Journal of Apicultural Research 52(4): (2013)
DOI 10.3896/IBRA.1.52.4.14

REVIEW ARTICLE

Standard methods for toxicology research in *Apis mellifera*

Piotr Medrzycki[1*], Hervé Giffard[2], Pierrick Aupinel[3], Luc P Belzunces[4], Marie-Pierre Chauzat[5], Christian Claßen[6], Marc E Colin[7], Thierry Dupont[8], Vincenzo Girolami[9], Reed Johnson[10], Yves Le Conte[4], Johannes Lückmann[6], Matteo Marzaro[9], Jens Pistorius[11], Claudio Porrini[12], Andrea Schur[13], Fabio Sgolastra[12], Noa Simon Delso[14], Jozef J M van der Steen[15], Klaus Wallner[16], Cédric Alaux[4], David G Biron[17], Nicolas Blot[17], Gherardo Bogo[1], Jean-Luc Brunet[4], Frédéric Delbac[17], Marie Diogon[17], Hicham El Alaoui[17], Bertille Provost[7], Simone Tosi[1] and Cyril Vidau[18]

[1]Agricultural Research Council, Honey Bee and Silkworm Research Unit, Bologna, Italy.
[2]Testapi, Gennes, France.
[3]INRA, UE1255 Entomologie, Surgères, France.
[4]INRA, UR 406 Abeilles & Environnement, Avignon, France.
[5]ANSES, Unit of Honey bee Pathology, Sophia-Antipolis, France.
[6]RIFCON GmbH, Hirschberg, Germany.
[7]Montpellier SupAgro, USAE, Montpellier, France.
[8]Qualilab, Olivet, France.
[9]Department of Agronomy Food Natural Resources Animals and Environment, University of Padua, Legnaro (PD), Italy.
[10]Department of Entomology, The Ohio State University - Ohio Agriculture Research and Development Centre, Wooster, Ohio, USA.
[11]Julius Kühn Institut, Institute for Plant Protection in Field Crops and Grassland, Braunschweig, Germany.
[12]Department of Agroenvironmental Sciences and Technologies – Entomology, University of Bologna, Bologna, Italy.
[13]Bee Department, Eurofins Agroscience Services EcoChem GmbH, Niefern-Öschelbronn, Germany.
[14]Centre Apicole de Recherche et Information (CARI), Louvain La Neuve, Belgium.
[15]Wageningen University, Biointeractions and Plant Health, Wageningen, The Netherlands.
[16]Apicultural State Institute, University Hohenheim, Stuttgart, Germany.
[17]Laboratoire Microorganismes: Génome et Environnement, CNRS UMR 6023, Université Blaise Pascal, Clermont-Ferrand, France.
[18]Laboratoire Venins et Activités Biologiques, EA 4357, PRES-Université de Toulouse, Centre de formation et de recherche Jean -François Champollion, Albi, France.

All authors except those listed first and second are alphabetical.

Received 4 July 2012, accepted subject to revision 7 December 2012, accepted for publication 17 July 2013.

*Corresponding author: Email: piotr.medrzycki@entecra.it

Summary

Modern agriculture often involves the use of pesticides to protect crops. These substances are harmful to target organisms (pests and pathogens). Nevertheless, they can also damage non-target animals, such as pollinators and entomophagous arthropods. It is obvious that the undesirable side effects of pesticides on the environment should be reduced to a minimum. Western honey bees (*Apis mellifera*) are very important organisms from an agricultural perspective and are vulnerable to pesticide-induced impacts. They contribute actively to the pollination of cultivated crops and wild vegetation, making food production possible. Of course, since *Apis mellifera* occupies the same ecological niche as many other species of pollinators, the loss of honey bees caused by environmental pollutants suggests that other insects may experience a similar outcome. Because pesticides can harm honey bees and other pollinators, it is important to register pesticides that are as selective as possible. In this manuscript, we describe a selection of methods used for studying pesticide toxicity/selectiveness towards *Apis mellifera*. These methods may be used in risk assessment schemes and in scientific research aimed to explain acute and chronic effects of any target compound on *Apis mellifera*.

Footnote: Please cite this paper as: MEDRZYCKI, P; GIFFARD, H; AUPINEL, P; BELZUNCES, L P; CHAUZAT, M-P; CLAßEN, C; COLIN, M E; DUPONT, T; GIROLAMI, V; JOHNSON, R; LECONTE, Y; LÜCKMANN, J; MARZARO, M; PISTORIUS, J; PORRINI, C; SCHUR, A; SGOLASTRA, F; SIMON DELSO, N; VAN DER STEEN, J J M; WALLNER, K; ALAUX, C; BIRON, D G; BLOT, N; BOGO, G; BRUNET, J-L; DELBAC, F; DIOGON, M; EL ALAOUI, H; PROVOST, B; TOSI, S; VIDAU, C (2013) Standard methods for toxicology research in *Apis mellifera*. In *V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS* BEEBOOK*, Volume I: standard methods for* Apis mellifera *research*. *Journal of Apicultural Research* 52(4): http://dx.doi.org/10.3896/IBRA.1.52.4.14

Métodos estándar para la investigación toxicológica en *Apis mellifera*

Resumen

La agricultura moderna a menudo implica el uso de plaguicidas para proteger los cultivos. Estas sustancias son dañinas para los organismos objetivo (plagas y patógenos). Sin embargo, también pueden dañar a animales que no son objetivo, como artrópodos polinizadores y entomófagos. Obviamente los efectos secundarios indeseables de los plaguicidas sobre el medio ambiente deben ser reducidos al mínimo. Las abejas occidentales (*Apis mellifera*) son organismos muy importantes desde el punto de vista agrícola y son vulnerables a los impactos inducidos por los plaguicidas. Contribuyen activamente a la polinización de los cultivos y de la vegetación silvestre, lo que hace posible la producción de alimentos. Como *Apis mellifera* ocupa el mismo nicho ecológico que muchas otras especies de polinizadores, la pérdida de las abejas melíferas causada por contaminantes ambientales sugiere que otros insectos pueden experimentar un resultado similar. Ya que los plaguicidas pueden dañar a las abejas y a otros polinizadores, es importante registrar los plaguicidas que sean lo más selectivos posible. En este artículo, se describe una selección de los métodos utilizados para el estudio de la toxicidad y el efecto selectivo de los plaguicidas hacia *Apis mellifera*. Estos métodos se pueden utilizar en sistemas de evaluación de riesgo y en la investigación científica para explicar los efectos agudos y crónicos en *Apis mellifera* de cualquier compuesto objetivo.

西方蜜蜂毒理学研究的标准方法

摘要

现代农业经常会使用农药以保护作物。这些物质对害虫和病原菌等靶标生物有害。但是它们也会对诸如授粉昆虫和食虫节肢动物等非靶标动物带来危害。显然，农药对环境的不良副作用应该减少到最低。从农业的角度看，西方蜜蜂是一种重要生物，同时它也极易受到农药的影响。它们对种植的作物和野生植物的授粉发挥了积极的作用，使得粮食生产成为可能。当然，因为西方蜜蜂与很多其它授粉物种处于同一个生态位，由环境污染造成的蜜蜂损失表明其它昆虫可能也在遭遇同样的经历。由于农药会危害蜜蜂和其它授粉昆虫，因此注册登记选择性尽量强的农药显得尤为重要。本文我们选择描述了一些研究针对西方蜜蜂的农药毒性和选择性的方法。这些方法可以应用于风险评估方案和旨在评估某种化合物对于西方蜜蜂的急性和慢性作用的科学研究。

Keywords: COLOSS, *BEEBOOK*, *Apis mellifera*, honey bee, pesticide, exposure, residue, lethal, sublethal, field, semifield, laboratory

Table of Contents

Table of Contents Cont'd

Table of Contents Cont'd

1. Introduction

The presence of toxic substances in the environment may be an important factor contributing to the poor health of honey bee colonies globally. Agrochemicals are of particular interest because they often are accused of causing sublethal effects in individual bees and the bee colony, possibly even leading to the loss of entire colonies and even apiaries (Maini *et al.*, 2010; Desneux *et al.*, 2007).

Honey bees are excellent bioindicators of environmental pollution (Celli and Maccagnani, 2003). Thus, it is easy to imagine that wild

pollinators (or other animals occupying the same ecological niche) present in polluted areas will suffer outcomes similar to those experienced by honey bees in the area. For this reason, the research community should work to limit the hazard of toxins to honey bees and, by doing this, will help to protect wild pollinators.

The risk assessment addressing the potential risk for pollinating insects from the use of Plant Protection Products (PPPs) is comprised by oral and contact LD_{50} (Lethal Dose that kills 50% of the population), toxicity exposure ratio (TER) and results of semi-field and field trials (e.g. direct or delayed bee mortality) highlighting the impact on brood development, foraging abilities, etc.

The registration of agrochemicals requires that specific toxicological tests be performed on honey bees, such as those required by the US Environmental Protection Agency (US EPA, 1996) and the European Organisation for Economic Co-operation and Development (OECD, 1998a; OECD, 1998b). These tests must follow specific protocols in order to (1) assess the level of selectiveness of the pesticide to honey bees and (2) satisfy a given country's pesticide regulatory requirements. They must be performed in Good Laboratory Practices (GLP).

The present chapter is not a proposal of guidelines but rather a compendium of methods for testing toxic effects of agrochemicals and other compounds on honey bees. These methods may be used in scientific studies and in official risk assessment schemes where appropriate or where consistent with a given government's requirements. To be used for the latter, the test should undergo regulatory testing and risk assessment systems in order to be properly validated. Nevertheless, both OECD 75 (tunnel test) and acute toxicity standards (OECD 213 and 214) have not been ring-tested despite that they are referenced by all OECD members as standard methodologies.

2. Common terms and abbreviations

Here are some abbreviations and definition of terms used in this manuscript listed in alphabetical order.

Acute oral toxicity: the adverse effects occurring within a maximum period of 96 h of an oral administration of a single dose of test substance.

Acute contact toxicity: the adverse effects occurring within a maximum period of 96 h of a topical application of a single dose of test substance.

AI: active ingredient - the substance composing a commercial formulation of a pesticide which has the desired effects on target organisms.

BFD: Brood area Fixing Day (see sections 5.2.2. and 5.2.3.)

CEB: Biological Tests Commission (Commission des Essais Biologiques), of the French Plant Protection Association (AFPP - Association Française de Protection des Plantes)

Dose (contact): the amount of test substance applied. Dose is expressed as mass (µg) of test substance per test animal (honey bee) or per mg body weight (in non-*Apis* bees).

Table 1. Possible honey bee behavioural effects due to exposure to pesticides in individual tests. Note: "freeze" and "paralysis" bees may be recorded as dead bees at a certain point and later as living bees.

Effect	Looks like	To be recorded as:
Dead	Immobile, no reaction to stimuli such as touching with forceps	Mortality, number of bees
No effect	Bees having normal behaviour	NE, number of bees observed
Freeze	Motionless bees caught in action and looking active such as attached to feeder, standing on the floor but actually completely inactive.	F, number of bees observed
Paralysis	Motionless on the floor of the test cage, responding to stimuli by moving leg, antenna etc.	P, number of bees observed
Spasm	Crawling bees, movement uncoordinated	S, number of bees observed

Dose (oral): the amount of test substance consumed. Dose is expressed as mass (µg) of test substance per test animal (honey bee), or per mg body weight (in non-*Apis* bees). In tests with bulk administration the real dose for each bee cannot be calculated as the bees are fed collectively, but an average dose can be estimated (total test substance consumed/number of test bees in one cage).

EEC: European Economic Community.

ED_{50}: median effective dose - term extending LD_{50} (see below in this section) to the effects other than mortality, e.g. behaviour (see Table 1 and Scheiner *et al.*, 2013)

EFSA: European Food Safety Authority - an agency of European Union (EU) risk assessment regarding food and feed safety. In close collaboration with national authorities and in open consultation with its stakeholders, EFSA provides independent scientific and clear communication on existing and emerging risks. (from: EFSA)

EPPO: European and Mediterranean Plant Protection Organisation - an intergovernmental organisation responsible for European cooperation in plant protection in the European and Mediterranean region. EPPO's objectives are to: (1) protect plants; (2) develop international strategies against the introduction and spread of dangerous pests; and (3) promote safe and effective control methods. EPPO has developed international standards and recommendations on phytosanitary measures, good plant protection practices and on the assessment of PPPs. (from: Wikipedia)

GAP: Good Agricultural Practices - specific methods which, when applied to agriculture, create food for consumers or further processing that is safe and wholesome. The Food and Agricultural Organization of the United Nations (FAO) uses GAP as a collection of principles to apply for on-farm production and post-production processes, resulting in safe and healthy food and non-food agricultural products, while taking into account economic, social and environmental sustainability.

GLP: Good Laboratory Practices - a set of principles that provides a framework within which laboratory studies are planned, performed, monitored, recorded, reported and archived. These studies are undertaken to generate data by which the hazards and risks to users,

consumers and third parties, including the environment, can be assessed for pharmaceuticals (only preclinical studies), agrochemicals, cosmetics, food additives, feed additives and contaminants, novel foods, biocides, detergents etc. GLP helps assure regulatory authorities that the data submitted are a true reflection of the results obtained during the study and can therefore be relied upon when making risk/ safety assessments. (from: Medicines and Healthcare products Regulatory Agency-UK)

HQ: Hazard Quotient. See section 8.4.2.1.

ICPPR: International Commission for Plant-Pollinator Relationships (formerly ICPBR: International Commission for Plant-Bee Relationships) - an international commission aimed to: (1) promote and coordinate research on the relationships between plants and pollinators of all types. (insect-pollinated plants, bee foraging behaviour, effects of pollinator visits on plants, management and protection of insect pollinators, bee collected materials from plants, products derived from plants and modified by bees); (2) organise meetings, colloquia or symposia related to the above topics and to publish and distribute the proceedings; and (3) collaborate closely with national and international institutions interested in the relationships between plants and bees, particularly those whose objectives are to expand scientific knowledge of animal and plant ecology and fauna protection.

IGR: Insect Growth Regulator - a chemical substance used as an insecticide that inhibits the life cycle of an insect. Normally the IGRs target juvenile harmful insect populations while cause less detrimental effects to beneficial insects.

LD_{50} / LC_{50}: median lethal dose / concentration - a statistically derived single dose /concentration of a substance that can cause death in 50% of animals when administered by the contact or oral route (according to the test), or combined (like in brood test). The LD_{50} value is expressed in µg of test substance per test animal (honey bee), or per mg body weight (in non-*Apis* bees). The LC_{50} value is expressed in concentration units, like mg of test substance / kg or L of the diet (pollen, syrup, honey). For pesticides, the test substance may be either an AI or a formulated product containing one or more than one AI. See section 8.2.1.2.

Moribund bee: a bee is considered moribund when it is not dead (it still moves) but is not able to deambulate actively and in an apparently "normal" way.

Mortality: an animal is recorded as dead when it is completely immobile upon prodding (Ffrench-Constant and Rouch, 1992).

NOAEC: Non Observable Adverse Effect Concentration. See section 8.4.3.

NOAEL/NOAED: Non Observable Adverse Effect Level/Dose (these are two synonyms). See section 8.4.3.

OECD: Organisation for Economic Co-operation and Development - an international economic organisation of 34 countries aimed to stimulate economic progress and world trade.

PER test: Proboscis Extension Reflex (see Scheiner *et al.*, 2013)

PPP: Plant Protection Product - active ingredient of a chemical or biological nature and preparation containing one or more active ingredients, or formulated preparation of microorganisms, put up in the form in which it is supplied to the user, intended to: (1) protect plants or plant products against all harmful organisms or prevent the action of such organisms; (2) influence the life processes of plants, other than as a nutrient, (e.g. growth regulators); (3) preserve plant products; (4) destroy undesired plants; or (5) destroy parts of plants, check or prevent undesired growth of plants. PPPs include: fungicides, bactericides, insecticides, acaricides, nematicides, rodenticides, herbicides, molluscicides, virucides, soil fumigants, insect attractants (e.g. pheromones used in control strategies), repellents (bird, wild life, rodent, insect repellents), stored product protectants, plant growth regulators, products to improve plant resistance to pests, products to inhibit germination, products to eliminate aquatic plants and algae, desiccants and defoliants to destroy parts of plants, products to assist wound healing, products to preserve plants or plant parts after harvest, timber preservatives (for fresh wood), additives to sprays to improve the action of any other PPP, additives to reduce the phytotoxicity of any other PPP. They do not include: fertilizers, timber preservatives (for dried wood). (from: EEC and EPPO)

RQ: Risk Quotient. See section 8.4.2.2.

SSST: Systemic product as Seed and Soil Treatment

Sub-lethal dose/concentration: the dose/concentration inducing no statistically significant mortality.

Sub-lethal effects: the effects of a factor (e.g. intoxication) which was administered at such a low level that the mortality was not significantly higher than in negative reference. These (generally negative) effects can have either behavioural (disorientation, problems with memory, etc.) or physiological nature (pharyngeal gland development impairment, thermoregulation problems, etc.).

TER: Toxicity Exposure Ratio – the ratio between a toxicity index (LD_{50}, LC_{50}, NOAEL…) and the predicted bee exposure in field conditions following a treatment.

3. Effects of toxic substances on adult worker bees: individual assays

This section describes methods for determining the toxicity of test compounds on adult bees in instances where the insects have no possibility of interacting with the hive. The bees are treated individually or within small experimental groups of individuals. The individual adult honey bee is the experimental unit.

3.1. Introduction

3.1.1. Definitions of poisoning and exposure

Poisoning is generally defined as injury or impairment of organ function

or death, following exposure to any substance capable of producing adverse effects (Hodgson, 2004). The toxin can have local and/or systemic effects for varying periods of time. Depending on the severity of the effects, poisoning can be considered acute or chronic, both types with varying degrees of intensity. Often, acute poisoning leads to a rapid death.

Exposure is the encounter of the living organism with the poison. It may be characterised by many parameters: duration, number of replications, interval of time, routes of penetration into the body etc. The evaluation of exposure is the key point in experimental toxicology to provide valuable data.

3.1.2. Exploration of acute poisoning using the lethality criterion

Lethality is the most common experimental criterion in bee toxicology. In toxicological tests, an insect usually is considered dead when it exhibits "no movements after prodding" (see section 2). Using this criterion, investigators often use correlation metrics to link the lethality and dose of a toxic substance to a test subject. This assumes that the group of subjects to be tested are randomly selected from a population with a normal distribution (Gaussian) susceptibility to the toxic substance.

The cumulative distribution of the normal probability density is an increasing sigmoidal function (Wesstein, http://mathworld.wolfram.com). In matter of toxicology, the consequence is that the theoretical dose-cumulated lethality (% lethality) relation is a sigmoid ranging from 0% to 100% lethality. To transform the sigmoid into a straight line, Bliss (1934) proposed to use the logarithm of the doses in X axis and the probability units or probits in Y axis, the probit being the percentage of killed individuals converted following a special table. At the present time, a nonlinear regression analysis (Seber and Wild, 1989) can be more relevant and efficient, particularly when using statistical analysis software.

Laboratory experiments to establish the dose-lethality relation involve the administration of increasing doses to groups of selected subjects and the count of the two categories of subjects (dead or alive) after a specified time interval (Robertson *et al.*, 1984). Replications are needed to estimate the variability of each point representing the lethality associated to a particular dose.

From a theoretical point of view, by considering the cumulative distribution function (sigmoid) and its fluctuations due to the experimental replications, the less variable point is the inflection point, in other words the 50% lethality point and its associated dose, the 50% lethal dose or LD_{50} (Finney, 1971). On the contrary, the most variable ones are the extremes of the sigmoid graph. Consequently, when the estimation of the LD_{90} is required, e.g. efficiency of an insecticide against pests, special designs must be used to guarantee its precision (Robertson *et al.*, 1984). From an experimental point of view, the graph of the cumulative distribution function is not necessarily sigmoidal. For instance, after one imidacloprid contact exposure, Suchail *et al.* (2000) evidenced that mortality rates were positively correlated with doses lower than 7 ng/bee and negatively with doses ranging from 7 to 15 ng/bee. In this situation, the calculation of any lethal dose with the log-probit model is incorrect.

When considering beneficial insect such as bees, the doses which cause slight mortalities (e.g. LD_5, LD_{10}, LD_{25}, etc.) are more pertinent, even if the variability of these LDs due to the toxin is difficult to distinguish from that of the natural mortality deduced from the control groups (Abbott, 1925). This variability is not to be rejected, because its very existence in experimental conditions suggests that the same variability also exists in field conditions.

The variability created by the replications refers mainly to the assumption concerning dealing with the random selection of the subjects and the normal distribution of population from which the subjects are chosen. The variability induced by the replications, meaning that the experiment is identically repeated several times, provides additional information on the reproducibility of the experiment.

For a set of given experimental conditions often recommended by precise guidelines, the LD_{50} should be as reproducible as possible (i.e. with a minimum variability.) Conversely, when the experimental conditions are modified, the LD_{50} correspondingly changes. Zbinden and Flury-Roversi (1981) noted that "every LD_{50} value must thus be regarded as a unique result of one particular biological experiment".

3.1.3. Factors influencing the dose-lethality relation

The scientific literature provides numerous examples of abiotic or biotic factors able to influence the dose-lethality relation.

3.1.3.1. Active ingredient and chemical formulation

An AI is a molecule able to bind on specific receptors of target organisms and produce adverse effects (Hodgson, 2004). Generally the chemical formula is only mentioned, without respect of the spatial arrangements. However, pyrethroids have isomers with varying levels of toxicity (Soderlund and Bloomquist, 1989). The same findings are true for some enantiomers, which have identical physical-chemical properties, but different biological activities (Konwick *et al.*, 2005).

To be used in laboratory conditions, the AI should be formulated as simply as possible, generally with one solvent. The commercial formulation spread in field conditions is more complex because surfactants, stabilizing agents, dispersants, sometimes synergists (Bernard and Philogène, 1993) are added after dilution of the AI. The commercial formulation is targeted at the improvement of the AI activity in time and/or in toxicity. Certain mixtures of AI have synergistic effects, i.e. insecticide and fungicide at sub-lethal doses (Colin and Belzunces, 1992). Some AIs are converted under biological or environmental conditions into products (metabolites) that are often higher in toxicity than the parent compound (Ramade, 1992; Nauen *et al.*, 1998; Suchail *et al.*, 2001; Tingle *et al.*, 2003).

3.1.3.2. Physical formulation

Generally, the higher the concentration of the AI in the formulation, the finer the required dispersion of the formulation in the field. Target application sites can be treated with the same dose of AI in different ways. For instance, the same dose can be sprayed (one method of product delivery) after final dilution in one hundred litres of water for a tractor-drawn device or in three litres of water or oil (ultra-low volumes) by aeroplane. Depending on the spraying method, the concentrations are not identical and the diameter of the droplets ranges between 1 micron to hundreds of microns. Consequently, the delivery method makes the penetration of the AI into the body of living organisms and its toxicological effects different (Luttrell, 1985). In the same order of size as for droplets (1 to 100 µm), plastic micro-capsules are conceived to extend the effective life of AI by releasing slowly through pores of the plastic walls (Stoner *et al.*, 1979). Nanoparticles are patented but their biological and environmental fates are poorly documented (Hodgson, 2004).

3.1.3.3. Temperature and hygrometry

For many substances, a linear relation links ambient temperature and LD_{50}s, negatively for DDT and most of pyrethroids (Ladas, 1972; Faucon *et al.*, 1985), positively for organophosphates and carbamates. Hygrometry is a factor of variation but its true impact on the impact of toxic substances is poorly documented.

3.1.3.4. Exposure features

First, dose and concentration are both to be considered. Local and general consequences on a living organism are quite different if the same dose is concentrated in one microlitre or if diluted in one millilitre. Depending on the toxin, repellent effects could occur at certain concentrations. Inversely, the forced contact with these concentrations would be able to induce local necrosis, with general consequences. Second, the route of administration is important to overall toxicity because it modulates the rapidity and the extension of the toxin in the living organism as well as the triggering of the detoxification pathways. Third, there is a higher probability of poisoning the longer the duration of the exposure to an AI (and/or its toxic metabolites) (Hodgson, 2004). Finally, the temporal features of the exposure often influence the severity of the poisoning. For example, Brunet *et al.* (2009) demonstrated that a dose applied daily for five days can induce higher mortality than a dose five times higher but administered one time.

3.1.3.5. Sex, age and caste

The sex, age, and caste of the insect can influence the impact of the toxin on the individual. For insects, males generally are more susceptible to insecticides than females and newly emerged adults often are more susceptible than older ones (Hodgson, 2004). After emergence, the age-susceptibility relation is variable depending on the target species and toxin. These factors are tightly linked in a social insect colony like the honey bee colony since one female is responsible for egg production while many others perform other activities (some depending on age). To a lesser extent, the same occurs with males; the young male bees remain in the hive while older ones fly outside (Tautz, 2009). The susceptibility to toxins increases with age when bees are nearly inactive gathered in a winter cluster, (Wahl and Ulm, 1983). Thus, it can be more pertinent to consider the social function of the individual than its sex and age when considering toxin impacts on the organism.

3.1.3.6. Weight and diet

The weight of an individual is an important factor influencing the LD_{50} and it is often negatively correlated with toxin susceptibility. Food deprivation can increase the susceptibility of individuals to toxins, with the protein content of the diet being of particular influence (Zbinden and Flury-Roversi, 1981). For honey bees, the amount and quality of pollen ingested in the first days of life can affect the pesticide susceptibility of young and older worker bees independently of their weight (Wahl and Ulm, 1983).

3.1.3.7. Health

The health of the individual or colony can influence the level of poisoning, especially regarding aggravation by or recovery from the toxin. For the honey bee, contact with the toxin can be more frequent during certain activities (for instance, foraging or nursing), thus requiring an acceptable state of health if the impacts of the toxins are to be overcome. The penetration kinetics of the toxin is made easier when injuries are present, for instance broken setae or loss of the epicuticular waxes. The integrity of the intestinal wall and the quantity/ quality of the gut flora play an important role in the penetration of the toxin into the body via the digestive route. The fat bodies can trap lypophilic toxins and are important sites of detoxication. Furthermore, the pathogenic action of parasites or microbes influences the severity of poisoning if it modifies the penetration abilities of the toxin, the detoxication capacities, and/or the proteic and energetic metabolisms (Hodgson, 2004). In particular, the interactions between *Nosema* spp. and insecticides have been documented (Ladas, 1972; Alaux *et al.*, 2010; Vidau *et al.*, 2011). Conversely numerous pesticides can have extended general effects, for instance if they inhibit neurosecretion or cellular energy production, impairing the physiology of all the tissues. Bendahou *et al.* (1997), for example, showed that pyrethroids act by decreasing lysozyme concentration and phagocytosis capabilities, thus explaining the observed upsurge of Chronic Bee Paralysis Virus or other diseases in studied honey bees.

3.1.3.8. Genetics and resistance

At the individual level, subspecies and strains of honey bees are not equally susceptible to a given dose of AI (Ladas, 1972; Suchail *et al.*, 2000). Moreover a colony is not genetically homogeneous because of the coexistence of half-sister workers. Part of the tolerance to insecticides

is due to genes encoding detoxifying enzymes. However there are significantly fewer genes encoding three major superfamilies of these enzymes in *Apis mellifera* than in other insect groups such as drosophila. Thus the honey bee would have great difficulty to metabolize certain pesticides (Claudianos *et al.*, 2006), making the resistance uncertain and non-uniform across races/subspecies.

3.1.3.9. Density of subjects

The dose-lethality relation typically is determined after submitting small groups of caged subjects to doses of a toxin. Sautet *et al.* (1968) indicated that the susceptibility to DDT increased positively with the number of caged mosquitoes, thus suggesting that individuals within a treatment group are not independent. For honey bees where social interactions occur, Dechaume-Moncharmont *et al.* (2003) concluded that "bees do not die independently of each other" for a continuous chronic exposure.

3.1.3.10. Conclusion

In conclusion, the variation between factors influencing the dose-lethality relation are so numerous, the difference between the lowest and the highest LD_{50} values can be more than a hundred of times (NRCC, 1981). Consequently, the concept of acute toxicity testing must not be restricted to one determination of the LD_{50} but extended to many others, reflecting the biotic and abiotic factors of toxicity variation. In the preliminary evaluation of a compound's toxicity, it is important to establish the dose-lethality relation for the parent molecule and its by-products at three temperatures: internal body temperature for flying (37°C), low wintering bee temperature (12°C, see Stabentheiner *et al.*, 2003), and one intermediate.

Insect death is not always the best determinant of acute toxicity because the moment of insect death often is imprecise, for example when confused with a severe knock-down that fails to result in death (Moréteau, 1991). For insects, the evaluation of acute toxicity would be more accurate if based on the apparition and intensity of severe clinical signs such as intense trembling, paralysis, feeding or warming inabilities, etc. (Vandame and Belzunces, 1998).

3.1.4. Exploration of sub-lethal poisoning

The link between the dose-lethality relation in laboratory conditions and the acute toxicity in field conditions is neither direct nor simple, nor can it be blindly guided by the "useful rule of thumb way of determining the anticipated toxicity hazards of a pesticide to honey bees in the field" (Atkins *et al.*, 1973). For example, this rule stipulates that "since the LD_{50} of parathion is 0.175 µg/bee, we would expect that 0.17 lb/acre of parathion would kill 50% of the bees foraging in a treated field crop at the time of the treatment or shortly afterwards", without mentioning the possibility of sub-lethal toxicity. So the following question remains: can the sublethal toxicology be deduced from the dose-lethality relation?

In the log-probit model itself, the extreme values of the dose-% lethality relation cannot be derived from the LD_{50} and the slope of the regression line (Robertson *et al.*, 1984). Moreover the log-probit model is not necessarily the most adapted model for the dose-lethality relation. For the lowest LD values, the log-probit model is questioned by Calabrese (2005), who mentioned the frequency of the hormesis phenomenon, that is "a modest treatment-related response occur(ing) immediately below the No Observable Effect Level". Consequently, special designs are needed to estimate the low doses effects.

In this complex domain, mortality is not the best criterion for determining toxic effects. During its adult life, the worker bee must be physically able to fly and has to use functional short and long term memories to communicate, care the larvae, form the winter cluster and perform many other social functions. Thus a panel of markers of behavioural, physiological, and molecular origins can provide substantial information in matter of sub-acute poisoning (Desneux *et al.*, 2007). Each sublethal individual assay is important so one can know if the adult bees are capable of accomplishing one of the activities essential for perpetuating the bee colony and maintaining its ecological role (Brittain and Potts, 2011).

3.2. Laboratory methods for testing toxicity of chemical substances on adult bees

3.2.1. Oral application

This method was never ring-tested but was several times reviewed by OECD, EPPO and CEB. It is considered validated.

3.2.1.1. Introduction

The determination of acute oral toxicity on honey bees is required for the assessment and evaluation of chemicals prior to their registration as pesticides (Regulation EC No 1107/2009 of the European Parliament and of the Council of 21 October 2009). In this way, the acute oral toxicity test is conducted to determine the toxicity of all types of compounds to bees (pesticides, specifically, are tested as AIs or as formulated products). The methodology outlined in this section is a general approach of the laboratory test with oral applications and does not present all the details of the referenced guidelines.

Usually an oral exposure study is intended to determine the LD_{50} (see section 8.2.1.2.) and the results are used to define the need for further evaluation. Although the LD_{50} is a common aim of these studies, oral exposure tests can be used to determine NOAEL (see section 8.4.3.). When the LD_{50} cannot be determined because a given compound has a low toxicity, a limit test may be performed in order to demonstrate that the LD_{50} is greater than the standard value of 100 µg of AI/bee.

Data from oral LD_{50} calculations can be used to generate HQ for each compound of interest (see section 8.4.2.1). The LD_{50} calculation provides a raw value only. This result has to be related to the exposure of honey bees in field conditions.

- When the HQ < 50, the product can be considered of low acute risk to adult worker honey bees when ingested. The HQ does not predict product toxicity to brood or the occurrence of any sub-lethal effects on adults or brood.
- When the HQ > 50, more tests are required in semi-field or field conditions for a better evaluation of impact (cf. European scheme for the assessment of impact of PPPs - Guidelines commonly used refer to EPPO (2010b), OECD (1998a) and French CEB (2011). All are similar with main differences occurring on number of the number of replicates.

3.2.1.2. General principle

- Worker honey bees that are all aged or young emerged honey bees that are 1 to 2 days old are kept in laboratory boxes and fed with a sucrose solution for one day.
- Following this, they are exposed to a range of doses of the test substance dispersed in the sucrose solution.
- Usually mortality is recorded up to 48 h and values are used to calculate the LD_{50} with a regression line (see section 8.2.1.2.). Mortality can be recorded after 4 hours to look at an eventual acute effect, and is then recorded at 24 and 48 hours and compared with control values for assessment. When mortality continues to increase, the test can be extended to 72 or 96 hours. In the case of chronic oral toxicity, data are recorded up to 10 days of daily exposure with low doses.

3.2.1.3. Experimental conditions and modalities

3.2.1.3.1. Establishing the hoarding cages

1. Adult honey bees should be collected per Williams *et al.*, 2013. They should be from a single colony in order to provide a similar status regarding origin and health.
2. Upon collection, the adult bees should be kept in hoarding cages that have a syrup feeder. For convenience, plastic containers are recommended as they can be discarded after use in order to avoid contaminations. Glass, wooden or iron boxes that have been used before are not recommended for reuse unless the process of cleaning and sterilization is validated under Good Laboratory Practices. The boxes can be created per Williams *et al.*, 2013.
3. The cages should be individually identified and placed in incubators or in a dedicated controlled room.
4. The cages should be stored at 25 ± 2°C and > 50% rH.
5. Each cage should contain at least 10 bees (EPPO, 2010a; OECD, 1998a). The CEB (2011) recommends 20 bees and up to 50 bees in some specific chronic tests.

3.2.1.3.2. Identifying and replicating the treatment modalities

The number of modalities is defined by the objectives of the study and includes at least the following groups:

1. A control - untreated sugar water, often containing the solvent used to dissolve the test compound in the treatment doses. The control provides the evaluation standard in the assessments.
2. The toxic reference - This reference verifies bee sensitivity to toxic compounds. The toxic standard validates the test. Dimethoate is the main toxic standard used and provides a high subsequent mortality at known doses. It is usually administered at 2-3 doses to cover the expected LD_{50} value. The expected oral LD_{50} for dimethoate ranges from 0.10 to 0.35 µg AI/bee.
3. The test compound at five doses.

Consequently, there are at least 9 "groups" for each study (the control, the toxic standard administered at 3 doses, and the test compound administered at 5 doses). Each group should be replicated three times (i.e. with 3 hoarding cages of 10 to 20 bees) (EPPO 2010a; OECD, 1998a, 1998b). The CEB (2011) guideline requires three "runs" of three replicates/run (3 x 3).

3.2.1.3.3. Substance administration

1. Starve the bees for 1-2 hours before the test so that all bees will feed once the study begins.
2. All bees in a cage are exposed to one of the test substances dispersed in a sucrose solution by being allowed to feed *ad libitum*. The sucrose solution is mixed at 500 g sugar to 1 l distilled water.
3. The number of doses and replicates tested should meet the statistical requirements for determination of LD_{50} with 95% confidence limits. A preliminary test (range finder) is usually conducted with a dose range of factor 10 in order to determine the appropriate doses for the formal test (1, 10, 100, 1000, etc.). Secondly the acute toxicity test is conducted with five doses in a geometric series with a factor 2 in order to cover the range for the LD_{50} (ex; 100, 200, 400, 800, etc.).
4. Bees are provided with 10 µl/per bee of the sucrose solution containing the test substance at the different concentrations. In each test group, the feeder is removed from the box when empty (within 2-4 hours) and replaced with another one containing untreated sucrose solution.
5. In all groups, the eventual remaining treated diet is weighed and replaced with untreated sucrose solution after 6 hours; the amount of treated diet consumed per group is recorded.

Table 2. Example of data sheet: both mortality, number of living bees and abnormal behaviour of living bees are recorded simultaneously. For behavioural effects see Table 1.

Contact/Oral LD_{50} test honeybee (*Apis mellifera* L.)

Test substance: Concentration:
Start date: Administration: oral / contact (cancel)

For contact administration: Start time: Pipet nr:

For oral administration: Start feeding time: Weight feeding device: End feeding time: Weight feeding device: Amount consumed: Remarks consumption: Remarks trophallaxis:

General remarks:

Date	Time	Observation	Test cage / replicate 1	2	3	Initials
		Mortality				
		N. living bees				
		Behavioural effects				
		Mortality				
		N. living bees				
		Behavioural effects				
		Mortality				
		N. living bees				
		Behavioural effects				

Note: behavioural effects = NE (no effect), F ("Freeze"), P (paralysis), S (spasms), other

3.2.1.4. Mortality assessment

1. In all treated and control groups, mortality (see section 2) is recorded at 4, 24 and 48 h post exposure. Data should be summarised in tabular form, showing for each treatment group, as well as control and toxic standard groups, the number of bees used, mortality at each observation time, and number of bees with adverse behaviour (Table 2). Any abnormal effects observed during the test are recorded in order to inform about possible subletal effects (Table 1). When mortality continues to increase after 48h, it is appropriate to extend the duration of the test up to 72 or 96 hours.
2. For the validity of the test, mortality in the negative (untreated) reference should be < 10% (OECD, 1998a; CEB, 2011) or 15% (EPPO, 2010b) and the mortality of the toxic standard dimethoate (positive reference) should meet the specified range: almost 50% with the lower dose (0.10 µg AI/bee) to 80-100% for the higher dose (0.35 µg AI/bee). Data from tests failing to meet these standard criteria should not be used.
3. Mortality data are submitted to a statistical analysis. The LD_{50} has to be calculated (see 8.2.1.2.) for each recommended observation time (i.e. 24h, 48h and if relevant, 72h, 96h) based on mortality data corrected for control mortality using Abbott's formula (see 8.4.1.).

3.2.1.5. Extension to other tests

Although the acute oral toxicity test provides an LD_{50} value, this result is not sufficient to appreciate other kinds of pesticide impacts. The oral toxicity test is nevertheless being adapted in other trial protocols related to honey bees. Notably, it is being refined to determine contact toxicity, chronic oral toxicity, seed dust effects, etc. and its evolution is certain to continue.

3.2.2. Topical application

The method outlined in this section (acute contact LD_{50}) is based on the OECD guideline 214 (OECD, 1998b) to which later recommendations from EPPO Bulletin 40 (EPPO, 2010b) are added. This method was never ring-tested but was several times reviewed by OECD, EPPO and CEB. It is considered validated.

3.2.2.1. Introduction

Two approaches to determine the contact toxicity of a PPP can be distinguished; a practical approach simulating the contact between a PPP and a honey bee in the field and an academic one assessing the LD_{50}. The academic approach is the one presented in this section as it is part of the risk assessment according to the OECD and EPPO guidelines used for legislation of PPP's worldwide.

3.2.2.1.1. Field simulated contact toxicity

To place the contact toxicity of pesticides briefly in a historic framework, two protocols are described briefly. In Stute (1991), the contact toxicity of PPPs applied as a spray, was assessed by exposing the bees to a 150 cm^2 paper, contaminated with twice the recommended field application rate of the target pesticide. The PPPs to be applied in a dusted form were administered using a Lang-Welte-Glocke to cover the surface completely and homogeneously. Johansen (1978) assessed the contact toxicity by placing bees in a bell-jar duster loaded with 200 mg pesticide and administered the pesticide onto the bees via vacuum and subsequent imploding incoming air to disperse the pesticide homogeneously over the bees. Both the Stute (1991) and Johansen (1978) tests provide general information about toxicity. However, in both cases the amount of the PPP actually administered to the bees was unknown. This makes it hard to do further calculations about risk assessment. The other two methods imitating field contact exposure are described in section 3.2.3.

3.2.2.1.2. Contact LD_{50}

The acute contact toxicity test is conducted to determine the inherent toxicity of pesticides and other chemicals to bees. The results of this test are used to define the need for further evaluation. The contact LD_{50} is part of the tiered approach; from laboratory to semi-field to field. The tiered approach is implemented in the EU. The contact LD_{50} is assessed for the risk assessment of sprayed PPPs to adult worker bees. The result, a certain dose expressed as µg or ng AI or formulation per bee or per gram of bee is an academic parameter and does not express the hazard of the product in the field. This depends on the concentrations and the field application and is assessed in the HQ

(EPPO, 2010b) or RQ (EPHC, 2009) (see 8.4.2.). When an HQ calculation results in a value lower than 50, the risk to bees is considered to be low. When performing acute contact studies, a toxic standard (positive reference, such as dimethoate) should be used. The results from the test with the toxic standard provide information on potential changes in sensitivity of the test organisms (in time) and consequently the suitability of these populations for further testing. Additionally, information on the precision of the test procedure is generated.

3.2.2.2. Description of the method

3.2.2.2.1. Outline of the test

1. The AI or formulation of a PPP is tested.
2. The PPP is dissolved in acetone if possible. Other solvents should be used only in instances where the compound is insolvable in acetone and these alternative solvents are known to be harmless to bees.
3. When formulations are tested (rather than AIs), they should be water and if needed, an appropriate wetting agent added. If a wetting agent is applied, it should be applied in the positive and negative reference as well.
4. The test substances are administered to anaesthetized bees (Human *et al.*, 2013) in a 1 µl droplet on the dorsal thorax of individual bees.
5. After treatment, the bees are provided *ad libitum* with freshly made sucrose-solution 50% (w/v) and checked daily for mortality and behaviour (see Table 2).

3.2.2.2.2. Collection of bees

Adult worker bees used for this protocol should be collected per Williams *et al.*, 2013. Other special considerations:

1. Adult worker bees of the same race.
2. The bees should be collected in the morning of use or in the evening before the test and kept under test conditions to the next day.
3. Bees collected from frames without brood are suitable.
4. Collection in early spring or late autumn should be avoided, as the bees have an altered physiology during this time.
5. If tests are to be conducted in early spring or late autumn, the bees can be emerged in an incubator and reared for one week with "bee bread" (pollen collected from the comb) and sucrose solution.
6. The bees should not have a treatment history or originate from colonies that have been treated with chemical substances such as antibiotics, anti-*Varroa* agents, etc. Bees can be used from colonies that have been treated with these substance longer than 4 weeks before bee collection.

3.2.2.2.3. Test cages

1. Easy to clean and well-ventilated cages should be used. For recommendations on cage types and maintaining bees in laboratory cages, see Williams *et al.*, 2013.
2. The cages should be lined with filter paper to avoid contamination of the bees from vomit and faeces. Groups of ten bees per cage are preferred.
3. The size of test cages should be appropriate to the number of bees (Williams *et al.*, 2013).

3.2.2.2.4. Handling and feeding conditions

1. Handling procedures, including treatment administration and general observations, may be conducted under daylight conditions.
2. Sucrose solution in water with a final concentration of 50% (w/v) should be used as food for the adult bees and provided *ad libitum* during the test using a feeder device.

3.2.2.2.5. Preparation of bees

1. The collected bees may be anaesthetized with carbon dioxide or nitrogen for application of the test substance (Human *et al.*, 2013). The amount of anaesthetic used and time of exposure should be minimised.
2. Moribund bees, affected by the handling or otherwise, should be rejected and replaced by healthy vital bees before starting the test.

3.2.2.2.6. Preparation of doses

1. The test substance is to be applied as solution in acetone or as a water solution with a wetting agent. As an organic solvent, acetone is preferred but other organic solvents of low toxicity to bees may be used (e.g. dimethylformamide, dimethylsulfoxide). If others are used, they must be administered in the negative reference.
2. For water dispersed formulated products and highly polar organic substances not soluble in organic carrier solvents, solutions may be easier to apply if prepared in a solution of a commercial wetting agent to an extend the product dissolves (e.g. Agral, Citowett, Lubrol, Triton, and Tween).

3.2.2.3. Procedure

3.2.2.3.1. Test and control groups

1. The number of doses and replicates tested should meet the statistical requirements for determination of LD_{50} with 95% confidence limits (OECD, 1998b). Normally, five doses in a geometric series, with a factor not exceeding 2.2, and covering the range for the LD_{50}, are required for the test.

Table 3. Test scheme for the acute contact LD_{50} test. "conc." = concentration.

Test solution	**Replicate 1 (colony X)**	**Replicate 2 (colony Y)**	**Replicate 3 (colony Z)**
Test conc. 1	conc. 1	conc. 1	conc. 1
Test conc. 2	conc. 2	conc. 2	conc. 2
Test conc. 3	conc. 3	conc. 3	conc. 3
Test conc. 4	conc. 4	conc. 4	conc. 4
Test conc. 5	conc. 5	conc. 5	conc. 5
Positive control conc. a	conc. a	conc. a	conc. a
Positive control conc. b	conc. b	conc. b	conc. b
Positive control conc. c	conc. c	conc. c	conc. c
Negative control [solvent: acetone (or other), water, or water with wetting agent]	solvent	solvent	solvent

However, the number of doses has to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results.

2. A range-finding test preceding the actual toxicity test enables one to choose the appropriate doses.

3.2.2.3.2. Replicates

1. A minimum of three replicate test groups, each of ten bees, should be dosed with each test concentration. Bees in a single cage (a single replicate group) should be from the same colony, with a different colony being used to populate each cage.
2. The three replicates per dose of the PPP tested are treated with the same preparation of the test solution with a specific concentration, i.e. not a newly prepared test solution for each replicate group (Table 3).

3.2.2.3.3. Toxic reference

1. A toxic (positive) reference should be included in the test series.
2. At least three doses should be selected to cover the expected LD_{50} value.
3. A minimum of three replicate cages, each containing ten bees, should be used with each test dose.
4. The preferred toxic reference is dimethoate. Gough *et al.* (1994) evaluated the use of dimethoate as a reference compound for acute toxicity tests on honey bees. The results of 63 contact tests of technical dimethoate were evaluated, using the 95% confidence linear regression of logit transformation on log10 dose (µg/bee), adjustments using Abbott's correction. The contact LD_{50} assessed with six concentrations in acetone, control acetone and administration on the thorax was 0.16 (min 0.11, max 0.26) µg AI/bee. LD_{50} values ranging from 0.075 to 0.30 µl AI/bee in groups of 10 bees should be considered as valid results of the toxic standard Dimethoate. The LD_{50} (48 h) was similar to 24 h. For the contact LD_{50} tests, the contact LD_{50} (24 h) should be in the range of 0.10-0.30 µg AI/bee.
5. A dose range of 0.075 to 1.0 µg/bee is recommended and results falling in this range validate the test.
6. Other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

3.2.2.3.4. Administration of doses

1. Anaesthetized bees (Human *et al.*, 2013) are individually treated by topical application.
2. The bees are randomly assigned to the different test doses and controls.
3. A volume of 1µl of solution containing the test substance at the suitable concentration should be applied with a validated micro applicator to the dorsal side of the thorax of each bee.
4. Other volumes may be used, if justified.
5. After application, the bees are allocated to test cages and supplied with sucrose solutions (50% w/v).

3.2.2.3.5. Test conditions

1. The bees should be held in the dark in an experimental room at a temperature of 25 ± 2°C.
2. The relative humidity, normally around 50-70%, should be recorded throughout the test.

3.2.2.3.6. Duration and observations

1. The number of dead or affected bees (see Table 1) is counted at 4 h after dosing and thereafter at 24 h intervals for up to 48 h or longer if mortality is still increasing (> 15% increase in mortality in the 25-48 h period).
2. Additional assessments at shorter intervals may be useful in specific cases.
3. It is appropriate to extend the duration of the test to a maximum of 96 h.
4. Mortality is recorded daily and compared with values from the positive and negative references.
5. All abnormal behavioural effects observed during the testing period should be recorded.

6. Therefore the total number of bees having yes/no effect should be recorded at each recording. These data allow the calculation of ED_{50}.

3.2.2.4. Calculation of the LD_{50}

The results are analysed in order to calculate the LD_{50} at 24 and 48 h and, in case the study is prolonged, at 72 h and 96 h. The mortality data should be analysed using appropriate statistical methods (LD_{50} calculated based on data corrected for control mortality, see 8.2.1.2. and 8.4.1.)

3.2.2.5. Limit test

In some cases (e.g. when a test substance is expected to be of low toxicity), a limit test may be performed using 100 µg AI/bee in order to demonstrate that the LD_{50} is greater than this value. The same procedure outlined in section 3.2.2.2. should be used, including three replicate test groups for the test dose, the relevant controls, and the toxic reference. If mortality occurs, a full study should be conducted. If sublethal effects are observed, these should be recorded.

3.2.2.6. Validity of the test

The test is valid if:

1. The LD_{50} of the toxic standard meets the specified range (see section 3.2.2.3.3.)
2. Control mortality in 48 h ≤ 15% (EPPO, 2010b).

3.2.2.7. Data and reporting

3.2.2.7.1. Data

- The LD_{50} is expressed in µg AI test substance or µg formulation/bee.
- In case the LD_{50} is applied for the HQ calculation (see 8.4.2.), the LD_{50} of the AI should be used.
- Data should be summarised in tabular form, showing for each treatment group, as well as control and toxic standard groups, the number of bees used, mortality at each observation time, and number of bees with adverse behaviour (see Table 1).

3.2.2.7.2. Test report

The test report must include the following information:

3.2.2.7.2.1. Test substance

- physical nature and relevant physical-chemical properties (e.g. stability in water, vapour pressure);
- chemical identification data, including structural formula, purity (i.e. for pesticides, the identity and concentration of AI).

3.2.2.7.2.2. Test bees

- scientific name, race, approximate age (in weeks), collection method, date of collection;
- all relevant information on colonies used for collection of test bees, including health, any adult disease, any pre-treatment, etc.

3.2.2.7.2.3. Test conditions

- temperature and relative humidity of experimental room;
- housing conditions including type, size and material of cages;
- methods of administration of test substance, e.g. carrier solvent used, volume of test solution applied, anaesthetics used;
- test design, e.g. number and test doses used, number of controls; for each test dose and control, number of replicate cages and number of bees per cage;
- date of test.

3.2.2.7.2.4. Results

- results of preliminary range-finding study if performed;
- raw data: mortality at each concentration tested at each observation time;
- graph of the dose-response curves at the end of the test;
- LD_{50} values, with 95% confidence limits, at each recommended observation time, for test substance and toxic standard;
- statistical procedures used for determining LD_{50};
- mortality in controls;
- other biological effects observed and any abnormal responses of the bees;
- any deviation from the Test Guideline procedures and any other relevant information.

3.2.2.8. Recommendation

It may be useful to have the test solutions analysed to verify the concentrations administered.

3.2.3. Toxicity of residues on foliage

3.2.3.1. Testing toxicity of contaminated dust from pesticide-dressed seed by indirect contact

3.2.3.1.1. Introduction

In some cases, the indirect toxicity tests can be preferred to topical tests because they better simulate the field conditions of the exposure and provide fast and applicable data (see 3.2.2.1.1.). In the indirect or residual toxicity tests, bees enter in contact with the test substance by walking on contaminated substrate in a hording cage (Williams *et al.*, 2013). The "OPPTS 850.3030 Honey bee toxicity of residues on foliage" is the unique official guideline designed to develop data on residual toxicity to honey bees for spray products but no official methods are available to test contaminated dust in laboratory. In fact, individual compounds can show different levels of toxicity depending on formulation (spray *vs.* dust for example) but, specific tests should be adopted to estimate the toxicity of powder products when pesticides are applied as seed treatment.

Table 4. Example of the calculation of dust and AI quantity to distribute in the bottom surface of the hoarding cage.

Quantity of AI deposited during sowing on the ground at 5 m (µg/m²) **A**	**Percentage of AI in the dust obtained by Heubach cylinder** **P**	**Quantity of the AI-containing dust deposited on the ground at 5 m (µg/m²)** **Q = A*100/P**	**Surface of the bottom of the hoarding cage (cm²)** **S**	**Quantity of the AI-containing dust (in µg) on the surface of the hoarding cage** **D = Q*S/10,000**	**Concentrations**	**Quantity of the AI-containing dust per cage (µg in 0.01 g of talc)**
2.25	33%	6.82	56.72	0.039	x 1000	39
					x 100	3.9
					x 10	0.39
					x 1	0.039

Several bee mortalities in Europe and USA have been linked with contaminated dust dispersed during maize sowing operations (Alix *et al.*, 2009; Bortolotti *et al.*, 2009; Pistorius *et al.*, 2009; Krupke *et al.*, 2012). Pesticides can be dispersed by air during sowing operations when pesticide-dressed seeds are used and contaminated dusts can subsequently deposit on soil and vegetation, posing an exposure risk to foraging bees (Greatti *et al.*, 2003, 2006). In this section, a method to test the impact of contaminated dusts on honey bees is proposed.

3.2.3.1.2. Test procedures

3.2.3.1.2.1. Background

This protocol follows the method of Arzone and Vidano (1980) applied for spray products but adapted to soil/seed treatments. This method has been applied in Italy in order to investigate the effects of pesticides drifted from maize seed dressing on honey bees when bees forage in the edge of the maize field during sowing operation (APENET, 2009, 2010; Sgolastra *et al.*, 2012).

3.2.3.1.2.2. Dust extraction

1. Dust from maize-dressed seeds is obtained by Heubach method. This method is commonly performed to measure the seed dustiness (Heimbach, 2008). In the Heubach method, treated seeds are mechanically stressed inside a rotating drum. A vacuum pump produces an air flow through the rotating drum, the connected glass cylinder and the attached filter. By the air flow, abraded dust particles are transported out of the rotating drum through the glass cylinder and subsequently through the filter unit. Fine dust particle (Ø < 0.5 mm) are deposited onto a filter while coarse non-floating particles are separated and collected in the glass cylinder.
2. The dust retained by the Heubach cylinder filter and the other particles extracted with Ø < 45 µm should be used in the toxicity test. Fine and coarse dust particles are mashed and sieved with a precision 45 µm mesh sieve in order to use only small particles for the test, which are more likely to drift.

3.2.3.1.2.3. Dosages

As a worst case, the quantity of contaminated dusts deposited on the ground during sowing at a maximum of 5 m distance from the edge of the field should be used. The distance was chosen based on the previous results of field studies (APENET, 2010) where the amount of the AIs deposited on the ground during sowing at 5, 10, 20 m distances from the field's edge was measured and a decline in pesticide concentration was observed as distance increased (APENET, 2009, 2010). The dose of AI deposited on the ground was measured in field studies following the indication of the agricultural industry in agribusiness field trials, which in turn were taken over from a methodology designed to study liquid pesticide drift (BBA, 1992; APENET 2009, 2010).

3.2.3.1.2.4. Contaminated dust preparation

1. The AI-containing dust, obtained from dressed seed with Heubach cylinder (see 3.2.3.1.2.2.), is analysed. The percentage of AI content in the dust is used to calculate the quantity of dust to distribute on the surface of the bottom of the hoarding cage (Table 4).
2. To allow homogeneous dispersal of dust on the cage substrate in proportion to the quantity of AI deposited at 5 m, it is necessary to mix the dust with an inert material (talc) through geometric dilutions, starting from a dose that is 1000 times more concentrated. An appropriate quantity of talc is used as a dispersing agent in order to reach the desired concentration (Table 4).

Talc has been suggested as a dispersing agent because it is a common mineral material, not toxic to bees, usually added to seed boxes to reduce friction and stickiness, and to ensure smooth flow of seed during planting. Krupke *et al.* (2012) found that waste talc expelled during and after sowing represents a route of pesticide exposure for bees.

3.2.3.1.2.5. Substrate

1. Leaves collected from a plant that is as far as possible from

possible pollution sources. Other removable substrates (e.g. plastic or Plexiglas surface) may also be used.
2. Before the test, samples of leaves can be analysed for the residues in order to exclude previous contaminations.

3.2.3.1.2.6. Dust application

1. 0.01 g of total dust (the AI-containing dust plus the talc powder) per cage should be distributed on the leaves (Table 4). This quantity was considered adequate for a homogeneous distribution on the surface of approximately 50-70 cm^2. For bigger cages, a proportionally higher amount should be used.
2. A small sieve obtained from a modified Eppendorf tube can be used as shown in Fig. 1.

3.2.3.1.2.7. Control

A negative (untreated) reference is required during the test. The control substrate should be treated with pure talc. Control and treated bees should be kept under the same laboratory conditions (see section 3.2.3.1.2.12.).

3.2.3.1.2.8. Exposure to test substance

1. Forager bees, collected per Williams *et al.* (2013) are exposed to the dust by walking for 3 h on treated apple leaves or other substrate, placed on the bottom of a standard hoarding cage (e.g. 13 x 6 x 11 cm or one from Williams *et al.*, 2013).
2. The leaves are removed from the cage after 3 h.

3.2.3.1.2.9. Number of animals tested

Usually 10 bees per cage should be used.

3.2.3.1.2.10. Number of replicates

3 to 5 cages per treatment (see section 8.4.4.)

3.2.3.1.2.11. Duration of the test

At least 3 days or when the control mortality is >20%.

3.2.3.1.2.12. Test conditions

1. During the trials, the cages containing the bees should be maintained in a darkened incubator at 25 ± 1°C and with 60 - 80% RH.
2. Each cage should be equipped with a dispenser containing sugar solution for the bees (50% w/v). It is important to avoid the dropping of the sugar solution on the treated surface during the exposure period.

3.2.3.1.2.13. Endpoints

1. Cumulative mortality is assessed, then LC_{50} is calculated (see 8.2.1.2.) and any noted sub-lethal effects are registered (see Table 1).
2. The PER assay (Scheiner *et al.*, 2013) can also be performed after bees have been exposed to contaminated dust for 3 h following the above test procedure (APENET, 2010).

3.2.3.2. Testing contact toxicity on bees exposed to pesticide-contaminated leaves

3.2.3.2.1. Introduction

The assessment of the toxicity of residues on foliage to bees can be managed with several methodologies related to the mode of action and the way of application. From 1998 to 2003 the subject of high bee mortalities during spring when sowing of seeds is common became an important topic. On a review of different hypotheses, it was decided to investigate the ability of seeder machines to leave dust residues in the environment, a suspicion identified because of the use of insecticide coated seeds in southwest France. Consequently, it became necessary to determine if increased bee mortalities were related to the dust from coated seeds or alternative routes of exposure.

Crops of maize and sunflower were suspected to trigger such mortalities because of the numerous surfaces and AIs of the insecticide seed protection. As mortalities were mainly located in apiaries of this area, a major link was established with the sowing time of sunflowers.

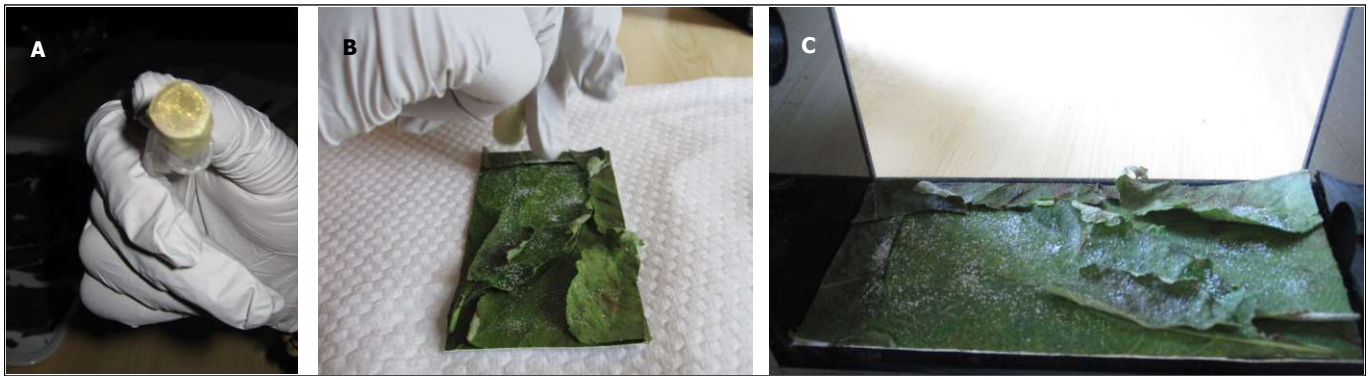

Fig. 1A. Small sieve obtained from a modified Eppendorf tube (the bottom is removed from the tube and replaced with screen mesh); ***B.*** Dust application on the apple leaves; ***C.*** Leaves placed in the bottom of the hoarding cage.

The following field-lab protocol was developed after initial tests of dust emission.

1. In indoor conditions, non-moving seeder machines are used to collect dust from different varieties of seeds and dressings. Seeders are equipped with filters that permit one to analyse the source and quantity of dust when working.
2. Coated seeds are classified from a screening with different kinds of varieties as well as different dressings for the same variety.
3. Among all dressing coated seeds, two modalities are selected for comparison of pesticide impact on honey bees. One concerns the low level of dust emission and is expected to have a minor impact when contacting honey bees. The second modality focuses on higher dust emission data and is tested for assessment of an eventual impact to honey bees.
4. The field part of this protocol aims to collect dust from a sowing operation in agricultural conditions. Fields of at least two hectares are separate from one another by about three kilometres in order to avoid a cross-contamination under wind conditions. These fields are bordered by a hedge on the edge of plot so that the wind creates turbulence on site. Dusts are expected to drop to the ground instead of being borne away. Dedicated sentinel plants are arranged on the ground to catch dust. They must have hairy leaves with good hair disposition on the upper leaf surface such as with *Tibouchina* (Order: Myrtales; Family: Melastomataceae) or other ornamental plants. They are placed in fields before sowing starts and they remain in the field for 2 days post sowing.

3.2.3.2.2. Methodology

1. The design includes 4 treatment groups:
   - the 2 sunflower varieties,
   - the untreated control
   - toxic standard (positive reference with dimethoate at 400g AI/ha)
2. The untreated control and the toxic standard are kept in an open space close to the laboratory.
3. The control group receives no treatment. There is no "dusted" toxic reference; thus to ensure bee sensitivity and to validate the design, the toxic reference is treated with a liquid spray of dimethoate (i.e. Dimezyl 1 l/ha = 400 g AI/ha).
4. In this method, the four treatment groups do not have the same route of exposure; the two varieties with coated dressings are tested from dust issued from agricultural practices whereas the toxic standard is a spray and the control is untreated or water treated.
5. Assessments are conducted under controlled conditions where bees are exposed to foliage in hoarding cages similar to LD_{50} tests (see Williams *et al.*, 2013 and section 3.2.3.1.2. of this manuscript). Sentinel plant foliage is collected 2 hours after seed sowing to look for acute toxicity effects on bees.
6. The surface in each hoarding cage is covered with foliage taken from sentinel plants. The surface of foliage is exactly adapted in number of cm^2. Twenty honey bees are introduced into all hoarding cages and are allowed to contact the leaves from the sentinel plants. Bees are taken from one single and healthy beehive and dispatched in the 4 groups and containers at random and per Williams *et al.*, 2013.
7. The foliage from the sentinel plants is removed after 24 hours but bees are left in boxes for 2 additional days; thus the test duration is 72 h. Then the laboratory part of this methodology is very similar to standardized LD_{50} test: CEB 230 (CEB, 2011), EPPO 170 (EPPO, 2010), OECD 214 (OECD, 1998b), with mortality assessments at 4 hours, 24, 48 and 72 h after exposure.
8. From the raw data, the average mortalities are calculated in three (3) replicates of each treatment group using usual formulas in statistical analysis (see section 8.4.1.).
9. These results are validated by mortality at 24 hours of 0% in the control and over 90% in the toxic standard.
10. Item modalities induce intermediate mortalities close to the control or higher according to the amount of dust in contact with bees.
11. Assuming no cross contamination is possible, some lethal effects are observed on bees following the use of one treated seed, and absolutely no effect for the other one.

3.3. Field methods for testing toxicity of chemical substances on individual adult bees

3.3.1. In-field exposure to dust during sowing

3.3.1.1. Introduction

It has been shown that bees can be contaminated with potentially lethal doses of insecticide simply by flying in the vicinity of a pneumatic drilling machine using seeds coated with insecticide (Marzaro *et al.*, 2011; Girolami *et al.*, 2012). The fragments of this coating are emitted into the atmosphere and constitute a toxic cloud the size of which may be estimated at some tens of metres in diameter. Only bees in flight were considered when reporting these observations about powdering, not bees possibly exposed to powder that fell to the ground and could contaminate on contact.

The following reported techniques presuppose an evaluation of the contamination, mortality and chemical analysis of a single bee. Once the bees are treated with powder, one must avoid the possibility that the bees in the same cage could contact and exchange contaminants, thus altering the results. For this reason, bees were kept separately one per cage. The test reports the evaluation of the acute toxicity which can cause the death of bees between 24 and 48 h

and for maximum practicality should be conducted under normal laboratory conditions (see section 3.3.1.3. below).

3.3.1.2. The management of the bees after exposure

1. In the contamination trials (be it in free flight or in mobile cages as reported below), the bees should be placed singly in small cages with a cubic steel skeleton of 5 cm and all the six sides enclosed entirely in tulle (with mesh of 1.1 mm) (Fig. 2).
2. The bees should be fed with small drops of honey during the period of observation. The honey can be placed on the top of the cage.
3. Additionally, so as to avoid honey dissolving, soiling and to prevent rapid ingestion, parallelepipeds of sponge can be placed on each cage. These can be 2 x 2 x 1 cm and made of normal, non-soluble domestic sponge soaked in 0.5 ml of honey.
4. The cages are ideal for observation when placed in a transparent container (for example, a polystyrene box 24 x 35 x 10 cm) sufficient to contain 12 small cages with a sheet of absorbent paper underneath (Fig. 3). The cages should be kept raised above the base of the cages by means of a net of folded metal. This device was used to prevent accumulations of honey on the base of the cages and to prevent the cage from contacting other liquids.

Fig. 2. Cages employed to expose bees to seed drill emissions and to evaluate survival after exposure.

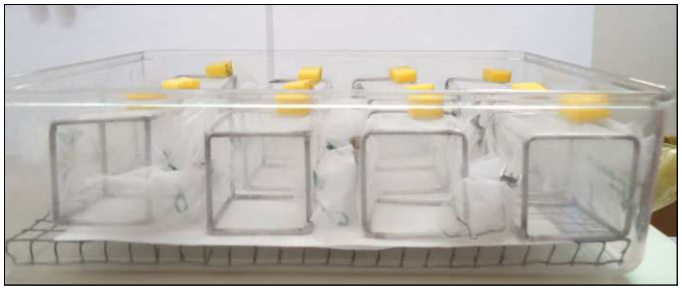

Fig. 3. Transparent polystyrene container with 12 small cages. Feeders placed on the cages are shown.

3.3.1.3. Study conditions

1. The containers with cages are kept at 23 ± 2°C with natural light, or added artificial light, in cloudy condition during the day.
2. The containers should be closed with a sheet of transparent plastic.
3. It is possible to keep the bees at a relative humidity close to saturation simply by wetting an absorbent sheet of paper on the bottom of the container with distilled water.

This system enables the evaluation of the influence of high humidities without wetting the cages. Thus, bees are prevented from contact with the water. Because of the high humidity, the sponges soaked with honey should be replaced every 6-12 h; otherwise, the bees continue to suck at the light with an increase of mortality possible in untreated controls.

3.3.1.4. Capturing the bees

3.3.1.4.1. Inducing the bees to visit the dispenser

In order to apply the trials in free flight, the bees must be conditioned to visit a dispenser simulating normal foraging trips.

1. In order to condition the bees rapidly to take sugar solution (about 50% w/v) from a dispenser placed not less than 30 metres from the apiary, a little flat dispenser with sugar solution is first placed on a running board (the dispenser must be refilled for minimum 2 days).
2. When the bees become accustomed to feed and crowd on the dispenser, it can be placed some metres of distance from the hive. The change of position must be gentle to keep bees from flying away.
3. To achieve visits from a particular hive, the above method can be employed using an isolated hive.
4. Once the bees associate the dispenser with the sugar solution, it is possible to put the dispenser with bees in a cage and transport them even hundreds of metres away.
5. When the bees are freed from the cage, some of them associate with the new position of the dispenser and indicate it to their companions once they re-enter the hive.
6. After the hive is conditioned to the required distance, it is possible to attract hundreds of foraging worker bees by replacing the sugar solution once daily. This is better done at the same hour each day.
7. The solution can be quickly and practically produced by mixing equal quantities of water and sucrose (approximately 50% w/v).

3.3.1.4.2. Collecting bees for use during the study

This topic is reviewed in detail in Williams *et al.*, 2013.

1. The most accurate method of collecting the bees is to put them singly, at the dispenser, into glass test tubes with a diameter greater than 1 cm and 10 cm in height (Falcon vials).

2. The collection can be accelerated by the use of the end section of an "insect vacuum" (Fig. 4).
3. For safety reasons, the vacuum necessary to suck a bee into the tube can be provided by an electric pump. If done manually, a fine, soft mesh should be placed at the mouth of the insect vacuum and a second protective diaphragm over the mouth of the test tube. This should be a thin, fine mesh.
4. It is necessary to limit captured bee exposure to any sort of rubbery material where they could insert their sting and die.
5. If it is not necessary to capture the bees singly at the dispenser (for example in the free flight trials), the bees may be caught en masse in a 20 cm tulle cage (or similar), placing it at the entrance to the hive (Fig. 5) (section 4.3.3.2 in Williams *et al.*, 2013).
6. The dispenser should be withdrawn from the cage, the cage closed and taken to the laboratory.
7. At the laboratory, the bees may be fed with honey placed on the upper part of the cage (Fig. 3).
8. The bees may be transferred from the cage to be kept singly in the laboratory, as described for the capture at the dispenser (step 1 above).
9. It is ideal that the bees not used at the end of the trial be freed to be renewed on successive days of experimentation.
10. Wherever possible, the powdering trials should be conducted using bees collected at the dispenser, avoiding using bees collected with an entomological net in front of the hive. This ensures that no juvenile bees are captured and used during the study.
11. If necessary, in the winter, bees can be caught in front of the hive, taking care to catch those bees returning to the hive (thus, certainly foraging worker bees) and not those exiting the hive who could be solely engaged in orientation flights. Nevertheless, it should be noted that winter bees normally should not be used for standardized ecotoxicological testing.

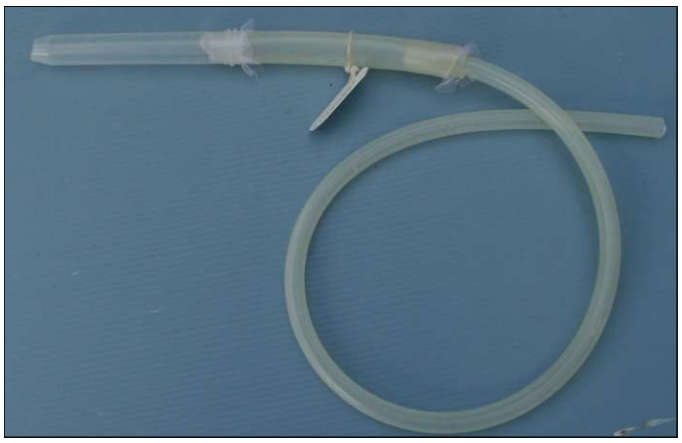

Fig. 4. Insect vacuum (aspirator) used to capture the bees. The two mesh diaphragms safely prevent the bee from being sucked into the mouth of the operator.

Fig. 5. Capture of bees from the hive. Method described in section 4.3.3.2 in Williams *et al.*, 2013.

3.3.1.5. Trials in mobile cages

1. In trial set up to evaluate the presence, consistency, extent and duration of the toxic cloud surrounding pneumatic seed drills during the maize sowing season, and using seed coated with insecticide, the powdering was evaluated by means of an aluminium bar 4 m long, to which cages, each containing a single bee, were attached every 0.3 m (12 in total) (Figs. 2 and 6).
2. The bar was supported at each end by a vertical pole of 2.5 m. The bar was passed by two people at a fast walking pace (6-8 km/h) by the side of the drilling machine, at a variable height according to how the exhaust air was emitted from the machine, taking into account that bees fly predominantly at 1-3 m over ploughed land (unpublished data). The cages may be numbered considering the progressive distances from the drill.
3. The people with the bar followed and passed the tractor on the right hand side (in the first 30 m of the plot) (Fig. 6). The tractor then reduced speed and waited while the people with the bar made a U-turn and again passed the machine, once more at working speed, on the left hand side. In this way, the bees were twice exposed to the cloud in a similar way to foragers in free flight making a round trip over the sowing area.
4. To evaluate the mortality, once the bees had been exposed to the insecticide dust in a cage in the field, they were transferred (inside the same cage) to a room at a controlled temperature (22 ± 1.5°C) and in conditions of high humidity (Girolami *et al.*, 2012b but see section 3.3.1.2.-3.3.1.3.).

3.3.1.6. Trials in free flight

This method is used to evaluate the effect of direct exposure of a bee in flight to the powder emitted by the drill while sowing coated maize

Fig. 6. Exposure of bees using the mobile cage method.

seed. Such a method is needed to test the hypothesis that bees, in repeated flights to flowering plants, can be expected to fly over plots being sown with coated maize seed and become lethally poisoned with powder acquired during the flight.

1. Bees from 4 hives can be conditioned to visit a feeder some 25 cm in diameter, containing a sucrose solution (50 w/v). The feeder can be progressively distanced from the hives up to a final distance of 100 m (see section 3.3.1.4.1.). Observing the bees, it is possible to count hundreds of bees flying, at an average height of 2 m, to and from the hives to the food source.
2. From the beginning of the sowing and at succeeding 15 minute intervals, bees can be caught in test tubes at the feeder and placed singly in small tulle cages (5 x 5 x 5 cm) and fed with a drop of honey placed on the mesh of the cage, and periodically renewed (every 6-12 h).
3. 24 samples can be captured at each time period, the first when the tractor starts and then every 15 minutes thereafter.
4. Each sample of 24 bees can be taken in cages to the laboratory and kept at a conditioned temperature of 22 ± 1.5°C (see section 3.3.1.2.-3.3.1.3.).
5. For each time interval, 12 cages chosen at random are kept at laboratory humidity and the remaining 12 cages placed in a box at high humidity close to saturation (>95%). The raised relative humidity was obtained by placing the cages in a transparent plastic box sealed, but not hermetically, with a sheet of Plexiglas, and by placing a sheet of wetted absorbent paper at the base. The walls and the cover were sprayed with water and the cages were raised with a strip of polystyrene so that the bees could not get wet from any water that might remain on the base (Girolami *et al.*, 2012a).

3.3.1.7. Collection and analysis of data

1. For both tests (mobile cage and free flight), the comparison between bee survival at the beginning of the trial, i.e. before the start of drilling and after every 15 minutes is obtained (for a maximum of four samples, but are sufficient two samples).
2. To compare different bee samples (treatments, humidity levels and collection times), the null hypothesis that the mortality is independent on the considered parameters should be tested using a chi-squared goodness-of-fit test.
3. To verify the influence of relative humidity, the cages with the bees, are randomly divided and held in laboratory or high humidity (see section 3.3.1.3.).
4. In the mobile cage test, the distance from the driller, which causes no acute bee mortality, also can be estimated.
5. This method of bee mortality evaluation in the field (in particular the mobile cage) is an innovative biological test that can be applied to verify the efficiency of driller modifications.

4. Effects of toxic substances on bee colonies

This section describes methods of testing effects of toxic substances on honey bee colonies. The experimental unit consists of the colony or its different components (brood, stores, bee community etc.). If the observed subjects are not the colonies but single bees, these are free to interact with the entire colony. This assures that the bee behaviour is as natural as possible.

4.1. Introduction

The honey bee colony can be considered as a superorganism including numerous bees of different castes, ages and sex acting together to develop the nest. The evolutionary success of honey bee colonies is based on social organization between the workers and the queen for colony growth and development. The social organization is based on division of labour that depends on individual endogenous biotic factors like hormonal, genetic, immune and neurobiological backgrounds and on exogenous biotic factors like chemical communications, social immunity and behavioural interactions, with all of these factors capable of being modulated by the external environment.

Bearing in mind the complexity of the functioning colony, when significant variability in the response to toxic substances of bees is demonstrated using cage experiments, it is reasonable to expect that the difference in response will be even greater between bees in cages and in natural conditions. Depending on the questions to be addressed, it may be necessary to consider working either at individual (cage) or colony level. Thus, for studying the molecular effects of a toxin on bees, cage experiments using very controlled environment may be the best choice. However, in the end, the effect of the toxin in the real life of the bee, i.e. in natural conditions, should be addressed, even if it is much more difficult to manage honey bee colonies than cages.

Ideally, studies on the effects of toxic substances at the colony level require contiguous treated and non-treated areas of a field where

colonies can be placed. Unfortunately, these protocols are not easy to use as the bees will forage in both non-treated and treated areas. Moreover, the sites at which bees can forage in field conditions are not controlled at all, even when colonies are placed close to the observation areas. Thus, it is proposed to observe the behaviour of foragers directly on the target crops, in addition to overall colony development or in semi-field trials (in tunnels), to determine the effect of treated crops on honey bee colonies in semi-controlled conditions. These semi-field trials are informative, but with the bias that usually the colonies do not develop as well as colonies placed in natural conditions. Another approach consists of mimicking the exposure to a substance on the field crop by forced in-hive feeding with syrup or pollen patties and observing the colony development and the impacts on individuals using various investigation methods. This approach can be used to test the effects of acute, chronic, lethal or sub-lethal exposures to different substances. Different parameters can be studied using those methods for testing the toxins on bees at the colony level: individual adult and brood mortality, clinical symptoms or colony development. However, individual observations on behaviour are particularly interesting for gathering information on sublethal effects of the toxins. Different technologies such as honey bee counters, RFID labelling or harmonic radars have been proposed for this purpose.

This section gives information on techniques used to study the effects of toxic substances, including dusts dispersed during sowing and systemic substances distributed in plant matrices, at the colony level. Different field or semi-field protocols are described and in the future could be the basis of procedures used in the risk assessment of pesticides.

4.2. Determining pesticide toxicity on bee colonies in semi-field conditions

4.2.1. Introduction

After the determination of LD_{50}'s on individual honey bees in laboratory conditions, it is necessary to enlarge the assessment of pesticide impacts using outdoor tests at the colony level. These higher-tiered semi-field tests are performed under insect-proof tunnels. A key characteristic of such tunnels, which are similar to those used for the production of some vegetable crops, is that they must be of sufficient size to permit "normal" bee activity (flight and foraging). Tunnels should be at least 120 m²-150 m² (7-8 m x 20 m) and covered with a net that allows wind and rain into the tunnel to duplicate natural climatic conditions. In contrast, small cages of 9 m² (3 x 3 m) typically dedicated to plant selection cannot be considered for semi-field tests for various methodological reasons. The available space is too small and the numerous limited bees cannot fly around the queen -less one-frame hive.

Semi-field studies under insect-proof tunnels are largely based on the existing French CEB protocol n 230 (CEB, 2011). This kind of a test is intended to assess effects from a worst-case exposure scenario, where bees are confined to plants treated with a pesticide. Such studies under insect-proof tunnels are used to determine the following parameters:

- daily mortality,
- foraging activity and repellence effects,
- brood development,
- colony strength,
- behaviour of forager bees,
- residues on apiarist matrices (bees, honey, brood, wax…)

4.2.2. Tunnel description

1. The tunnels (Fig. 7) are placed side by side and separated from each other by a minimum distance of 2 m. All tunnels have the same orientation for common disposal. The tunnel nets are stretched out and embedded alongside the tunnel, thus creating a closed environment limiting foragers' flights. This space appears nevertheless sufficient after adaptation. Rain and wind, though weakened, are able to pass through the net. Temperature is sometimes a little higher in the tunnel than outside, but generally, there is small difference between the two environments (± 1°C).
2. Attractive plants are grown under tunnels in order to trigger foraging activity. These include *Phacelia tanacetifolia,* oilseed rape (*Brassica napus*) or mustard (*Sinapis alba*). When the trial is dedicated to behaviour assessment, sunflowers are convenient for their large flowers where forager bees can be easily observed. In the special case of the use of a pesticide against aphids on cereals, the crop should be winter wheat where bees are attracted by the daily spray of a sugar solution simulating the aphids' honeydew.
3. Inside each tunnel, 4 plots of the same size (2m x 8m) are delimited and separated by areas covered with a film of synthetic material, where vegetation has been removed (Lane 1 to Lane 6, see Fig. 8). The dimensions of these plastic-covered areas are adapted to the tunnel dimensions but the peripheral paths (Lane 1, Lane 3, Lane 4 and Lane 6) are at least 1m wide. The 4 plots (T1 to T4) receive foliar applications. The same relative plot position is adopted in all tunnels.
4. The hives (see section 4.2.5.) are placed in the central parts of the tunnels (Lane 2), as shown in Fig. 8. The entrance of the hive is directed towards the water supply on the central path. After placing the colonies in the tunnels, a water source is provided on the central path. The water source is removed during the foliar application.
5. After a few days of confinement, foraging bees' activity is adapted to the considered area.

Fig. 7. Example of a tunnel used for semi-field toxicity tests.

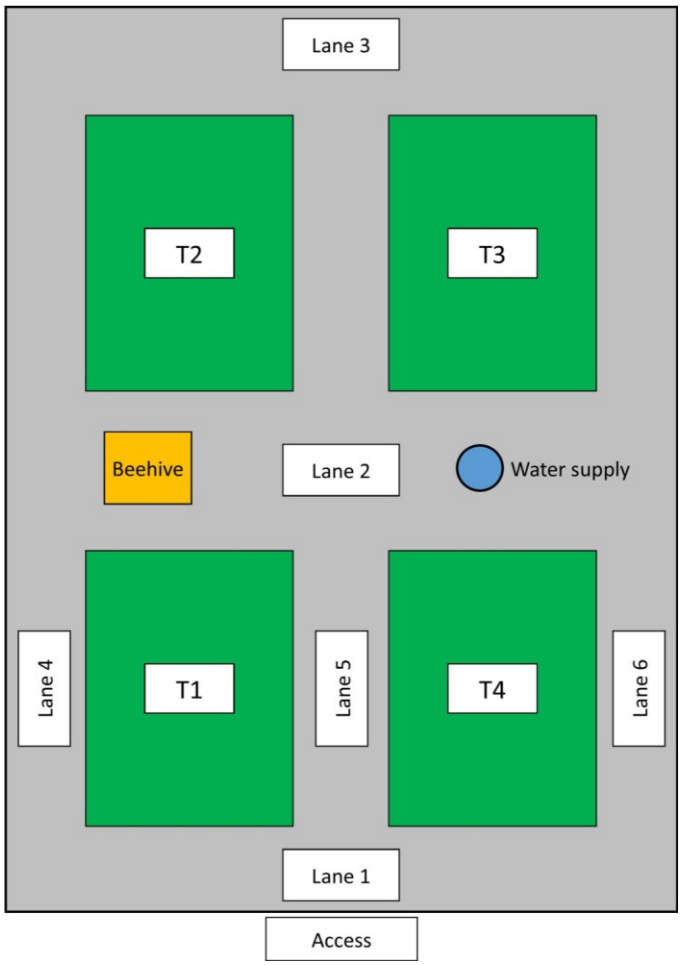

Fig. 8. Tunnel design of 4 plots to be treated and dedicated covered plastic lanes to collect dead bees.

4.2.3. Mortality assessment

1. By agreement, daily mortalities are collected all over the dedicated surfaces of plastic covered lanes. Bees dying among the crops are not collected.
2. Dead bees are collected every day in the morning in order to be accurate, and data express the mortality of the previous day. Additionally, bees can be collected twice on the treatment day (D0 in the morning, and D0+ in the evening in order to look at an eventual acute effect). The total mortality rate recorded in a tunnel for a given day results from adding up mortality rates observed in each of the six plastic lanes in the tunnel (lane 1 to lane 6).
3. During the first days, as well as in the control tunnel, mortality could be considered "normal" without, therefore, being natural. Bees hurt themselves against the net when introduced in the tunnel or when trying to escape. They try to locate themselves above the hive and at both ends of the tunnel. So in all tunnels, part of the recorded mortality during the first days is linked to biological and technical reasons. The impact of substances should be considered over this level and is usually recorded in the control.

4.2.4. Foraging activity assessment

1. Foraging activity is observed on all the crop plots during the trial. It is possible to adapt the time of counting to the environment of the trial and to active foraging periods. All the bees present on the crop plots are considered as forager bees. They are all counted one after the other. Counts can be shifted if activity is not considered satisfying (late activity due to morning mist or disturbed by rainfall, etc.).
2. Assessments are managed at least once a day, except on the day of application where assessments are recorded twice before application with one count just before, and three times after with one count 35 min after application.

4.2.5. Hive description

1. A first selection of the hives is made before experimentation in order to choose appropriate colonies. At least two apiarist visits are needed in the beginning and at the end of experimentation, in order to assess colony development. Parameters taking into account include adult bee population and the quantity of brood the quality of the brood (different stages observed), and amount of reserves (see Delaplane *et al.*, 2013b).
2. The structures of colonies are comparable to each other at the beginning of the test period. Colonies are homogenous regarding population, colony strength, food storage, brood and preparation. Beehives, each with a colony of approx. 15,000 to 20,000 bees (see Delaplane *et al.*, 2013b), are local bred. The colonies have queens of the same maternal origin and the same age, one to two years old. Preparation of the

colonies starts in an appropriate temporal distance to the beginning of the study. The colonies are established in Dadant hives with 6 to 10 frames comprising 4-5 frames for brood of all ages, and at least 1 storage frame and 1 empty frame. Hives are introduced into the tunnels 2 to 5 days before crop plot treatments during flowering. In case of applications before flowering, the hives are established in the tunnels during early flowering.

4.2.6. Treatment methodology

1. After hive settlement under the tunnels, the bees will forage on crop plots and strength parameters can be assessed (Delaplane *et al.*, 2013b) for 2 to 5 days until decreasing mortalities are homogeneous within modalities.
2. The number of semi-field tunnels is defined by the objectives of the study and includes at least 4 tunnels:
   - two tunnels for the pesticide in question
   - control tunnel (negative reference)
   - reference tunnel (positive reference)
3. The tested pesticide has to be applied in two modalities. The first duplicates GAP (i.e. applied according to label) and the second includes "the worst case of exposure". Therefore the first pesticide application occurs during flowering but when bees are not present in order to avoid contact with forager bees (after bee flight generally at night). The second tunnel receives a pesticide application while the bees are foraging on the test crop. To ensure adequate bee exposure for the second modality, there should be at least 5 forager bees/m² crop at the time of the foliar application
4. In the negative reference tunnel, the test crop plots are treated with water in order to determine any physical effect of the spray.
5. The reference tunnel (positive reference) exists to demonstrate bee sensitivity to a pesticide and to validate the trial. Dimethoate (400 g AI/ha) should serve as the toxic standard in the reference tunnel. It provides a high peak in mortality after application. It is, therefore, possible to add replicates of these four initial tunnels in a single study, or to conduct the study again in other conditions.
6. When the semi-field test is used to determine the behaviour of forager bees exposed to no foliar pesticide application (i.e. coated seeds or soil treatment), the test design has no toxic reference (positive reference, one does not exist) and only two modalities are needed (treated and negative reference).

4.2.7. Applications

1. Foliar applications are conducted after the stabilisation of daily mortalities in 2 to 5 days.
2. The four crop plots inside a tunnel receive the treatment, first the water control, then the study item, and the toxic reference at the end.
3. The application is conducted using a 2m long side sprayer boom set with nozzles.
4. The test pesticide and positive reference are applied with an air sprayer.
5. Spraying is performed at a steady speed that guarantees a homogenous deposit level over all sprayed areas. The application is performed with a volume of solution of nominally 200 l/ha at a pressure of 1-2 bar. Walking speed is established during the calibration procedure. The calibration procedure of the equipment used for the application is documented in the raw data.

4.2.8. Comparison of impacts

1. The use of the control and the toxic reference provides predictable impacts to which the impact of the test pesticide can be compared. Mortality is standard and predictable in the control though the foraging activity to the flowering crop may vary with climatic conditions.
2. Colony strength and development (measured per Delaplane *et al.*, 2013b) should be similar at the beginning and end of the experimental phase under the tunnel.
3. On the contrary, the reference dimethoate 400 g AI/ha induces a high mortality the day after application and continues for several days. During the same time, the count of forager bees (see section 4.2.4.) drops to zero because of the pesticide's high repellent effect.

4.2.9. Extension to other topics in semi-field tests

Foliar application on flowering crops is the main classic topic addressed using semi-field tests. However, as previously mentioned, it is possible to perform semi-field tests with other special aims:

- forager behaviour on treated sunflowers: =observe specific parameters associated with individual forager bees (mobile/ immobile, cleaning signs, clinic intoxication signs, etc., see Scheiner *et al.*, 2013)
- brood parameters associated with foliar applications and specific assessment along a 21-day brood cycle (see sections 5.2.2.2.5.4. and 5.2.3.). The OECD (2007) guidance document highlights the problems caused to brood development: assessment of the brood, including an estimate of adults, the area containing cells, eggs, larvae and capped cells (termination of the brood development and eventual compensation).
- residue studies in controlled conditions in pollen, nectar, dead bees, as well as in honey wax, soil and plant (flowers or the whole plant).

4.3. Testing toxicity on bee colonies in field conditions

4.3.1. Problems related to the experimental design

4.3.1.1. Introduction

In the current EPPO guideline (EPPO, 2010a), the field test is designed as the higher tier for the bee risk assessment of PPPs. In fact, according to the EPPO, field tests provide the most reliable risk assessment because it is based on data gathered under conditions which are most similar to agricultural practice. However, field studies are not often repeated because of the complexity of their establishment and their high cost. Only replicates over time can be conducted but, they are subjected to climate variations. Moreover, several methodological limitations, especially related to honey bees' underexposure, make it difficult to assess the realistic risk of a given pesticide to bees using field tests. In this section, the problems related to the experimental design of the field test and how to deal with these problems are discussed. The recent considerations from the EFSA Opinion on the risk assessment of PPPs on bees were taken into account (EFSA, 2012).

4.3.1.2. Replicates

Field studies are more difficult to conduct than semi-field and laboratory studies. One of the main critical points concerns the replicates. In fact, it has always been affirmed that one replicate consists in more colonies located in a single area. Nevertheless this assertion is controversial. In fact, in a field study it is always very difficult to replicate the same environmental conditions in independent trials (it is necessary to have no interference between treated/ untreated colonies and replications). For these reasons, in the field every single colony needs to be considered a replicate. In this way, a field experiment using about 10 colonies per apiary can be considered adequate. Furthermore, if it was impossible to find two experimental fields in the same conditions for the comparison of the treatments, then it should be allowed to perform the test on a single plot (before and after the chemical treatment in the same field).

4.3.1.3. External factors

The results of the field studies can be affected by several factors outside the intrinsic toxicity of the substance. This includes the attractiveness of the target crop and the other plants surrounding the test field, the weather conditions during the experimental test, and the modality of the treatments. Honey bees forage an average of 1.5 km radius around their nest (Crane, 1984). However, this can extend to > 9 km under stressed food conditions (Seeley, 1985). For this reason, it is possible that bees from colonies in treated fields could forage in untreated areas and *vice versa*, thus underestimating pesticide exposure. In order to reduce this "dilution factor", the colonies in the test field should be isolated from other important blossoms and the test crop should be very attractive to bees (see section 4.3.1.4.). Ploughed fields, rivers and highways can be used as natural barriers to isolate the test fields. The negative reference field (if present) should be located at least 4 km from the treated field and in an area with similar climatic and landscape conditions.

4.3.1.4. Application of treatment

EPPO guideline 170 (EPPO, 2010a) suggests to make treatments using the formulated product applied on the blooming crop (e.g. rape, mustard, *Phacelia* or another attractive crop to bees). The product should normally be applied at the highest dose recommended for practical field use. EPPO guideline 170 suggests treating a crop area of about 1 ha. This field range may be sufficient if the crop is very attractive to bees, with high nectar and pollen production, and a high number of flowers per area unit. However, this treated test area is much smaller than the mean foraging area (700 ha) and the level of exposure could be considerably underestimated. An area of at least 2 ha should be used in field tests and it should be isolated from other flowering crops in the bee foraging area. Otherwise, the plot size will be increased proportionally so as to maximise the exposure of foraging bees.

4.3.1.5. Colonies

The colonies should have queens of the same age (1-2 years) and from the same mother origin. Colonies should be homogeneous in size (adult bees and brood – Delaplane *et al.*, 2013b), in brood composition (about same number of young and capped larvae) and in food supply among treatments. The colonies should be visited regularly, at least once or twice a week, for purposes of monitoring the health status and should be free of pathogens before the pesticide application (see Volume II of the *BEEBOOK* for methods to choose colonies that are free of the various pests/diseases). Each colony should have a bee population that covers at least 7 to 10 frames, containing at least: 5 brood frames, 2-3 frames of food, and 1-2 empty frames in order to allow colony growth. The hives should be placed in the edge of the field from 7 to 5 days before the application of the pesticide to the crop to allow the colony to adapt to the surroundings. In order to prevent the bees from foraging in another field, the installation of the hives should be made at the beginning of flowering and a minimum of 7 days before pesticide application. In order to consider the inter-colony variability, at least 10 hives equipped with dead bee traps should be installed in each field.

4.3.1.6. Level of exposure

An important issue in field studies is to demonstrate that all age cohorts of bees (forager and in-hive bees), have been exposed to the test pesticide at the level from which we want to protect them when considering the worst case exposure scenario. For spray products, three exposure routes should be considered: oral, contact and inhalation. Honey bees can be exposed orally through nectar, pollen, and water but also directly during flight or when walking on contaminated

substrates. These exposure routes should be considered both for forager and in-hive bees, even if in-hive bees are exposed mainly through residues in the food. The contact and inhalation exposures for in-hive bees should be assessed only in certain cases (e.g. fumigant and liposoluble products with high wax-affinity).

In order to determine if the experimental conditions in the field tests allow one to achieve the target exposure level, several observations and analysis should be performed. For forager bees, the level of exposure can be assessed by observing the number of bees on the test crop, the number of bees entering the nest with pollen loads and the flight activity (e.g. counting the number of bees exiting from the nest in 30 seconds (Porrini, 1995). Confirmed contact with the treated crop can derive from the palynological analysis of the pollen load (see Delaplane *et al.*, 2013a). Pesticide residues should be analysed in honey bees, as well as in the plant matrices (nectar, pollen and guttation droplets) and in the hive (honey, wax, stored pollen and larvae) in order to know the amount of the target pesticide potentially available for forager and in-hive bees following the "destiny" of the compounds from the plant to the hive. For systemic compounds or for pesticides sprayed during bloom, residue analysis should be always carried out in the hive matrices. These analyses can be used to know the potential exposure routes for bees and their duration over time.

4.3.1.7. Mode of assessment and recording

Meteorological data should be recorded at appropriate interval during the whole trial period. These data should include at least: temperature, relative humidity, rainfall and wind speed and direction. All parameters should be assessed at least from 7 days before to 15 days after pesticide application. Post-application assessment should last at least two brood cycles; this evaluation should be extended in case of residues in wax, honey or pollen. In any case, the colonies should be monitored until the following spring, when bees have consumed the food stores.

All parameters should be recorded at least for 7 days after treatments or during the whole exposure period (blooming) for systemic products. After that, assessments should be limited to determining colony size (Delaplane *et al.*, 2013b) until 42 days after treatment (two complete brood cycles). Because time of the day can affect several bee parameters (e.g. flight activity), assessments should be performed approximately at the same time of day.

4.3.1.8. Interpretation of results

4.3.1.8.1. Simultaneous trials

In case the treatment and the control trials were carried out simultaneously, in two different fields, the study could be considered valid if it meets the following conditions:

- before application, the mortality and the foraging activity among the hives of the two treatments are similar and standard (mortality comparable to that detected in the same period in hives located in the same area in good health conditions and without environmental stress);
- in the untreated field, the mortality and the sanitary status of the colonies are comparable before and after application;
- weather conditions during the test allowed normal foraging behaviour.

4.3.1.8.2. Consecutive trials

In case the treatment and the control trials were carried out consecutively (control trial: first week, treatment trial: second week), in the same field, the study could be considered valid if it meets the following conditions:

- before application, the mortality and the foraging activity of the hives are standard (mortality comparable to that detected in the same period in hives located in the same area in good health conditions and without environmental stress);
- weather conditions during the trials are similar;
- the tested crop's attractiveness to bees is higher, compared to the surrounding area, during the trials.

4.3.1.8.3. Data processing

Appropriate statistical analysis should be done for each assessed parameter in order to detect differences between treatments and among days, in particular before and after pesticide application (see Pirk *et al.*, 2013). The magnitude and the duration of the effects should always be detected for following parameters:

- bee mortality and behaviour deviance (see Table 1),
- strength of the colony and honey production (Delaplane *et al.*, 2013b),
- bee activity (Scheiner *et al.*, 2013).

Moreover, an analysis of the statistical power to detect a certain magnitude of effect should be provided in the test (Cresswell, 2011). In fact, the hazard of a pesticide should be defined in terms of magnitude and of temporal scale. For instance, in the treated fields, the bee mortality is increased *x* times compared with the control for *y* days. This information can be of use to the risk manager for mitigation actions (see section 8.4.4.).

4.3.2. Forced in-hive nutrition

4.3.2.1. Introduction

Forced in-hive nutrition has been used to investigate the distribution of a xenobiotic within the colony (honey bees) and within the hive (beeswax, pollen, honey) and determine the effects of exposure on honey bee colonies and the development of honey bee colonies.

The selection of the conditions to conduct tests with honey bee colonies is driven by the goal of the experiment. When studying pesticides, the exposure – acute or chronic - is the first parameter to determine. Secondly, experimental conditions have to be chosen for the observation of the targeted parameters such as the mortality of honey bees (adults and larvae), the behaviour of honey bees (Scheiner *et al.*, 2013), the presence/absence of bee pests and diseases (see *BEEBOOK* Volume II, and typical bee disorders (absence of eggs, absence of foraging activity, etc.).

Forced, in-hive nutrition has been used to study veterinary drugs given to colonies (antibiotics and acaricides (Adams *et al.*, 2007)), pesticides used for plant protection (Faucon *et al.*, 2005; Pettis *et al.*, 2012) and the effects of various diets, whether artificial or natural, on colony development (Mattila and Otis, 2006b). The last point does not imply the study of any AI but has generated many publications describing how to artificially feed colonies. These publications also described the parameters observed to assess colony development and some biological traits of honey bees: estimation of the number of populated frames; estimation of the total comb area with sealed brood, open brood (eggs and larvae), stored pollen, or stored honey (see Delaplane *et al.*, 2013b); assessment of worker longevity, monitoring of behaviour –including memory through the use of PER reflex (proboscis-extension response)- and foraging pattern (see Scheiner *et al.*, 2013); measurement of protein content of workers; and the measurement of *Nosema* spore levels in workers (Mattila and Otis, 2006a; Mattila and Otis, 2006b; Mattila and Otis 2007; DeGrandi-Hoffman *et al.*, 2008; Mattila and Smith 2008; Avni *et al.*, 2009, Fries *et al.*, 2013).

4.3.2.2. Methods

4.3.2.2.1. The use of test syrup

There are multiple reasons for using syrups (sugar water) in the study of honey bee colonies. In this section, we will only focus on syrup use to study pesticide effects on colony or pesticide repartition within the colony. The use of syrup to distribute an AI for varroa control such as the trickling method (pouring syrup directly onto the bees between the frame spaces with a syringe) will not be reviewed but can be found in Dietemann *et al.*, 2012.

4.3.2.2.1.1. For pesticide studies

Only a few studies report the use of supplemented syrup to study the influence of pesticide on the colonies maintained in field conditions. Faucon and collaborators (Faucon *et al.*, 2005) studied the effect of imidacloprid exposure on colonies by feeding them with two concentrations of the pesticide diluted into syrup. One litre of syrup was given to each colony twice a week during two months. Bee activity, bee mortality, colony weight, honey production, observation of disease symptoms and pesticide repartition within the colony were assessed.

In 2007, the European Commission indicated that some guidelines related to setting maximum residue limits (MRL) should be produced for pesticides in honey within the EU regulation framework (EC-396/2005) using colonies fed with supplemented syrup. The working group led by French Food Safety Agency - AFSSA (now incorporated in French Agency for Food, Environmental and Occupational Health Safety - ANSES) identified a gap in the regulation when pesticide residues may arise in honey through residues present in feeding stuffs. MRLs established in this case should in principle be set on the basis of appropriate supervised residue trials data. Therefore the group produced a document including a protocol to study the transfer of pesticide residues from syrup to honey (AFSSA, 2009). The principle of the test is based on spiked sugar syrup placed in a colony feeder. The honey bees collect it and store it in the cells of beehive frames. After transformation, the ripe honey is analysed to determine the "residue" of the tested AI. Control syrup is spiked with the solvent used to dilute the test compound. The quantity of syrup given to each colony depends on the strength of the tested colony. A quantity of 5l for a colony of 10 combs and 20,000 honey bees is considered sufficient. Syrup is distributed in the feeder all at once. In this protocol, only residues in honey are assessed. However, it is possible to adapt other observation concerning the biological traits of honey bees if needed.

4.3.2.2.1.2. For antibiotic studies

When experiments are set to study antibiotics, they usually aim at documenting the repartition of antibiotic residues within the apicultural matrices. Antibiotics are mixed with syrup made usually with sucrose. Syrup can be poured into frames (Adams *et al.*, 2007), or fed to the colony with through feeders. Control colonies are fed with non-supplemented syrup (Martel *et al.*, 2006).

4.3.2.2.2. The use of pollen patties

Patties have been used mainly to document the influence of diet on colony development. In some experiments, they have been use to investigate the effects of chronic pesticide exposure on honey bee health (Pettis *et al.*, 2012). Patties are principally made with some kind of protein (commercial products or pollen collected by honey bees) and sugar (syrup or honey) (Mattila and Otis 2006a; Degrandi-Hoffman *et al.*, 2008). Quantities given to colonies are dependent on the purpose of the experiment and on the size of the colony. When patties are used for pesticide studies, they are spiked with the given AI. In the latter case, it is recommended to sample the fresh patties and analyse it for pesticide levels to insure the proper delivery of the target dose to the colony.

4.3.3. Dust dispersion during sowing

4.3.3.1. Introduction

In contrast to targeted spray applications, where bees are exposed in the treated crop, exposure of bees to dusts is caused by dusts in the seed bag and dusts abraded from the seeds which are emitted into the environment during loading of sowers and during sowing and drift into neighbouring flowering crops. The contamination of nectar and pollen in adjacent field crops and contact exposure to dusts on the treated plants are the most important routes of exposure of bees to dusts. To achieve a realistic pesticide exposure to bees foraging on flowers from bee attractive plants located next to fields sown with pesticide-treated seeds, specific requirements in terms of study design, test item application, and field experiment establishment need to be met.

As no commercial machinery for a targeted dust application on flowering crops is available, it is not possible to administer precisely target doses of AI/ha on flowering crops. Most field trials are conducted by sowing treated seeds and measuring drift into neighbouring areas. To achieve meaningful results, appropriate establishment of trials with sowing and drift of dusts into adjacent crops must be accomplished and one must generate proof of achieving the targeted exposure to bees. While the development of appropriate methods for dust trials continues, experimental designs that allow assessing pesticide effects on bee colonies have been effective and are described in this section.

4.3.3.2. Methods and general requirements for dust exposure field studies

4.3.3.2.1. Requirements for establishment of field trials

4.3.3.2.1.1. Set up and location of bee hives

Field colonies should be set up directly at the field border and sowing activity should be carried out during full bee flight to ensure bees will be exposed by flying through dust clouds during sowing.

4.3.3.2.1.2. Seeds

1. Seed treatment quality data should be obtained before the trial. As the treatment quality may vary between seed treatments and batches, a poor seed treatment quality should be used as a worst case scenario. The total emission from the sowing machine is influenced by the dust abrasiveness (Heubach-value) as well as by the content of AI in dust. The seed quality used for trials needs to be documented for both, amount of dust and content of AI, before the trial starts and given in the report. Since 2008 the Heubach-Dustmeter test method (Heimbach, 2008) was introduced and proposed as a standardized measure of dust abrasion. The Heubach method mainly detects fine dust particles which are most prone for drifting.
2. Residue analysis of the AI in the dust needs to be given in the study as well as information on the AI and the treatment rates.
3. Furthermore, dusts may be present at the bottom of the seed bags. Thus, before the trials, seed bags should be checked to determine if any dusts remain at the bottom. All contents from the bag should be filled into the driller.

4.3.3.2.1.3. Amount of seeds used per hectare

The amount of seeds used per hectare influences the emission for the field sown into neighbouring areas. Therefore the amount of seeds drilled per hectare (amount filled into the drillers minus amount still in the driller after the sowing) needs to be calculated and reported.

4.3.3.2.1.4. Machinery and modifications of sowing machines

1. The machinery used will influence the potential emission. Depending on the crop, mechanical or pneumatic seeders are used for sowing of different crops. Mechanical seeders usually release only small amounts of dusts which is in contrast to precision airplanters with pneumatic vacuum singling of seeds. A number of sowing machines and their accessory kits regarding the potential for dust emission during sowing have been tested for their dust emission potential. Compared to unmodified standard equipment, the drift of these models with deflectors was at least 90% reduced.
2. Depending on the study aim, it should be decided if deflectors should be used. All details on the machinery and deflectors used for sowing need to be documented and given in the report. Preferably tested sowers should be used (e.g.http://www.jki.bund.de/no_cache/en/startseite/institute/anwendungstechnik/geraetelisten/abdriftmindernde-maissaegeraete.html). For dust drift trials, different machinery types, e.g. pneumatic or mechanic sowing machines, may be used depending on the study aim. Also deflectors may be used depending on study aim. All details about the machinery used need to be given in reports.

4.3.3.2.1.5. Location of fields

An isolated location ensuring exposure of bees in an attractive, exposed crop adjacent to the sowing needs to be chosen. As with all standard field tests, it should be ensured that no other bee attractive crops are present in a range of at least 2 km to ensure maximum exposure.

4.3.3.2.1.6. Soil conditions

Humid soil surface is more likely to retain dust particles on the field sown. As a worst case situation, a dry soil surface is recommended which will allow dust particles to travel and drift even after having touched the soil surface. Soil condition and soil humidity for the time of the sowing have to be reported.

4.3.3.2.1.7. Wind conditions, direction, weather conditions

1. The field site needs to be carefully chosen as it should be determined that sufficient drift directed into the exposed flowering crop occurs.
2. Wind speed and wind direction especially during sowing needs to be documented and reported. For achieving the worst case exposure, fields should be established to ensure that all dusts drift into the flowering crop. Since it is not possible to predict the wind direction several days before start of the experiment, it is recommended to have flowering neighbouring crops on two sides, representing two main wind directions. The trial set up and the availability of uncontaminated forage needs to be carefully considered in the interpretation of the results.
3. Other weather conditions before, during and after sowing have to be reported in the same way as for experiments with spray applications.

4.3.3.2.1.8. Sowing

The sowing area should be sufficiently large. Dust drift may travel far wider than spray drift. Therefore the sowing width should be sufficiently wide (about 50 m or more). The start and end of the sowing area has to be reported.

4.3.3.2.1.9. Foraging conditions during full bee flight

To ensure the exposure of flying and foraging bees to the pesticide, sowing should be done during full bee flight activity when bees are actively foraging on the crop neighbouring the sowing area to ensure the worst case exposure to contaminated plant surfaces, nectar, pollen, and to dusts present in the air during the sowing process.

4.3.3.2.1.10. Crop for sowing

As the seed treatment quality and the potential of crop exposure may vary greatly between different crops, the crop needs to be selected according to the study aim.

4.3.3.2.1.11. Flowering adjacent crops

Adjacent to the sowing area, a bee attractive crop (e.g. Winter Oilseed Rape, *Phacelia* or Mustard) is needed. The crop should be at full flowering (BBCH 65-67).

4.3.3.2.1.12. Residue samples (plants, bees, bee matrices) proof of exposure

1. To demonstrate the exposure achieved in the contaminated adjacent crop, Petri dishes with wet filter paper should be placed at least at 1, 3, 5, 10 and 20 m in free cut areas (on at least 30 m length) in the neighbouring crop.
2. Also, flower samples may be taken very carefully to avoid a loss of dust particles.
3. Foraging bees returning to the hive should be collected for residue analyses of nectar and pollen.
4. Additionally, samples of fresh nectar in combs, freshly stored pollen, honey and bee bread or other matrices (e.g. Royal Jelly) may be obtained.
5. Because soil particles may drift during sowing, a residue analysis of the upper soil layer is recommended.

4.3.3.2.2. Setup of field trials using other devices for a direct dust application

A few testing facilities have developed machinery for a direct application of dusts in field trials. As only small amounts of contaminated dust containing insecticides are emitted during sowing operations, only very small amounts of these dusts have to be applied homogenously. To ensure a good dispersion of small amounts of insecticidal dusts during application in the field, an inert filling material may be necessary. Different materials may be used for filling purposes. Small dust particles of soil seem to represent real field situations best and are recommended. A good mixing of the contaminated dust and the filling material needs to be ensured. It is important to ensure that appropriate particle sizes of dusts and of the filling material are used. In semi-field trials with manual application of dusts on flowering crops, it has been demonstrated that smaller particles, e.g. below 160 µm, result in higher effects. Small particles are also more likely to drift into adjacent crops. See section 3.2.3.1.2.4. of the present manuscript for the method.

4.3.4. Foraging on a treated crop

4.3.4.1. Returning foragers as a tool to measure the pesticide confrontation and the transport into the bee colony

After the application of a pesticide in blooming cultivations or orchards, forager bees might be contaminated during their flight (Schur and Wallner, 1998). Also systemic pesticides may reach nectar and pollen of seed treated plants or after spray applications before the blooming stage (Wallner, 2009). The bee body itself and the collected goods contain residues of the applied ingredients.

Residue analysis with honey showed that this bee product is inadequate to measure the realistic level with which single bees are confronted. During honey preparation, honey bees have a remarkable influence on the residue level in honey. Reduction factors up to 1000 times have been shown between the nectar contamination and prepared honey. Based on the lypophilic character of the pesticide, colonies are more or less successful at reducing the contamination level. As a general rule, harvested honey is less contaminated than harvested nectar (Wallner, 2009). Therefore honey cannot be used to access the pesticide levels that bees have to handle on their flights. A much better tool, even to demonstrate that there was a contact to sprayed fields, is the analysis of returning foragers and their loads (Reetz *et al.*, 2012). This can be done in field experiments as well as in tent tests with reasonable plot sizes.

Besides the analysis of returning foragers at the hive entrance, it is also possible to collect bees directly from plants or flowers. In this case, a 12 Volt vacuum, which can be run with a car battery, is useful (Wallner, 1997). Residue analysis is performed on the basis of single bees (pollen loads or honey stomach content) or pooled groups of one sampling date.

4.3.4.1.1. Reasons for collection of forager bees

- Residues at worst case level (no dilution, nectar present in the crop)
- In combination with sampling plants/flowers and matrices from the bee hive (honey, pollen, bee bread), the route of transfer of residues from a pesticide in the bee hive can be demonstrated
- Determination of realistic residue values for the risk assessment and further evaluations/studies (e.g. bee brood study in lab)
- Assessment of exposure in the field via pollen source determination

- Assessment of exposure to contaminated water sources, e.g. guttation (Reetz *et al.*, 2011).

4.3.4.1.2. Collection of forager bees in tunnel tents or in the field

On each sampling day, one sample of approximately 300-600 forager bees will be taken per hive. At each sampling, the hive entrances will be sealed before the sampling and the forager bees will be subsequently collected as they return to the hive e.g. by suction with a vacuum, by brushing them into a box filled with dry ice, or by using a pair of tweezers. After each sampling interval, the hive will be re-opened allowing honey bees to return to and leave the hive.

Directly after sampling, each sample will be divided into two sub-samples (A and B). Each sub-sample should approximately 150 bees, one for preparation (A) and one as a retained sample (B). To avoid squeezing during storage and shipment, the bees will be transferred into containers. If <300 bees are collected per hive and sampling day, then sub-sample A will be composed of up to 150 bees with any remainder being allotted to sub-sample B. Details of the approximate numbers of bees collected for each sub-sample will be recorded in the raw data. Each sub-sample will be labelled uniquely.

All samples will be chilled during transport to the freezer and subsequently will be stored deep frozen at ≤-18 °C. Storage conditions will be recorded by use of a data logger or a min/max thermometer and will be documented in the raw data.

4.3.4.1.2.1 Preparation of the honey stomachs

The forager bees collected as described above will be stored deep frozen (≤-18°C) in separate containers for each treatment group until preparation in house of the honey stomachs. In principle, it is possible to determine the nectar source of single bees with pollen analysis of the honey stomach content. Successful foragers could be identified by their body weight before the preparation process.

The preparation of the honey stomachs from forager bees will be done as follows (see Carreck *et al.*, 2013 for more information):

1. All bees of one sample will be allowed to thaw for a few minutes.
2. Bees will be fixed at their thorax and their abdomens will be stretched flat with a pair of tweezers.
3. The abdomens or the tergite plates will be removed, so that the honey stomachs will be free.
4. The honey stomach will be held at the lowest part of the oesophagus (see Carreck *et al.*, 2013).
5. The main front part of the oesophagus should be removed.
6. The honey stomach will be held with a pair of tweezers at the small remaining part of the oesophagus.
7. The total weight of the honey stomachs will be determined.
8. The honey stomach contents from one sampling time, treatment and replicate hive will be pooled to get at least 0.2g per sample. The number of prepared bees per sampling time, treatment and replicate, will be recorded. The nectar sample will be transferred into the freezer immediately after the preparation of one forager bee sample.
9. Bees from the control sampling will be processed first. Once this task has been completed, the process will be started with the last sampling.
10. After preparation, the contents of the honey stomachs will be stored separately for each sample at ≤ -18°C.

4.3.4.1.2.2. Preparation of the pollen loads

The preparation of the pollen loads will be carried out as follows (see Delaplane *et al.*, 2013a and Carreck *et al.*, 2013 for more information):

1. All bees from sub-sample A are kept on a deep frozen metal plate (≤ -18°C).
2. The pollen loads will be detached from the legs of the forager bees and placed into a vial.
3. All pollen loads from sub-sample A will be collected and pooled in order to get at least 100 mg of pollen for residue analysis. If < 100 mg is obtained from sub-sample A then sub-sample B will be prepared. If this is the case, all bees of sub-sample B will be prepared in the same way as sub-sample A and added to sub-sample A. The total number of prepared bees and the sub-samples used will be recorded.

The pollen samples will be unfrozen during the preparation of one sub-sample. The bees and pollen will be transferred back to the freezer immediately after the preparation of one sub-sample. Each sub-sample will be labelled 3 times and will include at least the information given below. All samples will be frozen at ≤ -18°C outside of the sample preparation time.

4.3.5. Systemic toxins expressed in plant matrices

4.3.5.1. Introduction

Systemic products have the capacity to enter into the plants independently of their application pattern. Commercial products containing these AIs exist for treatments of seeds, soils, for applications as spray or directly to the roots or bulbs. Other application patterns may render systemic any AI, as is the case of stem injections. Pesticide formulations may contain other AIs or co-formulants that increase the systemicity of the AI under study (Dieckmann *et al.*, 2010).

This section focuses on the proposal of a protocol evaluating the impact on honey bees exposed to the pollen and nectar coming from a crop that has received a treatment different from spraying with systemic products in field conditions. Exposure to guttation water or honeydew would require specific modifications of the methodology. Therefore, it should be dealt with separately.

Different methodologies for different application patterns: a different section should deal with the study of the impact of pesticides with systemic properties applied on spray.

4.3.5.2. Application of systemic products as seed and soil treatment (SSST), bulbs or root bathing

4.3.5.2.1. Introduction

The methodology presented here focuses on the exposure of bees to contaminated flowers resulting from treated plants (as seed and soil treatments, bulbs or roots bathing). Observations are done at the level of the colony and only individual observations on bees are included insofar as they may affect colony development. In principle, guttation water would not be a major source of exposure given that normally these droplets occur mainly in early plant developmental stages (Girolami *et al.*, 2009; Tapparo *et al.*, 2012). However, the individual geographical and meteorological conditions of each area should be considered to exclude this potential exposure route.

The EFSA has published an extensive review about the risk assessment of pesticides on bees (EFSA, 2012). In this document, a thorough analysis has been conducted concerning the adequacy of the international standards (EPPO, 2010a) recommended for field-testing to the exposure of bees to systemic pesticides. The following recommendations are based on the limitations identified on the EFSA document.

4.3.5.2.2. Principle of the trial

Beehives come from a similar background, the same apiary or constituted in the same way. Their health status and strength are evaluated before the beginning of the trial. Then they are placed on the test fields as soon as the crop presents a number of flowers enough to allow the visit of foragers (5 to 15% of the flowers are flowering). The crop must have been treated at the time of seeding/planted when it starts to bloom. After the flowering period, the colonies are returned to a common area where they will remain until the following season.

The observation of effects continues during and after bloom. The monitoring can be extended until the spring of the following year. Especially when the tests is run during the period of production of winter bees, this monitoring until the spring becomes more relevant. Ideally, the generic observations on the full colonies should be complemented with individual tests studying the impact of sublethal doses on bees, e.g. homing flight tests or with more specific observations (fecundity, growth and development of individual honey bees), though many of the sublethal effects may be captured in the full colony assessments.

4.3.5.2.3. Preliminary steps

Seeding/planting/pesticide application should follow GAP. Bee colonies should be conducted following Good Beekeeping Practices. A flight entrance observation system (e.g. Floriade), which includes a climate control station as well as bee tracking system, could be placed in the area of testing. It should collect the meteorological data (temperature, relative humidity and rainfall) and provide information about the bees' activity all along the duration of the trial. Should such a system not be available, alternatives should be found to collect the mentioned data (meteorological data, foraging activity, etc.).

4.3.5.2.4. Environment of the trial

The aim of the information collected from the environment of the colonies under study is identifying potential interferences of the exposure of bees to the AI or potential synergies in their action on bee colonies.

It is well known that bees cover wide surfaces when foraging, mean distances being around 1.5-3 km, extreme distances being around 10 km (Vischer and Seeley, 1982; Winston, 1987; Seeley, 1995; Steffan-Dewenter and Kuhn, 2003), average surface ranging from 7 to over 100 km^2. International standards, however, normally recommend a treated area of 2,500 m^2 or 1 ha.

With the help of satellite imaging or similar, the environment of 3 km around the placement of the colonies could be audited and noted. All software should be up-to-date. Whenever possible, any chemical treatments happening in this area should be registered and considered for the study.

4.3.5.2.5. Trial plots: experimental and control

4.3.5.2.5.1. Crops planted in the trial plots

In order to increase the likelihood that bees will forage in treated plots, crops attractive to bees should be used. Special attention should be put on the nutritional value of the pollen of the chosen crop. Rich pollens as that of oilseed rape or *Phacelia* may mask the effects of the exposure to the pesticide. Ideally, an attractive crop with pollen of lower nutritional value would better evidence any toxicological problems (e.g. sunflower). For regulatory purposes, the crop for which the authorisation is to be requested should be used.

4.3.5.2.5.2. Size of the trial plots

Trial plots should be a minimum of 5 ha. Should this not be the case, testers should make sure that the treated crop represents a major nutritional source for the colonies of the test during the crop flowering period. Treated seeds or granules with the formulated product can be used as well. It should contain the highest dose recommended for field application. Should less attractive crops be used, specific attention should be put on assuring that exposure occurs.

4.3.5.2.5.3. Location of the colonies at the trial plots

One can possibly increase exposure by placing the colonies on the edge of the field. Studies have shown that pesticides affect the navigation capacity of foragers. By bringing the colonies closer to the field, the distances foragers need to cover might not require as much flight effort. Similarly, bees foraging close to their hive would not need to consume part of the nectar they collect to obtain energy for returning to the hive. Therefore, effects on foragers might be underestimated.

Pesticide exposure has been shown to hinder homing flight and affect foraging behaviour (Vandame *et al.*, 1995; Bortolotti *et al.*, 2003; Colin *et al.*, 2004; Karise *et al.*, 2007; Yang *et al.*, 2008; Decourtye *et al.*, 2011; Henry *et al.*, 2012; Scheinder *et al.*, 2012). Therefore, field trials should be complemented with methodologies specifically evaluating these behaviours. For further information on the protocols to run these tests, see Scheiner *et al.*, 2013. Specific methods can evaluate the impact of pesticide exposure on fecundity, growth and development of individual honey bees (Dai *et al.*, 2010). The development of the colony can be assessed per Delaplane *et al.*, 2013b.

4.3.5.2.5.4. Distance between trial plots

The distance between treatment plots and control ones should be enough to avoid the exposure of the latter to the AI. Therefore, a distance of at least 6 km is desirable. Otherwise, environmental conditions should remain comparable for all plots.

Should the minimum distance of 3 km not be achieved, residue analyses of the contents of the honey stomach of foragers or pollen clusters returning to the hive would provide information about the existence of cross foraging (i.e. bees foraging on the plots not designated for them). Palynological studies can as well help in this task. For method on recovering the honey stomach, see section 4.3.4.1.2.1. or Carreck *et al.*, 2013. Potentially, the same procedure could be developed for the study of the exposure of bee colonies to pesticides in water sources around the apiary.

4.3.5.2.6 Colonies used

Queen-right colonies are used for the trial. Queens should be daughters of one queen of the same age. Ideally, colonies with no remarkable problems (i.e. free of pests/diseases/hive abnormalities) for at least one brood cycle previous to the beginning of the trial should be used.

4.3.5.2.6.1. Colony health status

Colonies should be regularly monitored for the occurrence of diseases (including varroa infestation level, see Dietemann *et al.*, 2013) and any clinical sign should be noted. Prior to the exposure to pesticides, no clinical signs should be observed. Colonies should not be taken if they have received a treatment against varroa in the last 4 weeks prior to the trial. If the varroa treatment is administered during the trial period, the treatment protocol (date of the treatment, product, duration, quantity applied and efficacy observations) should be noted.

Delaplane *et al.*, 2013b describes recommendations concerning colony size, which should be as homogeneous as possible. As field tests should resemble as much as possible realistic conditions, colonies' population would differ depending on the time of the year in which the trial would occur. Colonies of 15,000 individuals would be characteristic of a beginning of the season or overwintering period, while colonies of approx. 50,000-60,000 individuals would be characteristic of the middle of the season (EFSA, 2012). These estimations however, might vary geographically. The evolution of the colony health status along the trial is one of the observations described later in this method.

4.3.5.2.6.2. Number of colonies/replicates – statistical power

6 to 10 colonies per treatment group (exposure/control) should overcome the inter-colony variability (EFSA, 2012). The number of replicates per trial depends on the magnitude of effects that the test should detect. The statistical power of the test should always be calculated (see Pirk *et al.*, 2013).

4.3.5.2.6.3. Colony placement and equipment

Colonies will be placed all together at an environment free of pesticides where they will be monitored at least 7 days before flowering. If necessary, colonies can be fed with syrup to avoid starvation. The colony should not be exposed to contaminants in syrup. Residue analyses or tracking the syrup origin may help providing this information.

When the crop starts blooming (5 to 15% flowers of the crop have bloomed), colonies will be placed on the edge of the plots. Observations of the colonies will start 7 days before the expected time of flowering.

Pollen traps can be installed in 3 or 4 colonies per treatment group. Each colony should have dead bee traps. Devices like colony scales, bee counters or bee-tracking systems (e.g. Floriade, etc.) may provide extra information on the evolution of the colony throughout the trial (see Human *et al.*, for information on using pollen traps, dead bee traps, and for weighing colonies).

4.3.5.2.7. Duration of the test

Colonies remain on the edge of the field for the period of blossom. However, observations of the their evolution will be extended up to at least 42 days after the placement on the edge of the fields under study. This is the time of two complete brood cycles.

After blooming, they should be moved to an environment where they would overwinter together on the reserves they have accumulated during the trial period. The environment of the colonies should provide enough sources of pollen and nectar to survive. If necessary, colonies can be fed with syrup. This can be done making sure that the colony has consumed first its reserves collected during the exposure period. The colony should not be exposed to further contaminants contained in syrup. Residue analyses or tracking the syrup origin may help providing this information.

The colonies should be monitored through the following season. In the event that pesticide residues are still present in the colony at this time, the monitoring should be extended in the new season. A residue analysis of beekeeping matrices would enable one to know when the exposure of the colonies to the AI has occurred over the winter. It should be noted that these are test conditions. In reality, colonies might be exposed to larger amounts of AI over longer periods or to a mixture of AI.

4.3.5.2.8. Bees' exposure

The exposure of bees to AI following SSST is more difficult to control than that following spraying of non-systemic products. This is because blooming does not occur in the whole surface at the same time and because during the blooming period one cannot say if bees are only going to forage in the treated crop. Therefore, special manipulations need to be performed to ensure the level of exposure achieved by the colony as a whole. The control of the colony's food intake is one parameter that can be achieved.

For this purpose, pollen pellets should be collected with pollen traps installed at the entrance of the colonies prior the blooming of the first flowers of the crop and every 2-3 days during the blooming period (see Human *et al.*, 2013). Samples of at least 5 g of pollen should be collected and kept in hermetic conditions, adequately labelled and immediately frozen. Samples are stored at least at -18°C before analysis.

Pollen from the comb should be collected once before the beginning of the crop bloom and once a week following it. If the samples were taken by cutting a piece of comb, wax samples would be readily available. Otherwise, wax samples should be taken as well on the same days and immediately frozen. Samples are stored at least at -18°C before analysis.

Foragers returning to their hive should be collected (see section 4.3.4.1.2.) at the entrance of the colony to undergo residue analysis of the content of their honey sac. Approximately 50 foragers should be collected prior to the blooming and every 2-3 days during the blooming period. Samples should be kept in hermetic conditions, adequately labelled and immediately frozen. Samples are stored at least at -18°C before analysis.

Honey samples should be collected once before the blooming of the crop and once a week after.

Dead bees should be counted daily from the period starting before the bloom and 42 days after it. Dead bee traps (Human *et al.*, 2013) will be cleaned every evening and samples of bees should be collected from the bee traps before sunrise. The collection period goes from just before the start of blooming and is conducted every 2 days during the blooming period. Samples should be kept in hermetic bags, appropriately labelled and immediately frozen (stored at least at -18°C before analysis).

The quantity of sample per beekeeping matrix hereby proposed is indicative. It should be checked with the laboratory in charge of residue analyses prior to the beginning of the test.

Prepupae should be counted daily, in the same way as dead bees. Bee traps will be cleaned every evening and samples of bees should be collected from the bee traps before sunrise. They can be collected from the bee traps every 2 days and kept in hermetic bags, appropriately labelled and immediately frozen. Another option is the sampling of larvae directly from the comb once before the blooming of the crop and once a week after. Again samples should be kept in hermetic bags, appropriately labelled and frozen in case analyses should be delayed.

4.3.5.2.8.1. Pollen analyses

The origin of pollen in the pollen pellets can be identified through their colour and their palynologic analysis (see Delaplane *et al.*, 2013a). Pollen provides a good tool to monitor the environment of the colony. Palynologic analysis should as well be carried out in honey samples. Therefore, in the week previous to the expected blooming of the treated and control crop and once weekly during this period, pollen samples should be taken with the help of pollen traps (see Human *et al.*, 2013). Pollen origin analysis can be used to complete the information on the environment collected from the satellite images.

4.3.5.2.8.2. Residue analyses

Residue analyses of the previously mentioned matrices should be performed for both treatment and control colonies. Two different analyses could be envisaged, one specific on the AI under study for which the lowest possible LOD and LOQ should be used, and a multi-residue analysis of the most common AI used in the area. The former should be systematically performed when conducting field studies. We do not provide a method for residue analyses as such analyses are typically outsourced to analytical labs.

4.3.5.2.8.3. Reserves of the colonies at the beginning of the trial

It is necessary to reduce as much as possible the content of previous food reserves in hives so that the exposure to the AI present in the field can be maximised. That is why one could remove the frames containing mainly food reserves from colonies before the crop blooms. This could lead colonies to starve in the days immediately following the removal of the food. Consequently, the health of the colony should be monitored closely.

4.3.5.2.9. Observations

4.3.5.2.9.1. Controls

The experimental design allows two kinds of controls: internal and external ones. Each colony serves as its own control (internal control), by comparing its evolution before the exposure to the AI and after it. Additionally, the evolution of the treatment colonies would be compared to that of the control ones (external control).

4.3.5.2.9.2. Brood and reserves content

The surface of brood and reserves should be monitored before, during and after the trial (see Delaplane *et al.*, 2013b). Estimation of colony strength parameters should be performed close before the crop bloom and one week after. Given that the reserve frames should have been removed before the study, there should be visual controls of the food content of the colony. The observation should be repeated once weekly

up to the 42 days of the duration of the trial. In case a more intensive data gathering method is used (e.g. the Liebefelder method presented first in Imdorf, 1987 and described in Delaplane *et al.*, 2013b), one could reduce the data collection to every three weeks.

4.3.5.2.9.3. Interpretation of residual information

The information of the residue content in the nectar and pollen brought back to the control and treatment colonies allows one to determine the quality of the control. Additionally, it would provide an estimation of the level of exposure and the comparison of the level of contaminated and non-contaminated food arriving to each colony.

The results of the residue analyses of larvae and dead bees from the trap would provide an indication of the level of exposure that in-hive individuals face. The result of the residue analyses of in-hive stored pollen and honey and the wax would provide an indication of the level of exposure of in-hive bees and of a potential long-term exposure.

4.3.5.2.9.4. Toxicological endpoints

In this section we focus only on the colony as experimental unit. Therefore, the endpoints chosen in this section are directly linked with colony status. Further methodologies could be developed in the field to complement these observations, as is the case of homing flight tests or fecundity tests.

4.3.5.2.9.4.1. Mortality trend

Dead bees can be counted using bee traps placed in front of the hive (Human *et al.*, 2013). If a bee counter is used instead (an electronic device that counts bees exiting and entering the hive), the number of bees leaving the colony and not returning should be determined. These observations should be compared at a certain time of the day with a specific duration (e.g. every morning from 7 to 8 am).

These observations should be done on a daily basis from one week before the colonies are placed in the field until the end of blossom of the treated/control crop. Afterwards, the observations can be done on a weekly basis up to the 42 days.

4.3.5.2.9.4.2. General evolution of the colony during the test

Special attention should be put on the strength and vitality of the colony (see Delaplane *et al.*, 2013b). Should scales be placed on the colonies of study, weight evolution could be used as well as variable to compare treatment and control colonies (Human *et al.*, 2013). The same could be done in case bee counters are installed.

These observations should be done on a daily basis from one week before the day the colonies are placed into the field and until the end of blossom of the treated/control crop. Afterwards, the observations can be done on a weekly basis up to the 42 days.

4.3.5.2.9.4.3. Behavioural observations

The aim of the present protocol is not to evaluate effects on specific behaviours (e.g. homing flight, thermoregulation, etc.), but to observe any alterations on the general behaviour of the colony during the test and after the test. For this reason, any qualitative modification as trembling, aggressiveness, disorientation, apathy, etc. observed at the flight board, outside or within the hive during the test should be noted. Additionally, during a longer period (until next season), abnormalities in the reproduction cycle of the colony should be noted (e.g. supersedure of the queen, problems on egg-laying capacity, etc.). Finally, observations of the flight activity and the foraging behaviour around the hive should be done and alterations should be noted.

There is a wide room for improvement of the behavioural observations that could be done in field test. Namely, specific behavioural traits would increase the accuracy of the observations. The present protocol should be modified in the future as soon as there are advances in methodologies.

4.3.5.2.9.4.4. Colony health

In principle, only colonies not showing disease signs should be included into the experiment. Then pathological signs, their date of appearance and severity should be noted (see *BEEBOOK* Volume II for information on this). The health status of the colony should be monitored from one week before the day the colonies are placed into the field and extended up to the overwintering. The appearance of pathological signs in the treatment colonies, but their absence in the control ones, could be due to a synergic effect pathogens-pesticide.

4.3.5.2.9.4.5. Brood surface and quality

The different observations developed on the brood surface should allow identifying eventual deficiencies in the egg-laying capacity of the queen or the brood success. Any alteration (e.g. mosaic brood, dead larvae/nymphs, increase of pathologies affecting brood, etc.) should be noted, both in quantity and quality. Protocols for brood evolution and monitoring are described in Delaplane *et al.*, 2013b. The assessment of the duration of a brood cycle would be indeed, very interesting from the point of view of the interactions between the pesticide and pathologies. Dead larvae in the bee trap should as well be noted.

4.3.5.2.10. Validity of the trial

Positive residue analyses in samples of pollen or nectar brought back to the control colonies would render the test as invalid. Negative residue analyses in samples of pollen or nectar brought back to the treatment colonies would render the test invalid. Prior to the treatment (before the blooming period) the mortality and behaviour of the colony (incl. foraging activity) should be not statistically differ between

treatment and control groups. Should this not be the case, the study would be invalid.

The evolution of mortality and the different observations described above do not change in the case of the control fields both before and after exposure to flowers. Different crops are susceptible to being treated with the same AI. This could extend the exposure of the colonies under study in time and quantity. Similarly, the different blooms happening in the surroundings of the colonies under testing may dilute the exposure quantities. The purpose of this protocol is to evaluate the effect of on bee colonies of a specific AI applied to a specific crop at a specific time in the year. The uncertainty of the representativeness of the results of the trial to reality is therefore high.

5. Effects of toxic substances on honey bee brood

5.1. Introduction

Honey bee brood may be exposed to pesticides through nectar and pollen collected by foragers. Effects on brood may vary according to the nature of the compound and its concentration in pollen and nectar (Aupinel *et al.*, 2007a, 2007b). Lethal or sublethal effects can be expected throughout the colony life, according to the number of larvae affected, the mode of action and its consequences on bees. Considering that colony survival depends on the adult population directly linked to brood health, it is evident that the effects of pesticides on brood have to be seriously considered.

5.2. *in vivo* larval tests

5.2.1. Oomen test

This test, even if never ring-tested, is a requirement in Europe and it is based on the method outlined in Oomen *et al.* (1992).

- In this in-hive method, experimental units are free flying colonies.
- The artificial contamination with AI is ensured using a syrup feeder of 1 litre fitted to the hive for 24 hours.
- Brood development is followed by weekly inspection of individual brood cells.
- Due to environmental variations, this method may not be easily reproducible since the test product may be stored in the combs and not immediately dispensed to the brood by nurse bees. It may also be diluted by external nectar. No quantitative data can be provided by this test due to the fact that exposure is not controlled.

5.2.2. Semi field test

This in hive method was devised by Schur *et al.* (2003) and is recommended by OECD.

5.2.2.1. Introduction

The European regulatory framework (Directive 91/414/EEC, Regulation 1107/2009/EC) requires data to evaluate the risk of pesticides on the honey bee brood. Beside the possibility to run studies under laboratory conditions, there are 2 publications available to run higher tier studies (e.g. semi-field and field) in order to evaluate the potential impact of a pesticide on the honey bee brood development.

The "in-hive field test" published by Oomen *et al.* (1992), is carried out with free-flying bee colonies, which are fed with contaminated sugar solution. One litre of sugar solution is mixed with a certain amount of pesticide and offered to the bee colonies over a short time period. The brood development is followed by weekly assessments of individual marked brood cells. Such kinds of tests are qualitative test methods or screening tests in order to evaluate the question, whether PPPs are causing harmful effects on the bee brood or not.

A quantitative test method closer to the real field scenario is the semi-field brood test according to the OECD Guidance Document 75 (OECD, 2007). Within this test design a PPP is sprayed directly on a flowering crop and the bee colonies are forced to forage for nectar and pollen in tunnel tents. Thus the bee brood contacts contaminated food and the development of the bee brood in single cells is followed regularly over one complete brood cycle from an egg to a worker bee.

A third possibility to evaluate the risk of PPPs to the bee brood under field conditions is a honey bee field study based on the EPPO 170 (EPPO, 2010a) guideline in combination with detailed brood assessments according to the OECD Guidance Document 75. In the following paragraphs the main focus will be directed to the test method under semi-field conditions.

5.2.2.2. Material and methods of a semi-field brood test

1. Similar as for standard studies based on the EPPO 170 guideline; small healthy honey bee colonies are initially placed in tunnel tents (herein after named tunnels) shortly before full flowering of the crop, a few days before application of the test chemical.
2. Following exposure of the bees in the tunnel for the period of flowering of the crop (e.g. at least 7 days after application of the product), the hives are placed outside the tunnels for the remaining time of the study and are free to forage in the field.
3. It is important to check that the neighbouring environment within a radius of 3 km is free from bee attractive main crops (e.g. sunflower, maize, oil seed rape, fruit orchards) as well as the test substance or other compounds.
4. Mortality of honey bees, flight activity (Human *et al.*, 2013), and condition of the colonies and development of the bee brood (Delaplane *et al.*, 2013b) are evaluated several times over a period of at least 4 weeks after the initial brood assessment.

5. Results are evaluated by comparing the treated colonies with the water-treated colonies and with the reference chemical-treated colonies.

5.2.2.2.1. Design of the test

1. A test includes at least 3 treatments:
   - Test chemical
   - Reference chemical or positive reference: An IGR known to produce adverse effects on honey bee brood (e.g. Fenoxycarb (CAS. 121-75-5), rate: at least 150 g/ha)
   - Control: The plants are treated with tap water (water volume: 200-400 L/ha in case of *Phacelia* as test plant)
2. All spray applications should be done with the same water volume. It is suggested to run the test with at least three replicates for better statistical analysis. Thus, in total at least nine tunnels are established for one test. However, it is also possible to increase the number of replicates to four per treatment group in order to increase the stability of the test.

5.2.2.2.2. Preparation of the colonies

1. The OECD 75 recommends using small healthy honey bee colonies (e.g. Mini Plus, nuclei, etc.) for the test, but it is also possible to use small commercial bee colonies. However, the size of the colonies should be adapted to the size of the crop area within the tunnels.
2. All colonies of one set or study have to be produced at the same time from colonies headed by sister queens to guarantee that the colonies in all variants are uniform as far as possible (Delaplane *et al.*, 2013b). The colonies must be headed by sister queens which are the progeny of the same queen and mated at the same place in order to minimise genetic variability.
3. The bee colonies should be free of clinical symptoms of disease (e.g. *nosema*, *Amoeba*, chalkbrood, sacbrood, and American or European foulbrood) or pests (*Varroa destructor*): see *BEEBOOK* Volume II. The colonies should be free of unusual occurrences (e.g. presence of dead bees, dark-"bald"-bees, "crawlers" or flightless bees, unusual brood distribution patterns or brood age structure).
4. After establishment of the colonies within the tunnels, all hives are equipped with a dead bee trap at the entrance to count the number of dead bees (Human *et al.*, 2013).
5. The colonies should be established in the tunnels shortly before full flowering of the crop and at least three days before application in order to allow the bees to adapt to the conditions in the tunnels.
6. The colonies should be exposed to the treated crop in the tunnels for a period of at least 7 days after the application.

5.2.2.2.3. Test conditions

1. As mentioned in section 5.2.2.2.2., the size of the tunnels should be adapted to the size of the used colonies, but a minimum size of 40 m² floor space is recommended in the OECD 75 guidance document. The minimum height of the tunnels should be 2.5m, to guarantee an unhindered flight of the bees. The covering gauze should have a maximal mesh size of 3mm. The test crop should be attractive to honey bees. Suitable are for example *Phacelia tanacetifolia*, *Sinapis arvensis* and *Brassica napus*.
2. During the whole testing period, the colonies should be supplied with water. A water feeder should be placed into each tunnel as water supply for the bees. During product application, the water feeder should be removed from the tunnel.

5.2.2.2.4. Application

1. The applications should be performed with a boom sprayer with calibrated nozzles according to GAP.
2. The spraying should normally be performed at the time of full flowering of the crop and during high bee flight for worst case conditions or, if required (e.g. for testing of residual or delayed action), in accordance with the intended use pattern of the product.
3. The wind speed should not exceed 2m/sec outside the tunnel.
4. Test products should normally be applied at the highest field rate (ml or g/ha) intended for the registration of the product in order to produce a worst-case exposure for the bees.
5. During the applications in the tunnels the water containers should be taken out of the respective tunnels and the bee colonies should be covered with a plastic sheet until the end of application to avoid direct contamination.

5.2.2.2.5. Assessments

The total observation period of the colonies is at least 28 days.

5.2.2.2.5.1. Meteorological data

During the whole testing period, the following meteorological data should be recorded daily (ideally inside the tunnel):

- temperature (min, max and mean)
- relative humidity (min, max and mean)
- rainfall (total daily)
- wind speed (only during application inside and outside the tunnel)
- cloudiness (during assessment).

Table 5. Time schedule for hive mortality assessment in semi-field brood tests:

DBA = days before application, DAA = days after application.

Timing	**Evaluation of number of dead honey bees**
At least 3DBA to 1DBA	Once a day, if possible at about the same time
0DBA	Once shortly before application
0DAA	2 hours after application 6 hours after application
1 to 7DAA	Once a day, if possible at about the same time
Outside the tunnels:	
8 to 27(±2)DAA	Once a day, if possible at about the same time at monitoring site (dead bee trap only)

5.2.2.2.5.2. Mortality of honey bees

1. Mortality of honey bees should be assessed on sheets suitable for the collection of dead bees (e.g. linen sheets) which are spread out in front of the hives and at the front, middle and back of the tunnels. From experiences with semi-field studies in general, it is known that most bees which are dying in the crop area can be found in the front and back corner of the tunnels. The middle linen is necessary as a path for walking during the application.
2. Before the start of the test, such paths should be created in each tunnel by removing of the plants and by smoothing the ground. Subsequently, the paths are covered with the aforementioned sheets in order to facilitate the collection of the dead bees in the crop area.
3. Additionally the dead bees are noted and counted in the dead bee traps which are fixed at the entrance of the hives. The assessments could be done according to the Table 5.
4. The assessments of the number of dead bees should be conducted at approximately the same time in the morning in order to cover the same time span from one day to another. During each assessment, the number of dead bees should be differentiated into adult worker bees, drones, freshly emerged bees, pupae and larvae.

Table 6. Time schedule for flight activity assessment in semi-field brood tests:

DBA = days before application, DAA = days after application.

Timing	**Evaluation of number of forager honey bees/1 m² and observation of behaviour**
At least 3DBA to 1DBA	Once a day during flight activity of the bees
0DBA	Once shortly before application
0DAA	4 times during the first hour after application 2 hours after application 4 hours after application 6 hours after application
1DAA	Three times during flight activity of the bees (preferably in the morning, midday and afternoon)
2 to 7DAA	Once a day during flight activity of the bees

5.2.2.2.5.3. Flight activity and behaviour

1. Flight activity could be recorded on a 1 m² area, at 3 different places in each tunnel according to the time table presented in Table 6.
2. At each assessment time, the number of bees that are both foraging on flowering plants and flying around the crop are counted for a short time period (for example 10-15 seconds depending on the crop) per marked area.
3. During the assessments of flight intensity, the behaviour of the honey bees in the crop and around the hive should be observed with respect to the following criteria:
   - aggressiveness towards the observer
   - guard bees attacking and/or preventing returning bees from entering the hive
   - intensive flying activity in front of the hives without entering the hive
   - intoxication symptoms (e.g. cramping, locomotion problems)
   - clustering of large numbers of bees at the hive entrance.

5.2.2.2.5.4. Brood assessments

5.2.2.2.5.4.1. Condition of the colonies

1. The condition of the colonies is assessed once before the application and several times after the application according to the following time schedule:
   - BFD (brood area fixing day), first assessment
   - Application at +2 days (±1 day) after BFD
   - + 5 days (±1 day) after BFD
   - + 10 days (±1 day) after BFD
   - + 16 days (±1 day) after BFD
   - + 22 days (±1 day) after BFD
   - + 28 days (±1 day) after BFD.
2. For the condition of the colonies the following parameters are assessed in order to record effects of the test chemical:
   - Colony strength (number of bees per Delaplane *et al.*, 2013b)
   - Presence of a healthy queen (e.g. presence of eggs)
   - Pollen storage area and area with nectar or honey (per Delaplane *et al.*, 2013b)
   - Area containing cells with eggs, larvae and capped cells (per Delaplane *et al.*, 2013b).

The coverage of a comb can be estimated assuming that a comb is covered by 120 bees per 100 cm² if bees are sitting very close to each other (Imdorf and Gerig, 1999; Imdorf *et al.*, 1987). The estimations will be done for all combs (both sides) in each hive. The assessment of the areas containing brood and food can be done by estimating subareas of 100 cm². Afterwards the number of cells per brood stage/food stock is calculated assuming that 100 cm² of the

comb comprise 400 cells (Imdorf and Gerig, 1999; Imdorf *et al.*, 1987). These estimations will be done for all combs (both sides) in each hive.

5.2.2.2.5.4.2. Development of the bee brood in single cells

The time schedule of the brood assessment days was chosen in order to check the bee brood at different expected stages during the development as mentioned in the Table 7.

1. The application in the tunnels should be performed shortly after BFD (within 2 days afterwards).
2. In contrast to the method described in the OECD Guidance Document 75, it is now common to use the digital photo method (Jeker *et al.*, 2011 but see section 5.2.3. of the present manuscript) to follow the development from an egg to the adult honey bee. In the following text, this method will be used to describe the system.
3. The development of bee brood is assessed in individual marked brood cells of all colonies within a study. At the assessment before the application (BFD) one or more brood combs should be taken out of each colony, marked with the study code, treatment group, hive number, comb number, comb side and BFD date, and photographed with a digital camera. In the laboratory, all photos are transferred to a personal computer and areas with at least 100 cells containing eggs are marked on the screen. The exact position of the markers and of each cell and its content should be stored in a computer file that serves as a template for later assessments. The same cells are assessed on each of the following assessment dates (Table 7). Thus, the development of each individually marked cell throughout the duration of the study can be determined (pre-imaginal development period of worker honey bees typically averages 21 days).
4. For the evaluation of the different brood stages of single marked cells, the recorded growth stages are transformed into values counting from 0 to 5 as listed below:
   - 0: termination/breakup of the development (e.g. nectar or pollen found in a cell, if in the previous assessments the presence of brood was recorded)
   - 1: egg stage
   - 2: young larvae (L1 or L2)
   - 3: old larvae (L3 to L5)
   - 4: pupal stage (capped cell)
   - 5: empty after hatching or again filled with brood (eggs and small larvae)
   - N: cell containing nectar
   - P: cell containing pollen

   Cells filled with nectar and pollen after the termination of brood development in the respective cell (counted 0) may be identified by an "N" and "P" in the following assessments; the respective cells have to be excluded from further calculations, but should be included in the overall evaluation in the end.

Table 7. Time schedule of the brood assessment in semi-field brood tests:

BFD = brood area fixing day. *Assessments will be performed outside the tunnels at the monitoring location.

Timing	**Determined brood stage in marked cells**
BFD (1-2 days before application)	Egg
Timing	**Expected brood stage in marked cells**
5(±1) days after BFD	Young to old larvae
10(±1) days after BFD*	Capped cells
16(±1) days after BFD*	Capped cells shortly before hatch
22(±1) days after BFD*	Empty cells or cells containing eggs, young larvae, nectar or pollen

5. Based on the numbering described above, mean values (indices) can be calculated for each colony and assessment day.
6. Assuming that at the first assessment only eggs will be marked, the index is one. An increase of the brood index during the following assessment can be observed, if a normal development of the brood is presumed. This increase is caused by the development from eggs to larval stages, from larvae to pupae and from pupae to adults. Details of the evaluation of the results are presented by Schur *et al.* (2003).

5.2.2.3. Evaluation of the results of the semi-field test

The influence of the test product can be evaluated by comparing the results in the test chemical treatment to the water-treated control and to the reference chemical treatment, and furthermore by comparing the pre- and post-application data regarding:

1. Mortality (dead adult bees, pupae and larvae) within the crop area (linen sheets) and in the dead bee traps (per day and over time after application during bee exposure).It is of interest if an increase in the number of dead pupae is noticed or if malformations of the dead pupae or young dead bees are observed. In case of fenoxycarb in the reference treatment group, an increase in the number of dead pupae can be observed 10-12 days after application. This factor should be considered when demonstrating its sensitivity to bees.
2. Flight intensity in the crop (mean number of forager bees/m² flowering *P. tanacetifolia* after application)
3. Behaviour of the bees on the crop and around the hive
4. Condition of the colonies (strength (number of bees) of the colonies, presence of a healthy queen, mean values of the different brood stages per colony and assessment date, per Delaplane *et al.*, 2013b)
5. Development of the bee brood (brood indices) in > 100 cells:
   - Brood-index:

     The brood-index is an indicator of bee brood development and facilitates comparison between different treatments. It is calculated for each assessment day and colony. For all cells containing the expected brood stage at the respective day, the assessed value (1-5) could be used.

For all cells that do not contain the expected brood stage, 0 is used for calculation. All values per hive and assessment day are summed and divided by the number of observed cells in order to obtain the average brood-index.

- Compensation-index:
  The compensation-index is an indicator for recovery of the colony. It is calculated for each assessment day and colony. The values of all individual cells in each treatment, assessed at the respective day for each hive, could be summed and divided by the number of observed cells in order to obtain the average compensation-index. By that, the compensation of bee brood losses is included in the calculation.
- Brood termination rate:
  Percentage of marked cells where a break (i.e. no successful development) of the bee brood development is recorded, i.e. the bee brood did not reach the expected brood stage at one of the assessment days or food was stored in the cell during BFD +5 to +15.
  Specific statistical analysis for bee trials in semi-field and field conditions are still under development. In general, it is recommended to follow the OECD guidelines (OECD, 2006) and Becker *et al.*, 2011.

5.2.2.4. Discussion and conclusion

Based on the OECD Guidance Document 75 (OECD, 2007), numerous studies were performed and it became obvious that the brood termination rate (= mortality of bee brood in selected cells on combs) was subject to a certain degree of variation, e.g. resulting in replicates with increased rates up to 100% in the control and reduced rates in the reference item group down to 21% (Pistorius *et al.*, 2011). Additionally, a high variation between replicates within a respective treatment group occurred sometimes. The variability which was distinctly more present under semi-field conditions compared to a field method (Oomen *et al.*, 1992) complicates the interpretation of results regarding potential brood effects of a test item with the outcome that some studies were regarded as invalid. The time between BFD and the following assessment on BFD +5 days turned out to be the most critical for such variations. Due to these variances, no definite conclusions regarding potential brood effects were possible in such cases, and the studies needed to be repeated.

In 2011, possible causes and improvements for the existing method were shown by Pistorius *et al.* (2011) and at the ICPBR (now ICPPR) meeting in Wageningen. Attempts to improve the methodology were initiated by the Working Group "Honey bee brood" of the German AG Bienenschutz. In 2011, honey bee brood studies adapted to these identified possible improvements, resulting in better results compared to historical data (for details see Pistorius *et al.*, 2011).

Based on the analysed results, the working group recommended to improve the method by using bigger colonies with more brood, using 4 instead of 3 replicates for better interpretation of data, starting the study early in the season, avoiding major modifications of the colonies shortly before application and using larger tunnels with effective crop areas preferably > 80 m². To carry out quicker brood cell assessments to reduce stress for the colonies, it is recommended to use digital photo brood assessment as described in section 5.2.3., which allows marking a higher amount of cells (e.g. 200 to 400 cells).

In the overall outcome of the studies of the German working group, the combination of the suggested improvements showed a reduction in the breakup rate of the brood development in single cells and in the variability of the results in the control group (Pistorius *et al.*, 2011). However, it also showed that even when fulfilling all the described improvements, it may happen that the brood mortality increases to such a high level, that an evaluation of the test product data still is not possible.

Since the bee colonies are kept under semi-field conditions with restriction in their normal collection and flying behaviour, they generally are sensitive to any interference from outside. Therefore, one should avoid stressing the bees too much during the assessments as well as before set-up of the colonies in the tunnels.

For this reason, it is important to analyse the importance of additional factors in the future in order to be able to improve semi-field studies and studies under field conditions, where the detailed brood assessments are integrated into the study design.

5.2.3. Evaluation of honey bee brood development by using digital image processing

5.2.3.1. Introduction

Evaluations of potential effects on honey bee brood are an important part of the registration process of PPPs. The recently used methodology to investigate bee brood development under realistic exposure conditions are semi-field studies according to Schur *et al.* (2003) (see section 5.2.2. in this manuscript) superseded by the OECD Guidance Document No. 75 or field studies according to Oomen *et al.* (1992) (see section 5.2.1. in this manuscript). Originally, at least 100 brood cells have to be marked and evaluated on acetate sheets with overhead markers for both methods. This is time consuming. The disadvantages of the "acetate method" are the restricted number of cells that can be marked and the long "off-hive-time" of the brood combs. Therefore a digital image processing method was developed (Wang & Claßen, 2011, Jeker *et al.*, 2012;) to reduce the "off-hive-time" of the single brood combs and therefore the stress for the whole honey bee colony. In principle, the use of digital image processing allows one to evaluate the development of an unlimited number of brood cells resulting in increased statistical power. Further, the digital method allows one to

re-evaluate the brood development of single cells in the case of uncertainties.

5.2.3.2. Material and methods

5.2.3.2.1. Photographing of the brood combs at the field site

1. Before taking photos, each brood comb must be marked with the hive description, treatment group, study code, comb number & side and BFD date (BFD0 is the day of the first photographing, one to two days before treatment application).
2. Further (depending on the image processing software), markers have to be defined that allow the program to recover the single brood cells or it has to be ensured that fixed points of the comb (e. g. the edges of the comb) are photographed at the BFDs.
3. After marking the combs, the photos should be taken with a high resolution camera. To standardise the photos of the different combs at the different BFDs, a "photo box" should be used which allows photographing the combs under the same parameters (e.g. distance, focal length). Additionally the camera should support a "live view mode" which is useful to ensure that the photos are of a high quality and facilitate the setting of the camera. The results are most favourable when the photographed combs are located in the centre of the brood area.

5.2.3.2.2. Evaluation of the brood combs at the laboratory

1. The first step at the laboratory is to set the markers or fixed points with the respective image processing program.
2. Afterwards brood cells containing eggs are chosen. To achieve better results, the cells of choice should be on combs containing nectar and pollen and located close to the centre of the combs and not near the edges. At the following BFDs, the image processing program is able to recover the cells marked at BFD0 by use of the markers or fixed points.
3. At the following BFDs (BFD5, 10, 16, 22), the contents of the brood cells are evaluated according to the respective test method (for a demonstration see the online demo video at Rifcon, 2012).
4. During and after the study, the image processing programs are able to calculate all relevant parameters such as brood termination rate, compensation index and brood index (see section 5.2.2.3.). The results of the single cells are presented tabular or in an image gallery for an easier comparison of the respective brood cells.

5.2.3.3. Discussion and conclusion

The digital image processing (Wang & Claßen, 2011, Jeker *et al.*, 2012) improves the evaluation of the honey bee brood development. It reduces the stress for the honey bee colony as well as unnatural influences on the brood development caused by long lasting manual assessments. Due to the fast and standardised photo taking procedure, a high photo quality can be guaranteed and the number of brood cells to be evaluated is almost unlimited. Nevertheless, practical experience has proved that the evaluation of a high number of brood cells is time-consuming and thus it was suggested that the evaluation of 200 to 400 brood cells should be sufficient (Pistorius *et al.*, 2012). Future innovations could produce a more automated evaluation (e.g. automatic determination of the brood stages) and also the exact determination of the brood and food status on colony level.

5.3. *in vitro* larval tests

Aupinel *et al.* (2005) devised a standard *in vitro* test usable for any research topic on larvae (Crailsheim *et al.*, 2013) and more specifically for brood risk assessment (Aupinel *et al*, 2007b). This test has already been ring-tested (Aupinel *et al*, 2009) with the participation of 7 laboratories originating from 6 countries that satisfied the 2 criteria of validity: control mortality lower than 15% at D6 and successful emergence of worker adults in at least the control group. This test, based on an individual rearing method permits one to control exactly the individual exposure with a high reproducibility. It provides quantitative oral toxicity data on honey bee brood. It is designed for *in vitro* treatments of AIs or formulated pesticides. Adopted in France by the CEB, it was validated at OECD and will be recommended in the near future as a guideline for acute exposure at D4 and lethal effect at D7. Chronic exposure and observations on pupae and adult stages will be referenced as guidance.

5.3.1. The rearing method

The rearing method used for this test is detailed in Crailsheim *et al.* (2013), summarised in Fig. 9, and outlined in the steps below.

1. For one replicate, larvae are collected preferably from a unique colony. If two colonies are necessary, larvae originated from both colonies must be distributed in two samples of equal size (24 larvae) in each plate. The colonies have to be healthy and must not show any visible clinical symptoms of pests, pathogens (see *BEEBOOK* Volume II) and/or toxin stress.
2. Tests are performed with summer larvae during a period from the middle spring to the middle autumn (the exact time of year varies by location).
3. In case of sanitary treatment (i.e. products added to the hive for purposes of disease/pest control), the date of application and the kind of product has to be noted. No treatment should be applied within the 4 weeks preceding the beginning of experiments.
4. The queen is confined in its own colony in an excluder cage containing a comb with emerging worker brood and empty cells for less than 30 hours in order to obtain a large number of fresh laid eggs. According to queen vigour, the queen's isolation time can be reduced in order to minimize variability in larval size (age).

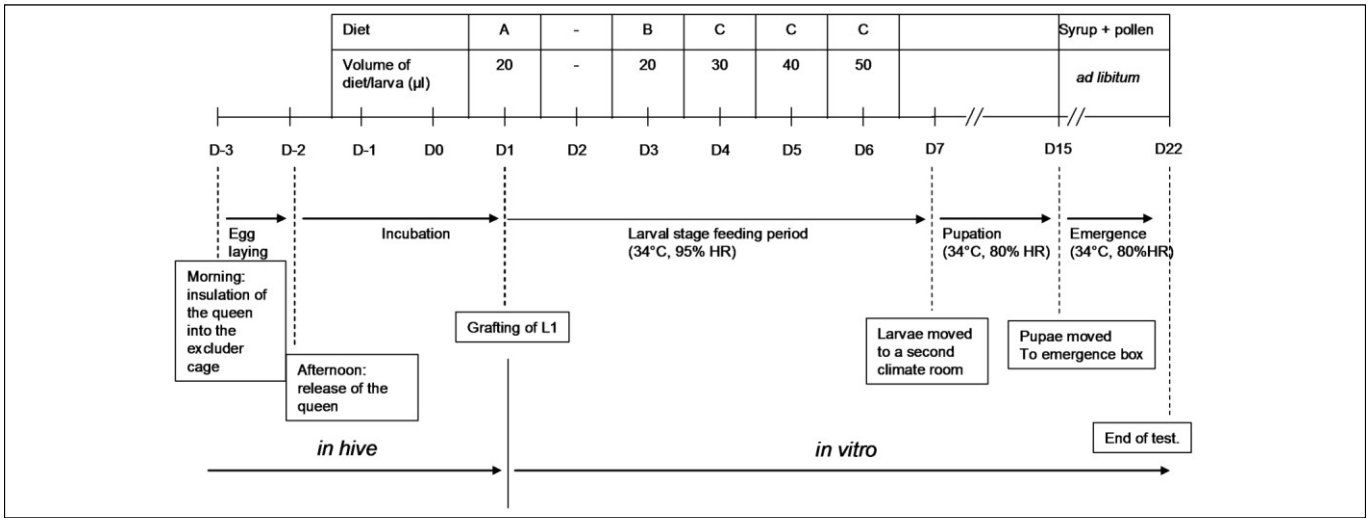

Fig. 9. Steps of a brood *in vitro* test.

5. To ensure one obtains enough larvae, it is recommended to isolate the queens in 2 or 3 colonies in the eventuality that one queen lays few or no eggs.
6. The queen is removed from the cage and the caged comb is left in the hive for 3 days until the larvae hatch.
7. At day 1 (D1, Fig. 9), the comb containing fresh laid eggs is carried from the hive to the laboratory (regulated at a constant temperature of 25°C if possible), in a special wooden container in order to avoid temperature variation and to transfer the larvae into individual rearing cells. We recommend crystal polystyrene grafting cells (ref CNE/3, NICOPLAST Society), having an internal diameter of 9 mm.
8. Before use, the cells are submerged for 30 min in 0.4% MBC (methyl benzethonium chloride) in water, and then dried in a laminar-flow hood. MBC can be replaced by chloride tablets generally used for nursing bottle sterilisation.
9. Each cell is placed into a well of a 48-well tissue culture plate, which was previously half filled with a piece of dental roll wetted with 15.5% glycerol in 0.4% MBC.
10. The young larvae are transferred with a grafting tool (a thin paint brush for example) from the frame into individual plastic cells previously filled with 20 µl of diet A (Table 8).
11. The larvae are fed once a day (except day 2) with a micro-pipette. Diet composition varies according to larval age (Fig. 9, Table 8). The diet is warmed at 34°C prior to each use.
12. The plates are placed into a hermetic Plexiglas desiccator (NALGENE 5314-0120 or 5317-0180 or similar, according to the required volume), provided with a dish filled with K2SO4 saturated solution in order to maintain a water-saturated atmosphere.
13. The desiccator is placed into an incubator at 34 ± 0.5°C. This parameter is crucial considering that susceptibility to a compound may vary significantly according to temperature (Medrzycki *et al.*, 2010).
14. At D7 (pre pupa stage), the plates are transferred into a hermetic container containing a dish filled with a saturated NaCl solution in order to maintain 80% relative humidity. The container is then placed into an incubator at 34°C.
15. At D15, each plate is transferred into a crystal polypropylene box (11 x 15 x 12 cm) with a cover aerated with a wire mesh, and containing a piece of comb with a small plastic royal pheromone diffuser in its centre (Bee Boost®), fixed with a wire.
16. Emerging bees are fed with syrup and pollen powder delivered using bird feeders or similar structures. The boxes are kept in the hermetic container.

Table 8. Composition of the diets provided to larvae (Aupinel *et al.*, 2005, summarised in Crailsheim *et al.* 2013). (Example: to prepare 20 g of diet A (Crailsheim et al., 2013). - Mix 1.2 g glucose, 1.2 g fructose and 0.2 g yeast extract into 7 ml water, and then adjust until 10 ml with water. Mix 10 g of this solution with 10 g of royal jelly.

Diet	**A**	**B**	**C**
Royal jelly (%)	50	50	50
Yeast extract (%)	1.0	1.5	2.0
D glucose (%)	6.0	7.5	9.0
D fructose (%)	6.0	7.5	9.0
Dry matter (%)	29.6	33.1	36.6

5.3.2. Toxicity testing

1. The experimental unit is the 48-larvae plate. For each test, the following treatments should be used:
   - control without solvent (1 plate),
   - control with solvent (1 plate) if necessary,
   - 5 treatments, i.e. the 5 doses or concentrations to be tested (1 plate per treatment),
   - reference treatment with dimethoate (1 plate).
   - 1 additional plate (totally or partially filled with larvae, according to the number of available, remaining larvae) can be used at D4 in the acute toxicity test to replace the larvae which died before D4.

One test has a minimum of three replicates with different larvae origin and new tested solutions for each replicate.

2. The tested pesticide is preferably dissolved in water. If it is not soluble in water at the experimental concentrations, one can use another solvent such as acetone. In that case, it is necessary to prepare a second negative reference fed with diet containing the solvent at the same concentration as in the treated samples.
3. Dilutions of the stock solutions are made with osmosed water, using disposable pipette tips equipped with a filter.
4. The rate of the tested solution in the diet must not exceed 10% of the final volume. In all cases, it is necessary to use a constant volume for the different treatments in order to have a constant rate between the diet and the test pesticide solution.
5. The toxic reference is dimethoate:
   - in acute toxicity test: 3 µg/larva mixed with diet C and provided at D4,
   - in chronic toxicity test: mixed with the three diets at the constant concentration of 20,000 µg/kg diet.
6. In an acute toxicity test, larvae are treated at D4 with diet C containing the preparation to test at the suitable concentration. For a chronic toxicity test, larvae are treated every day (except D2) with the diets containing the preparation to test at a constant concentration.
7. In order to assess the adequate LD_{50} range, it is recommended to run a preliminary experiment where doses of the test preparation may vary according to a geometrical ratio from 5 to 10.

5.3.3. Results

1. Mortality can be defined according to the following criteria:
   - Larva: an immobile larva or a larva which does not react to the contact of the paintbrush is noted as dead.
   - Pupa: a non-emerged individual at D22 is noted as dead during pupal stage.
   - Adult: an immobile adult which does not react to a tactile stimulation is noted as dead.
2. Mortality is checked at the following moments:
   - Larva: At the feeding moment, dead larvae are systematically removed for sanitary reasons. Specific mortality checks are made according to the type of test. In the test where exposure is at D4 (acute toxicity), a first mortality check is made at D4 in order to replace the dead larvae before they have started consuming the diet containing the insecticide. Then one should note the mortality at D5, D6 and D7. In the test with chronic exposure, mortality is noted at D7.
   - Pupa: Non emerged bees are counted at D22.
   - Adult: Alive adult bees and dead adults which have left their cell and show a normal development are both counted at D22.
3. Sublethal effects such as development length, prepupa weight, wing malformation, adult survival, etc. can be noted. It is recommended to weigh prepupa without removing them from the rearing plastic cell. Adults can be kept in the emergence boxes with *ad libitum* food for behaviour observations or longevity assessment.

5.3.4. Statistical analysis

1. The validity of a test depends on some data validity range.
2. In negative reference samples, larval mortality (number of dead larvae/48), pupal mortality (number of dead pupae at D22/number of alive pre pupae at D7) and adult mortality (number of dead emerged bees at D22/total number of emerged bees) must be lower or equal to 15% for the assessment of LD_{50} or LC_{50}, or 20% for the assessment of NOAEL or NOAEC. In case of higher mortality in the control sample, the replicate is invalidated.
3. The mortality rate with positive reference (dimethoate) must be:
   - higher than or equal to 50% at D6 for larvae exposed to 3µg/larva at D4
   - higher than or equal to 50% at D7 in chronic exposure of larvae to the concentration 20,000µg/kg diet.
4. The calculated LD_{50} and LC_{50} must in each case be between the two extreme tested doses. They must not be extrapolated out of the tested limits.
5. Any deviation from the above conditions will invalidate the test.
6. LD_{50} and LC_{50} are calculated from mortalities expressed in percentage of the reference populations after an adjustment according to the Abbott or Shneider-Orelli formula (see section 8.4.1.).
7. The results will be analysed using regression model with high adjustment level, which can be checked with the determination coefficient value (Abbott, 1925).
8. Basing on the same raw mortality data, the NOAEL and NOAEC are assessed (see section 8.4.3.).

5.3.5. General discussion

More research has been published on *in vitro* brood feeding test. Descriptions of laboratory methods have been provided over almost half a century (Weaver, 1955; Rembold and Lackner, 1981; Wittmann and Engels, 1981; Vandenberg and Shimanuki, 1987; Davis *et al.*, 1988; Czoppelt, 1990; Engels, 1990; Peng *et al.*, 1992; Malone *et al.*, 2002; Brodsgaard *et al.*, 2003). These methods generally provide LD_{50} or LC_{50} for the treated larval stage. In 1981, Wittmann and Engels suggested to use the *in vitro* brood feeding test as a routine method for screening insecticides and classifying chemicals according to their toxicity to larvae. Considering both the laboratory toxicity of a product to larvae and exposure data of brood to this product in natural conditions, the *in vitro* larval feeding test seems an appropriate starting point of the brood risk assessment, in other terms a tier 1 study. However, objections have been raised against the *in vitro* method and its regulatory use, in

particular doubts on the standardisation of the protocol, criticisms on the frequent high mortality and the presence of intercasts in the control samples. The difference of food quality and mode of dispensing between natural (Haydack, 1968) and artificial conditions described by authors may account for these weaknesses. See a detailed review of *in vitro* larval rearing in Crailsheim *et al.*, 2013.

6. Effects of toxic substances on queen bees and drones

6.1. Introduction

Although the honey bee queen is the only reproductive female in a colony, therefore responsible for the colony sustainability, very few toxicological studies are dedicated to this key member of the social structure. The scientific literature devoted to poisoning of drones is nearly non-existent.

6.2. Mortality and poisoning signs in honey bee queens

Most of the information on pesticide impacts on colonies comes from experimental protocols performed in field conditions, protocols not focused on the effects of pesticides on the queens. In such studies, standardized colonies are fed with sugar syrup or pollen patties contaminated with different pesticides at different concentrations. The administration of contaminated food was regularly repeated over a period of several weeks on colonies in the field.

When pollen patties were contaminated with micro-encapsulated methyl-parathion (Penncap-M), an organophosphate insecticide, and given to colonies in field conditions, Stoner and Wilson (1983) noticed that queens were superseded or died more frequently in the treated groups than in untreated ones (43.3% versus 25%, respectively), without clear relation between concentration and queen problems. When colonies were fed with sugar syrup contaminated with 10 ppm dimethoate, another organophosphate insecticide, Stoner *et al.* (1983) observed that queens died but were not replaced.

Two hypotheses involving the nurse bees were proposed to explain the queen death. The toxin, carried by the sugar syrup, contaminated the crop of the workers and particularly that of the nurse bees. When they offered the glandular secretions to the young larvae or to the queens, they regurgitated contaminated matters at the same time (Davis and Shuel, 1988). Consequently, the queen can be poisoned directly (fed contaminated food) or the queen can reject the contaminated food and suffer from malnutrition. Both hypotheses could result in a situation where the queen drastically decreases egg production. A reduction in egg production generally triggers queen elimination (supersedure) by worker bees. In the case of carbofuran, a carbamate insecticide (Stoner *et al.*, 1982), heavy losses of young bees by poisoning occurred.

6.3. Reduction in egg production

Although often neglected, plant foodstuffs harvested by workers can harm colonies and potentially impact queen physiology. When the nectar and pollen of *Aesculus californica* (California buckeye) is intensively harvested, returned to the hive and consumed, queens lay only male eggs and can be superseded. The poisoning stops generally at the end of buckeye bloom (Vansell, 1926). A deleterious compound of the nectar was suspected but not isolated.

Johansen (1977) mentioned that queens may be affected by insecticides and behave abnormally. For instance, they may produce a an abnormal brood pattern. This was the case with ovicidal effects of certain herbicides. When package bees containing a laying queen were fed with the 2, 4, 5 T and 2, 4 D herbicides at 100 mg/kg, some of the eggs were unable to hatch, thus presenting as a bad brood pattern (Morton and Moffett, 1972).

Bendahou *et al.* (1999) suggested a reduction in the amount of vitellogenin in eggs (see: Tufail and Takeda, 2008) explained a low hatch rate of eggs, and consequently, the resulting high frequency of supersedure observed in colonies fed weekly with sugar syrup including 12.5 µg/l of cypermethrin, a pyrethroid insecticide.

Dai *et al.* (2010) validated that the hatch rate of eggs can be reduced when queens are fed sublethal doses of bifenthrin and deltamethrin, both pyrethroid insecticides. Moreover, the daily number of laid egg was reduced 30 to 50% for bifenthrin and deltamethrin, respectively.

Ovicidal effects, suggested by egg replacement in the cells, can occur after exposure to IGR insecticides such as fenoxycarb or diflubenzuron (Thompson *et al.*, 2005). The maximum replacement rate measured in the first week after treatment was 60% and 90% for fenoxycarb- and diflubenzuron-treated colonies respectively. No queens successfully mated and laid eggs when treated with fenoxycarb.

Other IGR insecticides acting on the Juvenile Hormone III titre in the haemolymph, were shown to inhibit vitellogenin synthesis (Pinto *et al.*, 2000).

The questions of side-effects of acaricide treatments on queen egg laying success were investigated for fluvalinate and coumaphos. After treating queens and attendant bees placed in Benton mailing cage with specially designed strips of fluvalinate for three days, Pettis *et al.* (1991) observed no differences in colony acceptance of queens, brood viability or supersedure rates. After moderate queen larvae exposure to fluvalinate in a starter/finisher colony, Haarmann *et al.* (2002) confirmed the statistical absence of differences compared with the control group of newly mated queens, with queen weight, ovary weight and the number of sperm.

Coumaphos, another acaricide/insecticide, was shown to be more toxic than fluvalinate by Haarmann *et al.* (2002). They contaminated frames of grafted cells placed in starter colonies for 24 h, with two plastic strips each containing 1.360 g of coumaphos. Afterwards, queen cells were raised in finisher colonies. At the end of the experiment, queen cells contained 8 to 28 mg/kg coumaphos depending on the

presence or absence of contact of the strips with the grafted cell frames. In coumaphos treated groups, the queen and ovary weights were significantly lower. After artificial contamination of the wax of queen cups with 100 mg/kg of coumaphos, Pettis *et al.* (2004) showed a negative effect on young queen acceptance and on their weights.

6.4. Inability to requeen

In cases where supersedures failed, some authors focused their experiments on the ability of orphan colonies to rear new queens. Before aerial application of fenthion, an organophosphate insecticide, Nunamaker *et al.* (1984) placed orphan colonies in a pasture due to be treated. After treatment, they noticed that some new queens emerged at a later date, compared with control colonies, but neither egg-laying queens nor eggs were found in the exposed colonies.

When Stoner *et al.* (1985) fed nurse colonies for queen rearing purposes with sugar syrup contaminated at 5 mg/kg of acephate, an organophosphate insecticide, for several weeks, most of the queen cells aborted. To observe the effects of 4 insecticides (fenoxycarb, diflubenzuron, tebufenozide, azadirachtin), known as IGR insecticides, on newly emerged queens, Thompson *et al.* (2005), transferred queen cells in nuclei containing about 1000 worker bees and supplied them with contaminated fondant. In the fenoxycarb treated group, the emerged queens showed virgin queen characteristics but none of them successfully mated or laid eggs. These authors were also interested in the effects of the molecules on the drones. They concluded that the number of mature drones was reduced in the diflubenzuron treated colonies and even absent from some fenoxycarb ones.

6.5. Conclusion

Studies are needed to assess pesticide impacts on reproductive activity in the colony, that is to say, the physical and physiological integrity of the queen and drone bees. Methods using a strict control of the toxin exposure of queens and drones must be preferred to field conditions where the exposure of the foragers is always questionable because of the difficulty to locate the foraging sites. Effects on daily egg-laying rates, egg hatch rates, number and viability of the spermatozoa in the queen spermathaeca (see Cobey *et al.*, 2013), and in the seminal vesicles of the mature drone should not be overlooked and may be captured in overall risk assessments of brood and population development in higher tier testing. Nevertheless, specific guidelines may be needed to take into account these criteria in the evaluation of toxicity of any AI or commercial formulations.

7. Evaluation of synergistic effects

7.1. Laboratory testing for interactions between agents

7.1.1. Introduction

The theoretical basis for interpreting interactions between agents is rooted in the history of testing combinations of chemical poisons, such as pesticides, but this theoretical framework is broadly applicable to many biotic and abiotic factors that may interact in bees (section 3 of this manuscript). Bliss (1939) recognized three basic types of interactions between agents that can be observed: Independent Joint Action, Additive Joint Action and Synergistic Action (Robertson *et al.*, 2007).

The simplest interaction between agents, and the implicit null hypothesis in experiments testing for interactions, is termed "Independent Joint Action". In independent joint action, the different agents act on bees through different modes of action and no combinatorial effects are observed. The more highly toxic agent in a combination is understood to cause the observed mortality (or other toxicological endpoint) and the observed mortality is indistinguishable from mortality when the more toxic agent is administered alone.

An agonistic interaction occurs when the toxicity of two agents applied together is higher than that of either agent when applied alone. If an agonistic interaction is observed and agents are known to work through similar modes of action, then the term additive toxicity is used. For example, if bees are exposed to different pyrethroid pesticides which share the same mode of action, then the observed toxicity is a sum of the doses of the different pyrethroid pesticides (e.g. tau-fluvalinate and bifenthrin, Ellis and Baxendale, 1997). Differential potencies between different agents with similar modes of action may need to be taken into account (Robertson *et al.*, 2007).

Agonistic interactions may also be synergistic in nature when the toxicity of a combination of agents cannot be predicted from knowledge of the toxicity of each agent alone. Synergistic interactions do not generally occur at the active site (but see Liu and Plapp, 1992), but instead occur when one agent affects the absorption, distribution, metabolism or excretion of the other agent, rendering it more toxic to bees. For example, piperonyl butoxide acts synergistically with both thiacloprid (Iwasa *et al.*, 2004) and tau-fluvalinate (Johnson *et al.*, 2006) by inhibiting the metabolism of these pesticides and greatly increasing their toxicity to bees.

Antagonistic interactions, where a combination of agents is less toxic than each agent alone, may also be observed.

The potency of an interaction can be substantially affected by the ratio of the different agents, for example the level of exposure to coumaphos affects bees' susceptibility to tau-fluvalinate (Johnson *et al.*, 2009). A range of ratios between agents can be explored using the methods described.

7.1.2. Model synergists

Model synergists are chemical tools that are useful for determining the biological basis of synergistic interactions. Model synergists are not overtly toxic to bees at the doses used, but can greatly alter the toxicity of other agents by changing the absorption, disposition, metabolism or excretion of the second agent.

Commonly used inhibitors of detoxicative metabolism include piperonyl butoxide (PBO), which inhibits cytochrome P450 monooxygenase enzyme activity, S,S,S-tributylphosphorotrithioate (DEF), which inhibits carboxylesterase activity and diethyl maleate (DEM), which inhibits glutathione S-transferase activity. These inhibitors are applied topically to the thoracic notum at sublethal doses of 10 µg (PBO and DEF) or 100 µg (DEM) dissolved in 1 µl of acetone 1h prior to treatment with a second chemical agent (Iwasa *et al.*, 2004; Johnson *et al.*, 2006).

The membrane-bound Multi Drug Resistance transporter can be inhibited by feeding bees verapamil at a concentration of 1mM dissolved in 50% sucrose syrup (Hawthorne and Dively, 2011).

7.1.3. Response variables

Acute mortality is the most commonly used response variable when looking for interactions between agents (section 3). Acute mortality is appropriate when one of the agents to be tested is an insecticide that will reliably kill bees using standard acute testing protocols (Section 3.1-3.3). The protocols listed all assume that mortality is the response to be measured, but this may not be an appropriate response if the agent under study is not acutely toxic to bees or if a binary sublethal effect is of interest.

7.1.4. Experiments testing for interactions

7.1.4.1. Discriminating dose bioassay

The simplest experiment involves treating bees with a single dose, termed the discriminating dose, in the presence and absence of another agent. It is important that an appropriate discriminating dose is chosen that will allow for any changes in toxicity to be detected. Discriminating dose experiments have been extensively conducted in *Varroa destructor* to determine acaricide resistance (Elzen *et al.*, 1998), and have been used in honey bees as well (Hawthorne and Dively, 2011). A significant drawback to the discriminating dose approach is that the full dose-response curve is not explored and it is impossible to differentiate between interactions affecting the slope and the intercept of the dose -response curve.

1. Preliminary toxicity bioassays are performed singly on both agents to be tested. This bioassay can use adults treated through oral exposure (section 3.2.1.), topical exposure (section 3.2.2.) or exposure on foliage (section 3.2.3.).
2. The dose of the first, less toxic, agent should be chosen using the dose-response curve generated in step 1. Either this "non-killing" dose should be chosen so that it is the maximum dose that can be delivered that does not cause mortality different from control, or it should be an environmentally relevant dose determined through chemical analysis or predicted exposure.
3. The discriminating dose of the second, "killing" agent is chosen using the dose-response curves generated in step 1. The appropriate discriminating dose depends on the expected outcome of the interaction between the two agents – if antagonism is expected, then the LD_{90} or LC_{90} of the more toxic agent should be used. If synergism is expected, then the LD_{10} or LC_{10} is appropriate. If there are no *a priori* expectations the LD_{50} or LC_{50} should be used. An environmentally relevant dose, based results of chemical analysis or predicted exposure, may also be used.
4. To test for interactions bees are treated as recommended for oral, topical or foliage exposure (sections 3.2.1.-3.2.3.), except that only four groups of bees are used. Bees are then exposed to either the "non-killing" dose of the first agent (Step 2) or a control in combination with, or followed by, the discriminating dose of the second "killing" agent (Step 3), or a control. If the two agents cannot be delivered in combination (e.g. an oral "non-killing" agent and a topical "killing" agent) then the "non-killing" agent should be administered 1 h (topical or foliage) or 24 h (oral) prior to administration of the "killing" agent.
5. Testing in Step 4 is repeated to produce 5 replicates. The proportion of bees dying is transformed using the arcsine square root method, then a simple t-test or ANOVA is used to determine the statistical significance of observed differences in mortality (Hawthorne and Dively, 2011).

7.1.4.2. Comparison of dose-response curves

A superior method for detecting interactions can also be detected by comparing the complete dose-response curves of an agent in the presence and absence of a second agent. This approach allows complete characterization of the dose-response curve, including slope, intercept and LD_{50} or LC_{50} (Johnson *et al.*, 2006, 2009).

1. Preliminary toxicity bioassays are performed and the "non-killing" dose of the first agent is determined (steps 1-2 in the section 7.1.4.1.).

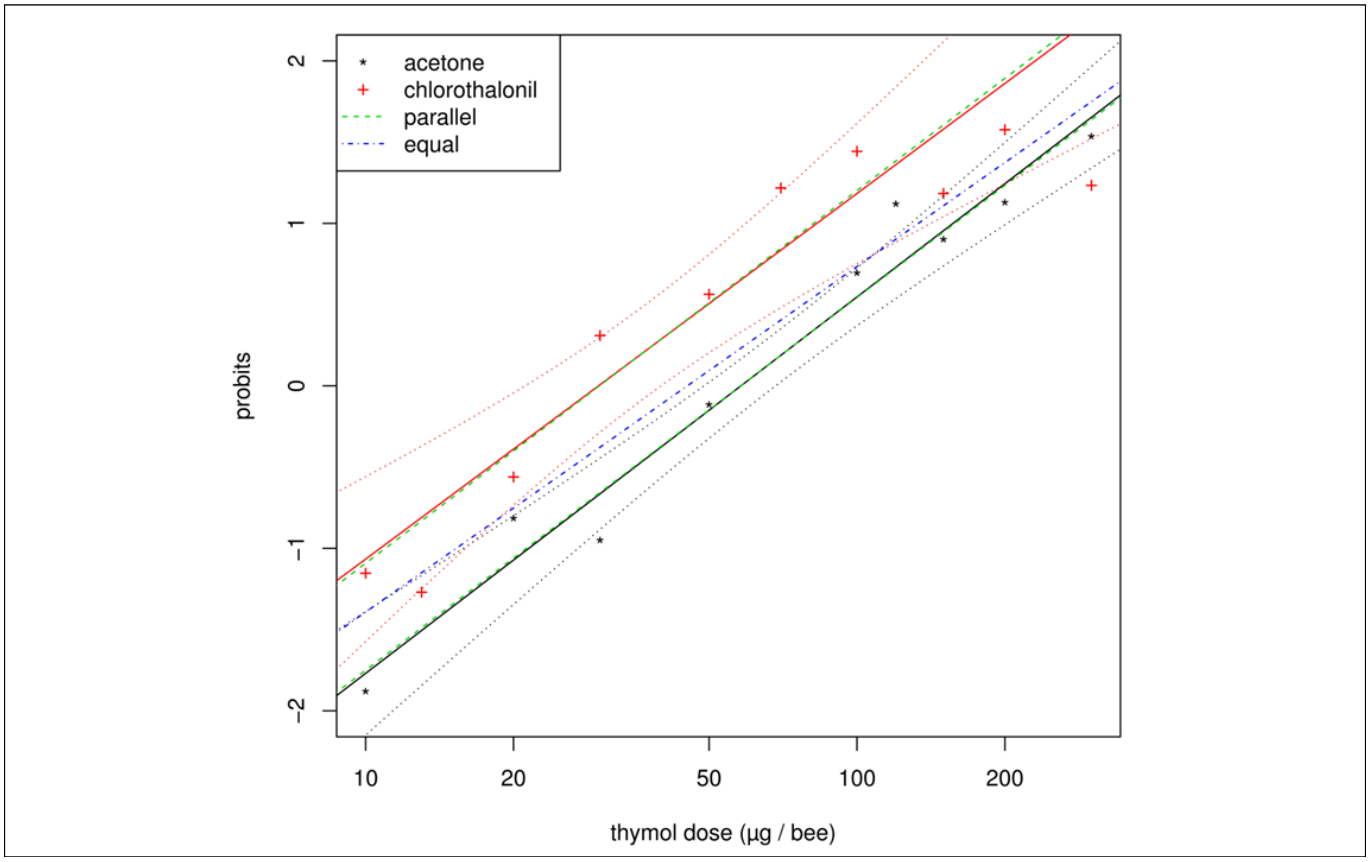

Fig. 10. Test for synergistic interaction between thymol (an acaricide) and chlorothalonil (a fungicide) in bees. Symbols indicate raw mortality data for groups of bees treated with acetone ("*", control, N = 864) or chlorothalonil ("*", N = 467). Solid black and red lines are fit independently to data for acetone and chlorothalonil treatments, respectively. Curved dotted lines correspond to 95% confidence intervals. Dashed green lines were generated using a model where the slope is identical for both lines. The "Test of Parallelism" is a likelihood ratio test between the green lines and the red and black lines (deviance = 0.035, df = 1,17, p-value = 1). The single dashed blue line represents a model fit to pooled data for both treatment groups. The "Test of Equality" is a likelihood ratio test between the blue line and the red and black lines (deviance = 10.449, df = 2,18, p-value < 0.0001).

2. The dose-response of the second "killing" agent is determined by treating bees as recommended for oral, topical or foliage exposure (sections 3.2.1.-3.2.3.), with the exception that all bees are treated with a uniform dose of the "non-killing" agent before, or simultaneous with, administration of a the recommended series of doses of the "killing" agent. A control dose-response series, in which bees are not exposed to the "non-killing" agent at all, is also performed for comparison.
3. Each dose-response series should be repeated at least 3 times.
4. For analysis, the doses are transformed on a log scale and the mortality is transformed on a probit scale, and a dose-response line is fit (Fig. 10). Comparison of the dose-response curves can be performed using commercially available software such as PoloPC (Robertson *et al.*, 2007) or using 'glm' in the R statistical package (R Development Core Team, 2010) (see section 7.3. for a sample script).
5. Three different tests are available to determine the presence of a significant interaction between agents by comparing dose -response curves.
   - Comparison of the overlap of 95% confidence intervals around the calculated the LD_{50} or LC_{50}. The LD_{50} or LC_{50} values, and accompanying 95% confidence intervals, are calculated from the log-probit lines using Fieller's method, with correction for heterogeneity where appropriate (Finney, 1971). If the confidence intervals do not overlap, then the treatments are deemed significantly different. However, this test has been criticized for being overly conservative (Payton *et al.*, 2003), it does not generate p-values and there is no method for correcting for multiple comparisons.
   - A ratio test comparing the ratio of the LD_{50} or LC_{50} derived from the pair of dose-response curves can be performed.

This test will produce the synergism or antagonism ratio and the associated 95% confidence interval. If the confidence intervals do not overlap "1", then the treatments are deemed significantly different (Robertson *et al.*, 2007). The ratio test does not generate a p-value and there is no method to correct for multiple comparisons.

- Interactions can be determined by comparing the dose-response lines using a test analogous to ANCOVA (Johnson *et al.*, 2013). Models are fit using 'glm' in R with all data from both dose-response curves. For the full model, the second "killing" agent serves as the covariate, and the presence or absence of the "non-killing" agent serves as a categorical factor. The interaction between the "killing" agent dose and "non-killing" agent is then compared using two simplified models with the explanatory power of the terms in the models assessed through a process of model simplification in reference to the likelihood ratio (Savin *et al.*, 1977). The first simplified model leaves out the interaction term and, when compared with the full model, tests for differences in slope between the dose-response lines. The second simplified model leaves out the "non-killing" factor entirely and tests for evidence of an agonistic or antagonistic interaction between the two agents. Model comparison using the likelihood ratio generates a p-value which may be adjusted for multiple comparisons using the Bonferroni correction for multiple comparisons.

7.2. Laboratory approach to study toxico-pathological interactions in honey bees

7.2.1. Introduction

Pesticides and pathogens are two categories of environmental stressors that may contribute to the decline of honey bee populations (vanEngelsdorp and Meixner, 2010). However, if their separate impacts on the honey bee are relatively well studied, knowledge on their interactions are somewhat lacking. Pioneer studies on toxico-pathological interactions have been conducted on the association of *Nosema* and chronic bee paralysis virus (CBPV) with organophosphate, organochlorine and pyrethroid insecticides (Ladas, 1972; Bendahou *et al.*, 1997). These studies focused on the acute exposure to insecticides regardless of their chronic toxicity. However, the introduction of systemic insecticides, such as phenylpyrazoles and neonicotinoids in the mid 1990's renders more relevant the studies on chronic exposures to pesticides by oral route.

A new laboratory approach to study the chronic toxicity of insecticide has offered the possibility to explore the interactions between pathogens and pesticides during chronic exposures (Suchail *et al.*, 2001). Studies on the joint exposure to *Nosema* and systemic insecticides have revealed that toxico-pathological interactions may elicit damaging effects on the bees, even when both stressors have no or limited effects on bee mortality (Alaux *et al.*, 2010; Vidau *et al.*, 2011). Two approaches have been used to study the effects of pesticide-pathogen associations. The first carries out simultaneous exposures to the pathogen and the pesticide and is particularly suitable to reveal antagonistic, additive and synergistic effects (Alaux *et al.*, 2010). The second involves sequential exposures to the pathogen and the pesticide and is particularly relevant to investigate the sensitization to one stressor by another (Vidau *et al.*, 2011; Aufauvre *et al.*, 2012).

The toxico-pathological interactions have been observed in laboratory conditions but the few attempts to demonstrate them in field conditions were not always as successful as expected (Wehling *et al.*, 2009; Pettis *et al.*, 2012). However, workers reared in brood frames containing high levels of pesticide residues exhibited a higher sensitivity to *Nosema* infection (Wu *et al.*, 2012). Hence, since such interactions were observed for humans and other species in their living environment, there is no reason to think that they do not occur in field conditions (Arkoosh *et al.*, 1998; Lewis *et al.*, 2002, Bauer *et al.*, 2012). Thus, in many cases, colony diseases could have been triggered by pollutants in healthy carriers.

7.2.2. Materials

7.2.2.1. Honey bees

Traditionally, the effects of pesticides are investigated in honey bee foragers that are the individuals first exposed to pesticides. Considering the contamination of pollen and honey by systemic insecticides, all individuals may be potentially exposed by ingestion of a contaminated food. Thus, the exploration of the toxico-pathological interactions has also been studied in cohorts of young isolated bees of known age, which represent a relatively homogeneous biological material. A sufficient amount of honey bee colonies not infected by *Nosema*, as confirmed by PCR and using primers previously described (Martin-Hernandez *et al.*, 2007), must be selected in order to obtain the desired number of emerging bees. To make the collection of emerging bees easier, queens can be isolated 20 days before the start of the experiment, using a queen excluder grid during 24 hours.

To fully sustain their physiological maturation after emergence, bees ingest pollen during the first days of their life. Pollen is the natural source of proteins for bees but the risk of contamination by pesticides cannot be ruled out (Chauzat *et al.*, 2006; Mullin *et al.*, 2010). A chemical analysis should normally yield information on the pesticide residues present in the pollen. However, the limit of detection of pesticides achieved with multi-residue methods are above 2 µg/kg for a large number of substances. Thus, a substance may be not detected but might still induce toxicity below its limit of detection. In addition, pathogens, notably *Nosema* and viruses, can be found in the pollen (Higes *et al.*, 2008; Singh *et al.*, 2010). For this reason, pollen is replaced by yeast extracts for protein supply. Commercial protein supplies can be used.

The day before starting the study, frames of sealed brood are sampled from colonies, put in boxes and placed in an incubator in the dark at 34°C with 80% relative humidity.

The day of the study, emerging honey bees (0-1 day) present in the boxes are collected, confined to laboratory cages (e.g. Pain type, 10.5 x 7.5 x 11.5 cm) in groups of 30-50 (see Williams *et al.*, 2013), and maintained in the incubator for different periods of time at 30-32°C and 70-80% relative humidity. To mimic the hive environment, a little piece of wax and a Beeboost® (Pherotech; Delta, BC, Canada) releasing one queen-equivalent of queen mandibular pheromone per day, are placed in each cage.

7.2.2.2. Pesticide

Stock solutions of pesticides in 100% DMSO will be diluted to obtain the required concentration of pesticide and 0.1% DMSO final concentration in 50% (w/v) sucrose syrup.

7.2.2.3. Food supply

Sucrose solution for experimental treatments (pathogens and pesticides) is made with sucrose and distilled water (50%; w/v). Proteins (Provita'bee) and candy (Apifonda®) can be purchased from beekeeping suppliers.

For more details on laboratory rearing methods see Williams *et al.*, 2013.

7.2.3. Joint action of pathogens and pesticides

1. The day of the study, emerging honey bees (0-1 day) present in the boxes are collected and distributed in different experimental groups: (i) uninfected controls, (ii) infected with the pathogen only (e.g. *N. ceranae*), (iii) uninfected and chronically exposed to the pesticide at different doses, and (iv) infected with the pathogen and chronically exposed to the pesticide at different doses. Emerging bees can be handled relatively easily because they are quiet and neither sting or fly.
2. Honey bees are first individually infected by feeding with 3 µl of a freshly prepared 50% (w/v) sucrose solution containing the appropriate inoculum of the pathogen. Feeding is performed by holding each bee with its mouthparts touching the sucrose droplet at the tip of a micropipette (Malone and Gatehouse, 1998). This induces the extension of the proboscis and allows the bees consuming the entire droplet. Non-infected bees are similarly treated with the sucrose solution devoid of pathogen.
3. Bee are then confined to laboratory cages in groups of 30-50, and maintained in the incubator at 30-32°C and 80% relative humidity.
4. Honey bees are chronically exposed to pesticides for different periods of time by ingesting *ad libitum*, 10 h per day, 50% sucrose syrup containing, 1% (w/v) proteins, the pesticide at the appropriate concentration and 0.1% DMSO. The remaining 14 h, bees are fed with Candy and water *ad libitum*.
5. During the experiment, each cage is checked every morning and dead honey bees are removed and counted. The food, containing or not the pesticide, is freshly prepared and renewed daily. The actual insecticide consumption is quantified by measuring the daily amount of sucrose syrup consumed per bee.

7.2.4. Sensitization to pesticides by a previous exposure to pathogens

1. Bees are distributed in different experimental groups:
   - uninfected controls,
   - infected with the pathogen only (e.g. *N. ceranae*),
   - uninfected and chronically exposed to the pesticide at different doses 10 days post-infection (d.p.i.),
   - infected with the pathogen and chronically exposed to the pesticide at different doses 10 d.p.i.
2. Honey bees are first individually infected with the pathogen (see section 7.2.3.). If studies are conducted on emerging bees, go to step 3. If studies are performed on aged bees, go to step 5.
3. Studies on emerging bees. Honey bees are individually infected by feeding with 3 µl of a freshly prepared 50% (w/v) sucrose solution containing the appropriate inoculum of pathogen. Emerging honey bees are then fed during 10 days with 50% (w/v) sucrose syrup supplemented with 1% (w/v) protein 10 h per day and thereafter with candy and water *ad libitum* 14 h per day. Each day, feeders are replaced and the daily sucrose consumption is quantified.
4. Ten days after infection, honey bees are chronically exposed for 10 days to the pesticide by ingesting *ad libitum*, 10 h per day, 50% (w/v) sucrose syrup containing 1% proteins, the pesticide at the appropriate concentration and 0.1% DMSO. Honey bees not exposed to insecticides are fed *ad libitum* with sucrose syrup containing 1% proteins and 0.1% DMSO. Then, bees are fed with candy and water *ad libitum* 14 h per day.
5. Studies on aged bees. At a given post-emergence time, caged bees are CO2-anaesthetized, put individually in infection boxes consisting of ventilated compartments (3.5x4x2 cm) and starved for 2 h. Each compartment is supplied with a tip containing the appropriate inoculum of pathogen in 3 µL of sucrose syrup (non-infected bees are similarly treated with sucrose syrup devoid of pathogen).
6. Infection boxes are placed in the incubator and 1 h later, bees that have consumed the total pathogen solution are again encaged (50 bees per cage). Bees are then fed during 10 days with 50% (w/v) sucrose syrup supplemented with 1% (w/v) proteins 10 h per day and thereafter with candy and water *ad libitum* 14 h per day. Each day, feeders are replaced and the daily sucrose consumption is quantified.
7. Ten days after infection, honey bees are then exposed for 10 days to the pesticide (see step 4 above).
8. Throughout both types of experiments, each cage is checked every morning and dead honey bees removed and counted. The food, containing or not the pesticide, is freshly prepared

and renewed daily. The actual insecticide consumption is quantified by measuring the daily amount of sucrose syrup consumed per bee.

9. At the end of the experiment (20 d.p.i.), surviving honey bees can be subjected to investigations or may be quickly frozen and set aside for subsequent analysis.

7.2.5. Notes

- To analyse honey bees at a second post-infection time, the number of cages for each modality must be multiplied by two.
- To avoid any bias due to the weather or season on bee physiology, mortality, physiological and chemical investigations should be performed at the same time.
- Honey bees must be handled with a soft insect holding forceps to avoid physiological damages.
- The experimental design may be modified to change the day of infection, the starting day and the duration of exposure to pesticide, and the sequence of exposure to stressors.
- It is proposed to expose the bees to the pesticide 10 h per day in order to avoid overexposure not compatible with environmental exposures (Suchail *et al.*, 2001). However, bees can be exposed continuously to the pesticide.
- The levels of exposure to pesticides are relatively easy to determine on the basis of pesticide residues in pollen, nectar and honey. However, for the pathogens, it is impossible to determine an infectious level that could be representative of an environmental exposure or a pathological situation. Thus, the inoculum has to be determined by the experimenter on the basis of the objectives intended.

7.3. R script for testing synergistic interactions

See online Supplementary Material.
(http://www.ibra.org.uk/downloads/20130809/download)

8. Introduction to the use of statistical methods in honey bee studies

This paper is not written to describe all the possible statistical tests but to provide some information on common statistics used on honey bee toxicological studies. For more information on using statistics in honey bee studies, see Pirk *et al.*, 2013.

8.1. Foreword

Statistics for experimental design are performed to describe the results and to help clarify a conclusion giving a probability to accept or reject a hypothesis which is in many cases a hypothesis of no differentiation. For most bee study plans or protocols, the variables are mainly counting. Very few are issued from a quantified continue measure such as weight, length, etc. These measured variables can be mortality counts, foraging counts, behavioural counts such as toxicity signs or brood development, etc. These observed counts are raw data issued from experimenter observations in a laboratory box or cage, in a tunnel (semi-field condition), in a field, or directly in a hive. For these counts, two main situations are observed. In the first case, the size is exactly known as when a LD_{50} study is performed in cages with ten or twenty bees, or in a hive for a brood development study, 100 individual brood cells per hive are identified. In the second case, the size is not known. An estimation of population is made in the hive, and the counting is performed on the foraging activities or a counting of the dead bees is performed in the tunnel or in the field.

For most situations, several dose modalities are studied. The experimental design at a minimum includes a negative reference group as a sentinel to measure the experimental background noise (untreated or water treated control). A positive reference group is also often included to measure an experimental bias of no response (i.e. dimethoate). These two kinds of control permit one to validate (or invalidate) the study. Formal criteria are predefined in protocols.

An experimental test item modality is included in the experimental design. At least one modality is studied. The experimental design will include at minimum two or three groups, or product modalities, and up to ten or more product modalities. These modalities are usually independent. The same hive is not observed under several doses or product modalities but the hives are observed several times; then the counting is repeated. If the same modality is studied several times, replicates are observed and can be compared.

8.2. Statistical tests and situations

8.2.1. Honey bee tunnel study

In this study, one hive is observed during several days and several times a day, before and after product applications. The hive population is estimated before its introduction into the tunnel and at the end of the study. Foraging activity and mortality are counted. Indexes are computed as mortality index or forager mortality index for each treatment group: negative reference, positive reference and sponsor's product groups.

If they are no replicates in the study design, the best statistical approach is to compare study index with an historical positive reference index in a database. A control chart with statistical intervals at two levels of significance can be executed and study computed index can be positioned in this control graphic. A decision can be taken about the sponsor's product classification. It is in or outside the statistical bars.

If the study design includes replicates, indexes can be computed in each treatment group at one or several days and index results become study data for parametric or non-parametric analysis of variance.

Table 9. Example of BFD values for a numerical example (see section 8.2.2.).

		Before Exposure	3 days after	7 days after	14 days after	19 days after	Total
Control Group	**H1,1**	1.0	1.9	3.7	3.8	4.7	15.1
	H1,2	1.0	2.2	3.5	3.7	4.4	14.8
	H1,3	1.0	2.1	2.7	2.9	3.2	11.9
	H1,4	1.0	1.9	3.7	3.8	4.7	15.1
	H1,5	1.0	1.8	3.0	3.6	4.5	13.9
	H1,6	1.0	2.0	3.1	3.5	4.0	13.6
	TOTAL	6.0	11.5	18.9	21.0	25.0	82.4
Test Group	**H2,1**	1.0	1.5	2.5	3.1	3.8	11.9
	H2,2	1.0	1.4	2.7	3.0	3.6	11.7
	H2,3	1.0	1.7	2.0	3.3	3.5	11.5
	H2,4	1.0	1.3	2.1	3.4	3.7	11.5
	H2,5	1.0	1.5	2.5	3.0	3.9	11.9
	H2,6	1.0	1.8	2.6	2.9	3.5	11.8
	TOTAL	6.0	9.2	14.4	18.7	22.0	70.3
Total Groups		12.0	20.7	33.3	39.7	47.0	152.7

8.2.1.1. Honey bee brood development

The study is performed usually in field conditions or in semi-field conditions and the study design includes replicates: several hives are observed under the same modality. Indexes are computed from at least a 10 x 10 section of capped brood cells for each hive and for several days during the brood development as a repeated measure.

In this case, a repeated measures ANOVA can be performed to compare results between negative reference and one or several test item modalities. The statistical design is a factor group (modality) and a factor time (repeated measures). Each hive is a basic unit. This statistical analysis permits one to assess factors as group factors but also interactions between factors which could be interesting for the experimenter to assess a slow rate in the brood development.

A second statistical approach is to perform the statistical analysis on the raw data of each cell. In every modality and every hive, each cell among the 100 selected cells is observed during the brood development. A quotation of the development status is assessed by the experimenter. Each cell is a basic unit. The statistical design is a factor group (modality), a factor time (repeated measures), and a factor hive. Multiple interactions between the factors can be computed and statistically assessed. This study design which includes each cell quotation in the statistics permits to increase the statistical power (statistical packages are available to perform this kind of analysis). ANOVA parametric or non-parametric without or with transformation on the data can be performed.

8.2.1.2. LD_{50} determination

The study design is clearly defined in EPPO (2010b), OECD (1998a), or CEB (2011) guidelines. Well known statistical regression analysis from BLISS and LITCHFIELD and WILCOXON (Siegel and Castellan, 1988) and more recent publications lead to perform regressions with dose transformation as logarithm and probit or logit transformation on the response rate.

Dose-response curves at each recommended observation time should be plotted and the slopes of the curves and the median lethal doses (LD_{50}) with 95% confidence limits are calculated (Abbott, 1925). The LD_{50} is determined by the equation of the linear regression. Raw data provide dispersed values which need to be corrected by the control (see section 8.4.1.), then the 50% mortality is calculated with the equation type $y = ax + b$.

In some cases a lack of fit can be observed due to no dose related response. It depends on S shape component or an asymptotic data trend (Winer *et al.,* 1991). Non-linear standard or modified GOMPERTZ regression may give a better fit on experimental data.

Generally for the LDs calculation, different statistical softwares (both commercial and open source) are used. The computer-aided procedure performs the calculations automatically, thus helping to prevent errors.

8.2.2. Brood development index (numerical example)

The numerical example is a factorial experiment in which the factor product has two levels (p): control level and test level. The factor repeated measures has five levels (q): before exposure, three days after exposure, seven days, fourteen days, and nineteen days after exposure. There are six hives (n) in each product modality. In this design, each hive is observed under one modality of the factor product. There are 6 independent hives in every treatment group. The number of hives is twelve (2 x 6). The statistical model has npq = 60 data: n = 6, p = 2; q = 5. Example data are reported in Table 9.

8.2.2.1. Analysis of variance for numerical example

The test calculations are reported in the Table 10. In this example, factor group and factor repeated measures show a *P* value via a Fisher less than the classical level of significance (0.05): Group ($p = 0.0019$) and repeated measures R ($p < 0.00001$). These observed probabilities do not permit one to accept a null hypothesis of equality between the

Table 10. Analysis of variance for the example reported in Table 9. Formulae used: (1)= G^2/npq= $152.7^2/60$; (2)= Σx^2= 1^2+ 1.9^2+……+3.5^2; (3)= (ΣAi^2)/nq= (82.4^2 + 70.3^2)/30; (4)=(ΣRj^2)/np= (12.0^2+20.7^2+……+47.0^2)/12; (5)=[Σ ($ARij^2$)]/n= (6.0^2+11.5^2+…..+22.0^2)/6; (6)=(ΣHk^2)/q= (15.1^2+14.8^2+ ……11.8^2)/5

Source of variation	Computational formula	Sum of square	df	MS	F	(probability)
Between Hives	(6)-(1)	3.84	(pn-1) = 11	0.35		
Group (Product)	(3)-(1)	2.44	(p-1) = 1	2.44	17.48	(p = 0.0019)
Hives within groups	(6)-(3)	1.40	p(n-1) = 10	0.14		
Within Hives	(2)-(6)	70.23	pn(q-1) = 48			
Repeated	(4)-(1)	66.92	(q-1) = 4	16.73	274.70	(p < 0.0001)
Interaction Group x R	(5)-(3)-(4)+(1)	0.88	(p-1)(q-1) = 4	0.22	3.61	(p = 0.0132)
R x Hives within groups	(2)-(5)-(6)+(3)	2.44	p(n-1)(q-1) = 40	0.061		

levels inside each factor. However the experimenter is not authorised to conclude the main factors because the interaction between the factors is significant (p = 0.0132). This statistical observation shows that the mean time profiles are not parallel between both groups (control and test product). The experimenter does analyse this interaction for instance with comparisons between groups at each time of measure.

8.2.2.2. Interaction statistical analysis

An analysis of variance is performed at each time, using a variance error which is computed from both the variance error of the main ANOVA described previously in the table (hives within group, [R x hives] within groups). This computation is performed for comparisons between groups. This combined mean square error with pq(n-1) = 50 degrees of freedom is 0.0766. This degree of freedom must be corrected because this common error comes from two sources of heterogeneity. This correction from SATTERTHWAITE gives the degree of freedom of 43 instead of 50 theoretical degrees.

All kinds of comparisons between both groups will be performed with the same common variance error.

- The comparisons at each level (time of measure) give the statistical results:
- 3 days after exposure: (MS = 0.4408; F = 5.75, observed probability p = 0.0209).
- 7 days after exposure: (MS = 1.6875; F = 22.03, observed probability p < 0.0001).
- 14 days after exposure: (MS = 0.4408; F = 5.75, observed probability p = 0.0209).
- 19 days after exposure: (MS = 0.7500; F = 9.79, observed probability p = 0.0031).
- 7 days after exposure, the comparison between means conduct to reject the null hypothesis with a probability < 0.0001. This observed probability is between 0.01 and 0.05 after 3 days and 14 days. 19 days after exposure, this observed probability is between 0.001 and 0.01.

All the statistical conditions for this statistical model are assumed to be obtained.

8.3. Conclusion

The experimenter needs to use statistical tests to help him make a decision (Fig. 11). A statistical analysis can be conducted only if it is included in the experimental design defined during the drafting of the study protocol. Without *a priori* conception, the statistical performance is frequently poor and the conclusions can be biased.

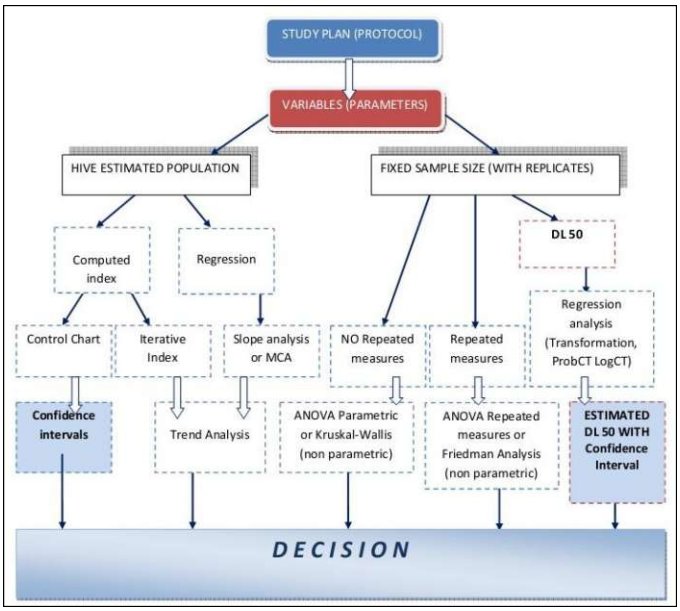

Fig. 11. Statistical decision chart.

8.4. Formulas and procedures frequently used in toxicological studies

8.4.1. Correction of the mortality rates

The mortality ratio is corrected on control mortality with the Henderson-Tilton formula.

$$The\ Henderson-Tilton\ \ formula: \left\{1-\frac{Ta}{Ca}*\frac{Cb}{Tb}\right\}$$

If the parameter comprises live individuals and uniform numbers of bees per treatment (test and control), the Abbott formula is used.

$$The\ Abbott\ formula: \left\{\frac{Ca-Ta}{Ca}\right\}$$

If the parameter comprises mortality ratios and a uniform start, the Schneider-Orelli formula should be applied.

$$The\ Schneider-Orelli\ formula: \left\{\frac{b-k}{1-k}\right\}$$

Abbreviations:

Tb = number of live bees before treatment

Ta = number of live bees after treatment

Cb = number of live bees in control before treatment

Ca = number of live bees in control after treatment

b = ratio of dead bees in treatment

k = ratio of dead bees in control

8.4.1.1. Example correction for control mortality

Tb	number of live test bees before treatment	10
Ta	number of live test bees after treatment	5
Cb	number of live control bees before treatment	10
Ca	number of live control bees after treatment	9
b	ratio not corrected test mortality	0.50
k	ratio control mortality	0.10

correction formulas for control mortality	**not corrected mortality ratio**	**formula**	**corrected mortality ratio**	**percentage (*100)**
Abbott's formula	0.50	((Ca-Ta)/Ca)	0.44	44.44%
Schneider-Orelli	0.50	((b-k)/(1-k))	0.44	44.44%
Henderson-Tilton	0.50	1-((Ta/Ca)*(Cb/Tb))	0.44	44.44%

8.4.2. Calculation of the HQ and RQ

8.4.2.1. Hazard Quotient HQ (EPPO, 2010b)

$$HQ = \frac{application\ rate\ (g\ AI/ha)}{acute\ LD_{50}\ (\mu g\ AI/bee)}$$

The critical HQ < 50 indicates low risk.

8.4.2.2. Risk Quotient RQ (EPHC, 2009)

$$RQ = \frac{application\ rate\ (g\ AI/cm^2)}{acute\ LD_{50}\ (\mu g\ AI/bee)}$$

Assuming the surface area of a honey bee is 1 cm^2

8.4.3. NOAEL and NOAEC

In individual laboratory assays, The NOAEL and NOAEC are the highest dose (in acute toxicity tests) and concentration (in chronic toxicity tests), respectively, which do not induce mortality significantly higher than that observed in controls. The statistical comparison between uncorrected mortality in the treated sample and in the control is performed using the Chi^2 test. The highest dose/concentration where bee mortality is not significantly different (p = 0.05) from the control is considered as NOAEL/NOAEC (respectively).

8.4.4. Power of a test

The power of a statistical test is the probability that the test will reject the null hypothesis when the null hypothesis is false (Type II error). Conventionally, statisticians require that the power of a test to detect a treatment effect of a specified magnitude is 80% but it may depend on the magnitude of the effects that it is required to detect.

9. Acknowledgements

The COLOSS (Prevention of honey bee COlony LOSSes) network aims to explain and prevent massive honey bee colony losses. It was funded through the COST Action FA0803. COST (European Cooperation in Science and Technology) is a unique means for European researchers to jointly develop their own ideas and new initiatives across all scientific disciplines through trans-European networking of nationally funded research activities. Based on a pan-European intergovernmental framework for cooperation in science and technology, COST has contributed since its creation more than 40 years ago to closing the gap between science, policy makers and society throughout Europe and beyond. COST is supported by the EU Seventh Framework Programme for research, technological development and demonstration activities (Official Journal L 412, 30 December 2006). The European Science Foundation as implementing agent of COST provides the COST Office through an EC Grant Agreement. The Council of the European Union provides the COST Secretariat. The COLOSS network is now supported by the Ricola Foundation - Nature & Culture.

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