AMIGA

Assessing and Monitoring the Impacts of Genetically modified plants on Agro-ecosystems

Collaborative Project: Medium-scale focused research project

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Deliverable 6.1 **Report on standardised laboratory test methods and effects of different AMIGA GM pollen on *in vitro* reared pollinators**

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Status: preliminary - Statement of confidentiality: two publications are under preparation based on the work carried out (Chapter 2 & 4), and therefore according to the accepted standards for scientific publications, the Consortium will retain the present report confidential until the scientific papers will be accepted for publication by peer-reviewed scientific journals.

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NON TECHNICAL SUMMARY

The development of robust and reproducible methods for testing potential detrimental effects of genetically modified (GM) crops on pollinators is one of the central goals of AMIGA WP6. In the last two years significant steps in the area of pollinators` safety have been achieved by the development of improved environmental risk assessment (ERA) approaches.

The honey bee, *Apis mellifera* is currently used as indicator species for pollinators worldwide. The rearing of larvae in the laboratory (*in vitro*) is a highly effective ERA methodology that complements field experiments at the colony (*in vivo*) level, which can be influenced by external factors. The use of controlled laboratory conditions, the high reproducibility and the defined amounts of ingested test doses by the larvae provide important standards for ERA.

In this project we improved and standardised an *in vitro* rearing method for honey bee larvae. This method enables us to test the exposure of bee larvae to single or a mixture of GM proteins under controlled laboratory conditions (Hendriksma et al. 2012; chapter one of the present report). The recently published method is characterised by low control mortalities and a reduction of experimenter driven impact on the results. It has the potential to become a standard method of regulatory ERA schemes of GM crops for honey bees.

The developed methods have been used in the framework of AMIGA to analyse the dose-dependent effects of Bt maize pollen on honey bee larvae (Steijven et al., in prep, chapter two). Feeding experiments with different amounts of Bt pollen are reflecting natural and thus highly relevant exposure scenarios. As two new and informative data endpoints, Bt effects on pollen digestibility and larval developmental time were incorporated. The approach is novel and could close an important knowledge gap in ERA of GM crops. It is recommended to add dose-dependent analyses of potential GM pollen effects on bee larvae to the standard methods in regulatory ERA schemes for pollinators.

In addition to the *in vitro* rearing tests for honey bees, we have adapted and developed *in vitro* larvae rearing protocols for bumble bees (*Bombus terrestris*, chapter three) and solitary wild bees (*Osmia* spp., chapter four) to further minimise environmental risks potentially posed by genetically modified insect resistant plants to other important non-target pollinators.

In 2012 and mainly in 2013, we received and stored Bt maize pollen from three European AMIGA fields (Sweden, Denmark, and Slovakia) and potato pollen (DuRPh) from the Netherlands and non-transgenic potato pollen from Finland. This will allow to assessing for potential region-specific effects of GM pollen by using our new *in vitro* rearing methods.

Co-authors of the two publications under preparation not belong to either WUE or UNIBO are member of a scientific cooperation without financial involvement in AMIGA.

POLICY RELEVANCE

The scientific results in this deliverable could trigger the adoption of new actions relevant for regulatory purposes. Taking into account the economic value of pollination services in agricultural landscapes to ensure crop productivity and food security, robust and diversified methods to identify potential risks of GM crops for pollinators are required.

Thus, it is pivotal to establish robust environmental risk assessment (ERA) methods for major groups of bee pollinators. Standardised risk assessments based on laboratory conditions will help to minimise risks posed by genetically modified crops and/or chemical Plant Protection Products (PPPs) to ensure the abundance and health of managed pollinators, as well as a species rich and abundant fauna of wild non-*Apis* pollinators.

According to the Regulation EC No 1107/2009 of the European Parliament and the Annex II of Directive 2001/18/EC, ERA should consider the possible environmental impact resulting from new PPPs as well as direct and indirect interactions of GM plants with non-target organisms (NTOs). The directive 2001/18/EC also clearly points out that harmonised procedures and criteria for the case-by-case evaluation of potential risks arising from the deliberate release of GMOs into the environment must be established.

The European Food Safety Authority (EFSA) has recently finalised the Guidance Document (GD) on risk assessment of PPPs on focal bee pollinators, comprising honey bees, bumble bees and solitary bees (EFSA, 2013). The goal of this GD is to give a framework by which PPPs can be evaluated for their potential risk to bee pollinators providing the same level of environmental safety for honey bees, bumble bees and solitary bees. So far only the honey bee has been incorporated in the regulatory process of new PPPs. Interestingly, even the suggested methods for honey bees do not cover testing of simple exposure scenarios like a direct pollen *in vitro* test for bee larvae. In other words, the impact of pollen containing systemic pesticides or insecticidal GM protein on honey bee larvae has yet not been considered for a harmonised standard test method. There is also not a single standardised test protocol available for non-*Apis* bees (EFSA, 2013). In order to extend the ERA of PPPs or GM crops to other focal bee pollinators, new test species and species specific methods have to be included in the EFSA GD.

The presented methods and protocols are more than first steps to fill in the gap identified and described in the EFSA GD. Our first published honey bee *in vitro* rearing method promotes testing the risks of single and multiple purified insecticidal proteins, which allows a more general view on crop independent risk assessment of certain transgenic active ingredients e.g. Cry proteins. The second method provides event and variety specific data about dose depending toxicity of plant produced transgenic agents on sensitive bee larvae. It could easily be transferred to crop pollen samples treated with systemic insecticides such like the very controversial discussed neonicotinoids.

The presented *in vitro* rearing protocols of non-*Apis* bees are a good starting point for suggestions in accordance with international harmonization of regulatory environmental risk assessment studies.

Introduction

The honey bee is commonly used in environmental risk assessments (ERA) of GM plants. Although recent reviews did not find significant effects of Cry toxins on honey bees in laboratory settings, standardised laboratory and field methods to assess the impact of GMO-based toxins on larval stages and colonies of honey bees and other pollinator species are currently lacking. Further, the potential combined effects of different environmental stressors and diseases with GM crop toxins are also not yet explored. Few studies on pollinator behaviour, population dynamics of pollinators and pollination services provide insight how GM crops might change the occurrence of pollinators and the security of pollination services in agricultural landscapes. Impacts of GM crops might include not only direct toxic effects but also lower pollen production or attractivity. Foraging distances of honey bees determine the spread of GM pollen across agricultural landscapes and potential contamination of honey and other bee products but variation of foraging distances in different bio-geographical regions and environmental settings is unexplored.

AMIGA WP6 will provide the link between laboratory experiments and field release experiments. The development of robust and reproducible methods for testing potential detrimental effects of genetically modified (GM) crops on pollinators is one of the central goals of WP6. The rearing of larvae in the laboratory (*in vitro*) is a highly effective ERA methodology that complements field experiments at the colony (*in vivo*) level, which can be influenced by external factors. However, recently used methods in risk assessments of GM insect resistant plants for honey bees do not show a high capability to be standardised. In this project we improved and standardised an *in vitro* rearing method for honey bee larvae. In addition *in vitro rearing* methods for non-honey bee pollinators are urgently required in ERA. Therefore, we have adapted and developed *in vitro* larvae rearing protocols for bumble bees (*Bombus terrestris*) and solitary wild bees (*Osmia* spp) to further minimise environmental risks potentially posed by genetically modified insect resistant plants to other important non-target pollinators. In WP 6 we addressed the following main objectives summarised in Deliverable 6.1:

- To develop and establish highly standardised laboratory testing methods in ERA of honey bees, bumble bees, and solitary bees;
- To analyse toxicity of GM pollen from different crops and bio-geographical regions

Chapter 1
Effects of multiple Bt-proteins and GNA-Lectin on *in vitro* reared honey bee larvae

The honey bee is a key non-target arthropod in environmental risk assessments of genetically modified crops. We analyzed for the first time combined effects of three Bt-proteins conferring insect resistances, and a CP4-protein conferring a herbicide resistance as simultaneously expressed in one GM-maize. Furthermore, the biosafety of Galanthus nivalis agglutinin (GNA-Lectin), a candidate protein for pest control was tested. Under worst-case exposure scenario, by using controlled in vitro larvae rearing, the combination of Bt-proteins showed no adverse effects on bee larvae. In contrast, the GNA-Lectin was toxic at a 144h-LD50 of 16.3 µg/larva. The prepupal weight was found to differ between the larvae collection days and between the colonies used for the experiment, explaining up to 5 times more data variance than the protein treatments (N=709 prepupae). In conclusion, neither single nor a mix of different Bt-proteins were found

harmful to honey bee larvae. The published honey bee *in vitro* rearing method promotes testing the risks of single and multiple purified insecticidal proteins, which allows a more general view on crop independent risk assessment of certain transgenic active ingredients e.g. Cry proteins.

Chapter 2
Dose dependent effects of stacked Bt maize pollen on *in vitro* reared honey bee larvae

A publication is under preparation based on the work carried out:

Steijven, K. Steffan-Dewenter I., Härtel S. (2014). Dose dependent effects of stacked Bt maize pollen on *in vitro* reared honey bee larvae. To be submitted to Ecotoxicology and Environmental Safety

In agricultural landscapes of many countries of the world, honey bees (*Apis mellifera*) are exposed to a number of different genetically modified pollen. In order to assess environmental risks of transgenic crops for pollinators, first tier laboratory risk assessment studies with a great potential of standardisation are highly needed. Here we conducted an *in vitro* larvae rearing experiment to test the potential effects of genetically modified (GM) stacked Bt maize pollen on honey bee larvae. The test substance was administered in increasing doses to be able to detect possible dose dependent effects. Feeding experiments with different amounts of Bt pollen are reflecting natural, and thus highly relevant, exposure scenarios for pollinators. In addition to the rather crude endpoint 'survival' we also measured several sub-lethal effects; pollen digestibility, prepupal weight, and development. There were no differences in digestibility. Increasing amounts of Bt pollen did not have a negative effect on the survival of honey bee larvae. Interestingly increasing amounts of multi-floral pollen did have a negative effect on honey bee larvae survival. In prepupal weight we find contradicting results; prepupal weight decreased at high doses of Bt pollen when compared to the multi-floral pollen treatment. There are negative dose dependent effects for juvenile development in bees that were fed Bt pollen as well as in bees that were fed multi-floral pollen. However these effects are not significantly different from the negative controls. We propose that in Environmental Risk Assessments (ERA) of transgenic plant products for honey bees a range of GM pollen doses is tested, similar as is done in toxicological studies of chemical plant protection products. After all 'the dose makes the poison'. Moreover, this study highlights the importance of including other endpoints except survival, as responses may differ. And finally the importance of multiple negative controls in the design is discussed, as to be able to make a biological relevant interpretation of your results and distinguish between transgenic effects and variety or species effects. The described approach has the potential to become a standard method in regulatory ERA schemes for pollinators, since dose dependent effects of GM pollen on bee larvae are demonstrable. Moreover, the test method could easily be adjusted to test the effects of systemic insecticides incorporating the natural exposure pathway via the pollen

Chapter 3
Protocols for standardised laboratory test methods for *in vitro* reared bumble bees (*Bombus terrestris*)

Recent Environmental Risk Assessment (ERA) concerning adverse effects on pollinators considers mostly *Apis mellifera*. The honey bee is an important model species since *Apis mellifera* is a global player and occurs all around the world, a large body of knowledge about her biology already exists and honey bees are an important generalist pollinator for myriad of wild and cultivated plants. However, it is important to realise they are not the sole pollinating insect. For example various bumble bees play an important role in crop pollination; 35 out of an estimated 150 crops thought to be insect pollinated (in Europe) benefit from bumble bee pollination. Also many wild plants profit from bumble bees' ability to start foraging earlier in the day and under harsher weather conditions. And in comparison to many other solitary wild bee species they are able to forage in a much larger range, which is especially important in a patchy landscape where certain species grow in relative isolation. Moreover, bumble bee workers tend to forage faster than honey bees. As such bumble bees provide a very important ecosystem service. And as an integral part of agro-ecosystems they are also exposed to agrochemicals intended for pest-insects, such as pesticides or Bt toxins expressed by transgenic plants. This protocol describe a method to test the effects of chemical pesticides such as imidacloprid; as well as insecticidal plant products, such as pollen containing Bt proteins; and purified Bt proteins, on bumble bees. The in vitro method is based on the formation of micro-colonies in a lab setting, which first of all enables the control of numbers and age of individual eggs (or larvae) and workers, and secondly provides the opportunity to stratify the genetic background. Moreover, one can control environmental factors such as temperature, humidity and food resources.

Chapter 4
Protocol for testing toxicity on solitary bee larvae (*Osmia* spp.)

A publication is under preparation based on the work carried out:

Sgolastra F., Tosi S., Medrzycki P., Porrini C., Burgio G. (2014) An *in vitro* method for testing toxicity on solitary bee larvae: the case study of spirotetramat on *Osmia cornuta*. Submitted to Apidologie

This protocol is proposed in order to test the effects (single or multiple) of toxic compounds (i.e. pesticides, Bt toxin protein in purified form) in vitro by adding them to the mass pollen provision of the solitary bees *Osmia cornuta* (Latreille). The following method is mainly based on the protocol developed by Konrad et al. (2008), for *O. bicornis* L, and adapted to *O. cornuta* according to Sgolastra et al. (in prep.). Similarly to honey bee larvae in vitro test, this protocol is developed in order to test the toxicity in larvae of solitary bees conducting the experiment in a reproducible and standardised way. In fact, this method allows to define exactly the quantity of testing compound up taken by a single larva and to standardise the rearing conditions of temperature during development and wintering, which is not feasible in the in vivo method. The

method aims to study the lethal and sub-lethal effects following exposure of larvae to a toxicant (particularly pesticide active ingredient or Bt toxin protein in purified form) at the environmental residue concentration (ERC). Moreover, it can be used to calculate a dose response curve in order to determine the LC50 or LD50 of a testing compound. The data should be used in an appropriate Environmental Risk Assessment scheme for solitary bees.

Chapter 1: Effects of multiple Bt-proteins and GNA-Lectin on *in vitro* reared honey bee larvae

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Abstract

The honey bee is a key non-target arthropod in environmental risk assessments of genetically modified crops. We analyzed for the first time combined effects of three Bt-proteins conferring insect resistances, and a CP4-protein conferring a herbicide resistance as simultaneously expressed in one GM-maize. Furthermore, the biosafety of *Galanthus nivalis* agglutinin (GNA-Lectin), a candidate protein for pest control was tested. Under worst-case exposure scenario, by using controlled in vitro larvae rearing, the combination of Bt-proteins showed no adverse effects on bee larvae. In contrast, the GNA-Lectin was toxic at a 144h-LD50 of 16.3 µg/larva. The prepupal weight was found to differ between the larvae collection days and between the colonies used for the experiment, explaining up to 5 times more data variance than the protein treatments (N=709 prepupae). In conclusion, neither single nor a mix of different Bt-proteins were found harmful to honey bee larvae.

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1. Introduction

The Western honey bee (*Apis mellifera* L.) is a main pollinator species of agricultural crops and wild plants worldwide (Klein et al., 2007; Potts et al., 2010). By feeding on pollen and nectar, honey bees can be exposed to insecticidal proteins expressed by genetically modified (GM) crops (Duan et al., 2008; Romeis et al., 2008; Malone and Burgess 2009). Transgenic gene products expressed in insect-resistant GM crops can confer protection against specific herbivorous pest insects. In particular, the expression of Cry-proteins derived from the bacterium *Bacillus thuringiensis* (*Bt*) is increasing in commercially cultivated GM crops (James, 2010). Cry-proteins typically affect the larvae of susceptible holometabolous insects by a lethal damage to the peritrophic membrane within the gut (De Maagd et al., 2001). Recent developments in crop biotechnology focus on multi-insect resistant crops with high expression levels, producing a number of different insecticidal proteins at the same time (James, 2010). In general, the stacking of traits in one event aims to enhance the protection against target pest insects by causing additive or synergistic toxicity effects (Wolt, 2011). Target lepidopteran pest insects are reported to be synergistically affected by the different combinations of Cry1Ab, Cry1Ac, Cry1F and/or Cry2Ab2 (Lee et al., 1996; Stewart et al., 2001; Khasdan et al., 2007; Sharma et al., 2010).

Pollen is the main protein source for honey bees. A colony can accumulate up to 55 kg of pollen per year (Seeley, 1985), and nurse bees consume 3.4 to 4.3 mg of pollen per day (Crailsheim et al., 1992). Most of the pollen is used to produce food for the larvae in their hypopharyngeal gland, but it was shown that nurse bees do not pass Bt-proteins on to larvae via their food secretions (Babendreier et al., 2005). Thus, the exposure of Bt-protein to larvae is limited to direct pollen feeding, which was found to be about 2.0 mg for maize pollen per larva during their development time (Babendreier et al., 2004). It thus appears that exposure of larvae towards transgenic products is lower than for adult bees. However, larval stages generally show a higher susceptibility to Bt-proteins than adults, with neonate larvae being more sensitive than older larval instars (Glare and O'Callaghan, 2000; Yao et al., 2008). Hence, we follow the idea of testing the potentially most sensitive life history stage for Bt-proteins (Romeis et al., 2011), i.e. honey bee larvae.

Bt-crops expressing single Cry-proteins were not found to impact honey bees during a recent meta-analysis (Duan et al., 2008). However, no studies assessing the risk of simultaneously expressed Cry-proteins on honey bees have been conducted until now. To assess the biosafety of pollen-rewarding transgenic crops with multi insect resistances, the protein expression of a stacked Bt-maize variety "Mon89034xMon88017" was taken as a reference model. Combined effects of four transgenic proteins were tested individually, and in combinations that are proportional to the expression levels in stacked Bt-pollen: Cry1A.105, Cry2Ab2, Cry3Bb1 against major lepidopteran and coleopteran pest insects and the CP4-epsps protein conferring herbicide resistance. The arthropod-active protein Lectin (Babendreier et al., 2008; Jaber et al., 2010) was also tested for toxic effects on honey bees since it is a future pest control candidate for expression in e.g. maize and rapeseed.

We used a concentration gradient which exceeds the estimated environmental concentration (EEC) by a multi-fold, and performed experiments that took into account protein interactions, the colony background of test individuals, thereby effectively monitoring honey bee biosafety.

2. Material and Methods

2.1. In vitro larvae bioassay

The rearing of larvae was performed under controlled laboratory conditions following the methods of Aupinel et al. (2007) and Hendriksma et al. (2011) (Suppl. P). These methods were adopted to test for the first time effects of mixed transgenic proteins on *in vitro* reared larvae. The test larvae originated from six donor honey bee colonies with naturally mated non-sibling queens (*Apis mellifera carnica)*. On June 23rd and June 25th 2009, queens were trapped on artificial combs within their colonies (Cupularve, Nicoplast©, Maisod, France). We further refer to the material and methods section in Hendriksma et al. (2011), for the first instar larvae collection (D4; age mean 10:29 h) and the details of *in vitro* rearing (D5-D9).

The larvae finished their *in vitro* diet at day 10 and terminated digestion by a molt and defecation of the intestinal tract. By day 11, the larvae were stretched and passive, which is indicative for the prepupae phase. To assess lethal effects, the survival of larvae was noted daily, and moribund larvae were removed, as recognised by occasional black or white sub-dermal necrotic stains or a visible loss of turgor. Potential sub-lethal effects were monitored on day 11, by weighing each prepupa on an analytical microbalance to the nearest 0.001 g (Hendriksma et al., 2011).

To reflect transgenic protein exposure by GM pollen consumption, eight treatments were established by mixing different proteins into the semi-artificial diet of second instar larvae on day 5. The diet was ingested by the larvae during the subsequent days. All protein treatments were made up to account for a concentration gradient (Table I). The bioassay was conducted with larvae, which were sampled on two successive days (N=755 larvae). Considering the different colony backgrounds, the larvae were equally distributed over the concentration gradient within each treatment, with mean 18 replicate larvae per individual treatment dose.

2.2. Protein treatments

The Bt-protein resistances by Cry1A.105 and Cry2Ab2 target a wide range of common lepidopteran pests (e.g. armyworms *Spodoptera sp*., black cutworm *Agrotis ipsilon*, corn borers e.g. *Ostrinia nubilalis* and corn earworms e.g. *Helicoverpa zea*). Cry3Bb1 confers resistance against coleopteran pests like the Western, Northern and Mexican corn rootworms *Diabrotica spp.* (Coleoptera: Chrysomelidae). A non-insect related protein EPSPS of *Agrobacterium sp.* strain CP4 was tested as transgenic protein conferring resistance to glyphosate, the active ingredient of the herbicide Roundup.

For each protein, a stock diet was made with a maximum treatment dose, of which an exponential concentration gradient was made by repetitively diluting each stock solution with

basic diet with the factor 1/10. All the diets were made on the first day of larval collection, stored at 6° Celsius and warmed up to 35° Celsius before application. The stock diets with the transgenic proteins were made by a replacement of the water fraction in the diet with buffer solutions containing the purified transgenic proteins (obtained from Monsanto Company, St. Louis, USA and stored at -80°C preceding application). The transgenic protein stock diet contained per 10 µl: 3.2 µg Cry1A.105 [treatment 1], 0.124 µg Cry2Ab2.820 [2], 3.0 µg Cry3Bb1 [3], 6.4 µg CP4 epsps [4] or 7.03 µg Cry1, Cry2, Cry3 and CP4 in the proportion as in 2 mg Mon89034xMon88017 pollen [5] (Monsanto Company, 2009). At the volumetric maximum, treatments [1, 2, 3] exceeded an environmental exposure concentration (=EEC) of 2 mg pollen by 186 times, and the treatments [4, 5] by 18.6 times (Technical Dossier {Part I} of the summary {Part II} of Monsanto Company, 2009; Table I).

Buffer chemicals may cause effects on larvae as well, thus zero concentration controls for transgenic protein treatments were diets with buffer solution [B1/B2/B3/B4/Bmix] (Table I). The mixed buffer treatment [6] is the direct control of the stacked protein treatment [5], containing the identically proportioned buffer-mix. Bovine Serum Albumin (BSA) was applied as a non-insecticidal protein control [7]: maximally 8% solid protein (w/w). Additionally, Snowdrop Lectin (GNA; *Galanthus nivalis* L. agglutinin) was used as another class of transgenic pest control proteins [8] (Romeis et al., 2003; Babendreier et al., 2008) at maximally 0.8% (w/w) solid protein (Table I). The buffer chemicals and the two control proteins were ordered at Sigma-Aldrich Chemie Gmbh, Munich, Germany.

2.3. Statistics

Four variables of possible influence on the data were considered: 8 Treatments, 1 Gradient, 6 Colonies, and 2 Trials (larval sampling days). The concentration gradient with the dosage levels d * $10^{-\infty}$, 10^0, 10^1, 10^2, 10^3, 10^4 was Log-transformed into the progressive values 0, 1, 2, 3, 4, 5 to correct for the exponential progression. This allowed testing Gradient as a standardised continuous linear variable, since treatment doses [treatments 1-6] were all in proportion to each other, reflecting the transgenic protein concentrations within stacked Bt-pollen. Larval survival and prepupae weight were the tested response variables; the dose-response tests were performed by regression over the concentration gradient. By the use of the interaction term Treatment*Gradient, treatment specific dose-response effects could be compared. All variables and all meaningful interactions were tested and successively rejected from the models when they were insignificant (α=0.05). For all *Post hoc* tests, such as in the comparison of 1 treatment with 7 other treatments, the significance of *P*-values was determined at $\alpha = 0.05$, applying Bonferroni corrections on the *P*-values for the number of comparisons.

The survival of larvae was analyzed with proportional hazards regression models (*Coxph*: Cox and Oakes 1990; Fox 2002) using the open source statistic software R, version 2.11.1 (R Development Core Team, 2010). This regression on survival dynamics over time can take multiple explanatory variables into account, and has the option to include a random factor to correct for non-independence within the data-set (Zuur et al., 2009; Hendriksma et al., 2011) (Table IIA). In case of toxicity, LD_{50} values were calculated, taking into account the colony

dependence of test individuals (Hendriksma et al., 2011), with 95% confidence intervals determined by Fieller's method (Finney, 1971; Niu et al., 2011). The prepupae weight analysis was performed using linear models (*lm*: Chambers, 1992, ANOVA, type-III) to measure Treatment, Colony, Gradient and Trial effects (Table IIB).

3. Results

3.1. Survival rates

The three tested Bt-proteins Cry1A.105 (n=109), Cry2Ab2 (n=110), Cry3Bb1 (n=109) [treatments 1-3] did not show insecticidal effects on developing honey bee larvae, with survival rates between 95.5% and 100% per test gradient (Table III). Even at the highest test concentration, 186 times exceeding the EEC, no susceptibility to any of the three Bt-proteins was found (survival 100% [1], 94.4% [2], 100% [3]). Similarly, for the CP4 protein treatment ([4] 92.5%, n=93), and the combination of all four transgenic proteins containing all three Bt-proteins ([5] 97.8%, n=92) the survival was high, and remained unaffected even at the highest concentration tested (Fig. 1).

The buffer mix [6] with 96.6% survival was not significantly different from the five transgenic protein treatments ($\chi^2 \leq 5.0$, P-value ≥ 0.18). With mean mortality rates of ≤ 7.5%, no treatment specific dose-response effects were found within the tested groups [1-7] ($\chi^2 \leq 1.17$, P-value ≥ 0.19, Table III).

In contrast, GNA-Lectin [8] showed a significant increase in larval mortality over the concentration gradient (Suppl. S: R^2=0.52, χ^2 = 67.0, P<0.001, n=93). GNA-Lectin [8] killed all test larvae at the highest dose of 5‰ w/w (LT_{100}=144 hours; n=20; Fig.1). The 96h and 144h LD_{50} values were indicated 39.1 µg and 16.7 µg dietary Lectin protein per larva respectively (with 95% CI's resp. 30.4 - 51.9 and 13.5 - 20.8 µg / larva). A *post hoc* test over all treatments, and an additional test on the highest applied doses only (Fig. 1), confirmed that Lectin was the only treatment causing mortality (Table III). It is important to note that the experiment had a low residual background mortality of mean 3.5% (26/735 larvae; excluding the highest dose of the Lectin treatment).

Neither the colony background of test organisms (χ^2 = 3.59, *d.f.* = 5, P = 0.61) and their potential interaction with treatments (χ^2 = 37.0, *d.f.* = 35, P = 0.38), nor the two trials (χ^2 = 0.70, *d.f.* = 1, P = 0.40) were found to affect survival of honey bee larvae. Only the Treatment*Gradient interaction was found to be significant, driven by Lectin [8] as sole discriminate treatment (*Post hoc* P-values < 0.001; Suppl. S).

3.2 Prepupae weights

The mean prepupal weight was in range of 138.9 to 143.6 mg (Table S1), showing no differences between treatments (P=0.66; Table IIB). The applied factor Gradient did not affect prepupal weight (P=0.08; Table IIB), showing the absence of dose related effects within treatments (Fig. 2; *Post hoc* P>0.13). However, between treatments dose response differences were present (P=0.008; Table IIB), with CP4 [4] and the protein mix [5] showing contrasting responses in

comparison to Cry2 [2], (Fig. 2, *Post hoc P*-values < 0.012, Suppl. W). We like to point out that neither the buffer control [6], nor the BSA control protein were different from the single Bt, or mixed transgenic protein treatments [1-5]. The 1.5 mg difference in prepupal weight between the larvae collection days was found significant (P=0.006; Table IIB). Similarly, a colony effect was found statistically significant (P=0.002; Table IIB), with a mean weight differences of 3.8 to 4.6 mg between colonies (*Post hoc P*-values < 0.029, Suppl. W).

Within the prepupae weight data, no explanatory variable, nor any interaction between variables, substantially contributed to the explanation of variance ($R^2 \leq 0.026$, Table IIB). Finally, a low weight of prepupae was not found to correlate with a higher larval mortality rate ($F_{(1,40)}$=0.16, P=0.69; R^2=0.004) .

4. Discussion

4.1 Toxicity of Cry-proteins and Cp4

The cultivation of GM-crops with insect resistances requires comprehensive biosafety assessments, with robust and highly standardised bioassays for main non-target organisms. We used a sensitive and well suited *in vitro* larvae rearing method to study single and multiple insect resistant Bt-crop effects on the main pollinator *Apis mellifera*. The three tested purified Bt-proteins, expressed in the pollen of the reference maize variety "Mon89034 x Mon88017" did not affect survival rates and weight gain of second instar larvae, even at Bt-toxin amounts exceeding a normal 2 mg Bt-maize pollen EEC by 186 times. Thus, stacking of three Bt-toxins showed no lethal or sub-lethal effects on honey bee larvae. Nonetheless, unknown subtle Bt-effects may have remained unrevealed by this study.

Our tested Cry1A.105 toxin is a "chimeric" protein, developed by recombining *cry1Ac*, *cry1F* and *cry1Ab* genes of different *Bacillus thuringiensis* strains. Compared to the native proteins, chimeric proteins are designed to have an increased toxicity and have a broadened range of target pest insects (Pardo-López et al., 2009; Pigott et al., 2008). Regulatory agencies may omit additional biosafety tests on chimeric proteins, if and when the predecessor proteins were assessed to be safe. However, as reduced selectivity and increased toxicity may not only affect target insects but also non-target insects, extrapolating risks of novel chimeric proteins based only on the data of the predecessor proteins could be misleading. Nevertheless, our data show that this chimeric Cry1A.105 protein is not directly harmful to *A. mellifera* larvae.

Recently conducted pollen feeding trials, in which *in vitro* reared third instar larvae were exposed to 2 mg pollen of the Bt-maize variety "Mon89034 x Mon88017" during 5 days, showed 100% survival (Hendriksma et al., 2011b) and thus are fully in line with results from worst case exposure scenarios obtained in the present study. Similarly, the overall mean weight of prepupae and also mean prepupal weight at the highest applied purified protein doses are in perfect range with the pollen feeding test (Hendriksma et al., 2011b). Our results on single Bt-proteins further complement the less standardised colony level studies on single Cry1Ab or Cry1F maize pollen (Hanley et al., 2003) and the purified Cry3B protein (Arpaia, 1996), for which also no deleterious effect by Cry-protein were found on honey bee brood. A recent *in vitro* study on the effect of

purified Bt-protein Cry1Ac (50µg) on Africanised honey bees reported no effect on larval survival rates, development time, or adult body mass (Lima et al., 2011). Together with our results on Cry1A.105 and earlier studies, a high Cry1 protein safety range for *Apis mellifera* larvae can be confirmed. While numerous studies have been conducted on Cry1 Bt-toxins, few studies have been done on Cry3 Bt-toxins and hardly any on potential risks of Cry2 Bt-toxins on bees (Malone and Burgess, 2009). Thus our results add valuable information here.

Similar to the results obtained for single Bt-proteins, the transgenic mix of proteins as expressed in Bt-pollen [5] did not affect larval survival or prepupal weight, not even at the highest concentration doses applied. Two observed dose response differences, with CP4 [4] and the protein mix [5] showing contrasting responses in comparison to Cry2 [2], were not substantiated by individually significant dose response effects. In addition, the biological non-toxicity of all applied transgenic treatment concentrations has been underlined by very low explanatory values ($\leq$ 2.6%; Table IIB), and the fact that the protein treatments [1-5] did never differ from the buffer control [6], or BSA [7] as non-toxic control. We conclude that the observed treatment differences were biologically irrelevant.

In general, the stacking of traits in one event aims to enhance the protection against target pest insects by causing additive or synergistic toxicity effects (Wolt, 2011). The uptake, transportation or degradation pathways within organisms are commonly involved at toxicant synergies (Andersen and Dennison, 2004). This typically addresses the mode of action of Bt-proteins, disrupting the intestinal systems of target arthropods. Target pest insects are reported to be synergistically affected by combinations between Cry1Ab, Cry1Ac, Cry1F and/or Cry2Ab2 (Lee et al., 1996; Stewart et al., 2001; Khasdan et al., 2007; Sharma et al., 2010). If susceptible to Bt-proteins, even to a small extent, non-target organisms need consideration on synergistic toxicity issues. However, the data presented here do not support any susceptibility of honey bee larvae to any of the three Cry toxins tested. Consequently, in our case study on mixed Bt effects on bees, additional mixed toxicity evaluations were regarded as irrelevant (e.g. testing on additivity of effects, or on synergistic or antagonistic effects). Our findings corroborate recent statements from EFSA that interactions among Cry1A.105, Cry2Ab2, Cry3Bb1, and CP4 EPSPS are unlikely, based on the known function and mode of action of these proteins (EFSA, 2010).

New to honey bee risk assessment is the testing of a purified transgenic CP4-EPSPS protein, both singly and mixed with the three Bt-toxins like it would appear in the transgenic maize event. The *Agrobacterium sp.* strain CP4 derived EPSP-synthase is tolerant to the herbicide glyphosate (Padgette et al., 1995). Because it replaces the intolerant synthase, CP4-EPSPS enables continuation of amino acid biosynthesis after glyphosate-herbicide treatment of plants (Steinrücken and Amrhein, 1980). Neither a mechanism, nor evidence exists that the CP4-EPSPS protein is harmful to animals, plants or other life forms (Peterson and Shama, 2005). Our results further indicate that the CP4-protein does not pose a risk to pollinating insects when it is expressed in pollen of transgenic plants.

A number of Bt-crops are assessed safe for *A. mellifera*, apparently due to missing receptors for the respective Bt toxins (Duan et al., 2008; Malone and Burgess, 2009). Even a Hymenopteran active Bt-strain (PS86Q3; active to sawflies *Diprion pini* and *Pristiphora abietin)*

was not found to affect honey bee larvae (Porcar et al., 2008). Nevertheless, a case by case risk assessment on future Bt-crops is mandatory, since Bt-products yet to be developed may pose new risks to bees (Romeis et al., 2006).

4.2 Lectins

In contrast to all other treatments, snowdrop derived Lectin (GNA) elicited mortality of all larvae at the highest concentration level (0.8% w/w in 10µl diet, 0.08 mg per larva). This could be relevant for honey bees because GNA is regarded as a candidate for expression in transgenic crops like maize and rapeseed to confer resistance against pest insects (e.g. Romeis et al., 2003; Lehrman, 2007; Babendreier et al., 2008). In comparison, 1.0% GNA mixed into sucrose solution fed to the parasitic Hymenopterans *Aphidius colemani*, *Trichogramma brassicae* and *Cotesia glomerata*, also reduced the survival of test-individuals by 58%, 39% and 56% respectively (Romeis et al., 2003).

A dietary pollen feeding test (1.5% w/w) expressing transgenic pea Lectin up to 1.2% of total soluble protein in oilseed rape pollen, revealed no negative effect on honey bee larvae (Lehrman, 2007), which is likely due to the relative low quantity of protein exposed. At the dose of 0.08% GNA in the diet we found no lethal effects and also no indication of a sublethal inhibition of larval feeding. This result contrasts to mason bee larvae *Osmia bicornis*, which showed an inhibited food intake and had a reduced survival at 0.1% GNA in the diet (Konrad et al., 2008). Similarly, 0.1% GNA mixed into sucrose solution and fed to bumble bee *Bombus terrestris* workers and drones also showed reduced survival rates (Babendreier et al., 2008). A similar Lectin (Wheat germ agglutinin; WGA) was described affecting adult honey bee midgut esterase and protease activity at 0.1% WGA feeding (Belzunces et al., 1994).

An explanation for not finding sublethal effect at 0.08% (8µg/10µl) is that above mentioned studies fed the concentration constantly, while in the present study the honey bee larvae were exposed to it in one dietary application. In this case, an assumption of chronic exposure would better fit our data to the other mentioned studies; No effects at 0.005% [8µg GNA/ total 160µl diet], and all individuals dead at 0.05% [80µg GNA/160µl].

In general, for potential GM crops expressing Lectins, the risk will depend very much on the exposure levels (Babendreier et al., 2008; Malone and Burgess, 2009). Despite the fact that Lectin expressing GM-crops are not commercialised, bees may already be exposed to Lectins (Babendreier et al., 2008). Leek (*Allium porrum*) nectar can contain 0.02% of a mannose-binding Lectin, similar to GNA (Peumans et al., 1997). As this concentration lies close to the effect range of about 0.1% as recorded in the above mentioned studies, a potential insecticidal risk is not excluded. Thus, risks of transgenic plants expressing lectins for honey bees need to be addressed for all melliferous, as well as all polleniferous crops.

4.3 Methodological strength

In comparison to the reported 19% background mortality at testing Cry1Ab over the larval phase by Lima et al. (2011), the 0% mortality for Cry1A.105 fed larvae (n=105), and a 3.5% general background mortality is a notable improvement for environmental risk assessment studies. The

low mortality rate is linked to the non-grafting approach of following the basic idea of minimizing contact with the larvae in order to optimise rearing success (Suppl. P) (Hendriksma et al., 2011a).

By following the basic idea of reflecting the natural exposure pathways, we started the Bt-protein applications at the second instar stage. This includes a safety margin, since exposure for young larvae is negligible because pollen are only in the larval food from the third instar stage onwards (Simpson 1955, Jung-Hoffmann 1966) and Bt-protein is not secreted via nurse bee feeding glands (Babendreier et al., 2005). Hive experiments reported similar weights of prepupae but revealed higher weight ranges (Babendreier et al., 2004). They found mean weights of 132 to 155 mg for fully grown larvae (Δ 23.0 mg), also with a significant difference among colony backgrounds. This proves the *in vitro* bioassay to produce data in a representative range, with all level means in the range of the empirical data (Table IV).

The general question of whether laboratory studies on transgenic insecticidal crops can be extrapolated to the field situation has been recently addressed by Duan et al. (2010). They showed that indeed laboratory studies on GM crops show effects that are either consistent with, or more conservative than, those found in field studies, provided that ecologically relevant routes of exposure have been addressed properly. Since we here have included a wide range of concentrations including worst case scenarios, it is concluded that our results are likely conservative, leaving a safety margin.

4.4 Conclusions

Under worst case exposure scenarios, Bt-proteins Cry1, Cry2 and Cry3 and the CP4-protein were not found to be toxic to developing honey bee larvae, and mixed toxicity effects were not indicated. The results presented in our case study on developing honey bee larvae extend the biosafety of single Bt proteins to multiple Bt proteins. In contrast, GNA-Lectin caused acute mortality among larvae, stressing the risk for beneficial insect pollinators in the agricultural landscape when GNA would be expressed in melliferous and/or polleniferous GM crops.

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Figure 1. Survival of *in vitro* reared honey bee larvae following treatments on day 5 (D5) with the highest protein concentrations tested: [1] 3.2 µg Cry1A.105, [2] 0.124 µg Cry2Ab2, [3] 3.0 µg Cry3Bb1, [4] 6.4 µg CP4-epsps, [5] 6.8 µg Stacked Mix, [6] Buffer Mix, [7] 800 µg BSA, [8] 80 µg GNA-Lectin.

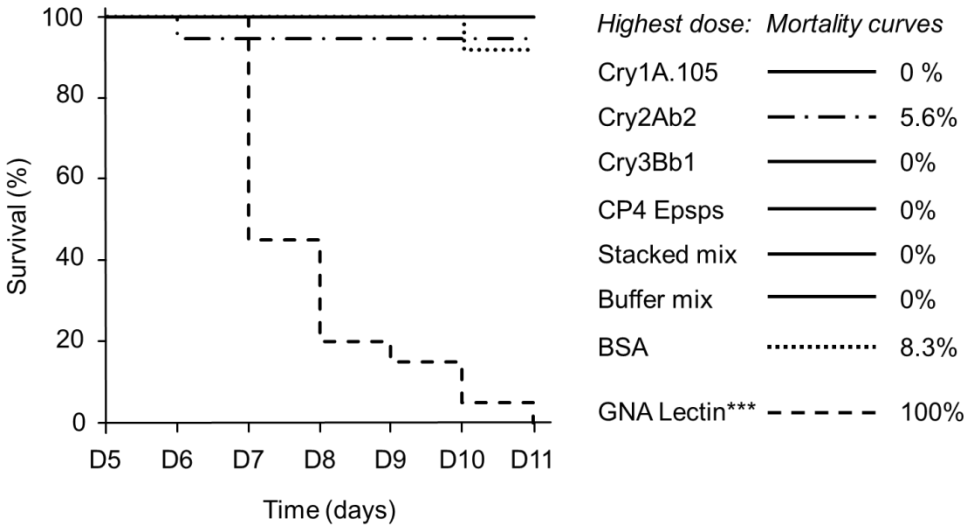

Figure 2. Prepupal weight of protein exposed larvae (n=709). Dose response effects of transgenic proteins on the prepupae weight of *in vitro* reared honey bee worker larvae are shown. Dotted lines indicate non-significant dose response result for each treatment at increasing concentrations (for treatment details see Table I and for statistics Table IIB and Table IV). Note that at the highest Lectin concentration, all test-individuals had died (~).

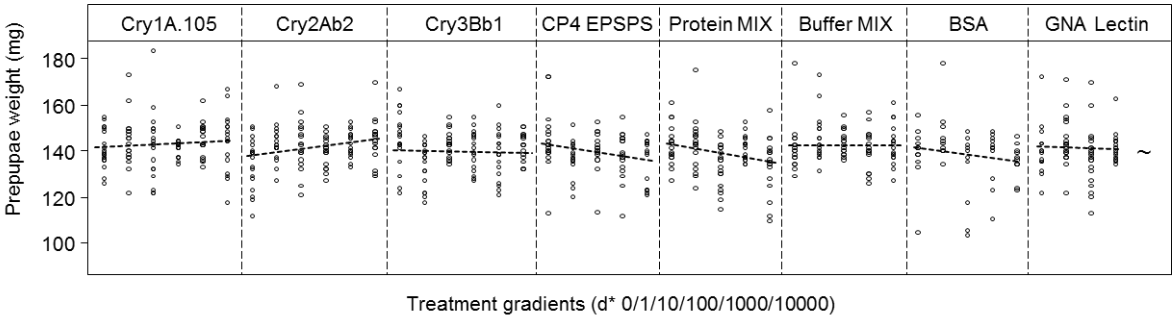

Table I. Feeding treatments of *in vitro* reared honey bee larvae for Bt-protein bioassays. Transgenic proteins were tested individually [1, 2, 3, 4] and combined [5], in proportions as in pollen of stacked "Mon89034 x Mon88017" maize. A mixed buffer gradient [6] was used as control for the mixed protein treatment. Bovine Serum Albumine (BSA) was used as neutral (non-toxic) protein control [7] and GNA-Lectin as a further pest control protein [8]. Specific buffer solutions of purified and combined proteins were used as d*0 concentration.

Treatment	n (D5)	Dose [d]	Conc. gradient [a] (field dose)	Controls	Highest dose	In pollen (dwt;fwt)[b]	Tested conc. [b]
[1] Cry1A.105	109	0.32 ng	0 / 1 / 10 / 100 / 1000 / 10000	10^4 Buffer Cry1	3.2 µg / 10µl	32.0 ng ; 17.2 ng	0 - 186 EEC
[2] Cry2Ab2	110	0.012 ng	0 / 1 / 10 / 100 / 1000 / 10000	10^4 Buffer Cry2	0.124 µg / 10µl	1.24 ng ; 0.66 ng	0 - 188 EEC
[3] Cry3Bb1	109	0.30 ng	0 / 1 / 10 / 100 / 1000 / 10000	10^4 Buffer Cry3	3.0 µg / 10µl	30.0 ng ; 16.0 ng	0 - 188 EEC
[4] CP4-epsps	93	6.4 ng	0 / 1 / 10 / 100 / 1000	10^3 Buffer CP4	6.4 µg / 10µl	640 ng ; 340 ng	0 - 19 EEC
[5] Stacked Mix	92	7.4 ng	0 / 1 / 10 / 100 / 1000	10^3 B(1+2+3+4)	7.03 µg / 10µl	703 ng ; 374 ng	0 - 18 EEC
[6] Buffer Mix	92	B[5]	0 / 1 / 10 / 100 / 1000	No additive	B[5] / 10µl		
[7] BSA	62	800 ng	0 / 1 / 10 / 100 / 1000	No additive	800 µg / 10µl		
[8] GNA-Lectin	92	80 ng	0 / 1 / 10 / 100 / 1000	No additive	80 µg / 10µl		

[a] Treatment doses applied within a one-time 10µl diet of second instars at the second day (D5) of *in vitro* rearing. The indicated field exposure (EEC) is equivalent to 3.8 mg stacked Bt-maize pollen. The zero concentration doses are treatment specific, and indicated in the 'Controls' column.
[b] Data Monsanto 2009

Table II. Summary statistics of protein Treatments, Gradient, Colony origin and Trials on (A) mortality rates of *in vitro* reared honey bee larvae (n=755) and (B) weight of prepupae (n=709). *P*-value significances are based on an α-level of 0.05, and labeled as trend '.' for $P<0.10$, '**' for $P<0.01$ and '***' for $P<0.001$.

A) Survival *Coxph* regression model ($R^2 \leq 0.55$)				χ^2	*d.f.*	*P*-value
Treatment				57.5	7	< 0.001***
Gradient (as linear variable)				16.2	1	< 0.001***
Treatment:Gradient				60.9	6	< 0.001***
Colony (as random factor)				4.1	1	
B) Prepupae weight model (R^2 = 0.081)	*d.f.*	*SS* (78626)	R^2	*F*-value		*P*-value
Treatment	7	530	0.7%	0.71		0.66
Colony	5	1966	2.5%	3.70		0.003 **
Trial	1	795	1.0%	7.48		0.006 **
Gradient (as linear variable)	1	325	0.4%	3.06		0.081 .
Treatment:Gradient	7	2054	2.6%	2.76		0.008 **
Residuals	687	72956	92.8%			

Table III. Mortality of 755 *in vitro* reared honey bee larvae. Second instar larvae were exposed to a protein dose within their diet (D5) and monitored for survival of test individuals up to the prepupae stage where larvae finish eating and growing (D11). The tabulated statistics for the gradient were based on individual treatment subsets. Color coding is used to visualise potential patterns in mortality (white 0%, light gray <10%, dark gray >10%, black 100% mortality).

Treatment	d*	0	1	10	100	1000	10000	Total mortality	Gradient	R^2	*P*-value
[1] Cry1A.105		0%	0%	0%	0%	0%	0%	0 % (0/109)	$\chi^2 = 0$	0	1
[2] Cry2Ab2		5.00%	0%	5.60%	5.60%	5.60%	5.60%	4.5 % (5/110)	$\chi^2 = 0.21$	0.040	0.64
[3] Cry3Bb1		5.30%	5.30%	0%	0%	0%	0%	1.8 % (2/109)	$\chi^2 = 1.71$	0.089	0.19
[4] CP4 epsps		5.00%	16.70%	11.10%	5.30%	0%		7.5 % (7/93)	$\chi^2 = 1.14$	0.115	0.29
[5] Stacked MIX		0%	5.30%	0%	5.60%	0%		2.2 % (2/92)	$\chi^2 = 0.00$	0.041	0.97
[6] BUFMIX		0%	0%	11.10%	5.60%	0%		3.4 % (3/88)	$\chi^2 = 0.23$	0.070	0.63
[7] BSA		7.70%	8.30%	8.30%	0%	8.30%		6.6 % (4/61)	$\chi^2 = 0.08$	0.090	0.78
[8] GNA-Lectin		6.70%	0%	4.80%	5.30%	**100%**		24.5 % (23/94)	$\chi^2 = 27.6$	0.524	< 0.001*

Table IV. Prepupal weight effects over the gradients, per treatment. The overall mean weight per treatment is given (with the standard deviation). Indicated in the matrix are mean prepupae weights per treatment dose. The gradient follows the exponentially increasing low dose 'd'. The range of effects per treatment is indicated with a light gray shade for minima values and a dark grey shade for the maxima values. Symbol † indicates that all test-individual have died (at the highest level of Lectin) for which no data on the weight of prepupae available.

Proteins	n	Weight (mg) ± SD	d * 0	1	10	100	1000	10000	R^2	t-value	*P*-value
[1] Cry1A.105	109	143.6 ± 10.0	141.7	144.8	142.6	142.1	146.1	144.6	0.008	0.92	1.0
[2] Cry2Ab2	110	142.0 ± 9.3	135.7	143.9	143.3	140.4	143.7	145.4	0.184	2.25	0.20
[3] Cry3Bb1	109	140.6 ± 11.3	145.6	135.1	143.8	137.4	139.1	142.6	0.088	-0.44	1.0
[4] CP4 Epsps	93	139.8 ± 10.3	145.1	137.4	140.9	139.2	136.1		0.059	-2.23	0.21
[5] Stacked mix	92	139.5 ± 10.2	143.3	143.4	134.5	142.2	134.7		0.068	-2.42	0.13
[6] Buffer mix	92	142.9 ± 10.8	142.3	143.1	144.4	141.5	143.3		0.000	-0.01	1.0
[7] BSA	62	138.9 ± 12.4	139.6	147.6	132.6	138.6	136.5		0.138	-1.75	0.65
[8] GNA Lectin	92	141.6 ± 10.7	141.2	144.8	138.1	142.6	†		0.001	-0.35	1.0

Supplement S: *Post hoc* survival statistics; between treatment differences

In *Coxph* models, four test variables of possible influence on the data were considered: Treatment (8 levels), Gradient (1 level), Colony (7 levels), Trial-days (2 levels). As fixed factors, Trial-days, Colony and the Colony-Treatment interaction were rejected for being insignificant; Table IIA. Dose-response effects tested with the interaction term *factor(treatment)*gradient*, alongside the single factors *treatment* and *gradient* (with colony used as a random factor). Symbols: * = significance; *ns* = insignificance; *xxx* = *Coxph* model 'indigestion' for a 100% survival rate (no mortalities, thus no ability to compare the dynamics on survival). These *P*-value significances were determined according to a sequential Holm-Bonferroni procedure using an α-correction considering the number of comparisons per level (significances indicated in yellow). Solely GNA-lectin showed to be toxic. General: GNA dose-response effect differed to all other individual treatments with $\chi^2 \geq 11.0$, $P < 0.001$; *Bonferoni α/7*.

Post hoc Survival	Treatment 2		Treatment 3		Treatment 4		Treatment 5		Treatment 6		Treatment 7		Treatment 8				treatment		gradient		interaction		High dose: treatment		
	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P									X^2	P	
Treatment 1	6.98	0.008	2.87	0.090	10.38	0.001	3.10	0.078	5.00	0.025	8.01	0.005	84.09	0.000	factor (treatment)	23)	0.250	ns	0.630	ns	0.060	ns			
	0.23	0.631	3.19	0.074	1.81	0.178	0.04	0.840	0.01	0.935	0.41	0.523	21.98	0.000	factor (gradient)	36)	0.540	ns	0.330	ns	0.080	ns	13)		xxx
	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	interaction	35)	0.160	ns	0.140	ns	0.170	ns	14)		xxx
																24)	0.970	ns	0.190	ns	0.180	ns	15)		xxx
Treatment 2			1.33	0.248	0.71	0.401	0.73	0.392	0.10	0.755	0.34	0.561	43.19	0.000	factor (treatment)	46)	0.070	ns	0.050	ns	0.270	ns	34)		xxx
			0.24	0.625	0.24	0.624	0.32	0.570	0.43	0.513	0.02	0.898	21.37	0.000	factor (gradient)	27)	0.400	ns	0.620	ns	0.270	ns	35)		xxx
			3.44	0.064	1.20	0.274	0.03	0.874	0.01	0.912	0.35	0.552	22.68	0.000	interaction	45)	0.220	ns	0.480	ns	0.400	ns	45)		xxx
																16)	0.560	ns	0.900	ns	0.550	ns	16)		xxx
Treatment 3					3.23	0.072	0.00	0.968	0.37	0.541	1.97	0.160	55.66	0.000	factor (treatment)	36)	0.080	ns	0.360	ns	0.610	ns	36)		xxx
					3.97	0.046	1.69	0.194	0.94	0.332	2.21	0.137	15.54	0.000	factor (gradient)	46)	0.810	ns	0.310	ns	0.760	ns	46)		xxx
					1.20	0.273	1.80	0.180	2.97	0.085	1.86	0.173	30.49	0.000	interaction	57)	0.180	ns	0.810	ns	0.810	ns	56)		xxx
																17)	0.180	ns	0.810	ns	0.810	ns	17)	1.76	0.184 ns
Treatment 4							3.00	0.083	1.48	0.223	0.06	0.806	16.27	0.000	factor (treatment)	56)	0.620	ns	0.760	ns	0.820	ns	37)	1.83	0.176 ns
							0.83	0.362	0.50	0.480	1.03	0.311	27.48	0.000	factor (gradient)	25)	0.390	ns	0.570	ns	0.870	ns	47)	1.83	0.176 ns
							0.26	0.608	0.72	0.397	0.09	0.762	37.68	0.000	interaction	26)	0.760	ns	0.510	ns	0.910	ns	57)	1.83	0.176 ns
Treatment 5									0.25	0.619	1.80	0.179	35.56	0.000	factor (treatment)	13)	0.090	ns	0.070	ns	0.000	X	67)	1.83	0.176 ns
									0.09	0.762	0.06	0.806	43.97	0.000	factor (gradient)	15)	0.080	ns	0.840	ns	0.000	X	12)	1.33	0.249 ns
									0.05	0.822	0.06	0.809	10.99	0.001	interaction	16)	0.030	ns	0.940	ns	0.000	X	23)	1.39	0.239 ns
Treatment 6											1.80	0.179	32.08	0.000	factor (treatment)	12)	0.008	*a/4	0.630	ns	0.000	X	24)	1.39	0.239 ns
											0.06	0.806	43.62	0.000	factor (gradient)	17)	0.005	*a/5	0.520	ns	0.000	X	26)	1.39	0.239 ns
											0.06	0.809	12.70	0.000	interaction	14)	0.001	*a/6	0.180	ns	0.000	X	25)	1.39	0.239 ns
																18)	0.000	*a/7	0.000	X	0.000	X	27)	0.07	0.791 ns
Treatment 7													17.37	0.000	factor (treatment)	18)	0.000	*	0.000	*	0.000	*	18)	46.98	0.000 *
													39.83	0.000	factor (gradient)	48)	0.000	*	0.000	*	0.000	*	38)	48.47	0.000 *
													22.34	0.000	interaction	38)	0.000	*	0.000	*	0.000	*	48)	48.47	0.000 *
																28)	0.000	*	0.000	*	0.000	*	58)	48.47	0.000 *
																78)	0.000	*	0.000	*	0.000	*	68)	48.47	0.000 *
																68)	0.000	*	0.000	*	0.000	*	78)	32.47	0.000 *
																58)	0.000	*	0.000	*	0.001	*	28)	38.13	0.000 *

Excellence in survival rate [1] was indicated, given the contrasts to treatments [2], [4] and [7]. The general low mortality was reported, but the mentioning of these significances was omitted because it had no eco-toxicological relevance: No dose-response effects were involved thus no toxicity was present [1-7].

Nonetheless, it does indicate a methodological strength: Cry [1] treatment mortality of mean 0% was significantly lower than [2], [4] and [7] (resp. mean 4.4%, 7.5% and 6.6%) and therefore we choose to mention it supplementary, as an indication of *Coxph* model strength.

Supplement W: *Post hoc* statistics on prepupal weight

Contrasts are directly derived from the summary output. They are directly bound to Table IIB.
A correction to compensate for multiple comparisons, to obtain the final comparative results. (multiplying *P* with #, and then tested against alfa = 0.05)

Trials	(2) 1 comparison, no correction	
Colonies	correct for 5 comparisons per colony (6)	#5
Treatments	correct for 7 comparisons per treatment (8)	#7
Gradient	correct the 8 times use to predict treatments (8)	#8
Interaction	correct for 7 comparisons to others, per treatment (8)	#7

Day / trial effect

Day 1 and 2	-2.74	0.006 **

Δ 1.5 mg between days
D1: mean 140.7 mg ± 10.3 SD
D2: mean 142.2 mg ± 11.0 SD

Dose response effects

	t-value	*P*		
Gradient 1 Cry1	0.92	1.0	*T~G*	*y = 0.53x + 142.3*
Gradient 2 Cry2	2.25	0.20	*T~G*	*y = 1.31x + 138.8*
Gradient 3 Cry3	-0.44	1.0	*T~G*	*y = -0.27x + 141.3*
Gradient 4 CP4	-2.23	0.21	*T~G*	*y = -1.72x + 143.3*
Gradient 5 MIX	-2.42	0.13	*T~G*	*y = -1.86x + 143.2*
Gradient 6 BUFMIX	-0.01	1.0	*T~G*	*y = 0.05x + 142.8*
Gradient 7 BSA	-1.75	0.65	*T~G*	*y = -1.41x + 141.8*
Gradient 8 LECTIN	-0.35	1.0	*T~G*	*y = -0.37x + 142.2*

Differences between colonies

	C2	C3	C4	C5	C6	*mean*	*t-value*	*Colonies*
Colony 1: 139.7 mg ± 9.9 SD	1.0	1.0	0.003 **	1.0	0.12	*Δ 4.6 mg*	*3.42*	*C4/C1*
Colony 2: 140.4 mg ± 8.5 SD		1.0	0.024 *	1.0	0.45	*Δ 3.9 mg*	*2.83*	*C4/C2*
Colony 3: 140.6 mg ± 11.1 SD			0.029 *	1.0	0.51	*Δ 3.8 mg*	*2.77*	*C4/C3*
Colony 4: 144.3 mg ± 10.5 SD				0.006 **	1.0	*Δ 4.2 mg*	*3.26*	*C4/C5*
Colony 5: 140.1 mg ± 13.2 SD					0.19			
Colony 6: 142.8 mg ± 8.7 SD								

Treatment differences

		T2	T3	T4	T5	T6	T7	T8
T1 Cry1	143.6 ± 10.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T2 Cry2	142.0 ± 9.3		1.0	0.53	0.57	0.75	1.0	1.0
T3 Cry3	140.6 ± 11.3			1.0	1.0	1.0	1.0	1.0
T4 CP4	139.8 ± 10.3				1.0	1.0	1.0	1.0
T5 MIX	139.5 ± 10.2					1.0	1.0	1.0
T6 BUFMIX	142.9 ± 10.8						1.0	1.0
T7 BSA	138.9 ± 12.4							1.0
T8 LECTIN	141.6 ± 10.7							

Differences in dose-responses

	G:T2	G:T3	G:T4	G:T5	G:T6	G:T7	G:T8		*t-value*
Interaction Gradient : T1 Cry1	1.0	1.0	0.14	0.09	1.0	0.34	1.0		
Interaction Gradient : T2 Cry2		0.40	0.012 *	0.007 **	1.0	0.060 .	1.0	*T2/T4*	*3.14*
Interaction Gradient : T3 Cry3			0.92	0.68	1.0	1.0	1.0	*T2/T5*	*3.29*
Interaction Gradient : T4 CP4				1.0	0.87	1.0	1.0	*T2/T7*	*2.66*
Interaction Gradient : T5 MIX					0.66	1.0	1.0		
Interaction Gradient : T6 BUFMIX						1.0	1.0		
Interaction Gradient : T7 BSA							1.0		
Interaction Gradient : T8 LECTIN									

Chapter 2:

A publication is under preparation based on the work carried out:

Steijven, K. Steffan-Dewenter I., Härtel S. (2014). Dose dependent effects of stacked Bt maize pollen on *in vitro* reared honey bee larvae. To be submitted to Ecotoxicology and Environmental Safety

Dose dependent effects of stacked Bt maize pollen on *in vitro* reared honey bee larvae

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Running title: Dose depending effects of GM pollen

Keywords: *Apis mellifera*; *Bacillus thuringiensis*; environmental risk assessment; genetically modified crops; pollen

Abstract

In agricultural landscapes of many countries of the world, honey bees (*Apis mellifera*) are exposed to a number of different genetically modified pollen. In order to assess environmental risks of transgenic crops for pollinators, first tier laboratory risk assessment studies with a great potential of standardisation are highly needed. Here we conducted an *in vitro* larvae rearing experiment to test the potential effects of genetically modified (GM) stacked Bt maize pollen on honey bee larvae. The test substance was administered in increasing doses to be able to detect possible dose dependent effects. Feeding experiments with different amounts of Bt pollen are reflecting natural, and thus highly relevant, exposure scenarios for pollinators. In addition to the rather crude endpoint 'survival' we also measured several sub-lethal effects; pollen digestibility, prepupal weight, and development. There were no differences in digestibility. Increasing amounts of Bt pollen did not have a negative effect on the survival of honey bee larvae. Interestingly increasing amounts of multi-floral pollen did have a negative effect on honey bee larvae survival. In prepupal weight we find contradicting results; prepupal weight decreased at high doses of Bt pollen when compared to the multi-floral pollen treatment. There are negative dose dependent effects for juvenile development in bees that were fed Bt pollen as well as in bees that were fed multi-floral pollen. However these effects are not significantly different from the negative controls. We propose that in Environmental Risk Assessments (ERA) schemes of transgenic plant products for honey bees a range of GM crop pollen doses is tested, similar as is done in toxicological studies of chemical plant protection products. After all 'the dose makes the poison'. Moreover, this study highlights the importance of including other endpoints except survival, as

responses may differ. And finally the importance of multiple negative controls in the design is discussed, as to be able to make a biological relevant interpretation of your results and distinguish between transgenic effects and variety or species effects. The described approach has the potential to become a standard method in regulatory ERA schemes for pollinators, since dose dependent effects of GM pollen on bee larvae are demonstrable. Moreover, the test method could easily be adjusted to test the effects of systemic insecticides incorporating the natural exposure pathway via the pollen.

Introduction

Honey bees are among the most important pollinators in the world. Numerous wild plant species benefit from their pollination service. As a super-generalist and due to their global occurrence they are able to pollinate a multitude of wild plants, as well as crops. They are managed not only for their honey, but also to enhance crop yields and it has been shown they are able to increase yields of 96% of all animal pollinated crops (in Klein et al. 2007). In recent years there have been various reports of honey bee declines: in the USA a decline of 35.8% over the years 2007 to 2008 has been recorded (vanEngelsdorp et al. 2008), in central Europe honey bees have declined with 25% over the period 1985-2005 (Potts et al. 2010). Since honey bees occur globally, this also means they co-occur with many genetically modified organisms (GMO's). Currently one of the most common and most widely grown genetically modified crops is Bt maize. Bt stands for *Bacillus thuringiensis*, a bacterium which expresses proteins that are toxic to specific insects depending on the strain (Schnepf et al. 1998). The mode of action of this protein is that it binds to a receptor in the insect's gut, after which it starts to form pores in the gut (Schnepf et al. 1998). One of the Bt maize crops currently being cultivated worldwide is a stacked Bt maize (Mon89034xMon88017); 'stacked' referring to the fact that it has multiple transgenes that enable the plant to produce toxins against multiple pest insects, along with a gene that makes the plant resistant against glyphosate (the active ingredient of the herbicide Roundup®).

Pollen is the sole protein source for honey bees and it is essential for the growth of the larvae. Nurse bees are the "turntable" for proteins within the colony (Crailsheim und Brodschneider 2013; Hendriksma et al. 2013). They feed the larvae with protein rich jelly. The larvae receive the main part of proteins via this glandular secretion, but a minor part of proteins is also provided in form of pollen grains (Haydak 1943, Babendreier et al. 2004). According to Babendreier et al (2004) only 5% of the total amount of protein needed for the development of a worker larvae is derived from pollen fed directly to the larvae. In the literature the amount of pollen that is directly mixed into the worker jelly varies, probably depending on pollen type being investigated and quantification method, ranging from 1.5 mg to 9.4 mg per larva (Hanley et al. 2003, Babendreier et al. 2004, Hrassnigg and Crailsheim 2005, Keller et al. 2005).

As the honey bee is a very important pollinator in many agro-ecosystems, it is of the utmost importance that the possible effects of GMO's on honey bees and honey bee health are thoroughly studied. Thus far studies in which honey bee colonies are exposed to Bt pollen or their insecticidal proteins have shown no effects on differences in immature stages/brood development, worker survival, bee weight, hypopharyngeal gland size, colony performance, syrup consumption, olfactory learning abilities, hemolymph protein content, superoxide dismutase activity (associated with environmental stress), or foraging activity (Hanley et al. 2003, Malone et al. 2004, Babendreier et al. 2005, Liu et al. 2005, Ramirez-Romero et al. 2005, Rose et al. 2007, Liu et al. 2009, Dai et al. 2012). But note that Ramirez-Romero et al (2005) observed hives under controlled conditions and showed a decline in foraging activity of worker honey bees during and after exposure to Cry1Ab, the most used Bt protein. Since Bt crops are targeted against the larval stages of the pest insects, and the larval phase is likely to be the most sensitive

phase (Romeis et al. 2011), it is vital to do controlled studies of their effects on the larvae of honey bees as well, instead of just looking at the colony level or the adult bees.

In toxicology the first principle is that the poison is the dose, not the substance in itself, and this principle is well implemented in ecotoxicological studies. Nonetheless, in the field of Environmental Risk Analyses of GMO's the implementation of this principle is not yet standard practice. Therefor we tested in this study the effect of increasing doses of transgenic pollen. Except for the mortality and prepupal weight, we included the digestibility and the delay in development as two novel sub-lethal endpoints. The impact of GM pollen or pollen treated with systemic pesticides on honey bee larvae has yet not been considered for a harmonised standard test method. The used *in vitro* rearing method based on the protocols of Aupinel et al (2005, 2007, 2009) and Hendriksma et al (2011a, b) has the potential to become a standard pollen test for transgenic or chemical plant protection products.

Materials and Methods

In vitro larvae rearing

We conducted an in *vitro larvae* rearing experiment with honey bee larvae from 11 different colonies from our own apiary (University of Würzburg). For the first two trials (19 and 30 July 2012) we obtained first instar larvae using an artificial comb (Cupularva queen rearing system by Nicotplast ©, Maisod, France) inside the hives, according to the method described by Hendriksma et al (2011a). Larvae were moved to the lab on the first day after hatching, placed in sterile 48-wellplates and kept in a desiccator within a brood stove (35 °C and 95% RH). Later on in the season bees were less cooperative with the artificial comb; either queens were reluctant to lay their eggs on the artificial comb, or laid eggs were removed by workers directly after hatching. So for three more trials later on in the season (10, 11 and 25 September 2012), honey bee larvae were collected via grafting. For more detailed information on the in vitro rearing of honey bee larvae we refer to the review by Crailsheim et al (2013) "Standard methods for artificial rearing of *Apis mellifera larvae*".

Pollen grains are already found in cells of very young larvae (larval weight up to 50 mg, probably 1st and 2nd instar larvae), however the numbers of pollen grains are very low (Malone et al. 2002). Moreover, maize pollen are relatively big and we suspected younger instar larvae would not be able to ingest the pollen grains, therefor we decided to start our experimental treatments two days after the larvae were moved to the lab. Larvae were fed according to an adjusted version of the feeding protocol of Aupinel et al (2005, 2007, 2009) on the first day of feeding, i.e. the fourth development day (D4), larvae received 20 µl of diet A, on D5 larvae were not fed, from D6 until D9 larvae received an increasing amount of Diet C (20, 30, 40, 50 µl respectively) spiked with pollen (figure 1).

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
EGG			L1	L2	L3	L4	L5		PP		PUPA									

Figure 1: The development cycle of a honey bee; Day one (D1) the egg is laid, the larva hatches after three days (on D4). Larvae are fed in the lab from D4 until D9. Pollen treatments are added to the larval food on D6 until D9. During its larval phase it moults 5 times (larval instars L1 to L5) after which it turns into a prepupa (D10 and D11). At this point the experiment was terminated. After the prepupal phase bees pupate after 10 days (on D21) adults eclose from their cells.

Pollen

Five different pollen types were fed to the larvae. We compared stacked transgenic Bt pollen (DKc7565, cultivar novelis, Monanto) to several negative controls; its near-isogenic line (DKc5340), another conventional maize variety (Benicia), and multi-floral pollen (bee pollen), and one positive control; toxic pollen from the tropical plant Heliconia *jacquinii*. The five different pollen types were fed at five increasing doses; 0, 1, 2, 5, and 10 mg larva^{-1}, fed additively over four consecutive days (D6-D9). This way, larvae received a fresh portion of pollen every day. Colony backgrounds and pollen types were distributed over all the well plates.

The three types of maize pollen were collected from an experimental field near Braunschweig (Germany) in 2009. Maize was grown in a randomised block design with eight replications. Before storage at -80 °C the pollen was pooled and sieved with a 0.32 mm sieve.

Multi-floral pollen was collected using a pollen trap in front of two beehives in apiary at Würzburg University (Germany). Returning forager bees climb through the narrow holes of the pollen trap and in doing so they lose their pollen pellets. After a few hours the pollen pellets were collected and stored at -20 °C.

Toxic pollen was collected from flowering *Heliconia jacquinii* plants, from the tropical greenhouse of the Botanical garden of Würzburg University. In contrast to maize flowers, During the flowering period twice a week individual flowers with dehiscent anthers were collected and suspended in demineralised water and shaken thoroughly. After flowers were taken out the suspended pollen was put in Eppendorf tubes and centrifuged for half a minute at a low velocity (as not to break the pollen). The top layer of water was removed and thick suspension of pollen placed overnight in a drying oven at 30°C. The water part on top was removed and the remaining substance was dried in a brood stove for 24 hours at 30 °C, before storage at -80 °C.

Dissection of larvae and digestion rate

A subsample of 3-4 larvae per experimental treatment per repetition was taken at least six hours after the last feeding, provided that some larvae would remain to follow the mortality. Especially in treatment groups that were fed higher doses of toxic pollen this was not possible, as all larvae had already died at that point. Larvae were first frozen before weighing and dissection. Larvae were pinned on a paraffin preparation plate and carefully dissected ventrally. Both mid,- and hind gut were taken out entirely and suspended in 200 µl of 0.5 M glucose solution, after which they are stored at -20 °C until pollen counting. The content of each sample is gently pipetted up and down 10 times and vortexed for 30 seconds before pollen counts, to rupture the gut tissue and create a suspension with uniform distribution. A Neubauer hemocytometer with two counting chambers is used to quantify the digestion rate. For the first fifty pollen grains the digestion is scored according to the remaining pollen grain in the pollen; 0 – 10 % remaining pollen grain is scored as ‘fully digested’, 10 – 90 % remaining pollen grain is scored as ‘partly digested’, and

more than 90 % remaining pollen grain is scored as 'undigested'. This procedure is repeated once, thus resulting in four subsamples per larva gut (two times two counting chambers).

Endpoints

Throughout larval development (until D11) the mortality was recorded and dead larvae were removed. Dead larvae can be recognised by discolourations or immobility, i.e. no signs of respiration (Crailsheim et al. 2013). On the ninth development day (D9) a subsample was taken to determine the digestion of the pollen grains. At the end of the experiment (D11) all remaining specimens were weighed and their development stage was recorded, ranging from L1 (1st instar larva) to PP (prepupa; also see figure 1).

Statistical analysis

All statistical tests were performed using R statistical software (R core development Team 2011). Digestion rate was quantified using a weighted mean of scored digestion values. The number of pollen kernels in each class (i.e. fully, partly or not digested) was multiplied by the mean digestion of that class to come to a weighted percentage as a quantification of digestion rate. To compare the digestion rates between the three maize varieties a nested mixed effects linear model (package lmer4) was made. Pollen type, dosage and their interaction were included as fixed factors. Colony background and wellplate nested within date were included as random factors. Multiple comparisons of factor levels were done with a Tukey post hoc test, corrected for multiple comparisons with the Benjamini Hochberg procedure (Benjamini and Yekutieli 2001). To analyse the mortality we did cox proportional hazards regressions with mixed effects (Therneau 2011). Pollen type and dose, and the interaction between the two were included as fixed factors. Colony background, wellplate, grafting method and date (nested within grafting method) were included as random factors. Multiple comparisons between the relevant levels of both factors were done by constructing a contrastmatrix. Adjustment for multiple comparisons were done by the Benjamini Hochberg procedure (Benjamini and Yekutieli 2001). Prepupal weight was also analysed with a nested mixed effects linear model (package lmer4). In this model we included pollen type as a fixed factor, dosage as a covariate, and their interaction. Grafting method, date and wellplate (nested) and colony background were included as random factors. The post hoc analysis was done by constructing a contrastmatrix. Adjustment for multiple comparisons were done by the Benjamini Hochberg procedure (Benjamini and Yekutieli 2001). To compare the development stages we did a cumulative link mixed model fitted with the Laplace approximation (package ordinal). Pollen type, dose and their interaction were included as fixed factors. Colony and wellplate nested within date were included as random factors. Graftin method did not improve the model and was therefore excluded from this analysis. Multiple comparisons were derived by changing the reference factor level and we did adjust p values via the Benjamini Hochberg procedure.

Results

Digestion

All maize pollen types were equally well digested, irrespective of the amount of pollen they were fed (figure 1; table 1). There was no interaction between pollen type and dosage (ANOVA; χ^2 = 4.3941, df = 6, p-value = 0.6235), nor was there a significant effect of dosage on the digestion (ANOVA; χ^2 = 1.0831, df = 3, p-value = 0.7812). Pollen type did seem to have a significant effect on digestion (ANOVA; χ^2 = 6.2575, df = 2, p-value = 0.04377), however, when we account for multiple comparisons in the post-hoc analyses, no significant differences could be detected (see table 1). Overall we found 6.7 % were fully digested pollen grains 32.9 % were partly digested and the biggest percentage, 60.5 %, remained undigested, resulting in a weighted average digestibility of 17.2 %.

Table 1: Multiple comparisons between the three different maize pollen types fed to the larvae (post hoc by Tukey, adjusted by Benjamini Hochberg procedure).

	Estimate	Std. Error	z value	P value
Bt_stacked - Benicia	3.452	1.804	1.914	0.0835
Comparator - Benicia	4.249	1.801	2.360	0.0549
Comparator - Bt_stacked	0.797	1.678	0.475	0.6349

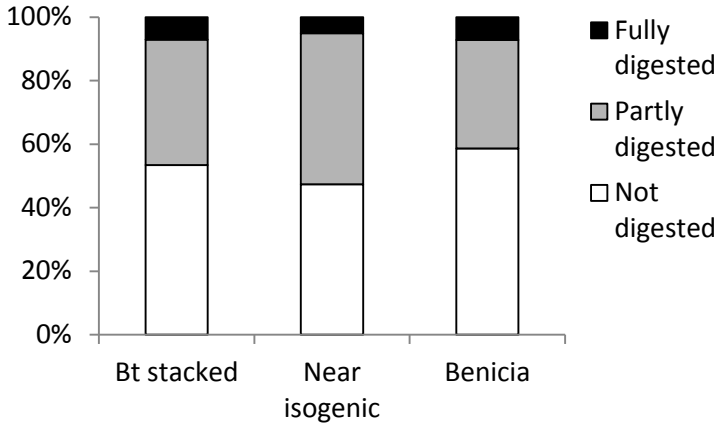

Figure 2: Digestion rate for the three different types of maize pollen used in this experiment. Data for all dosage levels (1 mg, 2 mg, 5 mg and 10 mg) are pooled.

Survival analysis

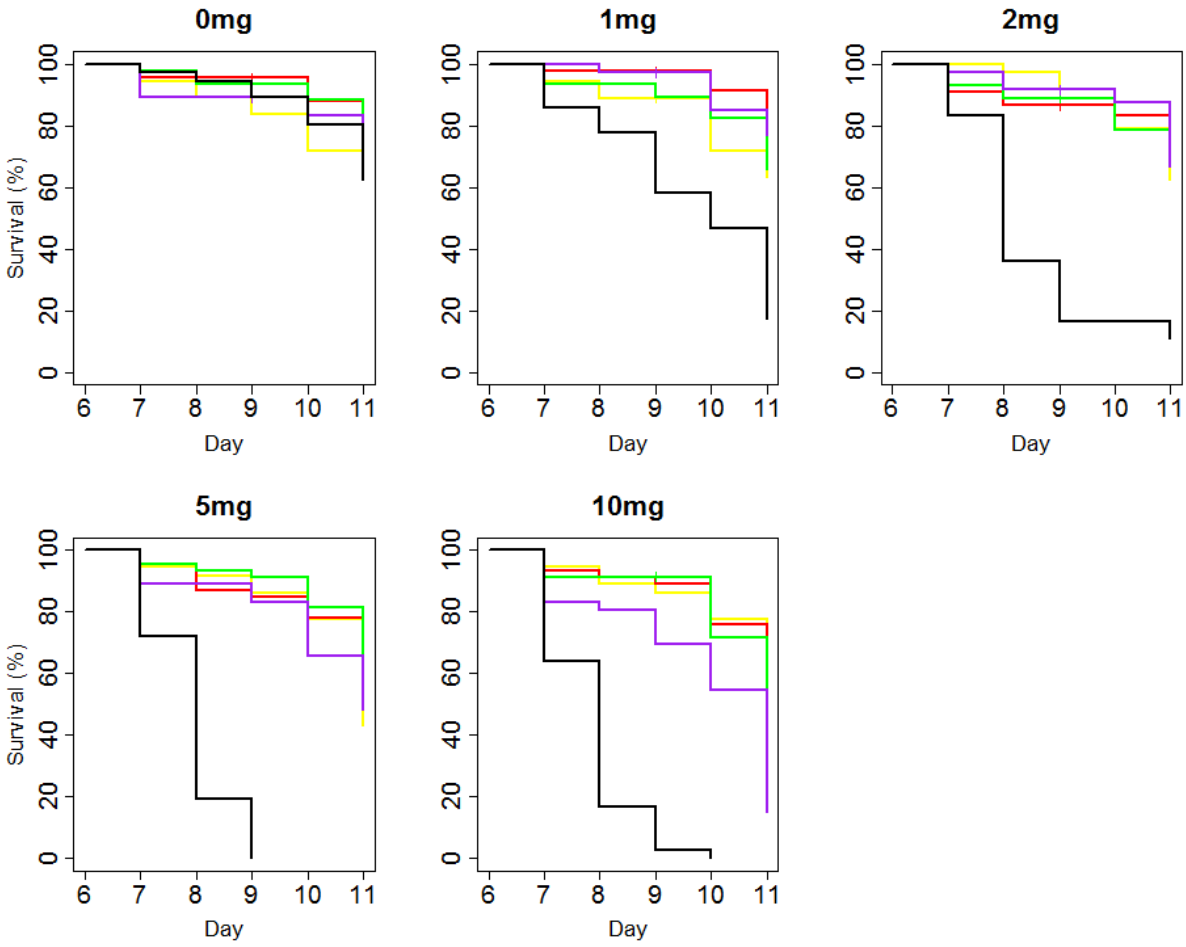

Figure 3: Survival curves for in vitro reared honey bee larvae. X axis show the development day counted from the day the eggs were laid, y-axis shows survival in percentage. Pollen treatments were administered from day six until day 9, the last day of feeding. Mortality was recorded for two more days, until pupation. Black = Toxic pollen (Heliconia *jacquinii); Red = stacked Bt pollen; Green = near isogenic line; Yellow = Benicia pollen (conventional maize); Purple = Multi-floral pollen.*

The random variables accounted in total for 94.8 % of the variance of data (Grafting method – 68.2 %; colony background – 21.5 %; date - 5.1 %; well plate – 0.04 %). Each pollen type was fed at 0 mg (background mortality), 1, 2, 5, and 10 mg. Note that all groups in the 0 mg group received the same base diet, without any pollen. Survival curves for the different pollen types are depicted per dose level in figure 3.

For all pollen types except Benicia (the conventional maize variety) there is a dose dependent effect on the survival of the larvae (table 2), i.e. with more pollen fed less larvae survive until the end of the experiment. Larvae fed with toxic pollen (the positive control) had lower survival compared to when they were fed maize pollen (Bt, near-isogenic or Benicia pollen), but there is no significant difference between the toxic treatment and the multifloral treatment. The larvae fed with multifloral pollen also had lower survival than the larvae fed with Benicia pollen.

Table 2: Outputs for multiple comparisons of the interaction effect between pollen type and dosage (correction with Benjaminin Hochberg procedure) on survival.

Hypothesis	Estimate	Std. Error	z value	p value	significance
slopeMF - 0 == 0	0.19214	0.03634	5.287	9.33e-07	***
slopeBt - 0 == 0	0.14553	0.03330	4.370	6.20e-05	***
slopeC - 0 == 0	0.10444	0.03603	2.898	0.00938	**
slopeBen - 0 == 0	0.06216	0.03760	1.653	0.13410	NS
slopeTox - 0 == 0	0.23833	0.02609	9.135	< 2e-16	***
slopeBt - slopeMF == 0	-0.04660	0.04549	-1.024	0.35265	NS
slopeBt - slopeC == 0	0.04109	0.04571	0.899	0.38453	NS
slopeBt - slopeBen == 0	0.08338	0.04670	1.785	0.11131	NS
slopeBt - slopeTox == 0	-0.09280	0.03795	-2.445	0.02715	*
slopeC - slopeBen == 0	0.04228	0.04863	0.870	0.38453	NS
slopeC - slopeTox == 0	-0.13389	0.04031	-3.322	0.00268	**
slopeC - slopeMF == 0	-0.08770	0.04760	-1.842	0.10907	NS
slopeBen - slopeTox == 0	-0.17618	0.04140	-4.256	7.82e-05	***
slopeBen - slopeMF == 0	-0.12998	0.04854	-2.678	0.01588	*
slopeMF - slopeTox == 0	-0.04620	0.03985	-1.159	0.30792	NS

Prepupal weight

Depending on the pollen type being fed to the larvae, an increasing amount had a negative effect on the prepupal weight (ANOVA; $\chi^2 = 88.245$, df = 4, p-value = 2.2e-16). This is very evident for the positive control, the toxic *Heliconia* pollen (figure 3, table 4). Note that we do not have any measurements for larvae that were fed 5 or 10 mg of toxic pollen, this is simply because those larvae died before reaching the prepupal stage. Nonetheless a regression could be made for the remaining data points. More interestingly the prepupal weight of the larvae fed with Bt maize also decreases at higher doses. This interaction effect differs significantly from 0, but no significant difference between Bt maize and the two other maize varieties (near isogenic line and Benicia) could be detected (table 4). Larvae that were fed Bt maize had a significantly lower prepupal weight compared to larvae that were fed multi-floral pollen (table 4).

Table 4: Outputs for multiple comparisons of the interaction effect between pollen type and dosage (correction with Benjaminin Hochberg procedure) on the sub-lethal effect prepupal weight.

Hypothesis	Estimate	Std. Error	z value	p value	significance
slopeMF - 0 == 0	0.6530	0.9750	0.670	0.580406	NS
slopeBt - 0 == 0	-34.910	0.7064	-4.942	1.93e-06	***
slopeC - 0 == 0	-10.686	0.6147	-1.739	0.111973	NS
slopeBen - 0 == 0	-14.735	0.7309	-2.016	0.073003	.
slopeTox - 0 == 0	-473.815	50.938	-9.302	< 2e-16	***
slopeBt - slopeMF == 0	-41.440	10.988	-3.771	0.000348	***
slopeBt - slopeC == 0	0.4048	0.8472	0.478	0.632757	NS

slopeBt - slopeBen == 0	-20.176	0.9213	-2.190	0.053510	.
slopeBt - slopeTox == 0	438.904	51.161	8.579	< 2e-16	***
slopeC - slopeBen == 0	0.4048	0.8472	0.478	0.632757	NS
slopeC - slopeTox == 0	463.128	51.038	9.074	< 2e-16	***
slopeC - slopeMf == 0	-17.216	10.569	-1.629	0.129168	NS
slopeBen - slopeTox == 0	459.080	51.154	8.974	< 2e-16	***
slopeBen - slopeMF == 0	-21.265	10.910	-1.949	0.076915	.
slopeMF - slopeTox == 0	480.344	51.365	9.352	< 2e-16	***

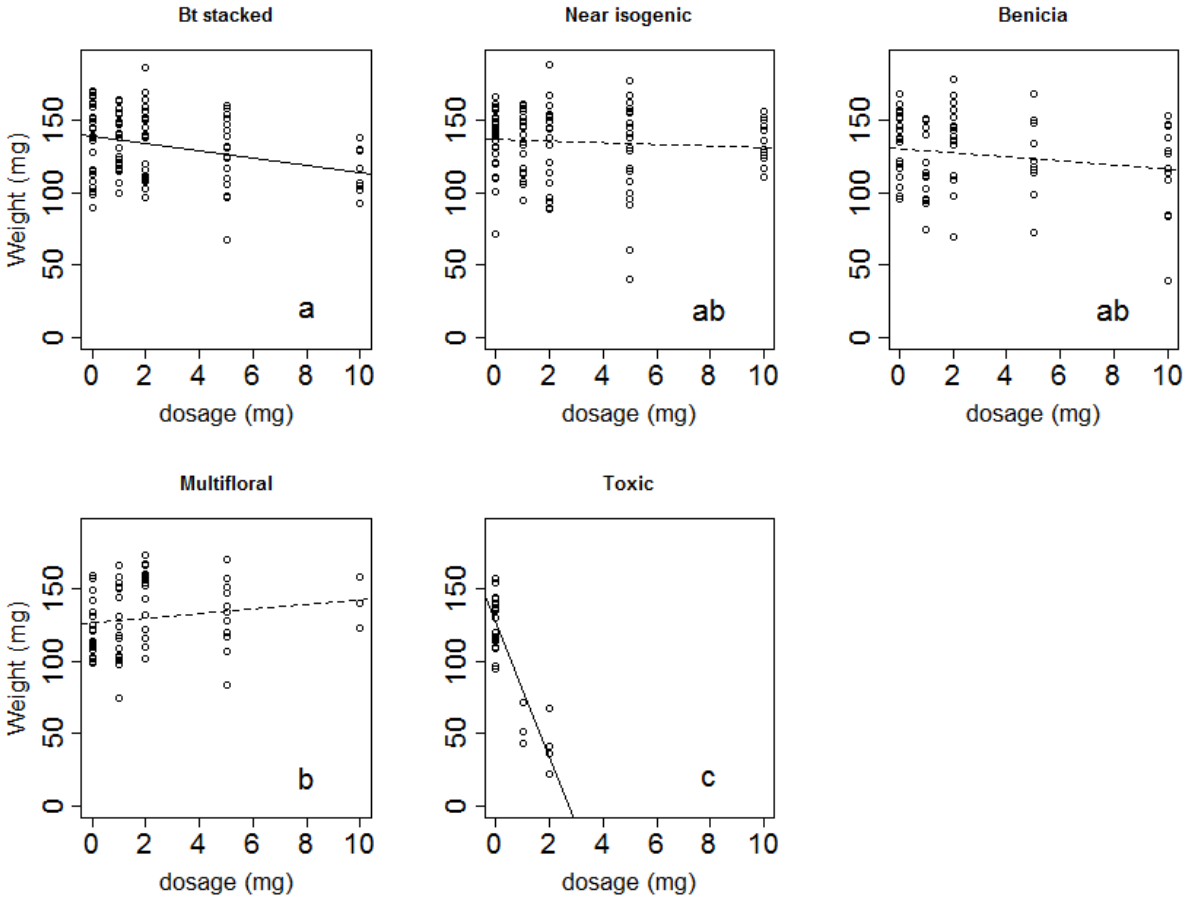

Figure 4: Prepupal weight (y-axis) in response to pollen type and dosage (amount of pollen fed in larval phase (mg); x-axis). Each graph shows the dose-response regression line for a specific pollen type. Only the pollen treatments 'Bt stacked' and 'Toxic' showed a significant dose dependent response. Significant differences between pollen treatments are indicated with letters.

Developmental delay

The expected development stage at day 11 would be the prepupal stage (also see fig 1). We found in all treatment groups, regardless of amount of pollen fed, a proportion of larvae that had not yet reached the prepupal stage. For the control group that received only the artificial diet (not spiked

with pollen, a.k.a. the 0 mg pollen treatment) 22.9 % to 37.1 % of the larvae had not yet reached the prepupal stage (figure 5).

At higher doses of toxic pollen, our positive control, larvae are more likely to be in a smaller developmental stage (see table 5 and figure5). None of the larvae fed with toxic pollen had reached the prepupal development stage at the end of the experiment. Larvae that were fed toxic pollen were still in the 4^{th} or 3^{rd} larval instar. Note that we do not have any values for the larvae that were fed 5 or 10 mg of toxic pollen. These larvae had already died at the end of the experiment.

With increasing amounts of pollen being fed also the larvae that received Bt pollen and multifloral pollen were more likely to remain smaller. The effect is less pronounced though (see figure 5). Of the larvae that received multifloral pollen a bigger proportion remained 5^{th} instar larvae with increasing amount of pollen fed. The larvae that received Bt pollen also show a negative dose dependent effect; more smaller larvae at higher amounts of pollen. When larvae received 10 mg Bt pollen, 30.4 % were 5^{th} instar larvae, 17.4 % were 4^{th} instar, and 8.7 % were still 3^{rd} instar.

However the dose dependent effect in the toxic pollen treatment was the only one that was significantly different from the other treatments. No significant differences were detected when comparing the Bt, - or the multifloral treatment with the other treatment groups or among each other (table 5).

Figure 5: Development stages of larvae at the end of the experiment, at D11. The x-axis depicts the pollen type, and the amount of pollen (in mg) fed to the larvae. Y-axis shows what percentage belongs to which development stage.

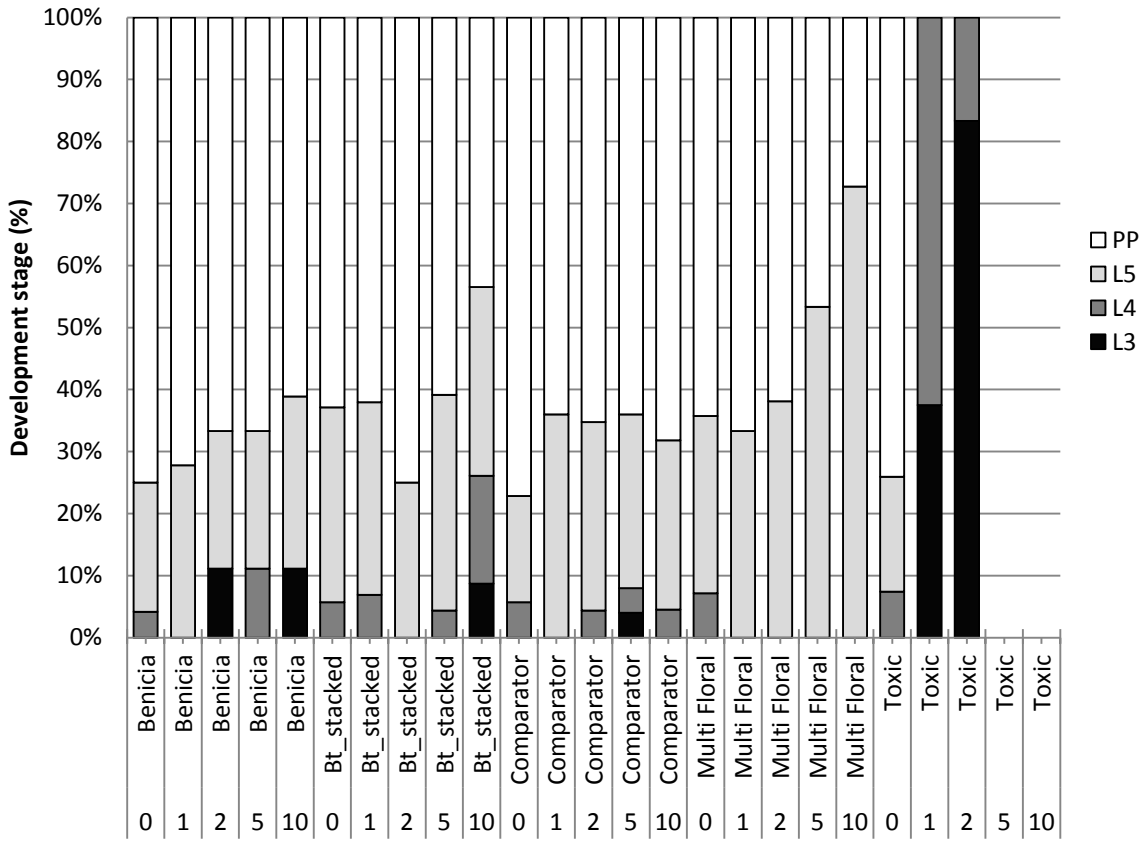

Table 5: Outputs for multiple comparisons of the interaction effect between pollen type and dosage (correction with Benjamini Hochberg) on the sub-lethal effect 'development'.

hypothesis	Estimate	Std. Error	z value	p value	p adj (BH)	sign
slopeMF==0	-0.21092	0.07458	-2.828	0.00468	0.01003	*
slopeTox==0	-57.970	0.8671	-6.685	2.30e-11	2.53e-07	***
slopeBen==0	-0.09156	0.07405	-1.236	0.216	0.30459	NS
slopeC==0	-0.04913	0.06216	-0.790	0.4293	0.49535	NS
slopeBt==0	-0.19560	0.05852	-3.343	0.00083	0.002075	**
slope Bt - slope MF==0	-0.01532	0.08784	-0.174	0.86158	0.86158	NS
slope Bt - slope C==0	0.14647	0.08152	1.797	0.07239	0.133	NS
slope Bt - slope Ben==0	0.10404	0.08924	1.166	0.24367	0.30459	NS
slope Bt - slope Tox==0	-560.140	0.86527	-6.474	9.57e-11	3.24e-10	***
slope C - slope Ben==0	-0.04243	0.09213	-0.461	0.6452	0.69129	NS
slope C - slope Tox==0	-574.787	0.86811	-6.621	3.57e-11	2.53e-07	***
slope C - slope MF==0	-0.16179	0.09236	-1.752	0.0798	0.133	NS
slope Ben - slope Tox==0	-570.544	0.86847	-6.570	5.05e-11	2.53e-07	***

slope Ben - slope MF==0	-0.11936	0.09843	-1.213	0.225	0.304588	NS
slope MF - slope Tox==0	-558.608	0.86525	-6.456	1.08e-10	3.24e-10	***

Discussion

Environmental risk assessments of transgenic plants need robust test approaches that allow for biological relevant interpretations. We investigated whether there is a dose dependent effect of transgenic Bt pollen on multiple endpoints of *in vitro* reared honey bee larvae. First of all we looked at survival, in addition we also measured several sub-lethal effects such as prepupal weight and the novel endpoints digestibility of pollen, and developmental delay. Transgenic crops expressing Bt proteins do so in all plant products, and therefore the entire plant becomes potentially insecticidal. As such we treated the pollen as were it an insecticide and to investigate its potential toxic effects on honey bees we administered it in naturally relevant increasing doses, as is common practice in ecotoxicological studies.

To assess whether the honey bee larvae are actually able to digest the administered pollen treatments, and as such become exposed to potentially toxic proteins therein, we evaluated the digestion rate of pollen kernels as found in the gut of the larvae. We found that, irrespective of the amount of pollen being fed, the Bt pollen were equally well digested as the two other maize pollen treatments. This means that larvae feeding on this pollen would be exposed to any potential toxic compounds within the pollen. Moreover, the transgene does not affect the digestibility of the pollen kernels, as the digestion rate of the transgenic pollen did not differ from the digestion rate of the near-isogenic line. Nor does the digestibility of the Bt maize differ from the other, conventional, maize variety (Benicia). Malone and Burgess (2009) argue that honey bees, and quite possibly other Hymenopterans as well, lack the appropriate gut receptor to be able to bind the Cry proteins that are expressed by Bt crops. Binding to this receptor is what preludes pore formation in the insects' gut, which is the mode of action of Bt crops for its target insects (Schnepf et al. 1998). Our finding is coherent with this argumentation, as the digestibility of the pollen does not decrease at higher amounts of pollen fed. In other words; gut functioning is not impaired by the Cry proteins in the Bt pollen.

In comparison to other studies our digestion values are rather low. Babendreier et al (2004) found that on average 74.5 % of pollen grains were fully digested, 23,3 % were partly digested and 2,2 % remained undigested. In comparison, we found 6.7 % were fully digested pollen grains 32.9 % were partly digested and the biggest percentage, 60.5 % remained undigested. Hendriksma et al (2011b) found an overall average weighted digestion rate of 62.7 % in adult honey bees. These values for digestibility are in line with what Schmidt and Buchmann (1985) described for the digestibility of a mixture of pollen of 77%. We however found an average weighted digestibility of 17.2 %. This is a remarkable difference that can however easily be explained. In the mentioned studies digestion of pollen grains was measured in larvae that were held under semi-field conditions, whereas we reared our larvae *in vitro*. This might result in differences in time between last feeding of pollen and the measurements. But more importantly our *in vitro* reared larvae were fed fresh pollen harvested by hand, and not processed by nurse

bees and fed via trophallaxis. Hence, they never came into contact with nurse bees, nor did their food go through the stage of being stored as beebread inside the hive. Due to missing these two steps *in vitro* reared larvae might lack proteolytic enzymes needed to break down the pollen kernels. Our pollen kernels could have a more hard-wearing exine-structure compared to pollen that has been stored as beebread, as this undergoes biochemical changes due to microbial activity (Roulston and Cane 2000) which probably also alters the digestibility. For another bee plant, *greatheadii* var. *davyana,* it has been shown that the nutritional content of its pollen kernels varies significantly between fresh collected pollen and bee stored pollen (Human and Nicolson 2006). Nonetheless, our larvae were able to partly digest the pollen that was added to their diet, and such they were exposed to their protein content via a rather natural exposure route. This indicates that gut functioning of bee larvae was not adversely affected by the three ingested Cry proteins. Probably honey bee larvae do not have the gut receptors necessary to activate the insecticidal mode of action, which is gut perforation (Malone 2009). This is highly relevant in terms of biosafety assessments, because the larval stage is theoretically the most Cry protein sensitive stage during bee development (Glare and O'Callaghan 2000, Schmidt et al. 2009, Romeis et al. 2011).

Interestingly we found poor survival at high doses of multi-floral pollen, our negative control that mimics a bee's natural, divers pollen diet most accurately. Perhaps our bee collected pollen mixture contained pollen toxic to bees/bee larvae (Kempf et al. 2010, de Assis Junior et al. 2011). Another possibility is that the pollen mixture contained (a cocktail of) pesticides (Pochi et al. , Rortais et al. 2005, Bernal et al. 2010, Genersch et al. 2010, Blacquiere et al. 2012, Pohorecka et al. 2012, Stoner and Eitzer 2012, Byrne et al. 2013), that had a negative influence on survival at higher doses. A third option is that the multi-floral, bee-collected pollen was infected with honey bee pathogens (Graystock et al. 2013a). In a colony setting nurse bees might not have fed these kinds of pollen mixtures in such high quantities (vanEngelsdorp et al. 2009). These possible explanations are of high relevance for the environmental safety of bee pollinators. In order to be able to include a multi-floral pollen treatment as a negative control, one could sterilise the pollen substrate by freezing and micro-waving it (Graystock et al. 2013b).

We showed that even at high doses Bt maize pollen does not have a negative effect on the survival of honey bee larvae. Our finding is in accordance with other studies that show no impact of transgenic Bt maize pollen (Hanley et al. 2003) or Bt cotton pollen (Liu et al. 2005) on larval/prepupal survival.

Mortality and body weight of adult nurse bees (Hendriksma et al. 2013) nor the microbiota in the gut (Geng et al. 2013, Hendriksma et al. 2013) are adversely affected by stacked Bt maize pollen.

A meta-analysis that encompassed 25 datasets also found no negative effects of transgenic Bt plant products on honey bees (Duan et al. 2008). These studies were carried out on colony level, and few studies exist that investigate Bt effects on honey bee larvae in a standardised laboratory setting. In an *in vitro* larvae rearing experiment by Hendriksma et al (2011b) all larvae from the Bt treatment survived the entire duration of the experiment (D11). Also in prepupal weight they did not find an effect of the transgenic pollen. They did, however, only test one amount of pollen (2mg administered on D6), where we tested a gradient between 0 mg and 10 mg of pollen, fed on

4 consecutive days, to be able to find dose dependent effects. The study by Hendriksma et al (2011b) only consisted out of a small sample size (n = 121) compared to our experiment with 1001 larvae that were compared in the survival analysis, of which 383 larvae were included the analysis of the sub-lethal effect weight, which suggests that our results are more robust. We did detect a negative effect of Bt maize pollen at high doses on prepupal weight. Bt did not have a significant negative effect when compared with the other two maize treatments, but prepupal weight was significantly lower at high doses for 'Bt'-larvae compared to 'multi-floral'-larvae. This suggests that in general maize pollen is a poor protein source for honey bee larvae. This is in accordance with the findings of Höcherl et al (2012) which showed reduced brood rearing in colonies that had maize pollen as sole protein source (but see Rose et al. 2007). Note however that larvae had poor survival at high doses of multi-floral pollen, which makes it more difficult to interpret the differences in prepupal weight between larvae fed with Bt pollen and with multifloral pollen. There are contradicting responses to increasing amounts of multi-floral pollen being fed; on the one hand larval survival goes down, whereas at the same time the prepupal weight increases at higher amounts of pollen being fed.

As we have shown that the larvae were able to digest the three maize pollen types equally well irrespective of the amount fed (dosage), the gut functioning is most likely not the mechanism explaining why Bt pollen fed larvae showed a dose dependent decrease in prepupal weight. Since the mode of action of the Cry proteins expressed by the Bt pollen is to disrupt the gut (Schnepf et al. 1998), we conclude that the found dose dependent effect on weight is most likely not a insecticidal Bt effect. Rather, we suggest that pleiotropic effects play a role. The transgenic constructs in the genome of the stacked Bt maize-plant might have altered certain phenotypic traits that consequently have an effect on the prepupal weight. Such pleiotropic effects have been shown for other study mechanisms investigating the potential effects of transgenic plant material (e.g. Escher et al. 2000, Wandeler et al. 2002, Jensen et al. 2010, Knecht and Nentwig 2010, Zurbrügg et al. 2010).

Additionally to the prepupal weight we also assessed the development of the larvae by scoring in what development stage they were at the end of the experiment, at D11. We found that in general larvae had delayed development, as in all groups a portion of the larvae had not yet reached the prepupal stage. The last three repetitions of our experiment were conducted late in the bee-season (September) and perhaps this could have influenced our data. Colonies start producing winter bees in August (Mattila et al. 2001), thus the larvae that we used might have already been winter bees that take longer to develop. We found a clear signal of delayed development in our positive control group, the toxic pollen. Those larvae were clearly underdeveloped (L3 or L4 larvae instead of prepupae) at the end of the experiment, and moreover, we found a dose dependent effect. In other words, with more toxic pollen significantly more larvae remained smaller. Even though we found a similar dose dependent effect for larvae that were fed Bt pollen and multifloral pollen, these effects were not biologically relevant, since they did not differ significantly with the other treatments.

In contrast to experiments in colonies (*in vivo*), which can be biased by many uncontrolled factors, the rearing of larvae in the laboratory *in vitro* is a highly effective ERA methodology. The use of controlled laboratory conditions, the high reproducibility and the defined amounts of ingested test doses by the larvae are standards which cannot be realised by simple *in vivo* experiments. Our results exemplify that different endpoints respond differently to test substances. It is therefore vital that multiple endpoints are quantified in an Environmental Risk Assessment. Our study shows the importance of testing different doses of potentially toxic plant products, and the inclusion of sufficient negative controls, in order to draw conclusions in a biological relevant context (also see Rauschen et al. 2009, Hendriksma et al. 2011b). A meta-analysis evaluating extrapolations from laboratory studies on Bt proteins showed that most lab studies are either consistent with, or more conservative than field-level studies (Duan et al. 2010), and the researchers stressed the importance of testing ecological relevant exposure routes like we did by directly feeding pollen. Since the digestion of the pollen we fed was relatively low compared to field situations, it is safe to assume our findings are on the conservative side as well.

Conclusion

According to the European Directive 2001/18/EC, ERA should consider the possible environmental impact resulting from direct and indirect interactions of GM plants with non-target organisms. The directive 2001/18/EC clearly points out that harmonised procedures and criteria for the case-by-case evaluation of the potential risks arising from the deliberate release of GMOs into the environment must be established. The importance of having harmonised risk assessment of PPPs for focal bee pollinators was recently underlined by a new Guidance Document (GD) of (EFSA) the European Food Safety Authority (EFSA, 2013). Our presented method and analyses provide a good basis for a standardised test protocol for assessing the risks of GM pollen or pollen containing systemic pesticides on *in vitro* reared honey bee larvae. First of all we propose testing a gradient of GM crop pollen doses, as is done in any well designed (eco) toxicological study. This enables to identify dose dependent effects. Secondly, we strongly recommend that an experimental design testing effects of transgenic pollen or systemic pesticides comprises a positive control, and several negative controls. In the case of GM pollen the near isogenic line of the GM plant should be included, in order to be able to attribute a potential effect to the transgene. At least one cultivar of the same plant should be included to relate a potential effect to any biological variance that naturally occurs within that species (also see Rauschen et al. 2009, Hendriksma et al. 2011b). Without that drawing biological relevant conclusions would be limited. And a multi-floral diet should be included as a control that approaches the natural diet, enabling a comparison with the natural situation. Finally, we stipulate that an evaluation of mortality alone does not suffice to evaluate potential risks of test substances, be it GM pollen or pollen containing systemic insecticides. As clearly shown by our study, the response of sub-lethal effects might vary from the more crude measure of mortality. Thus, sub-lethal response variables

should be included, weight being straight forward, easy to standardise, example. We suggest the test protocol to be validated in an international ring test within different European laboratories.

Acknowledgements

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Chapter 3: Protocols for standardised laboratory test methods for *in vitro* reared bumble bees (*Bombus terrestris*)

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Introduction

Recent Environmental Risk Assessment (ERA) concerning adverse effects on pollinators considers mostly *Apis mellifera*. The honey bee is an important model species since *Apis mellifera* is a global player and occurs all around the world, a large body of knowledge about her biology already exists and honey bees are an important generalist pollinator for myriad of wild and cultivated plants. However, it is important to realise they are not the sole pollinating insect. For example various bumble bees play an important role in crop pollination; 35 out of an estimated 150 crops thought to be insect pollinated (in Europe) benefit from bumble bee pollination (according to Corbet et al (1991) and Delaphane and Mayer (2000) in (Goulson 2003). Also many wild plants profit from bumble bees' ability to start foraging earlier in the day and under harsher weather conditions (Corbet et al. 1993). And in comparison to many other solitary wild bee species they are able to forage in a much larger range (Steffan-Dewenter and Tscharntke 1999), which is especially important in a patchy landscape where certain species grow in relative isolation. Moreover, bumble bee workers tend to forage faster than honey bees (reviewed in Goulson 2003). As such bumble bees provide a very important ecosystem service. And as an integral part of agro-ecosystems they are also exposed to agrochemicals intended for pest-insects, such as pesticides or Bt toxins expressed by transgenic plants.

This protocol describe a method to test the effects of chemical pesticides such as imidacloprid; as well as insecticidal plant products, such as pollen containing Bt proteins; and purified Bt proteins, on bumble bees. The *in vitro* method is based on the formation of micro-colonies in a lab setting, which first of all enables the control of numbers and age of individual eggs (or larvae) and workers, and secondly provides the opportunity to stratify the genetic background. Moreover, one can control environmental factors such as temperature, humidity and food resources.

Life history of Bombus terrestris

Bumble bee queens lay 4-16 eggs on a batch of bee pollen (pollen with nectar). She keeps them warm with her body at about 30°C. Later workers also help in keeping the brood warm. Eggs hatch after 4 days. It is said that in the beginning queen needs to visit 6000 flowers a day to have enough nectar to warm her eggs. Every time she leaves the nest, the eggs cool down, so it is important that the nest is located close to enough nectar producing plants.

The entire development cycle from egg to adult takes 4-5 weeks (figure 1), the first four days as egg, after that they spend half their time as larva (+-2weeks), half their time as pupa. Bumble bee larvae have a blind gut, which means they do not have to defecate in their larval phase. They defecate only once before pupation, and their faeces is used in the making of the cocoon. The queen usually lays another batch of eggs while the first batch is still in the larval stage.

In a series of studies by A. D. Brian (in Goulson 2003) it was found that of the eggs laid by the queen 71% hatch, of these larvae 75% go on to become pupae, and of the pupae 90% hatch out as adult bumble bees. So only about 47% of the eggs laid go on to produce adult bumble bees. Adult workers live for about 4 weeks. The first two broods consist completely out of worker bees, during the third brood however the queen switches to laying unfertilised eggs (also termed the switch point), form which the first reproductives, the males will hatch (Duchateau and Velthuis 1988). Later on workers start to oviposition, aggression between among workers and between workers and the queen rises, and is paired with oophagy (competition point). In this stage the queen lays fertilised eggs again from which the next generation of queens will hatch. *Bombus terrestris* can reach a colony size of approximately 400 individuals. After mating takes place only the fertilised queens overwinter, and in spring they will found new colonies.

Commercial mother colonies and how to keep them

Standard mother colonies can be obtained from Koppert B.V. from which the micro-colonies will be formed (the experimental unit). Worldwide Koppert has liaison offices through which bumble bee hives can be ordered. In case you want to do an experiment on workers it is advisable to order very small, i.e. young colonies. The first couple of batches of eggs the queen lays are always diploid; i.e. worker bees. After two generations of workers have been made, the queen switches to producing the next generation of reproductives.

Each colony comes in a plastic container within a cardboard box (figure 2) that contains a feeder with sugar solution (usually sufficient for the lifetime of the colony, when additional resources from flowering plants are available) and a pollen supply for a few days. The nest is covered with cotton wool for insulation. This obstructs an open view on the colony-development; therefor it is necessary that the entire colony will be moved to a new hive that will be kept in a climate chamber.

Laboratory hives (figure 3) consist of two wooden boxes (22 x 22 x 12 cm) mounted on a ground plate. One box functions as nesting area, the other as feeding area. A hole (⌀ 1.5 cm) in the middle connects the two areas. Either side of both boxes should have ventilation holes (⌀ 3 cm), covered with metal mesh. Both areas are covered with Plexiglas lids. Both lids have a hole (⌀ 6 cm) that, in the feeding area, functions as a dispenser holder, and in the nesting area offers easy access to the brood cells and worker bees.

When bumble bee hives have arrived over the mail, allow bees to calm down for about an hour prior to relocating them into the laboratory hives. Use organic cat litter as a substrate on the bottom of the new laboratory hive (1 – 2 cm). Work in a closed of room under red light. Open the box, and the plastic container, and carefully remove the cotton wool from the nest. Alternatively check whether your provider can send you hives without this insulation (or order directly from Koppert in the Netherlands, see appendix for contact details). Then start removing bees one by one, using tweezers and place them in the feeding area of the new nest box. Make sure the entrance to the brood area is closed (slide a piece of cardboard between the two sides of hive). Once you find the queen, keep her separate, e.g. in a drosophila glass. Once all workers are transferred, close the Plexiglas lid on the feeding side of the hive. Now you can carefully transfer the nest with its brood cells and honey pots to the nesting area. Release the queen on the nest. Close nesting side of with the Plexiglas lid and remove the cardboard separation so that workers can freely move between the two sides.

A feeder for sugar solution (either the sugar solution that comes with the packaged hives, or ApiInvert) is easily constructed from a 100 ml polyethylene flask; punch 6 to 8 holes the small end of the flask, dispense the flask upside down in the feeding area. Place a petri dish underneath, to avoid dripping. Bees can feed *ad libitum* from this feeder. Additionally one teaspoon of pollen needs to be given once a day, pollen can be directly fed into the nesting area.

Set-up and rearing methods of micro-colonies

Small mother colonies (Koppert, see previous paragraph) will be kept in a climate chamber (27°C and 65% RH; Yoon et al. 2011). Development of mother colonies will be recorded twice daily, once in the morning, once in the evening. This means that a map is made of the number of egg cups, brood cells and honey pots and adjusted accordingly so the age of egg cups or larval cells is precisely known. In each colony freshly hatched worker bees will be marked and their hatch day and time will be recorded. From the mother colonies micro-colonies are constructed. Usually eggs cups are constructed on top of a wax cell containing a pupa (Perenboom 2013). Each micro colony will be formed around one pupa with an egg cup. One pupa with egg cup will be removed from the mother colony on the 8^{th} or 9^{th} development day, so the larvae will be either second or early third instar. The number of eggs in the egg cup will be counted and redistributed over a number of artificial egg cups made by beeswax (depending on the number of treatments to be tested), and closed with a layer of bumble bee wax, then placed in the micro colony container (for this a simple plastic box, layered with some tissue, suffices). For every egg one young worker will be added to perform the brood care. No queen will be added, queens are kept in the mother colonies. In comparison to the method described by Babendreier and co-workers (2008) this design of micro colony formation enables us to also study effects on bumble larvae. It is likely that younger test animals, i.e. in the larval phase, respond more sensitive to potential toxins, thus is it highly relevant to take the effects on them into account within the scope of an Environmental Risk Assessment. Micro colonies will be provided with a sugar solution (ApiInvert) and a pollen

diet ad libitum, which will be fed to the larvae by the workers. Depending on the test substance, transgenic pollen, or an insecticidal toxin, such as a pesticide or purified Bt toxin, the mode of application will either be via the sugar solution, or via the pollen.

Treatment application

Transgenic pollen

In case the toxicity of transgenic pollen is investigated, pollen can be directly provided to the micro-colonies. The advantage of feeding pollen directly, as compared to feeding the purified proteins, is that the exposure route is more natural. In a natural setting, bees would also be exposed to plant products, such as nectar and pollen. Since bumble bees have an extremely high polymorphism, also food requirements may vary a lot. In a study by Peerenboom and co-workers (2003) bumble bee larvae were reared with a 50% sugar solution (glucose : fructose = 1:4 in water) which was mixed with fresh pollen (35% v/v; Pereboom 2000). Individual larvae received between 311 and 1275 µl of food during the entire larval development (Pereboom et al. 2003). Based on these numbers we calculated that the required amount of pollen should range between 68 and 223 mg (based on a specific weight of mixed floral bee pollen of 0.22 g/ml, also see table 1).

When testing transgenic pollen the experimental design should include two negative controls; one being multi-floral pollen (can be obtained from honey bee hives with use of a pollen trap, or ordered from beekeeper); the other one being the near-isogenic line of the transgenic plant (identical cultivar, without the transgene). Ideally one would include a positive control of known toxic pollen, which is not always feasible (see appendix II for a list of potentially toxic pollen types). A positive control may also consist out of a toxic compound, such as dimethoathe. The dose at which it should be administered should sufficiently high that 50 %, or more, of the test animals die. The LD50 for *Bombus terrestris* lies at 0.86 (0.35-2.12) µg a.i./g insect (Roessink 2013). The exact LD50 (48 hours) should be quantified during a ring-test (also see last paragraph).

Transgenic proteins or pesticides

When testing purified *cry* proteins or pesticides application should be via the provided sugar solution. Cry proteins are dissolved in a buffer solution. Note that one needs to control for possible effects of the buffer solution by adding another control that contains the solution without the transgenic proteins. Also some pesticides may not be soluble in water. An organic solvent such as acetone may be used in such a case. Again one needs to control for the possible effect the solvent might have (also see Wilkins et al. 2012).

Experimental unit

Per micro-colony 10 to 20 larva can be tested, depending on the desired resolution of the measured effect-size. Since individuals kept in one test-cage are not independent from each other, the experimental unit is the micro-colony. Per treatment group at least 10, preferably 20 micro-colonies should be formed. This can be a logistical challenge (getting enough same age larvae and workers at the same time), therefore we propose repeating the experimental set-up until the desired sample-size is achieved.

Data collection

The following data endpoints should be recorded:

- mortality rate (number of dead bees during development from egg/larva to adult);
- percentage of pupation (percentage of single wax cells at day 16 of the development cycle, also see figure 1)
- duration of development cycle (from 1st instar larva until eclosion)
- percentage of eclosion

On an individual level following endpoints can be measured:

- post-emergence longevity
- adult bee size (quantified by measuring proxies such as intertegular span and wing length)

Note that the experimental unit is still the micro-colony, so this needs to be taken into account in the statistical analysis. Values of individual bees should be grouped into one average per micro-colony.

Statistical analyses

Group differences are tested using ANOVA's. If assumptions of parametric tests are not met, a non-parametric analysis is performed (e.g. U-test or Kruskal-Wallis).

Test validation

The test is considered valid when in the negative control the pupation rate is 70 % (would 75% under natural conditions) eclosion rate (percentage of hatched adults in relation to the total number of pupae) is 85% (would be 90% under natural conditions). In the positive control, the mortality rate during larval development (from 1st instar larva until eclosion) should be higher than or equal to 50%.

Ring-test suggestion

For a sufficiently robust and reproducible risk assessment it is preferable to use internationally agreed guidelines wherever available. For this reason a ring-test of this protocol should be carried out. One important output of such a ring test should be what dose is needed of a specific toxin, i.e. dimethoathe, to obtain at least 50 % mortality, for the positive control. In toxicological studies that use honey bees as test animals, often dimethoathe is used as positive control (Aupinel et al. 2009).

Figures and Tables

Table 1. Pollen requirements based on the amounts studied in Pereboom *et al* (2000)

	pollen requirements (mg)	
#larvae in experimental unit	min	max
10	684	2805
11	753	3086
12	821	3366
13	889	3647
14	958	3927
15	1026	4208
16	1095	4488
17	1163	4769
18	1232	5049
19	1300	5330
20	1368	5610

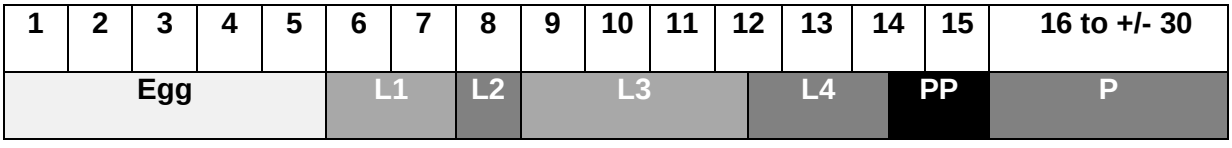

Figure 1. Development stages of *Bombus terrestris* from egg to pupa (figure adjusted from Cnaani et al. 1997). Duration of development cycle is variable and very dependent on temperature. Adult's eclose on approximately the 30th day adults eclose from wax cells.

Figure 2. Koppert B.V. Natupol beehive

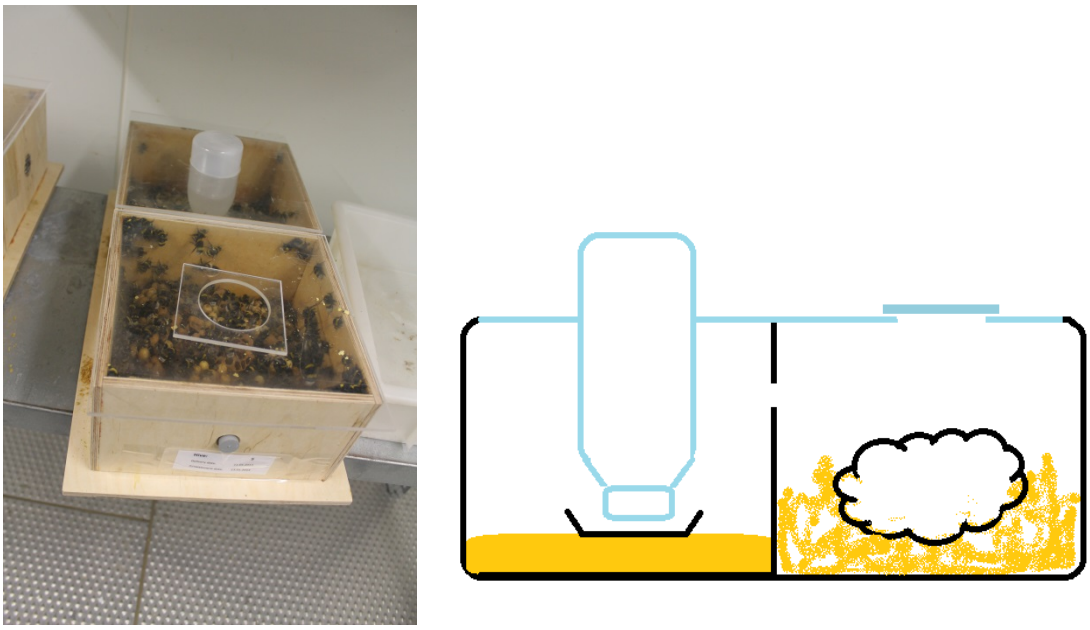

Figure 3. Laboratory hive consisting of two compartments; one for feeding (behind, or left in the abstraction); one where the nest is located. The top is covered with a Plexiglas lid

Figure 4. Example of a box for micro-colonies.

APENDICES

Appendix I: contact details Koppert B.V.

Koppert B.V.

Veilingweg 14

Postbus 155

2650 AD Berkel en Rodenrijs

The Netherlands

tel. +31 10 514 04 44

fax. + 31 10 511 52 03

email: info(at)koppert.nl

Chamber of commerce # 27216926
VAT number NL003657061B01

Appendix II: potentially toxic pollen for Bumble bees (these pollen are known to have toxic properties against honey bees)

- *Digitalis spp.*
- *Ranunculus spp.*
- *Zigadenus*
- *Heliconia*
- *Spathodea campanulata*
- *Ochroma lagopus*
- *Aesculus californica*
- *Zigadenus paniculatus*
- *Zigadenus vevenosus*
- *Cucurma*
- *Alpinia*
- *Hedychium*
- *Dichorisandra*
- *Zingiber*
- *Globba*
- *Etlingera*
- *Elettaria*
- *and Costus*
- *Tilia tomentosa Petiolaris*
- *Kalmia latifolia*
- *Aconitum*
- *Aesculus*
- *Andromeda*
- *Corynocarpus*
- *Hyoscyamus*
- *Polygonum*
- *Rhododendron*
- *Scolypoda*

This protocol was developed in the framework of the AMIGA project. We thank J. J. Pereboom for discussions and support.

Chapter 4:

A publication is under preparation based on the work carried out:

Sgolastra F., Tosi S., Medrzycki P., Porrini C., Burgio G. (2014) An in vitro method for testing toxicity on solitary bee larvae: the case study of spirotetramat on *Osmia cornuta*. Submitted to Apidologie

Protocol for testing toxicity on solitary bee larvae (*Osmia* spp.)

Sgolastra F., Tosi S., Medrzycki P., Porrini C., Burgio G

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Introduction

This protocol is proposed in order to test the effects (single or multiple) of toxic compounds (i.e. pesticides, Bt toxin protein in purified form) *in vitro* by adding them to the mass pollen provision of the solitary bees *Osmia cornuta* (Latreille). The following method is mainly based on the protocol developed by Konrad *et al.* (2008), for *O. bicornis* L, and adapted to *O. cornuta* according to Sgolastra *et al.* (in prep.). Similarly to honey bee larvae *in vitro* test, this protocol is developed in order to test the toxicity in larvae of solitary bees conducting the experiment in a reproducible and standardised way. In fact, this method allows to define exactly the quantity of testing compound up taken by a single larva and to standardise the rearing conditions of temperature during development and wintering, which is not feasible in the *in vivo* method.

The method aims to study the lethal and sublethal effects following exposure of larvae to a toxicant (particularly pesticide active ingredient or Bt toxin protein in purified form) at the environmental residue concentration (ERC). Moreover, it can be used to calculate a dose response curve in order to determine the LC50 or LD50 of a testing compound. The data should be used in an appropriate Environmental Risk Assessment scheme for solitary bees.

Life history of Osmia cornuta

O. cornuta (Latreille) is a Palearctic mason bee found in central and southern Europe, Turkey and parts of North Africa and the Middle East. The adult insects (first males, then females) emerge from the cocoons and fly in early spring. After mating, females start nesting in pre-established cavities, in which they build series of cells delimited with mud partitions. Each cell is provisioned with a mass of pollen and nectar, on top of which an egg is deposited. In each nest, the deeper

cells are bigger and are occupied by female offspring (diploid eggs) whereas the proximal cells are smaller and are occupied by male offspring (haploid eggs). The nesting females are active for about 3 weeks. By mid-summer, fifth-instar (larva) bees complete consumption of the pollen-nectar provision, defecate, and spin a cocoon with silk-like strands. In this stage (prepupa) bees enter in a summer diapause for ~1 month. In late summer, the insects complete their development and the adults eclose within the cocoon. They remain inside their cocoons in a dormant stage (winter diapause) throughout the winter period and they emerge in the spring as temperatures increase (fig. 1). *Osmia cornuta*, as well as the sister species *O. bicornis*, can be considered a suitable test species in the regulatory risk assessment because it shows several behaviour and life cycle traits representative of many species of solitary bees nesting above the ground (Bosch *et al.*, 2008).

Bee population and rearing methods

Bees are obtained from a population of *O. cornuta* nested in a field of oilseed rape (*Brassica* spp.) or other attractive crops (e.g. *Phacelia tanacetifolia*) with reduced chemical applications (organic) or at least with monitored cropping system (know plant protection actions). The target crop should provide enough pollen and nectar resource for *O. cornuta*. Orchards should be avoided because their flowering period is usually too short to cover the whole nesting period. In fact, a lower number of cells with a female-based sex ratio will be produced in case of an early interruption of the nesting period. Mixed fields with several successive flowering crops should be avoided as well, because the differences in pollen quality among provisions can affect the results. A minimum of 2-hectare field is required in order to obtain an adequate quantity of progeny. In spring, during the flowering of the test crop, a minimum 350 females and 700 males per hectare (parental population) are released in a nesting shelter in the edge or in the middle of the field. Parental population should be obtained by a local population of *O. cornuta* reared under standardised conditions during wintering in order to synchronise the emergence period with the flowering crop. The nesting shelter consists of wooden blocks with 144 drilled holes per block (fig. 2). The number of blocks should be enough to ensure two nests per nesting female. Each hole is 15 cm long and accommodates a 8 mm inside-diameter paper straw. Two weeks after the beginning of the nesting period the nests are checked and newly-plugged paper straws (completed nests) are pulled out of the wooden block and brought to the laboratory. Nests are than dissected and provisions with eggs or first instar larvae (~1-day-old) are individually placed in 48-well culture plates (fig. 3). Eggs and provisions are randomly assigned to each treatment. Eggs are sexed based on provision size and cell position within the nest (females are produced deeper in the nest and are assigned larger provision). Sex is confirmed after emergence of the bees from the cocoons (fig. 4). Eggs are dated assuming a cell production rate of 1 cell/day per nesting female. The culture plates with the larvae are maintained in an incubator at 25-26 °C and 55-65% RH (no light). Larval development is observed daily until cocoon spinning. In late September or early October, bees are transferred at 12 °C for one week and then at 2-4 °C for 160-180 days in order

to simulate the wintering. We provided a range of 20 days but the duration of wintering periods can be prolonged or shortened by a month without serious consequences for bee survival and post-emergence vigour (table 1). The following spring, the bees are individually caged in transparent polystyrene containers (diameter 5.5 mm, height 3.5 mm) covered with a plastic lid and incubated at 24±1 °C. Bees are checked daily to assess the date of emergence from the cocoon and the longevity.

Overall, the optimal development and wintering conditions should be adopted in the test in accordance with the species and the geographic origin of the test population. Here we presented the optimal rearing conditions for a *O. cornuta* population from southern Europe. In general, *O. bicornis* (=*O. rufa*) and *O. cornuta* populations from northern Europe, which nest later in the season, require lower temperatures and longer over-wintering period (Sgolastra *et al.*, 2012). In table 1 a summary of the test conditions used in other studies for *O. bicornis* and *O. cornuta*.

Mode of provision contamination

The tested toxicant is distributed within the mass provision as evenly as possible without touching and moving the attached egg. The test product will be dissolved in water reaching the desired concentration and 10 µL for females or 5 µL for males of this test solution is provided into either a longitudinal fissure or a hole previously made in the provision mass (fig. 3). In case the tested toxicant needs to be distributed in a large amount of solution it is proposed to apply 50 µL and 25 µL of the test solution, respectively for females and males, in the bottom of the wells in contact with the provision. Both modes of provision contamination can be used in case of multiple exposures.

The concentration of toxicant used in this study should be determined according to the environmental residue concentration (ERC). In case of a dose-response test aiming to assess the LD50 or LC50, 5 doses or concentrations should be tested ranging from 10 to 100% obtained mortality with no more than twofold dilutions between doses. A negative control with larvae fed provisions containing only water should be included in each test, and a positive control with a standard toxic compound for larvae (e.g. IGR, dimethoate) should be added (see ring-test suggestions).

Test solutions

The tested toxicant is normally dissolved in osmosed water, however, for poorly soluble chemicals, a solvent may be used (i.e. acetone) to prepare the stock solution. In such case, a solvent-control must be added to the diet instead of the regular water-control. The amount of organic solvent, if used, should be equal in all treatments and kept as low as possible anyway not exceeding 1% of the provision’s weight.

Experimental unit

The experimental unit is the individual bee. Since different sensitivity can be predicted between sexes, both male and female bees should be used. A minimum of 20 eggs/larvae per sex and treatment is required. Because each nest is usually provisioned by a single female, to account for genetic similarities, egg or larva for each sex should be distributed among treatments, so that no treatment received more than one individual from the same nest.

Data collection

The following data endpoints should be recorded:

- mortality rate (number of dead bees during development from egg/larva to adult);
- larval development period (number of days from egg to the beginning of the cocoon spinning);
- cocooning duration (number of days from the beginning of the cocoon spinning and the cocoon completion);
- emergence time after wintering period (days required to emerge when incubated at 24 °C);
- post-emergence longevity in spring (days from the emergence until death at 24 °C). The longevity of the bees after emergence without feeding is used to estimate the vigour of the bees (as a measure of remaining energy reserves).
- weight loss during wintering. Bees are weighed within their cocoons (without faecal particles) before and after wintering. Cocoon weight is measured at the end of the study and subtracted from the previous weight measurements.

Statistical analyses

t-test or ANOVA are used to test for differences in the above mentioned variables between treatments, separately for males and females. Bee mortality until spring is analysed, separately in males and females, using a Logit model analysis. Percent weight loss should be arcsine-transformed before the analysis. If assumptions of parametric tests are violated, a non-parametric analysis is performed (e.g. U-test or Kruskal-Wallis). Post-emergence longevity can be analysed by Cox proportional hazard models (log-likelihood test) or other survival analysis.

Test validation

The test is considered valid when in the negative control the development mortality (percentage of bees failing to reach the adult stage) and the wintering mortality (percentage of bees dead

before emergence) is lower than 20%. In the positive control, the mortality rate during larval development should be higher than or equal to 50%.

Ring-test suggestion

In the regulatory risk assessment it is preferable to use internationally agreed and adopted guidelines wherever available, in order to ensure a sufficiently robust risk assessment. In this framework, the study should be sufficiently reliable, repeatable and reproducible. For this reason a ring-test of this protocol should be carried out. In particular, it is necessary to determine the positive control test compound and its dose that causes a mortality rate higher or equal than 50%. At the moment the compound and the concentration used for the *in vitro* larva chronic toxicity test with *Apis mellifera* (dimethoate, 20,000 µg/Kg diet) seem a good starting point (Aupinel *et al.* 2007).

Figures and Tables

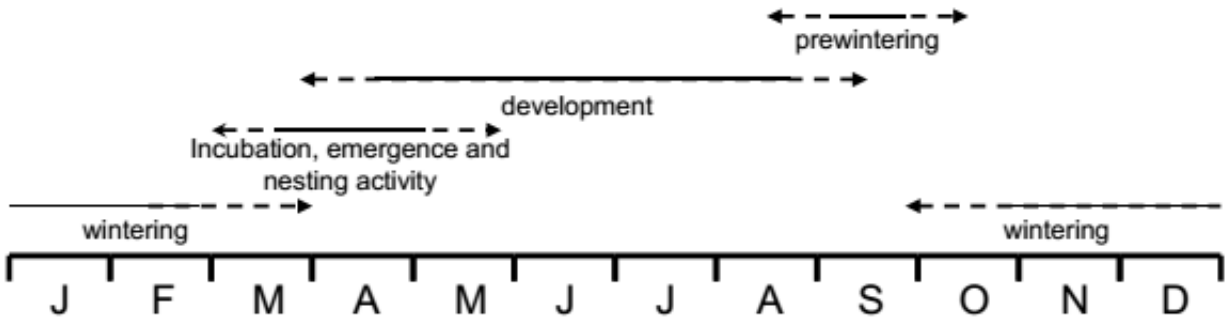

Figure 1. Life cycle phenology of *Osmia* spp.

Figure 2. Wooden blocks with 144 drilled holes per block. Paper straws are visible inside the holes.

Figure 3. Larvae and mass provisions individually placed in 48-well culture plates.

Figure 4. *Osmia cornuta* (left) and *O. bicornis* (right) with their cocoons. In both species females (left) are larger than males (right).

Table 1. Development, pre-wintering, wintering and incubation conditions in several *in vitro* rearing studies with *Osmia cornuta* and *O. bicornis*. [* indicates the optimal condition in the studies where more than one treatment was used]

	O. cornuta	*O. bicornis*
Development conditions	• 23.5±1 °C, 55-65% RH, no light (Sgolastra *et al.* in prep); • 25* °C, no light (Sgolastra *et al.* 2012); • 20:30 °C [12:12 h thermoperiod], 50-70% RH, no light (Bosch and Vicens, 2002)	• 20±1 °C, 75±5% RH, no light (Konrad *et al.*, 2008); • 10-25* °C [fluctuating regimes: temperature follows a sine curve and reaches the eponymous minimum and maximum values once in 24 h] (Radmacher and Strohm, 2011)
Pre-Wintering duration and conditions (acclimatization before wintering) and wintering start date	• On 24 September, 7 days at 12±1 °C, no light (Sgolastra *et al.*, in prep.); • 15 September* (~15 days from adulthood) as wintering initiation date (Bosch and Kemp, 2004); • On 31 October as wintering initiation date (Bosch and Vicens, 2002)	• After 120 days from the toxin application, 15 days at 14±1 °C, 85±5% RH, no light (Konrad *et al.,* 2008); • On 2 October as wintering initiation date (Radmacher and Strohm, 2011);
Wintering duration and conditions	• 180 days at 2±1 °C, 65±10% RH, no light (Sgolastra *et al.*, in prep.); • From 94 to 184* days at 3 °C, no light (Bosch and Kemp, 2004); • 138 days at 3 °C (Bosch and Vicens, 2002)	• 150 days at 3±1 °C, 65±10% RH, no light (Konrad *et al.*, 2008); • 165 days at 4 °C (Radmacher and Strohm, 2011);
Incubation conditions	• 24±1 °C, no light (Sgolastra *et al.,* in prep.); • 20 °C, no light (Bosch and Kemp, 2004)	• 25±5 °C, 16L:8D (Konrad *et al.*, 2008).

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