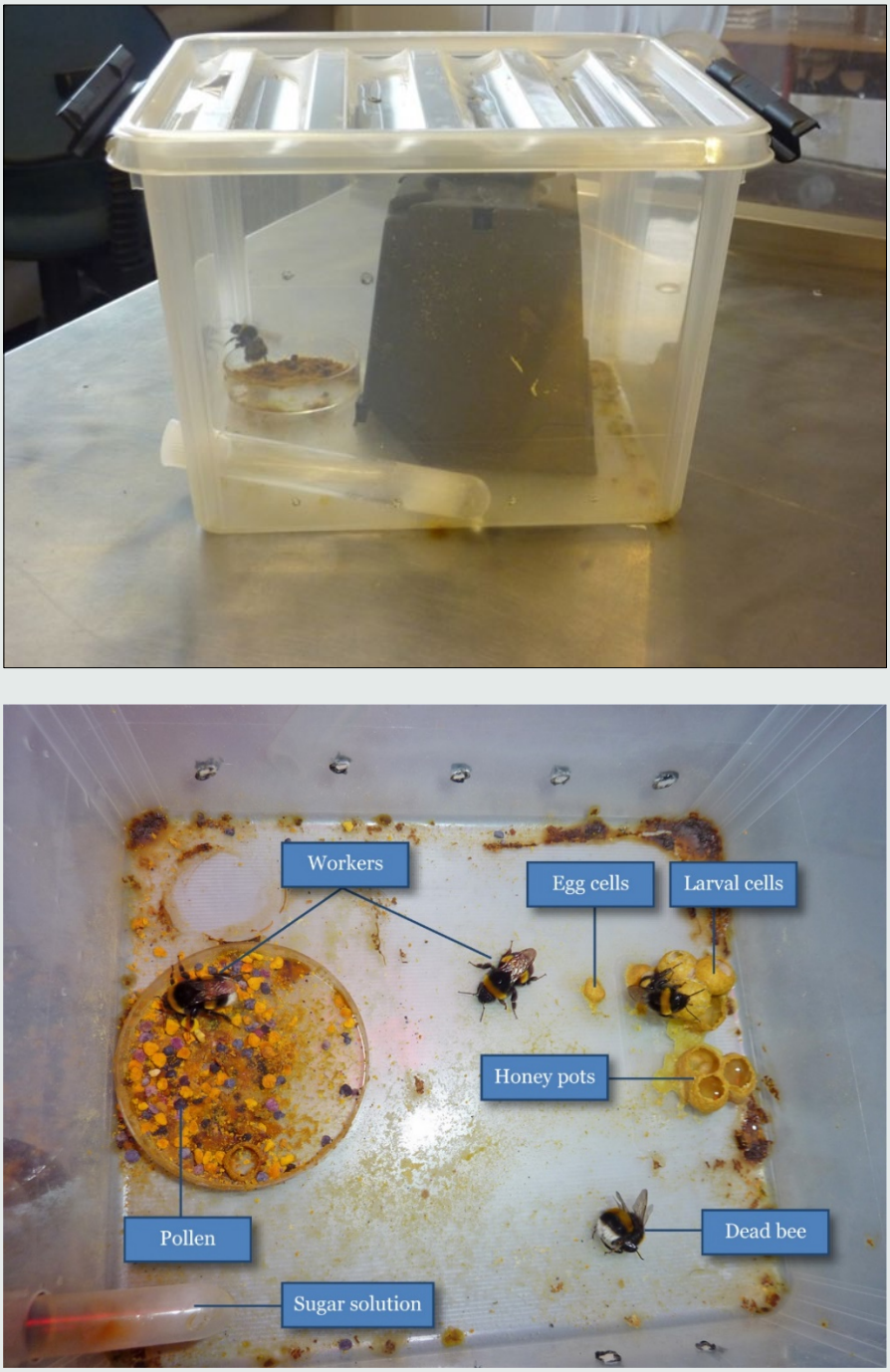

FIGURE 1. Side and top view of micro-colony in box. The flowerpot in the upper photo was omitted from the final design. A number of possible end-points for acute and chronic tests are marked

2.1.2 Range-finding, pesticide dosages

The choice of concentrations for the acute toxicity tests was based on a range-finding test with a broad range of concentrations and a limited number of bees, typically 5-10 per concentration, as well as on the results obtained in a previous project (Bruus et al. 2013).

2.1.3 Tests with pesticide and pathogenic fungus

In order to study whether bumble-bees infested with parasites may be more sensitive to pesticide exposure, a series of tests with combinations of the pathogenic fungus *Beauveria bassiana* and the three pesticides were performed.

Pathogenic fungus

In order to study whether bumble-bees infected with pathogens may be more sensitive to pesticide exposure, a series of tests with combinations of the entomopathogenic fungus *Beauveria bassiana* and the three pesticides were performed.

Fungus materials

The commercial product BotaniGard® WP22 (Borregaard Bioplant, Aarhus, Denmark) containing a wettable powder formulation of *B. bassiana* strain GHA was used. The bag containing the product was placed at 5 °C and retained its quality in terms of germination and purity for approximately six months. Spore suspensions were prepared by immersion of spore powder in 0.2% sterile Tween-20, serial dilution after thorough vortexing and spore enumeration in a Fuchs-Rosenthal counting chamber, followed by final adjustment of the spore concentration. Suspensions were stored at 1-5 °C for up to 24 h before use. Prior to use, the percentage germination was recorded 18h after plating spore suspensions onto 2% Potato Dextrose Agar plates incubated at 22 °C. Only spore suspensions with >92% germination were used. All bees dying in the experiments were surface disinfected separately by immersion 1 min in 1% sodium hypochlorite followed by rinsing 3 times in sterile water. Disinfected cadavers were then placed on moist filter paper in petri dishes sealed with parafilm, and after 5 days at room temperature the bees were checked for outgrowth of *B. bassiana* under a stereo microscope (Figure 2).

FIGURE 2. Bumble-bee killed by *B. bassiana*

Inoculation of bees with fungus

Based on the fact that bumble-bees in nature are known to frequently harbour various pathogens, which are prevalent in the populations continuously throughout the season, we decided to first treat bees with the fungus and then apply the pesticide. The timing of the pesticide application (6 days after fungus treatment) was selected to allow the fungus to cross the cutic-

ular and epidermal barriers and to activate cellular and humoral defence systems before exposure to the pesticide. A similar approach was taken by Alaux et al. (2010) and Vidau et al. (2011), where honeybees were exposed orally to *N. ceranae* 10 days before pesticide exposure.

Bees were treated topically by placing 50µl spore suspension in a 5 ml Eppendorf tube, adding a bee that had been lightly anaesthetized by CO2 and turning the tube upside down several times. The movements of the awakening bee in the tube and the relatively large volume of the droplet of spore suspension ensured that the bee was completely covered in inoculum. It was then gently transferred back to the micro-colony from where it originated. Control groups were treated likewise with a drop of 0.2% Tween-20. This approach was used throughout the following experiments.

Range finding for fungus (preliminary test)

This pilot experiment aimed at establishing a crude dose-response in workers. There were 5 dosages, including the control: 0 (control, 0.2% Tween-20), 1 (103 conidia/ml; 20 per bee) 2 (105 conidia/ml; 2.000 per bee), 3 (107 conidia/ml; 200.000 per bee), 4 (109 conidia/ml; 20.000.000 per bee). For each treatment, there were 5 bees per micro-colony and 4 replicate colonies. The experiment was repeated twice, ran over 20 days, and mortality was recorded at intervals throughout this time period.

Acute toxicity tests with fungus and pesticides

One day after the establishment of micro-colonies, the bumble-bees were exposed to three levels of *Beauveria bassiana* (0, 200 or 2000 conidia per bee), as described above.

One week after establishing the micro-colonies, the bees were exposed to pesticides. A range of ideally five concentrations, including a control, were prepared as aqueous solutions with 0.05% Dancon F to eliminate the surface tension (Table 2). In a few cases, the number of available micro-colonies forced us to reduce the number of concentrations tested. The pesticide was applied by pipetting 2µl on the thorax of the bees. The numbers of dead and living bees were counted after 24 and 48 h. Each pesticide dosage was tested in four replicates, and the entire test was run three times.

TABLE 2. Pesticide dosages (µg a.i./bumble-bee) tested in combination with three fungus levels (0, 200 or 2000 conidia per bee) in three runs of 48 h acute tests with topical exposure to pesticides. In each run, there were four replicates per dosage, each consisting of a micro-colony with five workers

Pesticide	Run1	Run 2	Run 3
Biscaya	0^c-10^c-25-50-100^c	0^c-10^c-25-50-100^c	0^c-25^c-50-100^c
Fastac	0^c-0.1^c-0.25-0.5-1^c	0^c-0.2^c-0.4-1-2^c	0^c -0.4^c -1-2^c
Karate	0^c-0.025^c-0.1-0.4^c	0^c-0.05^c-0.1-0.2-0.8^c	0^c-0.1^c-0.2-0.8^c

c: also tested without fungus (controls)

2.1.4 Tests with pesticide and starvation

In order to study whether starved bumble-bees may be more sensitive to pesticide exposure, a series of tests with combinations of starvation and the three pesticides was performed.

How to starve bees?

Since bumble-bees depend on a sugar source to survive from day to day, restricting their access to sugar seems a risky business, and we therefore chose to start by starving the bumble-bees by depriving them of pollen. A series of pilot studies were performed in order to es-

tablish how we could starve the bees without killing them, but despite the pilot studies, starvation methods had to be changed between the different runs of the tests.

Acute toxicity tests with starvation and pesticides

The time schedule for tests of starvation in combination with pesticides followed the scheme described for the tests with the pathogenic fungus, i.e. establishing micro-colonies, introducing starvation the following day, exposing bees to pesticide after one week, and estimating effects 24 and 48 h later.

Based on the pilot experiments, a layout with two levels of starvation was chosen: 1) No pollen on days 3 and 7 the week before pesticide exposure (starvation level 1), 2) no pollen on days 3, 4, 6 and 7. After pesticide exposure, all bees had access to pollen (starvation level 2). This test design was used for the first test run, which involved Biscaya and Fastac. However, despite the pilot experiments, it turned out that we had to starve the bumble-bees more severely in order to get just the slightest effect of starvation. Hence, in the second run of the tests starvation was introduced as 1) no pollen on days 3, 4, 6 and 7 the week before pesticide exposure (starvation level 2), and 2) no pollen at all (starvation level 3). This set-up was run with all three pesticides. Finally, in the third run of the test, in which only Karate was included due to lack of bees, sugar starvation was included in addition to pollen starvation, so that the bees were deprived of sugar every second day from 9 a.m. to 3 p.m. (starvation level 4). In addition, no pollen was supplied to half the micro-colonies (starvation level 5). The sugar starvation scheme was carefully tested in preliminary experiments in order to find a level that would cause no or very low mortality due to sugar starvation or thirst. In all cases, the test included control micro-colonies that were not starved, so that we could compare the effect of pesticide with the effect of starvation and the effect of starvation + pesticide. The pesticide dosages applied are presented in Table 3. Each dosage was established in four replicates (micro-colonies) per test run.

TABLE 3. Pesticide dosages (µg a.i./bumble-bee) used in combination with starvation for the acute tests. The tests were run twice with each pesticide and four replicates per dosage, each consisting of a micro-colony with five workers

	Run 1	Run 2
Biscaya	0^c-20^c-40-60-80-160^c	0^c-20^c-40-60-80-160^c
Fastac	0^c-0.2^c-0.5-1-2^c	0^c-0.2^c-0.5-1-2^c
Karate	0^c-0.05^c-0.1-0.4-0.8^c	0^c-0.05^c-0.1-0.4-0.8^c

c: also tested without starvation (controls)

2.2 Statistical analyses

Differences in initial mortality (before pesticide exposure) were not tested due to very low numbers of dead bees. Effects of pesticide dosage and pathogenic fungus or starvation, respectively, were tested separately for each pesticide and repetition in time (due to different ways of starving the bees) by PROC GENMOD in SAS. Starvation levels and pathogen levels were considered categorical variables, whereas dosage was tested both as a categorical and a regression variable. The categorical test allows comparison (contrasts) of dosages with controls. The response variable (death after 48 h) was considered a binary variable (a bee was either alive or not), and each micro-colony had five bees that were either alive or dead; hence, percentage dead bees was binomially distributed. In the analyses of pooled data sets from all repetitions of a given tests, the replicates from the different repetitions were treated as replicates of the same test. Results were evaluated at a 5 % significance level.

Dose-response relations were calculated with dosage as a regression variable. The dose-response curves presented are all based on a sigmoid dose-response relation,

$$P = \frac{e^{(\alpha+\beta\times dosage)}}{1+e^{(\alpha+\beta\times dosage)}}$$

where P is the probability of the response (mortality, no. honey pots etc.), α is the estimated intercept and β is the estimated slope. Ideally, α should be zero, but in several cases an underlying mortality (as seen in the untreated controls) was present. In addition, forcing the estimated dose-response curve through origo inevitably results in moving the curve to the right, i.e. a sub-optimal fit to the data points and a higher LD_{50} value.

2.3 Results of short-term laboratory tests

2.3.1 Pesticide alone

The effects of pesticide alone (Figure 3) were extracted from the experiments with starvation and fungus by choosing the micro-colonies that were neither starved nor infested with fungus. A clear dose-response relation was seen for all three insecticides, and the calculated LD50 levels, i.e. the dosages causing a 50 % mortality, are presented in Table 4.

TABLE 4. Estimated LD_{50} levels (dosages causing 50 % mortality) for the three pesticides tested without interactions from starvation or fungus infestation. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Pesticide	LD_{50} (µg/bee)	95% confidence limits
Biscaya	116	73-190
Fastac	1.4	0.94-2.2
Karate	0.59	0.38-0.91

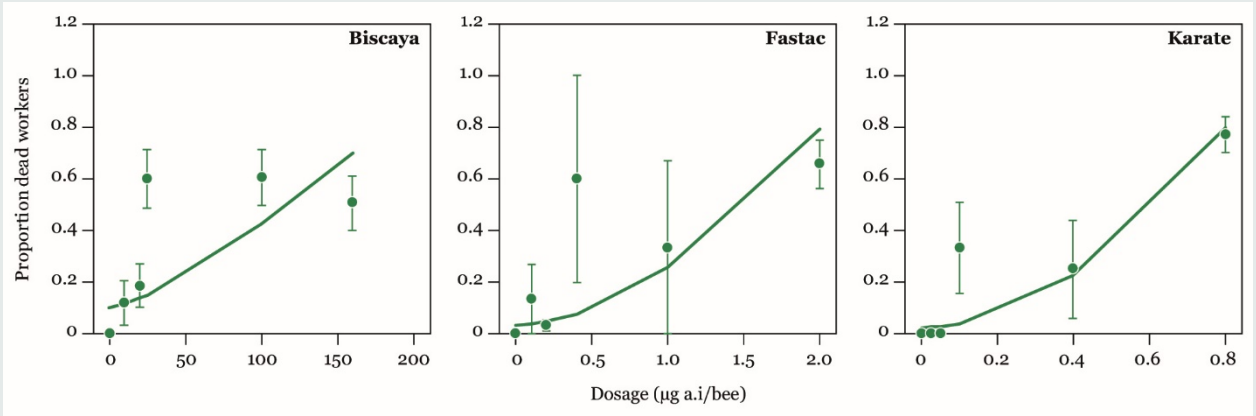

FIGURE 3. Effects of Biscaya (left), Fastac (centre) and Karate (right) on adult survival 48 h after pesticide exposure. Data points represent means +/- SEM of 8-20 replicates per dosage. The lines represent the modelled sigmoid dose-response relation

2.3.2 Interaction with pathogenic fungus

Range-finding: Optimal fungus level (preliminary test)

All fungus levels caused mortality higher than the control, with the highest level leading to the fastest mortality and a survival rate of 0% after 8 days of incubation at 27 °C (Figure 4). We aimed at selecting a level that affected all treated bees, i.e. that would activate their immune

reactions, while at the same time not killing them prior to or very soon after pesticide exposure. It was also important to be able to verify the efficacy of the fungus as measured as sporulation from cadavers, although a low level of fungus would be expected to result in few dead bees and, thus, low numbers of cadavers with sporulating fungus. At the lowest fungus level (20 conidia per bee), there were a total of 33 dead bees in the three repeats of the experiment, and two of these produced sporulation of *B. bassiana*. For the second lowest level (2.000 conidia per bee), the numbers were 40 dead, 14 of which produced fungus outgrowth. Based on these results, it was decided to conduct the acute test with two levels of 200 and 2.000 conidia per bee, respectively, and to use 2.000 conidia per bee in the lab and semi-field long-term experiments. It was also decided to apply the pesticides 6 days after fungus treatment, i.e. at a time when approximately 20% of the bees treated with low levels of conidia had died.

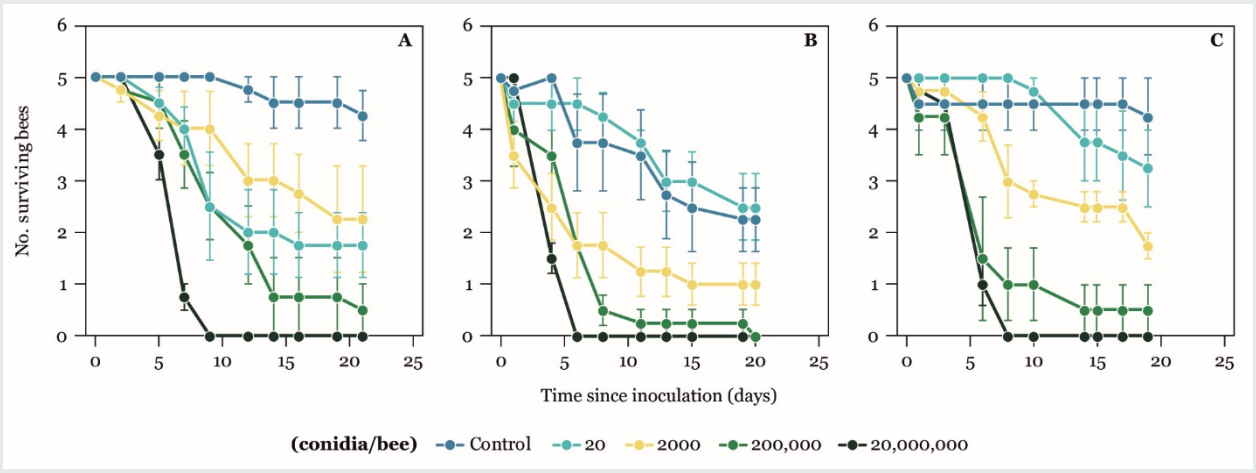

FIGURE 4. Survival of *B. terrestris* workers inoculated topically with spore suspensions of *B. bassiana*. Levels: 1 (20 conidia/bee), 2 (2000 conidia/bee), 3 (200.000/bee), 4 (20.000.000/bee). Figure illustrates course of survival (mean of 4 replicates +/- SEM) for the three experimental runs

Test with Biscaya and fungus

In all three repetitions of the test, bumble-bee survival was significantly lowered by Biscaya dosage (Figure 5, Table 33).

When all three repetitions were tested together, Biscaya dosage and fungus level were found not to interact (p=0.17). Biscaya dosage had a significant effect on survival, whereas there were no effects of fungus level. Consequently, LD_{50} levels did not differ significantly between fungus levels (Table 5), and LD_{50} was found to be app. 80 µg/bumble-bee.

TABLE 5. . Estimated LD_{50} levels for the effect of Biscaya at the different levels of fungus infestation. The bumble-bees were exposed to Biscaya one week after fungus infestation, and mortality was estimated 48 h later. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Fungus level	LD_{50} (µg a.i./bee)	95% confidence limits
0	82	43-138
1 (200 conidia per bee)	76	40-128
2 (2000 conidia per bee)	77	54-110

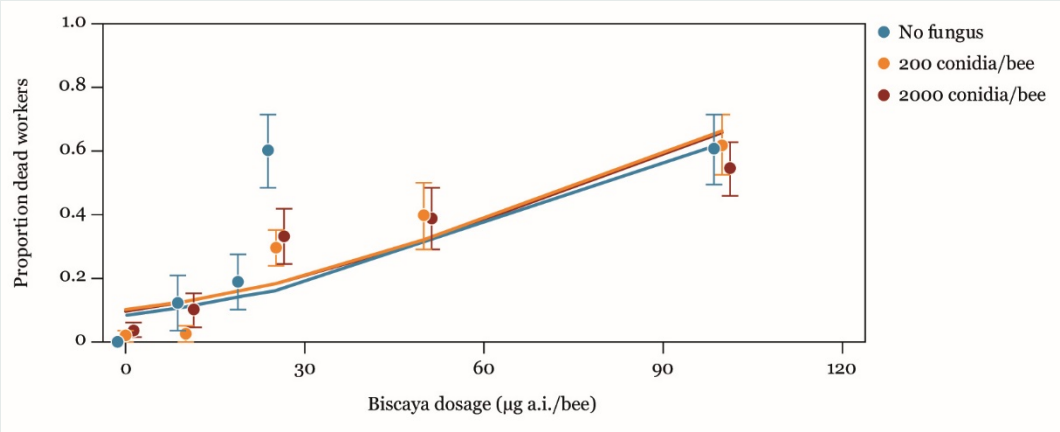

FIGURE 5. Bumble-bee mortality after exposure to a combination of the pathogenic fungus *B. bassiana* and the insecticide Biscaya. Points represent mean +/- SEM of 12 micro-colonies per dosage. Lines show the corresponding sigmoid fits

Test with Fastac and fungus

Worker survival was significantly lowered with increasing pesticide dosage. Fungus treatment and Fastac dosage did not interact significantly, but in the second and third repetition there was a significant effect of fungus level, survival being lower at the higher fungus level (Figure 6, Table 34).

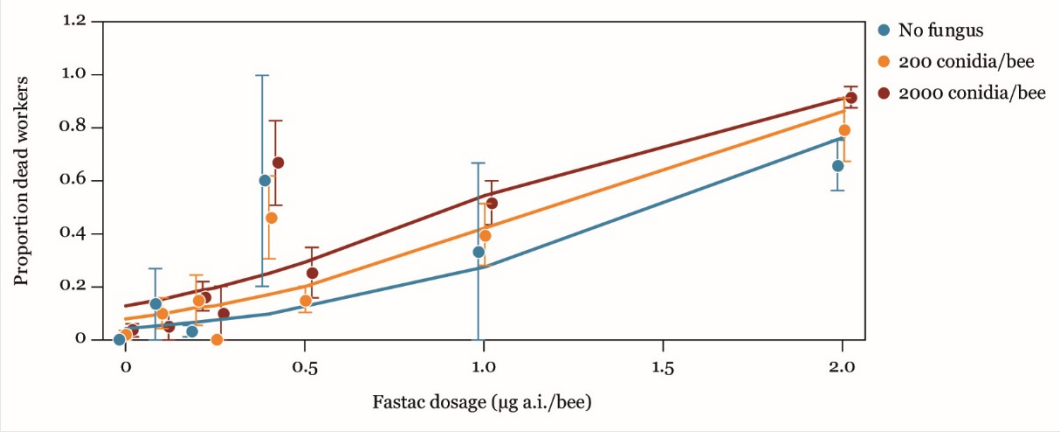

FIGURE 6. . Bumble-bee mortality after exposure to a combination of the pathogenic fungus B. bassiana and the insecticide Fastac. Points represent mean +/- SEM of 12 micro-colonies per dosage. Lines show the corresponding sigmoid fits

When all three repetitions were tested together, significant effects of both Fastac dosage and fungus level were identified. Although LD_{50} tended to decrease with increasing fungus level, this was not significant, since the 95% confidence levels overlap (Table 6).

TABLE 6. Estimated LD_{50} levels for the effect of Fastac at the different levels of fungus infestation. The bumble-bees were exposed to Fastac one week after fungus infestation, and mortality was estimated 48 h later. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Fungus level	LD_{50} (µg/bee)	95% confidence limits
0	1.5	0.83-2.3
1 (200 conidia per bee)	1.1	0.64-1.9
2 (2000 conidia per bee)	0.91	0.63-1.3

Test with Karate and fungus

In all repetitions of the test, increasing Karate dosage resulted in a significantly lowered survival (Figure 7). In the third repetition, there was a significant effect of fungus level, but not in the first and second (Table 35).

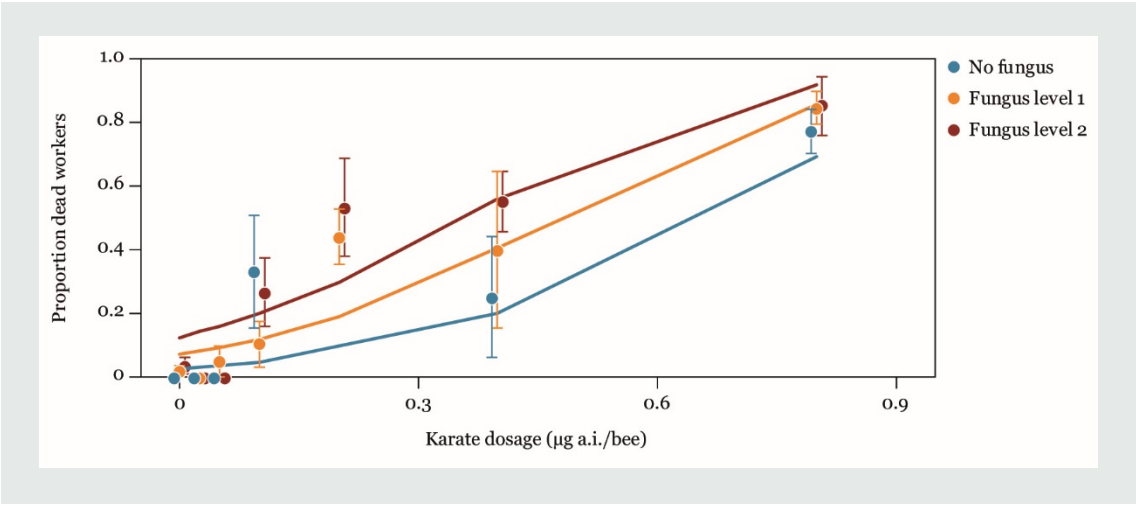

FIGURE 7. Bumble-bee mortality after exposure to a combination of the pathogenic fungus *B. bassiana* and the insecticide Karate. Points represent mean +/- SEM of 12 micro-colonies per dosage. Lines show the corresponding sigmoid fits

Overall, when all three repetitions were tested together, both Karate dosage and fungus level were found to affect survival significantly. LD_{50} tended to decrease with increasing fungus level (Table 7), but this was not significant.

TABLE 7. Estimated LD_{50} levels for the effect of Karate at the different levels of fungus infestation. The bumble-bees were exposed to Karate one week after fungus infestation, and mortality was estimated 48 h later. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Fungus level	LD_{50} (µg/bee)	95% confidence limits
0	0.65	0.40-1.0
1 (200 conidia per bee)	0.47	0.27-0.75
2 (2000 conidia per bee)	0.36	0.25-0.51

2.3.3 Interaction with starvation

As described previously, the tests including starvation were only run twice per pesticide due to unexpected effort needed in the range-finding of a suitable procedure for starving bees. De-

spite the extended range-finding work, it was necessary to adjust the procedure for introducing starvation in the different runs of the final tests in order to ensure a small, but significant, effect of starvation itself.

Test with Biscaya and starvation

In both test runs, Biscaya dosage significantly lowered bumble-bee survival. Starvation and Biscaya dosages did not interact significantly ($p>0.5$), and starvation alone did not have significant effects (Table 36, Figure 8). The same response pattern was seen when the two test runs were analysed together.

Accordingly, the estimated LD_{50} values did not differ significantly between starvation levels and test runs (Table 8).

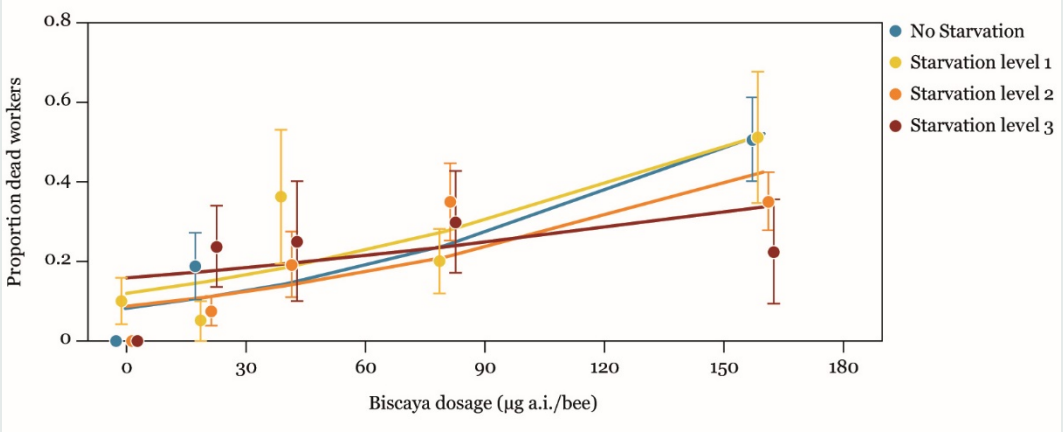

FIGURE 8. Bumble-bee mortality after exposure to a combination of starvation and the insecticide Biscaya. Points represent mean +/- SEM of 8 replicates per dosage. Lines show the corresponding sigmoid dose-response fits

TABLE 8. Estimated LD_{50} levels for the effect of Biscaya at the different levels of starvation. The bumble-bees were exposed to pesticide one week after the introduction of starvation, and mortality was estimated 48 h later. Negative values of the confidence limits indicate that these could not be determined. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Starvation level	LD_{50} (µg/bee)	95% confidence limits
0	154	68-377
1	154	56-586
2	183	89-459
3	270	56-(-856)

Test with Fastac and starvation

Fastac dosage significantly lowered bumble-bee survival (Table 37, Figure 9), but no significant interaction between starvation and Fastac dosages was found ($p>0.3$), and starvation itself only affected survival significantly in the second test run.

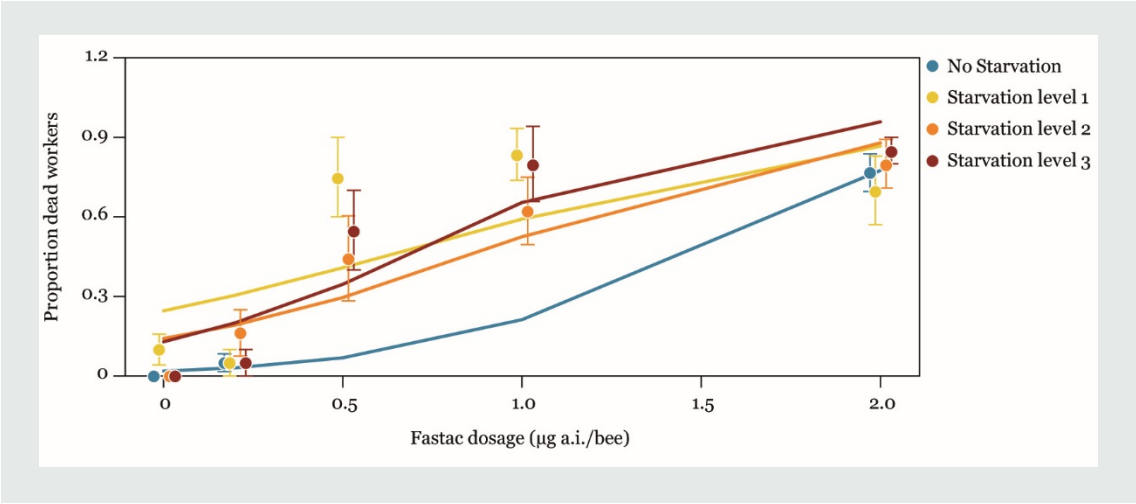

FIGURE 9. Bumble-bee mortality after exposure to a combination of starvation and the insecticide Fastac. Points represent mean +/- SEM of 8 replicates per dosage. Lines show the corresponding sigmoid dose-response fits

The estimated LD_{50} values tended to be lower when the bumble-bees were starved, but this was not significant (Table 9).

TABLE 9. Estimated LD_{50} levels for the effect of Fastac at the different levels of starvation. The bumble-bees were exposed to pesticide one week after the introduction of starvation, and mortality was estimated 48 h later. Negative values of the confidence limits indicate that these could not be determined. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Starvation level	LD_{50} (µg/bee)	95% confidence limits
0	0.74	0.71-0.77
2	0.4	0.2-1.1
3	0.3	0.1-0.7
4	0.2	(-0.005)-1.5
5	0.4	0.1-0.9

Test with Karate and starvation

In both test runs as well as in the pooled data set, starvation and Karate dosage significantly affected bee survival, but the two factors did interact (Table 38, Figure 10).

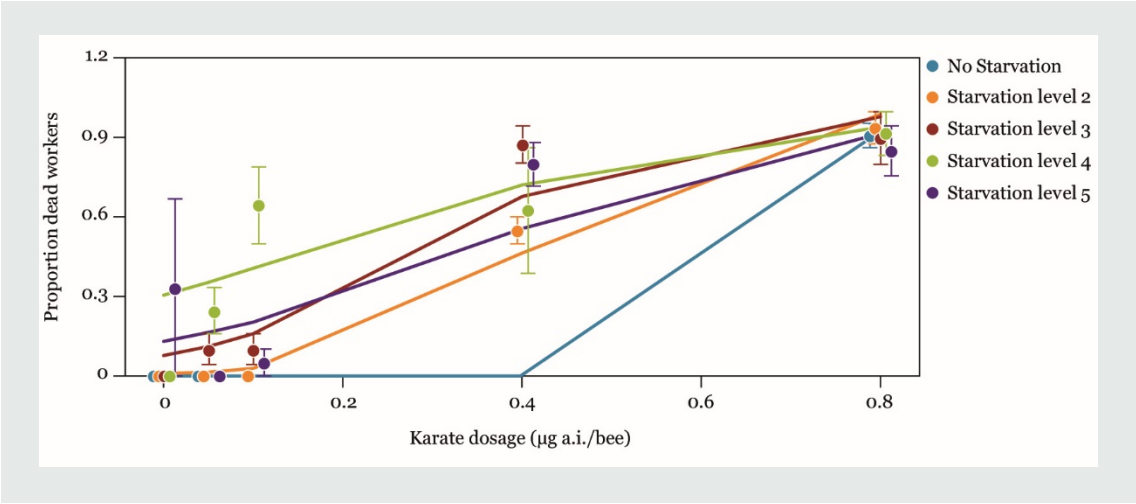

FIGURE 10. Bumble-bee mortality after exposure to a combination of starvation and the insecticide Karate. Points represent mean +/- SEM of 8 replicates per dosage. Lines show the corresponding sigmoid dose-response fits

The estimated LD_{50} level was lower for the most starved bumble-bees (starvation level 3) in the first test run (Table 10), while in the second run, with more severe starvation, no differences in LD_{50} levels could be detected, despite the interaction between starvation and pesticide effects.

TABLE 10. Estimated LD_{50} levels for the effect of Karate at the different levels of starvation. The bumble-bees were exposed to pesticide one week after the introduction of starvation, and mortality was estimated 48 h later. Negative values of the confidence limits indicate that these could not be determined. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Starvation level	LD_{50} (µg/bee)	95% confidence limits
0	0.74	0.71-0.77
2	0.4	0.2-1.1
3	0.3	0.1-0.7
4	0.2	(-0.005)-1.5
5	0.4	0.1-0.9

2.4 Discussion of short-term laboratory tests

Both in the short-term tests with fungus in combination with pesticide and in the tests with starvation and pesticide, clear effects of all three pesticides were seen. The response in the micro-colonies not exposed to fungus or starvation in the two series of tests did not differ significantly, which indicates that the response is rather robust. The LD_{50} values estimated here for Fastac and Karate are slightly higher than those reported by the review of Mommaerts and Smagghe (2011), who found LD_{50} values of 0.17-052 µg/bee for Fastac and 0.11-.022 µg/bee for Karate. No comparable LD_{50} values could be found for Biscaya.

The aim of establishing small fungus effects that could be combined with pesticide exposure to test possible interactions between the two types of stressors was fulfilled. The pathogenic fungus itself had slight effects in the tests with Fastac and Karate, but not in the one with Biscaya. This difference between the tests is difficult to explain, since the same two fungus levels were applied in all tests. Fungus and pesticides generally interacted, and the three repetitions per pesticide were conducted at different points of time, which ought to decrease the risk of experimental errors relating to preparation and application of the fungus inoculum. Similar

differences were seen for the effect of starvation. Although Biscaya and Fastac were tested in combination with the same levels of starvation, starvation itself had significant effects on mortality in the Fastac experiments, but not in the ones with Biscaya. It should, however, be noted that neither fungus nor starvation had significant effects on the estimated LD_{50} levels for the three pesticides.

The fact that we had to change the method for inducing starvation between the different runs of the test may be seen as an indication of the difficulty of establishing starvation in this simple laboratory set-up. One may argue that ideally starvation in short-tern acute tests should be established by lowering the availability of sugar solution and not that of pollen, since the bees did not reproduce during the 48 h test period and therefore did not need pollen. On the other hand, sugar starvation is a somewhat “risky business” because the worker bees will soon die if deprived of sugar, even if water is offered. In addition, one of the workers of the micro-colony will start to develop eggs immediately after the establishment of the colony (e.g. Mommaerts & Smagghe 2011) and, consequently, pollen starvation may be expected to affect development of eggs in the new queen and, hence, the function and survival of the micro-colony. At least, the results of our tests show effects of starvation, no matter if it is established through limitation of access to sugar solution or by depriving the bumble-bees of pollen.

3. Long-term laboratory tests – oral exposure

3.1 Methods

The general set-up was to collect worker bees from queen-right hives, place five in each container, let them establish the micro-colony for a week, and then expose them to orally to pesticide-contaminated sugar solution. Survival and sub-lethal end-points were estimated 14 d after pesticide exposure.

3.1.1 Reproductive end-points

As illustrated in the photo of Figure 1, micro-colony set-up in transparent boxes allows us to follow the production of honey pots, egg cells, larval cells and, in a few cases, drone off-spring. The eggs are placed in “lumps” of wax, and sometimes the wax layer is so thin that the individual eggs may be counted. However, we soon found that this is not always the case. The opening of a number of “lumps” revealed that on average each lump contained five eggs. Consequently, we proceeded by counting lumps of eggs and multiplying with 5.

3.1.2 Pesticide alone

Micro-colonies were established as described previously. Preliminary experiments of the consumption of sugar solution by “hungry” bumble-bees were used to establish the desired oral dosages. Accordingly, the dosages were based on the expectation that each bumble-bee would consume app. 0.09 g (=0.077 ml) sugar solution during the six hour exposure period.

One week after the establishment of micro-colonies, the bees were starved for two hours to ensure the consumption of pesticide-contaminated sugar solution, and thereafter the usual tube with sugar solution was substituted by an Eppendorf tube containing a known amount of pesticide-sugar solution. Six hours later, the tubes were swapped again, and the consumption of pesticide-sugar solution was determined. The bumble-bees were exposed to five pesticide dosages (Table 11) plus a control including the spreading and wetting agent Dancon F. Each dosage was replicated four times, and the test was run three times.

TABLE 11. . Pesticide dosages (µg a.i./bumble-bee) used for testing long-term effects in the laboratory. The test of each pesticide was repeated twice with four replicates per dosage per repetition

	Rep 1	Rep 2	Rep 3
Biscaya	0-5-25-50-100-200	0-5-10-25-50-100	0-5-10-25-50-100
Fastac	0-0.025-0.05-0.1-0.2-0.3	0-0.025-0.05-0.1-0.2-0.5	0-0.025-0.05-0.1-0.2-0.5
Karate	0-0.005-0.01-0.02-0.05	0-0.005-0.01-0.02-0.05-0.1	0-0.005-0.01-0.02-0.05-0.1

3.1.3 Pesticide and pathogen

One day after establishing the micro-colonies, the bees were inoculated with *Beauveria* as previously described. Only one inoculum level was used, 2000 conidia per bee. Infection was checked as described for acute tests. Pesticide exposure was established as described for pesticide alone, except that four dosages (Table 12) plus a control were used, and the test was run twice.

TABLE 12. Pesticide dosages used in combination with the pathogenic fungus for the laboratory test of long-term effects. The test of each pesticide was run twice with four replicates per dosage per repetition

	Rep 1	**Rep 2**
Biscaya	0-5-10-25-50	0-5-10-25-50
Fastac	0-0.025-0.1-0.5-2	0-0.025-0.1-0.5-2
Karate	0-0.05-0.1-0.2-0.4	0-0.05-0.1-0.2-0.4

3.1.4 Pesticide and starvation

The principles and time schedule of starvation followed the ones described for acute toxicity testing. Since these tests had to run for 14 days instead of 48 h, new long-term pilot studies were performed prior to the tests, with different cycles of pollen starvation and pollen availability. The resulting scheme used for the tests is presented in Table 13 Pesticide exposure as well as numbers of dosages, replicates and repetitions were similar to the ones used in the long-term laboratory tests with the pathogenic fungus.

TABLE 13. Cycles of pollen availability and starvation in the 14 d lab tests

Day	**Day no.**	**+/- pollen**	**Time of change**
Monday	-7 (start of micro-colony)	+	
Tuesday	-6	+	
Wednesday	-5	+/–	9 a.m.
Thursday	-4	–	
Friday	-3	–/+	3 p.m.
Saturday	-2	+	
Sunday	-1	+	
Monday	0 (test start, pesticide exposure)	+/–	9 a.m.
Tuesday	1	–	
Wednesday	2	–/+	9 a.m.
Thursday	3	+	
Friday	4	+/–	3 p.m.
Saturday	5	–	
Sunday	6	–	
Monday	7	–/+	9 a.m.
Tuesday	8	+	
Wednesday	9	+/–	9 a.m.
Thursday	10	–	
Friday	11	–/+	3 p.m.
Saturday	12	+	
Sunday	13	+	
Monday	14	End +/–	9 a.m.

3.2 Statistical analyses

Effects of pesticides on the consumption of pesticide-contaminated sugar solution were tested in SAS PROC GLM. Data for each pesticide and repetition in time was tested separately and pooled. At first, differences between control treatments (water with and without the spreading agent Dancon F) were tested to determine if a distinction should be made between the two types of controls. As for the acute toxicity tests, effects of pesticide dosage and starvation or pathogenic fungus, respectively, were tested separately for each pesticide and repetition in time (due to different ways of starving the bees) by PROC GENMOD in SAS. In the case of overall effects of pesticide, fungus or starvation, contrasts (differences) between the different dosages or levels of fungus or starvation and the controls were tested. LD_{50} values were established from the estimated sigmoid model parameters for intercept and slope. For sub-lethal end-points, data were fitted to a generalised exponential model with poisson distribution and log as link function, and ED_{50} values were estimated from the exponential model parameters.

3.3 Results of long-term laboratory tests

Results are reported below for pesticide alone and in combination with fungus and starvation, respectively. Although the number of dead larvae was registered in all tests running for 14 d, hardly any were found, and hence results are not reported for this end-point. All tables with the results of the statistical analyses are presented in Appendix B.

3.3.1 Pesticide alone

Since percentage live worker bees generally did not differ significantly between controls with and control without Dancon F, the two types of controls are treated as one in the tests for the individual repetitions. In the pooled analyses of the results for Biscaya and Fastac, controls without Dancon differed significantly from controls with Dancon with respect to adult survival. Consequently, data for controls without Dancon was omitted in the statistical analyses for Biscaya and Fastac, considering the fact that Dancon was added to all treatments containing pesticide. A similar procedure was followed to determine whether data for controls without Dancon F should be included in the statistical analyses for other end-points. In the analyses of the pooled data sets, across test repetitions, it was generally not possible to test for interaction between repetition and pesticide effects due to lack of variation.

3.3.1.1 Consumption

For the pooled data sets for Biscaya and Karate, significant differences in bumble-bee consumption of sugar solution during exposure were identified between controls with and without Dancon ($p \leq 0.0276$), and therefore data for controls without Dancon F were omitted. In tests with Biscaya, consumption was significantly lowered by pesticide dosage, while this was not the case for Fastac and Karate (Figure 11, Table 39).

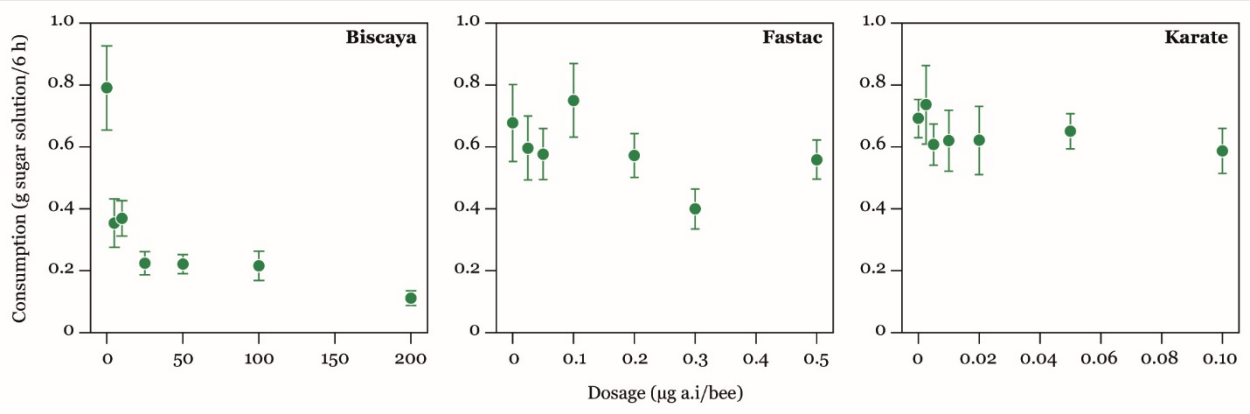

FIGURE 11. Consumption of sugar solution by bumble-bees during the 6 h pesticide exposure period. Data points show average +/- SEM of 12 micro-colonies per dosage for the pooled data sets, i.e. all three test repetitions

Biscaya

Adult survival

Adult survival was significantly affected by Biscaya dosage throughout the 14 d test period (Figure 12, Table 40) in all three repetitions of the test, even though the maximum dosage was halved in repetitions 2 and 3. The estimated LD_{50} levels did not differ between test repetitions (Table 14).

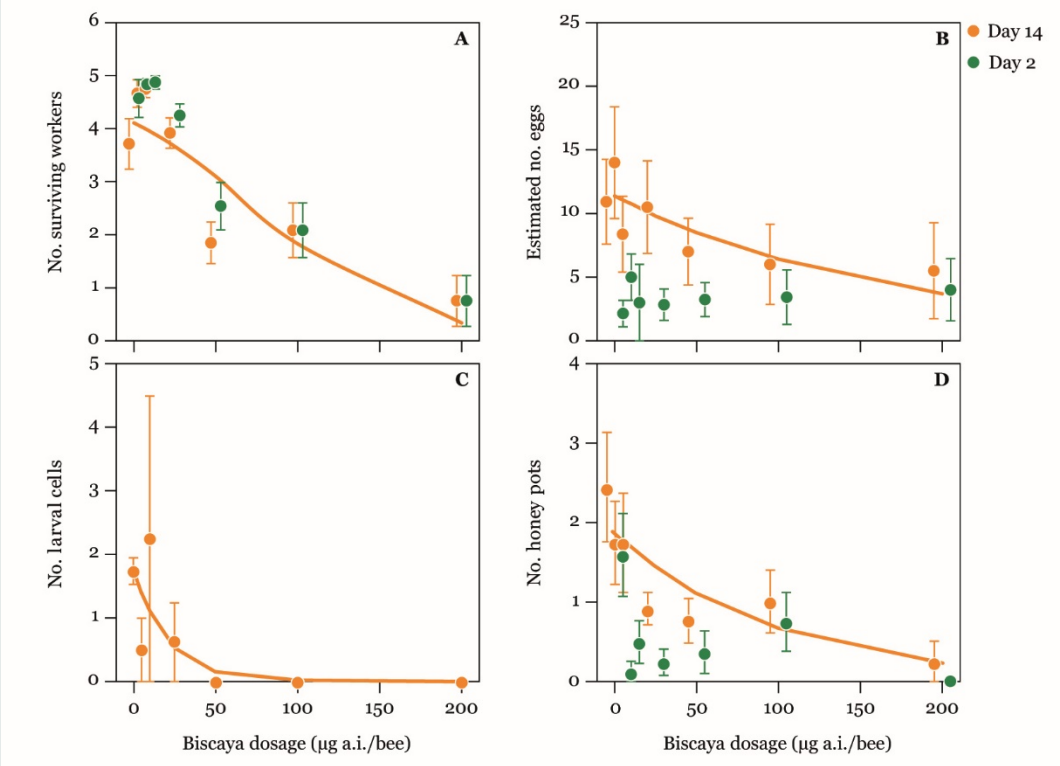

FIGURE 12. Adult bumble-bee survival (A), egg production (B), no. larval cells and no. honey pots 2 d and 14 d after Biscaya exposure of five bees per micro-colony. Points represent means +/- SEM of the pooled data set for all three repetitions (n=12). Lines represent sigmoid fits for the 14 d data

TABLE 14. Estimated LD_{50} values for day 14 of the three repetitions of the 14 d test with Biscaya. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Repetition	LD_{50}, µg a.i./bee	95% confidence limits
1	61	27-136
2	91	43-233
3	75	32-219

Number of eggs
In all repetitions of the test, significant overall effects of Biscaya dosage on the number of eggs produced were found on some days of inspection. In several cases, one or more treatments differed significantly from the control treatment, even if there was no overall effect (Figure 12, Table 41). When all data were analysed together, there was a significant interaction between test repetition and Biscaya dosage and, therefore, it was not possible to separate the two types of effect.

Number of larval cells
Analysis of the pooled data set detected significant effects of Biscaya dosage on the number of larval cells produced 14 d after exposure Apart from that, only very few micro-colonies developed larval cells and, consequently, hardly any effects of Biscaya on this end-point could be identified (Figure 12, Table 42).

Number of honey pots
The number of honey pots produced was significantly affected by Biscaya on day 14 in the analysis of the pooled data set (Figure 12, Table 43).

Effect levels for different end-points
In order to compare the sensitivity of different end-point to Biscaya exposure, the nominal (intended) exposure causing a 50% effect (LD_{50} and ED_{50}) was calculated (Table 15). While adult survival 1 and 14 days after exposure and the number of honey pots and eggs produced were affected at comparable Biscaya levels, the number of larval cells produced was apparently more sensitive to Biscaya exposure.

TABLE 15. Estimated ED_{50} values for various end-points 14 d after oral Biscaya exposure

End-point	ED_{50}, µg a.i./bee	95% confidence interval
Survival d 1	86	57-133
Survival d 14	73	45-123
No. egg cells	124	93-150
No. larval cells	14	9-19
No. honey pots	67	43-95

Fastac

Adult survival
When all three repetitions were pooled, a significant effect of Fastac on worker survival was seen both on day 2 and on day 14 after exposure (Figure 13, Table 44). Effects varied somewhat between test repetitions: In the first repetition, adult survival was significantly affected by Fastac dosage in the first days of the test period. One or two of the lower dosages differed from the control. After day 2, this effect disappeared. In repetitions 2 and 3, the maximum dosage was increased from 0.3 to 0.5 µg a.i./bee. In repetition 2, a significant effect of Fastac dosage was found on day 14, and the highest dosage caused a mortality significantly lower than the control. In repetition 3, significant effects were seen the first week of the test period,

and here the highest dosage consistently caused significantly higher mortality than the control. At the end of the test, however, this effect was no longer found. Individual LD_{50} estimates for the repetitions could not be established.

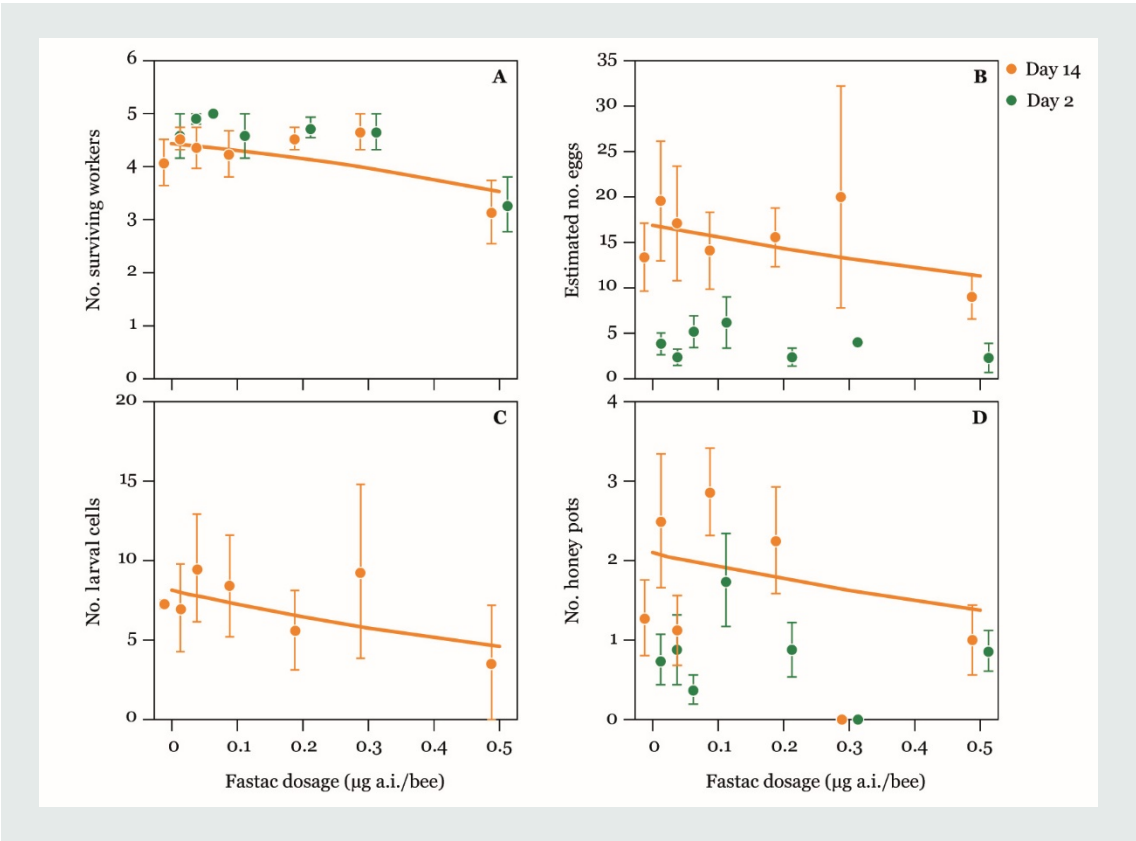

FIGURE 13. Adult bumble-bee survival (A), egg production (B), no. larval cells and no. honey pots 2 and 14 d after Fastac exposure. Points represent means +/- SEM of the pooled data set for all three repetitions (n=12). Lines represent sigmoid fits for the 14 d data

Number of eggs

The number of eggs produced was significantly affected by Fastac dosage 14 d after exposure in repetition 1 and 3 of the test and, in several cases, also earlier in the test period (Figure 13, Table 45). When all data were pooled, significant effects of Fastac dosage were detected throughout the test period, also on day 0. From day 5, a significant interactive effect of repetition number and Fastac dosage was seen.

Number of larval cells

At the end of the test period, significant effects were identified in repetitions 1 and 2 as well as the pooled data set, although only very few micro-colonies developed larval cells (Figure 13, Table 46).

Number of honey pots

In the first repetition of the test, very few honey pots developed. In the third repetition, the number of honeypots produced was significantly affected by Fastac dosage, whereas in the second repetition there was no overall effect of Fastac (Error! Reference source not found.). The analysis of the pooled data set showed a significant effect of Fastac dosage on days 5 and 14 (Table 47).

Comparison of end-points
Adult survival 1 and 14 days after exposure and the sub-lethal end-points were affected at comparable Fastac levels, as measured by the dosages causing a 50% affect (Table 16).

TABLE 16. Estimated ED_{50} levels for various end-points 14 days after oral Fastac exposure. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

End-point	ED_{50}, µg a.i./bee	95% confidence interval
Survival d 1	0.9	0.4-3.5
Survival d 14	0.9	0.4-4.1
No. egg cells	0.8	0.5-1.2
No. larval cells	0.6	0.4-0.9
No. honey pots	0.8	0.3-5.6

Karate

Adult survival
There were no significant differences in survival between Karate dosages on any of the days of observations (day 1, 2, 3, 5, 7 and 14 after Karate exposure, $p \geq 0.07$) when data from the individual tests repetitions were analysed separately. A pooled analysis detected significant effects of Karate dosage on day 5 ($p = 0.0496$), but not on any other day ($p > 0.13$) (Figure 14). Due to the lack of effects, LD_{50} values could not be estimated.

Number of eggs
The number of eggs produced was significantly affected by Karate dosage 14 d after exposure and, in several cases, also earlier in the test period (Figure 14, Table 48). On days 2-14, a significant interaction between test repetition and Karate dosage was observed, indicating that the effect of Karate differed between the repetitions.

Number of larval cells
Only few micro-colonies developed larval cells, but a significant overall effect of Karate was seen (Figure 14, Table 49) two weeks after exposure.

Number of honey pots
No overall effect of Karate dosage on the number of honey pots produced was found ($p \geq 0.11$, Figure 14).

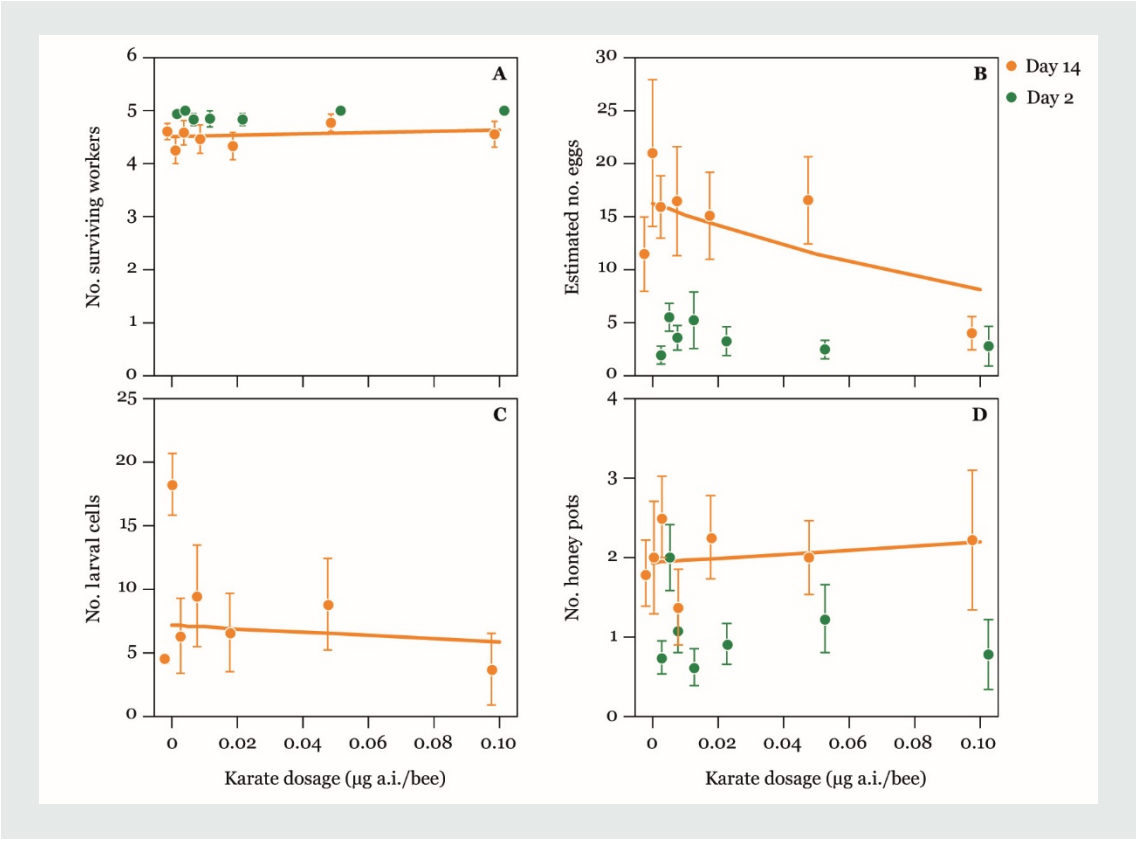

FIGURE 14. Adult survival (A), egg production (B), no. larval cells (C) and no. honey pots (D) 2 and 14 d after Karate exposure. Points represent means +/- SEM of the pooled data set for all three repetitions (n=12). Lines represent sigmoid fits for the 14 d data

Comparison of end-points

Fifty percent effect levels could only be established for the number of eggs and larval cells produced and, hence, a comparison of the sensitivity of different end-points is not possible (Table 17).

TABLE 17. Estimated ED_{50} levels for various end-points 14 days after oral Karate exposure. Negative figures indicate that value could not be determined. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

End-point	ED_{50}, µg a.i./bee	95% confidence interval
Survival d 1	(-0.12)	0.12-(-0.05)
Survival d 14	(-0.7)	0.2-(-0.2)
No. egg cells	0.098	0.074-0.12
No. larval cells	0.4	0.1-1.2
No. honey pots	(-0.5)	0.2-(-0.2)

3.3.2 Results of long-term laboratory tests with pesticide and fungus

Biscaya

Adult survival

Test of the pooled data found significant effects of Biscaya dosage on worker mortality throughout the test period (Figure 15, Table 50). A significant effect of the fungus was found in the overall test on day 7, but not on day 14.

There were no interactive effects of Biscaya and *Beauveria* fungus (p≥0.17). When the two repetitions were tested separately, no significant effects of Biscaya dosage were found in the first repetition, while in the second repetition significant effects were identified on some observation days (Table 50, Figure 15). In all cases, only survival at the highest dosage differed significantly from the control, or there were no significant differences between dosages. In the first repetition of the test, fungus treatment did not affect survival, whereas in the second repetition survival was significantly lower on days 7 and 14, when the bumble-bees were inoculated with *Beauveria*.

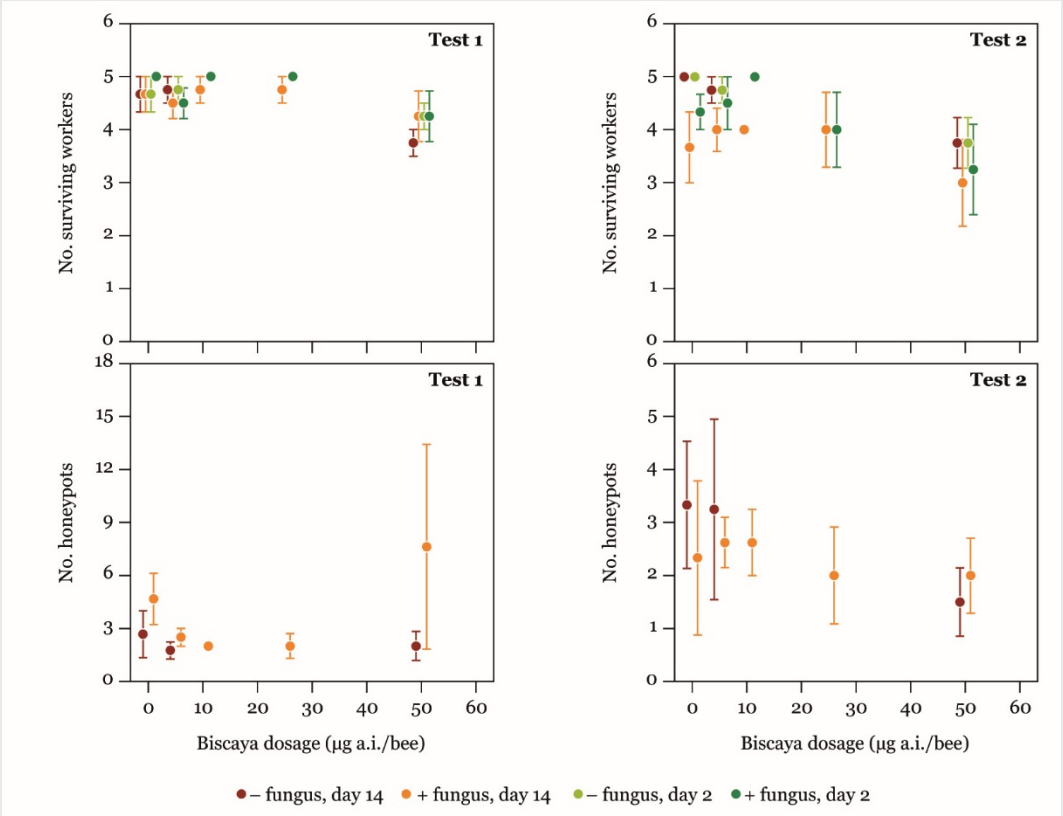

FIGURE 15. Adult bumble-bee survival d 2 and 14 after Biscaya exposure in combination with inoculation with the pathogenic fungus *B. bassiana*. Mean values +/- SEM of four replicates per dosage are shown for the two repetitions of the test

Egg cells

On day 2 and 7 after pesticide exposure, significant effects of the fungus on the number of egg cells present in the micro-colonies were found in the first repetition of the test, whereas in the second run and the overall test no significant effects of Biscaya and *Beauveria* were found (Table 51).

Honey pots

The test of the pooled data showed a significant effect of the fungus on the number of honey pots produced on day 14 (p=0.016), but not of Biscaya dosage. At the initiation of the tests, there were no effects of fungus or Biscaya exposure (p≥0.78). At the end of the tests, 14 d after pesticide exposure, significant effects of both fungus treatment and Biscaya dosage were found in the first repetition (p=0.0046, p=0.0018), but not in the second (p>0.5), although fungus treatment and Biscaya dosages were identical (Figure 15).

Larval cells

At the time of pesticide exposure, there were no larval cells in the micro-colonies. The analysis of the pooled data set found significant effects of the fungus on the number of larval cells on day 7 ($p=0.031$) and an interactive effect of Biscaya dosage and fungus on day 14 ($p=0.0037$). A significant effect of fungus as well as Biscaya was found on day 14 in the first repetition. On all other days of registration, no significant effects were found in either of the repetitions

Fastac

Adult survival

Generally, adult survival was significantly affected by Fastac, but only colonies treated with 2 µg/bee were significantly different from the control (Table 16, Figure 52). Fungus treatment did not affect worker survival significantly. Fastac and *Beauveria* treatment did not have interactive effects on worker survival ($p\geq0.12$).

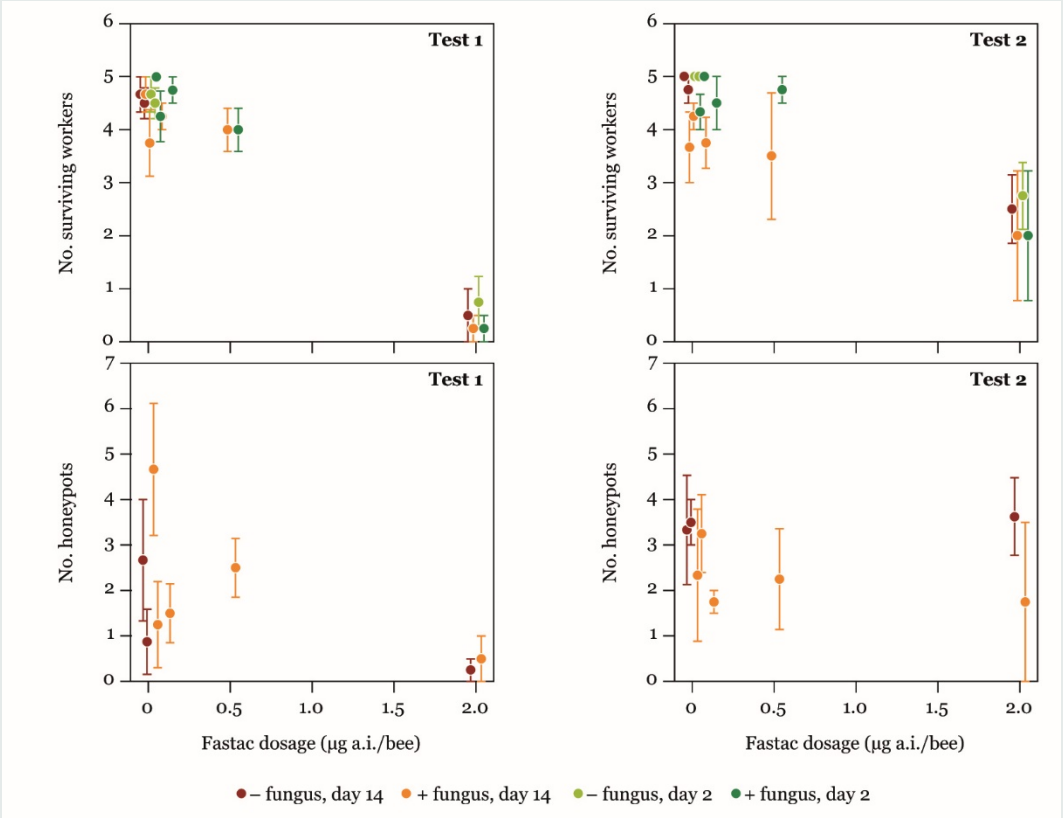

FIGURE 16. Adult bumble-bee survival d 2 and 14 after Fastac exposure in combination with inoculation with the pathogenic fungus *B. bassiana*. Mean values +/- SEM of four replicates per dosage are shown for the two repetitions of the test

Egg cells

No interactive effects of fungus and Fastac on production of egg cells were found ($p>0.2$). On the day of Fastac exposure, significant effects of fungus level were seen in both repetitions of the test (Table 53), but this effect did not last the entire test period. In the analysis of the pooled data set, no significant effects were identified.

Larval cells
No larval cells were seen until day 14 of the test. In the overall test, a significant effect of Fastac dosage on the number of larval cells was found on day 14 ($p=0.0006$), but not of the fungus treatment ($p=0.0530$). In the first repetition of the test, a significant effect of both Fastac dosage and fungus treatment was found ($p=0.0220$, $p=0.0111$), whereas in the second repetition no such effects were identified ($p=0.1510$, $p=0.0956$).

Honeypots
When the tests were started, micro-colonies did not differ significantly with respect to the number of honeypots present ($p>0.6$). When data for the two repetitions were analysed separately or pooled, treatment with fungus and Fastac did not induce any differences during the test 14 d period ($p>0.3$), and at the end of the test period, on day 14, there were still no significant effects of Fastac or fungus on the number of honeypots produced ($p>0.13$, Figure 16).

Karate

Adult survival
The analysis of the pooled data set found significant effects of Karate dosage on worker survival early in the test period, but not at the end of the experiment, whereas an effect of fungus treatment was found only at the end (Table 54). Karate and fungus exposure generally did not have interactive effects on adult survival. In the first repetition of the test, no effects of fungus or Karate were seen, whereas in the second repetition a pesticide effect was seen early in the test period, and an effect of fungus treatment from day 7 (Figure 17, Table 54). At the end of the experiment, an interactive effect of fungus treatment and Karate dosage was found.

Egg cells
No interactive effects of fungus and Karate on egg production were disclosed ($p>0.64$), and the number of eggs present in the micro-colonies did not differ at the time of Karate exposure ($p>0.7$). Neither fungus treatment nor Karate dosage caused any significant effects on the number of eggs produced during the 14 d test period ($p>0.15$ and $p>0.33$, respectively).

Larval cells
No larval cells evolved during the test period.

Honey pots
Only few honey pots were made in these tests (Figure 17). Karate and fungus exposure did not have interactive effects on the number of honeypots produced ($p\geq0.15$). At test start, micro-colonies did not differ significantly with respect to this end-point ($p>0.15$), and no significant differences occurred after Karate exposure ($p>0.17$, Figure 17).

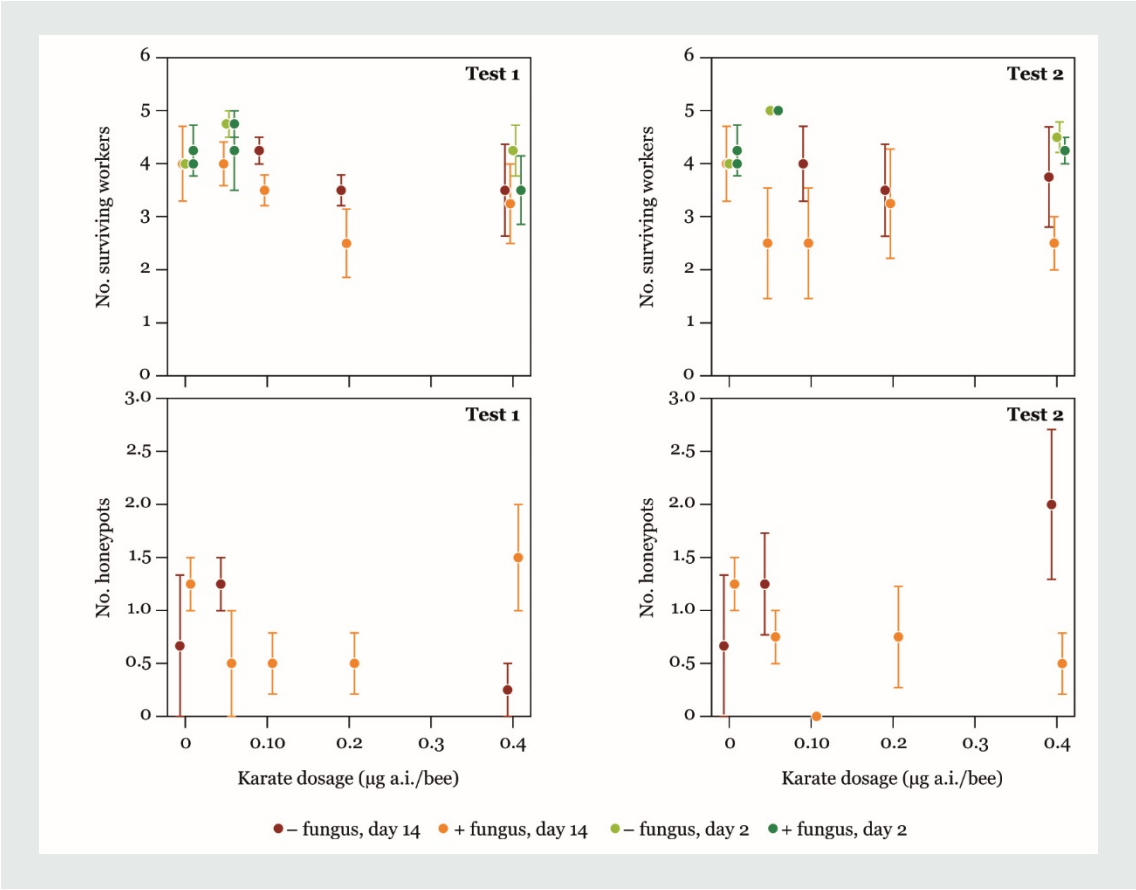

FIGURE 17. Adult bumble-bee survival d 2 and 14 after Karate exposure in combination with inoculation with the pathogenic fungus *B. bassiana*. Mean values +/- SEM of four replicates per dosage are shown for the two repetitions of the test

3.3.3 Results of long-term laboratory tests with pesticide and starvation

Biscaya

Worker survival

The general trend was that Biscaya dosage caused a significant decrease in adult survival, whereas starvation did not have significant effects (Figure 18, Table 55). There were no interactive effects of starvation and Biscaya dosage before day 14 ($p>0.17$).

Egg cells

At the end of the test, significant effect of starvation on the number of eggs produces was identified, whereas Biscaya did not cause any significant effects (Figure 18, Table 56). There were no interactive effects of Biscaya and starvation on the number of eggs produced during the 14 d test period ($p>0.32$).

Larval cells

Hardly any larval cells were developed, and therefore statistical analyses would not make sense.

Honey pots

In the analysis of the pooled data set for the two repetitions, a significant effect of starvation on the number of honey pots produced was found on day 14 ($p=0.045$, Figure 18). There were no interactive effects of Biscaya and starvation during the 14 d test period ($p>0.18$) and no significant effects of Biscaya on the number of honeypots produced.

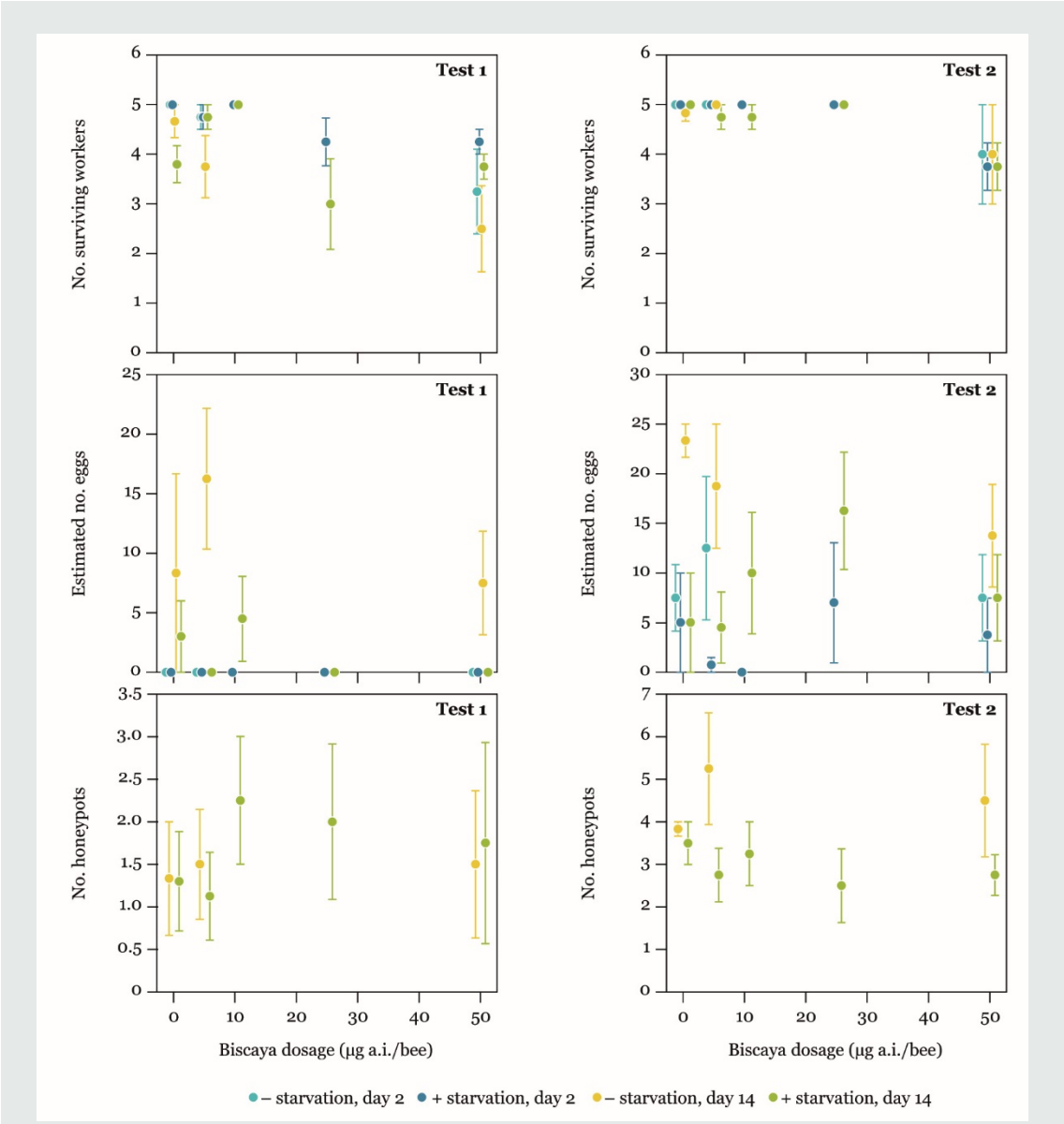

FIGURE 18. Effect of Biscaya and starvation on adult bumble-bee survival (top), no. eggs produced (middle) and no. honey pots (bottom) 2 and 14 d after pesticide exposure for the two repetitions of the test. Points represent mean +/- SEM of four replicates per dosage

Fastac

Worker survival

In the first repetition, significant effects of both starvation and Fastac dosage were found on day 14 (Figure 19, Table 57). In the second repetition, such effects were established already at the beginning of the test period. The effect of starvation was not found in the analysis of the pooled data set. Fastac dosage and starvation did not interact (p>0.33).

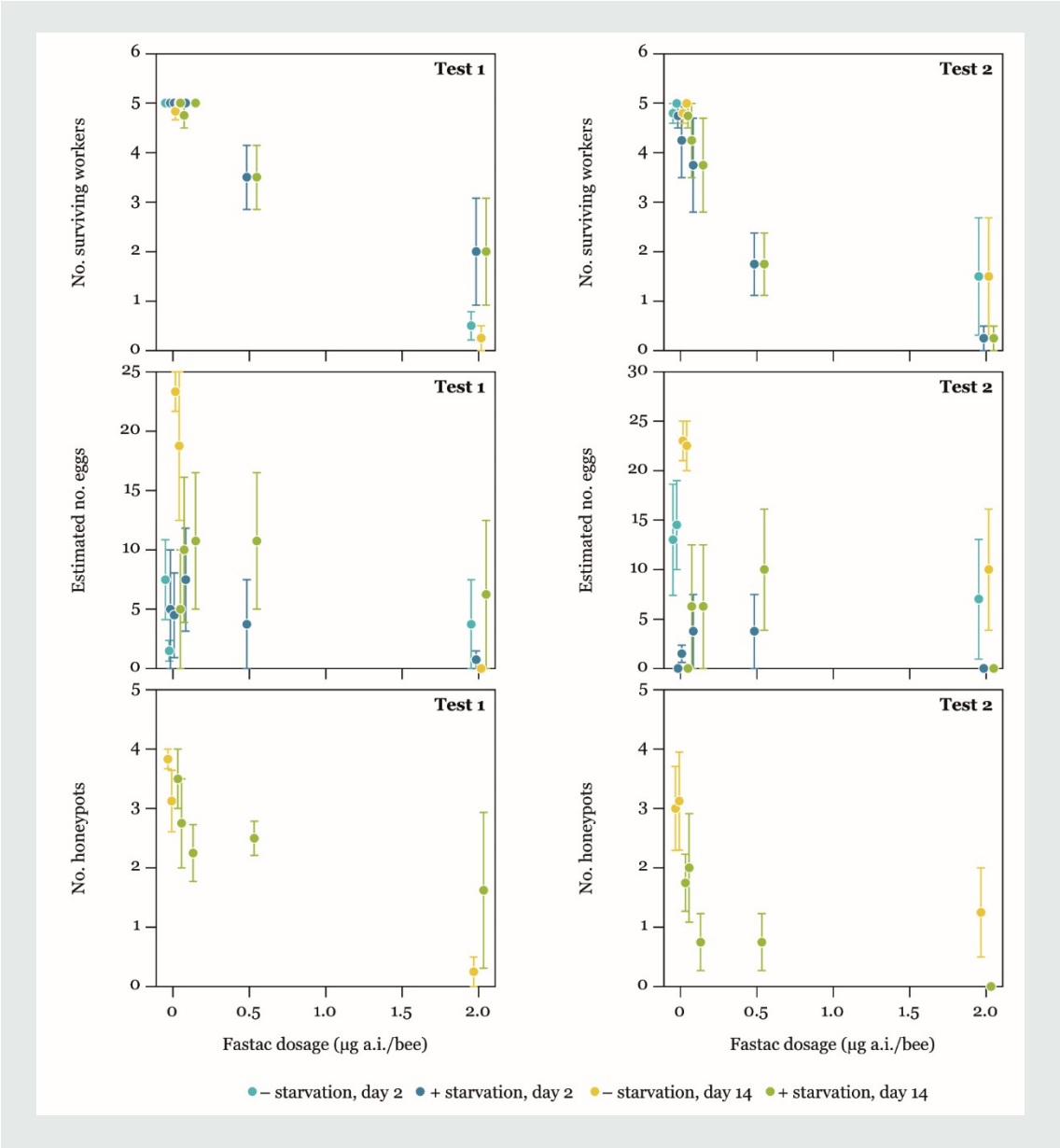

FIGURE 19. Effect of Fastac and starvation on adult survival (top), no. eggs produced (middle) and no. honey pots (bottom) 2 and 14 d after pesticide exposure for the two repetitions of the test. Points represent mean +/- SEM of four replicates per dosage

Eggs

Analyses of the pooled data set showed significant effects of starvation as well as Fastac dosage on the number of eggs produced, especially towards the end of the test (Figure 19). Starvation and Fastac dosage did not interact significantly ($p>0.40$), and there were no significant effects of Fastac dosage in individual repetitions (Table 58). However, in the second repetition of the test, starvation caused significantly different numbers of eggs (Table 58).

Larval cells

Very few eggs developed into larvae during the test period, and no significant effects of starvation or Fastac were detected ($p>0.52$).

Honey pots

From day 7, a significant effect of Fastac on the number of honey pots produced was found in the first repetition as well as in the pooled data set, while a significant effect of starvation was

found in the second repetition (Figure 19, Table 59). Fastac dosages and starvation did not interact (p>0.20).

Karate

Worker survival

Generally, both starvation and karate dosage had significant effects on bee survival (Figure 20, Table 60). In the first repetition of the test, an interactive effect of Karate and starvation was seen from day 2. Hence, the effects of the two factors could not be distinguished. In the second repetition, no interaction was seen (p>0.14), but throughout the test period a significant effect of Karate dosage was identified, and on some observation days an effect of starvation was also seen.

Egg cells

No interactive effects of Karate and starvation on the number of egg cells were detected (p>0.59), and neither of the factors caused significant effect (p>0.13, Figure 20).

Larval cells

No larval cells evolved during the 14 d test period.

Honey pots

Karate dosages and starvation did not interact (p>0.12), and neither Karate nor starvation caused significant effects on the number of honeypots in the micro-colonies (p>0.07, Figure 20).

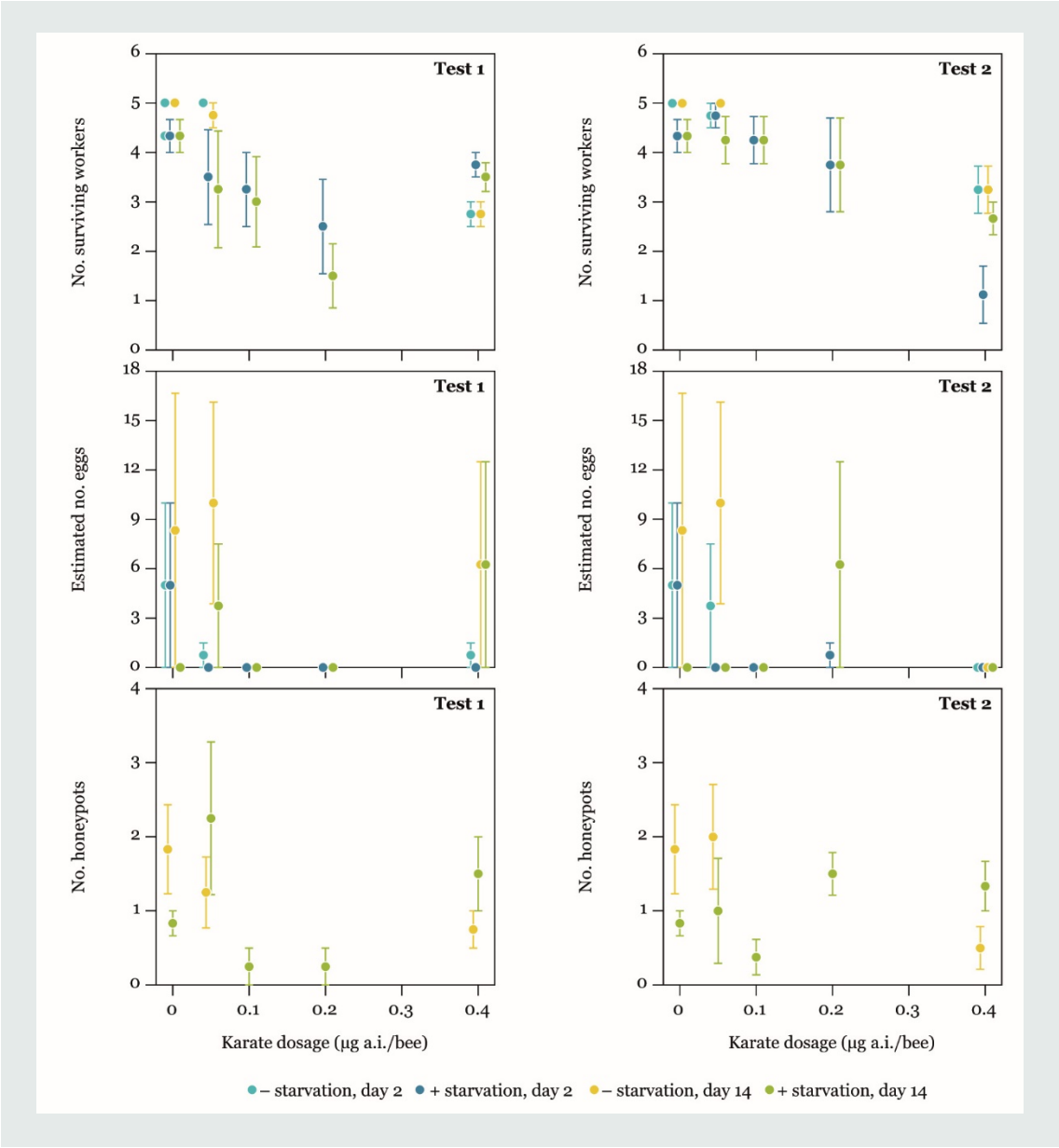

FIGURE 20. Effect of Karate and starvation on adult survival (top), no. eggs produced (middle) and no. honey pots (bottom) 2 and 14 d after pesticide exposure for the two repetitions of the test. Points represent mean +/- SEM of four replicates per dosage

3.4 Discussion of results of long-term laboratory tests

In the 14 d laboratory test where the bumble-bee were exposed orally, both Biscaya and Fastac caused effects on worker survival, egg production, the number of larval cells developed and the number of honey pots when pesticide effects alone were tested. The tested Karate dosages did not cause mortality, but, nevertheless, there were negative effects on the number of eggs and larvae. Generally, the observed effects were consistent in the three repetitions of the tests, except that the number of eggs produced interacted with repetition number, i.e. the response varied between repetitions.

In some cases, the spreading agent Dancon F caused effects on survival and the consumption of sugar solution. The substance was added to all pesticide solutions to ensure that the pesticides were properly mixed with the aqueous sugar solution. No literature could be found describing effects of Dancon F, but since it is a kind of detergent, it may have effects on the membranes of skin and intestines.

In the test with pesticide alone, Biscaya significantly reduced the consumption of sugar solution, while Fastac and Karate did not have such effects. We do not know whether the reduced consumption was caused by repellence or by effects on bumble-bee performance. However, such effects increases the uncertainty of actual exposure in experiments where the test animals are exposed orally to the tests substance.

Control mortality was generally low, which sustains the credibility of the obtained results and shows that the test set-up is useful for assessing both lethal and sub-lethal effects. A longer test period than the 2 weeks used here may increase the possibility of assessing effects on larval development and production of drones, provided that the micro-colonies can be kept sound as described in e.g. Mommaerts et al. (2010).

Generally, slight effects of pesticides, fungus and starvation as single factors were established as desired in order to make it possible to test if previous exposure to fungus or starvation increased the sensitivity of *B. terrestris* to the three insecticides.

When pesticide exposure was combined with previous inoculation with the pathogenic fungus, the general trend was that bumble-bees infested with the pathogenic fungus were not more sensitive to the tested pesticide dosages than bees not infested with fungus. Effects of the fungus itself were seen on the number of honey pots and larval cells produced in the tests with Biscaya, while in the tests with Karate an interactive effect of pesticide and fungus on bumble-bee survival was seen. In the tests with Fastac, no effects of the fungus were disclosed.

There was no general pattern in the effects of starvation on the bumble-bees; effects on worker survival were seen in the test with Karate, while in the tests with Biscaya and Fastac starvation had effects on the number of eggs produced and in Biscaya tests also on the number of honey pots. In a few cases, pesticide dosage and starvation had interactive effects, but that was not the general trend.

4. Semi-field tests – oral exposure

The aim of these experiments was to expose the bumble-bees to sub-lethal dosages of pesticide, fungus and starvation in order to estimate possible effects on reproduction and activity under more field-realistic conditions.

4.1 Methods

4.1.1 Experimental set-up

In this series of experiments, micro-colonies were exposed to pesticide, fungus and starvation in the same manner as described for the long-term laboratory experiments. The pesticide dosages used are shown in Tables 18-20. Initially, the intention was to place the micro-colonies in large greenhouse compartments with several micro-colonies in each compartment, but a preliminary experiment showed that the bumble-bees had problems finding their way back to their own box from the greenhouse compartment. In addition, many bees flew to the top of the greenhouse and never came back to the box. Consequently, we decided to confine the bees in a smaller, easily over-looked cage.

When the bumble-bee workers had been placed in the micro-colony box, the box was placed in a larger cage (94 cm by 70 cm by 100 cm) situated in a greenhouse (16:8 h light:darkness, target temperature 22 degrees centigrade, but with measured temperatures up to 40 degrees). The following week, the bees established their micro-colony and adapted to the new environment. Immediately upon pesticide exposure, the tube containing sugar solution was removed, so that the bees could move between the box and the surrounding cage. Pollen and sugar solution were supplied in the cage (see Figure 21), thereby forcing the bees to leave the box to feed.

Procedures for fungus inoculation and establishment of starvation were identical to those described for the long-term laboratory tests (paragraphs 3.1.3. and 3.1.4.), i.e. a fungus level of 2.000 conidia per bee was aimed at, and the bees were starved by depriving them of pollen part of the time.

TABLE 18. Pesticide dosages (µg a.i./bumble-bee) used in three repetitions of the semi-field test of pesticide effects

	Rep 1	Rep 2	Rep 3
Biscaya	0-5-10-25-50-100	0-2-5-10-25-50	0-2-5-10-25-50
Fastac	0-0.025-0.05-0.1-0.2-0.5	0-0.025-0.05-0.1-0.2-0.5	0-0.025-0.05-0.1-0.2-0.5
Karate	0-0.01-0.02-0.05-0.1-0.2	0-0.01-0.02-0.05-0.1-0.2	0-0.01-0.02-0.05-0.1-0.2

TABLE 19. Pesticide dosages (µg a.i./bumble-bee) used in the two repetitions of the semi-field test of pesticide effects in combination with the pathogenic fungus

	Rep 1	Rep 2
Biscaya	0-5-10-25-50	0-2-5-10-25
Fastac	0-0.025-0.05-0.1-0.2	0-0.025-0.1-0.5-2
Karate	0-0.01-0.02-0.05-0.1	0-0.01-0.02-0.05-0.1

TABLE 20. Pesticide dosages (µg a.i./bumble-bee) used in the two repetitions of the semi-field test of pesticide effects in combination with starvation

	Rep 1	Rep 2
Biscaya	0-2-5-10-25	0-2-5-10-25
Fastac	0-0.025-0.05-0.1-0.2	0-0.025-0.05-0.1-0.2
Karate	0-0.01-0.02-0.05-0.1	0-0.01-0.02-0.05-0.1

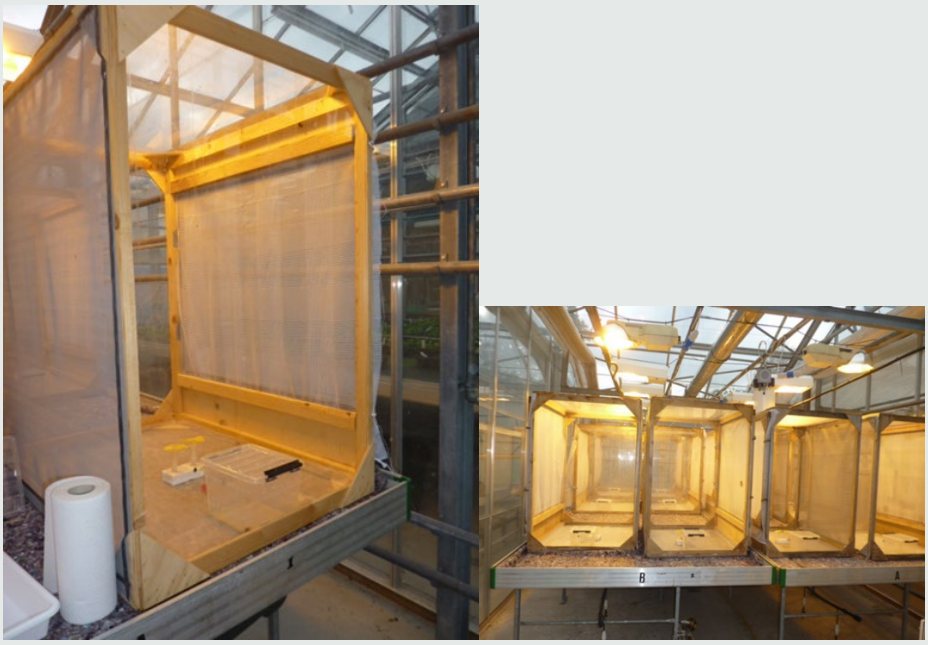

FIGURE 21. The semi-field set-up with micro-colonies in boxes placed in larger cages equipped with artificial flowers and pollen. The cages were kept in the greenhouse

4.1.2 Activity – RFID

Beside effects on reproduction, effects on bumble-bee activity were studied by applying RFID (Radio-Frequency IDentification) techniques. Just prior to pesticide exposure, the bumble-bees in selected boxes (six per experiment, corresponding with the capacity of the equipment) covering the planned range of pesticide dosages were equipped with two RFID tags. The tags were glued on thorax and abdomen of the bees after testing that the glue did not affect bee survival. A reader was mounted over the aperture of the box, so that the bees were identified and counted every time they passed. To test the functionality of the set-up, a tag mounted on a stick was used to activate the reader.

Principle

The principle of the RFID method is that a RFID tag, having a unique ID for only this specific tag, is attached to a movable object that can communicate the ID to a stationary reading station when it gets sufficiently close to this reader. The RFID tags used in the project are so small (1.2 mm x 1.2 mm) and light that they can be glued on the bumble-bee without posing a great burden (product: Microsensys). The station for reading is located at the entrance to the hive and the RFID tag read about 10 times per second while staying at approximately 1 mm from the reader. The reader is constructed as a small tunnel, and when the reader is mounted on e.g. a hive opening, the tag on the bee communicates with the reader every time the bee is going into or out of the hive. This reading indicates the ID for the tag, the time and the ID of the reading station that has read the tag. Two RFID tags were attached to each labelled bee with the use of super glue (Danlim) as outlined in Figure 22. The reason for using two tags per bee

was that we wanted to be able to determine whether the bee passes completely through the reader or just sticks its head into the reader, e.g. to guard the hive. Unfortunately, a rather large number of tags fell off the bees during the experimental period. However, in these cases usually one tag still remained so that it was still possible to record most of the movement of the bee. Five bees were labelled for each hive.

The raw data file coming from the reader has a large amount of redundant information, where, in some cases, the same bee is recorded repeatedly many times for the same passage or, in some cases, the bee may have been sitting in the channel for minutes or hours, causing the ID to be recorded 10 times per second. Hence, a data-filter is needed to remove redundant readings before interpretation is possible, and the following results are thus based on the following filter: After a tag ID is recorded, new recordings of the same tag will be neglected in the following time period of 5 seconds.

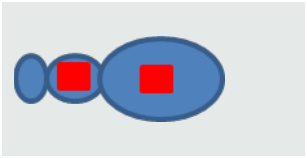

FIGURE 22. The two RFID tags were placed on thorax and abdomen of the bee

The records for a single ID are a series of clock readings, and the simplest version of output is the time since first reading, see the example of Figure 23. Normally, the first reading was obtained in the morning shortly after test start, but the time axes of the figure cannot be directly translated into time of day.

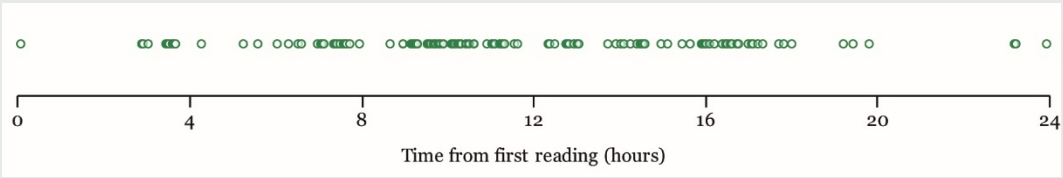

FIGURE 23. . Example of an RFID data set where a tag on the bumble-bee is read on the way in and out of the hive at particular points in time measured from the first reading

Since two tags are glued to each bee as illustrated in Figure 22, a pairwise set of data are recorded for each bee as illustrated in Figure 24.

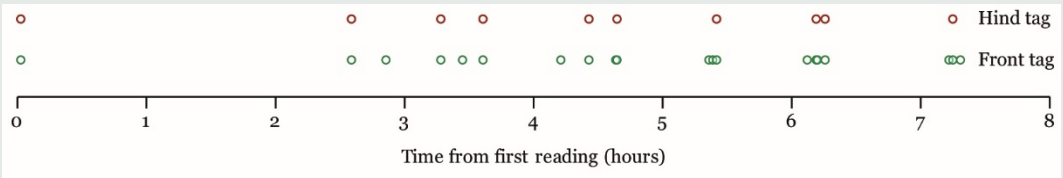

FIGURE 24. Example of the RFID tag in which records for both front and hind tags are shown for the same bee

Figure 24 shows that there are more readings for the front tag than for the hind tag, indicating that on several occasions the bee sticks its head into the channel of the reader a few times

before it decides to pass through. On that basis, it seems likely that data for the behind tag is better at describing a full passage compared to the front tag, which induces multiple readings without actually passing through.

The activity of the bee is reflected in the interval between two passages, so the purpose of making time recordings is to measure time intervals between passages. A time series of readings is therefore used to calculate the interval between two successive clock readings that maps the activity pattern of the bee.

This way of estimating bumble-bee activity gave us data on 1) the time spent inside/outside the hive-box, 2) the frequency with which the bees leave and return to the boxes. The method was compared to more traditional methods, where bees subject to RFID analyses were also observed directly at regular time intervals and their activities were noted (Appendix A).

4.1.3 Traditional activity measures as alternative/supplementary to RFID measurements

In one of the semi-field tests with Biscaya, an attempt was made to compare the RFID reading with a more traditional way of estimating effects on activity, i.e. the bees were observed for certain periods, and the time spent on different activities was noted.

The activity of individual bees was recorded at time intervals 0, 1, 2, 3, 5, 7 and 14 days after pesticide exposure. In order to differentiate between single individuals, these were marked with different colours prior to pesticide exposure. The colour code was combined with information about RFID tag number by “reading” each colour-marked and RFID-tagged bee with the RFID reader in a known order so that the RFID identity could later be obtained from the RFID file. Activity was observed for a 10 minute period just after exposure and, subsequently, in the time spans 9:30-11:30 and 15-17 series. Observations were noted as indicated in Table 21.

TABLE 21. Activity categories noted during the 10 minute observation periods and extracted from RFID reading. The time spent on each activity was also noted

Activity category	Description
Dose	Dose level as defined in the testing
crawling	Time spent on crawling around (s)
pfeeding	Time spent on feeding pollen from container (s)
resting	Time spent on resting (s)
buildingnest	Time spent on building nests (s)
cleaning	Time spent on cleaning the nest (s)
guarding	Time spent on guarding the entrance of the nest (s)
SumTime	Total time registered for the above activities (s)
form	The form factor for the fitted Weibull distribution for the RFID tag measured time intervals
level	The Weibull estimate level for the time intervals for the RFID tag measured time intervals
N	Total recorded number of RFID readings

4.2 Statistical analyses

Apart from the analyses of RFID data described elsewhere, the data from the semi-field experiments were analysed the same way as those from the long-term laboratory tests (paragraph 3.2).

4.3 Results of semi-field tests

Below, the results of semi-field tests with pesticides alone and in combination with fungus and starvation are presented. All tables with the results of the statistical analyses are listed in Appendix B.

4.3.1 Pesticide alone

Extremely few dead larvae were observed; hence, data for this end-point was not analysed.

Consumption

Generally, the consumption of sugar solution within the exposure period was affected by Biscaya dosage, but not by Fastac and Karate (Figure 25, Table 61). In the first and second repetition of the test with Biscaya, the possible difference in consumption of sugar solution with and without the spreading agent Dancon F was tested, resulting in a significant difference in the second repetition, but not in the first (Table 61). Even though the overall effect of dosage was only significant in the second repetition, there were significant differences between controls (water+Dancon or both water+Dancon and water alone) and one or more Biscaya dosages in all repetitions, which is reflected in Figure 25.

In the first repetition with Fastac, consumption was significantly higher by control bees than by bees offered Fastac-contaminated sugar solution, whereas in the second and third repetitions as well as in the analysis of the pooled data set no such differences were identified (Figure 25, Table 61). In the first repetition of the test with Karate, an increased consumption was seen with increasing Karate dosage, except for the highest dosage. In the other two repetitions and in the analysis of the pooled data set, no significant differences in consumption were seen (Figure 25, Table 61).

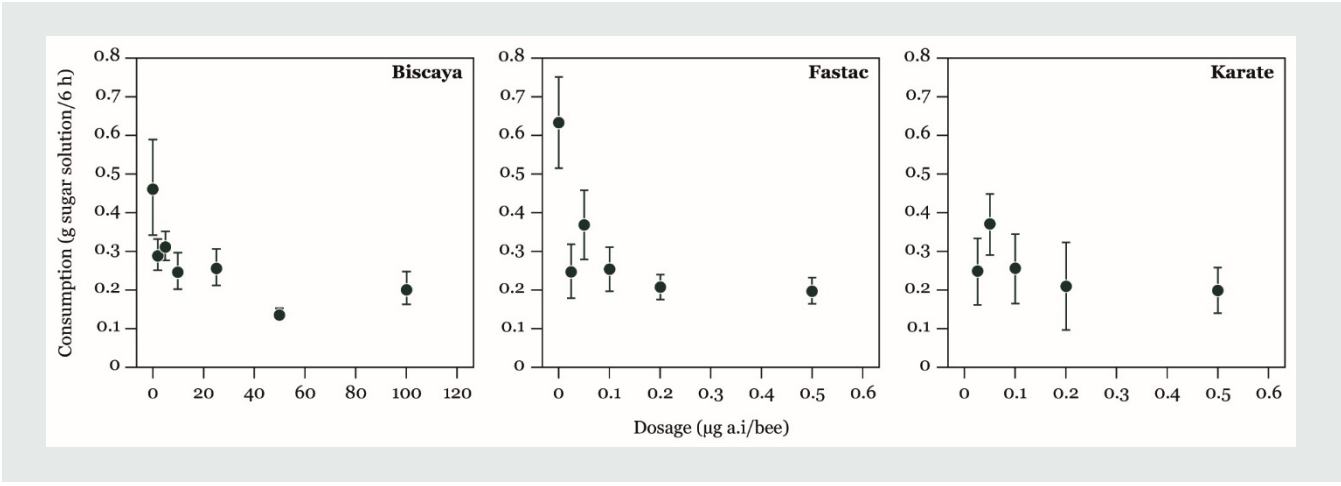

FIGURE 25. Consumption of sugar solution during the 6 h pesticide exposure period. Points represent means +/- SEM for the pooled data sets for all three repetitions per pesticide (n=12)

Biscaya

Worker survival

Biscaya dosage caused significant effects on adult survival on d 7 and 14 in all three repetitions of the test (Figure 26, Table 62). In the analyses of the pooled data set for all repetitions, only controls with Dancon F were included, as the results for controls with and without the spreading agent differed significantly. Due to interactive effects between repetition number and Biscaya dosage, these effects could not be separated.

An LD_{50} value of 26 [13-50] µg a.i./bumble-bee was estimated.

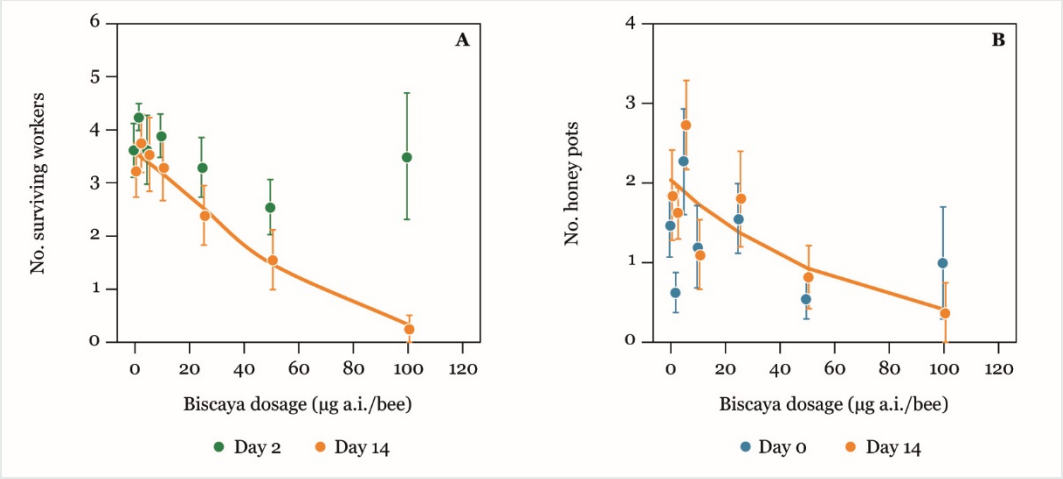

FIGURE 26. Adult bumble-bee survival 2 and 14 d after Biscaya exposure (A) and no. honey pots 0 and 14 d after exposure (B). Points represent means +/- SEM for the pooled data set for all three repetitions (n=12). The line shows the sigmoid fit of survival on day 14

Egg cells

No significant effects of Biscaya dosage were found in the individual test repetitions or in the pooled data set ($p>0.14$).

Larval cells

Hardly any larval cells developed, hence, analysis does not make sense.

Honey pots

The analysis of the pooled data set identified a significant effect of Biscaya dosage on day 14 ($p=0.0065$) (Figure 26). When the data from the three repetitions were analysed separately, Biscaya exposure did not have significant effects on the number of honey pots produced by the micro-colonies ($p\geq0.1381$).

Fastac

Worker survival

The number of adult bumble-bees was significantly lowered by Fastac exposure in all three repetitions of the test and in the analysis of the pooled data set (Figure 27, Table 63). An LD_{50} value of 0.2 [0.1-0.5] µg a.i./bumble-bee was estimated.

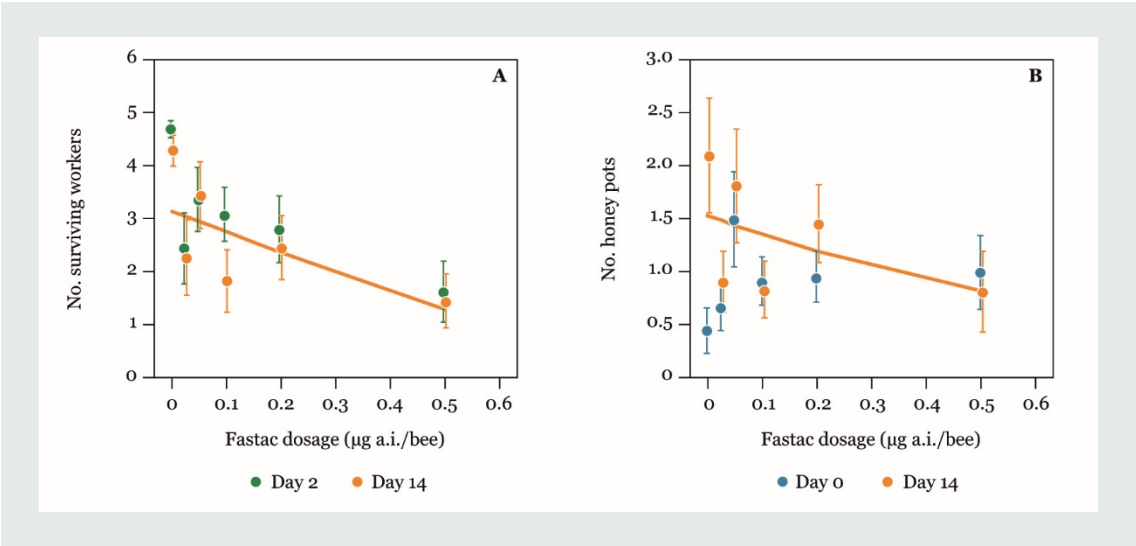

FIGURE 27. Adult bumble-bee survival day 2 and 14 after Fastac exposure (A) and no. honey pots 0 and 14 d after exposure (B). Points represent means +/- SEM for the pooled data set for all three repetitions (n=12). The line shows the sigmoid fit of survival on day 14

Egg cells

In the first repetition, no eggs were seen until day 14. In the second and third repetition, more eggs were produced, but there were no significant differences between micro-colonies exposed to different dosages (p>0.19).

Larval cells

Very few larval cells developed; hence, no statistical analyses are presented.

Honey pots

The analysis of the pooled data set found a significant effect on day 14 (p=0.0051) (Figure 27). In the individual repetitions of the test, the number of honey pots produced did not differ between micro-colonies treated with different levels of Fastac (p>0.1160), neither at the beginning of the test nor during the test period.

Karate

Worker survival

When data from the three repetitions were tested individually, adult survival was generally significantly affected by Karate (Figure 28, Table 64), although this effect was less clear in the first repetition of the test. The analysis of the pooled data set, however, did not identify significant effects of Karate exposure. An LD_{50} value of 0.02 [-0.06-0.13] µg a.i./bumble-bee was estimated, i.e. the lower 95% confidence limit could not be estimated.

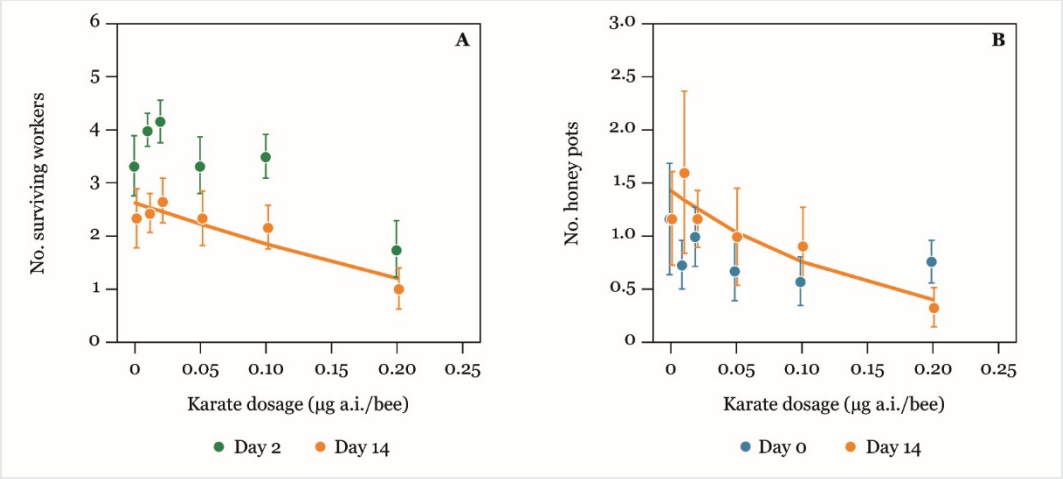

FIGURE 28. Adult bumble-bee survival day 2 and 14 after Karate exposure (A) and no. honey pots 0 and 14 d after exposure (B). Points represent means +/- SEM for the pooled data set for all three repetitions (n=12). The line shows the sigmoid fit of survival on day 14

Egg cells

Karate exposure did not result in significant differences in the number of egg cells produced ($p>0.52$).

Larval cells

Hardly any larval cells developed and, therefore, data have not been analysed.

Honey pots

No significant differences in the number of honey pots produced were detected during the 14 d test period, neither when data from the individual repetitions were analysed separately, nor when the pooled data set was analysed ($p>0.21$, Figure 28).

Activity measured by RFID

For all three pesticides, five bees in the cages exposed to different insecticide dosages were labelled with RFID tags, and the readers were recording during the entire experimental period of 14 days. The time intervals between readings for a single bee are presented in Figure 29.

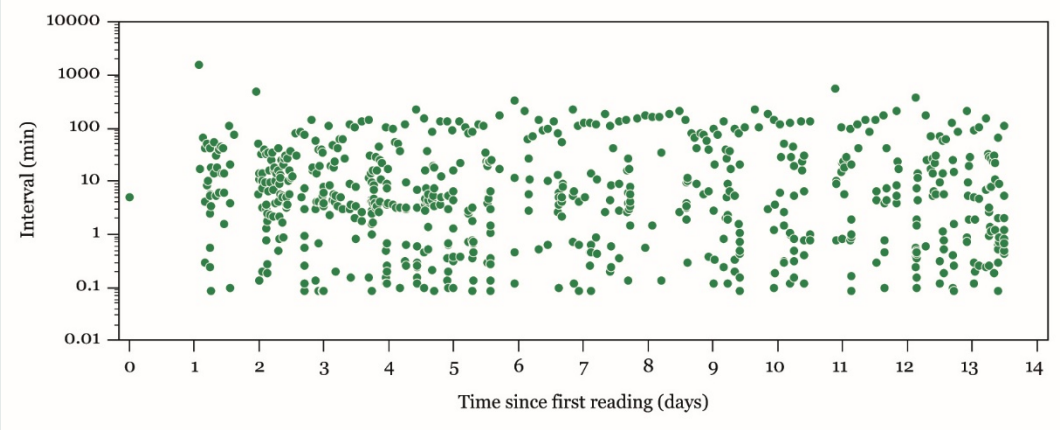

FIGURE 29. Records of time intervals between RFID reading for a single bee in the semi-field experiment

In order to find a useful way of describing the large number of collected data and relating it to pesticide exposure, we sought for a fitting distribution. It turned out that the time intervals found in the semi field experiments can be fitted rather closely to a Weibull distribution, see Figure 30. The Weibull distribution delivers two parameters from the data: Form (reflecting the variability in time interval size) and Level (reflecting the size of the time interval). There is a close and trivial relation between the total number of readings and the level because the time intervals will be large for few readings (time period divided into few intervals) compared to many readings (time period divided into many intervals). The form of the Weibull distribution can be interpreted as the variability, i.e. if the form value is large, it indicates a large variability of the time intervals. Thus, the form will depend on the behaviour of the bee that is not related to the number of recorded time intervals.

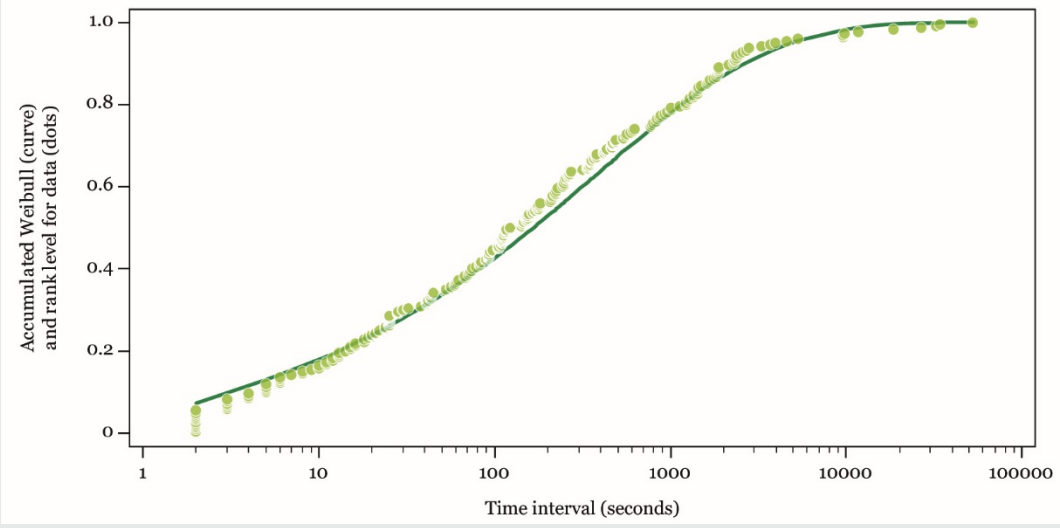

FIGURE 30. Weibull distribution (curve) fitted to time interval data for a single bee (dots)

Results for the semi-field tests of pesticide effects are shown in Table 22. The level and form parameters are estimated for the Weibull distribution for each bee. The mean values are shown for each colony and calculated based on all the labelled bees in the colony. Figure 31

shows the form factor as a function of dosage level for each pesticide. There seems not to be any causality between the form factor and dosage level, which means that the part of bee behaviour described by the form factor is apparently not influenced by pesticide dosage.

TABLE 22. MeanWeibull level and form estimates for bumble-bees exposed to different levels of Biscaya, Fastac and Karate

Colony Id	Pesticide	Dosage	Mean Level	Mean Form	Sum RFID readings	No. recorded bees
1099	Biscaya	0	5440.7119	0.467401072	282	4
410		0	1062.5317	0.498974401	572	5
1094		2	292.42755	0.461651307	4096	5
411		2	1086.3623	0.402630866	174	5
1095		5	1078.2197	0.402292579	1386	4
412		5	414.5773	0.480452582	3405	4
1096		10	31473.826	0.419059008	53	4
413		10	1213.762	0.47940082	1281	5
1097		25	2178.7502	0.330487758	92	1
414		25	1034.4442	0.479671925	860	3
1098		50	24236.625	0.521894529	217	2
408		50	2925.3376	0.317218274	5	1
1155	Fastac	0	1515.9507	0.425444886	6384	4
982		0	4422.1528	0.399402541	665	5
563		0	1369.6852	0.627553105	200	4
1146		0.025	3204.5183	0.338141888	16	2
977		0.025	535.42426	0.417810851	3643	5
564		0.025	1228.1505	0.59502242	364	6
1147		0.05	2099.5093	0.480475682	1311	5
978		0.05	1425.2543	0.349853985	507	4
566		0.05	1715.9896	0.581950863	123	6
1150		0.1	1641.3441	0.386858714	301	5
979		0.1	545.44843	0.422076273	146	5
562		0.1	1290.7875	0.571282052	214	4
1151		0.2	681.23474	0.436118281	2874	5
983		0.2	356.48679	0.382816535	1388	5
583		0.2	1376.0245	0.74035511	245	5
1152		0.5	705.53723	0.434899479	1426	4
981		0.5	1982.0123	0.5428339	677	5
1179	Karate	0	21451.467	0.626893461	22	2
1085		0	1025.5238	0.458998509	1907	4
745		0	2382.6511	0.734207253	32	3
1064		0.01	4414.5444	0.422741808	975	4
722		0.01	239.67007	0.39727746	109	2
1065		0.02	867.24982	0.502031565	3341	3
723		0.02	1275.1235	0.386782259	114	5
1175		0.05	1658.576	0.463588819	22	2
1066		0.05	902.32324	0.462307465	1668	5
724		0.05	834.04041	0.392483562	47	2
1177		0.1	1163.2568	0.400320083	98	2
1068		0.1	546.22394	0.484485358	2398	3
725		0.1	1914.3799	0.752864435	89	4
1178		0.2	6741.2305	0.606694221	6	1
1069		0.2	1488.486	0.449300845	639	3
726		0.2	938.15942	0.556868851	123	4

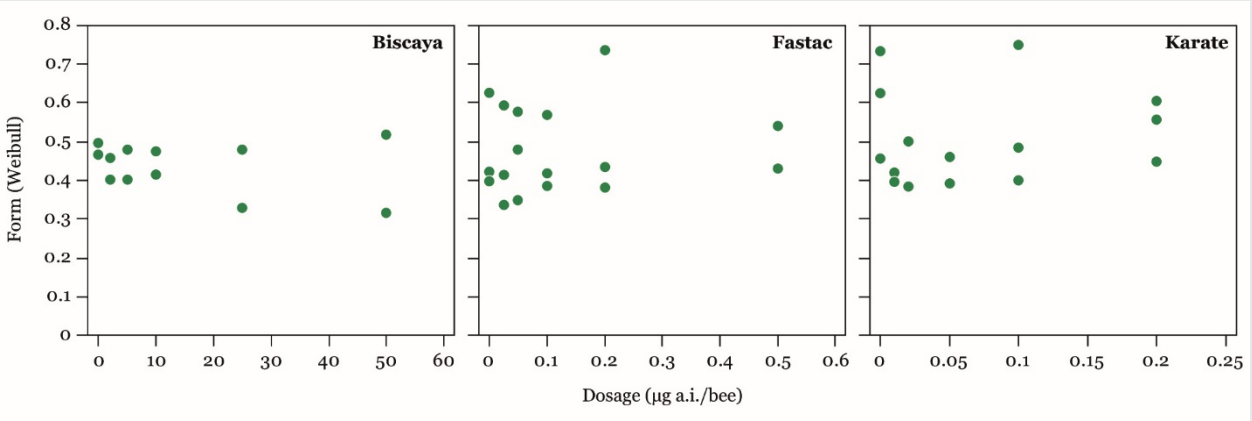

FIGURE 31. The Form factor from the Weibull fitting of time intervals shown as function of dosage level for each pesticide

A simple activity measure is the sum of RFID readings during the experimental period. This reflects overall activity, and the sum is shown for different dosage levels and for the different pesticides in Figure 32.

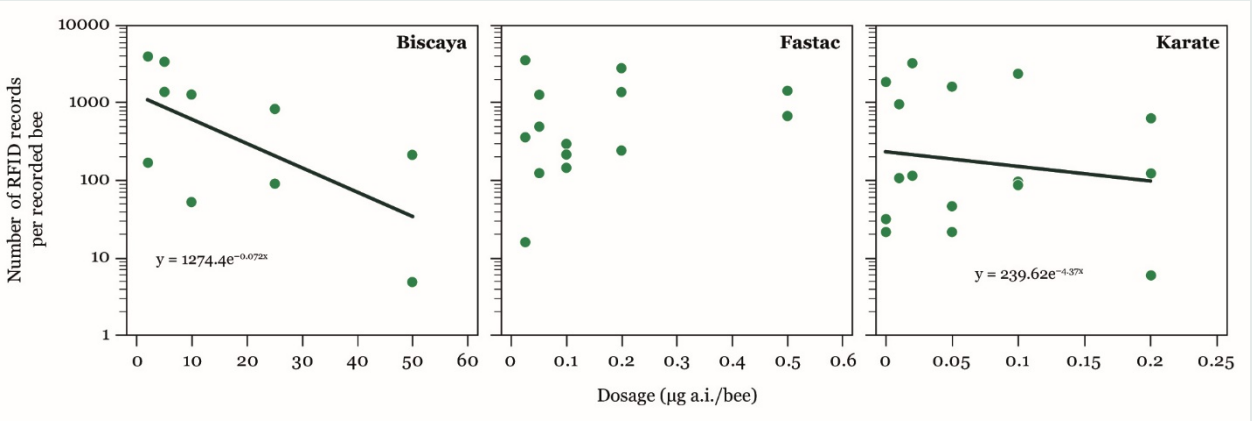

FIGURE 32. The sum of RFID readings per micro-colony shown as a function of dosage level at a log y-scale. Data for all three test repetitions are shown. Only dosages with registered activity are included. The lines show the fitted dose-response-relations

Figure 32 indicates that there is a dose-response relation for Biscaya, while for the other two pesticides the relation is less clear. Furthermore, data for Fastac tend not to have homogeneity of variance at the log y-scale.

If the dose-response is assumed to follow an exponential relation as indicated for Biscaya in Figure 51:

$$RFID = b \cdot e^{a \cdot x}$$

there is a simple method to calculate the ED_{50} for RFID:

$$ED50_{RFID} = -\frac{\ln 2}{a}$$

Thus, in the case of Biscaya the $ED_{50\ RFID}$ is ln(2)/0.072=9.6 µg a.i./bee.

The sum of RFID readings is a rough measure, however, as illustrated in Figure 33 for the colonies 1098 and 1009, where colony 1098 has received the highest Biscaya dosage tested, while colony 1099 is the control. In Figure 22, it is seen that the sums of RFID reading are close to being equal, since the two colonies have 217 and 282 readings, respectively. However, Figure 33 shows a different pattern, where the RFID reading from colony 1098 is mostly related to a single bee, while the reading for colony 1099 is a contribution from four bees.

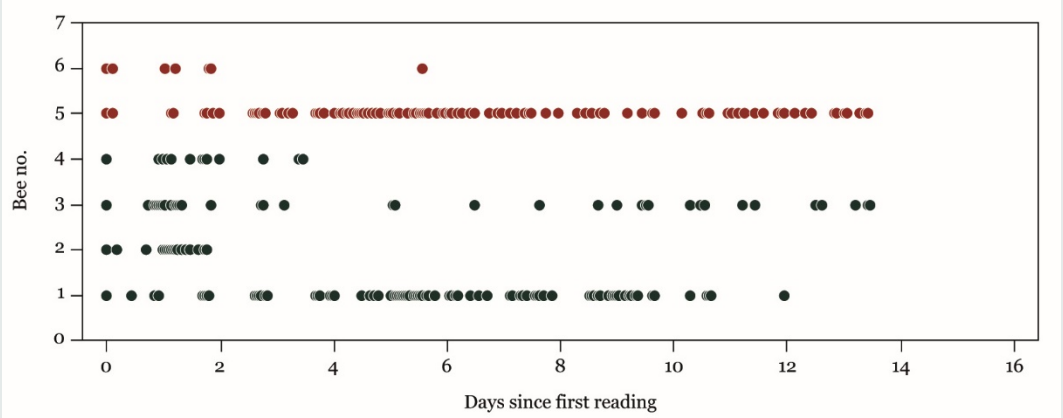

FIGURE 33. Single time recording for the control (colony 1099, black points) and Biscaya dosage level 50 (colony 1098, red points), respectively. Each horizontal line of points represents a single bee

Activity measured by more traditional methods

Data records of all activities for individual bees are seen in Appendix C. It should be noted that only data for micro-colonies where activity was actually seen is presented. Consequently, the highest Biscaya dosage shown is 25 µg a.i./bee, although dosages up to 100 25 µg a.i./bee were tested. In Appendix D, the correlation between the records for bee with RFID tags are shown. No correlation is observed between the records from the RFID reading described above (form, level, N) and traditional activity categories, except for a correlation between N and the total time spent on activities (SumTime). However, it seems trivial that the bees that have long time records also have been active and thus will have e high N value. Consequently, the RFID tag parameters have been removed to increase the data set to the individual bees that were not tagged, and the correlation is disclosed in Appendix E.

The relation between Biscaya dosage and selected activity categories is presented in Figure 34. For comparison, the RFID readings for the same micro-colonies were extracted from Figure 32 (shown in Figure 35). The selected activities seem to be negatively affected by Biscaya dosage, except for the time spent on cleaning. It is particularly important to note the decreasing relation between SumTime and Dose. At higher dosages, the activity is decreased. However, statistical assessment of significance in this testing is difficult due to the pseudo-replication of several bees from the same nest and the fact that the number of data points is too limited to make more complex statistical models.

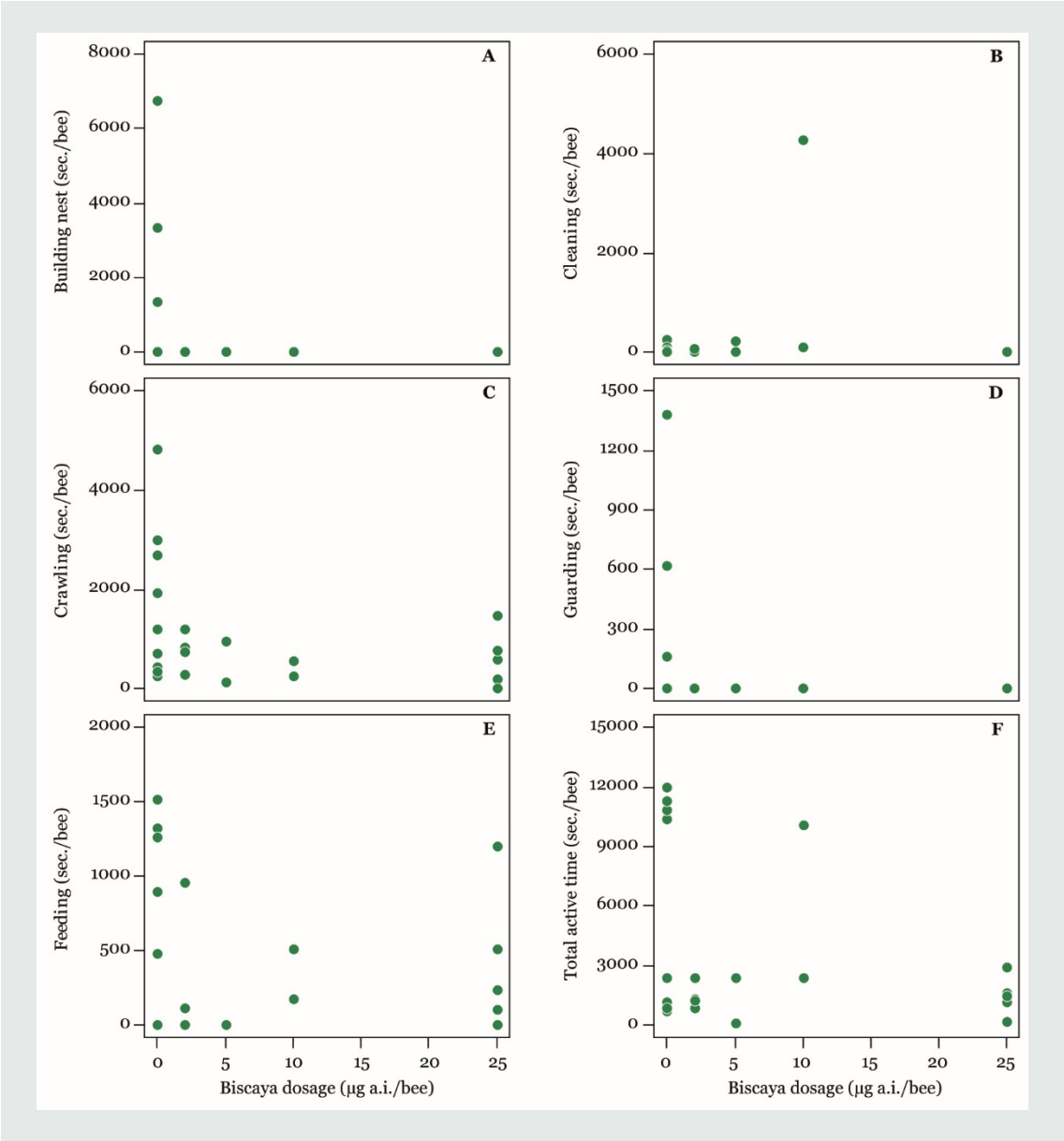

FIGURE 34. Time that individual bumble-bees spent on different activities as well as the total time spent on these activities as function of Biscaya dosage. Each point represents observations of the activity of a single bee for 110 minutes during the first two weeks after pesticide exposure

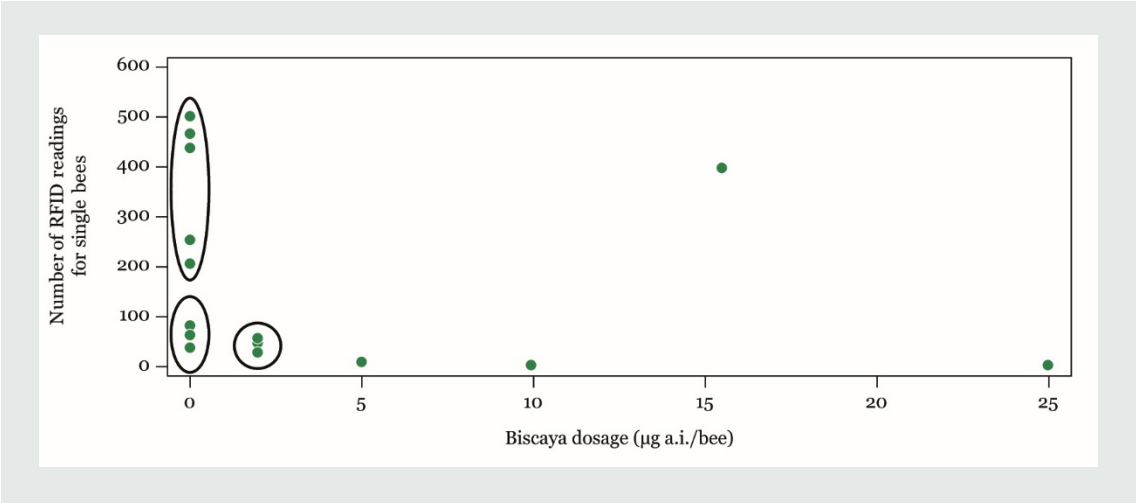

FIGURE 35. Relation between Biscaya dosage and no. RFID readings for single bees in micro-colonies where also other activity categories were estimated (cf. Figure 35). For control and lowest dosage, points representing bees from the same micro-colonies are indicated

4.3.2 Interaction with pathogenic fungus

No larval cells developed during the test period and no dead larvae were observed. Hence, these end-points were not analysed.

Biscaya

Worker survival

The number of adult bees was significantly affected by Biscaya dosage from the beginning of the test period, while fungus treatment had no significant effects. Later in the test period, interactive effects of pesticide and fungus were identified (Figure 36, Table 65).

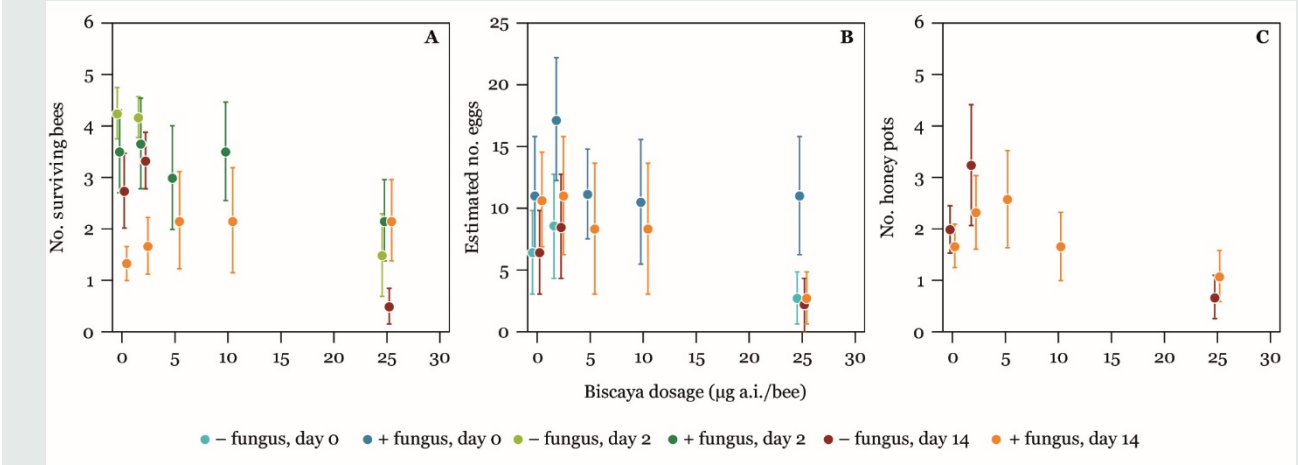

FIGURE 36. Effect of Biscaya and the pathogenic fungus *B. bassiana* on adult bumble-bee survival (A), no. eggs produced (B) and no. honey pots (C) 14 d after pesticide exposure. Points represent means +/- SEM of 8 micro-colonies per dosage

Egg cells

In the analysis of the pooled data set, there was a significant effect of the fungus on the number of eggs produced on day 7, but not on day 14 (Table 66). There were no interactive effects between pesticide and fungus ($p>0.9$). At the beginning of the test (i.e. a week after fungus

inoculation), the fungus treated micro-colonies differed from the controls, but after pesticide exposure no effects of pesticide or fungus were identified in the individual repetitions (Table 66).

Honey pots

The analysis of the pooled data set found significant effects of Biscaya dosage on day 7 (p=0.0238) and 14 (p=0.0222), but no effect of the fungus (p>0.3). Until the end of the test period of the first repetition, no significant effects of Biscaya or *Beauveria* on the number of honey pots in the micro-colonies were identified (p>0.05). On day 14, an overall effect of Biscaya was found (p=0.0094), although only micro-colonies treated with the lowest dosage differed significantly from untreated controls (p=0.021) (Figure 36). In the second repetition of the test, no significant effects of fungus or Biscaya exposure were found (p>0.5).

Fastac

Worker survival

Adult survival was significantly affected by fungus treatment during the entire test period, or fungus treatment and Fastac dosage interacted, while pesticide alone did not have significant effects (Figure 37, Table 67).

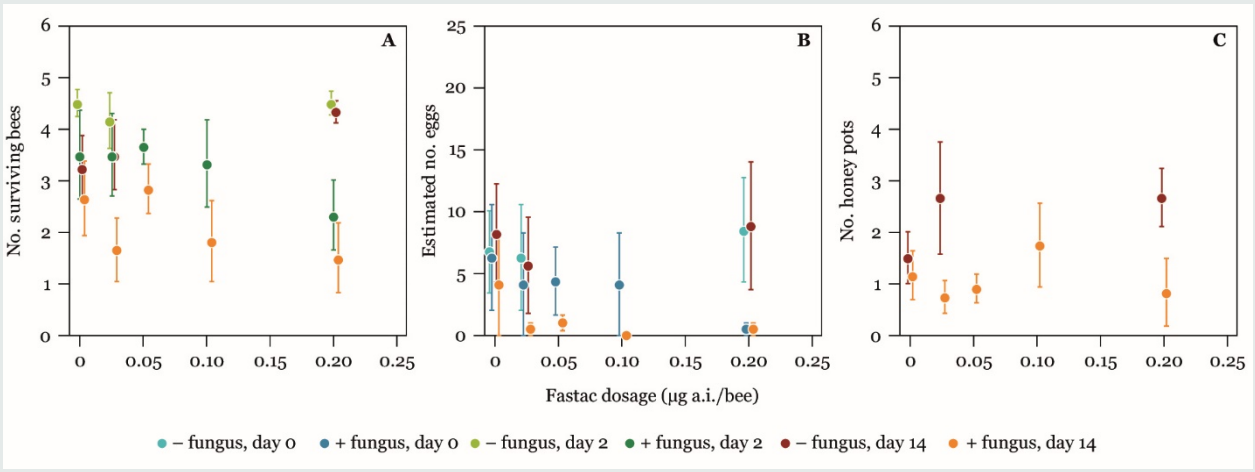

FIGURE 37. Effect of Fastac and the pathogenic fungus *B. bassiana* on adult bumble-bee survival (A), no. eggs produced (B) and no. honey pots (C) 14 d after pesticide exposure. Points represent means +/- SEM of 8 micro-colonies per dosage

Egg cells

In the analysis of the pooled data set, significant effects of fungus exposure were found on day 3 (p=0.043), day 7 (p=0.024) and day 14 (p=0.014) (Figure 37). There were no interactive effects between pesticide and fungus on the number of egg cells in the micro-colonies of the individual repetitions (p>0.9) and no significant effects of fungus or Fastac level (p>0.076), except for day 14 in the second repetition (p=0.046).

Honey pots

The analysis of the pooled data set found significant effects of *Beaveria* on days 7 and 14 (Figure 37, table 68). No interactive effects of fungus and Fastac treatment were seen (p>0.39). On day 7 in the second repetition of the test and on day 14 of the first repetition, significant effects of fungus treatment on the number of honey pots in the micro-colonies were identified.

Karate

Worker survival

The analysis of the pooled data set showed that Karate dosage and fungus level interacted significantly (Figure 38, Table 69). Two days after Karate exposure, significant differences in the number of surviving workers were found between micro-colonies exposed to different pesticide dosage in both repetitions of the test. However, these effects were not consistent throughout the 14 d test period, and in the first repetition Karate and fungus levels interacted at the end of the test.

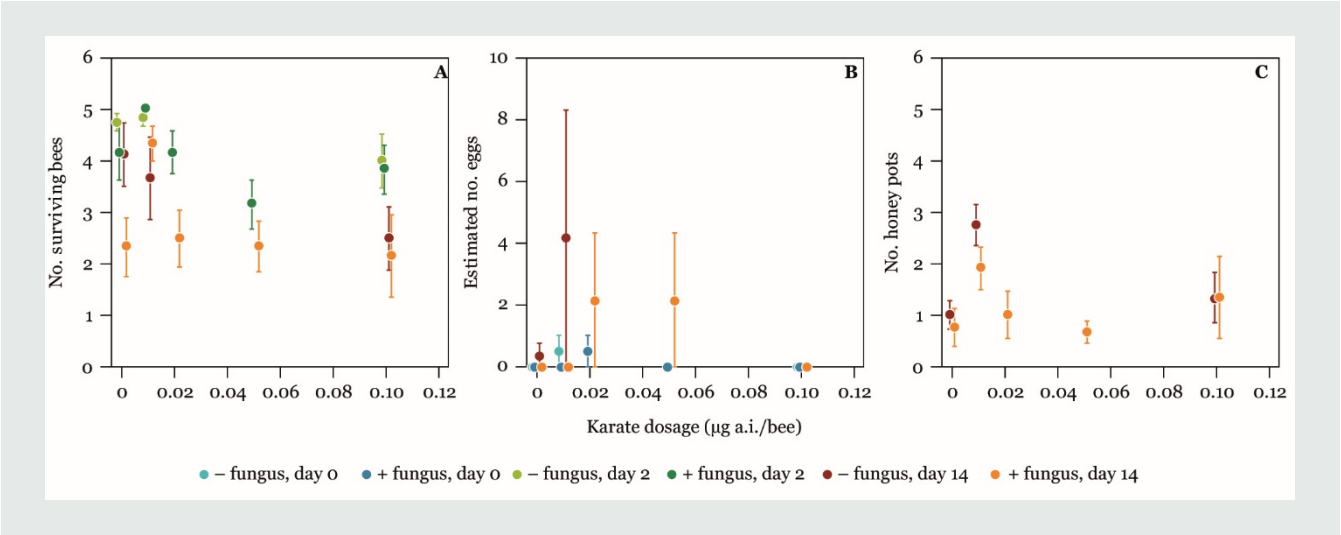

FIGURE 38. Effect of Karate and the pathogenic fungus *B. bassiana* on adult bumble-bee survival (A), no. eggs produced (B) and no. honey pots (C) 14 d after pesticide exposure. Points represent means +/- SEM of 8 micro-colonies per dosage

Egg cells

Only few egg cells were produced in this test, and no significant differences in micro-colonies exposed to different levels of Karate or fungus were detected in the individual repetitions or in the analysis of the pooled data set ($p>0.40$, Figure 38).

Honey pots

The analysis of the pooled data set identified significant effects of Karate dosage on day 3 ($p=0.0432$) and 14 ($p=0.0267$), but no significant effect of fungus treatment. In the individual repetitions of the test, there were no significant effects of fungus treatment or Karate dosage on the number of honey pots found in the micro-colonies ($p>0.08$) (Figure 38).

4.3.3 Interaction with starvation

Hardly any larval cells developed during the test period and no dead larvae were observed. Hence, these end-points were not analysed.

Biscaya

Worker survival

Two days after Biscaya exposure, significant differences in the number of surviving workers were found between micro-colonies exposed to different pesticide dosages in both repetitions of the test (Table 70). However, these effects were not consistent throughout the 14 d test period (Figure 39). Starvation did not significantly affect survival, and Biscaya dosage and starvation did not have interactive effects on the number of adult bees surviving ($p>0.12$).

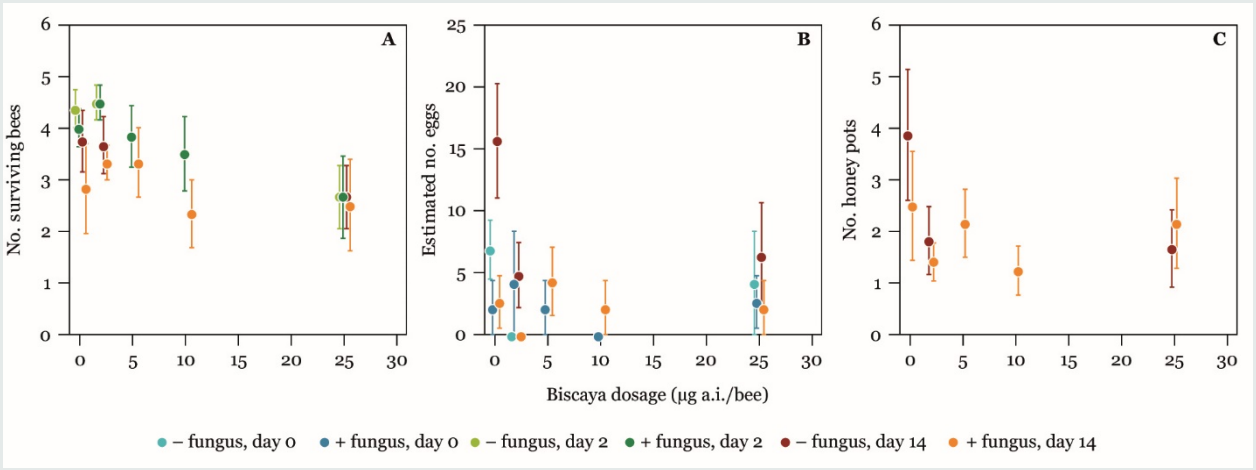

FIGURE 39. Effect of Biscaya in combination with starvation on bumble-bee survival (A), no. eggs produced (B) and no. honey pots (C) 2 and 14 d after pesticide exposure. Points represent means +/- SEM of 8 micro-colonies per dosage

Egg cells

The analysis of the pooled data set showed that starvation significantly reduced the number of eggs produced at the end of the test period, while Biscaya did not have significant effect on egg production (Figure 39, Table 71). Starvation and Biscaya did not have interactive effects on the number of egg cells produced ($p>0.24$). No effects of starvation were found in the second repetition, where only few eggs were seen.

Honey pots

Starvation and Biscaya did not have interactive effects on the number of honey pots present in the micro-colonies ($p>0.11$) and no effect of the two factors were detected during the test period, when data from the individual repetitions were analysed separately ($p>0.068$). The analysis of the pooled data set found a significant effect of Biscaya dosage on day 7 ($p=0.0199$), but not on day 14 ($p=0.0665$) (Figure 39). No significant effects of starvation were detected ($p>0.16$).

Fastac

Worker survival

The analysis of the pooled data set identified significantly interactive effects of starvation and Fastac on bumble-bee survival (Figure 40, Table 72). In the first repetition, starvation caused a significantly higher mortality among workers, whereas Fastac dosage did not affect survival.

Egg cells

Generally, there was a significant effect of starvation on the number of eggs produced, but no significant effect of Fastac dosage (Figure 40, Table 73). There were no interactive effects of Fastac and starvation ($p>0.58$).

Honey pots

In the first repetition and the analysis of the pooled data set, there was a significant effect of starvation (Figure 40, Table 74), while no significant effects of Fastac exposure were detected. There were no significant interactive effects of starvation and Fastac exposure on the number of honey pots in the micro-colonies ($p>0.39$).

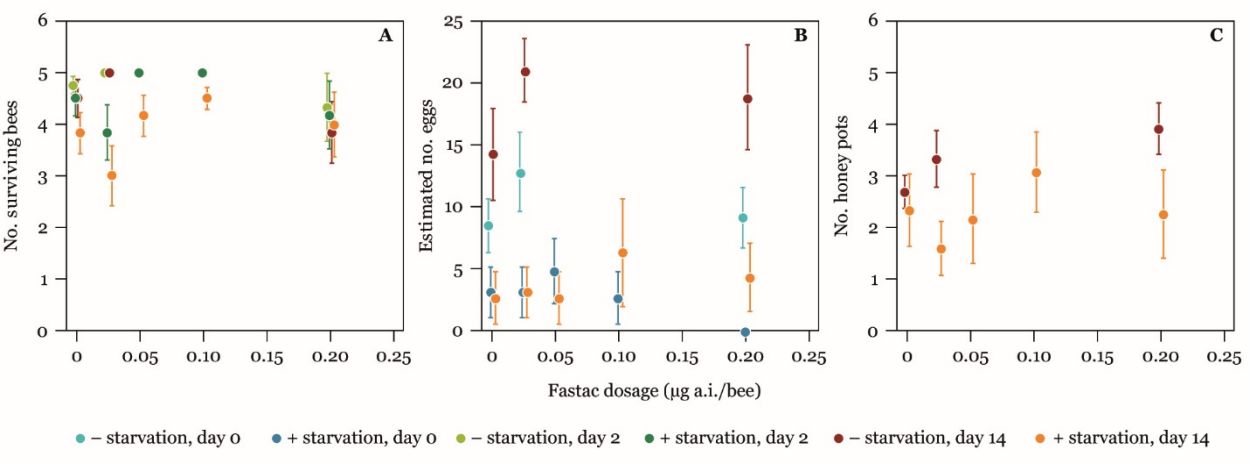

FIGURE 40. Effect of Fastac in combination with starvation on bumble-bee survival (A), no. eggs produced (B) and no. honey pots (C) 2 and 14 d after pesticide exposure. Points represent means +/- SEM of 8 micro-colonies per dosage

Karate

Adult survival

The analyses of the pooled data set identified significant effects of Karate dosage on day 2 ($p=0.0012$) and day 7 ($p=0.0357$), but not on day 14 ($p=0.1263$) (Figure 41). No significant effects of starvation were found ($p>0.08$). In the first repetition of the test, increasing Karate dosage caused significantly higher worker mortality throughout the test period ($p\leq0.0003$), while starvation did not cause any significant effects ($p>0.063$). In the second repetition, mortality was too low to allow testing, except for day 14 when no significant effects of dosage or starvation were detected ($p>0.15$).

Egg cells

On day 7 and 14, significant effects of starvation were identified, while there were no significant interactive effects on the number of eggs produced ($p>0.26$) and no significant effects of Karate dosage alone (Figure 41, Table 75).

Honey pots

No significant effects of starvation or Karate on the number of honey pots produced were found ($p>0.14$) (Figure 41).

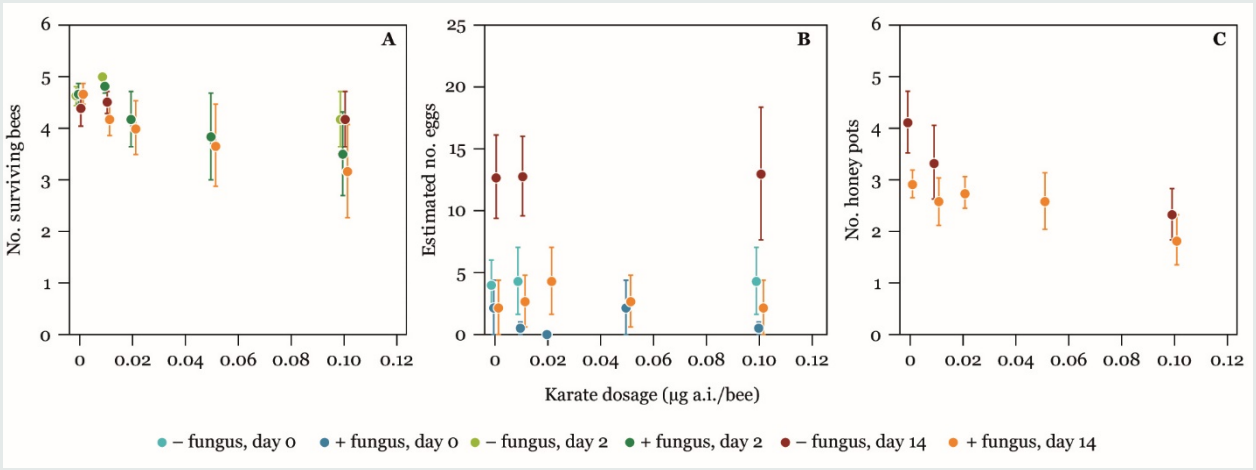

FIGURE 41. Effect of Karate in combination with starvation on bumble-bee survival (A), no. eggs produced (B) and no. honey pots (C) 2 and 14 d after pesticide exposure. Points represent means +/- SEM of 8 micro-colonies per dosage

4.4 Discussion of results of semi-field tests

As in the 14 d laboratory tests, Biscaya lowered the consumption of sugar solution by the bumble-bees and a similar effect was seen for Fastac. This may affect actual oral exposure in tests with Biscaya and Fastac. Biscaya and Fastac affected worker survival, while this effect was less evident for the tested range of Karate dosages. Consequently, LD_{50} values could be estimated for Biscaya and Fastac, whereas for Karate satisfactory confidence limits could not be calculated. The tested dosages of Biscaya and Fastac also had significant effects on the number of honey pots produced, while no effects were seen on the number of eggs. The number of larval cells was generally very low and, therefore, this end-point was not feasible to assess in the applied test set-up. Control mortality was higher than desired, especially in the tests with Biscaya and Fastac, which reduces the credibility of the tests and makes it more difficult to demonstrate pesticide effects. Elevated control mortality highly affects other end-points, especially if the egg-producing worker is killed.

When pesticide exposure was combined with inoculation with the pathogenic fungus, interactive effects were seen on bumble-bee survival, but not on the other end-points, which were generally not affected by the fungus.

In the tests that combined starvation with pesticide exposure, the two stressors generally did not interact. Starvation itself only affected survival in the test with Fastac, while the number of eggs produced was affected in all tests. Control mortality was generally low in these tests, which sustains the credibility of the results, and therefore it seems plausible that egg production may be affected by starvation, even though survival was unaffected.

Pesticide effects on bumble-bee activity were primarily measured by the use of RFID techniques. In order to make sense of the many readings, data was fitted to a Weibull distribution. The Weibull distribution is described by two parameters, the form parameter, which is a measure of variability of the length of the time intervals between readings, and the level parameter, which is a measure of the length of time interval between readings. No relation was found between the form parameter of this distribution and pesticide dosage, which indicates that the activity not described by the level parameter is not influenced by pesticide dosage. Since the level parameter is a measure of the length of the time interval between readings, it is closely related to the total number of RFID readings per bee. The sum of RFID readings was used to

assess the effect of Biscaya on bumble-bee activity, resulting in an estimated EC_{50} of 9.6 µg a.i./bee, which is lower than the LD_{50} value of 26 µg a.i./bee found in the semi-field test above. This indicates that the sum of RFID readings could be a sensitive end-point for toxic effects on bumble-bee activity. However, the estimate of the RFID effect level is too uncertain to say that this difference in effect levels between end-points is significant, and no LD_{50} values based on the number of reading could be established for Fastac and Karate. Furthermore, the sum of RFID readings is a somewhat “rough” end-point because it aggregates bumble-bee activity in the colony and does not reveal whether the activity was performed by one or several bees. If more detailed information is required, the number of active bees per micro-colony may be useful.

The more traditional method of observing bumble-bees in well-defined periods of time to assess effects on activity was performed for one of the tests, where also RFID techniques were applied. Some activity categories, e.g. nest building, crawling, pollen feeding and guarding, as well as the sum of these, decreased at increasing Biscaya dosages. Although a similar response was seen for the number of RFID readings for the same test, the only clear correlation between the “traditional” responses and the RFID readings was for the total time spent on activities. There are no indications in the comparison made here that traditional observation techniques reveal effects on activity not covered by the RFID techniques.

5. Field tests – oral exposure

5.1 Methods

In the field trial, we investigated how activity level and colony size (measured as colony weight) of full size hives of *Bombus terrestris* foraging in an agricultural landscape are affected by three different pesticides at different dosages. Bumble-bees were exposed to the pesticides orally by adding pesticides to the sugar solution they fed on. Experimental colonies were then placed in one of four different field localities, all in areas (within 2 km of the hives) where pesticide usage due to conventional farming was relatively low (Figure 42).

5.1.1 Field sites

The low pesticide load landscapes were located using the methods described in Kjær et al. (2008=. The four study sites were: Vejlsøvej (56° 9'16"N, 9°33'43"E), Funder (56° 9'7"N 9°23'21"E), Them (56° 5'40"N 9°31'22"), and Moesgaard (56° 5'7"N 10°13'34"E). The shortest distance between two study sites was 7.3 km. Although *Bombus terrestris* may fly up to 10 km if forced to (Goulson 2003), maximum average foraging ranges are reported at < 2km (Walther-Hellwig and Frankl 2000, Darvill et al. 2004, Knight et al. 2005, Osborne et al. 2008). Hence, the landscapes are considered to be independent of each other and bees are not expected to move among different study localities.

FIGURE 42. The four study localities of the field experiment marked with yellow pins

5.1.2 Queen-right colonies

We used commercial standard hives produced for open field pollination. These consist of an inner plastic box, where the sides of the boxes are perforated by numerous holes to provide sufficient ventilation. The inner box contained some nesting material and was connected to a container with sugar solution consisting of 70% invert sugar and 30% water. The sugar container and nesting box are placed in an outer, insulating Styrofoam box. The boxes have two entrance holes, one that can be used for both entering and exiting the box, while the other hole had an extension permitting ingoing, but not outgoing traffic.

In the days prior to the experiment, the standard hives were placed in a climate chamber with constant temperature and humidity (27 degrees centigrade, 60% air humidity) and provided with pollen every second day. At the onset of the experiment, boxes contained an egg-laying

queen and approximately 10 workers in addition to brood cells with eggs, larvae and pupae. Each hive was exposed to one dosage of one pesticide (or control with no pesticide), as summarised in Table 23. We had 10 replicates per treatment, totalling 120 hives. Three replicates were placed at each of the sites Them, Moesgaard and Funder, while the last replicate was placed at AU Vejlsøvej.

5.1.3 Pesticide exposure

Dosages (expected consumption of pesticide per bee during the exposure period) were calculated from the consumption of sugar solution and pesticide concentrations used in the lab experiments. In the lab experiments using micro-colonies, each bee consumed an average of 0.077 mL of sugar solution during the exposure time (6 hours). For simplicity, we assumed that bees of the standard hives used in the field experiment had the same consumption, trying to take into account that they would have other food sources available, but also a higher food intake in order to warm up the hive and feed the larvae. Thus, a pesticide dosage of 0.1 µg pesticide denotes that each bee is expected to consume 0.1 µg active ingredient during the exposure period.

TABLE 23. Nominal exposure concentrations of the field based on a theoretical consumption of 0.077 ml/bee in 6 h

Pesticide	µg a.i./bi	ml stock solution* pr. 500 ml solution
Biscaya	100	2.7100
	25	0.6775
	5	0.1355
	0	0
Fastac	0.5	6.49
	0.1	1.30
	0.025	0.325
	0	0
Karate	0.05	13.0
	0.01	2.60
	0.0025	0.649
	0	0

* Biscaya 240 mg a.i./ml, Fastac 0.5 mg a.i./ml, Karate 25 µg a.i./ml

A hive was exposed to a pesticide by removing the normal sugar container of the hive and replacing it with a sugar solution containing the pesticides at the specified concentrations. The exposure time was 1 day (24 hours) for Karate and Fastac and 14 days for Biscaya. However, for the Biscaya treatment at the Them locality (a certified organic farm), exposure time was only 1 day, i.e. exposure only took place in the greenhouse, whereby contamination of the organic field was prevented. After the exposure period, the hives were again provided with non-toxic sugar solution. Immediately after the first day of exposure (i.e. 7th-9th of May), hives were placed in the field (i.e. at three of the four study localities, the Biscaya treated hives had toxic sugar solution during the first two weeks of the experiment). At the field localities, the entrance/exit holes were opened and the bumble-bees were allowed to forage in the field.

5.1.4 Colony growth

Each nest was weighed by lifting out the inner plastic box that contained bees and nesting material. Weights were monitored initially on the day of exposure and, subsequently, every week for a total of eight weeks. Prior to weighing a hive, the exit (but not entrance) holes were closed for two hours or, alternatively, weighting was done in early morning before bumble-

bees started foraging. After weighing, the inner box was put back into the insulation box and the exit hole was re-opened.

5.1.5 Bumble-bee activity – RFID

Similar to the micro-colonies of the semi-field experiments, the activity of selected bees was measured with RFID techniques. Due to the much larger numbers of bees, only a fraction of the bees could be marked. At the Vejlsøvej and Funder locations, the activity of five bees from six hives was measured on two occasions - at Vejlsøvej 8/9 and 38/39 days after pesticide exposure, and at Funder 12/13 and 25/26 days after exposure. On each occasion, bees were caught by closing the outgoing flying aperture at sunset the day before, and then picking up emerging bees the next morning, when the aperture was re-opened, using forceps and a small plastic tube. The bees were anaesthetized with CO_2 and RFID tags were glued on as previously described. When the bees were caught, the outward aperture was closed again so that RFID readers could be mounted. Once they were in the right position, the aperture was again opened and the activity of the marked bees followed until sunset, when the procedure was reversed and the readers were removed.

5.1.6 Final condition of hives

At the end of the field trial, the inner box with the colonies was collected and frozen. Later, a picture was taken of the hive content and the following parameters registered: number of egg cells, honey pots, number of larval cells (in upper layer), number of putative queens (0 = no queen, 1 = one queen, 2 = several queens), number of adult bees, the total weight of bees and presence/absence of foreign animals (which may be parasites). Unfortunately, it turned out that many of the bees were dead at the time of collection and several hives were deteriorating. Therefore, we found the value of the obtained data low, and the data is not presented.

5.2 Statistical analyses

The following variables were analysed: absolute weight change ($w_t - w_0$, weight change from exposure day) and relative weight change (w_0/w_t) from onset of the experiment and days to maximum weight was obtained (t_{maxw}). These variables were considered to be normally distributed and were compared within each study site (categorical or fixed variable, depending on the analysis) or for all sites pooled. Analyses were conducted separately per pesticide and study site. In the latter analyses, dosage and weighting dates were categorical variables. Contrasts were estimated and tested against the control for each pesticide at each study site.

ANOVAs and mixed model analyses were done using the PROC GLM and PROC MIXED procedures in SAS.

Presence/absence of egg cell and honey pots (binary variables), number of queens (categorical variable) and number of larval cells, adult bees and weight of bees (continuous variables) in hives of different treatments (3 pesticides and 5 dosages including control) were analysed by chi-square tests and ANOVAs. The continuous variables were square root transformed to meet the assumptions of normality. Pairwise contrasts between different dosages of each pesticide and the control treatment with no pesticide were compared at each study site.

5.3 Results of field test

In the following, the effects of the three pesticides on relative bumble-bee hive weight and activity are shown. Results for absolute weight change are not presented, since they are almost identical to those for relative weight. As the initial hive weights are very similar, relative growth is also a good measure of growth rate. All tables with the results of the statistical analyses are presented in Appendix B.

5.3.1 Effects on hive weight

In general, hive weights increased initially, reaching a peak followed by a period with decreasing weights, ending at weights below the initial weight. At Moesgaard, the field trial was terminated after seven weeks (one week earlier than planned) because 18 of the hives had been destroyed, possibly by badgers. At Funder, seven hives were destroyed during the last two weeks of the experiment. Weights of the destroyed hives are included in the data set until the date the hives were destroyed.

In addition to the destroyed hives, a few hives did not develop. However, the majority of hives gained weight and reached a peak hive weight around 4-6 weeks into the experiment. After the peak, hive weight decreased steadily (Figures 43-45). At the end of the experiment, most hives were still alive, but at the end of colony life. Although inter-hive variation was low initially, variation among hives increased during the experiment in all treatments (different pesticides and dosages) and at all study sites.

Two-way ANOVAs of the effects of dosage, date and dosage*date interaction for each pesticide at each study sites showed no significant interaction effects, except for the case of Fastac at Moesgaard ($F = 2.05$, $P = 0.0248$). Hence, models without interaction effects were used for all but the latter case. Date had a significant effect for all pesticides at all study sites, while dosage had a significant effect for almost all pesticides and sites (only marginally significant for Biscaya at Them and Funder) (Table 76).

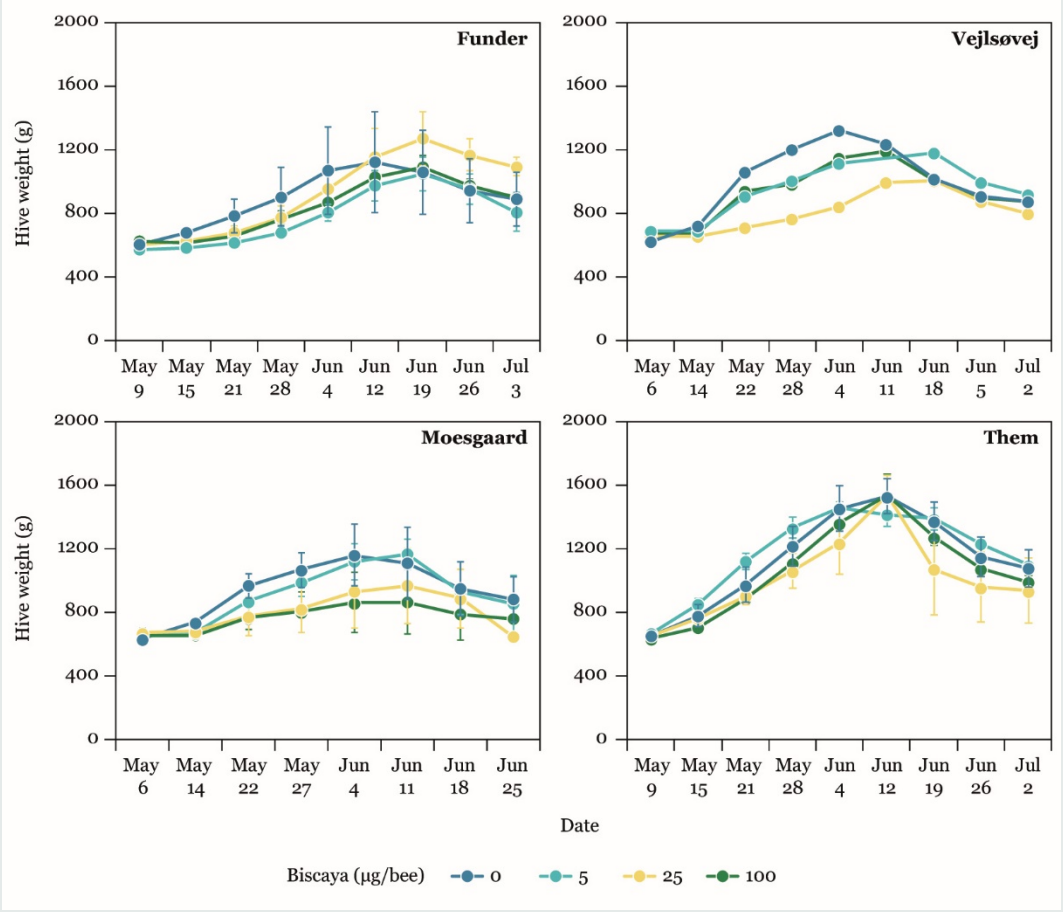

FIGURE 43. Average hive weights during the experimental period at the four field localities upon exposure to different Biscaya dosages. Points represent means +/-SEM. Hives at Moesgaard (three per dosage), Funder (three per dosage) and Vejlsøvej (one per dosage) were exposed for 14 d, while the hives at Them (three per dosage) were only exposed for 24 h

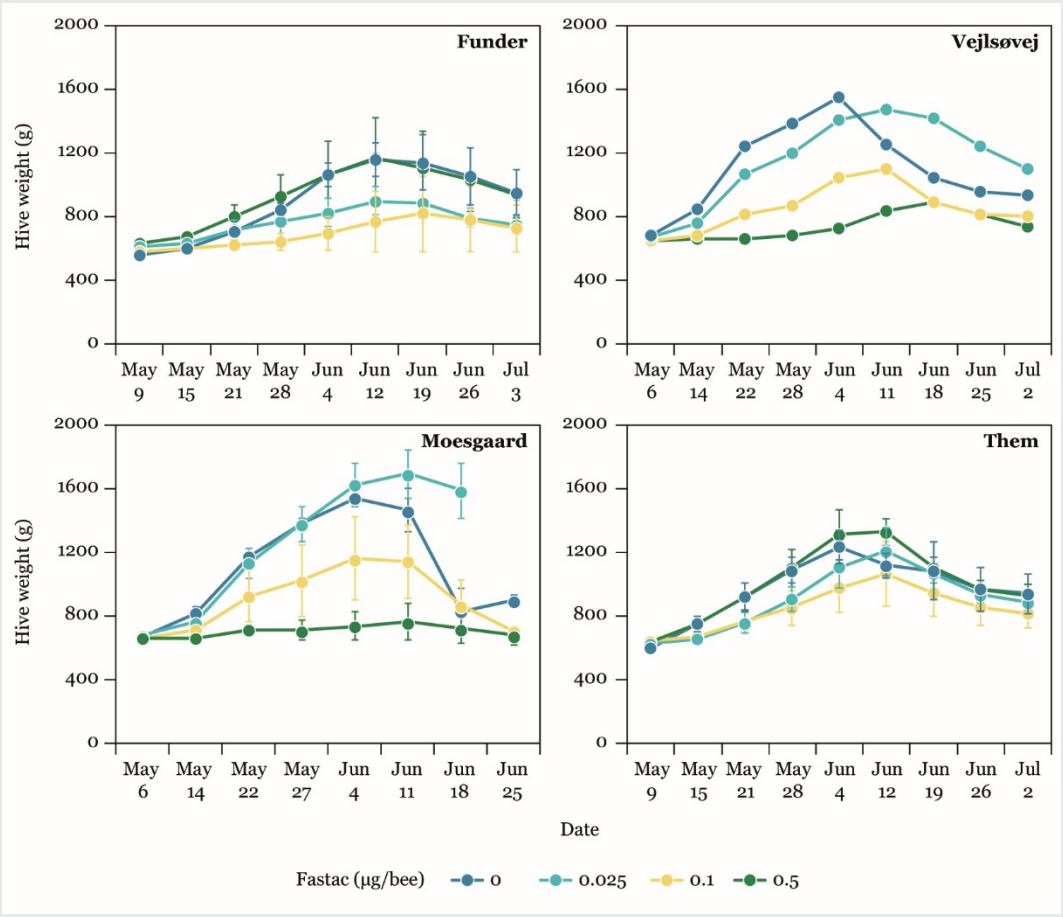

FIGURE 44. Average hive weights during the experimental period at the four field localities upon 24 h of exposure to different Fastac dosages. Points represent means +/-SEM of three hives per dosage for Funder, Moesgaard and Them, while at Vejlsøvej there was only one hive per dosage

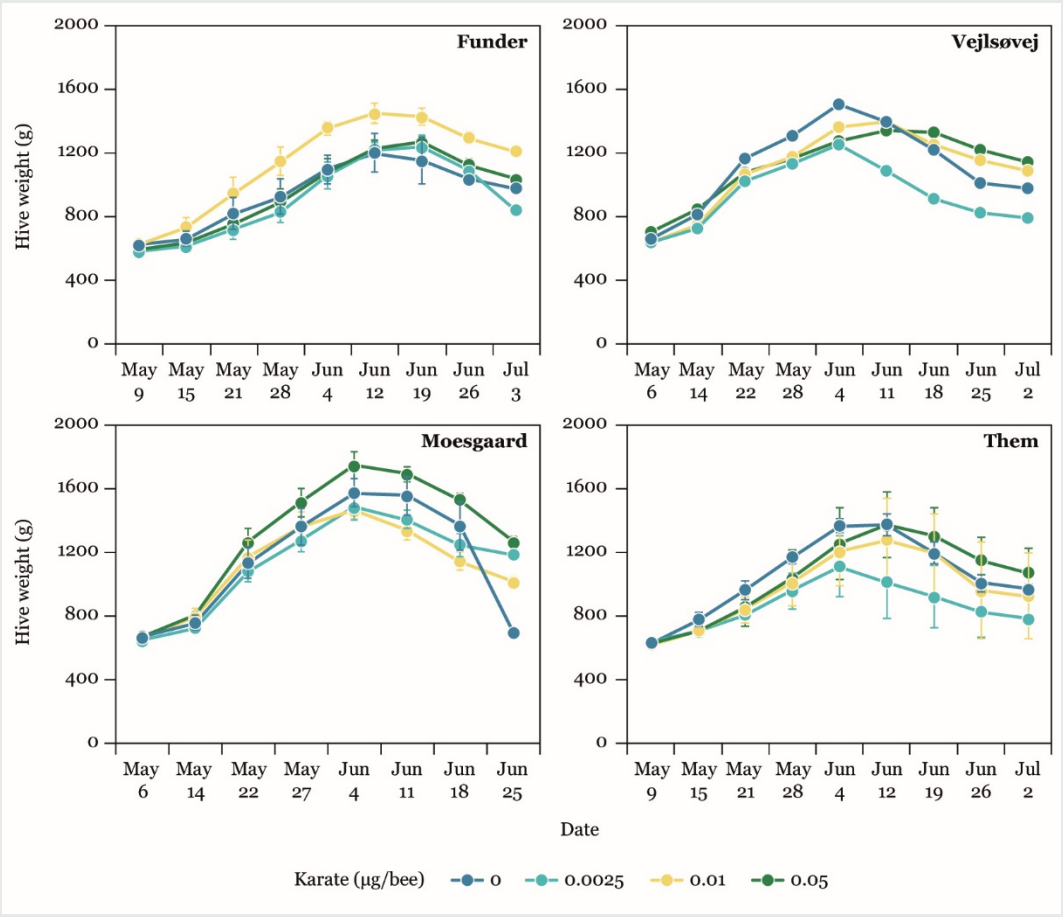

FIGURE 45. Average hive weights during the experimental period at the four field localities upon 24 h of exposure to different Karate dosages. Points represent means +/-SEM of three hives per dosage for Funder, Moesgaard and Them, while at Vejlsøvej there was only one hive per dosage

In most cases, the comparison of pesticide treatment with control hives (contrasts) showed no significant difference or significantly higher relative weights of control hives. However, in two cases (both for Karate) pesticide treated hives were significantly larger (Table 77).

When study sites were treated as a random effect and the pooled data set for all study sites were analysed, study site did not have a significant effect ($P>0.05$ for all three pesticides). However, both dosage and date were significant (Table 78). Comparisons of pesticide treatments against controls showed that relative hive weights of pesticide-treated hives were significantly lower than untreated control hives, except in the case of Karate (Table 79). For Biscaya, weight declined more at increasing pesticide dosages, while for Fastac weight decline was almost equal for the two highest dosages and a smaller weight change for the lowest dosage of pesticide. For Karate, weight change did not differ significantly from the control at the two highest dosages, while a small decline was detected for the lowest dosage (Table 79).

Because hives that initially had a low growth rate tended to peak in size later than fast-growing hives, it was suspected that pesticide-treated hives, in particular those at high dosages, were delayed in their development. However, ANOVA analysis of time until maximum hive weight was obtained was not significantly different among the different dosages for any of the pesticides at all study sites (Table 80).

As shown in Table 81, there was an overall significant effect of Biscaya in the first week after exposure when all sites were analysed together. The effect of Fastac persisted for half the study period, whereas Karate did not cause significant effects on hive weight.

5.3.2 Pesticide effects on bumble-bee activity

RFID recording was implemented at the field sites at Funder and Vejlsøvej. In hives covering the range of dosages for the three pesticides (one hive per dosage), five bees per hive were equipped with RFID tags. An example of a single bee record during one day is shown in Figure 46. It is clear that the bee has two repeated types of "activities", one lasting app. 40 min, and one lasting 5½ min. An educated guess would be that the two types of activity represent out-time and home-time, respectively. The home-time must be the short time value, as is unrealistic to assume that the bee only spends a few minutes outside the hive for foraging. The fact that a short interval always is recorded following a long interval shows a cycle of going out to forage and home to deliver what has been collected.

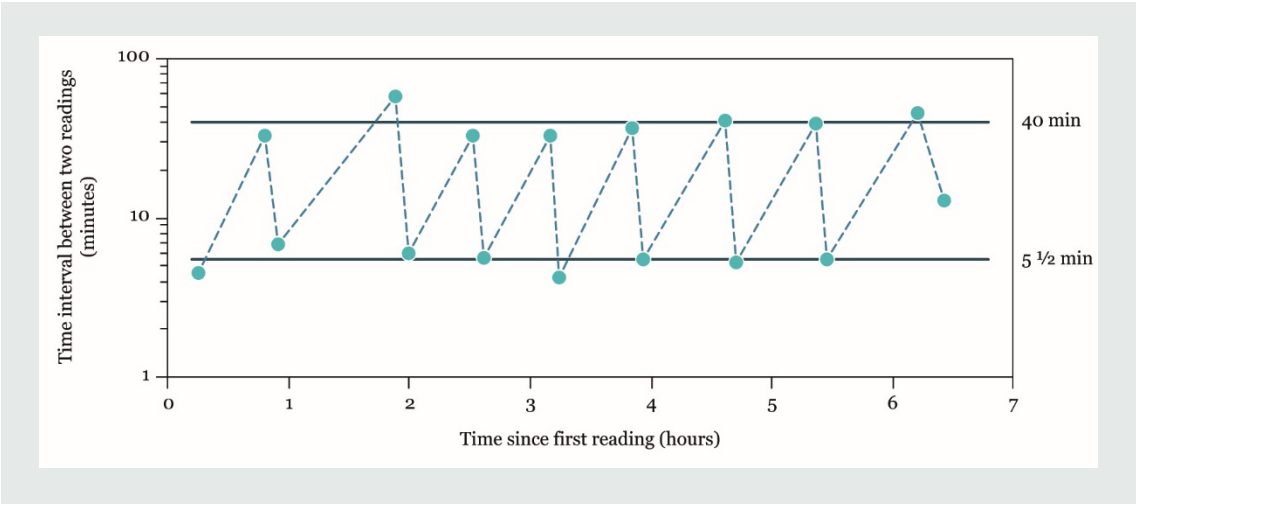

FIGURE 46. Time series of intervals between two time records for a single bumble-bee. The time intervals 5½ and 40 minutes are the mean values of the larger and smaller intervals, respectively

The result of one day of records for Hive F9 (Karate control) is shown in Figure 47, where the black dots represent the same bee as shown in Figure 46, and the other colours represent other bees. In this multiple bee plot, there is still a clear picture of a short time-activity followed by a longer-lasting activity. Some individual differences in behaviour are seen, where the bee displayed as black dots is rather constant in the long time activity and is making shorter trips than the bee displayed as red dots. This may, for instance, reflect foraging on different resources.

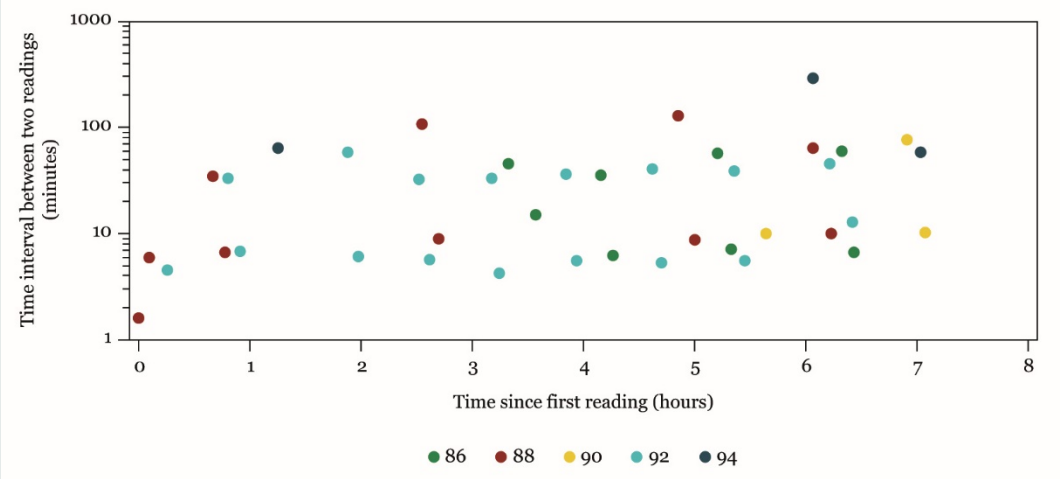

FIGURE 47. Time interval recordings for all five bees recorded in one hive (Karate control) in Funder having more than three readings. Each bee is represented by a specific colour

Some of the bees labelled with tags were not recorded by the reader or were just recorded a few times. There can be several reasons for these missing records. One possibility is that both the front and the rear tag are lost, or maybe the bee is not foraging during the recording period. All records from bees with more than 3 records are shown in Figure 48 for the Funder site. The recording period is two consecutive days, with one set of hives on day 1 and another set on day 2. Again, the bees display longer periods when they are out foraging and a shorter period in the hive.

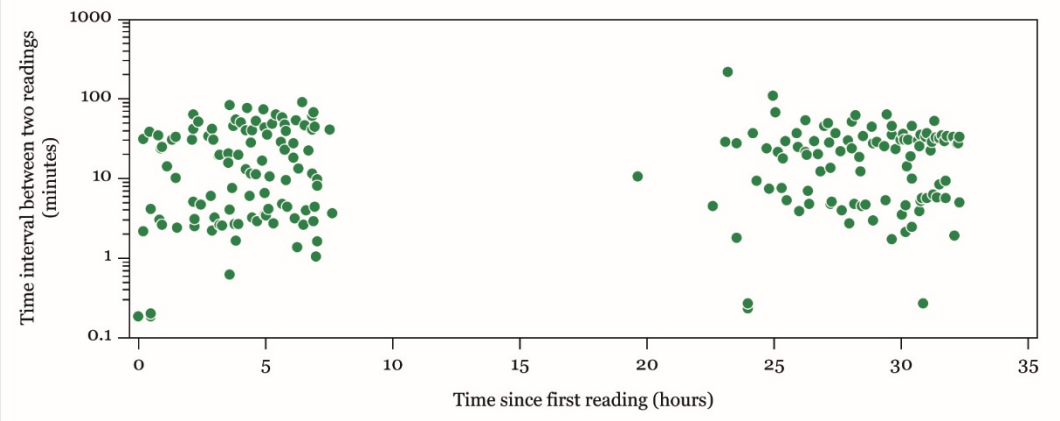

FIGURE 48. All RFID readings from bees at the Funder site with more than three time records. The two clouds of points represent two different sets of six hives each

The RFID records from the Vejlsøvej site (Figure 49) show the same picture as Funder.

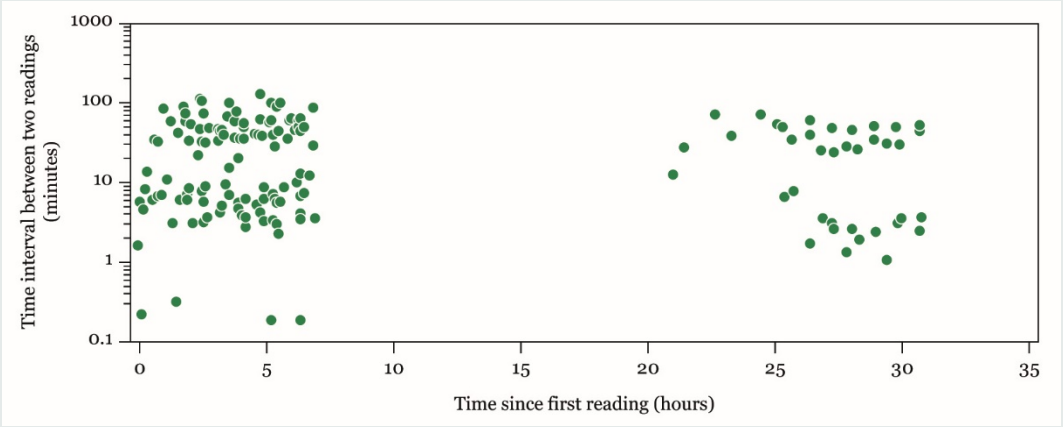

FIGURE 49. All RFID readings from bees in at the Vejlsøvej site with more than three time records. The two clouds of points represent two different sets of six hives

From this way of presenting the RFID data, it does not seem possible to identify a difference in pattern reflecting differences in pesticide dosage. This is illustrated in Figure 50, where the time interval recordings are labelled according to the pesticide and dosage, and there does not seem to be any pattern that reflects a causal relation to toxicity.

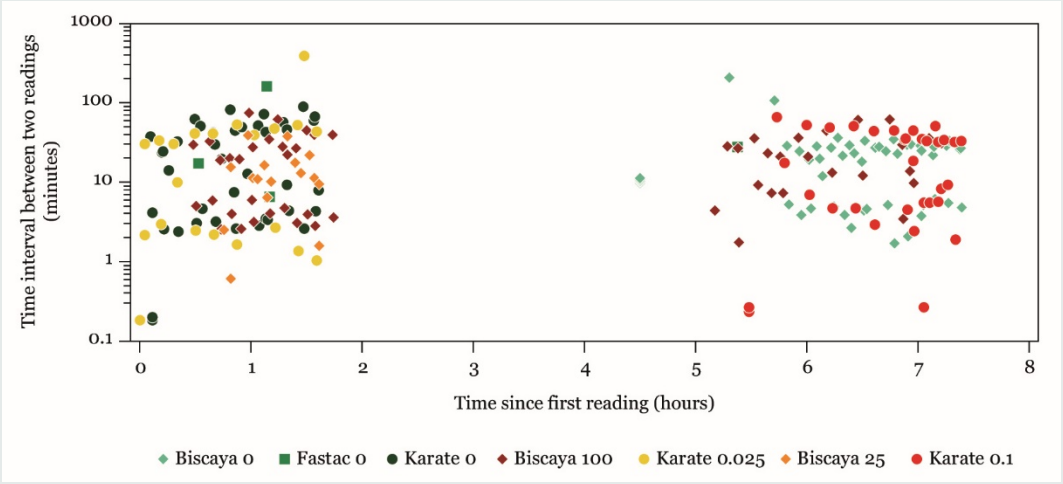

FIGURE 50. Time interval records for bumble-bee hives Funder. The labelling indicates the treatment of the hives of the specific bee

However, a simple measure may be the number of records for a hive, presuming that toxicity makes the bees less active and, thus, the total number of readings drops. The total number of records for bees exposed to different dosages of the insecticides Biscay, Fastac and Karate is shown in Table 24. Only data for the first round of RFID readings is shown. A large decrease is seen for the two highest dosages of Fastac, but not for other dosage levels and pesticides.

TABLE 24. Total number of RFID records for hives exposed to different dosages of Biscaya, Fastac and Karate. Data from both Vejlsøvej and Funder

Pesticide	Location	Dosage (µg a.i./bee)	Number of RFID readings	Days since pesticide exposure
Biscaya	Vejlsøvej	0	10	8
		5	51	9
		25	-	-
		100	47	8
	Funder	0	62	13
		5	16	13
		25	34	12
		100	76	12
Fastac	Vejlsøvej	0	14	9
		0.025	46	8
		0.1	0	8
		0.5	-	-
	Funder	0	6	12
		0.025	17	13
		0.1	0	13
		0.5	0	13
Karate	Vejlsøvej	0	22	9
		0.0025	40	9
		0.01	11	8
		0.05	26	8
	Funder	0	11	12
		0.0025	67	12
		0.01	48	13
		0.05	14	12

5.4 Discussion of results of field tests

Generally, there was a significant effect of the three pesticides on the growth of the bumblebee hives, but not consistently a clear dose-response relationship. This indicates that the three pesticides can potentially affect the development queen-right colonies, but, in some cases, the pesticide effect is low or the bees recover some time after pesticide exposure. There is, however, no indication of a delay in hive growth as a response to pesticide exposure. Since the hives at each site experienced identical surrounding conditions, it seems likely that the general internal conditions in the hive, e.g. the strength of the queen, has a large impact on the effect of pesticides.

The set-up used for field experiments in this project gives a good over-all indication of hive response to pesticide exposure. However, the set-up is vulnerable to predators such as badgers. This may be overcome by some kind of outer wire cage. Furthermore, we found it difficult to determine the timing of “harvesting” bees, larval cells etc. to give a more detailed description of hive fitness, even though we tried to time this “harvest” by evaluating hive development at the weekly weighings.

Because exposure time was longer in the field experiment than in the lab experiment, bees in the field trial may have consumed more sugar solution and, hence, higher pesticide dosages than the dosages calculated based on consumption during the lab experiments. On the other

hand, in the Biscaya treatments where bees were exposed for 14 days, bees were allowed to leave the hive and forage in a landscape with low pesticide load. Hence, these bees may have consumed slightly less of the pesticide-containing sugar solution in the hive. This is discussed further in Chapter 6.

The RFID readings obtained from some of the hives show the sampling cycle of the bees in the queen-right colonies. The cycles differ between bees, but apparently not as a response to pesticide exposure. The number of readings per hive seems to have some potential to reveal pesticide effects, since a dose-response relation was seen for Fastac. In order to establish a dose-response relation, more readers and more labelled bees would be required, and the latter depends on finding a more reliable way of gluing the tags onto the bumble-bees.

6. Pesticide analyses

6.1 Extraction and LC-MS/MS methods

A limited number of bees were analysed for pesticide content in order to get an idea of the actual exposure. From selected laboratory and semi-field experiments, extra bees were exposed to pesticide and then frozen immediately after exposure for later analysis of pesticide content.

To investigate the actual exposure of the bees in the field, we sampled bees from the highest and lowest concentration of each of the three pesticides in addition to control hives (with no pesticide). Each sample consisted of 3-10 bees, preferentially collected from different hives and different sites. Bees were caught at the exit hole of the hives immediately after pesticide exposure and after seven days. One sample was analysed for each pesticide, thiacloprid (Biscaya), α-cypermethrin (Fastac), and λ-cyhalothrin (Karate), at each pesticide dosage (control, lowest, and highest) and sampling time (0 or 7 days after exposure). Based on these results, a series of experiments were conducted evaluating the effects of pesticides and starvation of bumble-bees, as described in Section 6.2.

QuEChERS extraction of bumble-bees. Three to ten lyophilised (freeze-dried) bumble-bees were combined and homogenised for 30 s at 1500 strokes using a SPEX Sample Prep (Metuchen, NJ) Geno/Grinder before extraction by the QuEChERS method. Bumble-bees to be used for recovery experiments were spiked with 2.5 ng (thiacloprid and α-cypermethrin, active ingredients of Biscaya and Fastac) or 12.5 ng (λ-cyhalothrin, active ingredient of Karate) (total for five pooled bees) prior to homogenisation. To each sample of bees was added 4.5 mL (6.75 mL for samples over 0.8 g) extraction solvent (44:55:1 water/acetonitrile/acetic acid) before vortexing for 1 min. 1 g (1.5 g for samples over 0.8 g) magnesium sulphate and 0.25 g (0.375 g for samples over 0.8 g) sodium acetate were subsequently added and the sample vortexed again for 1 min. Samples were then transferred to 15 mL Falcon tubes and centrifuged at 4300 x *g* for 10 min. 1 mL of the supernatant was transferred to a 2 mL dispersive SPE tube for fatty samples (Agilent, Santa Clara, CA) and vortexed for 1 min. The tubes were centrifuged at 1850 x *g* for 2 min and the supernatant decanted into a vial for LC-MS analysis. Unfortunately, a centrifuge tube shattered during centrifugation causing the loss of one of the samples.

LC-MS analysis of bumble-bee extracts. LC-MS analysis of bumble-bee extracts was carried out on an LC-MS system consisting of an Agilent 1260 HPLC and a Sciex (Framingham, MA) 3200 QTrap mass spectrometer. A ThermoFisher (Waltham, MA) BDS Hypersil C18 column of length 250 mm, i.d. 2.1 mm, and particle size 5 µm was used for chromatographic separation. Compounds were detected using positive-mode electrospray ionisation and multiple reaction monitoring. Two separate methods were used for analysis of i) thiacloprid (Biscaya), and ii) α-cypermethrin (Fastac) and λ-cyhalothrin (Karate), respectively. A binary mixture of solvents was used for gradient elution. Solvent A consisted of 1% methanol in water, while solvent B consisted of 90% methanol and 10% water. Both solvents contained 10 mM ammonium acetate.

For the analysis of thiacloprid (Biscaya), the gradient was as follows: 0 min, 10% B, ramping to 100% B at 4 min before holding at 100% B until 20 min and returning to 10% B at 21 min and holding until 26 min. The injection volume was 2 µL, the flow rate was 200 µL/min, and the column temperature was maintained at 20°C and the following source-dependent MS parameters were used: curtain gas, 20 psi; CAD gas, medium; ion spray voltage, 4500 V; tempera-

ture, 475°C; gas 1, 50 psi; gas 2, 50 psi; interface heater, on. Compound-dependent parameters specific to thiacloprid were: Q1 m/z (parent ion), 253; Q3 m/z (fragment ion), 126; declustering potential, 56 V; entrance potential, 7.5 V; collision cell entrance potential, 18 V; and collision energy 27 V.

For the analysis of α-cypermethrin and λ-cyhalothrin, the gradient was as follows: 0 min, 10% B, ramping to 100% B at 2 min and holding until 17 min before returning to 10% B at 18 min and holding until 23 min. The injection volume was 25 µL, the flow rate was 200 µL/min, and the column temperature was maintained at 25°C. The following source-dependent MS parameters were used: curtain gas, 20 psi; CAD gas, medium; ion spray voltage, 4500 V; temperature, 475°C; gas 1, 50; gas 2, 50; interface heater, on. Compound-dependent parameters for α-cypermethrin were: Q1 m/z (parent ion), 416; Q3 m/z (fragment ion), 191; declustering potential, 56 V; entrance potential, 10 V; collision cell entrance potential, 20 V; and collision energy, 21 V. Compound-dependent parameters for λ-cyhalothrin were: Q1 m/z (parent ion), 467; Q3 m/z (fragment ion), 225; declustering potential, 20 V; entrance potential, 6 V; collision cell entrance potential, 40 V; and collision energy, 25 V.

Calibration curves were recorded in the range 0.6-625 ng/mL for thiacloprid, 0.5-500 ng/mL for α-cypermethrin and 0.2-50 ng/mL for λ-cyhalothrin and used to quantify the pesticides in the bumble-bee extracts.

6.2 Pesticide content of bumble-bees

For each type of experiment (laboratory, semi-field, and field) and for each pesticide (α-cypermethrin (Fastac), λ-cyhalothrin (Karate), and thiacloprid (Biscaya)), there was a rough correlation between the dosage of the pesticide applied in the experiment and the amount of pesticide measured in the bees.

Preliminary quantification was carried out on bees exposed to all three pesticides and, generally, higher dosages and shorter waiting periods before sampling resulted in higher measured concentrations of the pesticides (Table 25). The applied dosage of λ-cyhalothrin (Karate) was quite low, resulting in measured concentrations near the limit of detection, and this should be kept in mind when evaluating the results. For α-cypermethrin (Fastac) and thiacloprid (Biscaya), measured concentrations ranged from 0 to 11.5 µg/g. The highest concentrations, found in both cases immediately after exposure to the high dosage, were remarkably similar (10.8 µg/g for α-cypermethrin (Fastac) and 11.5 µg/g for thiacloprid (Biscaya)). Delaying sampling for 1 wk produced a concentration of α-cypermethrin (Fastac) lower by 98%. Unfortunately, the same measurement could not be made for thiacloprid (Biscaya), as the sample was lost during centrifugation. Exposure to the low dosage of pesticides produced values differing by a factor of 4 (0.5 µg/g for α-cypermethrin (Fastac) and 22 µg/g for thiacloprid (Biscaya)). Again, delaying sampling for 1 wk produced a large decrease, with almost no α-cypermethrin (Fastac) detectable and only 0.3% of the original amount of thiacloprid (Biscaya). It thus appeared from the initial quantification that uptake depended strongly on the applied concentration and that metabolism of the pesticide was complete within 1 wk. This was also consistent with the numbers recorded for λ-cyhalothrin (Karate), although the low quantities detected made that conclusion more uncertain.

TABLE 25. Preliminary quantification of pesticides in bumble-bees from field experiments. All bees had been exposed orally to pesticide for 24 h. The number of bees pooled for analysis varied between 4 and 10 per sample

Pesticide	Dosage (µg a.i./bee)	Sampling time (d after exposure)	Concentration (µg/g DW*)	Concentration (µg/bee)
Thiacloprid (Biscaya)	control	0	0.0004	0.00004
	5	0	2.2278	0.23700
		7	0.0086	0.00067
	100	0	11.5411	1.19543
		7	**	**
α-cypermethrin (Fastac)	control	0	0.0000	0.00000
	0.025	0	0.5305	0.05365
		7	0.0001	0.00002
	0.5	0	10.7879	0.96525
		7	0.2038	0.01370
λ-cyhalothrin (Karate)	control	0	0.0001	0.00001
	0.0025	0	0.0001	0.00001
		7	0.0002	0.00002
	0.05	0	0.0041	0.00035
		7	0.0001	0.00001

*DW = dry weight. **The sample was lost during centrifugation and could therefore not be quantified

Thiacloprid (Biscaya) was quantified in field experiments after application in nominal dosages of 5-100 µg a.i./bee, resulting in measured concentrations of 10-900 ng/g dry weight in the bees (see Table 26). The intermediate dosage of 25 µg a.i./bee produced the largest amount found in the bees relative to the amount applied, suggesting that the uptake became limited at higher dosages, perhaps because the bees avoid excessively pesticide-contaminated feed if they are able to do so.

TABLE 26. Quantification of thiacloprid (Biscaya) in bees from field experiments. Three bees were pooled per sample, and there was one sample per dosage

Dosage (µg a.i./bee)	Concentration (µg/g DW*)	Concentration (µg/bee)
0	0.0000	0.00000
5	0.0115	0.00088
25	0.7216	0.04727
100	0.8982	0.05470

*DW = dry weight

Thiacloprid (Biscaya) and α-cypermethrin (Fastac) were quantified in bumble-bees subjected to starvation in laboratory experiments. Higher dosages of pesticide applied produced higher concentrations in the bees, but the level of starvation also appeared to have an effect. At the low dosage of thiacloprid (Biscaya), the highest starvation level produced an elevated concentration in the bees, whereas the lower starvation level and the control were very similar. At the high dosage, the situation was reversed, as the concentration of thiacloprid (Biscaya) measured in the unstarved bees was approximately twice that measured at the other starvation levels. For α-cypermethrin (Fastac) the results were more consistent. At both the higher and lower applied dosages, the unstarved bees contained an intermediate level of pesticide. The lower starvation level resulted in a lower concentration of pesticide, while the higher starvation

level at both dosages resulted in a higher measured concentration of pesticide than either the control or the lower starvation level. The observed trends were true whether the data was reported per unit of dry mass of bees or per number of bees (see Table 27), which was always all five bees from a single micro-colony, although the weight varied considerably from micro-colony to micro-colony.

Thiacloprid (Biscaya), α-cypermethrin (Fastac) and λ-cyhalothrin (Karate) were all quantified in semi-field experiments where bees were exposed to either a low or a high dosage of each pesticide (see Table 28). As expected, a higher concentration of pesticide was measured in the bees exposed to higher dosages of pesticide through their feed, although the low dosage of λ-cyhalothrin (Karate) did not produce a measurable concentration in the bees. The concentration increase in the bees was not, however, equivalent to the increase in the feed. For thiacloprid (Biscaya), increasing the dosage by a factor of 25 only increased the concentration in the bees by a factor of nine, whereas for α-cypermethrin (Fastac), increasing the dosage by a factor of 20 increased the concentration found in the bees by a factor of nearly 30.

TABLE 27. Quantification of thiacloprid (Biscaya) and α-cypermethrin (Fastac) in laboratory starvation experiments. Five bees were pooled per sample and there was one sample per dosage

Pesticide	Dosage (µg/bee)	Starvation level	Concentration (µg/g DW*)	Concentration (µg/bee)
Thiacloprid (Biscaya)	20	0	52.5904	7.703
	20	2	41.1596	4.696
	20	3	118.2253	9.801
	160	0	1304.1450	95.411
	160	2	674.6074	57.989
	160	3	574.4841	43.845
α-cypermethrin (Fastac)	0.2	0	0.1969	0.0151
	0.2	2	0.1157	0.0062
	0.2	3	0.2281	0.0198
	2	0	18.9844	1.378
	2	2	8.1641	0.750
	2	3	139.2838	5.959

*DW = dry weight

TABLE 28. Quantification of thiacloprid (Biscaya), α-cypermethrin (Fastac), and λ-cyhalothrin (Karate) in semi-field experiments. Five bees were pooled per sample, and there was one sample per dosage

Pesticide	Dosage (µg/bee)	Concentration (µg/ DW*)	Concentration (µg/bee)
Thiacloprid (Biscaya)	2	1.0977	0.0869
	50	9.2286	1.6261
α-cypermethrin (Fastac)	0.025	0.0226	0.0030
	0.5	0.6679	0.0661
λ-cyhalothrin (Karate)	0.001	0.0000	0.00000
	0.2	0.4041	0.0332

*DW = dry weight

λ-cyhalothrin (Karate) was quantified in bumble-bees periodically starved in semi-field experiments (see Table 29). Two dosages were applied, and both dosages produced measurable amounts of pesticide in the bumble-bees. The applied dosages differed by a factor of 10, and the measured concentrations of pesticide differed by approximately a factor of 7, with the bumble-bees that were periodically starved containing about two thirds as much pesticide as those that were not, regardless of which dosage of pesticide was applied.

TABLE 29. Quantification of λ-cyhalothrin (Karate) in semi-field starvation experiments. Five bees were pooled per sample and there was one sample per dosage

Starved	Dosage (µg a.i./bee)	Concentration (µg/g DW*)	Concentration (µg/bee)
Yes	0.01	0.0167	0.0008
No	0.01	0.0241	0.0029
Yes	0.1	0.1131	0.0123
No	0.1	0.1790	0.0164

*DW = dry weight

6.3 Discussion - Pesticide dosage, extraction, and analysis

In the preliminary quantification of pesticides, it was observed that the concentration found in the bumble-bees themselves was related to the dosage to which the bees had been exposed. In the case of Biscaya, application of 5 µg a.i./bee resulted in detection of approximately 5% of that amount in the bees, while only around 1% was detected after application of 100 µg/bee. Similarly, for Karate less than 1% of the applied pesticide was detected in the bees. In the case of Fastac, dosages of 0.025 and 0.5 µg a.i./bee were applied, and in both cases approximately double the amount of the applied pesticide was found. Taken together, these results suggest that the consumption of pesticide-containing sugar solution by the bees was highly variable and pesticide-dependent, as was the absorption and metabolisation. The relationship between nominal and actual exposure is thus difficult to establish, potentially affected by variation in the actual exposure due to varying consumption of sugar solution by the bees themselves and by difficulty in measuring the actual exposure due to metabolisation of the pesticide by the bees during the exposure period.

It should be noted that regardless of these difficulties, the expected qualitative relationships between nominal and measured actual exposure held. For all three pesticides, the higher of the two nominal exposures produced the higher measurement of the actual exposure when the bees were sampled immediately following exposure. Furthermore, for all three pesticides delaying sampling by one week following exposure resulted in a lower measured actual exposure, with the exception of the lower dosage of λ-cyhalothrin (Karate), where the concentrations were near the limit of detection

After the preliminary determination of concentrations in bumble-bees, the average recovery and its uncertainty were determined before quantification of pesticides in bumble-bees exposed to pesticides in field and semi-field experiments as well as laboratory starvation experiments. Because the detection limit of λ-cyhalothrin (Karate) was higher than for the other pesticides, 12.5 ng was applied. Recovery was found to range from 57-103% (see Table 30), which was deemed acceptable, but the concentrations reported for the quantification experiments were not corrected for recovery. The entirety of thiacloprid (Biscaya) spiked to samples of 5 bees was recovered with very little variability, and the majority of α-cypermethrin (Fastac) and λ-cyhalothrin (Karate) was recovered, although with somewhat higher variability.

TABLE 30. Determination of pesticide recovery during analysis

Pesticide	Amount spiked (µg/bee)	Amount measured (µg/bee)	Recovery (%)	RSD (%)
Thiacloprid	0.0005	0.00051	103	4
α-cypermethrin F	0.0005	0.00025	57	16
λ-cyhalothrin	0.0025	0.00174	62	21

Following the preliminary quantification and the recovery estimation, four different experiments were carried out that involved quantification of pesticides to which the bumble-bees were exposed. The first was a field experiment in which bumble-bees were exposed to thiacloprid (Biscaya) at four dosages (see Table 28). The second was a laboratory starvation experiment in which bumble-bees were exposed to either thiacloprid (Biscaya) or α-cypermethrin (Fastac) at one of two dosages while suffering one of three starvation levels (see Table 29). The third was a semi-field experiment where bumble-bees were exposed to either thiacloprid (Biscaya), α-cypermethrin (Fastac), or λ–cyhalothrin (Karate) at one of two dosages (see Table 30), and the fourth was a semi-field starvation experiment where bumble-bees were exposed to λ–cyhalothrin (Karate). Across the total battery of experiments performed, the relation between the nominal exposure and the measured actual exposure was reasonably consistent. For thiacloprid (Biscaya), no more than 5% of the nominal dosage was measured in any of the field or semi-field experiments, while this increased to 25-50% in the only lab experiment conducted, indicating increased pesticide exposure due to restricted food choices. For α-cypermethrin (Fastac), the relation was more variable, with the expected exposure being sometimes exceeded by a factor of 2 or 3 in field and lab experiments, while just under 15% of the nominal exposure was measured in the semifield experiment. This suggested a potential undesired source of α-cypermethrin (Fastac) and highly variable feeding behaviour of bumble-bees at high dosages in laboratory conditions. For λ–cyhalothrin (Karate), across the field and semi-field experiments performed 8-33% of the nominal exposure was measured, except at very low dosages where less or none at all was measured, with no laboratory experiment being available for comparison.

The limits of detection for the pesticides were estimated at 12.5 ng / 0.5 g lyophilised bee material for λ-cyhalothrin (Karate) and 2.5 ng / 0.5 g lyophilised bee material for thiacloprid (Biscaya) and α-cypermethrin (Fastac) as the lowest concentration at which a signal could be reliably seen by the applied LC-MS method after spiking bee material with each pesticide. The recovery of each pesticide was estimated at this concentration (see Table 27), and although a higher recovery for α-cypermethrin (Fastac) and λ-cyhalothrin (Karate) would have been desirable, this could not be achieved, and the recoveries of 57% and 62%, respectively, were deemed sufficient for the present work. Full recovery of thiacloprid (Biscaya) was achieved, at 103%.

λ-cyhalothrin (Karate) was the only pesticide that presented a challenge to the method, in that the quantification results were at times near the detection limit, but all results are included for the sake of completeness. The detection limits of either 12.5 ng or 2.5 ng / 0.5 g lyophilised bee material corresponded to approximately 2.5 ng / bee for λ-cyhalothrin (Karate) and 0.5 ng / bee for thiacloprid (Biscaya) and α-cypermethrin (Fastac)and should be kept in mind when interpreting the quantification results.

7. Check for pathogens in test bumble-bees

7.1 Methods

Throughout the study, live bees taken from the main colonies or from among those surviving control treatments were killed by freezing briefly and then examined for presence of bumble-bee pathogens. For each bee, haemolymph as well as tissue from the midgut, the Malpighian tubules and fat body was mounted in a drop of 0.9% NaCl on a glass slide and checked in a compound microscope (400x magnification with phase-contrast and under direct light) for approximately 3 minutes to detect Nosema sp[1]., Crithidia sp[1]. and Apicystis bombi.

7.2 Presence of pathogens in test bees

A total of 109 live *B. terrestris* (workers (N=105) and queens (N=4)) sampled from production hives and experimental units throughout the duration of the project were screened for the presence of *Nosema* sp. and other pathogens. Three of the four queens contained pathogens (*Nosema* sp. (n=1), *Crithidia* sp. (n=1), and a mixed infection (n=1) and 68.5% (n=72) of the workers harboured pathogens (44.8% *Nosema* sp. (n=47)), 18.1% *Crithidia* sp. (n=19) and 5.7% contained both species (n6). The pathogen load was not quantified, but ranged from very few to massive numbers of spores and cells. *Apicystis bombi* was not found in any of the bees.

7.3 Discussion of the presence of pathogens

The high prevalence of bumble-bee pathogens found in test bees throughout the study period was unexpected, but is in accordance with Graystock et al. (2013), who screened commercially produced bumble-bee colonies that had been imported on the basis of being disease-free and found *Nosema bombi*, *Crithidia bombi* and *Apicystis bombi* to be prevalent. Rearings of insects generally are at risk of developing disease, and it is not realistic that pathogen-free bumble-bee colonies could have been obtained and maintained for the tests. High proportions of the test bees were infected by one or more pathogen and this could potentially affect their susceptibility to insecticides and to infection by *B. bassiana* (and to starvation), as they constitute another stressor. The pathogens differ in mode of infection and could influence the response of the bees differently. In any case, data obtained should be interpreted cautiously, as there may be further stressors involved, although their presence and potential effects on test insects remain poorly documented.

[1] Recently, several species of Nosema and Crithidia have been recorded from bumble bees (Piiroinen & Goulson 2016; Schmid-Hempel & Tognazzo 2010) and diagnosis thus cannot be made solely based on morphology.

8. General discussion

In this chapter, general aspects of the project are discussed, both concerning the applied methods and concerning the implications of the obtained results.

8.1 Effects of insecticides on consumption and, consequently, exposure

Both in the laboratory experiments and in the semi-field tests, reduced consumption of pesticide-containing sugar solution were observed in several cases - especially for Biscaya, but also, to some degree, for Karate and Fastac. For Biscaya, which contains the neonicotinoid substance thiacloprid, consumption during the six-hour exposure period was found to decrease at increasing pesticide dosages in some, but not all, cases. Hence, it is not clear if the effect on consumptions is due to a toxic effect, as would be indicated by a clear dose-response relation, or whether the pesticide may be repellent to the bumble-bees, which would be strongly indicated if all sub-lethal Biscaya dosages had the same effect on consumption. Kessler et al. (2015) found that both *B. terrestris* and honeybees prefer sugar solutions containing two other neonicotinoid pesticides, imidacloprid and thiamethoxam, whereas they found no preference for a third neonicotinoid, clothianidin. In addition, Kessler et al. found no stimulation of bees' gustatory neurons when exposed to the three neonicotinoids and concluded that bees cannot taste and control their intake of neonicotinoids. Despite this, they showed that overall food consumption was negatively affected by the two neonicotinoid pesticides. A Norwegian field study of Biscaya applied to red clover shortly before flowering showed that this pesticide had no repellent effect on honeybees and bumble-bees (Havstad et al. 2015).

The effect of Fastac and Karate on consumption is also unclear. In the laboratory tests, only high dosages tended to cause decreased consumption, whereas in the semi-field tests consumption by Fastac-treated bees was generally lower than in the controls and Karate only affected consumption significantly in some of the test runs. Some pyrethroid insecticides are known to be repellent to insects, including the pyrethroids lambda-cyhalothrin (the active ingredient of Karate) and alpha-cypermethrin (a.i. of Fastac) (e.g. Rasmussen et al. 2013; Thompson & Folkard-Ward 2001). However, a field study of several pesticides, including lamda-cyhalothrin, showed little or no repellent effects on honeybees (Fagúndez et al. 2016). Similarly, no repellent effect of alpha-cypermethrin was found for honeybees foraging in oilseed rape under field conditions (Karise et al. 2007).

In relation to test set-up, it may be recommended to take the possibility of effects on consumption and, thereby, on actual exposure into consideration, also when choosing between oral and topical exposure. As described in Chapter 6, part of the variation seen in the actual (measured) exposure with Biscaya may be explained by differences in consumption, which may affect test results if not taken into account. Exposure of queen-less micro-colonies of *B. terrestris* workers to a range of dosages of dietary imidacloprid showed a dosage-dependent decline in fecundity at environmentally realistic dosages in the range of 1μgL^{-1} (Laycock et al. 2012). At these dosages, brood production was reduced by one third. Imidacloprid reduced feeding on both syrup and pollen, and the authors argued that the detrimental effects of imidacloprid emerged principally from nutrient limitation imposed by the failure to feed.

8.2 Interactions between pesticides and pathogenic fungus

Effects of the entomopathogenic fungus *B. bassiana* were found notably on adult survival in laboratory tests, but we did not find a general, clear-cut trend that the pathogen stressor increased bumble-bees sensitivity to the tested insecticides. Significant interactive effects of

pesticides and fungus were found for adult survival in a few short-term laboratory tests, long-term laboratory tests and semi-field tests. However, such effects were only demonstrated in single experimental runs and not for all three pesticides in the acute tests and the long-term laboratory tests, while interactions between pesticide and pathogen were found for both Biscaya, Fastac and Karate in the semi-field tests. When looking at sub-lethal endpoints, no interactions were found between any of the pesticides and the fungus for formation of honey pots, larval cells or egg cells in any of the tests done in long-term laboratory conditions or under semi-field conditions (with a single exception). In accordance, in the acute test and the long-term lab test there were either no effect of the fungus itself, or the effect was only found on one day, in one of the tests or the effect was transient and disappeared over time. On the other hand, even though the fungus treatment had a significant effect on the formation of honey pots and egg cells in the semi-field experiment with Fastac, there were still no interactive effects with the pesticide. The highly variable data generated in these experiments reflects the difficulty in working with interactions where one component is a pathogen.

The dosages of fungus were selected with the aim to cause low mortality while at the same time causing psysiological stress to the surviving bees by activating the immune defence. Data on sporulating cadavers from the tests (data not shown) demonstrates that the fungus was indeed active and killed low numbers of bumble bees in all experimental runs. Under semi-field conditions, *Bombus terrestris* appears to be more susceptible to pesticide exposure when already exposed to a fungal pathogen, as documented by interactive effects on adult survival when combining pesticide exposure with the pathogen fungus. Apart from the effects caused by fungus and pesticide, the less controlled conditions may also cause extra stress on the bumble-bees.

In the literature, the highly cited pioneer paper by Alaux et al. (2010) is the first example of synergy between a honey bee pathogen and the neonicotinoid imidacloprid. However, the effects described in the paper are additive rather than synergistic, as pointed out by Thompson (2012). Likewise, the synergistic interaction between thiacloprid and *Nosema ceranae* reported by Vidau et al. (2011) seems not to be correct, as the amount of sugar water was not taken into account (as discussed in Thompson, 2012), whereas there is synergy between fipronil and the microsporidian fungus (Thompson, 2012). In any case, Retschnig et al. (2015) notice that interactions between pathogens and pesticides may have been overemphasized among researchers based on their finding no synergy between thiacloprid and *N. ceranae* in field studies with honey-bees. Furthermore, the evidence for synergistic interaction of *Crithidia* and bumble bees is rather limited at present, as only two published studies are found (Fauser-Mislin 2013; Baron 2014).

On the other hand, *B. bassiana* is frequently described as an entomopathogen that may interact synergistically with various pesticides (e.g., Furlong & Groden 2001; Farenhorst et al. 2010), and it cannot be ruled out that a different timing of the treatments or changing the sequence of the treatments would have influenced the outcome of our trials. Hence, in Furlong & Groden (2001), where *B. bassiana* and imidacloprid were combined against larvae of the Colorado potato beetle, three of four variations of sequence and timing of treatments caused synergistic interactions. The sequence proved to be of importance, while the timing - which differed by 24 h - did not. In addition, while Farenhorst et al. (2010) found synergistic interactions in all combinations of *B. bassiana* and permethrin tested against mosquitoes, the highest mortality level of the combined treatment was found when both fungus and pesticide was applied simultaneously, as compared to combinations where either fungus or pesticide were applied 2 days prior to the other treatment. This illustrates that small variation in experimental set-up can influence the outcome of these kinds of studies significantly.

Specifically concerning the timing of the treatment, pesticides may potentially influence the fungus inoculum negatively in pesticide-pathogen combination treatments, i.e. if the bumble-

bees were exposed to pesticide before fungus infection. However, in the acute test where pesticide was applied topically 6 days after topical application of fungus, the fungus would have germinated and eventually penetrated into the host several days before pesticides were applied to the dorsal thorax. In addition, fungus inoculum was applied to cover all surfaces of the bee, so in case the pesticide interacted with conidia deposited on the surface of the host, the interaction would be of limited importance for the outcome of the test. For the other tests where pesticides were applied via the sugar solution 6 days after topical application of the fungus, there should be no risk of negative interactions.

In order to study synergistic interactions, it is necessary to apply treatments causing relatively low mortality levels on their own. By choosing a relatively low dosage of fungus, there is a risk that a proportion of the bees are not sufficiently exposed to the fungus, as the aim is to cause stress to the bees by activating their defence reactions rather than killing them. The biology of the fungus allows for easy confirmation of infection in dead bees from bioassays, as visible fungus outgrowth is produced after surface disinfection, and this was used to verify that the fungus had killed a low proportion of the treated bees. No dead bees in other treatments produced fungus outgrowth. The defence systems of surviving bees are therefore expected to have been challenged by the treatment of fungus.

8.3 The influence of starvation - how to starve bumble-bees experimentally?

Despite a number of pilot studies prior to the first tests of pesticide effects, starvation proved difficult to establish in a way that caused small, but significant effects on survival, when tested alone. Hardly any sub-lethal effects of starvation were observed in the long-term tests, although several combinations of sugar and pollen starvation were tried, even when an interactive effect was seen on worker survival, as in the long-term laboratory tests with Biscaya and Karate. Of course, this does not mean that the two types of stressors do not interact under field conditions, but it points out the difficulty of elucidating such questions by experimental work and it may also indicate that sub-lethal effects do not occur at dosages that do not affect survival because the allocation of food to reproduction is prioritised.

We have not been able to find other studies that have tried to starve bumble-bee micro-colonies experimentally. We wanted to study whether this simple and easy-to-observe test design was also applicable to more complex tests, e.g. studies of interactive effects and semi-field tests. Our results indicate that if the minimum requirements of the bees are met, survival and reproduction will not be greatly affected within a 2 week test period. When starvation is established by reducing the access to pollen (described in Table 13), there is hardly any immediate effect on bumble-bee fitness in the 14 d laboratory tests. However, when it comes to the semi-field tests, where the same starvation procedure was used, interactive effects between pesticide dosage and starvation were seen on both mortality and, especially, on reproduction (no. eggs). This shows that starvation and pesticides may interact, but an experimental approach is difficult, at least when using small micro-colonies. It therefore seems tempting to conclude that studies involving starvation are better done with the larger, queen-right colonies. Although full colonies are more costly and difficult to observe, their size and colony composition of bees having different sizes and tasks not only increase the realism of the outcome of the experiments – they also offer a more robust system. Furthermore, the fact that they persist for a longer period also means that it is possible to starve the colony for longer periods. A recent project by Couvillon & Dornhaus (2010) not only presents an applicable set-up, but also reveals the interesting result that small bumble-bee workers (of the species *Bombus impatiens*) are hardier against starvation than larger specimens, which emphasizes the importance of including workers of different size in such experiments.

Another aspect of starvation that was revealed by the pesticide analyses in Chapter 6 is that starved bees may be exposed differently to a given pesticide dosage than unstarved bees. In

several cases, starved bees contained higher levels of pesticide, which may indicate that these bees ate a higher proportion of the ingested sugar solution than the well-fed bees, which may have chosen e.g. to regurgitate some of the pesticide-contaminated sugar solution into the honey pots.

8.4 RFID techniques for measuring activity

RFID measurements were included in the study in order to find out whether this technique is applicable as a standardised and less time-consuming method for assessing bumble-bee activity under semi-field and field conditions. Our results demonstrate that bumble-bee activity in micro-colonies under semi-field conditions seems to follow another “rhythm” than queen-right colonies in the field. In the field, RFID records documented that during daytime the bumble-bees generally used short periods in the hive and longer periods in the field collecting food. In contrast to this, there does not seem to be a clear division between a short and long time interval in the semi-field set-up. A possible explanation of this difference may be that the micro-colonies lack the steering mediated by the queen pheromones in the field. However, the semi-field set-up also differs in other respects that may affect worker behaviour, e.g. the availability of real flowers or a group of workers of different age and, therefore, with different tasks in the colony. There is an initial lag period of about one day in the semi-field set-up, during which there is hardly any activity. It seems likely that this lag has to do with the organization between the bees in the newly formed artificial colony once the aperture to the outer cage is opened, since this happened concurrently with pesticide exposure and the start of RFID measurements.

RFID data do not supply detailed information about the different activities in the bumble-bee micro-colony or hive. For comparison, an example of the kind of information that may be collected by a more classical way of quantifying activity is included in the project. Here, a number of micro-colonies were observed simultaneously, and the time spent on different kinds of activities could be quantified. On the other hand, once the RFID set-up is evolved to run more smoothly, activity may be quantified for a longer time-span using fewer man-hours. We found the total time spent on activity measured by traditional methods to be correlated with the total number of RFID readings and no indications that observations of the bees revealed effects not found by the RFID counting. A critical prerequisite for a more general application of the RFID techniques would be a better method for gluing the tags onto the bees. In addition, it may be worthwhile to elaborate on the reader design; if a double-reader were available, it would be possible to register automatically in which direction the bees pass, i.e. whether they enter or leave the hive.

8.5 Single repetitions versus pooled data sets

Except for the field trials, all experiments were repeated in time. In several cases, the outcome of a particular test varied between the individual repetitions, see for instance the effect of Karate on adult survival in semi-field tests (Table 64) and the effect of fungus infestation in the 14 d laboratory test including Biscaya (Table 50). Sometimes, the corresponding pooled analysis resulted in a more significant effect, while in other cases the opposite was seen, i.e. no significant effects were disclosed in the pooled analysis. Although it is not surprising that more data (e.g. the pooled data sets) leads to different conclusions than smaller data sets, the differences between repetitions of the same test document the high variability of such biological systems, even under controlled laboratory conditions. Hence, although annoying at first sight, the variability disclosed by establishing some of the replication as repetitions in time gave some insight that may not have been obtained had all replicates run simultaneously. This also sustains the procedure of running tests with replicates not only of dosage, but also in time, as a way to overcome the possibly unknown skewness introduced by circumstances that are not controlled by the test set-up.

8.6 Comparison of the different kinds of tests and end-points used in the project

A diverse set of test types have been applied in this project, from simple acute tests similar to those used in relation to approval of new pesticides, e.g. the 48 h honey bee tests (OECD 1998 a,b), over long-term laboratory and semi-field tests to tests with queen-right hives in the field. For the first three types of tests, i.e. the tests performed under more or less standardised conditions, results may be compared by looking at the estimated LD_{50} values, since this is the only end-point that all tests performed in the laboratory or in the greenhouse have in common. In Table 31, these values from the different chapters of the report are listed together. Although the estimates of effect levels for a given substance tend to overlap between the three test types, the overall trend is clearly that increasing the test period and the level of complexity (i.e. the realism) of the test design results in a lower LD_{50} values. This implies that bumble-bees may be more sensitive to pesticides in nature than in traditional, standardized short-term laboratory tests when exposed to similar pesticide dosages. However, the results of the field tests also imply that queen-right colonies may recover from effects caused by exposure to moderate pesticide dosages, although the end-points are not directly comparable with those of the other tests. The difference in effect levels between short-term and long-term laboratory tests is probably primarily due to exposure route (topical versus oral), since mortality generally only increased slightly with time in the long-term tests and test conditions were identical. Oppositely, test conditions varied between long-term laboratory and semi-field tests, whereas exposure method and duration did not. The differences in effect levels between these two test types thus express the impact of test condition.

TABLE 31. Comparison of estimated LD_{50} values for Biscaya, Fastac and Karate from different types of tests with bumble-bees. Negative figures indicate that the value could not be determined. Overlapping 95% confidence intervals indicate that LD_{50} estimates are not significantly different

Pesticide	Active ingredient	Test type	Estimated LD_{50}, µg a.i./bee	95% confidence limits
Biscaya	Thiacloprid	48 h lab	116	73-190
		14 d lab	73	45-123
		14 d semi-field	26	13-50
Fastac	Alpha-cypermethrin	48 h lab	1.4	0.94-2.2
		14 d lab	0.9	0.4-4.1
		14 d semi-field	0.2	0.1-0.5
Karate	Lambda-cyhalothrin	48 h lab	0.59	0.38-0.91
		14 d lab	-0.7	0.2-(-0.2)
		14 d semi-field	0.02	-0.06-0.13

In order to compare the sensitivity of different end-points, the estimated EC_{50} values for mortality, no. eggs produced and no. honey pots produced seem most applicable because the other end-points assessed in the tests (i.e. no. larval cells and no. dead larvae) often resulted in very few counts. Furthermore, this comparison of end-points only makes sense for the 14 d laboratory tests, since the outcome of the semi-field tests was much more variable with many instances of low or no counts of most sub-lethal end-points. Since the establishment of EC_{50} values was not very successful for Karate, we are left with the results for 14 d laboratory tests with Biscaya and Fastac (Table 15 and Table 16). Those results indicate that LD_{50} values (i.e. mortality) and EC_{50} values for sub-lethal end-points 14 d after pesticide exposure were almost identical, except for the number of larval cells produced by bees exposed to Biscaya. This is contradictory to our expectations that effects on reproductive end-point occur at sub-lethal dosages. However, if (as described by e.g. Mommaerts et al. 2006), only one of the five worker bees in the micro-colony produces eggs, and if this bee is the largest of the five, it may be

exposed to a lower pesticide dosage per unit body mass, provided that the consumption of sugar solution during pesticide exposure is not related to body mass.

Since the current evaluation procedure for pesticides is carried out using honeybees, but not bumble-bees or other bees, a comparison of the effect levels found in this study with the LD_{50} for honeybees seems appropriate. For thiacloprid, the active ingredient of Biscaya, the LD_{50} for oral toxicity is 17.32 µg/bee, and for contact toxicity 38.82 µg/bee. The corresponding LD_{50} values for alpha-cypermethrin (active ingredient of Fastac) are 0.059 and 0.033 µg/bee, respectively, and for lambda-cyhalothrin (active ingredient of Karate) 0.91 and 0.038 µg/bee. Hence, none of the LD_{50} or EC_{50} values found for *Bombus terrestris* in this project indicate that this species is more sensitive than honeybees to the three pesticides. It should be kept in mind, though, that in early spring the bumble-bee queen may be seen as a solitary bee, and any harm to her has a much more severe consequence for the future nest than any effect on single honeybee workers (Stoner 2014). Similarly, bumble-bee workers also present a higher relative value than honeybee workers because of their lower numbers.

When it comes to the applicability of the examined end-points for quantification and testing, worker survival, the number of honey pots produced and the number of larval cells developed, all are easy to observe and count, whereas the number of eggs produced is more difficult to estimate precisely because they are placed in small clusters inside lumps of wax. On the other hand, larval cells may require a longer test period than the 14 d used here in order to reach numbers high enough to satisfy the demand for statistical power. This also holds true for the number of emerging drones as a measure of reproductive success, as suggested by e.g. Mommaerts et al. (2010).

Both the long-term laboratory tests and the semi-field tests use micro-colonies as the experimental unit. In principle, this set-up is very simple, since it only requires that five worker bees from the same colony are placed together in a box and supplied with pollen and sugar solution. However, we soon found out that there are a number of challenges to cope with. First of all, the selection of workers from queen-right colonies may not always be straight-forward. From the literature, discrimination between workers on one hand and queens and drones on the other hand should be easy. However, it is our experience that drones can only be avoided with certainty if the number of antennal joints is counted. Therefore, we introduced a standard procedure of checking at least five bees every time we took out a new batch for setting up micro-colonies. If drones were detected among those five, this was taken as an indication that the queen-right colony had reached the reproductive stage where drones and sometimes queens are produced, and therefore the colony was discarded as supply for micro-colonies. Avoidance of queens when selecting bees for micro-colonies was a problem in those queen-right colonies that produced relatively large workers, since the obvious non-destructive way of distinguishing between queens and workers is the size of the animals. However, since queens are expected to occur at the same time as drones, the avoidance of drones ought to ensure the avoidance of queens too. Generally, it is our experience that the period in which the queen-right colonies only produce workers, but not drones or new queens, is highly variable, also under standardised conditions, where the supply of food seems optimal.

As previously described, we used a one- chamber design for the micro-colonies, where the bees lived and were fed in the same box. We do not claim that this works better than the two-chamber design used by others, as we did not try the alternative, but the one-chamber box was chosen because it is less space-consuming. Furthermore, we initially tested the effects of having a blinded compartment for nesting using a flowerpot where the bees could hide. However, we did not find any positive effects of this, and the main consequence was that it became more difficult to follow the production of eggs, larvae and honey pots. Hence, the boxes used for testing did not contain a blinded compartment. Mommaerts et al. (2010) were able to keep the micro-colonies sound for 11 weeks, and thereby more end-points may be evaluated, espe-

cially the number of drones produced. However, letting the test run for that long increases the space needed to run tests continuously, and we therefore chose to terminate the tests after 2 weeks.

The set-up for semi-field tests, although examined in preliminary experiments, turned out to vary greatly in performance. Sometimes, the bees seemed to be thriving in their butterfly cage universe, moving back and forth between the box, where they had established a well-functioning micro-colony before being allowed access to the outer world and the food supply outside the box. In other cases, some or all bees became more or less immobile, often just sitting on the inner frame of the butterfly cage apparently without seeking food. Since the cages were placed in a greenhouse where temperature and humidity were more variable than under laboratory conditions due to the influence from actual weather conditions, this may be part of the explanation. However, it seemed very difficult to predict a success or failure based on the actual weather conditions. In our opinion, an even more critical parameter for the success of this set-up may be how well-established the micro-colony was before they were allowed to leave the box and experience the larger cage. Although all micro-colonies had had a week to establish, as in Mommaerts et al (2010), before the aperture was opened and the test began, the degree of colony-establishment, as evaluated from the number of honey pots built and the presence of wax lumps with eggs, varied greatly. Apparently, this variance in colony-establishment was more crucial for the semi-field set-up than for the simpler laboratory set-up. As a consequence, control mortality became too high in several tests, which, of course, lowers the credibility of the results and impairs the identification of pesticide effects. Unfortunately, our attempts to contact other groups with experience in working with micro-colonies were unsuccessful. In a recent paper by Ceuppens et al. (2015), the micro-colonies were allowed two weeks to establish before test start, which may lower the number of non-functioning micro-colonies or at least make it easier to identify them.

Unlike the semi-field set-up, the application of commercially available queen-right hives in the field seems rather straight-forward, as long as the nests are not attacked by predators, e.g. badgers. The handling of the inner nest compartment for weekly weighing can be done by virtually anybody, whereas more expertise is needed to decide the right time for destroying the nest for determining the resulting production of new queens and drones at the culmination of the colony. In our study, we seem to have been waiting a little too long, since many of the nests appeared to have passed the climax, and the colonies were more or less dead already at the time we decided to kill them by freezing before taking them apart and counting what was found inside.

The RFID techniques applied in some of the tests gave results that indicate a potential of developing this approach into a sensitive and time-efficient method to disclose effects on bumble-bee activity. Tentatively, very low EC_{50} values was estimated for Biscaya compared to the other end-points measured, but the preliminary set-up used here does not allow us to evaluate the credibility of the effect level estimate. In a study involving exposure of *B. terrestris* to Imidacloprid both orally and topically and topically to λ-cyhalothrin, Gill & Raine (2014) found that imidacloprid increased the foraging activity as measured by RFID techniques, whereas the foraging efficiency decreased because the bees exposed to imidacloprid brought back smaller pollen loads. Bumble-bees exposed to λ-cyhalothrin (a.i. of Karate) did not increase their foraging activity, but unlike the control bees they did not increase their pollen load size with time. Unfortunately, the study design of Gill & Raine (2014) does not allow assessment of actual exposure because the bees could freely choose whether to get into contact with the pesticide-contaminated sugar solution and substrate.

8.7 Relevance of effect levels compared to likely exposure levels

When foraging, bees may be exposed to dietary trace residues of insecticides, and if the seeds or plants have been treated with a systemic insecticide, e.g. Biscaya or one of the other neonictinoid pesticides, the pollen and nectar brought back by the bees to feed the young may contain pesticide residues. Blacquiére et al. (2012) reviewed studies of neonicotinoid concentrations in pollen and nectar of plants from neonicotinoid-treated seeds and found up to 10 µg insecticide kg^{-1} in pollen and nectar, depending crop and insecticide. Similarly, Rundlöf et al. (2015) report that seed coating of oilseed rape seeds by the neonicotinoid clothiadinin and the non-systemic pyrethroid β-cyfluthrin results in concentrations of up to 13.9±1.8 ng a.i. g^{-1} and 10.3±1.3 ng a.i. ml^{-1} of clothianidin in pollen and nectar, respectively, and no β-cyfluthrin. In pollen collected from honeybee colonies in France, the concentration of imidacloprid varied between 1.1 and 5.7 ppb (Chauzat et al. 2006).

The reported uptake of neonicotinoids by plants grown in soils treated by label rates of the insecticides and translocation to nectar and pollen varies. Whereas Schmuck et al. (2011) found little uptake of imidacloprid from soils containing the neonicotinoid and no imidacloprid in pollen and nectar of sunflower plants grown in treated soil, Stoner and Eitzer (2012) report considerable uptake and relatively high concentrations of imidacloprid and thiamethoxam in pollen and nectar of squash (*Cucurbita pepo*) grown in treated soil. They found nectar concentrations of imidacloprid and thiamethoxam of 10± 3µg a.i. kg^{-1} and 11± 6µg a.i. kg^{-1}, respectively, and concentrations in pollen were 14± 8µg a.i. kg^{-1} and 12.9± 9µg a.i. kg^{-1} of imidacloprid and thiamethoxam, respectively.

In chronic feeding tests with honeybees, Decourtye et al. (2003) found that the lowest observed effect concentrations (LOEC) of imidacloprid on mortality were 24 µg a.i. kg^{-1} for winter honeybees exposed via sugar solution. However, the surviving bees showed reduced learning performances, with a LOEC of 48 µg a.i. kg^{-1} for winter bees and 12 µg a.i. kg^{-1} for summer bees. Since these concentrations lie within the range of concentrations found in nectar, effects in the field seem likely.

The LD_{50} for ingestion of sugar solution containing insecticide found in this study is based on the assumption that a bee consumes 0.077 ml (=0.09 g) sugar solution during the six hour exposure period. For thiacloprid, the LD_{50} was 73 µg per bee, and the effect concentration estimated from the RFID records was 9.6 µg thiacloprid per bee. In a comparable study with queen-less micro-colonies of *Bombus terrestris* and a thirteen-day dietary exposure to imidiacloprid at a range of dosages, Laycock et al (2012) found a dosage-dependent decline in fecundity for imidiacloprid in the range of 1 µg a.i. L^{-1}. The daily consumption of syrup in that study was calculated at approximately 300 mg per bee, equal to 0.236 ml/24 hours. The corresponding effect concentration is approximately 31 µg per bee, i.e. at the same range as we found for thiacloprid (Biscaya). There are not many studies of thiacloprid concentrations in crops, but Pohorecka et al. (2012) studied oil seed rape sprayed with this active ingredient and found maximum residues of 0.2 µg/kg in nectar and 1.0 µg/kg in pollen. Based on concurrent studies with honeybees, they concluded that the residues did not affect colony development. However, in a recent study Ellis et al. (2017) found that similar residues in raspberry nectar and pollen (561 and 771 ppb, respectively) increased bumble-bee mortality and colony growth in queen-right hives exposed in the field. These effect values are much lower than the ones found in the present study, since for instance the low tentative EC_{50} value for activity measured by RFID, 9.6 µg/bee, at a consumption of 0.09 g is equivalent of 107,000 ppb. However, the effect concentrations of the two studies cannot be compared directly because the bumblebees in Ellis et al. (2017) were exposed for a much longer period.

The other two insecticides studied in the present project, Fastac and Karate, are not systemic pesticides, i.e. they are not taken up by the plant and distributed to e.g. pollen and nectar.

Both insecticides are honeybee-labelled, which implies that they are not used on flowering crops during the active hours of honeybees. Even though an international review (Sanchez-Bayo & Goka 2014) found λ-cyhalothrin (a.i. of Karate) in 6% of pollen samples (but not in nectar), bumble-bees are probably most likely to be exposed to Fastac and Karate by direct over-spray during field treatment or by crawling on newly sprayed plants, although sometime bumble-bees are active outside the working hours of honeybees. Calculations based on the highest allowed dosages of Fastac and Karate (0.225 l/ha and 0.4 kg/ha, respectively) multiplied by the content of the active ingredients lead to estimated exposure levels of 11.25 and 10 ng/cm^2, respectively. If a bumble-bee has an exposed area of 2 cm^2, exposure levels of 0.022 and 0.02 µg/bee may be expected from over-spray with Fastac and Karate, respectively. Compared with the effect levels found in this study, effects of Karate seem very likely, whereas the expected Fastac exposure may be more harmless. However, it should be kept in mind that a bumble-bee flying through the spray cloud or crawling on newly-sprayed plants may experience higher exposure levels. No other studies of Fastac effects on bumble-bees were found. For λ-cyhalothrin (a.i. of Karate), Sanchez-Bayo & Goka (2014) estimated a 0.5% risk for *B. terrestris* of reaching LD_{50}, given the occurrence in 6% of the pollen samples, and Ceuppens et al. (2015) found sub-lethal effects on *B. terrestris* under laboratory and semi-field conditions at dosages down to 1/20 of the maximum allowed for field application in Europe.

9. Conclusions

The project has attempted to test three main hypotheses, and this has led to the following main conclusions:

(1) *Exposure to sub-lethal dosages of pesticides will result in significantly decreased reproduction and population growth in bumble-bees.* Sub-lethal effects were seen in both long-term laboratory tests, semi-field tests and the field study. A variety of sub-lethal end-points including no. eggs produced, no. larval cells and no. honey pots was most successfully quantified in the 14 laboratory tests, probably due to the more standardised conditions compared to the other types of tests. However, a comparison of effect levels reveals that the sub-lethal effects do not necessarily occur at lower dosages than lethal effects. Of course, an increased mortality is expected to cause a decrease in e.g. the number of offspring produced as a consequence of the lower number of adults present to perform this.

(2) *Tests of pesticide effects at different tiers will result in different effects levels for the same end-point because the level of complexity during testing will affect the response.* In the project, tests were carried out in different tier studies, ranging from acute toxicity tests in the laboratory to long-term exposure of standard hives under field conditions. Due to overlapping end-points, results from different tiers could be compared directly. Due to the failure of establishing clear dose-effect relations for some of the sub-lethal endpoints in some of the test types, the most obvious comparison across tests is effects on survival. The comparison of LD_{50} levels indicates that the bumble-bees become more sensitive to the three pesticides when the test period is extended, when exposure is altered from topical to oral, and when test conditions are less stable, but also more realistic. Generally, the end-points assessed in this project do not indicate that *B. terrestris* is more sensitive to the three insecticides than honeybees. However, it should be taken into consideration that effects on bumble-bee individuals may be more severe for the colony than is the case for honeybees because of the much lower number of individuals per colony. In addition, effects on behaviour may occur at lower levels than effects on survival and reproduction.

(3) *Bumble-bees subject to other stressors are more sensitive to pesticide exposure.* This was not the case in our study. Effects were seen of both starvation and the pathogenic fungus *B. bassiana*, but there was not a general, clear trend that the two stressors made the bumble-bees more sensitive to the tested insecticides. However, other ways of starving bees and other levels or species of pathogens may have other effects, also on the sensitivity of bees to pesticides.

10. Perspectives

10.1 Research perspectives

This project has aimed at comparing effects of pesticides on bumble-bees estimated at different levels of realism and complexity (tiers), including combinations with a pathogenic fungus and starvation. Thus, test types of increasing complexity were included. For the tier involving semi-field conditions, a new test design was tried, while the 48 h tests are rather similar to those used in routine testing of honeybees and the 14 d laboratory tests build on the suggestions of e.g. Mommaerts et al. (2006), although with substantial modifications. When it comes to semi-field test, there is still quite a long way to go before we have a well-functioning set-up that may be used on a regular basis. The tested design too often led to the bumble-bees remaining inactive outside the nesting box.

Similarly, some serious challenges must be overcome concerning the application of RFID techniques for assessing bumble-bee activity. Although it is in principle easy to obtain data at a far larger scale than by traditional observation methods, there are still several problems that need to be solved, including a safe, but still reliable way to glue the tags on the bumble-bees. It is, however, worth noting that this method seems at least as sensitive as more traditional and time-consuming observation methods.

The field approach used works quite well, provided that bumble-bee predators are kept away from the hives. Weighing the interior nest compartment of the hives on a weekly basis seems a robust approach that may be used to study the population growth of *B. terrestris* under many different conditions, not only in relation to toxic effects. On the other hand, quantification of other measures of bumble-bee fitness, e.g. the production of drones and new queens, requires that great care is taken when deciding the time for evaluating these end-points, since it can only be done in a destructive way.

The challenge of experimentally combining pesticide stress with other stressors, such as starvation and pathogens, in a controlled way remains, although the application of the fungus *B. bassiana* worked quite satisfactorily, and future studies may build on the experience with starvation obtained in the study.

The rather surprising finding that sub-lethal effects seem to occur at app. the same exposure levels as lethal effects definitely needs further attention in order to clarify whether it is a general phenomenon for bumble-bees. This is also the case for the strong indication that effect levels decrease at increasing complexity and duration of the tests.

10.2 Administrative perspectives

With this project, we add to the knowledge about extrapolation between tests of different complexity and between different end-points. Several suggestions for test methods have been tested, and suggestions for improvement are presented. For instance, the limitation of micro-colonies as test units is demonstrated by the difficulties of making the bumble-bees thrive under greenhouse conditions and the resulting high uncertainty. Similarly, the inclusion of other stressors, in particular starvation, turned out to be quite difficult and requires dedicated preliminary studies. Thereby, the project delivers input to further development of a future strategy for testing, as requested by EFSA and others.

Our results indicate that the sensitivity to pesticides increases if the complexity of the studied system increases. This sustains the demand for more realistic risk assessment and may have

consequences for the way available toxicity data should be interpreted. Thus, both exposure route (oral or topical), test duration, colony size and type (queen-right versus queen-less) and physical test conditions (stability of light, temperature and humidity as well as size and complexity of test arena) seem to be factors that may affect test outcome significantly. On the other hand, a comparison of our results for the three tested insecticides with the PPDB (Pesticide Property Data Base, http://sitem.herts.ac.uk/aeru/ppdb/en/atoz.htm) does not raise concern that the bumble-bees are not protected by the current evaluation procedure for these substances.

Comparison of our results with the experience from other studies shows that effects of Biscaya-contaminated pollen or nectar are only likely to occur if the bumble-bees feed on these sources for a long period of time, i.e. at least a couple of weeks. The implication of this for the risk assessment of thiacloprid should be evaluated by more focus on the food selection in different landscapes, including areas dominated by mass-flowering crops such as oilseed rape.

If further studies confirm that bumble-bee activity measured by e.g. RFID techniques is a sensitive end-point, inclusion of this method in higher tier tests may be considered.

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Appendix A. Alternative method to quantify activity

The number of flowers visited within 10 minutes was counted 0, 1, 2, 3, 5, 7 and 14 days after pesticide exposure. In order to differentiate between single individuals, these were marked with different colours prior to pesticide exposure. The colour code was combined with information about RFID tag number by “reading” each colour-marked and RFID-tagged bee with the RFID reader in a known order, so that the RFID identity could later be obtained from the RFID file. Activity was observed for a 10 minute period in the time spans 9:30-11:30 and 15-17, except for the first observation series just after exposure. Observations included time spent inside/outside box, feeding in artificial flowers and feeding on pollen.

TABLE 32. Observation and registration of various types of activities in six semi-field cages simultaneously. Example of data set

Colony	Date/Time	Temp	color	Box in/out	activity start	activity end	activity
1463	26.05.15, 19:10	30	black	in	19:10:00	19:12:00	crawling
1463	26.05.15, 19:10	30	black	in	19:12:00	19:20:00	lying on back
1463	26.05.15, 19:10	30	white	in	19:10:00	19:12:00	crawling
1463	26.05.15, 19:10	30	white	in	19:12:00	19:20:00	lying on back
1463	26.05.15, 19:10	30	green	in	19:10:00	19:20:00	lying on back
1463	26.05.15, 19:10	30	blue	in	19:10:00	19:20:00	lying on back
1463	26.05.15, 19:10	30	red	in	19:10:00	19:20:00	lying on back
1448	26.05.15, 19:11	30	black	in	19:11:00	19:21:00	dead
1448	26.05.15, 19:11	30	white	in	19:11:00	19:19:30	p-feeding
1448	26.05.15, 19:11	30	white	in	19:19:30	19:21:00	inactive
1448	26.05.15, 19:11	30	green	in	19:11:00	19:21:00	dead
1448	26.05.15, 19:11	30	blue	in	19:11:00	19:21:00	dead
1448	26.05.15, 19:11	30	red	in	19:11:00	19:21:00	inactive
1446	26.05.15, 19:18	32	black	in	19:18:00	19:28:00	p-feeding
1446	26.05.15, 19:18	32	white	in	19:18:00	19:28:00	inactive
1446	26.05.15, 19:18	32	green	in	19:18:00	19:20:30	inactive
1446	26.05.15, 19:18	32	green	in	19:20:30	19:28:00	p-feeding
1446	26.05.15, 19:18	32	blue	in	19:18:00	19:24:00	inactive
1446	26.05.15, 19:11	32	blue	in	19:24:00	19:28:00	p-feeding
1446	26.05.15, 19:11	32	red	in	19:18:00	19:28:00	inactive
1458	26.05.15, 19:19	29	black	in	19:19:00	19:29:00	p-feeding
1458	26.05.15, 19:19	29	white	out	19:19:00	19:23:00	crawling
1458	26.05.15, 19:19	29	white	out	19:23:00	19:29:00	lying on back
1458	26.05.15, 19:19	29	green	in	19:19:00	19:28:30	inactive
1458	26.05.15, 19:19	29	green	in	19:28:30	19:29:00	crawling
1458	26.05.15, 19:19	29	blue	in	19:19:00	19:29:00	crawling
1458	26.05.15, 19:19	29	red	out	19:19:00	19:27:30	crawling
1458	26.05.15, 19:19	29	red	in	19:27:30	19:29:00	crawling
1451	26.05.15, 19:31	30	black	out	19:31:00	19:41:00	crawling

1451	26.05.15, 19:31	30	white	in	19:31:00	19:36:50	p-feeding
1451	26.05.15, 19:31	30	white	in	19:36:50	19:37:40	inactive
1451	26.05.15, 19:31	30	white	in	19:36:40	19:40:00	p-feeding
1451	26.05.15, 19:31	30	white	in	19:40:00	19:41:00	inactive
1451	26.05.15, 19:31	30	green	in	19:31:00	19:35:20	p-feeding
1451	26.05.15, 19:31	30	green	in	19:35:20	19:35:40	inactive
1451	26.05.15, 19:31	30	green	in	19:35:40	19:39:30	p-feeding
1451	26.05.15, 19:31	30	green	in	19:39:30	19:41:00	inactive
1451	26.05.15, 19:31	30	blue	out	19:31:00	19:41:00	p-feeding
1451	26.05.15, 19:31	30	red	in	19:31:00	19:35:50	inactive
1451	26.05.15, 19:31	30	red	in	19:35:50	19:40:00	p-feeding
1451	26.05.15, 19:31	30	red	in	19:40:00	19:41:00	inactive
1467	26.05.15, 19:37	28	black	in	19:37:00	19:43:00	p-feeding
1467	26.05.15, 19:37	28	black	in	19:43:00	19:47:00	crawling
1467	26.05.15, 19:37	28	white	out	19:37:00	19:41:00	crawling
1467	26.05.15, 19:37	28	white	out	19:41:00	19:47:00	inactive
1467	26.05.15, 19:37	28	green	out	19:37:00	19:47:00	crawling
1467	26.05.15, 19:37	28	blue	out	19:37:00	19:47:00	crawling
1467	26.05.15, 19:37	28	red	out	19:37:00	19:47:00	crawling
1451	26.05.15, 19:51	28	black	out	19:51:00	20:01:00	crawling
1451	26.05.15, 19:51	28	white	in	19:51:00	19:53:50	p-feeding
1451	26.05.15, 19:51	28	white	in	19:53:50	19:54:30	inactive
1451	26.05.15, 19:51	28	white	in	19:54:30	19:56:00	p-feeding
1451	26.05.15, 19:51	28	white	in	19:56:00	19:56:40	inactive
1451	26.05.15, 19:51	28	white	in	19:56:40	20:01:00	p-feeding
1451	26.05.15, 19:51	28	green	in	19:51:00	19:57:50	p-feeding
1451	26.05.15, 19:51	28	green	in	19:57:50	20:01:00	inactive
1451	26.05.15, 19:51	28	blue	out	19:51:00	19:56:00	p-feeding
1451	26.05.15, 19:51	28	blue	out	19:56:00	20:01:00	crawling
1451	26.05.15, 19:51	28	red	in	19:51:00	19:57:50	p-feeding
1451	26.05.15, 19:51	28	red	in	19:57:50	20:00:00	inactive
1451	26.05.15, 19:51	28	red	in	20:00:00	20:01:00	p-feeding

Abbreviations:

p-feeding	feeding on pollen
f-feeding	feeding on flower
guarding	sitting right in front of exit
inactive	bee lies on side, not sure if dead
chewing wo fa	bee chews on wood frame

Appendix B. Results of statistical analyses

Appendix B.1 Acute toxicity

Appendix B.1.1 Pesticide and fungus

TABLE 33. Outcome of test of acute effects of fungus and Biscaya exposure. The test was run three times, each with three levels of fungus and three or four pesticide dosages plus a control in four replicates per repetition.

Repetition	Source	DF	X^2	$P > X^2$
1	fungus level	2	1.4844	0.4761
	pesticide dosage	4	28.7190	<0.0001
2	fungus level	2	0.0655	0.9678
	pesticide dosage	4	12.5648	0.0136
3	fungus level	2	2.9594	0.2277
	pesticide dosage	3	20.5025	0.0001
All	fungus level	2	1.87	0.3933
	pesticide dosage	4	204.76	<0.0001

TABLE 34. Outcome of test of acute effects of fungus and Fastac exposure. The test was run three times, each with three levels of fungus and three or four pesticide dosages in four replicates per repetition.

Repetition	Source	DF	X^2	$P > X^2$
1	fungus level	2	3.0082	0.2222
	pesticide dosage	4	16.61	<0.0001
2	fungus level	2	10.7424	0.0046
	pesticide dosage	4	18.3437	0.0011
3	fungus level	2	8.8194	0.0122
	pesticide dosage	4	20.1896	0.0002
All	fungus level	2	9.28	0.0096
	pesticide dosage	7	257.32	<0.0001

TABLE 35. Outcome of test of acute effects of fungus and Karate exposure. The test was run three times, each with three levels of fungus and three or four pesticide dosages in four replicates per repetition.

Repetition	Source	DF	X^2	$P > X^2$
1	fungus level	2	3.0155	0.2214
	pesticide dosage	3	9.4566	0.0238
2	fungus level	2	5.8969	0.0524
	pesticide dosage	4	27.5156	<0.0001
3	fungus level	2	12.2261	0.0022
	pesticide dosage	3	25.0591	<0.0001
All	fungus level	2	10.66	0.0048
	pesticide dosage	6	288.08	<0.0001

Appendix B.1.2 Pesticide and starvation

TABLE 36. Effects of Biscaya dosage and starvation on the survival of adult bumble-bees in the test runs. N.B. Starvation levels are not identical in the two runs of the test.

Test run	Source	DF	X^2	$P > X^2$
1	starvation level	2	1.55	0.4609
	pesticide dosage	4	43.72	<0.0001
2	starvation level	2	2.98	0.2250
	pesticide dosage	4	33.09	<0.0001
Both	starvation level	3	3.53	0.3174
	pesticide dosage	4	68.01	<0.0001

TABLE 37. Effects of Fastac dosage and starvation on the survival of adult bumble-bees in the test runs. N.B. Starvation levels are not comparable in the two runs.

Test run	Source	DF	X^2	$P > X^2$
1	starvation level	2	2.62	0.2704
	pesticide dosage	4	126.24	<0.0001
2	starvation level	2	11.44	0.0033
	pesticide dosage	4	138.25	<0.0001
Both	starvation level	3	3.38	0.3371
	pesticide dosage	4	245.71	<0.0001

TABLE 38. Effects of Karate dosage and starvation on the survival of adult bumble-bees in the test runs. N.B. Starvation levels are not comparable in the two runs.

Test run	Source	DF	X^2	$P > X^2$
1	starvation level	2	8.86	0.0119
	pesticide dosage	4	185.96	<0.0001
2	starvation level	2	7.63	0.0221
	pesticide dosage	4	108.79	<0.0001
Both	starvation level	4	21.90	0.0002
	pesticide dosage	4	286.48	<0.0001

Appendix B.2 Long-term laboratory tests

Appendix B.2.1 Pesticide alone

TABLE 39. Test of effect of pesticide dosage on consumption of pesticide-contaminated sugar solution. Pooled data from the three repetitions of the tests.

Pesticide	DF	F value	P>F
Biscaya	6	8.09	**<0.0001**
Fastac	6	1.06	0.3936
Karate	6	0.27	0.9507

TABLE 40. Outcome of analysis of effect of Biscaya dosage on adult survival 1-14 days after exposure.

Repetition	Day	DF	X^2	$P > X^2$
1	1	5	78.65	<0.0001
	2	5	78.65	<0.0001
	3	5	78.65	<0.0001
	5	5	78.65	<0.0001
	7	5	74.29	<0.0001
	14	5	86.54	<0.0001
2	1	5	32.72	<0.0001
	2	5	42.02	<0.0001
	3	5	42.02	<0.0001
	5	5	45.57	<0.0001
	7	5	38.17	<0.0001
	14	5	34.06	<0.0001
3	1	5	42.67	<0.0001
	2	5	39.31	<0.0001
	3	5	37.60	<0.0001
	5	5	31.72	<0.0001
	7	5	27.93	<0.0001
	14	5	18.93	0.0020
All	1	6	136.90	<0.0001
	2	6	131.86	<0.0001
	3	6	137.24	<0.0001
	5	6	132.67	<0.0001
	7	6	125.30	<0.0001
	14	6	112.20	<0.0001

TABLE 41. Outcome of analysis of effects of Biscaya dosage on the number of eggs produced in the micro-colonies during the 14 d test period.

Repetition	Day	Num DF	Den DF	F	P > F
1	0	5	19	2.35	0.0803
	1	-			
	2	5	19	1.57	0.2151
	3	5	19	3.63	0.0179
	5	5	19	4.18	0.0099
	7	5	19	9.03	0.0002
	14	5	19	10.52	<0.0001
2	0	-			
	1	5	19	1.47	0.2445
	2	5	19	0.87	0.5215
	3	5	19	2.35	0.0809
	5	5	19	1.78	0.1653
	7	5	19	2.34	0.0810
	14	5	19	3.42	0.0226
3	0	5	19	5.48	0.0027
	1	5	19	0.53	0.7489
	2	5	19	5.52	0.0027
	3	5	19	3.60	0.0184
	5	5	19	4.51	0.0070
	7	5	19	6.58	0.0010
	14	5	19	4.25	0.0092
All	0	4	38	4.25	0.0061[i]
	1	5	44	3.94	0.0048
	2	9	57	2.88	0.0072[i]
	3	9	57	3.96	0.0006[i]
	5	9	57	2.88	0.0071[i]
	7	9	57	4.45	0.0002[i]
	14	9	57	3.95	0.0006[i]

– not analysed due to lack of data or variation, [i] interactive effect between repetition no. and pesticide dosage; test values given for the interaction

TABLE 42. Outcome of analysis of effects of Biscaya dosage on the number of larval cells produced in the micro-colonies during the 14 d test period.

Repetition	Day	Num DF	Den DF	F	P > F
1	7	5	23	4.98	0.0031
	14	-			
2	7	5	24	0.00	1.0000
	14	5	24	0.01	1.0000
3	7	-			
	14	5	23	1.73	0.1676
All	7	6	52	4.78	0.0006
	14	5	53	2.43	0.0470

- Not analysed due to missing data or variation

TABLE 43. Outcome of analysis of effects of Biscaya dosage on the number of honey pots produced in the micro-colonies during the 14 d test period.

Repetition	Day	Num DF	Den DF	F	P > F
1	0	-	-	-	-
	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	5	-	-	-	-
	7	5	23	1.38	0.2696
	14	5	23	1.79	0.1553
2	0	-	-	-	-
	1	5	22	0.63	0.6797
	2	5	24	0.77	0.5814
	3	5	24	1.98	0.1184
	5	5	24	0.26	0.9315
	7	5	24	0.48	0.7857
	14	5	24	1.36	0.2726
3	0	5	23	1.49	0.2332
	1	5	23	1.00	0.4378
	2	5	23	0.94	0.4755
	3	5	23	1.19	0.3458
	5	5	23	1.25	0.3169
	7	5	23	0.64	0.6746
	14	5	23	1.42	0.2541
All	0	5	19	1.71	0.1804
	1	5	42	2.55	0.0423
	2	5	44	3.26	0.0137
	3	5	44	1.37	0.2537
	5	5	44	2.02	0.0943
	7	6	68	2.14	0.0599
	14	6	68	3.49	0.0046

– not analysed due to lack of data or variation

TABLE 44. Outcome of analysis of effect of Fastac dosage on adult survival 1-14 days after exposure.

Repetition	Day	DF	X^2	$P > X^2$
1	1	5	12.59	0.0276
	2	5	13.03	0.0231
	3	-	-	-
	5	5	10.00	0.0752
	7	5	6.76	0.2392
	14	5	4.80	0.4411
2	1	-	-	-
	2	-	-	-
	3	-	-	-
	5	-	-	-
	7	-	-	-
	14	5	21.64	0.0006
3	1	-	-	-
	2	5	17.93	0.0030
	3	5	18.35	0.0025
	5	5	20.20	0.0011
	7	5	16.42	0.0057
	14	5	8.25	0.1428
All	1	6	18.58	0.0049
	2	6	33.28	<0.0001
	3	5	45.24	<0.0001
	5	6	23.68	0.0006
	7	6	22.00	0.0012
	14	6	15.36	0.0176

– not analysed due to lack of data or variation

TABLE 45. Effects of Fastac dosage on the number of egg cells produced 0-14 days after exposure.

Repetition	Day	Num DF	Den DF	F	P > F
1	0	5	14	0.04	0.9989
	1	5	14	1.37	0.2920
	2	5	14	3.38	0.0327
	3	-	-	-	-
	5	5	14	4.47	0.0121
	7	5	14	11.95	0.0001
	14	5	14	22.63	<0.0001
2	0	5	18	2.71	0.0541
	1	5	18	2.64	0.0584
	2	5	18	1.13	0.3814
	3	5	18	2.89	0.0437
	5	5	18	4.09	0.0118
	7	5	18	6.74	0.0011
	14	5	18	0.86	0.5294
3	0	5	17	1.53	0.2315
	1	5	17	1.53	0.2315
	2	5	17	3.48	0.0239
	3	5	17	3.95	0.0147
	5	5	17	2.89	0.0459
	7	5	17	6.78	0.0012
	14	5	16	4.28	0.0116
All	0	6	60	3.59	0.0042
	1	6	60	5.16	0.0002
	2	6	60	6.15	<0.0001
	3	5	41	8.01	<0.0001
	5	9	49	2.48	0.0204[i]
	7	9	49	10.95	<0.0001[i]
	14	9	48	8.41	<0.0001[i]

– not analysed due to lack of data or variation, i interactive effect between repetition no. and pesticide pesticide dosage; test values given for the interaction

TABLE 46. Effects of Fastac dosage on the number of larval cells produced 0-14 days after exposure.

Repetition	Day	Num DF	Den DF	F	P > F
1	7	5	18	3.28	0.0280
	14	5	18	11.93	<0.0001
2	7	-	-	-	-
	14	5	22	3.24	0.0243
3	7	5	22	0.48	0.7905
	14	5	20	0.77	0.5830
All	7	6	45	2.94	0.0165
	14	6	71	4.68	0.0005

– not analysed due to lack of data or variation

TABLE 47. Effects of Fastac dosage on the number of honey pots produced 0-14 days after exposure.

Repetition	Day	Num DF	Den DF	F	P > F
1	0	-	-	-	-
	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	5	-	-	-	-
	7	5	18	0.74	0.6050
	14	-	-	-	-
2	0	5	22	0.37	0.8611
	1	5	22	0.15	0.9793
	2	5	22	0.46	0.8018
	3	5	22	0.78	0.5746
	5	5	22	0.58	0.7146
	7	5	22	0.61	0.6907
	14	5	22	2.07	0.1085
3	0	5	22	0.32	0.8947
	1	5	22	0.56	0.7304
	2	5	22	1.47	0.2410
	3	5	22	1.99	0.1197
	5	5	22	3.35	0.0213
	7	5	22	3.06	0.0301
	14	5	20	3.01	0.0347
All	0	5	50	0.40	0.8478
	1	5	50	1.10	0.3707
	2	5	50	1.71	0.1491
	3	5	50	2.22	0.0664
	5	5	50	3.10	0.0162
	7	6	73	1.59	0.1610
	14	5	48	3.86	0.0051

– not analysed due to lack of data or variation

TABLE 48. Outcome of analysis of effect of Karate dosage on the number of eggs produced 0-14 days after exposure.

Repetition	Day	Num DF	Den DF	F	P > F
1	0	5	16	2.49	0.0749
	1	5	16	1.14	0.3793
	2	5	16	0.83	0.5444
	3	5	16	0.28	0.9192
	5	5	16	2.04	0.1275
	7	5	16	3.48	0.0256
	14	5	16	6.40	0.0019
2	0	-	-	-	-
	1	5	13	0.00	1.0000
	2	5	13	8.42	0.0010
	3	5	13	8.90	0.0007
	5	5	13	0.50	0.7740
	7	5	13	0.00	1.0000
	14	5	13	8.71	0.0008
3	0	-	-	-	-
	1	5	31	1.92	0.1195
	2	5	31	2.99	0.0258
	3	5	31	5.54	0.0009
	5	5	31	13.70	<0.0001
	7	5	31	10.35	<0.0001
	14	5	31	9.19	<0.0001
All	0	5	16	2.49	0.0749
	1	6	71	2.33	0.0413
	2	9	60	4.43	0.0002[i]
	3	9	60	6.15	<0.0001[i]
	5	9	60	6.44	<0.0001[i]
	7	9	60	3.16	0.0035[i]
	14	9	60	8.71	<0.0001[i]

TABLE 49. Outcome of analysis of effect of Karate dosage on the number of larval cells evolved 0-14 days after exposure.

Repetition	Day	Num DF	Den DF	F	P > F
1	7	5	20	2.12	0.1051
	14	5	20	6.58	0.0009
2	7	-	-	-	-
	14	5	17	0.11	0.9897
3	7	-	-	-	-
	14	5	38	2.15	0.0803
All	7	5	20	2.12	0.1051
	14	6	86	20.37	<0.0001

– not analysed due to lack of data or variation

Appendix B.2.2 Pesticide and fungus

TABLE 50. Outcome of statistical analyses of effects of the pesticide Biscaya and the fungus *Beauveria* on survival of adult bumble-bees.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	2	Pesticide dosage	4	8.79	0.0666
		Fungus level	1	0.00	1.0000
	7	Pesticide dosage	4	5.83	0.2121
		Fungus level	1	0.10	0.7485
	14	Pesticide dosage	4	5.00	0.2876
		Fungus level	1	0.09	0.7634
2	2	Pesticide dosage	4	17.83	0.0013
		Fungus level	1	1.94	0.1634
	7	Pesticide dosage	4	9.78	0.0444
		Fungus level	1	6.21	0.0127
	14	Pesticide dosage	4	7.33	0.1195
		Fungus level	1	6.21	0.0127
Both	2	Pesticide dosage	4	22.75	0.0001
		Fungus level	1	1.12	0.2890
	7	Pesticide dosage	4	13.66	0.0085
		Fungus level	1	4.54	0.0331
	14	Pesticide dosage	4	11.71	0.0197
		Fungus level	1	2.91	0.0879

TABLE 51. Outcome of statistical analyses of effects of the pesticide Biscaya and the fungus *Beauveria bassiana* on the number of egg cells produced in micro-colonies.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	0	Pesticide dosage	4	4.1138	0.3908
		Fungus level	1	3.0670	0.0799
	2	Pesticide dosage	4	8.6833	0.0695
		Fungus level	1	4.3635	0.0367
	7	Pesticide dosage	4	6.8792	0.1424
		Fungus level	1	4.9849	0.0256
	14	Pesticide dosage	4	3.6176	0.4602
		Fungus level	1	3.7181	0.0538
2	0	Pesticide dosage	4	1.0484	0.9024
		Fungus level	1	0.1167	0.7327
	2	Pesticide dosage	4	3.2484	0.5172
		Fungus level	1	0.1138	0.7358
	7	Pesticide dosage	4	2.9940	0.5588
		Fungus level	1	0.7759	0.3784
	14	Pesticide dosage	4	1.7975	0.7729
		Fungus level	1	0.0288	0.8654
Both	0	Pesticide dosage	4	1.0204	0.9067
		Fungus level	1	1.2077	0.2718
	2	Pesticide dosage	4	7.0594	0.1328
		Fungus level	1	0.7529	0.3855
	7	Pesticide dosage	4	7.1025	0.1306
		Fungus level	1	0.4264	0.5138
	14	Pesticide dosage	4	3.8419	0.4278
		Fungus level	1	1.2823	0.2575

TABLE 52. Outcome of statistical analyses of effects of the pesticide Fastac and the fungus *Beauveria* on the survival of adult bumble-bees.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	2	Pesticide dosage	3	74.69	<0.0001
		Fungus level	1	1.15	0.2840
	7	Pesticide dosage	3	68.86	<0.0001
		Fungus level	1	1.92	0.1661
	14	Pesticide dosage	3	68.86	<0.0001
		Fungus level	1	1.92	0.1661
2	2	Pesticide dosage	3	44.48	<0.0001
		Fungus level	1	0.91	0.3413
	7	Pesticide dosage	3	28.89	<0.0001
		Fungus level	1	1.37	0.2426
	14	Pesticide dosage	3	21.15	<0.0001
		Fungus level	1	1.19	0.2749
Both	2	Pesticide dosage	3	104.68	<0.0001
		Fungus level	1	1.72	0.1898
	7	Pesticide dosage	3	84.76	<0.0001
		Fungus level	1	2.75	0.0970
	14	Pesticide dosage	3	76.61	<0.0001
		Fungus level	1	2.59	0.1079

TABLE 53. Outcome of statistical analyses of effects of the pesticide Fastac and the fungus *Beauveria* on the number of egg cells produced in micro-colonies.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	0	Pesticide dosage	3	5.3717	0.1465
		Fungus level	1	4.9048	0.0268
	2	Pesticide dosage	3	3.5053	0.3201
		Fungus level	1	0.6007	0.4383
	7	Pesticide dosage	3	2.6997	0.4403
		Fungus level	1	0.3850	0.5349
	14	Pesticide dosage	3	4.1407	0.2467
		Fungus level	1	0.1425	0.7058
2	0	Pesticide dosage	3	1.9134	0.5906
		Fungus level	1	5.9498	0.0147
	2	Pesticide dosage	3	2.2250	0.5270
		Fungus level	1	8.4382	0.0037
	7	Pesticide dosage	3	1.2033	0.7522
		Fungus level	1	3.0804	0.0792
	14	Pesticide dosage	3	3.8459	0.2786
		Fungus level	1	0.4671	0.4943
Both	0	Pesticide dosage	3	3.5286	0.3171
		Fungus level	1	0.6730	0.4120
	2	Pesticide dosage	3	3.4626	0.3256
		Fungus level	1	3.0861	0.0790
	7	Pesticide dosage	3	3.8884	0.2738
		Fungus level	1	0.8199	0.3652
	14	Pesticide dosage	3	6.8796	0.0758
		Fungus level	1	0.1649	0.6847

TABLE 54. Outcome of statistical analyses of effects of the pesticide Karate and the fungus *Beauveria* on survival of adult bumble-bee.

Repetition	Day	Source	DF	X^2	P > X^2
1	2	Pesticide dosage	4	7.56	0.1090
		Fungus level	1	0.34	0.5621
	7	Pesticide dosage	4	6.30	0.1782
		Fungus level	1	0.24	0.6227
	14	Pesticide dosage	4	6.99	0.1363
		Fungus level	1	0.20	0.6546
2	2	Pesticide dosage	4	14.61	0.0056
		Fungus level	1	0.44	0.5093
	7	Pesticide dosage	4	5.99	0.1996
		Fungus level	1	5.95	0.0147
	14	Pesticide dosage*Fungus level	2	8.96	0.0114[i]
Both	2	Pesticide dosage	4	18.55	0.0010
		Fungus level	1	1.36	0.2431
	7	Pesticide dosage	4	10.85	0.0284
		Fungus level	1	5.13	0.0235
	14	Pesticide dosage	4	4.63	0.3273
		Fungus level	1	10.16	0.0014

[i] Significant interaction between fungus level and pesticide pesticide dosage, p value for interaction shown

Appendix B.2.3 Pesticide and starvation

TABLE 55. Analysis of effects of Biscaya dosage and starvation on adult survival 2-14 days after pesticide exposure.

Repetition	Day	Source	DF	X^2	P > X^2
1	2	Pesticide dosage	4	21.01	0.0003
		Starvation level	1	1.73	0.1884
	7	Pesticide dosage	4	25.99	<0.0001
		Starvation level	1	1.57	0.2105
	14	Pesticide dosage*Starvation level	2	6.63	0.0364[i]
2	2	Pesticide dosage	-	-	-
		Starvation level	-	-	-
	7	Pesticide dosage	-	-	-
		Starvation level	-	-	-
	14	Pesticide dosage	4	16.67	0.0022
		Starvation level	1	0.20	0.6536
Both	2	Pesticide dosage	-	-	-
		Starvation level	-	-	-
	7	Pesticide dosage	3	17.13	0.0007
		Starvation level	1	0.51	0.4769
	14	Pesticide dosage	3	14.04	0.0029
		Starvation level	1	0.51	0.4769

- not analysed due to lack of data or variation; i Significant interaction between starvation and pesticide dosage, test values for interaction shown

TABLE 56. Analysis of effects of Biscaya dosage and starvation on the number of egg produced 0-14 days after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	0	Pesticide dosa-	4	0.0254	0.9999
		Starvation level	1	0.0366	0.8483
	2	-	-	-	-
		-	-	-	-
	7	Pesticide dosa-	4	0.8159	0.9363
		Starvation level	1	0.0237	0.8776
	14	Pesticide dosa-	4	3.1286	0.5365
		Starvation level	1	6.4398	0.0112
2	0	Pesticide dosa-	4	0.5205	0.9715
		Starvation level	1	0.4060	0.5240
	2	Pesticide dosa-	4	0.9534	0.9168
		Starvation level	1	2.1441	0.1431
	7	Pesticide dosa-	4	3.6382	0.4572
		Starvation level	1	0.7052	0.4010
	14	Pesticide dosa-	4	4.1166	0.3905
		Starvation level	1	8.8063	0.0030
Both	0	Pesticide dosa-	3	0.3667	0.9470
		Starvation level	1	0.2283	0.6328
	2	Pesticide dosa-	3	0.9614	0.8106
		Starvation level	1	1.9261	0.1652
	7	Pesticide dosa-	3	3.0428	0.3851
		Starvation level	1	0.1356	0.7127
	14	Pesticide dosa-	3	2.6787	0.4439
		Starvation level	1	3.5991	0.0578

- not analysed due to lack of data or variation

TABLE 57. Analysis of effects of Fastac dosage and starvation on adult survival 2-14 days after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	2	Pesticide dosage	-	-	-
		Starvation level	-	-	-
	7	Pesticide dosage	-	-	-
		Starvation level	-	-	-
	14	Pesticide dosage	4	98.25	<0.0001
		Starvation level	1	5.44	0.0196
2	2	Pesticide dosage	4	89.40	<0.0001
		Starvation level	1	6.96	0.0083
	7	Pesticide dosage	4	89.40	<0.0001
		Starvation level	1	6.96	0.0083
	14	Pesticide dosage	4	89.40	<0.0001
		Starvation level	1	6.96	0.0083
Both	2	Pesticide dosage	4	148.52	<0.0001
		Starvation level	1	0.27	0.6019
	7	Pesticide dosage	4	152.55	<0.0001
		Starvation level	1	0.08	0.7706
	14	Pesticide dosage	4	147.97	<0.0001
		Starvation level	1	0.27	0.6052

- not analysed due to lack of data or variation

TABLE 58. Analysis of effects of Fastac dosage and starvation on the number of eggs produced 0-14 days after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	0	Pesticide dosage	4	2.1049	0.7165
		Starvation level	1	0.0931	0.7603
	2	Pesticide dosage	4	2.0301	0.7302
		Starvation level	1	0.0572	0.8110
	7	Pesticide dosage	4	2.6049	0.6260
		Starvation level	1	1.9811	0.1593
	14	Pesticide dosage	4	7.6185	0.1066
		Starvation level	1	4.0901	0.0431
2	0	Pesticide dosage	4	1.0302	0.9052
		Starvation level	1	2.9951	0.0835
	2	Pesticide dosage	4	3.4062	0.4923
		Starvation level	1	7.6218	0.0058
	7	Pesticide dosage	4	6.9565	0.1382
		Starvation level	1	8.9922	0.0027
	14	Pesticide dosage	4	5.7814	0.2161
		Starvation level	1	9.7397	0.0018
Both	0	Pesticide dosage	4	1.9149	0.7514
		Starvation level	1	2.1439	0.1431
	2	Pesticide dosage	4	3.7527	0.4405
		Starvation level	1	5.6467	0.0175
	7	Pesticide dosage	4	14.5211	0.0058
		Starvation level	1	12.2625	0.0005
	14	Pesticide dosage	4	10.6421	0.0309
		Starvation level	1	10.6755	0.0011

TABLE 59. Analysis of effects of Fastac dosage and starvation on the number of honey pots produced 0-14 days after pesticide exposure.

Repetition	Day	Source	Num DF	Den DF	F	P > F
1	0	Pesticide dosage	4	27	0.18	0.9460
		Starvation level	1	27	0.35	0.5595
	3*	Pesticide dosage	4	27	1.28	0.3017
		Starvation level	1	27	0.01	0.9044
	7	Pesticide dosage	4	27	2.84	0.0434
		Starvation level	1	27	0.00	0.9611
	14	Pesticide dosage	4	27	3.07	0.0332
		Starvation level	1	27	0.18	0.6761
2	0	Pesticide dosage	4	27	0.05	0.9949
		Starvation level	1	27	1.07	0.3104
	3*	Pesticide dosage	4	27	1.31	0.2920
		Starvation level	1	27	4.12	0.0522
	7	Pesticide dosage	4	27	1.83	0.1518
		Starvation level	1	27	2.86	0.1024
	14	Pesticide dosage	4	27	2.61	0.0577
		Starvation level	1	27	4.88	0.0359
Both	0	Pesticide dosage	4	51	0.07	0.9911
		Starvation level	1	51	0.99	0.3234
	3*	Pesticide dosage	-	-	-	-
		Starvation level	-	-	-	-
	7	Pesticide dosage	4	51	3.06	0.0247
		Starvation level	1	51	0.98	0.3273
	14	Pesticide dosage	4	51	4.07	0.0061
		Starvation level	1	51	1.66	0.2030

- not analysed due to lack of data or variation; * no data for day 2

TABLE 60. Outcome of analysis of effects of Karate dosage and starvation on the survival of adult bumble-bees 2-14 days after pesticide exposure.

Repetition	Day	Source	DF	X^2	P > X^2
1	2	Pesticide dosage*Starvation level	2	13.07	0.0014 [i]
	7	Pesticide dosage*Starvation level	2	11.72	0.0028 [i]
	14	Pesticide dosage*Starvation level	2	8.65	0.0132 [i]
2	2	Pesticide dosage	4	58.91	<0.0001
		Starvation level	1	11.16	0.0008
	7	Pesticide dosage	4	17.86	0.0013
		Starvation level	1	2.02	0.1555
	14	Pesticide dosage	4	18.63	0.0009
		Starvation level	1	3.87	0.0491
Both	2	Pesticide dosage	4	50.32	<0.0001
		Starvation level	1	9.55	0.0020
	7	Pesticide dosage	4	28.26	<0.0001
		Starvation level	1	2.34	0.1259
	14	Pesticide dosage*Starvation level	2	9.29	0.0096 [i]

[i] Significant interactive effect of pesticide pesticide dosage and starvation – p value for interaction shown

Appendix B.3 Semi-field tests

Appendix B.3.1 Pesticide alone

TABLE 61. Results of analysis of the effect of pesticide dosages on the consumption of sugar solution during the 6 h exposure period. The right-hand column shows p values for the analysis of differences between pesticide controls with and without the spreading agent Dancon F.

Pesticide	Test repetition	DF	F	P > F*	Contrast water vs. water+Dancon (p-value)
Biscaya	1	6	2.26	0.0841	0.5409
	2	6	11.20	<0.0001	0.0002
	3	5	2.20	0.0971	-
	all	6	6.32	<0.0001	-
Fastac	1	5	6.02	0.0022	-
	2	5	1.90	0.1423	-
	3	5	0.77	0.5857	-
	all	5	5.10	0.0006	-
Karate	1	5	3.21	0.0286	-
	2	5	1.26	0.3199	-
	3	6	0.23	0.9567	-
	all	6	0.47	0.8314	-

*water and water + dancon tested as separate pesticide dosages if significantly different (p<0.05);
– water without Dancon not tested

TABLE 62. Outcome of analysis of effect of Biscaya dosage on the survival of adult bees 2-14 d after exposure.

Repetition	Day	DF	X^2	$P > X^2$
1	2	5	2.73	0.7419
	7	5	39.92	<0.0001
	14	5	25.06	0.0001
2	2	5	35.16	<0.0001
	7	5	32.51	<0.0001
	14	5	24.94	0.0001
3	2	5	9.24	0.0997
	7	5	13.54	0.0188
	14	5	13.93	0.0161
All	2	6	131.86	<0.0001
	7	9	18.93	0.0258 [i]
	14	9	30.00	0.0004 [i]

[i] significant interactive effect between repetition and Biscaya pesticide dosage – test values for interaction shown

TABLE 63. Outcome of analysis of effect of Fastac dosage on the survival of adult bees 2-14 d after exposure.

Repetition	Day	DF	X^2	$P > X^2$
1	2	5	36.03	<0.0001
	7	5	23.80	0.0002
	14	5	21.00	0.0008
2	2	5	35.06	<0.0001
	7	5	41.11	<0.0001
	14	5	45.69	<0.0001
3	2	-	-	-
	7	5	13.99	0.0157
	14	5	18.40	0.0025
All	2	6	33.28	<0.0001
	7	6	22.00	0.0012
	14	6	15.36	0.0176

- not analysed due to lack of data or variation

TABLE 64. Outcome of analysis of effect of Karate dosage on the survival of adult bees 2-14 d after exposure.

Repetition	Day	DF	X^2	$P > X^2$
1	2	5	6.23	0.2844
	7	5	15.38	0.0089
	14	5	3.91	0.5618
2	2	5	33.20	<0.0001
	7	5	34.12	<0.0001
	14	5	21.61	0.0006
3	2	5	38.79	<0.0001
	7	5	25.98	<0.0001
	14	5	15.72	0.0077
All	2	-	-	-
	7	6	5.41	0.4928
	14	6	4.35	0.6295

- not analysed due to lack of data or variation

Appendix B.3.2 Pesticide and fungus

TABLE 65. Outcome of analysis of effect of Biscaya dosage and inoculation with the pathogenic fungus B. *bassiana* on the survival of adult bees 2-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	P > X^2
1	2	Pesticide dosage	4	40.25	<0.0001
		Fungus level	1	0.06	0.8046
	7	Pesticide dosage*Fungus level	2	8.31	0.0157 [i]
	14	Pesticide dosage*Fungus level	2	14.47	0.0007 [i]
2	2	Pesticide dosage	4	19.05	0.0008
		Fungus level	1	0.52	0.4724
	7	Pesticide dosage	4	20.96	0.0003
		Fungus level	1	0.12	0.7249
	14	Pesticide dosage*Fungus level	2	7.42	0.0244 [i]
Both	2	Pesticide dosage	4	31.65	<0.0001
		Fungus level	1	0.48	0.4881
	7	Pesticide dosage*Fungus level	2	10.34	0.0057 [i]
	14	Pesticide dosage*Fungus level	2	19.41	<0.0001 [i]

[i] interactive effect between pesticide pesticide dosage and fungus level – test values for interaction shown.

TABLE 66. Outcome of analysis of effect of Biscaya dosage and inoculation with the pathogenic fungus *B. bassiana* on the the production of eggs 0-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	P > X^2
1	0	Pesticide dosage	4	5.6409	0.2276
		Fungus level	1	4.4652	0.0346
	7	Pesticide dosage	4	9.3244	0.0535
		Fungus level	1	2.7143	0.0995
	14	Pesticide dosage	4	7.5967	0.1075
		Fungus level	1	1.3033	0.2536
2	0	Pesticide dosage	4	2.7874	0.5940
		Fungus level	1	4.3823	0.0363
	7	Pesticide dosage	4	0.6429	0.9582
		Fungus level	1	3.5330	0.0602
	14	Pesticide dosage	4	0.5749	0.9658
		Fungus level	1	1.0824	0.2982
Both	0	Pesticide dosage	4	2.7951	0.5927
		Fungus level	1	5.2164	0.0224
	7	Pesticide dosage	4	6.1240	0.1901
		Fungus level	1	4.3656	0.0367
	14	Pesticide dosage	4	3.4751	0.4817
		Fungus level	1	1.1956	0.2742

TABLE 67. . Outcome of analysis of effect of Fastac dosage and inoculation with the pathogenic fungus *B. bassiana* on the survival of adult bees 2-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	2	Pesticide dosage*Fungus level	2	8.24	0.0163 [i]
	7	Pesticide dosage	4	2.85	0.5829
		Fungus level	1	11.68	0.0006
	14	Pesticide dosage*Fungus level	2	10.96	0.0042 [i]
2	2	Pesticide dosage	4	3.05	0.5503
		Fungus level	1	7.24	0.0071
	7	Pesticide dosage	4	5.06	0.2817
		Fungus level	1	14.85	0.0001
	14	Pesticide dosage	4	6.05	0.1952
		Fungus level	1	14.51	0.0001
Both	2	Pesticide dosage	4	3.83	0.4297
		Fungus level	1	17.13	<0.0001
	7	Pesticide dosage	4	4.33	0.3632
		Fungus level	1	25.56	<0.0001
	14	Pesticide dosage*Fungus level	2	7.88	0.0194 [i]

[i] interactive effect between pesticide pesticide dosage and fungus level – test values for interaction shown.

TABLE 68. Outcome of analysis of effect of Fastac dosage and inoculation with the pathogenic fungus *B. bassiana* on the production of honey pots 0-14 d after pesticide exposure.

Repetition	Day	Source	Num DF	Den DF	F	P > F
1	0	Pesticide dosage	4	19	0.85	0.5121
		Fungus level	1	19	3.30	0.0853
	7	Pesticide dosage	4	19	0.13	0.9679
		Fungus level	1	19	3.57	0.0742
	14	Pesticide dosage	4	19	1.64	0.2055
		Fungus level	1	19	7.67	0.0122
2	0	Pesticide dosage	4	19	0.86	0.5074
		Fungus level	1	19	0.16	0.6946
	7	Pesticide dosage	4	19	1.01	0.4284
		Fungus level	1	19	8.63	0.0084
	14	Pesticide dosage	4	19	0.27	0.8929
		Fungus level	1	19	2.46	0.1331
Both	0	Pesticide dosage	4	44	0.70	0.5961
		Fungus level	1	44	0.38	0.5395
	7	Pesticide dosage	4	44	1.06	0.3866
		Fungus level	1	44	12.20	0.0011
	14	Pesticide dosage	4	44	1.08	0.3771
		Fungus level	1	44	9.15	0.0041

TABLE 69. Outcome of analysis of effect of Karate dosage and inoculation with the pathogenic fungus *B. bassiana* on the survival of adult bees 2-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	2	Pesticide dosage	4	10.34	0.0351
		Fungus level	1	1.81	0.1782
	7	Pesticide dosage	4	2.91	0.5734
		Fungus level	1	0.38	0.5375
	14	Pesticide dosage*Fungus level	2	9.68	0.0079 [i]
2	2	Pesticide dosage	4	16.18	0.0028
		Fungus level	1	0.01	0.9246
	7	Pesticide dosage	4	12.28	0.0154
		Fungus level	1	2.34	0.1265
	14	Pesticide dosage	4	9.14	0.0578
		Fungus level	1	3.79	0.0517
Both	2	Pesticide dosage	4	20.97	0.0003
		Fungus level	1	0.92	0.3370
	7	Pesticide dosage	4	11.17	0.0248
		Fungus level	1	0.28	0.5935
	14	Pesticide dosage*Fungus level	2	9.38	0.0092 [i]

[i] interactive effect between pesticide pesticide dosage and fungus level – test values for interaction shown.

Appendix B.3.3 Pesticide and starvation

TABLE 70. Outcome of analysis of effect of Biscaya dosage and starvation on the survival of adult bees 2-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	2	Pesticide dosage	4	9.61	0.0475
		Starvation level	1	1.15	0.2841
	7	Pesticide dosage	4	8.83	0.0656
		Starvation level	1	0.56	0.4529
	14	Pesticide dosage	4	8.84	0.0653
		Starvation level	1	1.48	0.2243
2	2	Pesticide dosage	4	21.62	0.0002
		Starvation level	1	0.02	0.8902
	7	Pesticide dosage	4	16.58	0.0023
		Starvation level	1	0.26	0.6090
	14	Pesticide dosage	4	8.22	0.0839
		Starvation level	1	1.16	0.2824
Both	2	Pesticide dosage	4	25.47	<0.0001
		Starvation level	1	0.22	0.6356
	7	Pesticide dosage	4	17.68	0.0014
		Starvation level	1	0.57	0.4504
	14	Pesticide dosage	4	7.39	0.1164
		Starvation level	1	2.02	0.1554

TABLE 71. Outcome of analysis of effect of Biscaya dosage and starvation on the production of eggs 0-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	0	Pesticide dosage	4	3.2368	0.5190
		Starvation level	1	0.2978	0.5852
	7	Pesticide dosage	4	1.2975	0.8618
		Starvation level	1	3.8468	0.0498
	14	Pesticide dosage	4	8.3703	0.0789
		Starvation level	1	8.3859	0.0038
2	0	Pesticide dosage	4	0.0230	0.9999
		Starvation level	1	0.0229	0.8797
	7	Pesticide dosage	4	0.0230	0.9999
		Starvation level	1	0.0229	0.8797
	14	Pesticide dosage	4	0.0093	1.0000
		Starvation level	1	0.0118	0.9135
Both	0	Pesticide dosage	4	3.1777	0.5285
		Starvation level	1	0.2516	0.6160
	7	Pesticide dosage	4	1.1511	0.8861
		Starvation level	1	2.8300	0.0925
	14	Pesticide dosage	4	6.2405	0.1819
		Starvation level	1	7.1285	0.0076

TABLE 72. Outcome of analysis of effect of Fastac dosage and starvation on the survival of adult bees 2-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	2	Pesticide dosage	4	13.98	0.0074
		Starvation level	1	13.70	0.0002
	7	Pesticide dosage	4	6.28	0.1789
		Starvation level	1	15.22	<0.0001
	14	Pesticide dosage	4	5.59	0.2318
		Starvation level	1	16.09	<0.0001
2	2	Pesticide dosage	4	7.39	0.1167
		Starvation level	1	0.50	0.4797
	7	Pesticide dosage	4	6.74	0.1501
		Starvation level	1	0.06	0.7989
	14	Pesticide dosage*Starvation level	2	10.28	0.0059 [i]
Both	2	Pesticide dosage*Starvation level	2	6.15	0.0462 [i]
	7	Pesticide dosage*Starvation level	2	8.82	0.0121 [i]
	14	Pesticide dosage*Starvation level	2	13.52	0.0012 [i]

[i] interactive effect of pesticide pesticide dosage and starvation level – test values for interaction shown

TABLE 73. Outcome of analysis of effect of Fastac dosage and starvation on the production of eggs 0-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	$P > X^2$
1	0	Pesticide dosage	4	0.7898	0.9398
		Starvation level	1	4.5393	0.0331
	7	Pesticide dosage	4	2.3049	0.6799
		Starvation level	1	6.2087	0.0127
	14	Pesticide dosage	4	1.0648	0.8998
		Starvation level	1	0.0411	0.8393
2	0	Pesticide dosage	4	7.3976	0.1163
		Starvation level	1	9.0324	0.0027
	7	Pesticide dosage	4	0.7684	0.9426
		Starvation level	1	4.8512	0.0276
	14	Pesticide dosage	4	1.3982	0.8445
		Starvation level	1	4.7697	0.0290
Both	0	Pesticide dosage	4	4.0314	0.4018
		Starvation level	1	12.7243	0.0004
	7	Pesticide dosage	4	1.5899	0.8106
		Starvation level	1	11.1263	0.0009
	14	Pesticide dosage	4	1.8160	0.7696
		Starvation level	1	16.1255	<0.0001

TABLE 74. Outcome of analysis of effect of Fastac dosage and starvation on the production of honey pots 0-14 d after pesticide exposure.

Repetition	Day	Source	Num DF	Den DF	F	P > F
1	0	Pesticide dosage	4	19	0.47	0.7571
		Starvation level	1	19	5.61	0.0286
	7	Pesticide dosage	4	19	0.46	0.7607
		Starvation level	1	19	9.01	0.0073
	14	Pesticide dosage	4	19	1.75	0.1809
		Starvation level	1	19	8.88	0.0077
2	0	Pesticide dosage	4	19	0.21	0.9309
		Starvation level	1	19	0.01	0.9190
	7	Pesticide dosage	4	19	0.06	0.9933
		Starvation level	1	19	0.17	0.6866
	14	Pesticide dosage	4	19	0.18	0.9440
		Starvation level	1	19	0.21	0.6542
Both	0	Pesticide dosage	4	44	0.15	0.9599
		Starvation level	1	44	2.09	0.1551
	7	Pesticide dosage	4	44	0.14	0.9657
		Starvation level	1	44	2.51	0.1201
	14	Pesticide dosage	4	44	0.88	0.4840
		Starvation level	1	44	4.97	0.0309

TABLE 75. Outcome of analysis of effect of Karate dosage and starvation on the production of eggs 0-14 d after pesticide exposure.

Repetition	Day	Source	DF	X^2	P > X^2
1	0	Pesticide dosage	4	0.0141	1.0000
		Starvation level	1	0.0189	0.8906
	7	Pesticide dosage	4	1.0670	0.8995
		Starvation level	1	6.3054	0.0120
	14	Pesticide dosage	4	2.9997	0.5579
		Starvation level	1	0.0045	0.9466
2	0	Pesticide dosage	4	0.4578	0.9775
		Starvation level	1	1.2857	0.2569
	7	Pesticide dosage	4	3.7929	0.4348
		Starvation level	1	1.2867	0.2567
	14	Pesticide dosage	4	1.8205	0.7687
		Starvation level	1	7.6004	0.0058
Both	0	Pesticide dosage	4	0.1609	0.9969
		Starvation level	1	2.5876	0.1077
	7	Pesticide dosage	4	3.2541	0.5162
		Starvation level	1	4.8835	0.0271
	14	Pesticide dosage	4	0.5678	0.9666
		Starvation level	1	12.1369	0.0005

Appendix B.4 Field tests

TABLE 76. Two-way ANOVA of effect of date and pesticide dosage on relative hive weight (wt/w0) for each pesticide at each study site. No statistics are reported for Vejlsøvej due to only one replicate.

Pesticide	Study site	Source	DF	Type III SS	Mean Square	F Value	P > F
Biscaya	Moesgaard	Pesticide dosage	3	1.64978	0.54993	5.22	0.0026
		Date	6	2.32544	0.38757	3.68	0.0031
	Funder	Pesticide dosage	3	0.7874	0.2625	2.32	0.0808
		Date	7	7.6312	1.0902	9.65	<0.0001
	Them	Pesticide dosage	3	0.56430	0.18810	2.61	0.0573
		Date	7	10.49566	1.49938	20.84	<0.0001
Fastac	Moesgaard	Pesticide dosage	3	8.28062	2.76021	28.46	<0.0001
		Date	6	5.66727	0.94455	9.74	<0.0001
		Pesticide dosage*Date	17	3.38466	0.19910	2.05	0.0248
	Funder	Pesticide dosage	3	3.2671	1.0890	9.37	<0.0001
		Date	7	4.0596	0.5799	4.99	<0.0001
	Them	Pesticide dosage	3	1.70750	0.56917	7.60	0.0002
		Date	7	5.40182	0.77169	10.31	<0.0001
Karate	Moesgaard	Pesticide dosage	3	1.01295	0.33765	7.80	0.0001
		Date	6	11.93887	1.98981	45.99	<0.0001
	Funder	Pesticide dosage	3	1.5744	0.5248	7.87	0.0001
		Date	7	11.0715	1.5816	23.73	<0.0001
	Them	Pesticide dosage	3	1.17516	0.39172	3.71	0.0150
		Date	7	7.53069	1.07581	10.20	<0.0001

TABLE 77. Pairwise comparison (contrasts) of relative hive weight of each pesticide dosage against control. Red asterisks indicate that pesticide-treated hives were larger than control hives.

Study site	Pesticide	Contrast	Estimate	SE	t	P > |t|	
Moesgaard	Biscaya	5	-0.14372558	0.10	-1.42	0.1615	n.s.
		25	-0.32012335	0.10	-3.10	0.0028	**
		100	-0.35554177	0.10	-3.50	0.0008	**
	Fastac	0.1	-0.29929612	0.10	-2.87	0.0060	*
		0.5	-0.63641112	0.10	-6.51	<0.0001	***
	Karate	0.0025	-0.03175376	0.07	-0.47	0.6395	n.s.
		0.01	-0.08030464	0.07	-1.20	0.2333	n.s.
		0.05	0.21465555	0.07	3.21	0.0020	**
Funder	Biscaya	5	-0.11664129	0.10	-1.20	0.2327	n.s.
		25	0.06342588	0.10	0.65	0.5152	n.s.
		100	-0.16436101	0.10	-1.69	0.0940	n.s.
	Fastac	0.025	-0.40208930	0.10	-4.08	<0.0001	***
		0.1	-0.45601895	0.10	-4.63	<0.0001	***
		0.5	-0.15980061	0.10	-1.62	0.1082	n.s.
	Karate	0.0025	0.06629835	0.07	0.89	0.3762	n.s.
		0.01	0.33978598	0.07	4.56	<0.0001	***
		0.05	0.10445764	0.07	1.40	0.1647	n.s.
Them	Biscaya	5	0.00121653	0.08	0.01	0.9884	n.s.
		25	-0.19554906	0.08	-2.44	0.0172	*
		100	-0.06281637	0.08	-0.77	0.4456	n.s.
	Fastac	0.025	-0.20153769	0.08	-2.55	0.0126	*
		0.1	-0.34684076	0.08	-4.39	<0.0001	***
		0.5	-0.05809571	0.08	-0.71	0.4803	n.s.
	Karate	0.0025	-0.30263211	0.10	-3.01	0.0035	**
		0.01	-0.13265894	0.10	-1.35	0.1796	n.s.
		0.05	-0.02831148	0.10	-0.29	0.7690	n.s.

*P<0.05, ** P<0.005, *** P<0.0005

TABLE 78. Two-way ANOVA of effect of date and pesticide dosage on relative hive weight (wt/w0) for each pesticide for all study sites pooled.

Pesticide	Effect	DF	F	P > F	
Biscaya	pesticide dosage	3	5.28	0.0015	**
	date	7	22.32	<0.0001	***
Fastac	pesticide dosage	3	16.80	<0.0001	***
	date	7	16.06	<0.0001	***
Karate	pesticide dosage	3	4.96	0.0023	**
	date	7	49.00	<0.0001	***

no interaction effects were found; *P<0.05, ** P<0.005, *** P<0.0005

TABLE 79. Pairwise comparisons (contrasts) of relative hive weight of each pesticide dosage against control.

Pesticide	Contrast	Estimate	t	P > |t|	
Biscaya	5	-0.1091	-2.02	0.0444	*
	25	-0.1725	-3.22	0.0015	**
	100	-0.1946	-3.62	0.0004	***
Fastac	0.025	-0.1215	-2.06	0.0398	*
	0.1	-0.3680	-6.28	<0.0001	***
	0.5	-0.3117	-5.30	<0.0001	***
Karate	0.0025	-0.1054	-2.17	0.0308	*
	0.01	0.04633	0.96	0.3358	n.s.
	0.05	0.06476	1.36	0.1761	n.s.

*P<0.05, ** P<0.005, *** P<0.0005

TABLE 80. Analysis of number of days until maximum hive weight is obtained (model df = 3), including pairwise comparisons (contrasts) with untreated control hives.

Study site	Pesticide	N	F	P		R^2	contrasts to pesticide dosage 0
Funder	Biscaya	12	1.03	0.4309	n.s.	0.277922	all P>0.05
	Fastac	12	1.46	0.2961	n.s.	0.354140	all P>0.05
	Karate	12	0.67	0.5957	n.s.	0.20000	all P>0.05
Moesgaard	Biscaya	12	0.63	0.6158	n.s.	0.191123	all P>0.05
	Fastac	12	1.68	0.2469	n.s.	0.387002	all P>0.05
	Karate	12	0	1	n.s.	0	all P>0.05
Them	Biscaya	12	2.55	0.1393	n.s.	0.521739	all P>0.05 (but P=0.056 for Biscaya)
	Fastac	12	1.25	0.3548	n.s.	0.318900	all P>0.05
	Karate	12	0.44	0.7293	n.s.	0.159722	all P>0.05
Vejlsøvej	Biscaya	4	-	-	-	-	-
	Fastac	4	-	-	-	-	-
	Karate	4	-	-	-	-	-

Analyses could not be done for Vejlsøvej because of only one replicate per pesticide dosage

TABLE 81. Outcome of F test effect of pesticide dosage on hive weight change and relative hive weight per week after pesticide exposure, analysed on data pooled from all study sites (DF = 3 for each test value).

Week after exposure	Weight change						Relative weight					
	Biscaya		Fastac		Karate		Biscaya		Fastac		Karate	
	F	P > F	F	P > F	F	P > F	F	P > F	F	P > F	F	P > F
1	3.36	0.0291	4.75	0.0068	1.29	0.2933	3.72	0.0200	4.92	0.0058	1.32	0.2821
2	2.52	0.0735	3.06	0.0406	0.57	0.6367	2.78	0.0551	3.51	0.0250	0.57	0.6400
3	2.04	0.1258	3.49	0.0254	0.65	0.5884	2.24	0.0999	4.08	0.0135	0.63	0.6001
4	1.20	0.3242	3.14	0.0369	0.51	0.6809	1.35	0.2726	3.70	0.0203	0.42	0.7389
5	0.29	0.8331	2.06	0.1227	0.99	0.4092	0.35	0.7910	2.24	0.0999	0.64	0.5962
6	0.23	0.8718	2.16	0.1102	1.31	0.2852	0.26	0.8522	1.91	0.1452	0.87	0.4673
7	0.09	0.9631	1.14	0.3494	1.69	0.1933	0.12	0.9457	1.27	0.3030	1.39	0.2664
8	0.41	0.7481	1.45	0.2551	2.76	0.0673	0.38	0.7651	1.70	0.1945	2.23	0.1150

Appendix C. Comparison of methods for measuring activity

TABEL 82. Time spent on different activities by individual bees. The table includes data from RFID measurements (level, form, N) and from traditional observations of the bees

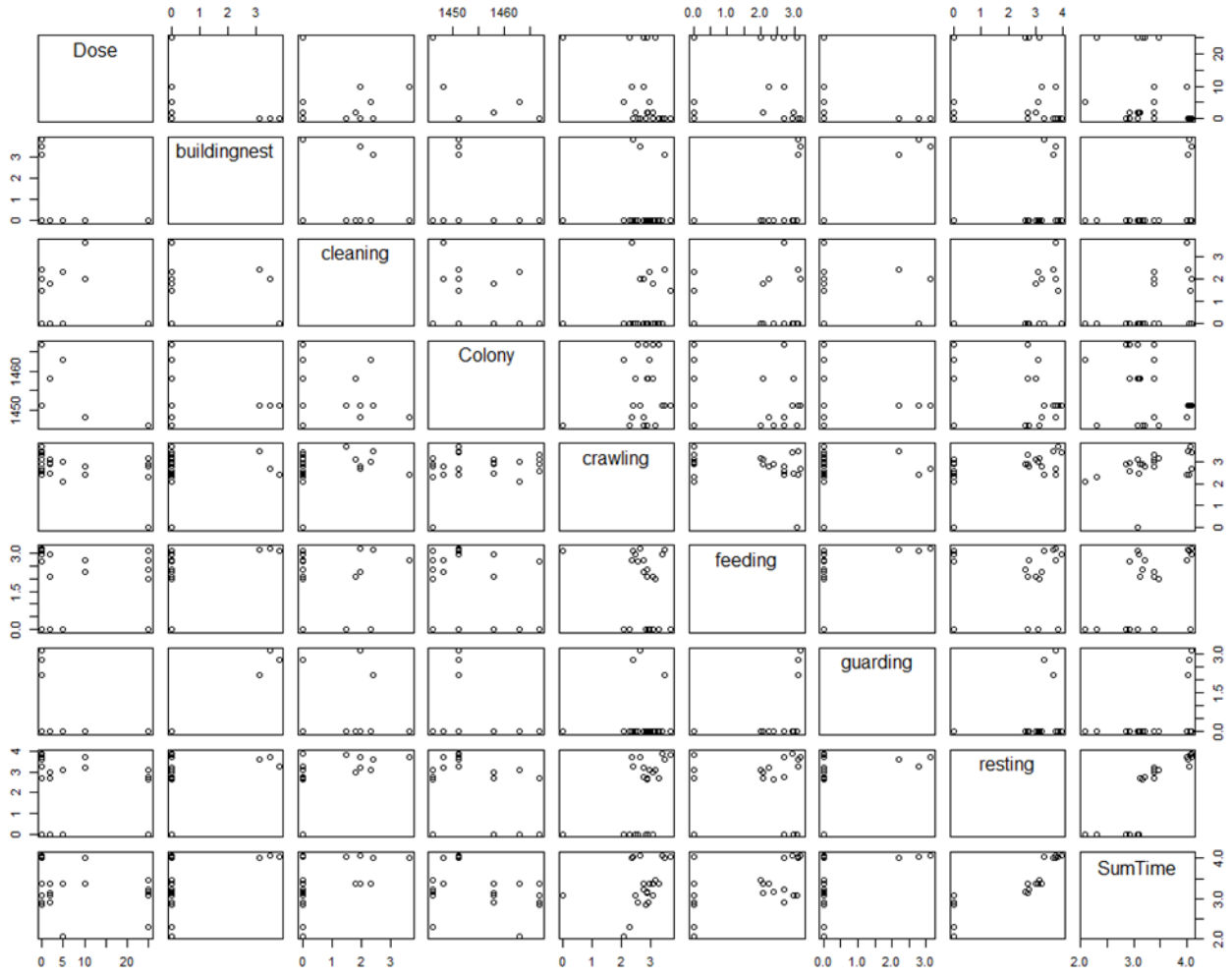
Colony	Dose	crawling	pfeeding	resting	buildingnest	cleaning	guarding	SumTime	level	form	N
1446	25	1464	100	1338	0	0	0	2902			0
1446	25	199	0	0	0	0	0	199	2073.88	0.485925	5
1446	25	599	508	539	0	0	0	1646			0
1446	25	0	1198	0	0	0	0	1198			1
1446	25	778	239	419	0	0	0	1436	5501.098	0.472563	4
1448	10	568	177	1553	0	90	0	2388	25170.37	1.101074	4
1448	10	239	509	5089	0	4257	0	10094			0
1451	0	2991	1317	4294	1356	249	159	10366	1390.776	0.485006	255
1451	0	447	1514	5175	3335	90	1379	11940	1263.044	0.494309	437
1451	0	249	1256	1916	6752	0	619	10792	2240.403	0.469928	207
1451	0	4807	0	6483	0	29	0	11319	910.6373	0.532935	466
1451	0	2683	898	8361	0	0	0	11942	613.0783	0.454919	502
1458	2	1198	0	0	0	0	0	1198	487.7998	0.515915	50
1458	2	838	0	0	0	0	0	838			0
1458	2	746	119	479	0	0	0	1344	384.8488	0.4663	57
1458	2	298	956	0	0	0	0	1254	696.1648	0.52382	28
1458	2	1197	119	1017	0	60	0	2393			0
1463	5	957	0	1225	0	208	0	2390	2618.83	0.531454	12
1463	5	119	0	0	0	0	0	119			0
1467	0	1199	0	0	0	0	0	1199	292.2951	0.512622	83
1467	0	718	0	0	0	0	0	718	422.5548	0.475957	40
1467	0	1199	0	0	0	0	0	1199	529.9277	0.621134	65
1467	0	359	478	0	0	0	0	837			0
1467	0	1917	0	479	0	0	0	2396			0

Appendix D. Correlations between activity measures for bees with RFID tags

Correlations between activity categories measured by observing the bees and by RFID techniques (form=the form parameter of the Weibull distribution, level=the level parameter from the same distribution, N=total n. RFID readings per bee). All variables, exclusive Dose and form, have been transformed by log(1+x) to avoid that a few data points dominate the plot scaling.

Appendix E. Correlations between activity measures – full data set

Scatter matrix showing pairwise correlations of all activity categories measured by observing the bees. This data set also includes data for bees that had lost both RFID tags. All variables, exclusive Dose and form, have been transformed by log(1+x) to avoid that a few data points dominate the plot scaling. This data set also includes data for bees that have lost both RFID tags, so this data extends the data set including RFID readings.



Pesticide Effects on Bumble-Bees

In the current standard risk assessment of pesticides, the risk of adverse effects on honeybees is assessed by short-term (48 hours) tests, in which only the effect on adult survival is measured. However, regulations based on these risk assessments may not protect these beneficial insects sufficiently. In order to investigate sub-lethal effects of pesticides, studies using lower dosages and measuring effects over a longer period are required.

Furthermore, in laboratory tests, conditions are generally optimal for the test animals, while in nature pesticides are seldom the only stressor of bees. In most landscapes in Northern Europe, periods of food scarcity occur, and bees are exposed to a range of different pathogens and parasites. Thus, an interesting aspect to investigate is whether bees exposed to other stressors, including nutritional stress or pathogens are more sensitive to pesticides than non-stressed bees.

Honeybees are not the only pollinating insects affected by pesticides. However, it is unknown whether the sensitivity of wild pollinators including bumble-bees, solitary bees, hover flies and butterflies differs from honeybees, which have a very different life history. Therefore, this project focusses on another common pollinator, the buff-tailed bumble-bee, *Bombus terrestris*. We have investigated the impact of three insecticides (Biscaya, Fastac and Karate), all of which are used in flowering crops that attract flower-visiting insects. Hence, foraging bumble-bees are expected to be exposed to pesticides by direct contact when handling sprayed flowers or by ingesting them with pollen and nectar.

Four types of tests were included in the study:

1. Short-term laboratory tests, in which bumble-bees are tested as in the honeybee acute tests. Results from these tests show mortality after 48 hours.
2. Long-term (14 d) laboratory studies that, in addition to mortality, assess effects on reproduction, such as numbers of eggs and larvae. In these studies, we used queen-less micro-colonies, which are easy to manage.
3. Semi-field tests, in which the bees in the micro-colonies did not only live in the small box, but had access to a larger cage in which they could move around and forage on artificial flowers. Effects were measured in the same manner as in the long-term laboratory tests.
4. Field studies, in which hives with queen-right colonies of bumble-bees were exposed by feeding them sugar solution containing pesticide and thereafter released into landscapes with a low pesticide load. The development of the bee families was measured by weighing the nests on a weekly basis for eight weeks.

In the laboratory and semi field tests, in addition to studying the effect of pesticides alone, we tested whether the sensitivity of bumble-bees to effects of pesticides increased when the bees were infected by an insect-pathogenic fungus or were starved prior to the experiments.

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