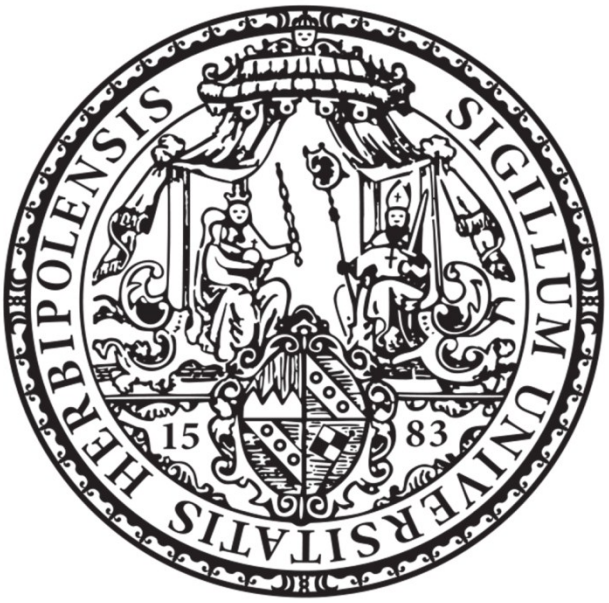
Non-target effects of a multiple insect resistant Bt-maize on the honey bee (*Apis mellifera* L.)



Dissertation in partial fulfillment of the
requirements for a Doctorate in Natural Sciences
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Verheert von Krieg und Pest, auf den Ruinen

Wächst Efeu, und im Efeu summen Bienen.

Hermann Hesse

L. Mossink, *lichtdrager* ©

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation mit dem Titel

Non-target effects of a multiple insect resistant Bt-maize on the honey bee (Apis mellifera L.)

selbständig, am Lehrstuhl für Tierökologie und Tropenbiologie (Zoologie III) der Julius-Maximilians-Universität Würzburg, unter der Anleitung und Betreuung durch Herrn Dr. Stephan Härtel und Herrn Prof. Dr. Ingolf Steffan-Dewenter angefertigt habe und dabei keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet habe. Ich erkläre außerdem, dass die vorliegende Dissertation weder in gleicher, noch in ähnlicher Form bereits in einem Prüfungsverfahren vorgelegen hat. Des Weiteren habe ich außer den mit dem Zulassungsantrag urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, 28.11.2011, Harmen P. Hendriksma

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The chapters within this thesis are based on the following manuscripts

Hendriksma HP, Härtel S (2010) A simple trap to measure worker bee mortality in small test colonies. Journal of Apicultural Research 49, 215-217.

Hendriksma HP, Härtel S, Steffan-Dewenter I (2011) Honey bee risk assessment: New approaches for *in vitro* larvae rearing and data analysis. Methods in Ecology and Evolution 2, 509–517.

Hendriksma HP, Härtel S, Steffan-Dewenter I (2011) Testing pollen of single and stacked insect-resistant Bt-maize on *in vitro* reared honey bee larvae. PLoS ONE 6, e28174, doi:10.1371/journal.pone.0028174.

Hendriksma HP, Härtel S, Babendreier D, Ohe von der W, Steffan-Dewenter I (Accepted with minor revisions) Effects of multiple Bt-proteins and GNA-Lectin on *in vitro* reared honey bee larvae. Apidologie, doi:10.1007/s13592-012-0123-3

Hendriksma HP, Härtel S, Von der Ohe W, Steffan-Dewenter I (in prep.) Exposure of flowering multiple insect resistant Bt-maize to *A. mellifera* colonies does not cause mortality over two successive generations of worker bees.

Küting M, Hendriksma HP, Härtel S, Dohrmann AB, Steffan-Dewenter I, Tebbe CC (subm.) Effects of genetically modified Bt-maize on pollen digestion and community structure of gut microbiota in honey bees.

Summary

I. Honey bee pollination is an ecologically and economically important ecosystem service. New methodological developments are needed to research the underlying factors of globally observed bee losses. The honey bee (*Apis mellifera*) is a key non-target arthropod species for environmental risk assessment of genetically modified (GM) crops. For GM-crop risk assessments, mainly methods for monitoring adult honey bees under laboratory conditions are documented. However, protocols with robust methods for standardized colonies or *in vitro* reared honey bee larvae are currently lacking.

II. We developed a hive entrance trap for small honey bee test hives (e.g., mating hives and nuclei) to study bee mortality due to toxicity. Efficiency of the trap, indicated by recapture of dead marked bees, was 93% ± 2.7% (mean ± s.e., n=9 colonies). During a four week semi-field experiment on biosafety monitoring of Bt-maize, 72 plastic traps were shown to work successfully. As the trap showed good performance and practicality over a long period of time, it has the potential to become a standard for small test colonies.

III. In a semi-field experiment, honey bee colony exposure to a multiple insect resistant Bt-maize (Mon89034xMon88017) and its near-isogenic line (DKc5143) was studied. A 'full life cycle test' over two successive generations of worker bees showed no significantly different honey bee life expectancy between the tested maize varieties. A higher pollen amount was collected from the near-isogenic maize variety, which also resulted in a higher number of new worker bees being born. However, the weight of newly hatched bees, and the amount of pollen units invested per new bee was found to be identical compared to Bt-maize. This indicated that the quality of the pollen was the same, and that a Bt-toxicity effect did not occur.

IV. A honey bee brood test under laboratory conditions was established, using a non-grafting method to enable collection of test-larvae without direct manipulation. The novel *in vitro* rearing approach showed a mere 3% background mortality upon the prepupae stage. A treatment with dimethoate significantly affected the larval survival and the weight of prepupae, with an acute 48h-LD_{50} toxicity value of 1.67µg/larva. The rearing method and statistical approaches can help to improve the quality of environmental risk assessment studies on honey bees.

V. Honey bee larvae are directly exposed to transgenic products by the consumption of GM pollen. The biosafety of pollen from two Bt-maize cultivars was tested on honey bee larvae under standardized *in vitro* conditions. One maize variety expressed a single transgenic Bt-protein, and the other three different Bt-proteins for pest insect control. The pollen was mixed into the diet of third instar honey bee larvae. In comparison to the pollen of three control maize varieties, neither single nor stacked Bt-maize pollen affected the survival of the larvae or the weight of the prepupae. In contrast, *Heliconia rostrata* pollen did cause significant toxic effects. On basis of our findings we argue that feeding GM pollen to *in vitro* reared honey bee larvae is well suited to become a standard bioassay in regulatory risk assessment schemes of GM crops.

VI. We analyzed combined toxicity effects on *in vitro* reared honey bee larvae by testing three purified Bt-proteins and the herbicide resistance protein CP4-EPSPS. These four transgenic proteins are simultaneously expressed in the pollen of Bt-maize Mon89034xMon88017. A worst-case scenario was considered, by testing dosages of up to 186-times the expected field exposure. In a bioassay exposing purified Bt proteins to second instar honey bee larvae, neither single nor a mix of different Bt-proteins were found to cause toxic dose-response effects on larval survival or prepupal weight. In contrast, the *Galanthus nivalis* agglutinin (GNA-Lectin), a candidate protein for use in commercial crops, was toxic at a 144h-LD_{50} value of 16.3µg/larva.

VII. Honey bee colonies were kept under semi-field conditions to study a potential insecticidal effect on nurse bees by a Bt-maize variety, in comparison to the near-isogenic variety and other maize controls. In particular, the digestion of maize pollen, the degradation of Bt proteins in the intestine, and the composition of the intestinal microflora were analysed. With T-RFLP (Terminal Restriction Fragment Length Polymorphism) the abundance and diversity of bacterial communities was genetically characterized. Differences in the bacterial communities based on the maize varieties were found, but the differences between the Bt variety and other varieties were not greater than the variability between the conventional maize varieties. The quantitative analysis showed neither differences in the density of the micro-organisms in the bee intestines, nor different pollen digestion rates among the maize varieties. Hence, an indication of adverse effects caused by Bt-maize was not provided. Considering that Bt-traits typically target insects from within their guts, this test approach is a sensitive and commendable method to monitor for subtle effects caused by Bt-crops.

VIII. In conclusion, multiple methodological developments were achieved; a mortality trap (II), a 'full life cycle test' (III), a novel *in vitro* rearing methodology (IV), a standardized *in vitro* test for Bt-pollen (V), a mixed toxicity test for purified transgenic proteins (VI), and a bacterial flora test with pollen digestion rate monitoring (VII). Overall, the studies did not indicate a detrimental effect caused by Bt-maize pollen, or by purified Bt-proteins at worst case exposure levels. Considering the risk for honey bees and larvae, we conclude that the tested Bt-maize Mon89034xMon88017 is not likely to cause harm to honey bee colonies. The study methods presented are highly recommended for future environmental risk assessment studies testing GM-crop biosafety on honey bees.

Zusammenfassung

I. Neue methodische Entwicklungen zur Untersuchung der Ursachen des weltweit beobachteten Bienensterbens sind nötig, um die lebenswichtige Ökosystemdienstleistung der Bestäubung zu gewährleisten. Die ökologisch und wirtschaftlich bedeutsame Honigbiene (*Apis mellifera*) ist ein wichtiger Nichtziel-Organismus im Zulassungsverfahren für gentechnisch veränderte Pflanzen. Bisher sind vor allem Methoden zur Testung erwachsener Bienen unter Laborbedingungen verwendet worden, aber für eine Risikobewertung mit Hilfe von standardisierten Bienenkolonien oder *in vitro* gezüchteten Honigbienenlarven sind keine robusten Methoden oder standardisierte Protokolle vorhanden.

II. Wir haben eine aus Kunststoff bestehende Fluglochfalle entwickelt, um die Mortalität innerhalb kleiner Honigbienen-Testkolonien zu studieren. Die Effizienz der Falle, gemessen durch den Rückfang von toten Bienen, betrug 93% ± 2,7% (Mittelwert ± SE, n = 9 Kolonien). Während eines vierwöchigen Halbfreilandversuches im Rahmen einer biologischen Sicherheitsüberwachung von Bt-Mais mit multiplen Insektenresistenzen wurde die Funktionalität der Falle in der Praxis bestätigt. Über einen langen Zeitraum zeigten die eingesetzten 72 Fallen eine hohe Praktikabilität und Robustheit. Damit hat die neu entwickelte Bienenmortalitätsfalle das Potential ein Standard für Versuche mit kleinen Versuchskolonien zu werden.

III. Innerhalb eines Halbfreilandversuches wurden die Entwicklung von Honigbienenkolonien in Feldern mit Bt-Mais mit multiplen Maiszünsler- und Maiswurzelbohrer-Resistenzen sowie der nah-isogenen Linie (DKc5143) verfolgt worden. Ein "Full-Life-Cycle" Test über aufeinanderfolgende Generationen von Arbeiterinnen zeigte keine Effekte auf die Lebenserwartung zwischen den getesteten Maisvarianten. Durch das Sammeln einer höheren Pollenmenge der nah-isogenen Linie wurde letztlich eine höhere Anzahl an Arbeiterinnen erzeugt. Jedoch war das Gewicht der geschlüpften Bienen und die investierte Pollenmenge pro Biene identisch gegenüber der Bt-Mais Gruppe. Dies weist darauf hin, dass die Qualität der Pollen vergleichbar war und keine Indikation von Toxizität vorlag.

IV. Ein standardisierter Honigbienen-Brut-Test wurde mit einer neuen Methode Honigbienenlarven zu sammeln (ohne die direkte Manipulation des Umlarvverfahrens) etabliert. Dieser Ansatz einer *in vitro* Aufzucht zeigte nur 3% Mortalität bis zum Alter der Präpuppe. Eine Behandlung mit dem Insektizid Dimethoat zeigte ein signifikant reduzierte Überlebensrate (48h-LD_{50}; 1,67µg/Larve) und einen Gewichtverlust im Präpuppenstadium. Unser Larvenzuchtprotokoll und die verwendeten statistischen Ansätze können dazu beitragen die Qualität der Biosicherheitsforschung für Honigbienen zu verbessern.

V. Da der Wirkmechanismus der Bt-Proteine gegen Insektenlarven gerichtet ist, setzt der *in-vitro*-Larventest an einer potenziell empfindlichen Phase der Honigbienenentwicklung an und kann deshalb besonders aufschlussreiche Ergebnisse liefern. Pollen der verschiedenen Maissorten

wurden direkt in das Larvenfutter (L3-Stadium) gemischt. Im Vergleich zu drei Kontroll-Mais-Sorten zeigte weder der getestete Bt-Maispollen mit einer Insektenresistenz, noch der Bt-Maispollen mit multiplen Resistenzen einen negativen Einfluss auf das Überleben von Larven oder dem Gewicht von Präpuppen. Im Gegensatz dazu hatte Pollen von *Heliconia rostrata* signifikante toxische Auswirkungen. Das direkte Füttern von Honigbienenlarven mit transgenen Pollen empfehlen wir als Standardmethode für die Zulassung von transgenen Kulturpflanzen.

VI. Unter kontrollierten Bedingungen wurden die kombinierten Effekte von drei Bt-Reinproteinen auf *in vitro* aufgezogene Bienenlarven untersucht. Die Larvenaufzucht wurde in einem "Worst-Case" Szenario getestet, welche bis zu 186-fach über der im Feld zu beobachtenden Dosis lag. Die einzelnen Proteine sowie ein Protein-Mix wurden dem Larvenfutter in fünf Konzentrationen (L2-Stadium) beigegeben. Eine Dosis-Wirkungsbeziehung wurde weder bei Einzel- noch bei einem Mix aus verschiedenen Proteinen gefunden. Es bestand kein signifikanter Zusammenhang zwischen den Proteinen, der Testdosis, der Sterblichkeit und/oder dem Präpuppengewicht. Im Gegensatz dazu war GNA-Lektin, ein neues Insektizidprotein und Kandidat für den kommerziellen Einsatz in Kulturpflanzen, mit einem 144h-LD_{50} Wert von 16.3µg/Larve giftig für Honigbienen.

VII. Unter Halbfreilandbedingungen wurde die Verdauungsfähigkeit von Maispollen, der Abbau von Bt-Proteinen im Bienendarm sowie der Einfluss von Bt-Mais auf die Zusammensetzung der Darmmikroflora von Ammenbienen analysiert. Mittels T-RFLP (Terminal restriction fragment lengths polymorphism) wurden die Abundanz und Diversität der bakteriellen Gemeinschaften genetisch bestimmt. Ein Sorteneffekt konnte festgestellt werden, aber die Unterschiede zwischen den Bt-Sorten und anderen Sorten waren nicht größer als die Variabilität zwischen den konventionellen Maissorten. Auch zeigte eine quantitative Analyse weder Unterschiede in der Besiedlungsdichte der Mikroorganismen noch in den verschiedene Pollenverdauungsraten zwischen den Maissorten. Damit gibt es keinen Hinweis darauf, dass Bt-Maispollen im Bienendarm schädliche Effekte verursachten. Dadurch, dass Bt-Proteine in der Regel gezielt den Darmtrakt von Zielinsekten schädigen, stellt unser Test eine empfindliche Methode zur Erfassung möglicher sublethaler Bt-Effekte dar.

VIII. In dieser Arbeit wurde eine Vielzahl an neuen methodischen Ansätzen für die Biosicherheitsforschung entwickelt: eine Mortalitäts-Falle (II), ein "Full-Life-Cycle" Test (III), eine robuste *in vitro* Aufzucht-Methodik (IV), ein standardisierter *in vitro* Test für Bt-Pollen (V), eine gemischte Toxizitätsprüfung für transgene Reinproteine (VI) und eine Überprüfung der Darmmikroflora sowie der Pollenverdauungrate (VII). Die Ergebnisse dieser Studien zeigten keine nachteiligen Wirkungen von Bt-Maispollen oder Bt-Reinproteinen im "Worst-Case" Szenario auf Honigbienen. In Anbetracht der Datenlage ist eine Schädigung der Honigbiene durch den getesteten Bt-Mais Mon89034xMon88017 unwahrscheinlich. Die Anwendung der Untersuchungsmethoden in zukünftigen Biosicherheitsstudien für transgene Pflanzen wird empfohlen.

I. General Introduction: Honey bees and GM Crops

Honey bees

Honey hunting and eating bee brood (*Apis sp.*) has been hominine behavior since the dawn of man (*Homo erectus*)(Skinner 1991). Ever since then, mankind has attempted to domesticate bees, by which the Western honey bee (*Apis mellifera* Linneaus) has now become the most representative species (Crane 1999). The distribution of *A. mellifera* is worldwide, brought to every inhabited continent by man due to their high value as livestock (Moritz et al. 2005). Beekeeping is commonly undertaken for the collection of honey, though brood and pollen are also edible bee products. Honey, propolis and bee venom are additionally valued for their medicinal purposes (Kwakman et al. 2011). Also beeswax is a renowned serviceable product, and royal jelly is valued for its alleged rejuvenating properties.

Despite all the valued beekeeping commodities, the prime commercial value of the honey bee is from pollination, and many apiaries specialize solely in this business (Free 1970, Calis and Boot 2010). Pollination is profitable because the production of many fruits, vegetables and oilseed crops are dependent on it. Pollination is also needed for the production of seeds for planting vegetables, and forage crops on which the cattle industry depends (Levin and Waller 1989). Recently, the economic value of worldwide insect pollination has been estimated at U.S. $217 Billion (Gallai et al. 2009). Pollination is an essential ecosystem service, as it actively sustains floral biodiversity. Therefore it secures food sources for many organisms, supporting the ecosystem.

The worldwide decline of pollinator populations over the last decades is a genuine concern (Steffan-Dewenter et al. 2005, Potts et al. 2010). Despite high concerns regarding honey bee colony losses, a conclusive clarification of causes has not yet been found (vanEngelsdorp et al. 2009, Neumann and Carreck 2010). Research is needed to clarify all potential threats to pollinators. In addition, pollinators need to be protected from harm, because their loss may critically affect ecosystem functions and threaten human food security (Allen-Wardell et al. 1998).

Environmental risk assessments

A substantial threat to honey bees is the use of pesticides (Desneux et al. 2007, Mineau et al. 2008). In addition to the application of chemical pesticides in agriculture, new pest control techniques based on the genetic modification (GM) of crops have been progressively developed over the last two decades. The DNA of a plant can be modified to introduce new traits like a transgenic protein based herbicide- or insect-resistance. In just 15 years, the total area of planted GM-crops worldwide has exceeded one billion hectares, used by 15.4 million farmers in 29 countries (James 2010). Presently, the application of herbicide resistance traits remains dominant (61%), though insect resistance traits are progressively applied, with a 21% growth between 2009 and 2010 (James 2010).

Before a new pest control product can be placed on the market (chemicals and GMO-crops), regulatory agencies first assess the risks of potential adverse effects to the environment. It commonly includes the biosafety testing on a range of beneficial arthropod species that fulfill important ecological functions such as biological control, pollination and decomposition. To establish adequate safety limits for pesticides under field conditions, standardized ecotoxicological tests are performed in the laboratory, and additional (semi-) field experiments may follow (OECD 1998a&b, EU 1991, EPPO/C0E 2000). For the environmental risk assessment (ERA) of GM-crops, the standard test methods are shortcoming, as GM-crop characteristics go far beyond the active ingredient that is responsible for the trait of concern (e.g. a transgenic protein). GM-crops are living organisms that are able to reproduce and propagate, and they may interact in many different ways with their environment (Hilbeck et al. 2008).

To date, most regulatory systems for insect resistant GM-crops have adopted the comparative approach, in which a GM-crop is compared with an equivalent non-transgenic variety (EFSA 2004). Further, the biosafety evaluation commonly involves a pre-release risk assessment, and a post-release monitoring of GM-crops (Romeis et al. 2006). Honey bee studies presented in this dissertation fall within the framework of a post-release biosafety research on a GM maize (*Zea mays*) expressing multiple Bt-genes (BMBF project 0315215E). In overview, the project includes the development of protein detection methods (DLR Neustadt), a quantification of transgenic-protein binding to soil particles (IBT Göttingen) and the extensive monitoring of biota: i.e. nematodes (IBN Regensburg), soil micro-organisms (vTI Braunschweig), maize litter decomposing micro-organisms (ZALF Müncheberg), earthworms and arthropods (e.g. butterflies, moths, bugs, cicadas and thrips) (RWTH Aachen University), beetles and spiders (LfL Freising), and honey bees (University of Würzburg).

Honey bees and Bt-maize

Maize or corn (*Zea mays*) is the most widely grown cereal crop in the world, with an approximate 150 million hectares planted and an approximate 800 million tons harvested each year (Naqvi et al. 2011). Currently, many cultivated maize varieties have single or multiple insect resistance traits, based on transgenic genes from the bacterium *Bacillus thuringiensis* (Bt). The genes induce the production of Cry-proteins, by which the crop is systemically toxic to herbivorous larvae of certain pest insects. Present day commercialized Bt-maize varieties target Lepidopteran pests (e.g. corn borers and armyworms) and/or Coleopteran pests (e.g. corn rootworms) (James 2010). In addition, an approximate 235 different Cry-protein holotypes have been identified to date, which also show specific toxicities for other species groups (e.g. Hymenoptera, Diptera and Nematoda) (Crickmore et al. 2011). In susceptible insects, Cry proteins cause midgut cell lysis and eventual death (De Maagd et al. 2001). Typically immature holometabolous insects show susceptibility to Cry-proteins, whereby neonates are found to be more sensitive than later instars (Glare and O'Callaghan 2000).

A meta-study on honey samples from 523 colonies (from Egypt, England, Scotland, Italy and Switzerland) indicated maize pollen as the most frequently occurring pollen (Keller et al. 2005). Hence, it is evident that honey bees have a high potential to be exposed to Cry-proteins via pollen when Bt-maize is legalized for application. As colonies store up to 20 kg maize pollen, stocks may represent a chronic exposure source within colonies (Odoux et al. 2004). Nectar or propolis are not present in maize, thus honey bee colony interaction with maize plants appears to be restricted to pollen. Maize pollen consumption rates by individuals, or indirect transgenic protein pass-over between individuals, have a limited documentation. One concise indication is reported, considering a 1720-2310 maize pollen consumption over the total larval development (Babendreier et al. 2004). In addition, ingested Cry-proteins by nurse bees were reported not to be passed on via feeding gland secretions (Babendreier 2005). This dissertation aims to contribute more quantitative data on maize pollen exposure under honey bee colony conditions, to substantiate the ERA on Bt-maize.

A considerable number of honey bee studies have been performed on Bt-pollen and purified Cry-proteins, either performed in a laboratory setting or within functional colonies (Duan et al. 2008, Malone and Burgess 2009), though a lethal or sublethal effect on brood or on worker bees has not been found. Only one study to date reported that high concentrations of purified Cry1Ab protein may affect food consumption or the learning processes of adult honey bees (Ramirez-Romero et al. 2008), but these results have not yet been corroborated by other studies (Rahn 2011, unpublished data).

Laboratory and semi-field tests

Standard ERA guidelines for tests follow a hierarchical (tiered) order. Tier 1 covers simple, short-term, low-cost tests under assumed worst-case conditions. They are performed commonly in a laboratory setting, with caged single-bees or groups of bees, testing topical and/or oral exposure (OECD 1998a&b, Romeis 2011). In the case of the GM-crop assessment of Bt-maize, the oral exposure pathway via pollen should be considered primarily, as Cry-toxicity is mechanistically limited to the midgut of susceptible insects. More importantly, since Cry-toxicity affects the immature insect growth stages (Glare and O'Callaghan 2000), young honey bee larva are especially amenable test-organisms, as they represent a most sensitive stage and are directly exposed to dietary pollen (Babendreier et al. 2004).

Within this dissertation, three chapters are devoted to *in vitro* experiments on honey bee larvae (IV, V and VI). The main methodology to rear larvae under standardized laboratory settings has been developed in the last 20 years, and has been found effective for testing chemical pesticides (Czoppelt and Rembold 1988, Davis et al. 1988, Oomen et al. 1992, Peng et al. 1992, Aupinel 2007). To date, no *in vitro* laboratory studies on the standardized feeding of pollen to larvae have been described. Alternatively, tests on larvae can be performed within colonies in which natural interaction among colony members occurs (Hanley et al. 2003). However, this interaction with nurse bees hampers the experimental standardization, with no guarantee that larvae are effectively exposed to a tested product (Hanley et al. 2003).

The general alternative for pollen feeding is the possibility of testing purified transgenic proteins, under laboratory (Lima et al. 2011) or colony conditions (Arpaia 1996). This approach is ideal for worst-case test scenarios. However, the proteins expressed in Bt-maize and the purified proteins derived by *E. coli,* can have different sizes (Andow&Hilbeck 2004) or other bio-chemical configurations (Kumar et al. 1996, Müller-Cohn et al. 1996). To account for the potential differences in bioactivity (Hilbeck et al. 2008), alongside the purified proteins also GM-crop products should be tested (i.e. Bt-maize pollen).

In the context of social insect behavior, a non-target test casus of honey bees is special. Bees behave different when grouped, compared to the behavior of individual bees. Naturally, larvae, nurse bees, forager bees, queens and drones interact, which is essential for colony stability and long term survival. Field and/or semi-field experiments complement the laboratory tests on individuals, and are essential to evaluate general colony performance within the environment. However, an open field approach has the downside that colonies cannot be forced to be exposed (Huang et al. 2004, Herrmann 2006 LWG), thus semi-field tests in tents or tunnels are a more preferable approach (Schur et al. 2000, Bakker and Calis 2003, Schmidt et al. 2003). The chapters II, III and IV of this dissertation are based on Bt-maize semi-field experiments during maize flowering, using standardized honey bee colonies kept in flight tents.

Cooperation, multidisciplinary research and novelty aspects

The non-target risk assessment of GM-crops is applied research, following a systematic inquiry, and involving a practical application of science. The six manuscripts bundled in this dissertation indirectly and/or directly address the solving of practical challenges using existing ideas, by combining different approaches and/or making new advancements. We succeeded in establishing a GM-crop monitoring over two successive field seasons of flowering maize, on a large scale. The preparation of a total of 150 standardized colonies was managed in fruitful cooperation with the LAVES bee institute (Dr. Werner von der Ohe). The massive experimental maize-field design was realized by the agricultural research station (Florian Hackelsberger) and the RTWH Aachen research group (Dr. Stefan Rauschen), which allowed us to focus on collecting a comprehensive data-set on honey bee colony development during the maize flowering season. My studies involved the development of new mortality monitoring techniques (Chapter II). Through cooperation with the Johann Heinrich von Thünen-Institut (vTI; Prof. Dr. ChristophTebbe), the semi-field experiments could be extended to a successive laboratory monitoring of longevity of a second generation of honey bees (Chapter III). In addition, a cooperation was possible on the measurements of qualitative traits in Bt-maize exposed bees, by an direct analysis of Cry-protein exposure, an analysis of the bacterial flora in the bee gut, and additionally checking the digestive capacity within the gut (Chapter VI).

Between the field seasons, a thorough improvement of *in vitro* analysis was established, rearing more than a 1000 larvae in the laboratory. The larvae tests are essential, because it is the larvae stage of target pest insects that is affected by Bt-toxicity traits. A first aspect was a methodological elimination of background mortality among larvae (Chapter IV). Secondly, a novel application of Bt-pollen feeding testing was realized (Chapter V), with additional worst case scenario testing of single and mixed transgenic proteins in purified form (Chapter VI). The latter in particular addresses the stacked aspect of multiple insect resistant Bt-maize, which to date has remained unaddressed for honey bees.

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II. A simple trap to measure worker bee mortality in small test colonies

This chapter has been published as: Hendriksma HP, Härtel S (2010) A simple trap to measure worker bee mortality in small test colonies. Journal of Apicultural Research 49, 215-217.

Keywords: honey bee, dead bee trap, mortality, ecotoxicology, risk assessment, pesticide testing, toxicity tests

Honey bee (*Apis mellifera*) colonies are widely used in ecotoxicological research and monitoring of environmental pollution to test for potential toxicity to non-target organisms. Collecting mortality data is undeniably important during the tests. We have developed a hive entrance trap for a risk assessment study in which pesticides are tested for toxicity to honey bee colonies. Our trap is used to monitor the mortality of bees which die within small test hives (e.g., mating hives and nuclei). Heavy wooden traps might accumulate toxins during multiple use in toxicological research, so our trap is made of plastic and has the advantage of being light, disposable and cheap. The material costs of one trap are about 1€. The trap is easy to build and is practical to use.

Our traps consist of two stacked white plastic 1 l (16 x 12 x 5.5 cm) containers, as used in take-away restaurants (Figs 1 & 2). The centres of the lids were cut out 1.0 cm from the sides (14 x 10 cm). Trap parts were fixed together with thermoplastic glue. A metal mesh (14.5 x 10.5 cm, 1.0 cm width) was attached over the opening in the upper lid. A lengthwise H-shaped slit (13 x 9 cm) was cut in the bottom of the upper container. This enabled the bottom to be opened in the centre by bending the flaps of the H-shaped slit downwards at an angle of 30°. The flaps protruded through the lid of the container below. A second metal mesh (13.5 x 9.5 cm, 1.5 cm width) was fixed to the bottom of the upper container, and the lid of the lower container was fixed underneath. This enabled easy removal and replacement of the bottom container to collect dead bees during experiments. A black marker was used to darken the lower container by blackening the remaining fringe of the transparent lower lid. This was done to allow living bees to exit the lower compartment by flying to the light in the centre. To enable rainwater drainage from the lower container, 4 mm holes were punched in each corner. A rectangular polystyrene block (12 x 5 x 1 cm) was glued to the front end of the upper container, which allowed smooth and firm attachment to the hive using adhesive tape (Fig. 2). A hole (1.5 x 1.5 cm) was cut through the upper container and the polystyrene at the hive entrance to enable bees to enter and exit the hive via the trap.

Figure 1. Drawing of the plastic dead bee trap.

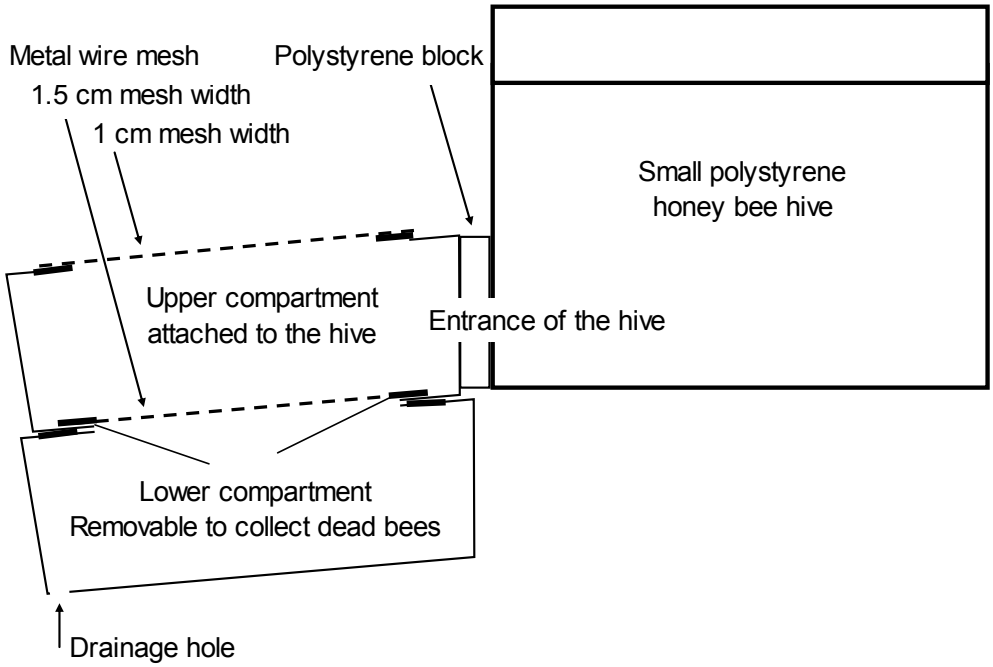


Figure 2. Photo of the plastic dead bee trap used in a semi-field trial on risk assessment of Bt-corn pollen.

Bees flew in and out of the hive through the upper compartment of the trap, passing through the wire mesh in the top lid. The 1 cm mesh width was no barrier for experienced foragers. Undertaker bees, on the other hand, which specialize in taking dead bees out of the hive (Visscher 1993), had great difficulty passing the wire mesh while carrying a dead bee. Dead bees dropped through the flaps into the lower compartment. Undertaker bees and scavengers such as wasps showed little or no interest in the removal of dead bees from the lower compartment. An important functional barrier of the trap was the 1 cm fringe of the upper lid, which prohibited crippled bees and undertaker bees carrying corpses from walking out of the trap.

We tested the traps' efficiency on 27 September 2008 by introducing marked dead bees into hives and monitoring the capture of the trap. We used mating hives (N=9) located in the botanical garden of the University of Bayreuth. Weather conditions were stable, with temperatures varying between 20°C during the day and 15°C during the night. Each standardized colony contained one queen and about 1000 workers. The polystyrene hives (Apidea™; 23x12x15 cm) contained three frames (10 x 10 cm) with food and brood in all stages. The queens (*A. m. carnica*) were sisters mated with the same drone population at a mating apiary of the bee institute in Celle, Germany.

We attached traps to the hives seven days prior to the experiment to let the honey bees adapt to the trap. One day before the experiment, bees were collected from the colonies, freeze killed, marked with a white pen on the thorax and kept fresh at 7°C. At 11:00 h we introduced 40 dead bees into each test colony under the lid. After 7 and 24 h, bees were collected from the trap. Every colony was checked afterwards for dead bees remaining within the hive.

Efficiency of the trap, indicated by recovery of the dead marked bees was 93 % ± 2.7 % (mean ± s.e., n=9 colonies). Since no marked dead bees were found inside the hives after the trial, 7 % of the test bees were considered as not trapped. 3 % ended up in the wire mesh of the upper compartment, which was easily accessible via the removable top lid, and the remaining 90 % ended up in the lower compartment from which the dead bees are collected. Of all captured test bees, 99 % were caught within 0 to 7 hours and 1 % in the remaining 7 to 24 hours of the experiment. Unmarked bees were also found in the traps (in range of 0 to 12 bees, with mean 3.7 ± 1.3 s.e.).

No entrance traps have previously been described in the literature for small polystyrene hives and nuclei (e.g., Apidea, Mini Bivo, Swi Bine, Mini-Mating Nuc). For full sized hives (e.g., Langstroth or Dadant type) traps such as the Gary, Todd, underbasket, barrier and the Münster trap have been described (Gary 1960, Atkins et al. 1970, Illies et al. 2002). Our preliminary results show that the efficiency of our plastic trap is comparable or even higher than these.

In practice, 72 plastic traps were shown to work successfully during a four week environmental risk assessment experiment (see Ch. III, and Härtel et al. 2009) (Fig. 2). The traps showed good performance over a long period of time and were practical to use. The removable bottom compartment and top lid were useful features of the trap, well suited for the risk assessment study, where traps had to be monitored daily to check possible toxicity effects. We therefore think that our trap has the capacity to be a standard trap for small honey bee colony mortality data collection.

III. Honey bee colony exposure to pollen of Bt-maize does not cause mortality over two successive generations of worker bees.

This chapter is in preparation for publication as: Hendriksma HP, Härtel S, Von der Ohe W, Steffan-Dewenter I, Honey bee colony exposure to pollen of Bt-maize does not cause mortality over two successive generations of worker bees.

Keywords: GMO, *Apis mellifera*, *Bacillus thuringiensis*, biotechnology, honey bee, mortality, risk assessment, stacked Bt maize, semi-field trial, dead bee trap

Summary: Honey bees (*Apis mellifera*) are a key non-target species in ecological risk assessment of genetically modified (GM) insect resistant crops. Under laboratory conditions, potential adverse effects on worker bees are well studied. However, within functional colonies, risk assessment data on the effect of Bt-pollen exposure are lacking so far. We analysed worker bee survival over two successive generations, under semi-field conditions with flowering maize plants as only available protein source. We compared a multiple insect resistant Bt-maize, with its near-isogenic line as the control maize variety. Mortality distribution within the worker force was monitored by i) the amounts of hive bees, ii) trapping of dead bees, and iii) a long term laboratory monitoring of survival rates of the next generation of workers. During the 3 week field experiment, no significant difference in worker bee mortality was observed. Interestingly, comparatively higher pollen amounts were collected from the isogenic line, and this significantly caused higher numbers of new worker bees being raised. But the amount of pollen units invested per new bee was found to be identical, indicating that the pollen quality was the same. The number of new bees born was directly related to the pollen quantity collected by colonies. Neither the weights of the freshly hatched bees, nor the survival rates among these individuals differed between the maize varieties. Considering the ecological risk assessment of Bt maize for honey bee colonies, we conclude that the tested stacked Bt-maize does not pose an increased mortality risk to two successive generations of worker bees.

Introduction

In 2010, more than 15 million farmers planted 148 million hectares of biotech crops, which accumulates up to one billion hectares planted since 1996 (James 2010). The genetic modifications (GM) are represented mostly by herbicide tolerances. However, insect resistance is the fastest growing trait, as indicated by a 21% growth between 2009 and 2010 (James 2010). Many agricultural crops, such as cotton, rapeseed, and maize, are genetically altered to produce toxins of the bacterium *Bacillus thuringiensis*. This will protect the plants against herbivorous larvae of specific pest moths and beetles. In this respect, a clear risk to the environment is potential collateral damage to non-target insects in the agricultural environment. In the evaluation and approval of new GM crops, these environmental risks should always be assessed scientifically and transparently, in a standardized and rational way (Romeis et al. 2008).

The honey bee (*Apis mellifera*) is a key test species within environmental risk assessment schemes (Malone and Pham-Delègue 2001, Marvier et al. 2007, Duan et al. 2008). Honey bees have a high ecological and economical value, being vital pollinators with a worldwide omnipresence in agricultural areas (Klein et al. 2007). The pest control by transgenic proteins can pose risks to pollinating insects when the insecticidal proteins are expressed within pollen. For honey bees, pollen is the only dietary source of protein and therefore pollen is of capital importance to the survival and reproduction of honey bee colonies (Dietz 1978).

A need exists for reliable test methods for honey bees as beneficial non-target species. For plants expressing transgenic pesticides, experimental protocols for a standardized monitoring of plant products (e.g. pollen) need to be developed (Stark et al. 1995, Hilbeck et al. 2000, Romeis et al. 2011). It is important to note that different life stages of bees, e.g. larvae, nurse bees and foragers, consume different amounts of pollen (Crailsheim et al. 1992, Babendreier et al. 2004, Rortais et al. 2005). Also, it is eminent that social insects have complex life history traits, which are difficult to simulate in a laboratory, thus biosafety tests under standardized colony conditions should play an essential part within risk assessments. Notwithstanding, colony level risk assessments under open field conditions are confounded by uncontrolled exposure rates. It is impossible to force colonies to forage exclusively on a certain field. Therefore, only semi-field conditions guarantee controlled colony exposure rates in an experimental risks assessment approach.

Only few reports exist on field experiments with Bt-maize pollen and honey bees (Schur et al. 2000, Rose et al. 2007). In addition, Bt-toxins as purified crystaline proteins have been tested in feeding experiments on larvae and adult bees (Arpaia 1996, Malone 2001). In general, harm by Bt-proteins is typically caused to target pest insects by lethal damage to the intestinal tract (Ingle et al. 2001). But to date, no lethal effect by Bt-pollen or high Bt-protein concentrations have been indicated on honey bees (Malone 2001, Marvier et al. 2007, Duan et al. 2008, Malone and Burgess 2009). The progressive development in GM crops is creating gaps in the biosafety data coverage. For instance, it has remained unstudied if honey bees are affected by a combination of transgenic traits.

Within this study, we performed a biosafety test on a stacked GM crop, using semi-field exposed honey bee colonies. The acute and chronic toxicity to Bt-maize pollen is examined, monitoring two generations of worker bees over 3 months. The Bt-maize studied was the variety Mon89034xMon88017, which has four transgenic genes incorporated in the genome. Within its pollen, three arthropod active Bt-proteins (Cry1A.105, Cry2Ab2, Cry3Bb1) and an herbicide tolerance protein (CP4 EPSPS) are expressed. This Bt-variety represents a number of novel developments in GM crop design, such as the combined expression of traits, and increased expression rates of transgenic proteins (Monsanto 2009).

Material and methods

Maize treatments and standardized honey bee colonies

The research maize field was planted on May 20, 2008 in Braunschweig, Germany. It included 40 systemic randomised plots 42x30 m (0.126 ha) and comprised eight replicates of five different maize treatments. The experiment focused on the stacked Bt-maize variety Mon89034xMon88017, and its isogenic line (DKc5143). These two varieties were flowering exactly at the same time. In each plot, a field cage 12 x 4 x 3 m (length, width, height) with a mesh size of 1.3 mm was set up. The field cages covering six rows of maize with 75 cm space between rows resulting in a total number of 485 ± 16 (mean ± sd) plants per cage. We estimated the produced amount of pollen per cage to be in a range of 1.46 kg (3.5 g/plant, Emberlin 1999).

Standardized honey bee test colonies were assembled at the LAVES Bee institute in Celle. Hives contained three empty frames (10x10 cm) and a feeder, all embedded into a Styrofoam box (Apidea^{TM}). Each colony was composed by joining 1 queen with approximately 1000 workers (114 gram bees ± 11 SD). These bees were a randomised mix of young worker bees from 10 colonies of pure *A. m. carnica* breeding lines. Also the queens were *A. m. carnica* breeding line offspring, and they were all mated at the same mating apiary with a controlled drone population. The colonies were set up 3 days before start of the

field experiment. After setup, the colonies were stored for two days in a cooling room with *ad libitum* sugar feeding. At this point, the test colonies were free of wax, brood, honey and pollen stores.

Two honey bee colonies were introduced into each cage when the ⅔rd of the plants started pollen shedding. Pollen shedding finished twelve days later. The hives were set up in field cages on poles in heights of approximately 1.6 m in a 2 m^2 plant free area. All test colonies had *ad libitum* access to water and were fed with same amounts of ApiInvert™ sugar solution over the entire experimental period. During the experiment, the near-isogenic line treatment contained 16 colonies, and the Bt-maize group 13 colonies, because in 2 hives the queens died at the start, and 1 hive was excluded because of internal damage after falling from the pole.

Study endpoints

Mortality of colony workers was monitored in two ways. Firstly, by checking the difference between the start- and end-weight of hive bees (July 31 - August 31) (Table 1; A). This indicated the quantity of bees that had not returned to the hive during the semi-field experiment. Secondly, the bees which died inside the hives were counted daily by means of a hive-entrance trap (Hendriksma and Härtel 2009) (Table 1; B). Also, during the experiment, every 5 days the colonies were checked for the amount of pollen cells stored in the combs (C). The cumulative number of cells was considered as indicator for the amount of collected pollen per colony over the semi-field experiment. Newly raised bees started to hatch in the last week of August, and their total number was counted as quantitative indication of colony performance (D). The number of pollen cells divided by the number of new bees was used as a measure of colony resource efficiency (E). This rate can be also considered as indicator for pollen quality. The new bees were collected on a daily basis, and weighed in the laboratory as a qualitative measure (F).

Per five individuals, the newly hatched bees were combined in clear plastic Petri-dishes (Dieteman et al. 2007), and incubated in a humidified brood stove at 28°C (Memmert, Germany). Each dish contained a filter (10cm diam.), two open tubes with tap water, and one tube with honey (1.5 ml, Eppendorf, Germany). The dishes were checked daily to remove dead individuals and to score mortality rates (Table 1; G). This monitoring of longevity was performed initially in a laboratory in Braunschweig, but was continued from the 16th of September onwards in Bayreuth under the same set of experimental laboratory conditions.

Statistics

All study endpoints were analyzed using the open source statistic software R, version 2.11.1 (R Development Core Team 2010). The maize treatment (Bt-maize or the near-isogenic variety) was examined as key explanatory variable in a number of different models. In addition to statistical results, all models are listed in Table 2. On the mortality rates of hive bees (A) an analysis of variance over the two maize varieties was performed with a linear model fit (*lm*; Chambers 1992). In relation to an approximate 1000 bees per colony at the start of the experiments, the dead bee trap mortality data (B; ‰) was analyzed with proportional hazards regression models (*Coxph*; Cox 1990, Fox 2002). This regression on survival dynamics included colony identity as a random factor, to correct for the non-independence within the data-set, by grouping bees to colony origin (Zuur et al. 2009, Hendriksma et al. 2011a). The pollen cell counts (C), new bee counts (D), and the breeding efficiency (E; the number of pollen cells used per newly raised worker) were similarly analyzed with a linear model fit, applying a square root transformation on the count data to meet ANOVA assumptions. Analysis of hatchment weights was performed with a linear mixed effects model (*lme*; Pinheiro et al. 2011), with weight data of individuals again grouped to colony background. Alike the mortality data (B), the longevity of young bees (G) was analyzed with a proportional hazards regression models on survival dynamics of individuals, with colony background as random factor (Cox 1990). The linear models were analyzed with a type-III ANOVA. The *R* package *gplots* was used for extracting 95% confidence intervals for error bars (Warnes 2011).

Table 1: Bt-maize semi field experiment on honey bee colonies, to monitor worker bees directly under pollen exposure conditions. The experiment was continued in the laboratory to monitor the survival of the new colony bees, which were raised on maize pollen proteins as sole dietary protein source available.

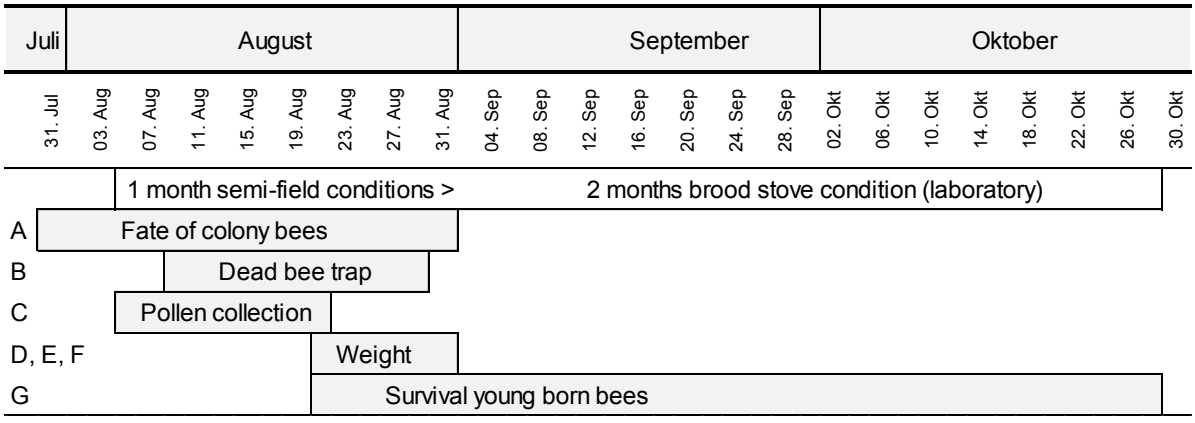


Table 2: The endpoints as been tested on the 3 month experiment, with statistical results of the comparison between varieties Bt-maize and the near-isogenic maize as control.

Endpoints	Bt-Maize	Near-isogene Maize	Value (*df*)	*P*-value	Models
A: Bee loss (%)	mean 52.6% ±7.2 SD	mean 54.4% ±6.4 SD	$F_{(1,27)} = 0.54$	= 0.46	*lm* on colony means
B: Trap deaths (days)	mean 4.4 /colony	mean 4.3 /colony	*Chisq* = 0.07	= 0.78	*Coxph* on individuals, *Rf*-colony
C: Pollen (numbers)	median 0, mean 1.8	median 5.6, mean 5.5	$F_{(1,27)} = 15.7$	< 0.001	*lm* on (sqrt) colony means
D: Young (numbers)	median 0, mean 1.0	median 4.4, mean 3.8	$F_{(1,27)} = 10.4$	= 0.003	*lm* on (sqrt) colony means
E: Breeding (rate)	mean 2.0 ± 1.2 SD	mean 2.0 ± 1.6 SD	$F_{(1,14)} = 0.00$	= 0.95	*lm* on (sqrt) colony rates #pol/#bee
F: Weight (mg)	87.1 ± 13% SD	86.3 ± 12% SD	$F_{(9,155)} = 0.02$	= 0.89	*lme* on individuals, *Rf*-colony
G: Survival (days)	median 34 days LT_{50}	median 35 days LT_{50}	*Chisq* = 3.6	= 0.06	*Coxph* on individuals, *Rf*-colony

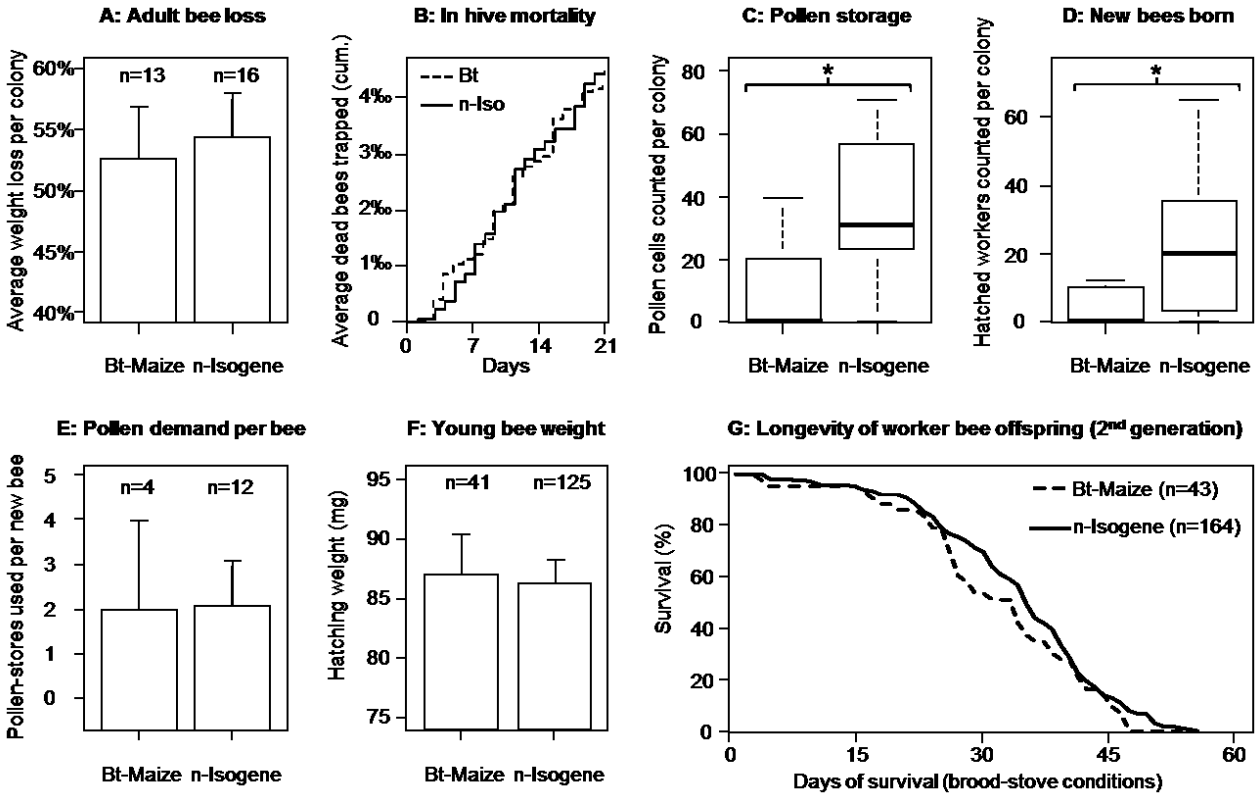


Fig 1: Results of a semi-field experiment on honey bee colonies in flowering maize fields. Mortality among worker bees was compared between a stacked Bt-maize and its near-isogenic line. Young bees were raised in the colonies on maize pollen as sole protein source. In the laboratory, the new bees were examined on their weight, and additionally monitored for survival, until the last bee died.

Results

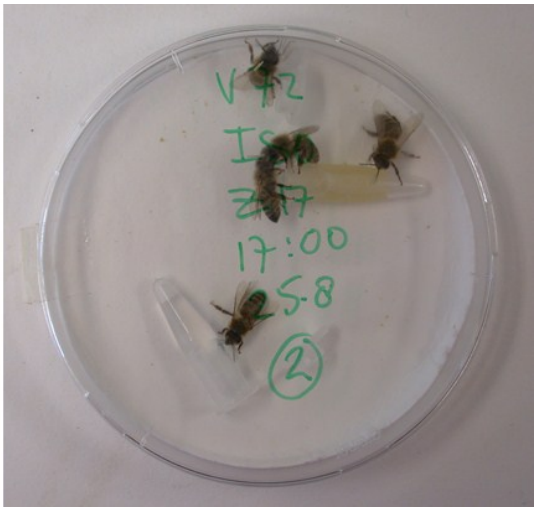
Semi field

The workers in colonies showed no differences in mortality between the two tested maize varieties. The overall loss of bees, as measured by their total weight over 4 weeks, was not significantly different between the maize varieties (Table 2; Fig1A). The amount of dead bees caught with the hive entrance traps was very similar between the treatments, with a difference of mean 0.1 bee per colony (not significant; Fig.1B). The forager bees in colonies exposed to the near-isogenic maize variety, stored significantly more pollen in comparison to colonies exposed to Bt-maize (Fig.1C). In addition, approximately 4 times more bees were raised, which was significantly higher compared to the Bt-maize treatment (Fig. 1D). However, the pollen resource efficiency showed that per colony a same mean amount of pollen per new bee was utilized (Fig.1E). It indicates that the nutritional quality of pollen was alike, which is additionally supported by the findings on the mean weights of the two young bee groups: at hatchment the difference was <1% (0.8 mg), which is not a significant difference (Fig.1F).

Pictures 1-3: The first picture shows a comb from a standardized colony at the start of the semi-field experiment, on a day where pollen was counted. The second comb picture is taken half-way the semi-field experiment where all pollen resources were invested in the brood (visible as pupae cells). The third picture was taken after emergence of new bees; hatched individuals were collected at a daily base, and were brought to the laboratory for weighing.

Survival of the second generation

The maize pollen exposed offspring showed under laboratory condition a considerable longevity, of up to 55 days. The lethality time at which 50% of the test individuals had died showed comparative survival rates: 34 days for Bt-maize colony individuals, and 35 days for individuals of the near-isogenic line (Table 2). The survival dynamics analysis showed that this difference was not significant (P=0.06; Fig 1G); it is a trend indication that near-isogenic line bees may live 2.9% longer.



Pictures 4 and 5: Picture 4 shows the inside of the incubator with a water container for humidification and stacks of plastic Petri-dishes containing bees. Picture 5 shows five bees in a Petri-disk, containing filter paper, a tube with water and one with honey. At a daily base the bees were successfully checked on survival, up to a maximum 55 days after incubation.

Discussion

Measurement on Bt effects

Pollinating insects are of high ecological and economical value, since pollination is an essential ecosystem service. It is unacceptable if honey bees, as key pollinators in the agricultural environment, were to be harmed by pollen of GM-crops. A main result of the present study is that the three different Bt-proteins simultaneously expressed in maize pollen cause no apparent chronic toxicity, either to adult bees or the developing brood. Our study results corroborate single Bt-protein studies, having also indicated no adverse effects, either on worker bees or on larvae (Arpaia 1996, Malone et al. 2001, Hanley et al. 2003, Robyn et al. 2007, Lima et al. 2011). The current study can be considered a realistic worst case test scenario, because only maize pollen was available to the test colonies. Hence, larvae were reared exclusively on maize pollen derived proteins. Target insect adults are found limitedly susceptibility to Bt-

proteins (Meisle et al. 2011), as compared to lethal effects to larvae (Vaughn et al. 2005, Clarck et al. 2006). Hence, especially testing of honey bee larvae stages should be considered for risk assessments on Bt-crops.

A report on tested Cry1Ab Bt-maize pollen on bees showed no adverse effects, neither by laboratory feeding for 35 days (47.2% survival of worker bees), nor in a maize field set-up with colonies fed with Bt-pollen cakes for 20 days (Robyn et al. 2007). The current laboratory results are comparable, with 50% survival at 34 and 35 days for Bt-maize and near-isogenic line treated bees respectively. In comparison, the present test lasted much longer, as we effectively combined a field and laboratory test into one experiment, lasting up to 85 days.

Another study tested Cry1A or Cry1F Bt-maize pollen by feeding to 4–5 day-old larvae within colonies (Hanley et al. 2003). The used method of adding pollen directly into the food of larvae is however not experimentally secure, because nurse bees can remove applied test doses (Hanley et al. 2003). A semi-field approach with colonies in flight cages is a potentially more reliable approach for a controlled pollen exposure experiment (Schur et al. 2003). As alternative to testing Bt-pollen, purified Bt-protein tests can be performed (Arpaia 1996, Malone et al. 2001, Lima et al. 2011). The ecological relevance of this approach can however be questioned, as this pathway of exposure does not mimic the way pollen is naturally processed by nurse bees, and then additionally fed to larvae. Nonetheless, purified protein studies enable to address Bt-protein safety at concentrations exceeding environmental exposure conditions by a multifold, which is a useful monitoring strategy to secure biosafety (Romeis et al. 2011).

Malone et al. (2001) reported an exponentially decreasing survival rate among Cry1Ba exposed bees (625 mg purified protein/g pollen food), as monitored under in-hive conditions. This mortality effect did not differ from controls, with a 5-10% contingent bees surviving successfully up to 45 days after emergence. Our newly emerged colony bees were monitored under laboratory conditions, and mortality progressed here with a sigmoidal curve (Fig. 1G). We did find a comparative longevity of at least 45 days, considering a last 10% contingent of surviving bees. The observed difference in curve progression (exponential *vs* sigmoidal) can be explained by experimental design. In contrast to our continuous laboratory monitoring, Malone et al. (2001) tested freshly hatched bees in the laboratory, and afterwards introduced these bees into colonies. At that point, many bees disappeared, as visible by an acute mortality increase.

Pollen availability

A striking result of our study is the difference in honey bee pollen resources, with a lower amount Bt-maize pollen collected in comparison to the near-isogenic line. The main explanation for this effect is a relative low quantity of Bt-pollen produced per plant. In 2008, the phenotypic development of the Bt-maize variety on the research field was inferior to its near-isogenic line (personal observation), which was corroborated in 2009 by a quantification of phenotypic traits, with a minor deviation found within the synchronization of flowering moments (Härtel et al. in prep.; Pictures 6 and 7). The qualification of lower pollen availability is arbitrary. It can be considered positive for environmental risk assessments that the transgenic protein exposure to bees is limited. But ecologically, low pollen availability within the agricultural landscape may impair the development of colonies, which can be considered also as a discernable effect.

A difference between the Bt-crop and its near-isogenic line may relate to the parental lineages. A direct non-transgenic parental control variety was absent, because the stacked Bt-maize was a hybrid of two other Bt-maize varieties. Hence, not having an identical offspring, a genotypic difference between the triple stacked Bt-maize and the near-best isogenic variety might have caused a variety based difference.

Picture 6: The semi-field study site in 2008 at the period of full-bloom: A randomized maize-plot design is shown, with each of the research plots (42x30m) containing a flight tent (12x4x3m) with approx. 485 flowering maize plants and 2 standardized honey bee colonies. It is clearly visible that phenology differences between the maize varieties existed, considering different shades of the maize-tassels.

Another explanation for the observed differences in pollen availability can be found in so-called pleiotropic effects, which relate genetic effects to phenotypic traits. For instance, after a transgenic gene introduction, the allocation of the plants biosynthesis resources may partly shift to the expression of transgenic proteins. Alternatively, it is possible that a gene introduction silences the expression of other genes (Joshi et al. 2011). In both examples the plant can be phenotypically affected, for instance by a reduction in growth.

Honey bees at a field of squash (*Cucurbita pepo*), were reported to visit flowers of a transgenic virus-resistant variety less, in comparison to conventional squash flowers (Prendeville and Pilson 2009). Here, causality was found by conventional female squash flowers being larger, producing more nectar than transgenic flowers. As a similar occurrence of a pleiotropic effect, this could relate to our result of different pollen quantity collected. Another study reported a similar inconsistency in pollen amounts, as observed in different pollen consumption rates between honey bees exposed to conventional and Bt-maize pollen (Robyn et al. 2007). In that case, the difference was suspected due to pollen quality differences, although it was additionally noted that the two pollen types were not collected at the same time (Robyn et al. 2007). The latter observation is indicative for a causal relation with phenology of flowering time. A pollen quality effect is less likely, in consideration of the present study: neither an affected resource-efficiency for breeding was found (Fig. 1E), nor a different mean weights of newly hatched bees, between the two maize treatments (Fig. 1F).

Picture 7: The semi-field study site in 2009, approx. 5 weeks before flowering. Again, considering the growth rate and size of plants, it visible that phenology differences between the maize varieties existed.

Considering a 13% increase of so-called stacked crops since 2009, different insect resistances and herbicide tolerance are increasingly applied in combinations (James 2010). Honey bee biosafety data on stacked Bt-crops has clearly fallen behind, as stacked GM crop reports on honey bees are absent to date. This hiatus has grown considerable, considering that in 2010 already 22% of global biotech crop area was occupied with double and triple stacked trait crops (James 2010). However, by finding no effects of a multiple insect resistant Bt-maize in this honey bee study, we corroborate that Bt-proteins are not generally toxic, but are restricted in their toxicity to specific insect classes. In our case study on Mon89034xMon88017 maize expressing Cry1A.105 and Cry2Ab2 against Lepidopteran pests, Cry3Bb1 against Coleopteran pests, and the CP4-epsps protein conferring glyphosate resistance, the transgenic proteins are apparently non-toxic for bees, or at least not expressed in concentrations that cause harm to honey bee workers or the developing brood.

Previous findings by Ramirez-Romero et al. (2008) indicated a potential disturbed learning performance at 600 ng Cry1Ab-protein per adult honey bee. Our tested Cry-protein dose was 773 ng per bee (considering a consumption of 62.4 mg stacked Bt-pollen per bee; 12.4 µg Cry-protein/g pollen; 4.24 µg Cry1/g, 1.19 µg Cry2/g, 6.95 µg Cry 3/g; Sauer and Jehle pers. comm.). At a comparable Cry-protein concentration, albeit with other types of Cry-proteins, we could not corroborate the occurrence of an affected learning performance of bees by the consumption of Mon89034xMon88017 maize pollen (Rahn 2011, unpublished data). A general biosafety for bees is likely for field exposure conditions with Mon89034xMon88017 Bt-maize, as lethal or sublethal weight effects or behavioral effects were absent.

Conclusion

In conclusion to our results, the monitoring of stacked Bt-pollen exposure to honey bee colonies found no discernable effects on worker bees. Neither an acute mortality effect on bees, nor a chronic effect on the next generation of worker bees is found. In addition, the current biosafety testing is addressing several methodological improvements. It is a first time honey bee test of a flowering GM-crop, expressing multiple Bt insect resistance proteins in pollen. We note that genetically modified crop varieties may show pleiotropic effects by a reduction of the amount of pollen, which in turn may affect colony performance within the agricultural environment. At the same time, we can conclude that pollen of the Bt-maize Mon89034xMon88017 is not toxic to worker bees, and colonies are likely to remain unharmed by the exposure to this maize variety.

IV. Honey bee risk assessment: New approaches for *in vitro* larvae rearing and data analyses

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Keywords: *Apis mellifera*, artificial comb, bioassay, Colony Collapse Disorder, dimethoate, ecotoxicology, grafting, larvae collection, mixed effect model, pollination

Summary

1. In order to sustain the vital ecosystem service of pollination, new methodical developments are needed for research on the underlying factors of globally observed bee losses. In particular, robust laboratory methods for assessing adverse effects on honey bee brood are required. In addition, from a statistical point of view, the shared origin of test individuals must be considered when analysing ecotoxicological data.

2. To improve honey bee *in vitro* rearing, we adopted a non-grafting method to collect honey bee larvae without direct manipulation. Linear mixed effects models to evaluate LD_{50}, larvae survival and prepupae weights integrated the colony background of larvae as a random factor into the statistical analyses. The novel rearing approach and appropriate statistical tools for data analyses are illustrated in an *in vitro* case study on acute oral dimethoate toxicity.

3. We recommend our honey bee larvae collection approach for *in vitro* larvae rearing applications, because of (i) a mere 3% background mortality upon the prepupae stage, (ii) a high quantitative capacity and (iii) because of robustness of performance which are great benefits for standardization.

4. Our analyses indicate clear adverse effects of dimethoate by a significant survival reduction and prepupae weight reduction. For second instars, the acute 48 hour LD_{50} was 1.67 µg dimethoate per larva.

5. We conclude that both our larvae collection method and the applied statistical approaches will help to improve the quality of environmental risk assessment studies on honey bees, to secure honey bee pollination and to sustain biodiversity.

Introduction

The worldwide losses of honey bee colonies have been raising genuine public concern. As global declines continue for many other social and solitary bees, the deterioration of insect-mediated pollination may critically affect agricultural and natural ecosystems (Fontaine et al. 2006, Potts et al. 2009). As a result, human food security is at stake since insect pollination is required for many kinds of seeds, fruits, vegetables and forage crops (Klein et al. 2007).

In recent years, researchers have reported numerous possible explanations for the phenomenon of disappearing honey bees, also known as Colony collapse disorder (CCD) or honey bee depopulation syndrome (HBDS). However, despite a high concern, conclusive clarification of CCD has not yet been found (vanEngelsdorp et al. 2009). To address the multiple open questions on colony losses, the development of effective and practical honey bee risk assessment approaches is imperative. Advances are needed in the development of field, semi-field and laboratory standard testing methods. On the other hand, appropriate multi-factorial data analysis methods have to be applied in order to integrate different explanatory variables, such as the genetic origin of honey bee colonies, pathogenic pressures, landscape structure and exposure to environmental pollution or agricultural pesticides.

The health of honey bee brood is a crucial factor for colony survival. During the larval phase environmental conditions play a formative role in the behaviour and longevity of bees (Becher et al. 2010). Feeding on pollen and nectar in the larval diet directly exposes larvae to the environment (Haydak 1970, Babendreier et al. 2004). Pollen or nectar containing pesticides may have detrimental consequences for the brood of a colony, therefore laboratory methods for assessing adverse effects on larvae development are required.

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 attractive assay because of controlled lab conditions, reproducibility and the defined amounts of ingested test doses by larvae (Aupinel et al. 2007). Biologically relevant *in vitro* assessment endpoints such as the survival and weight of test individuals can be monitored in a straightforward manner. This methodology could develop into a routine standard environmental risk assessment bioassay. However, many honey bee laboratories still face multiple challenges (COLOSS 2010), which shows the urgent need of further *in vitro* bioassay approaches. Many tests *in vitro* are hampered by occasional high mortality rates, a lack of standardisation and repeatability, as shown by the variance between different European laboratories in a standardized dimethoate LD_{50} ringtest (Aupinel et al. 2009). The causes of the observed variance may lie in practical

experimenter skills, the season, genetic variation and larval age heterogeneity at grafting (Aupinel et al. 2009).

Within our study we address many *in vitro* larvae rearing challenges: an easy collection of tests larvae, the use of age defined test larvae, a low larval mortality, the comparability between *in vitro* and *in vivo* developing larvae, standardisation of protocols, and the benefit of using up to date statistical applications. We introduce statistics which take into account that workers from one colony share the same environment and that they are all the progeny of a single mother queen. These facts have not yet been statistically implemented in ecotoxicological studies of honey bees. However, recent social insect studies correct for the multiple colony backgrounds among tested workers (Bocher et al. 2007, Koyama et al. 2007, Kasper et al. 2008, Muller et al. 2008, Zuur et al. 2009, Castella et al. 2010).

The main goal of this paper is to unite new approaches for *in vitro* larvae rearing. We present a novel artificial comb-based larvae collection method with the capacity to improve standardization between laboratories. We test the reliability and robustness of the new larvae collection approach. As the mechanical stress of traditional grafting is bypassed, the viability of collected individuals is optimized. The benefits of our method are illustrated in a case study, testing the reference insecticide dimethoate on honey bee larvae survival rates, weight and lethal dose values. The larvae collection method and the endpoint evaluation statistics will help to standardize *in vitro* rearing bioassays, in order to facilitate ecotoxicological studies on honey bees.

Materials and Methods

A new larvae collection protocol

First instar larvae were collected using artificial combs of the commercially available system for queen breeding (Cupularve Nicotplast©, Maisod, France). In preparation for *in vitro* rearing trials, artificial combs were mounted into wooden frames with honey comb. Each artificial comb (10x10cm) is made of plastic and has 110 honey bee cell sized holes (Pictures 1 & 2). Crystal polystyrene plastic queen cups were placed over the cells at the backside and covered with a transparent lid. In order to allow bees to become familiarized with the combs, the frames were introduced into colonies one week preceding the experiments.

On three days (12th, 23rd, 25th of June, 2009) queens were trapped on the artificial combs within their colonies by means of a plastic queen excluder lid. The queens of the test colonies were selected from six Upper Franconian apiaries to cover a variety of different *Apis mellifera carnica* genotypes. On day

four (D4), eggs started to hatch in the cups (Table 1). At mean 92:59 h ± 1:50 SD after queen enclosure (n=13), the cups with first instar larvae were collected from the colonies. Considering a 72 h development time of eggs (Bertholf 1925), the collected larvae had a mean chronological age of 10:29 h ± 0:55 SD and were at the biological age of being a first instar larvae.

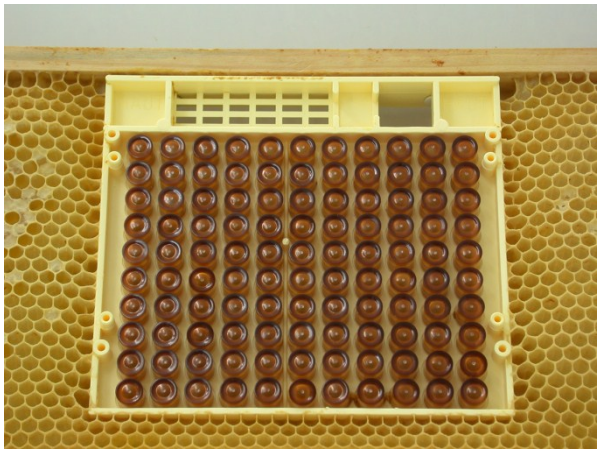
To indicate the swiftness of this larvae collection procedure; at the 28th of June it took two persons 45 minutes to collect 519 first instar larvae (mean 87 larvae per comb). At a speed of 12 harvested first instar larvae per minute, one person collected and replaced cells on the combs, while the other handled the combs at the colonies. Collected cups with larvae were placed in 48-well culture plates with a humidifying piece of dental cotton roll at the bottom of each well, wetted with a 0.4% Methylbenzethonium chloride, 15.5% Glycerol/H_20 solution (Aupinel et al. 2005, Aupinel et al. 2007). During collection, plates with larvae were stored in a 35° Celsius warmed polystyrene box for transport from the apiary to the laboratory. To quantify the efficiency of our first instar larvae collection method, survival of 1060 non-grafted larvae was evaluated over the initial 24 hours of *in vitro* rearing (see Table 1, D4-D5; Table 2, experiment 1a).

Protocol for in vitro rearing

Honey bees were reared in the laboratory according to the protocol of Aupinel et al. (2005&2007). The culture plates with larvae were kept in a hermetic plexiglass desiccator in an incubator at 35° Celsius. Larvae were fed over D4 to D9 with a 10, 10, 20, 30, 40, 50 µl semi-artificial diet, respectively. This diet consisted of 50% royal jelly (Le Rucher du Buzard certified organic apiary, Sospel, France) mixed with a 50% aqueous solution. The yeast extract / glucose / fructose proportion in the aqueous solutions was respectively 2/12/12 percent at D4 and D5; 3/15/15 percent at D6; and 4/18/18 percent at D7, D8 and D9. During larval development, relative humidity in the incubator was kept at 96% using a saturated solution of K_2SO_4. Further development upon hatching took place in 80% humidity, maintained using a saturated solution of NaCl. The temperature and humidity in the desiccator were continuously monitored with data loggers (MSR electronics, Henggart, Switzerland) to affirm consistent climatic conditions.

Table 1. Developmental stages of honey bees upon hatching (Bertholf 1925), with key days marked in grey: D4 for collection of first instars; D5 for dimethoate application; D11 for weighing prepupae (PP) and survival endpoint; D21 as checkpoint on the quality of hatched individuals.

D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21
egg			L1	L2	L3	L4	L5			PP	Pupae									

Pictures 1 & 2: The front-side of an artificial combs combination, and the backside which contains removable polystyrene cups, by which collection of first instar larvae is facilitated.

Performance of in vitro rearing

Successful larvae development upon the prepupae phase indicates a high performance of the *in vitro* rearing method. Therefore, the D4-D5 survival measurements of 1060 first instar larvae was followed by subsequent D5-D11 survival evaluation of 106 *in vitro* reared larvae (Table 2, experiment 1b). At D11, weight data (±1 mg) was collected by transplanting prepupae with soft metal tweezers into a new plastic queen cup on a microbalance, to enable a sound prepupae weight comparison between treatments (Table 2). Additionally, we show an artificial comb application to compare the weights of *in vitro* reared prepupae (laboratory) with *in vivo* prepupae which develop in parallel on the artificial combs inside honey bee colonies (Table 2, experiment 1c). A subsequent survival comparison over D11-D21 was used to measure mortality effects of the manipulation of prepupae for weighing (Table 2, experiment 1d). Therefore, the survival of unmanipulated prepupae was compared with survival of transplanted prepupae. The test individuals were daily monitored under a stereomicroscope to verify health: moribund and dead test individuals, recognized by occasional black or white sub-dermal necrotic stains or a visible loss of turgor, were removed. Additional quality checks on *in vitro* hatching honey bees (D21) were performed by measuring weight and checking the inter-caste characteristics of workers by a pollen comb inspection, as described by Allsopp *et al*. (2003).

Dimethoate toxicity test design

For ecotoxicological endpoint evaluation for an *in vitro* honey bee bioassay, a case study on dimethoate was performed. The acute oral toxicity of a dimethoate concentration gradient was tested on 99 *in vitro* reared larvae. The test doses were 0 / 0.2 / 0.8 / 3.2 / 12.8 µg larvae^{-1} (Aupinel et al. 2007&2009) on n=20±1 larva per dose. The dimethoate was obtained from Fluka Chemie, Switzerland. In contrast to

Aupinel et al. (2007&2009) we treated the second instar larvae (D5) by feeding dimethoate mixed in 10µl artificial diet. The test was split over two experimental periods: trial I starting on June 23rd and trial II starting on June 25th. Covering all treatment levels, larvae from 7 colonies were included.

The three evaluated toxicity endpoints were (i) the survival of larvae to indicate lethal effects, (ii) the weight of prepupae for indicating possible sub-lethal effects, and (iii) an 48 hour LD_{50} acute oral toxicity value of dimethoate for the exposed second instar larvae (see also: Statistical analyses).

Statistical analyses

One objective of this study is to apply the shared origin of honey bee workers in the analysis of ecotoxicological data sets. By using different packages of the open source statistic software R version 2.11.1 (R Development Core Team 2010), we evaluated the toxicity endpoints survival, weight and LD_{50}. Colony identity was always included as random factor in the models to compensate for the identical background of larvae. The dimethoate treatment gradient with five concentration levels was the key predictor. Trial was a fixed factor with two levels (starting on the 23rd or 25th of June) that was always tested, but was removed from the models when direct *P*-values or Likelihood ratio tests did not indicate it as a significant explanatory variable (Zuur et al. 2009).

Survival analysis: The larval survival was analysed with a Cox proportional hazards regression model (Fox 2002) using the R packages survival and survnnet (Ripley *et al*. 2004, Therneau 2009). The *P*-values from the Cox-model summary, which indicate differences between the control and the individual dimethoate levels, were corrected with the Holm-Bonferroni procedure (Holm 1979) to provide a multicomparison correction.

Prepupae weight analysis: To investigate whether our endpoint prepupae weight was affected by dimethoate, we applied a linear mixed effects model using the package lme4 (Bates & Maechler 2010). The treatment analysis was performed on the dimethaoate gradient as covariate predictor variable. The doses (+0.001µg) were $\log_{10}$ transformed to linearize the exponentially progressing gradient. Our prepupae weight model was checked visually on normality of the residuals by normal probability plots and we assured the homogeneity of variances and goodness of fit of the model by plotting residuals *versus* fitted values (Faraway 2006). The program R does not directly provide *P*-values for mixed effect models with the package lme4, so we extracted them using likelihood ratio tests (LRT) based on the changes in the deviance when an explanatory variable was dropped from the full model (Rödel et al. 2010).

LD_{50} analysis: As acute oral toxicity endpoints, LD_{50} values were calculated from survival data of a 48 hour dimethoate exposure to second instar larvae. A generalized linear mixed effects model (glmer) was fitted with colony identity included as a random factor. This approach is compared with a standard logit regression analysis, using a generalized linear model fit (glm). Treatment entered both models as covariate with $\log_{10}$ transformed dimethoate doses (+0.001µg) and for the survival data (dead or alive) the family function 'binomial' with the link function 'logit' were used.

Lethal dose values were calculated using the intercept (a) and treatment parameter estimate (b) from the models. At 50% mortality, the $\log_{10}$dose estimate is -(a/b) and the $LD_{50} = 10^{-(a/b)}$. The 95% confidence intervals of the mixed model LD_{50} value were calculated with Fieller's method (Finney 1971, Niu et al. 2010).

Table 2. Overview of performed experiments with the number of larvae and colonies used to evaluate the measured parameters survival, weight and LD_{50}.

	Treatment	n (larvae)	Colonies	Survival	Weight	LD_{50}
1a	non-grafting larvae	1060	7	D4-D5		
1b	larvae rearing	106	7	D5-D11	D11	
1c	in vivo / hive control	18	3		D11	
1d	weighting mortality	103	7	D11-D21	D21	
2	dimethoate gradient	99	7	D5-D11	D11	D5-D7

Table 3. Cox proportional hazards models with the colony of test larvae included as random factor, to compare survival dynamics of *in vitro* reared larvae between control and dimethoate treatments.

Dimethoate per larvae Level comparisons	*Df*	X^2	*Bonferroni-Holm* corrected α	*P*-value [1]
0 µg *versus* 0.2 µg	1	0.00	0.05	1.00 *ns*
0 µg *versus* 0.8 µg	1	4.55	0.025	0.034 *ns*
0 µg *versus* 3.2 µg	1	13.12	0.017	< 0.001 ***
0 µg *versus* 12.8 µg	1	14.72	0.0125	< 0.001 ***

[1] *P*–values are given, with indicators for high significance (***) and no significance (ns).

Results

Larval collection and in vitro rearing performance

The novel approach for larvae collection performed well, both quantitatively and qualitatively, considering that 1043 out of 1060 first instar larvae (98.4%) survived the first 24 hours of *in vitro* rearing (D4-D5). The robustness of our method is repetitively shown over the three collection days, by a survival rate of 100% (n=64), 97.6% (n=531) and 99.1% (n=465), respectively. Subsequently, of the 106 larvae of experiment 1b, 99.1% reached the fifth instar stage (D5-D9) and 97.2% the prepupae stage (D5-D11), indicating successful rearing performance over the larval stages. The mean weight of *in vitro* reared prepupae was 141.4 mg ± 1.4 SE (n=20). In contrast, *in vivo* prepupae reared on artificial combs in colonies were significantly heavier (mean 165.2 mg ± 2.84 SE; n=18) than *in vitro* prepupae (*t*-test: t=-7.758; Df = 36, P<0.001). The emergence rates of prepupae (D11-D21) were significantly affected by the manipulation of prepupae for weighing (Fig.1). Unmanipulated prepupae showed an emergence rate of 81%, whereas emergence rate of weighed prepupae was significantly lower at 30% (Cox proportional hazards regression: X^2=33.27; Df = 1, P<0.001). Hatched *in vitro* adults were all morphological workers having typical worker pollen combs, with a mean weight of 126.1 mg ± 5.0 SE (n=10).

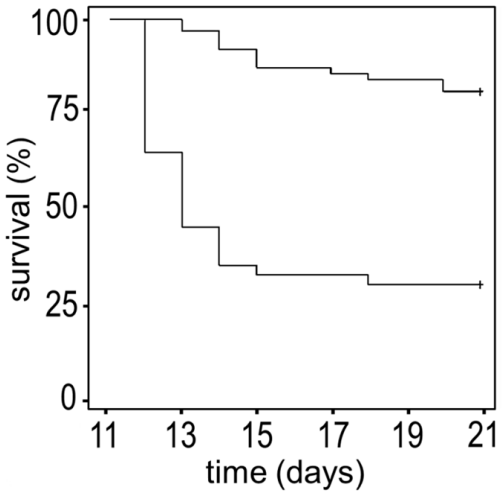


Figure 1. Influence of prepupae weighing on the survival of *in vitro* developing pupae. Shown is post weighing survival (> day 11) upon the hatching of bees at day 21 (n=63 unmanipulated *versus* 40 manipulated larvae).

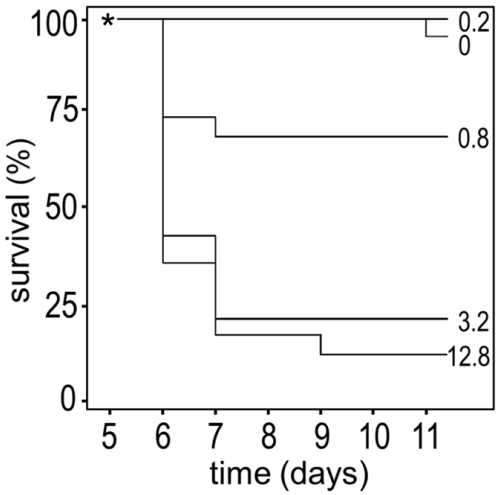


Figure 2. Survival characteristics of untreated and dimethoate treated larvae. The survival dynamics from the treatment day (D5) up to the prepupae phase (D11) are shown. The units 0, 0.2, 0.8, 3.2 and 12.8 indicate the dimethoate treatment doses in µg/larva.

Ecotoxicological analyses

The dimethoate treatment gradient clearly affected the endpoint survival over the larval phase (Fig. 2, Table 3 & 4). The post hoc comparisons among treatment levels showed that at 0.2µg dimethoate the survival among larvae was identical to the control larvae. A decrease in survival over our dimethoate gradient started as trend at the level of 0.8µg larvae^{-1} and at both the doses 3.2µg and 12.8µg dimethoate a significantly higher mortality rate compared to controls was found (Table 3, Fig. 2). A significant decrease of mean prepupae weight occurred with increasing dimethoate concentration (Table 4). Between the two experimental trials, starting on the 23rd or 25th of June, a difference in mean prepupae weight was found. The prepupae were mean 2.9% ± 2.0% SD (n=4 doses) significantly heavier in the second trial (Table 4). Acute larvae mortality by dimethoate exposure was indicated, as treatment was a significant factor in the generalized linear mixed effect model (Table 4). The mixed model approach showed a dimethoate 48 hour LD_{50} of 1.67 µg larvae^{-1} (n=99) with 95% confidence intervals of 0.84 and 3.30 µg larva^{-1} (Figure 4). The standard regression method resulted in a LD_{50} of 1.69 µg dimethoate (95% confidence interval of 1.03 to 5.48 µg larva^{-1}).

Table 4. Dimethoate test overview on models and results on the tested endpoints survival, weight and the 48 hour lethal dose value.

Endpoint	Model	Effects [1]	*Df*	X^2	*P*-value
survival	Cox ph	*fixed factor(treatment)*	4	67.4	< 0.001
D5-D11	regression model	*fixed factor(trial)*	1	0.2	0.64
prepupae	Linear mixed effects	*fixed(treatment)*	1	14.2	< 0.001
weight D11	model (with LRT [1])	*fixed factor(trial)*	1	4.3	0.038
48hr LD50	Generalized linear	*fixed(treatment)*	1	67.4	< 0.001
D5-D7	mixed effects model	*fixed factor(trial)*	1	1.7	0.19

[1] Effects for the predictor variables are derived from the model summaries or from Likelihood ratio tests (LRT).

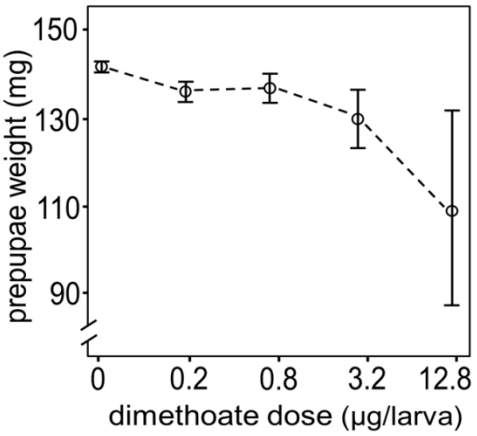


Figure 3. Effects of dimethoate treatments on prepupae weights of *in vitro* reared honey bee larvae. Prepupae weight sample sizes are respectively 20, 20, 13, 4, 2 over the dimethoate gradient 0 / 0.2 / 0.8 / 3.2 / 12.8 µg $larva^{-1}$, as applied in the diet of second instar larvae. The regression line over the log transformed dimethoate dose gradient, describes the significant trend for lower weight of prepupae at higher treatment doses (R^2=0.253; at X^2=14.2 with *Df*=1 and *P*< 0.001, with the colony identities considered as a random factor).

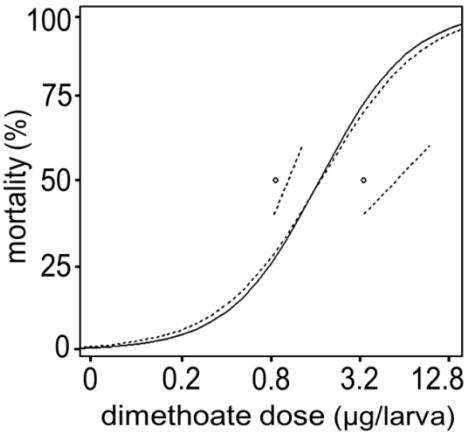


Figure 4. Dose response curves on larval mortality (n=99), 48h after dimethoate exposure. The x–axis shows the dose gradient, and the y–axis shows the probability of mortality. Model fits were performed on mortality (logit transformed) and dimethoate dose (+0.001µg and log10 transformed) to extract LD_{50} values. The dashed dose response curve shows the standard regression (glm) with 95% CI and the continuous curve shows the mixed model approach (glmer) with colony as random factor, and the fiducial limits indicated by 2 points.

Discussion

Larval collection and in vitro rearing performance

Improving methods in order to determine the underlying reasons for recent honey bee colony declines is imperative. Here we present effective approaches for larvae collection and data analyses, which can help facilitate *in vitro* rearing methodology and benefit the accuracy of honey bee risk assessment studies. We clearly show that larval collection with artificial combs generates very low mortalities and is practical, quick and easy. The new non-grafting approach helps to improve *in vitro* rearing techniques for fundamental and applied honey bee research. In addition, the use of the artificial comb system enabled us to include a number of further refinements, which result in a higher standardisation level of honey bee brood tests.

Of 1060 collected first instar larvae, 98.4% survived the first critical 24 hours of *in vitro* development. Since eggs and young larvae are physically vulnerable, it is to be expected that minimizing manipulation contributes to the survival of larvae. Where traditional grafting with brushes, needles or feathers may cause mechanical stress and mortality among larvae, the artificial comb based method enables first instar larvae collection without directly grafting larvae, by letting queens lay eggs directly into the test vessels for *in vitro* experiments. Depending on experience, the success of grafting is never guaranteed and often many eggs or larvae do not develop. Wegener et al. (2009) reported 75% of grafted eggs being retrieved as viable larvae. Evans *et al*. (2010) noted only 43% survival of needle-grafted eggs. Often, data on the collection success of test individuals remains unmentioned in papers on *in vitro* rearing (Aupinel *et al*. 2005/2007/2009, Behrens *et al*. 2007, Brodschneider *et al*. 2009, Jensen *et al*. 2009). It is common practise to replace unviable larvae by spare larvae at the moment of treatment to compensate for grafting mortality. By bypassing the grafting procedure we could solve this methodological shortcoming. In absence of grafting mortality, experimental time upon the prepupae phase is gained and thereby creating the opportunity to test the very sensitive first instar stage. In contrast to other artificial comb systems (Omholt *et al*. 1995, Aase *et al*. 2005), only the adopted Cupularve system allows the pragmatic collection of larvae within plastic cups, which are at the same time the perfect test vessels for *in vitro* rearing experiments.

As shown, 99% of *in vitro* fed larvae reached the fifth instar stage and 97% the prepupae phase. For a bioassay, this high survival rate is optimal in order to sensitively test acute or chronic mortality effects on developing larvae. Compared to 10% control larvae mortality in the chronic dimethoate test by Aupinel *et al*. (2007), our 2.8% background mortality upon the prepupae phase is a definite advancement.

In terms of standardization, our approach without direct human interference would guarantee a constant quality of *in vitro* larvae. As larvae collection is the primary step for *in vitro* rearing, the use of our robust approach would minimize experimenter generated variance. This is due to the dependency of traditional grafting on the experience of the human manipulator. Potentially the comparability between labs is greatly enhanced when studies adopt our approach to achieve general low background mortality. An essential challenge for honey bee laboratories is to overcome high mortality among controls (Janke et al. 2010). The validity criteria, that mortality should not exceed 15% at the end of acute 48 hour toxicity test (Aupinel et al. 2009), is by means of our methodology substantially secured. Thus, our results support the recommendation of the comb collection method to other laboratories that perform *in vitro* exposure bioassays.

Worth stipulating is the general practicability of using the non-grafting queen rearing system. It is a great advantage that grafting with needles is no longer necessary. Collection of larvae can be performed without much experience since the method is straightforward. Also the quantity of comb collected larvae indicates the potential for performing elaborate experiments. Our collected numbers of first instar larvae should amply suffice for extensive experimental designs. In 45 minutes, including hive handling time, over 500 larvae were collected. Evans et al. (2010) describes grafting 220 eggs in 30 minutes, which shows that our technique is comparable to the collection speed of experienced grafters. Compared to the procedure of Evans et al. (2010), basically gathering many eggs by hitting honey bee combs on the table, our mass collection method is considerably more refined.

We stipulate that quickness of collection is important, because it is likely a relevant factor to rearing successes. The larvae are less exposed to outside-hive conditions, which can be unfavourable due to dehydration, low temperatures, fungal contamination and UV-light.

A common rearing problem for the northern European bee-season is that early and late in the season, collection of viable larvae is inconvenienced due to a low amount of eggs, unfavourable grafting conditions and low willingness of bees to nurse young larvae (Aupinel et al. 2005&2009). The use of artificial combs might help by making *in vitro* rearing less season-dependent. Since queens are enclosed on the small comb area, searching time for collectable larvae is reduced. The swiftness of cup collection lessens damage to the larvae by unfavourable conditions. Optional additional sugar/protein feeding could stimulate queens to lay eggs and workers to nurse larvae on the combs.

Aupinel et al. (2005&2007) described the preference to confining queens for 30 hours for harvesting plentiful larvae. It is hypothesised that heterogeneity of larvae ages and a possible age depending susceptibility towards insecticides, can explain differences in test outcomes (Aupinel et al.

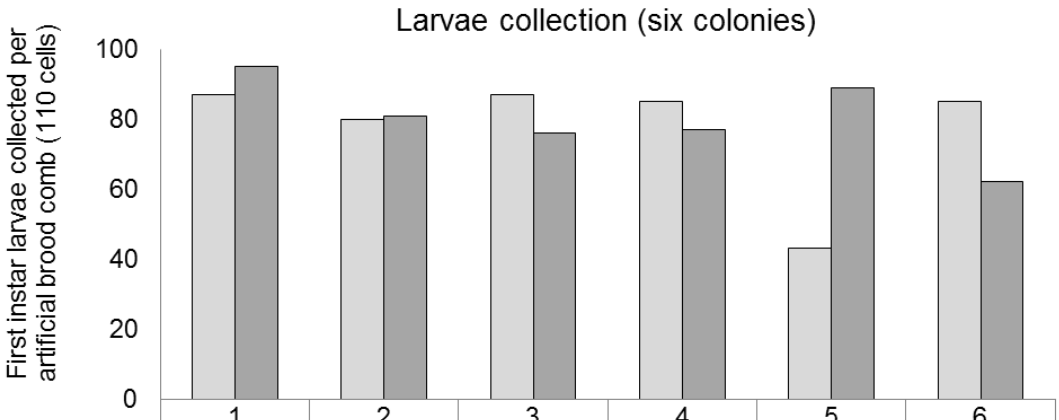
2009). Accordingly, we promote a higher age standardisation for *in vitro* test larvae, preferably an instar stage related age range (Table 1). By timing the harvest moment, we could control the age range of test individuals, limiting the maximum age, securing collection of first instar larvae. For smaller age variances, shorter queen enclosure time windows can be applied. For example, when queens are confined for 10 hours and larvae are subsequently collected after 87 hours, all larvae will be in the chronological age range of 5 to 15 hours. For queen enclosure time manipulation, the artificial comb is a practical tool. The queens in our experiments did not show reluctance in laying eggs, since all artificial comb cells were filled with larvae or eggs at the harvest moment (Fig. 5). Finally, the use of young queens is recommended since they have a high egg laying capacity.

Considering the low variance in prepupae weight data, the endpoint prepupae weight is exact and ideal for evaluating toxic effects on larvae. However, we like to point out that there is a clear mortality effect due to the manipulation of the prepupae (Fig. 1). Obviously, this developmental stage is extremely sensitive and the transplantation into a new cup causes lethal mechanical stress (see also the pictures 3 and 4). This implicates that direct manipulation of prepupae is unrecommended for prolonged studies until hatchment. For studies upon emergence, the hatching weight is the appropriate assessment endpoint. Alternatively, a weight assessment endpoint of lower quality could be achieved by a method given by Aupinel *et al*. (2005), measuring prepupae within their cells. However, this causes weight anomalies due to food residues, moulting skins and defecation products that are simultaneously weighed. To our experience, these residues are also breeding grounds for fungi. At the cost of a mortality effect, a cell transplantation can still benefit methodology, because the weight data is accurate and the chance of fungal infections is actively suppressed.

Prepupae reared in the laboratory were 3.4% more heavy in weight compared to in hive individuals as described by Wedenig *et al*. (2003). With a mean weight of 126 mg, hatched laboratory bees were 8% heavier compared to hatching hive bees, as reported by Bowen-Walker *et al*. (2001). Such differences can be explained by distinctive weight gain dynamics between *in vivo* and *in vitro* developing larvae, as described by Riesberger-Gallé et al. (2008).

Riesberger-Gallé *et al*. (2008) and Brodschneider *et al*. (2009) performed studies focusing on the quality of *in vitro* reared bees. *In vitro* honey bee risk assessments will be strengthened when test results are consolidated by proof that the development of test individuals was fully normal. We introduced with test 1c (Table 2) the innovative methodological control by artificial combs for in hive larval development, in parallel to laboratory *in vitro* developing larvae. Our preliminary results showed that test individuals from the same batch, simultaneously developing *in vitro* and *in vivo*, can be compared.

Considering that 97% of *in vitro* control larvae reached the prepupae phase, that the weight of prepupae and hatched honey bees was bordering normality and that hatched worker bees were lively and had no inter-caste characteristics, we conclude that the presented method of *in vitro* rearing is adequate.



	1	2	3	4	5	6
23th June	87	80	87	85	43	85
25th June	95	81	76	77	89	62

Fig. 5: Larvae collection graph. The Nicot/Cupularve system does not show queen reluctance to lay eggs in the artificial combs. In general, the artificial cells were filled with eggs and larvae. In only one case, we could only collect 43 first instar larvae of the wanted age (colony 5). But in the next trial, this queen was adapted to the comb condition and she performed above mean, resulting in 89 harvestable larvae. Despite this single observation, the between colony variance is low. Of these twelve queen enclosures, we successfully harvested mean 79 (median 83) first instars (originally published as an Appendix).

Pictures 3 & 4: A honey bee prepupa (left) and pupa (right), reared within the laboratory, *in vitro*.

Ecotoxicological analyses

In the dimethoate case study of *in vitro* reared larvae, mixed models were successfully applied for each toxicity endpoint: survival over the larval phase, prepupae weight and a LD_{50} value (Table 4). By including colony background as a random factor, all models allowed statements for colonies in general, at the cost of only one parameter for colony identity (Zuur *et al*. 2009). This is in contrast to the general approach in honey bee ecotoxicology to assess colonies individually. In comparison, an analysis with colony background as a fixed factor would have the disadvantage of coming at the cost of six degrees of freedom (Zuur *et al*. 2009). It would also have the model handicap of not holding for colonies in general, but only for each colony individual (Zuur *et al*. 2009). For this reason, as long as individual colony responses are not the focus of a project, random intercept models are to be preferred.

From a biological point of view, the colony is for social insects the principle level of reproduction, survival and homeostasis (Hölldobler and Wilson 2009). In addition, from a statistical perspective, honey bee worker data are considered nested because multiple observations are taken from the same colony (Zuur et al. 2009). Test-individuals from the same colony share (i) the same environment (stressors on test-larvae within colonies: bacterial / fungal / viral / chemical / nutritional / temperature) and they are related to each other (ii). Without considering tested colony mates as groups, models would fail to take into account a fundamental assumption of standard statistical models, the independence of errors (Crawley 2007).

Survival over the larvae phase was highly affected by dimethoate application in the case study (Table 3). Aupinel et al. (2007) found 5 days post treatment, 40% and 85% prepupal mortality for the treatments 0.8 and 12.8 µg dimethoate respectively. We observed 6 days post treatment, for the same treatment doses a mortality rate in the same range; 32% and 89% respectively (Fig. 2). As indicated by post hoc comparisons, the endpoint survival (from D5 to D11) is a clear and strong indicator of adverse effects to developing larvae. The Cox model uses chi-square statistics, which has limits to statistically discriminate differences when sample sizes are low. The tested sample size of 20 individuals per treatment level was at the boundary of indicating a possible treatment effect at 0.8µg $larvae^{-1}$. For this level in particular, a significant statistical discrimination was lost due to the sequential Bonferoni-Holm correction (Table 3). To optimise the statistical discrimination in similar test designs, the number of levels can be reduced and larvae sample sizes increased.

Our linear mixed effects analyses indicated a significant reduction of prepupae weight over the dimethoate concentration gradient (Fig. 3). Relative low weight of a prepupa is likely to be caused by low food uptake due to intoxication. Heterogeneity in age is unlikely a variable of influence on prepupal

weights because normally larvae finish the administered diets to the full extent by D10 and as no longer food is consumed, their weight is stable by D11. Thus, a developmental heterogeneity is evened out as younger larvae catch up on the older larvae in reaching their maximum weight as prepupae. Trial was however a significant predictor variable (Table 4). The 2.9% weight difference between the two experimental trials indicates that the methodology can be further standardized. We can recommend for example the use a multi-pipette to apply highly constant amounts of diet to larvae.

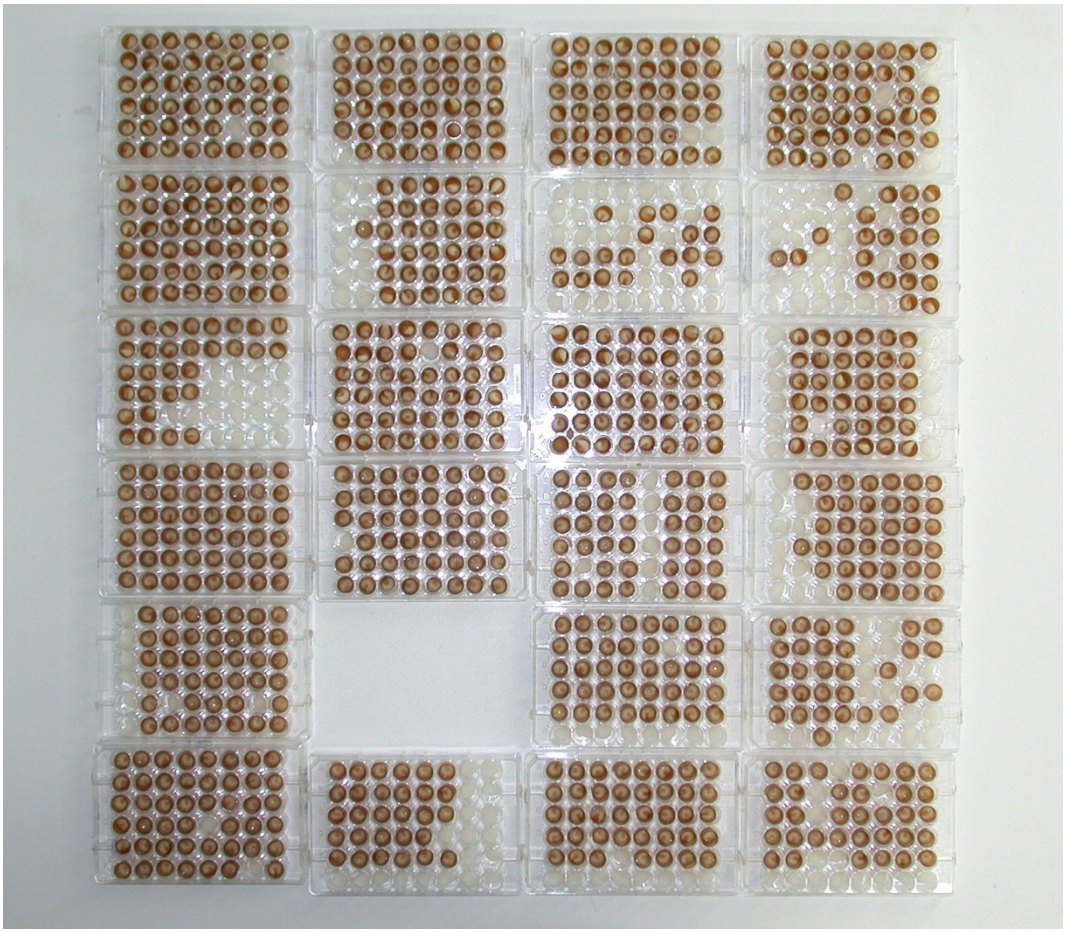
Our study provides a scientifically sound assessment of a standardized LD_{50} toxicity value. The generalized linear mixed effect model indicated a dimethoate LD_{50} value of 1.67 µg with a confidence interval between 0.84 and 3.30 µg $larva^{-1}$. This corresponds to the 1.93 µg $larvae^{-1}$ and a 1.0 to 3.0 µg $larva^{-1}$ confidence interval as reported by Aupinel et al. 2007. It lies also perfectly in range with the 1.5 to 3.1 µg $larvae^{-1}$ documented for a ring test carried out in seven different international laboratories (Aupinel et al. 2009). Hence, our methods and statistical approaches allow for an accurate and reproducible toxicity test. Adult bees by Hardstone & Scott (2010) were reported to have a dimethoate LD_{50} value of 1.62 µg g^{-1} body weight, which relates to acute 24h-LD_{50} values of mean 0.16 µg bee^{-1} for topical exposure and mean 0.18 µg bee^{-1} for oral exposure (Gough *et al*. 1994). In comparison, our LD_{50} at 1.67 µg $larva^{-1}$ is roughly 10 times higher, although the larvae are both topically and orally exposed.

The comparison of the standard method of linear regression (Aupinel et al. 2009) with our mixed model approach is visualised by the two dose response curves in Figure 4. The presented approaches of LD_{50} calculation are not considered significantly different because the 95% confidence intervals of the standard (glm) and the mixed effect (glmer) calculation are overlapping. The implementation of colony background in the LD_{50} analysis of this case study did not have a pronounced effect on the LD_{50} value, nonetheless the calculation correctly implemented the dependency of larvae originating from the same colony. Considering the dimethoate test design, a reserved stance towards individual colony LD_{50} values is recommended. Colony values must be considered with caution when larvae numbers throughout colonies are unbalanced, or when experimental designs do not provide high sample sizes per treatment level. Regressions with low sample sizes are sensitive to chance effects, resulting in a variance between lethal doses which is not necessarily colony or treatment related. Stochastically, a mixed model LD_{50} is more robust since the sample size is high, while colony backgrounds are included as a random factor.

As shown by the case study, tailor made analyses for a variety of *in vitro* toxicity test endpoints are possible. In general, the strength of such modelling statistics is the robustness and flexibility in dealing with e.g. pseudo replications, repeated measurements, abnormal distributions and imbalances within the dataset (Crawley 2007). It is innovative for honey bee bioassays that single effects, but also

multiple effects and also interactions can be evaluated by testing multiple explanatory variables. For example, this is suitable when analysing the interplay of different pesticides in varying doses on honey bees. CCD research should likewise benefit by the possibility of simultaneously analysing several potential stressors such as pesticides, parasites and malnutrition.

The comprehensiveness of bioassays benefits by inclusion of a high number of colonies and a broad range of backgrounds. This is in contrast with the widely observed limited number of test colonies in bioassays. As colony origin may cause variance in toxicity outcomes, low colony numbers in experiments could lead to possible under- or overestimation in toxicity assessments. Also to be considered is brood temperature related susceptibility towards toxicants (Medrzycki et al. 2010) and differences in susceptibility towards stressors between lineages (Behrens et al. 2007, Jensen et al. 2009). Also, brood development characteristics may differ between colonies (Collins 2004). An experimental bioassay design should address existing variations in susceptibility. Therefore it is best to include a multitude of colonies comprehending a wide range of genetic and phenotypic variation, as present in the honey bee population.



Picture 5: The methodology has a high quantitative capacity for ecotoxicological experiments.

Conclusions

To counteract the worldwide bee losses, the development and standardisation of effective risk assessment studies on honey bees is needed. Following the basic idea of minimizing contact with the larvae in order to optimize rearing success, a number of *in vitro* rearing challenges are solved. We state that our presented non-grafting approach, which to date is never applied for *in vitro* rearing, is highly efficient for larvae collection. The rearing method showed a strong quantitative capacity, which is usefull for ecotoxicological experiments (Picture 5). It has high potential for standardisation and method improvement of fundamental and applied *in vitro* rearing honey bee research. Contacting test individuals is harming them, as shown by the high mortality rate after weighing manipulation.

We presented in this paper suitable statistical methods for ecotoxicological data analyses for honey bee studies. As shown, multiple colony test designs can be evaluated using several explanatory factors on biologically relevant *in vitro* test endpoints, such as survival and weight. We conclude that a multi-colony approach with adequate statistical implementation to correct for highly related individuals benefits honey bee risk assessment studies.

V. Testing pollen of single and stacked insect-resistant Bt-maize on *in vitro* reared honey bee larvae

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Keywords: *Apis mellifera*; *Bacillus thuringiensis*; Bt-pollen; Environmental Risk Assessment; GM crops; *in vitro* bioassay; pollinators; regulatory standardization; toxic pollen; *Zea mays*

Summary: The ecologically and economic important honey bee (*Apis mellifera*) is a key non-target arthropod species in environmental risk assessment (ERA) of genetically modified (GM) crops. Honey bee larvae are directly exposed to transgenic products by the consumption of GM pollen. But most ERA studies only consider responses of adult bees, although Bt-proteins primarily affect the larval phases of target organisms. We adopted an *in vitro* larvae rearing system, to assess lethal and sublethal effects of Bt-pollen consumption in a standardized eco-toxicological bioassay. The effects of pollen from two Bt-maize cultivars, one expressing a single and the other a total of three Bt-proteins, on the survival and prepupae weight of honey bee larvae were analyzed. The control treatments included pollen from three non-transgenic maize varieties and of *Heliconia rostrata*. Three days old larvae were fed the realistic exposure dose of 2 mg pollen within the semi-artificial diet. The larvae were monitored over 120h, until the prepupal stage, where larvae terminate feeding and growing. Neither single nor stacked Bt-maize pollen showed an adverse effect on larval survival and the prepupal weight. In contrast, feeding of *H. rostrata* pollen caused significant toxic effects. The results of this study indicate that pollen of the tested Bt-varieties does not harm the development of *in vitro* reared *A. mellifera* larvae. To sustain the ecosystem service of pollination, Bt-impact on *A. mellifera* should always be a crucial part of regulatory biosafety assessments. We suggest that feeding GM pollen on *in vitro* reared honey bee larvae is well suited of becoming a standard bioassay in regulatory risk assessments schemes of GM crops.

Introduction

Pollinators provide key ecosystem services by maintaining both the biodiversity of wild plants and agricultural productivity (Klein et al. 2007, Potts et al. 2010) at an estimated value of US $217 billion yearly (Galllai et al. 2009). The most important pollinator species worldwide is the honey bee *Apis mellifera* (Free 1970), with populations present in all countries growing genetically modified (GM) crops (Ruttner 1988, James 2010). Hence, honey bees are a key non-target test species for assessing the potential adverse impacts of GM crops on pollinators (Duan et al. 2008, Malone and Burgess 2009).

Crops expressing insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (Bt) (Bt-proteins) are among the most widely cultivated GM crops worldwide (James 2010). A recent meta-analysis showed no adverse effects of Bt-crops on *A. mellifera* (Duan et al. 2008). All of the re-analyzed studies tested only the effect of single Bt-proteins. However, one future trend in plant biotechnology is the stacking of multiple resistance traits. An example is Bt-maize SmartStax™, released in 2010 in the USA with six different insect resistance genes for above- and below-ground insect control, with two additional herbicide tolerance genes (James 2010). Hence, regulatory authorities are in need of up to date test-standards, to guide robust first-Tier laboratory experiments to assess the risks of new GM plants to non-target organisms (Romeis et al. 2011).

Floral pollen is the sole protein source of *A. mellifera* colonies (Brodschneider and Crailsheim 2010) and pollen of a variety of important crops is collected by bee foragers (Malone and Burgess 2009). Adults and larvae of *A. mellifera* are directly exposed to transgenic material via pollen consumption of GM-crops, as planted in mass monocultures. On average, a worker consumes 3.4 to 4.3 mg of pollen per day (Brodschneider and Crailsheim 2010), with colonies accumulating up to 55 kg per year (Seeley 1985). Bees exposed to Mon810 maize pollen did not transmit quantifiable amounts of the Bt-proteins via their hypopharyngeal glands into the larval food they secrete (Babendreier et al. 2005). Nevertheless, pollen is also straightforwardly added by nurse bees to the larval food (Haydack 1970). It was reported that larvae consumed 1720-2310 maize pollen grains under semi-field exposure conditions, which is reflecting a worst case maize pollen exposure of 1.52-2.04 mg (Babendreier et al. 2004). In comparison, European butterfly larvae fed with pollen grains from the transgenic maize variety Bt-176 were lethally affected at much lower exposure doses: LD_{50} value of only 8 pollen grains per Diamond-back moth larva, and 32-39 pollen grains for Small tortoiseshells, Peacocks, European corn borers and Cabbage white larvae (Felke and Langenbruch 2005).

Bt-proteins confer plant-protection against herbivorous insects, with immature holometabolous pest insects showing a high susceptibility by a lethal damage to the gut (Glare and O'Callaghan 2010). This considering, especially young honey bee larva are amenable as non-target test organisms for GM crop pollen, because they represent a potentially sensitive life stage. In addition to larvae, young hive bees consume the most pollen within colonies (Haydak 1970), thus young bees are also amenable for precautionary tests on biosafety. Nonetheless, Bt susceptibility in target insect adults is considered limited (Meissle et al. 2011), in comparison to the lethal effects on larval stages (Vaughn et al. 2005, Clark et al. 2006). To date, only minor fractions of peer-reviewed pollen feeding studies assess the risks on honey bee larvae (Malone and Burgess 2009). Studies on Bt-pollen feeding to larvae have solely been performed within colonies (reviews Duan et al. 2008, Malone and Burgess 2009). In general, studies on the colony level are confounded by environmental influences and by nurse bees which remove the dietary treatments of the larvae. Thus, to be robust, laboratory bioassays need to exclude such uncontrolled factors as far as possible (Romeis et al. 2011). In this paper, to assess possible lethal and sublethal effects of GM crop pollen on the survival and prepupal weight of individual *A. mellifera* larvae, we adopted a controlled *in vitro* rearing bioassay (Aupinel et al. 2007, Hendriksma et al. 2011a). The test larvae were exposed by adding fresh Bt-maize pollen directly in their artificial diet. This approach simulates the natural way of pollen consumption, whereby pollen is digested within the gut and Bt-proteins get exposed. Mechanistically, this is of key importance as the lethality among target-organisms is caused by the disruption of the gut epithelium by Bt-protein-receptor interactions (De Maagd et al. 2001). This study fills an important gap in ERA's on bees, as laboratory feeding tests of Bt-pollen on bee larvae are completely lacking.

Materials and Methods

Pollen

Multiple pollen types were collected for the *in vitro* pollen feeding experiment (Table 1). Pollen of field grown maize varieties were collected by shaking flowering maize tassels in paper bags. The freshly collected maize pollen was sieved (Ø 0.32 mm). Preceding storage at -80° Celsius, the pollen was dehydrated for 24 hours at room temperature to prevent the grains to burst at freezing.

Pollen of the single transgenic Bt-maize event Mon810 (DKc7565, cultivar Novelis, Monsanto Co.) was collected on July 24th 2008 near Kitzingen (Lower Franconia, Germany). This maize variety expresses Cry1Ab proteins for the control of stemborers such as the European corn borer *Ostrinia nubilalis*

(Hübner) (Lepidoptera: Crambidae) at concentrations of 1–97 ng/g fresh weight (fwt) in pollen (Nguyen and Jehle 2007).

Pollen of a stacked Bt-variety was collected in the week of August 4th 2008 near Braunschweig (Germany). This maize variety expresses three genes for insect resistance and one gene for herbicide tolerance and was obtained by a traditional cross of the maize varieties Mon89034 and Mon88017. Line Mon89034 confers resistance to a wide range of butterflies and moths, such as the fall armyworm (*Spodoptera sp*.), the black cutworm (*Agrotis ipsilon*), the european corn borer (*Ostrinia nubilalis*) and the corn earworm (*Helicoverpa zea*) by expression of the Bt-proteins Cry1A.105 at a level of mean 4.24 µg/g (range 1.55-11.67, n=16, fwt in pollen) and Cry2Ab2 at a level of 1.19 µg/g (range 0.24-3.74, n=16, fwt in pollen). Cry1A.105 is a chimeric gene synthesized by combining 4 native Bt-gene domains of *cry1Ab*, *cry1F* and *cry1Ac* (Miranda 2008). This chimeric protein provides an increased activity against lepidopteran species compared to the original Cry1Ab protein as expressed in Mon810. The other parental line, Mon88017 (DKc5143), confers resistance to coleopteran pests, the Western, Northern and Mexican corn rootworms *Diabrotica spp.* (Coleoptera: Chrysomelidae) by the expression of the Bt-protein Cry3Bb1 at levels of 6.95 µg/g (range 1.11-13.13, n=16, fwt in pollen) (trademark YieldGard ® Rootworm) (see acknowledgement for the Cry-protein measurements in the stacked Bt-maize pollen). Mon88017 also expresses an *Agrobacterium sp.* CP4 derived 5-enolpyruvylshikimate-3-phosphate synthase (CP4 epsps) confering tolerance against glyphosate, the active ingredient of the herbicide Roundup (trademark Roundup Ready®) at an expression level of 170 µg/g (fwt in pollen).

Stacked Bt-maize pollen and also control pollen of three conventional maize varieties was collected in the week of August 4th 2008 near Braunschweig (Germany). These maize varieties were grown on an experimental field in a randomized block-design with eight replications. Samples were collected from all 30x40 m subplots, pooling the pollen into one representative sample per variety. The non-GM variety DKc5340 (Monsanto Co.) is near-isogenic to the tested stacked Bt-maize variety, DKc4250 (Monsanto Co.) is more distantly related and Benicia (Pioneer HiBred, Johnston, Iowa, USA) is totally unrelated to the stacked event (Table 1).

Pollen of the neotropical plant *Heliconia rostrata* was collected June 23rd 2009 from the greenhouse in the botanical garden of the University of Bayreuth (Upper-Franconia, Germany). The *Heliconia* family is known to have chemical defenses against herbivores (Auerbach and Strong 1981) and anecdotal brood mortality is known for *Heliconia* foraging honey bee colonies. The pollen of the flowers was collected in a 1.5 ml tube by shaking and scraping pollen from the anthers with a scalpel (45 mg pollen from 41 flowers).

In vitro larvae rearing and treatment applications

The rearing of larvae upon hatchment under laboratory conditions was performed following the protocols by Aupinel et al. 2007 and Hendriksma et al. 2011a. Six donor honey bee colonies were selected from different Upper-Franconian apiaries, choosing naturally mated non-sibling queens (*Apis mellifera carnica*). By means of an excluder lid, the queens were trapped within their colonies on artificial combs (Nicoplast©) (day 1; D1, 25th June 2009). After 91 hours, without grafting manipulation, larvae within plastic queen cups were collected from the combs. Considering a 72 hours development time of the embryos until the hatchment of eggs, the larvae had a mean chronological age of 9:30 hours (D4; min. 0 to max. 19 hours old) and were typically first instars (Hendriksma et al. 2011a).

The subsequent laboratory rearing was performed with larvae in queen-cups mounted in culture plates, placed in a hermetic plexiglass desiccator within an incubator at 35° Celsius. The larvae were fed once a day over D4 to D9 with a 10-, 10- 20-, 30-, 40-, 50-µl semi-artificial diet, respectively (Aupinel et al. 2007). The daily diets were administered with pipettes, adding each new diet into the diet in which individual larvae were floating. Each larva was fed the total amount of 160µl, since no diet was removed during or after the feeding period. The diet consisted of 50% royal jelly (Le Rucher du Buzard certified organic apiary, Sospel, France) mixed with a 50% aqueous solution. The yeast extract / glucose / fructose proportion in the aqueous solutions was respectively 2/12/12 percent at D4 and D5; 3/15/15 percent at D6; and 4/18/18 percent at D7, D8 and D9 (Aupinel et al. 2007). During larval development, relative humidity in the incubator was kept at 96% using a saturated solution of K_2SO_4. Further development upon hatching took place in 80% humidity, maintained using a saturated solution of NaCl. The survival of larvae preceding treatment was 97% (D4 to D6). For more details about the method please see Hendriksma et al. 2011a.

For each treatment, a stock solution of 50 mg pollen per 500µl D6-diet was made. This application is in agreement with empirical findings that the food of larvae contains pollen from the third instar stage onwards (D6) (Simpson 1955, Jung-Hoffmann 1966). In this way each 20µl treatment diet contained a 2 mg pollen dose per larvae (Babendreier et al. 2004). Mean pollen numbers per dose were obtained by 8 sample counts per stock solution using a Neubauer improved counting chamber and a light-microscope (Table 1). The larvae were only once given a dietary pollen dose (D6). Because the larvae did not finish their daily dietary amounts within 24h (Picture 1), the pollen were consumed over the remaining total exposure time, until the diet was completely finished at the non-feeding days D10/D11. The maize pollen varieties were tested on N=20 larvae per treatment (5 colonies x 4 larvae). *Heliconia*

pollen and a no-pollen control treatment were performed on N=10 and N=12 larvae respectively (Table 1).

The survival of larvae during the experiment was noted daily, to assess possible lethal effects of Bt-maize pollen during the 120 hours of dietary exposure. By weighing the prepupae after defecation (D11), a potential sublethal effect was monitored. As larvae defecate and molt their intestine at this stage, both the exposure and the potential Bt-protein-receptor based mechanism are physically terminated. Hence, the effective gain in weight can only measured after defecation. Every prepupa was transplanted with soft metal tweezers into a new clean cell on an analytical microbalance to measure the weight to the nearest 0.001 g.

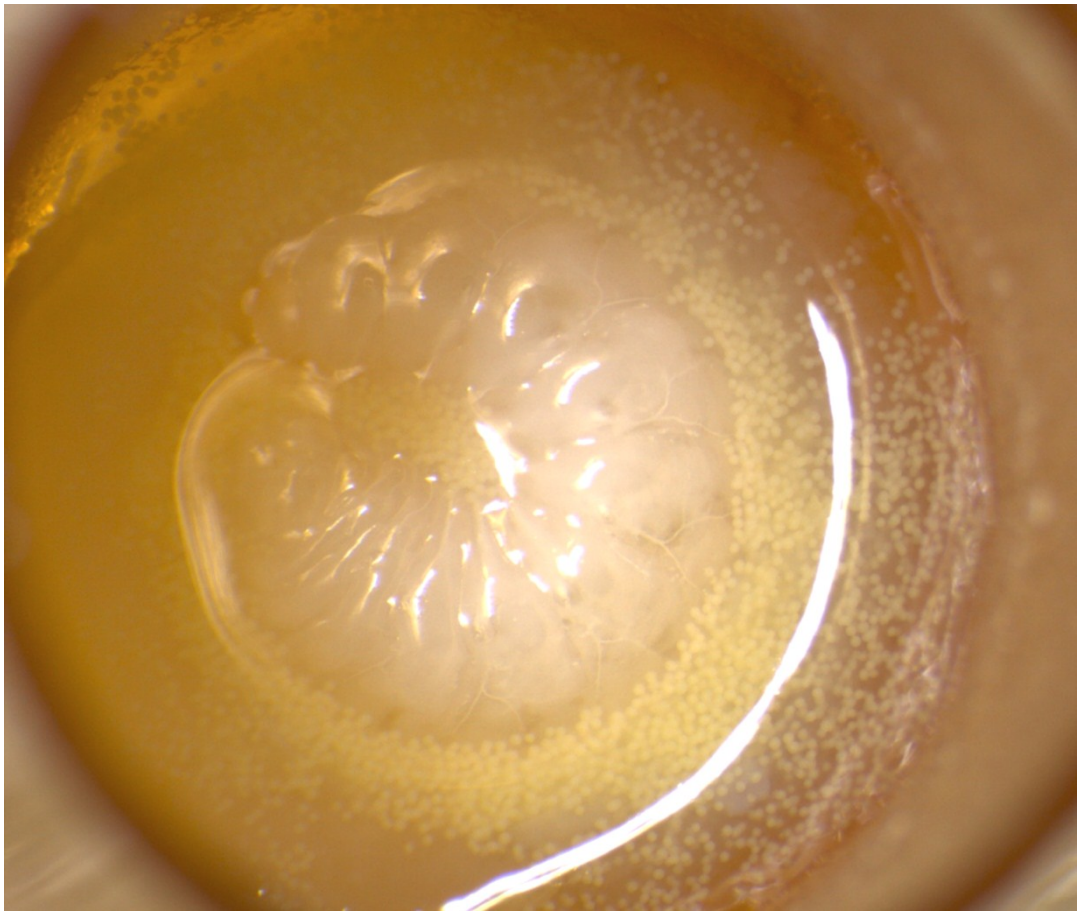
Statistics

The data were analyzed with mixed models using different packages of the open source statistic software R version 2.11.1 (R Development Core Team 2010). The identity of the replicate donor colonies was included as a random factor in the models to take the non-independence of larvae from individual colonies into account (Hendriksma et al. 2011a). Prepupae weights were analyzed with linear mixed effects models using the package *nlme* (Pinheiro et al. 2011). The survival dynamics of larvae were analyzed with Cox proportional hazards regression models (Fox 2002) using the R packages *survival* and *survnnet* (Ripley 2004, Therneau and Lumley 2009). A dynamic survival analysis is not applicable when all individuals of a group survive; in that case a Chi-square analysis was used. Three test levels were considered. An overall sort-effect was tested over the five maize varieties. All treatments were also tested individually, paired to one another, to indicate sort effects. The significance of *P* values (α=0.05) of multiple comparison were determined with an α-correction using the sequential Holm-Bonferroni procedure (Holm 1979). In case of no detectable difference, the treatment comparisons were summarized by evaluating the pooled data on Bt-maize pollen with control maize pollen data, also pooled.

Table 1. Feeding treatments of *in vitro* reared honey bee larvae for the Bt-pollen bioassay.

	Treatment [a]	Plant variety	Larvae	Colonies	Pollen/2mg
1	Transgenic maize	Stacked Bt; Mon89034xMon88017	20	5	1701
2	Transgenic maize	Single Bt; DKc7565	20	5	1750
3	Control maize	Near isogenic line; DKc5340	19	5	1784
4	Control maize	Distant related; DKc4250	20	5	1753
5	Control maize	Unrelated; Benicia	20	5	1722
6	No pollen control	-	12	6	0
7	Positive toxic control	*Heliconia rostrata* (H)	10	5	1600
1,2	Pooled Bt-maize	Transgenic maize (Bt)	40	5	1726
3,4,5	Pooled control maize	Control maize (C)	59	5	1753

[a] Treatment maize 1 expresses three Bt-proteins encoded by the genes *cry1A.105*, *cry2Ab2* and *cry3Bb1* from *Bacillus thuringiensis* that confer resistance against certain lepidopteran and coleopteran pests and additionally expresses the *CP4 epsps* gene for glyphosate-tolerance. Treatment maize 2 expresses a single lepidopteran specific Bt-toxin encoded by the gene *cry1Ab*. In addition, control treatments, tested plant varieties, number of larvae, colonies and counted pollen grains per 2 mg pollen treatment are indicated.



Picture 1: A honey bee larva (L4), consuming 2 mg maize pollen added into the semi-artificial diet.

Results

Survival

All 40 larvae fed with Bt-maize pollen survived the 120 hours of dietary exposure upon the prepupae phase (Fig.1). The survival rate of the conventional maize pollen fed larvae did not differ significantly from Bt-maize pollen fed larvae {C: 56 out of 59; 95%} (*Chisq* = 0.72, *df* = 1, *P* = 0.40). Of all the maize pollen fed larvae (N=99), in total 97% survived until the prepupal phase. Specific survival rates were: for stacked Bt-maize 100%, near-isogenic line 100%, Mon810 100%, DKc4250 95%, and for Benicia 90%. Thus, no significant difference among the five maize pollen varieties was found (*Chisq* = 5.41, *df* = 4, *P* = 0.28).

Among the larvae fed with diets without pollen, the individual survival dynamics and the survival rate of 92% did not differ compared to larvae fed with maize pollen enriched diets (all *P* values ≥ 0.64). In contrast, significantly fewer larvae survived the larval phase when they were fed with *H. rostrata* pollen compared to the other six treatments (*P* values ≤ 0.01, all significant with an α/6 sequential Holm-Bonferoni correction) (Fig.1).

Sublethal effects on the prepupae weight

With a mean of 142.3 mg, prepupae weights of Bt-maize pollen fed larvae were almost identical to the mean weight of conventional maize pollen fed larvae (142.6 mg; *t* = -0.20, *df* = 1, *P* = 0.82) (Table 2). A general variety-effect, considering possible differences between the five maize varieties, was not found (*F*=0.26, *df* = 4, *P* = 0.90) thus the weight distributions of the transgenic and non-transgenic maize pollen treatments were all alike (Fig. 2). Individual comparison shows that mean prepupae weights differed neither between stacked Bt-pollen and pollen from the near-isogenic line (*t* = 0.83, *df* = 33, *P* = 0.41), nor between the stacked Bt-variety and the single Bt-variety (*t* = 0.81, *df* = 34, *P* = 0.42) (Table 2). In contrast, *H. rostrata* pollen fed larvae showed a significantly lower mean prepupae weight compared to all the other treatments (mean 87.7 mg ± 21.0 SD; *P* values ≤ 0.001) (Table 2).

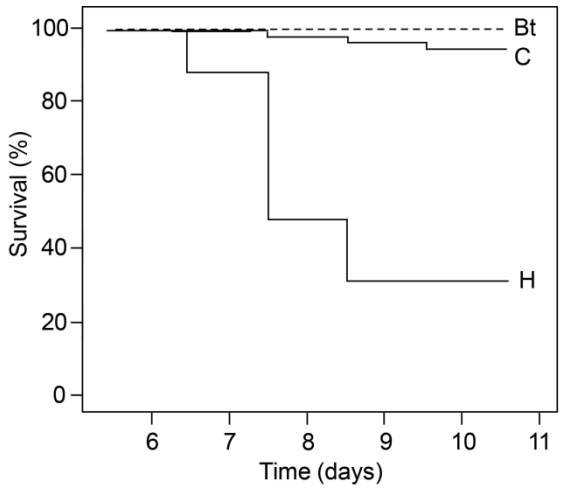


Figure 1. Survival analysis of honey bee larvae treated with pollen enriched diets. The dashed curve "Bt" indicates the 100% survival rate for Bt-pollen treated larvae (stacked Bt-maize expressing Cry1A.105, Cry2Ab2 and Cry3Bb1 and single Bt-maize expressing Cry1Ab were pooled; n=40 larvae). Curve "C" indicates survival for three conventional (control) maize pollen treatments (pooled n=59 larvae). No significant differences in survival rates were found among maize pollen treatments (neither individually, nor pooled). Compared to the other treatments, the larvae fed with the toxic *Heliconia rostrata* pollen (H; n=10) had a significantly lowered survival rate.

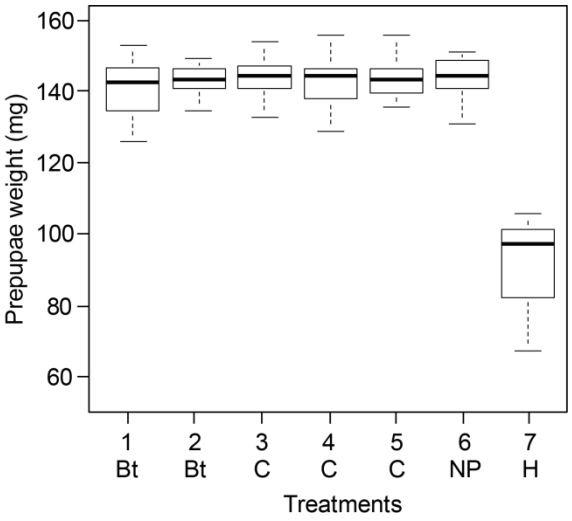


Figure 2. Prepupal weights (mg) of honey bee larvae fed with pollen. Treatments are Bt-maize pollen {Bt} (1 = stacked Bt-maize expressing Cry1A.105, Cry2Ab2 and Cry3Bb1; 2 = single Bt-maize expressing Cry1Ab) and non-GM maize pollen {C} (3 = near-isogenic line; 4 = distant related; 5 = unrelated) and two non-maize controls (6 = no pollen control {NP}; 7 = *Heliconia rostrata* {H}). The boxplots provide a graphical view of the median and quartiles with the error bars showing sample maximums and minimums. Prepupae weights did neither reveal a general Bt effect, nor single or stacked effects (GLMER: P values ≥ 0.41). *H. rostrata* pollen fed larvae had significantly lower weights compared to all other treatments (GLMER: P values ≤ 0.001).

Table 2. Prepupae numbers and weights after exposure to all individual dietary treatments, with a summarizing analysis for Bt-pollen (Bt) and non-GM pollen (C).

	Treatment	Prepupae weight Mean ± SD (n)	*P* values (GLMER with colony as random factor) 2	3	4	5	6	7	1,2	
1	Stacked Bt maize	141.4 ± 9.9 (20)	*0.42*	*0.41*	*0.90*	*0.88*	*0.86*	*<.0001**		
2	Single Bt maize	143.3 ± 4.9 (20)		*0.88*	*0.56*	*0.70*	*0.41*	*<.0001**		
3	Near isogene (stacked)	143.5 ± 4.9 (19)			*0.55*	*0.69*	*0.44*	*<.0001**		
4	Distant related maize	142 ± 10.5 (19)				*0.83*	*0.79*	*<.0001**		
5	Unrelated maize	142.4 ± 7.6 (18)					*0.64*	*<.0001**		
6	No pollen	140.6 ± 12.9 (11)						*0.0001**		
7	H: *Heliconia rostrata*	87.7 ± 21 (3)								
1,2	Bt: pooled Bt maize	142.3 ± 7.7 (40)								
3,4,5	C: pooled control maize	142.6 ± 9.1 (56)								*0.83*

* All *P* values are the results of paired tests: significances remain valid at the sequential Holm-Bonferoni correction of α/6 (considering the six comparisons per treatment).

Discussion

Honey bees are the most important pollinators in agricultural ecosystems. In order to minimize the environmental risks of cultivating GM crops and their discussed contribution of being an underlying factor of the globally observed bee losses, robust and highly standardized risk assessment methods for honey bees are imperative. Here we present an effective pollen based method to test the direct effects of GM crops on *in vitro* reared larvae. Our test system reflects the natural exposure under field conditions and is therefore highly recommended for regulatory studies.

Effects of pollen from single and multiple Bt-maize varieties on honey bee larvae

One recent trend in plant biotechnology is stacking of multiple insect resistance traits in a single cultivar (James 2010). Honey bees are exposed to mass flowering GM crops and not a single published study deals with the effect of stacked Bt-cultivars on bees. The results of this study did not indicate adverse effects of the consumption of single and stacked Bt-maize pollen on the survival and prepupae weight of *in vitro* reared *A. mellifera* larvae. At a realistic exposure dose, the 120 h survivorship of Bt-pollen treated larvae was 100% until the prepupae phase (Fig. 1). At the prepupae stage, where larvae had terminated feeding, digesting and growing, were no indications of a sublethal Bt-pollen effect on the weight of the prepupae (Fig. 2).

The outcome of our data on stacked Bt effects are in line with earlier brood tests under colony conditions on single insect resistant Bt-maize pollen (Hanley et al. 2003) or single purified Bt-proteins (Duan et al. 2008, Arpaia 1996). In contrast to these colony level studies, the current results are achieved by testing under controlled laboratory conditions, with minimum control mortality. Compared to single Bt-proteins in pollen or in purified form, our plant produced stacked Bt-proteins, with the chimeric Bt-protein Cry1A.105, indicate a similar level of safety. In accordance, a stacked maize variety, expressing Bt proteins VIP3A and Cry1Ab, also caused no adverse effects on the biodiversity of arthropods during a 3 year ERA field experiment (Dively 2005). A stacked cotton cultivar, expressing cowpea trypsin inhibitor (CpTI) and Cry1Ac in pollen carried no lethal risk for honey bees, though a worst case feeding regime did cause feeding inhibition (Han et al. 2010). However, in studies comparing Cry1 with transgenic protease inhibitors, it was found that only the latter was causing reduced feeding effects (Malone et al. 2001, Babendreier et al. 2005, Babendreier et al. 2008).

The stacking of insect resistance traits in one crop aims to enhance the effectiveness towards target pest insects, to cause an additive or synergistic toxicity. Among target pest insects, synergistic effects between e.g. Cry1Ab, Cry1Ac, Cry1F and/or Cry2Ab2 have been reported [*e.g.* Lee et al. 1996, Sharma et al. 2010). Involved in toxicant synergies are mostly uptake, transportation or degradation pathways (Andersen and Dennison 2004), causing a higher toxicity and a lower selectivity. Hence, potential synergistic effects on non-target insect also deserve consideration. The honey bee, a key non-target insect, has never shown lethality to Bt-proteins (Duan et al. 2008) and our data support the notion that, synergistic effects by stacking Bt-proteins at plant produced levels are unlikely a risk to bees. However, sublethal effects (Desneux et al. 2007) on feeding, learning performance and foraging behavior might occur (Ramirez-Romero et al. 2005&2007). Indeed, the *in vitro* approach covers the opportunity of testing of potential sublethal effects, by a subsequent behavioral tests on hatched bees (Brodschneider et al. 2009).

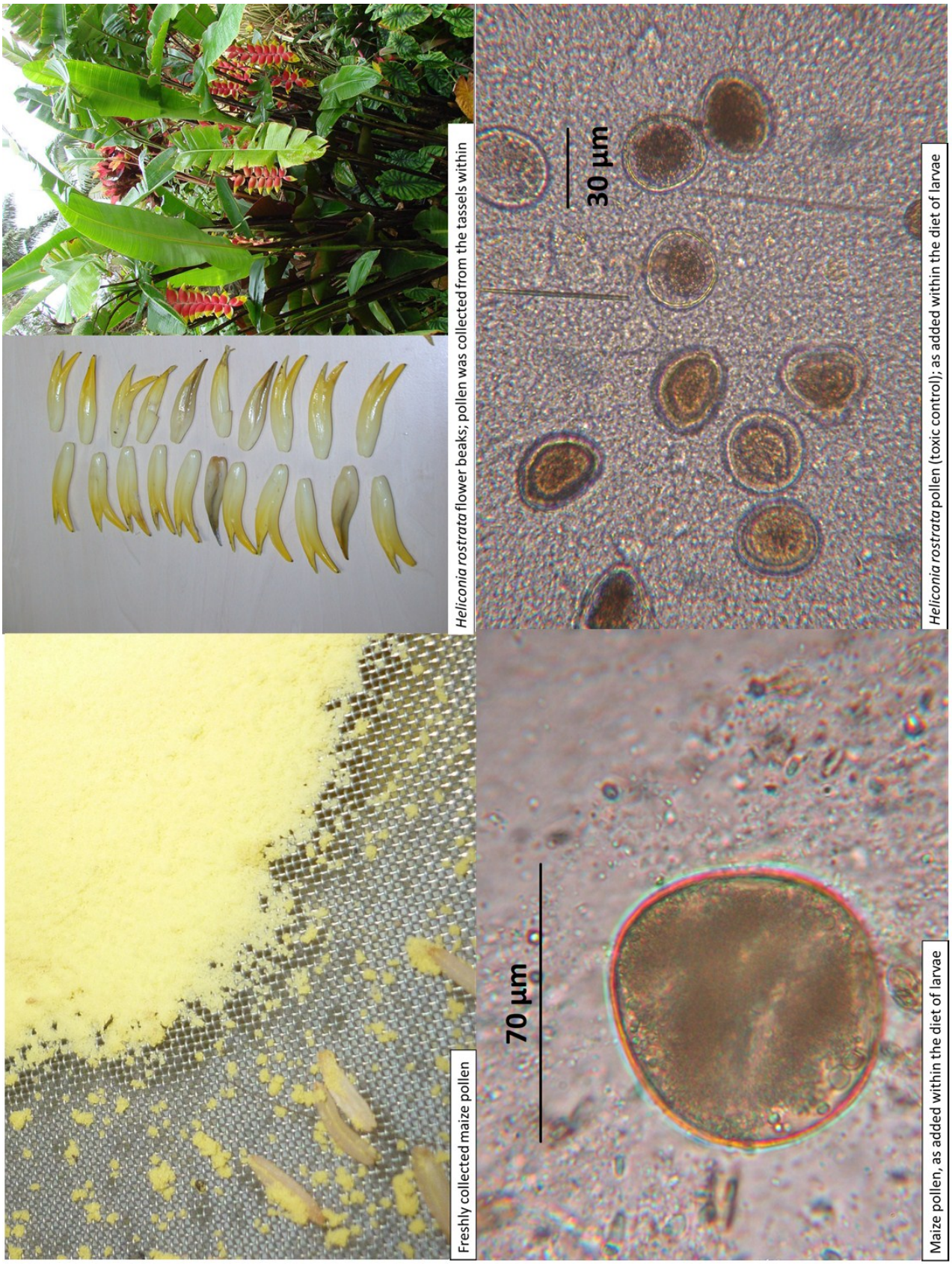
In order to examine a potential effect of increased protein expression levels, two Bt-maize varieties with different expression levels were compared. Bt-maize variety Mon89034 x Mon88017 has compared to Mon810 a 10^2 to 10^4 times increased Bt-protein expression level in pollen (see material and methods). Hypothetically, Mon810 could have had Bt-protein levels under a toxic threshold, but the larvae remain unharmed by the multifold Bt-protein of stacked Bt-maize pollen.

Pollen bioassays

The current bioassay tests GM plant material directly and realistically, by reflecting a natural consumption and digestion of pollen by *A. mellifera* larvae. It closes an important knowledge gap between *in vivo* colony experiments (e.g. Hanley et al. 2003, Arpai 1996) and *in vitro* experiments with purified transgenic proteins (Malone et al. 2002, Brodsgaard et al. 2003, Lima et al. 2011). Although purified proteins are ideal to test worst case exposure scenarios, the *E. coli* produced purified Bt-substances do not represent a field situation. And although field experiments have realistic pollen exposure conditions, a downside is a variety of uncontrolled environmental factors. In addition, pollinator field studies have to be synchronized to the flowering period and they are space and time consuming and therefore relatively costly (Romeis et al. 2008). Finally, within a bee colony many factors such as colony size, diseases, and nutrition could have an influence on the brood development. The presented bioassay minimizes environmental effects on larval development and allows a good control of dietary pollen amounts (Table 1).

The conventional non GM maize cultivars (Table 1) allow a secure assessment of the impact of the introduced transgenic traits (Rauschen et al. 2009). It makes assessments comprehensive, since it enables a reliable estimate of naturally occurring variation within the crop species. Though having tested the total of five maize varieties, no maize-sort related differences were found. Nevertheless, the toxic control treatment and the power analysis indicated that monitoring discernable effects of pollen on honey bee larvae was effective (Supplement Chapter V). The functionality of the pollen bioassay is proven by the feeding dose of 1600 *H. rostrata* pollen, which caused significant lethal and sublethal effects on larvae. This dose caused 50% of the larvae to die in 72 hours (LT_{50}) and 100% to die in 7 days (LT_{100}). The results demonstrate the usefulness of positive controls in order to i) validate the ingestion of pollen treatments, ii) to demonstrate the capacity of detecting treatment effects and iii) to allow comparisons with other studies (Romeis et al. 2011).

Precise and robust ERA methods are needed for honey bees (Hendriksma and Härtel 2010). Our bioassay is well suited to monitor environmental pollution of pollen or natural pollen toxicity. Of genuine concern are systemic, lipophilic chemicals (e.g. neonicotinoids) as used in agriculture, because the plant pollen are a carrier of pesticides into honey bee colonies. Such pesticides may cause (sub-) lethal effects and can be extremely persistent (Desneux et al. 2007). Our pollen test is widely applicable and it fits international tiered risk assessment schemes for regulatory biosafety assessments of any new transgenic trait. Hence, we propose the *in vitro* bioassay for consideration as a standard pre-release test for all polleniferous transgenic crops.



Supplementary pictures: Treatments *Heliconia rostrata* pollen and maize pollen (*Zea mays*).

Supplement Chapter V: POWER ANALYSIS

Statistical power was determined with the *pwr* package of the R software (Champely 2009). The mixed effect models with multi-comparisons, random effects and unequal sample sizes are represented by 2 power tests at a basic level by assuming a single comparison between a control and a treatment group with a same sample size. The survival power analysis was based on comparing a 100% survival of controls (proportion $p_0 = 1.0$) with a reduced treatment survival ($p_1 \leq 1.0$) using a one-tailed 2-proportions test. Accordingly, considering our empirically determined variance in prepupae weight data, a two-tailed t-test was used for power analysis on the determination of weight differences.

Considering the *H. rostrata*, the single maize pollen and the pooled Bt-maize treatment; with 0.8 power, sample sizes of 10 20 and 40 larvae can be used to indicate mortality effects >35%, >25% and >18% respectively (Fig 3a). Considering all maize pollen fed larvae (142 mg ± 8.5 SD, n=96), the 0.14% prepupae weight difference between the pooled Bt-maize and the controls maize treatments (Table 2; Bt *vs*. C) would need a sample size of >10.000 individuals for a significant result (α=0.05, 0.8 power). The bioassays' detection of 5.4% prepupae weight difference with 0.8 power at a sample size of 20 larvae per group, shows good prospects for indicating possible sublethal effects (Fig. 3b).

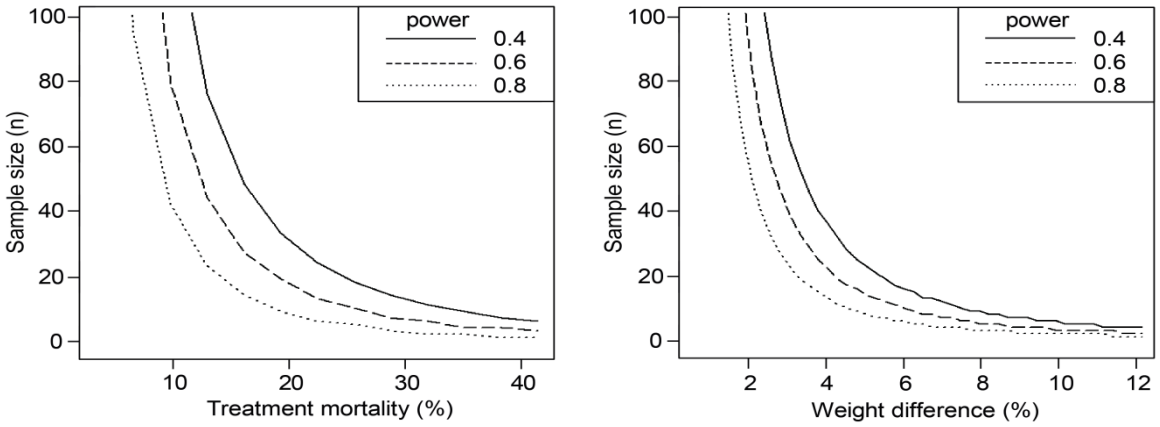


Figure 3: Power analysis for survival data (3a; left) and prepupae weight data (3b; right) of honey bee larvae reared on maize pollen enriched diets (3b). Both analyses are based on comparing a control and a treatment group with the same sample size. The survival power analysis (3a) was based on a one-tailed 2-proportions test on mortality rate differences. The weight difference power analysis (3b) was based on a two-tailed t-test on weight differences between the treatment group and the control. The sensitivity to measure the mg weight differences is relating to the general variance in weight of all maize pollen fed larvae (142 mg ± 8.5 SD, n=96). Determining treatment effects more sensitively at higher sample sizes, the curves indicate the level of power with dotted lines for 0.4, striped lines for 0.6 and a continuous line for 0.8 power at analysis.

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

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Keywords: *Apis mellifera*; *Bacillus thuringiensis*; environmental risk assessment; genetically modified crops; Cry-protein

Summary: 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 an 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.

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 (Syberg et al. 2009, 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 B-proteins than adults, with neonate larvae being more sensitive than older larval instars (Glare and O'Callaghan 2000). 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 multifold, and performed experiments that took into account protein interactions, the colony background of test individuals, thereby effectively monitoring honey bee biosafety.

Material and Methods

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. (2011a) (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. (2011a), 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 actively stopped digestion by a molt and defecation of the intestinal tract, which terminated the exposure to ingested products. 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 recognized by occasional black or white sub-dermal necrotic stains or a visible loss of turgor. Potential sublethal effects were monitored on day 11, by weighing each prepupa on an analytical microbalance to the nearest 0.001 g (Hendriksma et al. 2011a).

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.

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.

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 standardized 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. 2011a) (Table IIA). In case of toxicity, LD_{50} values were calculated, taking into account the colony dependence of test individuals (Hendriksma et al. 2011a), 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).

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 [d*0]	Highest dose	In pollen (d&fwt)[b]	Tested conc. [b]
[1] Cry1A.105	109	0.32 ng	d * 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	d * 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	d * 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	d * 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	d * 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]	d * 0 / 1 / 10 / 100 / 1000	No additive	B[5] / 10µl		
[7] BSA	62	800 ng	d * 0 / 1 / 10 / 100 / 1000	No additive	800 µg / 10µl		
[8] GNA-Lectin	92	80 ng	d * 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.

[b] Data Monsanto 2009

Results

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 $\leq 7.5\%$, 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 ($\chi^2 = 3.59$, *d.f.* = 5, $P = 0.61$) and their potential interaction with treatments ($\chi^2 = 37.0$, *d.f.* = 35, $P = 0.38$), nor the two trials ($\chi^2 = 0.70$, *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).

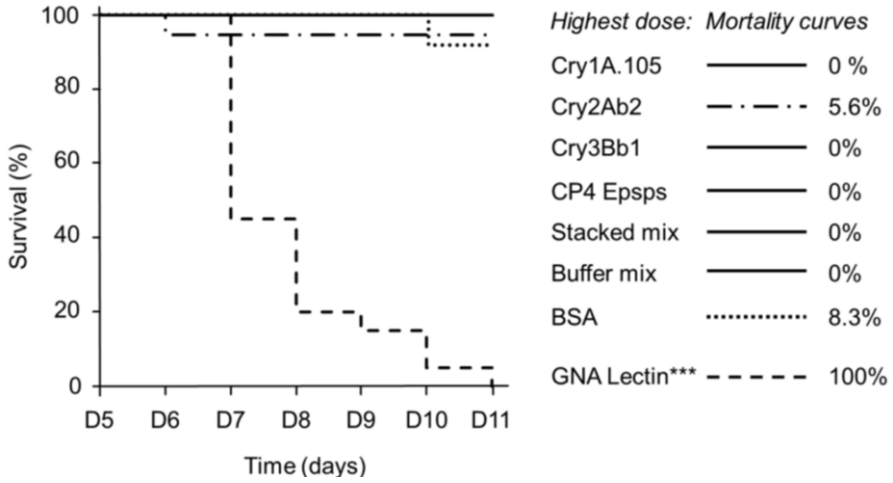


Figure 1. Survival of *in vitro* reared larvae following treatments on day 5 (D5) at highest concentrations.

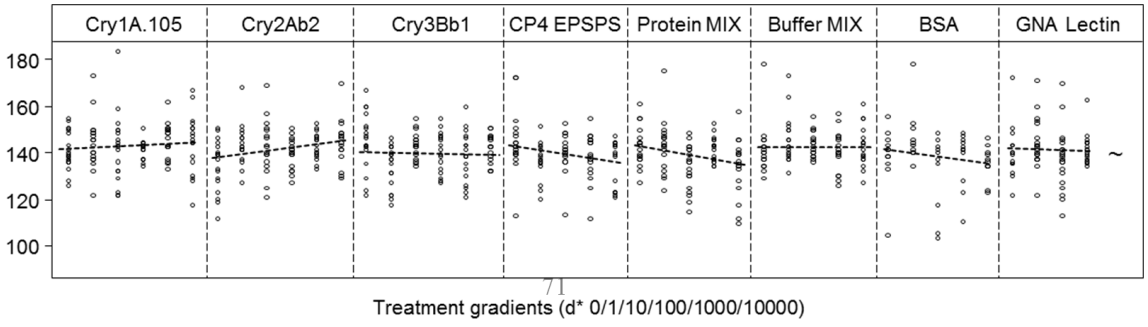


Figure 2. Prepupal weight of protein exposed larvae (n=709). Dose response effects of transgenic proteins on the prepupae weight of *in vitro* reared honey bee worker larvae are shown. Dotted lines indicate non-significant dose response result for each treatment at increasing concentrations (for treatment 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 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*	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%			

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) .

Discussion

Toxicity of Cry-proteins and CP4

The cultivation of GM-crops with insect resistances requires comprehensive biosafety assessments, with robust and highly standardized 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 sublethal 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 standardized 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 Africanized 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 (≤ 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).

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 bumblebee *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 commercialized, 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.

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 where minimizing contact with the larvae allows to optimize rearing success (Suppl. P) (Hendriksma et al. 2011a).

We started the Bt-protein applications at the second instar stage to reflect the natural exposure pathways in honey bees. 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 1995, 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.

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.

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 visualize 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 means (±SD) over the gradients, per treatment. Indicated in the matrix are mean prepupae weights per treatment dose. The gradient follows the exponentially increasing dose. 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)	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

Ch. VI, Supplement S1: *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 grey tone). 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* $\alpha/7$.

Post hoc Survival	Treatment 2		Treatment 3		Treatment 4		Treatment 5		Treatment 6		Treatment 7		Treatment 8		
	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)
	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)
	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	interaction
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)
			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)
			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
Treatment 3					3.23	0.072	0.00	0.968	0.37	0.541	1.97	0.160	55.66	0.000	factor (treatment)
					3.97	0.046	1.69	0.194	0.94	0.332	2.21	0.137	15.54	0.000	factor (gradient)
					1.20	0.273	1.80	0.180	2.97	0.085	1.86	0.173	30.49	0.000	interaction
Treatment 4							3.00	0.083	1.48	0.223	0.06	0.806	16.27	0.000	factor (treatment)
							0.83	0.362	0.50	0.480	1.03	0.311	27.48	0.000	factor (gradient)
							0.26	0.608	0.72	0.397	0.09	0.762	37.68	0.000	interaction
Treatment 5									0.25	0.619	1.80	0.179	35.56	0.000	factor (treatment)
									0.09	0.762	0.06	0.806	43.97	0.000	factor (gradient)
									0.05	0.822	0.06	0.809	10.99	0.001	interaction
Treatment 6											1.80	0.179	32.08	0.000	factor (treatment)
											0.06	0.806	43.62	0.000	factor (gradient)
											0.06	0.809	12.70	0.000	interaction
Treatment 7													17.37	0.000	factor (treatment)
													39.83	0.000	factor (gradient)
													22.34	0.000	interaction

Excellence in survival rate treatment [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.

Ch. VI, Supplement W1: *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

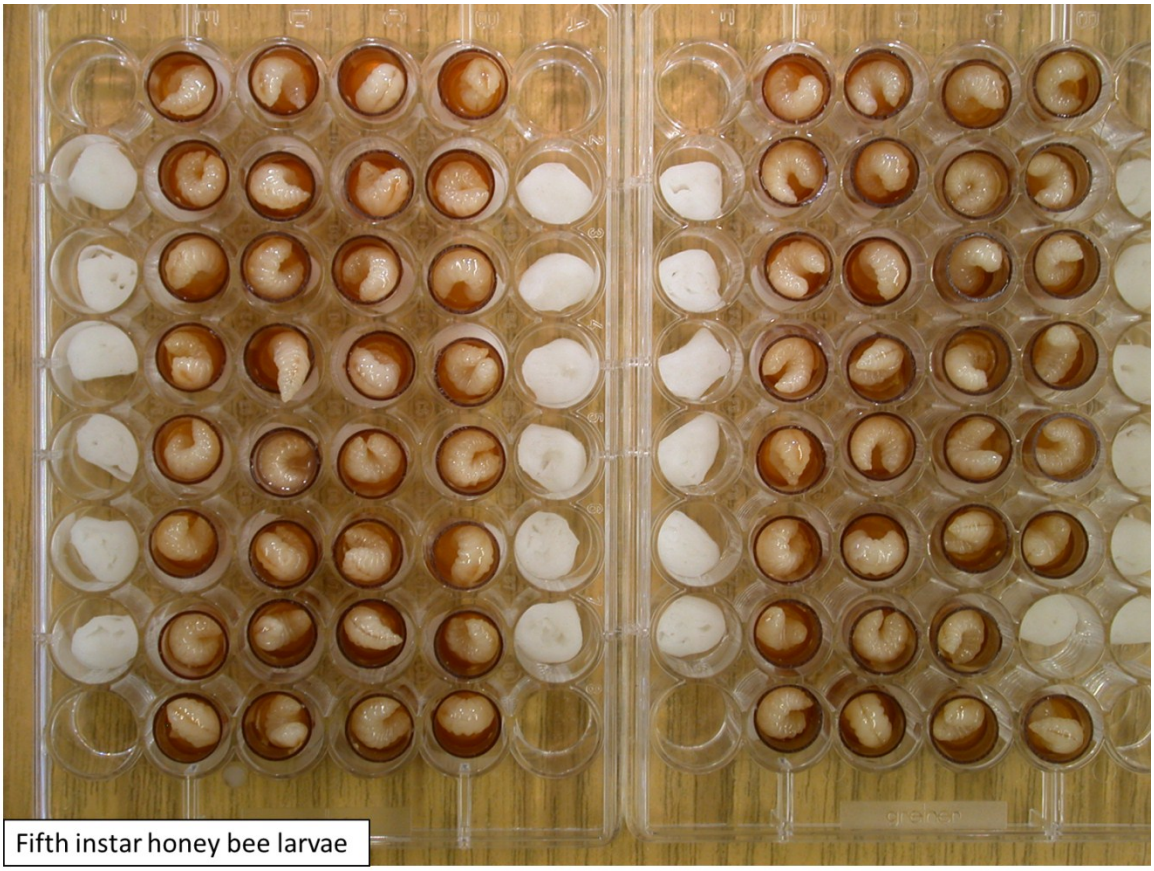
	C2	C3	C4	C5	C6	*mean*	*t-value*	*Colonies*
Colony 1: 139.7 mg ± 9.9 SD	1.0	1.0	0.003 **	1.0	0.12	*Δ 4.6 mg*	*3.42*	*C4/C1*
Colony 2: 140.4 mg ± 8.5 SD		1.0	0.024 *	1.0	0.45	*Δ 3.9 mg*	*2.83*	*C4/C2*
Colony 3: 140.6 mg ± 11.1 SD			0.029 *	1.0	0.51	*Δ 3.8 mg*	*2.77*	*C4/C3*
Colony 4: 144.3 mg ± 10.5 SD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

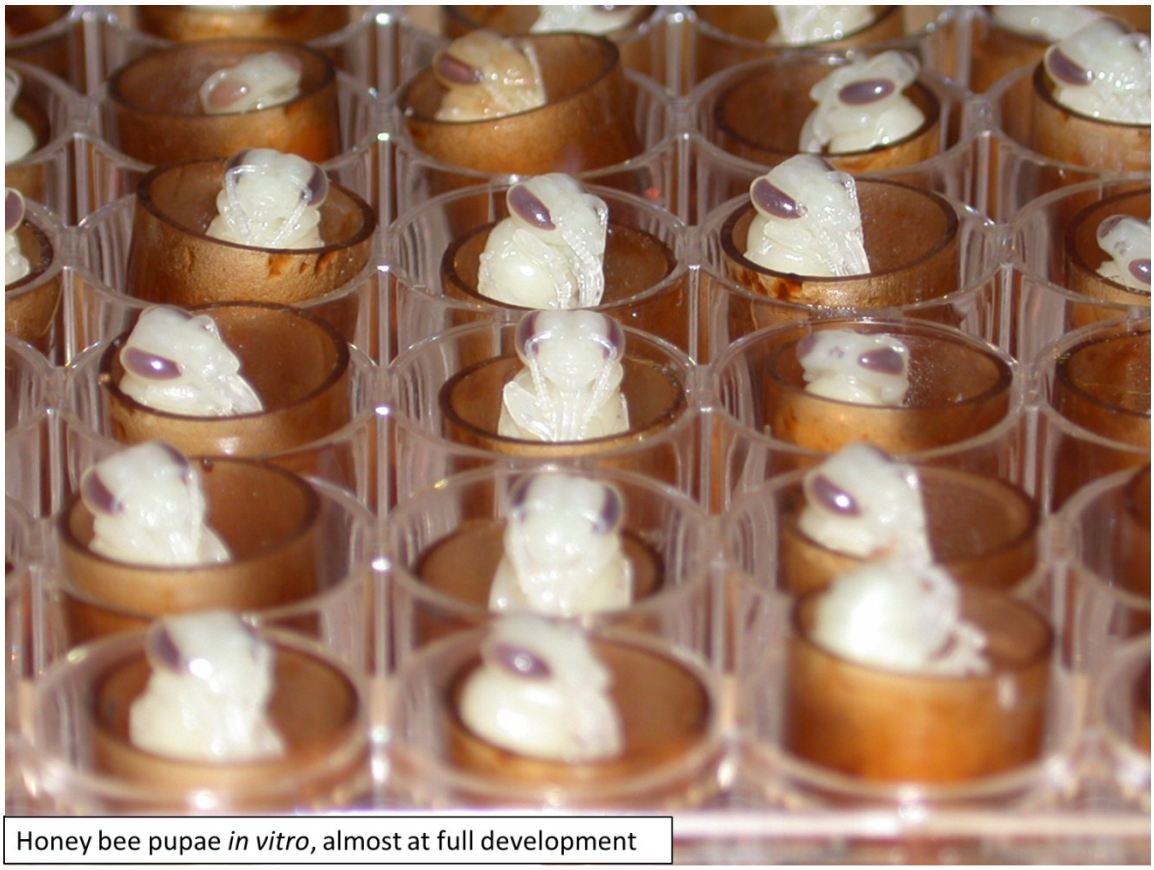
Treatment differences

		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									





Supplement P: Photographs honey bee larvea and pupae reared *in vitro*.

VII. Effects of genetically modified Bt-maize on pollen digestion and community structure of gut microbiota in honey bees.

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Summary: Biosafety research for transgenic crops rarely considers indirect effects on mutualistic microrganisms in non-target arthropods and responses of intact colonies of social insects. Further, no data for novel stacked Bt events are available. Here we analyzed genetically modified Bt-maize expressing three insecticidal proteins, Cry1A.105, Cry2Ab2, and Cry3Bb1 for adverse effects on honey bees (*Apis mellifera*) and their gut bacteria. During maize flowering, flight cages with standardized honey bee colonies were placed on an experimental field with Bt-maize Mon89034xMon88017 and two non-Bt maize varieties, respectively. As a control free-flying colonies were placed in a flowering *Phacelia* field. Nurse bees, which are known to process protein flows within colonies, were collected and analyzed after 10 days of exposure. Their mean body weight and the digestion rate of consumed maize pollen were unaffected by the particular maize varieties. Cry-proteins, quantified by ELISA, were mainly detected in the hindgut. The low Cry-protein concentrations found in the gut indicated their degradation by above 98%. Cultivation-independent analyses of PCR-amplified 16S rRNA revealed no differences in bacterial population sizes and bacterial community structure. Midgut communities harbored *Proteobacteria* while those in the hindgut contained *Lactobacillus* sp. and *Bifidobacterium* sp.. Natural bacteria related to *B. thuringiensis* caused the detection of Cry-proteins in bees not directly exposed to Bt-maize. Additional experiments indicated that already 50 sporulated cells of a *B. thuringiensis* ssp. *kurstaki* strain can suffice to explain positive results with the Cry1A.105-specific ELISA used. In conclusion, our data indicate that the combined expression of the three Cry proteins in Bt maize has no adverse effects on pollen digestion, community structure of symbiotic gut bacteria and gut functioning of honey bees.

Introduction

The release of novel GM crops requires an extensive environmental risk assessment of potential non-target effects. However, current risk assessment schemes do not consider effects on intact colonies of social insects and possible indirect effects on food digestion and mutualistic gut microorganisms. Insecticidal proteins derived from crystal delta endotoxins (Cry-proteins) of different subspecies and strains of the bacterium *Bacillus thuringiensis* are one of the major recombinant traits expressed in genetically modified (GM) crops to date (James 2010). In contrast to most conventional chemical insecticides which are sprayed, Cry-proteins are incorporated into the plant biomass and generally more specific towards certain insect groups, e.g., Cry1Ab against Lepidoptera, or Cry3Bb1 against Coleoptera. Due to this confinement, the use of Cry-proteins allows specific control of target pests with potentially less unintentional effects on non-target organisms (NTO) (O'Callaghan 2005). To confer resistances of a crop against several target pests, however, different Cry-proteins may need to be combined, which gives rise to genetically-modified stacked events. Examples already under cultivation in several countries are maize varieties (Bt-maize) such as Mon863xMon810, producing Cry1Ab and Cry3Bb1 proteins against both the European corn borer *Ostrinia nubilalis* (Crambidae, Lepidoptera) and the Western corn rootworm *Diabrotica virgifera* (Crysomelidae, Coleoptera), respectively. While Bt-maize producing single Cry-proteins (single events) have been evaluated for the environmental risks in detail, it is still an open debate whether the environmental safety of stacked events can be judged straightforward by evaluating properties of their single recombinant traits in an additive way, or whether the combination of different Cry-proteins may pose new risks for beneficial non-target organisms by unintended synergistic effects (De Schrijver et al. 2007).

One of the most important beneficial non-target insect to be considered for environmental risk assessment of insecticidal GM crops are bees, especially the honey bee (*Apis mellifera*, Apidae, Hymenoptera) (Duan et al. 2008; Romeis et al. 2008) with its high ecological and economic importance as pollinators and producers of honey (Klein et al. 2007; Malone and Pham-Delegue 2001; Steffan-Dewenter et al. 2005). Due the specificity of the currently used Cry-proteins towards Lepidoptera and Coleoptera in GM crops it was already assumed that there would be no immediate toxicity towards bees (Malone and Pham-Delegue 2001) and a recent meta-analyses of studies on the effects of purified or pollen enclosed single Cry-proteins concluded that there is to date no indication of chronic or acute toxicity neither for larvae nor adult bees (Duan et al. 2008). A feeding study with pollen from stacked Mon863xMon810 to caged worker bees showed no adverse effect on pollen consumption rates and body

weight (Lipinski et al. 2008), but it should be noted that Cry1Ab levels in pollen of Mon810 were very low (Babendreier et al. 2005) and, thus, response of bees to single and combined Cry-proteins at higher concentrations deserves further attention.

Honey bees are social insects and in order to search for potentially adverse effects, it appears sensible to conduct studies not with isolated bee individuals but with functional bee colonies under field conditions. Within functional colonies, a specifically high pollen exposure can be expected for nurse bees because of their central function to convert pollen into dietary proteins which they pass on to the bee brood. Due to this function, they consume the highest amounts of pollen. Exposure to potentially harmful pollen protein is also particularly high because nurse bees accumulate (digested) pollen material in their hindgut (Crailsheim et al. 1992; Brodschneider and Crailsheim 2010).

Therefore in this study we analyzed the effect of pollen from a stacked Bt-maize on nurse bees with special attention to their digestive tract. Since nurse bees will utilize the Bt-maize pollen as nutrient source, their Cry proteins must eventually be released into the gut lumen. In the digestive tract of target insects, Cry-proteins unfold their toxicity and thus, sublethal or indirect effects within NTOs such as honey bees may also occur here. The digestive tract of honey bees is functionally structured into different compartments including a midgut (ventriculum) and hindgut (rectum). A number of recent cultivation independent analyses have revealed that the gastrointestinal (GI) tract of bees harbors a bacterial diversity which seems highly conserved, independent of their geographical location (Babendreier et al. 2007; Jeyaprakash et al. 2003; Mohr and Tebbe 2006). The high similarity of the dominant bacterial community members in bees from different locations suggests a mutualistic relationship between these bacteria and their host (Martinson et al. 2011). Beneficial effects of gut bacteria for bees can be linked to contributing digestive enzymes, fermentation to acids, which can be utilized by the bees, or also to pathogen and parasite defense (Crotti et al. 2010; Dillon and Dillon 2004; Yoshiyama and Kimura 2009; Koch and Schmid-Hempel 2011). Due to this close relationship between gut microbiota and bees, alterations of the gut bacterial diversity may indicate environmental stress and/or occurrence of diseases (Hamdi et al. 2011) and, recently, such a response was in fact demonstrated for colony collapse disorder, a major threat to *A. mellifera* populations (Cox-Foster et al. 2007). Provided that the consumption of pollen from Bt-maize with Cry-proteins would have adverse effects on bees, as, e.g., caused by sublethal toxicity or even nutritional differences, these may therefore trigger a change in the community structure of gut microbiota and potential differences in the ability to digest pollen.

In this study, bee colonies were exposed to the stacked Bt-maize hybrid Mon89034xMon88017 during their flowering period in the field. The degradation of Cry-proteins within the digestive tract of the

bees was quantified by enzyme-linked immunosorbent assay (ELISA) targeting two of three different Cry proteins (Cry1A.105, Cry2Ab2 and Cry3Bb1) produced by the pollen. Control colonies were kept under the same conditions but with two conventionally bred maize cultivars and other colonies were kept close to a field with Lacy Phacelia (*Phacelia tanacetifolia*). The effect of the pollen consumption on gut bacteria was characterized independent of cultivation from directly extracted DNA of mid- and hindgut material, respectively. Bacterial populations were quantified by qPCR of their 16S rRNA genes and the structural diversity of the bacterial community was characterized by profiling, sequencing and phylogenetic analyses of the same genes. The results of this study were expected to give conclusive information on sublethal effects of cultivation of the stacked Bt-maize on bees.

Materials and Methods

Experimental field site and maize varieties

The experimental field with a total area of 6 ha was located on an agricultural site at the vTI (Johann Heinrich von Thünen-Institute) in Braunschweig, Germany. The field site consisted of 40 randomized field plots, each with an area of 30 m x 42 m. Five rows of plots were separated by 3 m plant-free stripes from each other, and the site was surrounded by an 8 m wide boarder of the conventionally bred maize cultivar Dkc4250. The plants cultivated on the plots were the Bt-maize hybrid Mon89034xMon88017, designated Bt, both in the genetic background of Dkc5143, the near-isogenic Dkc5143 without any genetic modification, both kindly provided by Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany, and the conventionally bred variety Benicia, kindly supplied by Pioneer Hi-Bred (Northern Europe Sales Distribution, Buxtehude, Germany). The Bt-maize event Mon89034 is genetically modified to produce the Cry1A.105 and Cry2Ab2 proteins while Mon88017 produces the Cry3Bb1 protein. As another recombinant trait, Mon88017 produces the enzyme 5-enolphyruvylshikimate-3-phosphate synthase originating from *Agrobacterium* CP4, which confers resistance to the herbicidal compound glyphosate. Cry1A.105 is a chimeric protein comprising domains of Cry1Ab, Cry1F and Cry1Ac (Miranda 2008). The concentration of the Cry-proteins in pollen from field grown Bt in 2009 (data from Sauer and Jehle, cited in Hendriksma et al. 2011 b) was 4.2 µg g^{-1} fresh weight for Cry1A.105, 1.2 µg g^{-1} for Cry2Ab2, and 7.0 µg g^{-1} for Cry3Bb1, respectively. The fresh weight of one maize pollen was set to 1 µg, which is in line with the 882 ng reported in another study (Babendreier et al. 2004), in order to calculate the amount of each particular Cry-protein per pollen. A flowering field with Phacelia (*P. tanacetifolia*) was located at 1,000 m distance from the experimental maize field.

Field exposure of bees

The maize varieties were sown on May 18, 2009. The flowering of Benicia started in the first week of August, followed by Bt and its near-isogenic line Dkc5143 one week later. During the mid of August, the varieties Bt and Dkc5143 peaked in their bloom.

Field cages (gauze with a mesh size of 1.3 mm) covering a 48 m^2 area with a height of 3 m, were installed on selected experimental plots of maize. For each of the three maize varieties tested, eight replicate cages were set up on different field plots. Each cage contained a total of 463 ± 27 maize plants, in six rows at a distance of 75 cm and a water source for bees. The total amount of available pollen per cage was estimated to be 2.7 kg (5.9 g/plant, Jarosz et al. 2003). The onset of colony exposition was synchronized to the phenology of the different test maize varieties. Five days before the maize flowering began (Benicia: August, 1; Bt, Dkc5143: August 8) standardized artificial swarms of *Apis mellifera carnica* were prepared without any pollen stores at the Institute for Apiculture, Celle, Germany. Each standardized colony contained one queen with approximately 1,100 workers (122.9 g bee biomass ± 7.2 SD, n=49 colonies). The polystyrene hives (24 x 15 x 17 cm, Apidea™ Vertriebs AG, Steinhausen, Switzerland) contained three empty frames (10 cm x 10 cm). All queens were sisters mated with a controlled drone population at an isolated mating apiary of the Institute for Apiculture. Within each hive, the bees had *ad libitum* access to a 72 % (wt/ vol) invert sugar (glucose, fructose) solution. As soon as 5 to 10 % of the total maize anthers were open, two honey bee colonies were placed into each flight cage.

Experimental set-up of the test bees

Freshly hatched worker bees (< 24 h) were collected from strong *A. mellifera carnica* donor colonies and marked with a pen. The integration into the test colonies was synchronized to the peak flowering time (BBCH 65) six days after colony exposure. At the point of introduction, the experimental bee colonies contained freshly build wax combs with maize pollen stores, stored sugar and open brood. After 9 days, all marked nurse bees were recollected from the colonies, individually weighed and frozen at -70°C.

Additionally, young worker bees from external colonies were analyzed as control group for Cry-protein detection within bee guts. This control group of bees had never been exposed to maize. All the samples originated from the Institute of Apiculture, and consisted of young in-hive bees from the donor-colonies which were used to make the experimental test colonies. On August 1, August 8 and September 28, each time eight bees were sampled immediately frozen and kept at -70°C until further analyses.

Preparation of nurse bees' gut contents

Immediately after thawing, the nurse bees were dissected. The midgut (ventriculus) and hindgut (rectum) were cut at the level of the ileum, and both were separately transferred to sterile 1.5 ml polypropylene snap cap tubes and kept on ice. A total of 300 µL sterile PBST buffer (137 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 10 mM KH_2PO_4, 0.5 % Tween-20, pH 7.4) was added, and the gut material was manually stirred with a sterile pipette tip to assure a high level of separation of pollen and gut bacteria from parts of the gut epithelium, followed by 20 s vortexing at highest setting (Vortex Genie 2, Scientific Industries, Bohemia, NY). A total of 50 µL aliquots of the suspensions were removed for pollen analyses and stored at -20°C. The remaining liquid was centrifuged at 16,200 x *g* and 4°C for 10 min and the supernatants were removed for immediate quantification of Cry-proteins while the pellets were resuspended in 300 µL of the sodium phosphate buffer provided with the "FastDNA SPIN kit for soil" (MP Biomedicals, Illkirch, France) for the DNA extraction. The suspensions were then stored at -70°C.

Quantification of pollen in bees' gut content

To determine maize pollen exposure to bees, the pollen amount in mid- and hindgut samples was quantified in four subsamples per sample by microscopic examination. Complete pollen grains and fragments larger than half of a pollen grain were counted at 100 x magnification in a Neubauer improved haemocytometer (Carl Roth, Karlsruhe, Germany).

Additionally, more than 40,000 maize pollen, originating from a total of 354 nurse bee hindgut samples, were analyzed for digestion efficiency. Depending on the level of digestion the pollen were scored as not digested (N, 0-10 %), partly digested (P, 10-90 %) or totally digested (T, 90-100 %). The scores were combined into a weighted average digestion rate per bee (Babendreier et al. 2004; Crailsheim et al. 1993). Honey bee worker data must be considered non-independent, when multiple observations are taken from the same colony (Zuur et al. 2009; Hendriksma et al. 2011a, b). Therefore the degree of maize pollen digestion was analyzed by a Linear Mixed Effects Model (LMM) to integrate the nested data structure.

Quantification of Cry-proteins

For quantification, a total of 100 µL of the supernatants obtained from the gut content in PBST buffer were immediately subjected to ELISA (enzyme-linked immunosorbent assay) targeting the Cry-proteins Cry1A.105 and Cry3Bb1, respectively. The test systems were double antibody sandwich ELISA, supplied by Monsanto (St. Louis, Missouri). Optical densities (OD_{450}) of standard Cry-protein dilutions were

measured at 450 nm to determine Cry-protein concentrations in unknown samples. The detection limit (DTC) was determined for each ELISA test plate as described elsewhere (Vogelgesang and Hadrich, 1998). The average DTC for Cry1A.105 was 0.56 ng mL^{-1}, corresponding to 0.17 ng Cry1A.105 per bee gut, and for Cry3Bb1 it was 0.40 ng mL^{-1}, corresponding to 0.12 ng Cry3Bb1 per bee gut, respectively. A one way analysis of variance (ANOVA) was used to compare for differences in Cry-protein contents higher than the DTC implementing the Holm-Sidak method for pairwise multiple comparisons (SigmaPlot, Systat Software, Erkrath, Germany). It was confirmed by testing, that both ELISA systems for Cry1A.105 and Cry3Bb1 did not cross react with the other two Cry-proteins expressed by Mon89034xMon88017.

The correlation of the number of pollen and the concentration of Cry1A.105 and Cry3Bb1 was established in analyses of 32 individual nurse bees and determined by a linear regression analysis in SigmaPlot (Systat Software). As a precondition for analyses, the normal distribution of data was confirmed by a Shapiro-Wilk test. The analysis was double-checked on non-detects (values below the DTC) bias, by omitting values < DTC, or their replacement by 0.55 x DTC or zero (Helsel, 2005).

DNA extraction and microbial community analysis

The frozen pellets containing the bee intestinal content including bacteria were thawed and 650 µL sterile PBST buffer was added. The suspensions were stirred and after centrifugation for 10 min at 100 x *g* the DNA was extracted from the supernatants using the "FastDNA SPIN kit for soil" (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. The extraction procedure included two bead beating steps of 45 s at 65 rev min^{-1} on a FastPrep-24 system (MP Biochemicals, Solon, Ohio) and an additional washing steps of the binding matrix with 1 ml 5.5 M guanidine thiocyanate (Carl Roth). DNA in the solutions was quantified with the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Epsom, United Kingdom). An aliquot of cells yielded 100 µl DNA solution of approx. 8 ng μL^{-1} DNA from midgut and 9.4 ng μL^{-1} from hindgut. The extracted DNA was stored at 4°C.

Analyses of the bacterial abundance and diversity

From each of the four treatments, 24 nurse bees were selected from different honey bee colonies for the analyses of their hind- and mid-gut contents, respectively. The abundance of the bacterial 16S rRNA genes was determined by a quantitative real-time PCR (qPCR) applying the Maxima SYBR Green/Fluorescein qPCR MasterMix (Fermentas GmbH, St. Leon-Rot, Germany) and 0.3 µM of each of the universal bacterial primers F27 (AGA GTT TGA TCM TGG CTC AG (Weisburg et al. 1991) and

Eub338rev (CTG CTG CCT CCC GTA GGA GT) which excluded 18S rRNA genes of maize and bees successfully. A total of 2 µL of template DNA, undiluted for midgut and 10^{-1} diluted (TE-buffer: 10 mM Tris, 1 mM EDTA, pH 8) for hindgut samples, were used in 25 µL reaction volume. All PCR were conducted in duplicates. Amplifications were carried out in a Bio-Rad MyiQ cycler (Bio-Rad, Munich, Germany) under the following conditions; 15 min at 95°C; 40 cycles of 35 s at 94°C, 35 s at 57°C, 45 s at 72°C, 15 s at 83°C; and a final step of 5 min at 72°C. Standard curves were obtained from 10-fold dilutions of the pGEM-T vector (Promega, Mannheim, Germany) containing the 16S rRNA gene of *Bacillus subtilis* BD466 (*Escherichia coli* positions 8 – 1513) (Brosius et al. 1981). Data were processed with the iQ5 software version 2 of the MyiQ cycler (Biorad).

Terminal restriction fragment lengths polymorphism (T-RFLP) was used to estimate the diversity of the dominant bacterial community members. The bacterial 16S rRNA genes were amplified by PCR using the Cy5-labeled forward primers 27F (Cy5-AGA GTT TGA TCM TGG CTC AG) and the unlabeled reverse primer 1378R (CGG TGT GTA CAA GGC CCG GGA ACG) (Heuer et al. 1997) resulting in PCR products corresponding to positions 8 to 1401 of the 16S rRNA gene of *E. coli* (Brosius et al. 1981). As for the qPCR system, primer F27 excluded the 18S rRNA genes. PCR were performed in a total volume of 30 µL containing 2 µL of template DNA, 0.5 mM of each primer (synthesized by biomers.net GmbH, Ulm, Germany), each dNTP at a concentration of 0.2 mM (Carl Roth), 2 U of Hot Star *Taq* Polymerase (Qiagen, Hilden, Germany), the corresponding 1 x PCR buffer (incl. 1.25 mM $MgCl_2$) and additional $MgCl_2$ to a final concentration of 2 mM. The thermocycling consisted of an initial denaturation and enzyme activation step for 15 min at 95°C followed by 30 cycles of 45 s at 94°C for denaturation, 45 s at 52°C for primer annealing and 2 min at 72°C for elongation. A final extension step was performed for 5 min at 72°C. The PCR products were purified after electrophoresis on 1 % agarose gels in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8) including ethidium bromide (0.5 mg L^{-1}), following the respective protocol of the PCR Clean-Up & Gel-Extraction System (SLG, Gauting, Germany).

The purified PCR products were digested overnight at 37°C in a total volume of 30 µL containing 15 µL of the PCR product, 10 U of the restriction endonuclease *Msp*I (Fermentas) and the corresponding reaction buffer. The restriction products were purified by ethanol precipitation with 150 µl ice cold 95 % ethanol and 3 µL 3 M sodium acetate (pH 4.6) for 1 h at -20°C. Precipitates were collected by centrifugation at 11,600 x *g* for 30 min at 4°C. The translucent pellets were washed with 100 µL of 70 % ice cold ethanol, collected by centrifugation for 10 min and dried at room temperature. The pellets were then dissolved in 30 µl of Sample Loading Buffer (SLB, Beckman Coulter, Krefeld, Germany) for

T-RFLP analyses.

The purified restriction fragments were analyzed with the CEQ8800^{TM} Genetic Analysis System (Beckman Coulter) using supply optimized by the manufacturer for this application, including GenomeLab^{TM} Capillary Array, Separation Gel, Separation Buffer, SLS and Size Standard Kit – 600. The sizes detection of the Cy5-labelled terminal restriction fragments (T-RFs) and the quantification of the T-RF signal (peak heights as relative fluorescence units, rfu) were automatically carried out by the CEQ8800^{TM} fragment analysis software (Version 9.0). The maximum bin width was set to 2.0 bp and the data were exported to MS-Excel for normalization in order to compensate for differences in PCR product quantity and T-RFLP profile intensity among samples. Relative abundances were generated as the sum of all peak heights in each profile was set to 100 %. Terminal restriction fragments (T-RFs) representing less than 4 % of the total peak heights were considered as background noise and excluded from the analysis remaining an average of approx. 90 % relative fluorescence units for each profile. After normalization rare peaks that occurred in less than 3 % of all profiles (less than 5 of 192 profiles) were removed from the analysis as such peaks were of low reproducibility considering 24 replicate profiles for each group.

The normalized T-RFLP data were imported into the program package BioNumerics 5.10 (Applied Maths, Sint-Martens-Latem, Belgium) as a character-type experiment. Pearson correlation was used to generate similarity matrices and the corresponding dendrograms were based on UPGMA (unweighted pair group method with arithmetic mean). In order to detect significant differences ($P < 0.05$) of the bacterial community structures, a permutation analysis was performed on the basis of the similarity matrices as described elsewhere (Kropf et al. 2007). Average similarities between the different crop varieties were calculated from the respective similarity matrix and ANOVA was applied to identify significant differences (SigmaPlot).

DNA-sequencing of bacterial 16S rRNA genes and phylogenetic analyses

In order to assign the dominant T-RFs detected in this study to known bacterial 16S rRNA genes, representative DNA solutions obtained from each group to be tested (mid- or hindgut material, caged and uncaged bees) were selected for cloning and DNA-sequencing. The amplification of the 16S rRNA genes was performed with unlabelled primers 27F and 1378R as described above. The PCR products were purified as mentioned before, ligated to the pJet1.2/blunt vector and transformed into competent cells of *E. coli* JM109 in accordance to the protocol supplied with the CloneJet^{TM} PCR Cloning Kit (Fermentas). Plasmids were purified following the protocol of the HighYield$^{®}$-Plasmid Mini Kit (SLG, Gauting, Germany). Cloned DNA sequences were then analyzed by T-RFLP to confirm their respective T-RFs.

Clones representing prominent T-RFs were further selected for DNA sequencing. In order to compare the T-RFs of the cloned sequences to the theoretical fragment sizes obtained by *in silico* analyzes of the DNA sequences, all PCR products had to be sequenced in forward orientation. Therefore, an additional PCR was performed with primers pJET1.2 forward (CGA CTC ACT ATA GGG AGA GCG GC) and 27F, to check the orientation of the PCR products in the cloning vector. PCR reactions were performed according to the PCR mixture described above in a total volume of 15 µL containing 1 µL of template DNA. The thermocycling was conducted as follows: Initial step for 15 min at 95°C, 25 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C, and a final incubation for 5 min at 72°C. The DNA sequencing was done by GATC Biotech AG (Konstanz, Germany) and the sequences were imported to the MEGA4 software (Tamura et al. 2007) for deletion of primer sequences and alignments. The sequences were compared to public databases using the BLASTN routine at NCBI (www.ncbi.nlm.nih.gov/BLAST). Sequences with closest relatives of less than 97 % identity to database sequences were furthermore analyzed for chimera with the Pintail tool (www.bioinformatics-toolkit.org). All sequences generated in this study were deposited in the EMBL Nucleotide Sequence Database.

Detection and quantification of Cry-proteins from bees not exposed to Bt-maize and from Bacillus thuringiensis strains

In order to analyze whether proteins related to Cry1A or Cry3B from *B. thuringensis* can naturally occur in the digestive tract of honey bees, a total of 24 bees which had never been in contact with Bt-maize, kindly provided by the Institute for Apiculture (see above), were analyzed for presence of such proteins using the two ELISA systems of this study targeting Cry1A.105 and Cry3Bb1, respectively. In addition, serial dilutions of four *B. thuringiensis* strains known to produce natural Cry1A proteins were analyzed in order to test if natural proteins would respond accordingly the ELISA system designed to detect the chimeric Cry1A.105, and if, to determine the threshold of detection. The bacterial strains, all obtained from the DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), were two *B. thuringiensis* ssp. *kurstaki* strains HD-1 (DSM 6102) and HD-73 (DSM 6101), producing Cry1Aa1, Cry1Ab3, Cry1Ab4, Cry1Ab10, Cry1Ac13, Cry1Ia3,Cry2Aa2, Cry2Ab1, Cry2Ab2 or Cry1Ac1, Cry1Ac7, Cry1Ac8 respectively (Crickmore et al. 2011) . Comparably detailed information was not available for *B. thuringiensis* ssp. *aizawai* another subspecies used as a positive control, represented by the two strains DSMZ HD-11 (DSM 6099) and HD-282 (DSM 6100), respectively. This subspecies is also the original donor organism from which the chimeric Cry1A.105 protein was generated. *Bacillus subtilis* 168 (DSM 402) was included as a negative control.

All strains were cultivated aerobically at 28°C in liquid nutrient medium supplemented with $MnSO_4$ for better sporulation (5 g L^{-1} peptone, 3 g L^{-1} meat extract, 60 µM $MnSO_4$, pH 7.0) and growth of the cultures was followed by enumeration of cells in a Thoma counting chamber (Carl Roth). After 5 d of cultivation at 28°C the liquid cultures shifted to 4°C without shaking to enhance sporulation. The efficiency of sporulation of the cultures was evaluated by phase contrast microscopy and it was found that nearly 100 % for all strains were sporulated after this procedure. After confirmation, the spore/cell suspensions were diluted in PBST buffer and used for the Cry1A.105 ELISA to determine immunoreactive Cry-protein equivalents. The amount of Cry-protein equivalents was correlated to the cell counts after 30 h of growth when the stationary growth phase had been entered 24 h before. Later on, spores and protein crystals were difficult to be differentiated by phase contrast microscopy and thus, the determination of spore numbers would have been biased. All *B. thuringiensis* strains were analyzed in triplicates.

Table S1. Exposure numbers of individual nurse bees to maize pollen in flight cages within experimental field plots of maize (number of sampled colonies; number of sampled nurse bees)

Treatment (variety)	Midgut	Hindgut	Exposure to maize pollen
Bt (maize)	0 (4; 16)	31 (8; 32)	96 %
Dkc5143 (maize)	1 (4; 4)	32 (8; 32)	100 %
Benicia (maize)	4 (4; 4)	32 (8; 32)	100 %
Phacelia	0 (4; 16)	6 (8; 32)	19 %

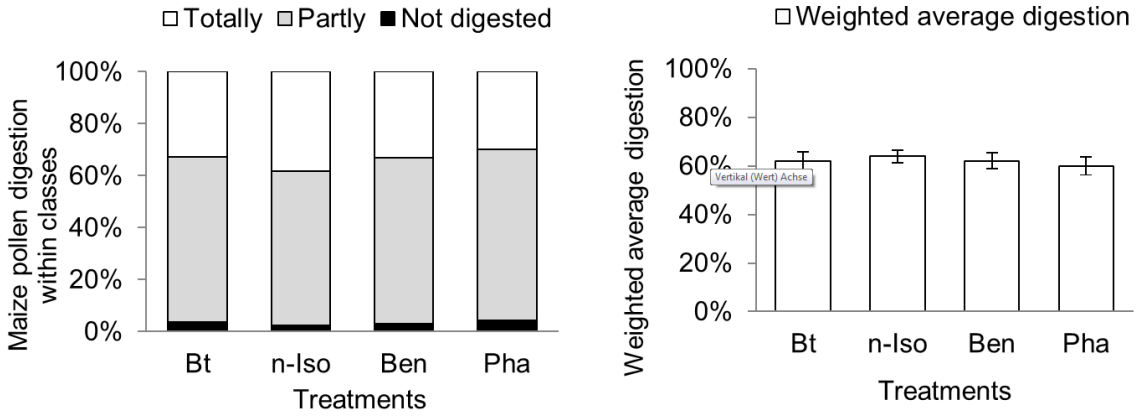


Fig. 1. Quantification of digested, partly digested and undigested maize pollen from the bee guts (shown left). The statistics were based on comparisons of the weighted average of maize pollen digestion (right hand side graph; with error bars indicating SD).

Results

Detection of maize pollen in the digestive tract and evaluation of their digestibility

The uptake of maize pollen by the nurse bees from colonies kept in cages of the particular maize field plots was high. Maize pollen was detected in the hindgut of 95 from 96 bees analyzed (Table S1). Prevalence of maize pollen was much lower in the midgut of the respective bees, where it was found only in 5 of the 96 individuals, indicating that pollen accumulated in the hindgut. Maize pollen were also found in colonies with free flying bees kept outside of the experimental maize field site next to the field with Lacy Phacelia (designated as treatment "Phacelia"), but the uptake of maize pollen was clearly lower, as it was only found in 6 of 32 bees analyzed. No maize pollen was detected in their midguts. The amount of maize pollen found in the hindgut of the nurse bees kept in Bt field plots was on average approx. 16,000 pollen grains, but the variability between individual bees was high, with a standard deviation of 80.9 %. Less pollen was detected in bees from the colonies kept outside of the maize field, where the mean number was 528 grains (± 71% SD). It should be noted that those maize pollen probably did not originate from plants of the experimental field site of this study, located in a distance of 1,000 m, because other maize fields where in closer vicinity.

The additional microscopic examination of the hindgut contents of 450 individual nurse bees revealed that 97% of the pollen analyzed was partly or fully digested (Fig. 1, Table S2). No differences in pollen digestibility were found between Bt, the near-isogenic counterpart Dkc5143 and Benicia, respectively. This also applied to the digestibility of the maize pollen from hindgut samples of nurse bees from the colonies with the free flying bees next to the *P. tanacetifolia* field.

Quantification of Cry-proteins

Nurse bees from colonies kept in the maize field plots as well as those from the free flying colonies were analyzed for the concentration of Cry1A.105 and Cry3Bb1 in their mid- and hindgut sections (Fig. 2). For the bees kept within Bt, 100 % of the analyzed hindgut samples were positive for Cry1A.105 while Cry3Bb1 was detected in 80 % of the samples. Average amounts of Cry1A.105 per hindgut were 0.91 ± 0.69 ng and for Cry3Bb1 0.29 ± 0.17 ng, respectively. The detection of Cry-proteins in the midgut was less frequent: A total of 66 % were positive for Cry1A.105 and 50 % for Cry3Bb1. In cases of positive detection, the respective amounts of the Cry proteins were not significantly different in mid- and hindgut, even though pollen numbers of the hindgut clearly exceeded those of the midgut. These results indicated that a proportion of Cry proteins were still detectable in the midgut, while the majority of pollen already

proceeded to the hindgut. Interestingly, the amounts of Cry1A.105 and Cry3Bb1 were comparable in the midgut, while significantly more Cry1A.105 than Cry3Bb1 was detected in the hindgut.

The relatively high variability of the Cry-protein concentrations which was 76 % (Cry1A.105) and 59 % (Cry3Bb1) in the hindgut of the nurse bees from the Bt plots can be explained by the different amounts of pollen ingested by the individual bees, as underlined by the significantly positive correlation of both Cry1A.105 and Cry3Bb1 to the respective pollen numbers in their hindguts (Fig. 3). Considering the concentrations of Cry-proteins of intact pollen (see Materials and Methods), the ingestion of 16,000 pollen grains per bee, digested on average by 62%, would have resulted in an expected release of 42 ng Cry1A.105 and 69 ng Cry3Bb1 into the gut lumen. However, the actual amounts detected were much smaller with 0.80 ± 0.62 ng for Cry1A.105 and 0.33 ± 0.21 ng for Cry3Bb1 in mid- and hindgut together. Based on these recoveries, degradation rates of the potentially released proteins during the gut passage were estimated to be 98.1 % for Cry1A.105 and 99.5 % of the Cry3Bb1, respectively.

Remarkably, Cry-proteins were also detected, even though less frequently, at comparable concentrations in bees with no direct exposure to Mon89034xMon88107 (Bt), i.e., from colonies of the neighboring non-Bt maize varieties Dkc5143 and Benicia, and also from the colonies of the free flying bees located outside of the maize field (Fig. 2). In contrast to the bees from the Bt plots, the two Cry-proteins from the other maize plots and those from the colonies outside were almost exclusively detected in hindgut samples, which were positive in 38 % of the maize field bees and in 60 % of the free flying bees for either one or both Cry-proteins, respectively. The result of the free flying bees was especially remarkable since in 68 % of the Cry-protein positive gut samples, no maize pollen grains were detected. Furthermore, there was no correlation between the concentrations of the Cry1A.105 and Cry3Bb1 proteins and the maize pollen numbers, as found for the bees from the Bt-exposed colonies (Fig. 3), suggesting other sources than pollen of Mon89034xMon88017.

Table S2. Digestion of maize pollen in hindgut samples of nurse bees analyzed in this study

Treatment	Colonies	Bees	Pollen*	Pollen percentage (±SD) in 3 digestion classes		
				Not	Partly	Totally
Bt	13	104	5,417	3.6 ± 3.9 %	63.4 ± 8.9 %	32.9 ± 8.3 %
Dkc5143	14	144	14,924	2.4 ± 2.3 %	59.5 ± 5.1 %	38.1 ± 5.3 %
Benicia	13	141	21,194	3.1 ± 3.3 %	63.8 ± 6.7 %	33.1 ± 6.7 %
Phacelia	9	61	86	4.2 ± 5.9 %	65.9 ± 7.2 %	30.0 ± 6.1 %

*Data of rated pollen used for the statistical analysis excluded individuals with <7 pollen grains.

Origin of Cry-proteins in bees

In order to explain whether the detection of Cry-proteins with the Cry1A.105 and Cry3Bb1 specific ELISA from gut contents of bees not directly exposed to Bt originated from maize pollen of Mon89034xMon88017 or from bacteria (*Bacillus thuringiensis*) present in the environment, a separate experiment with a control group was conducted. This control group, consisting of bees from colonies which had never been in contact with the respective or any other Bt-maize was analyzed for presence of Cry-proteins with the Cry1A.105 and Cry3Bb1 specific ELISA systems. From a total of 24 individual bees, hindgut samples of five bees were positive for Cry1A.105, and of six for Cry3Bb1. In the midgut, only two samples were positive for Cry3Bb1 and none for Cry1A.105. From bees with Cry proteins in the hindgut, four responded to Cry3Bb1 but not to Cry1A.105. Detection of Cry3Bb1 without detection of Cry1A.105 was never seen in bees from the Bt treatment, indicating that native bacterial expressed Cry-proteins might have caused the positive ELISA results with this control group.

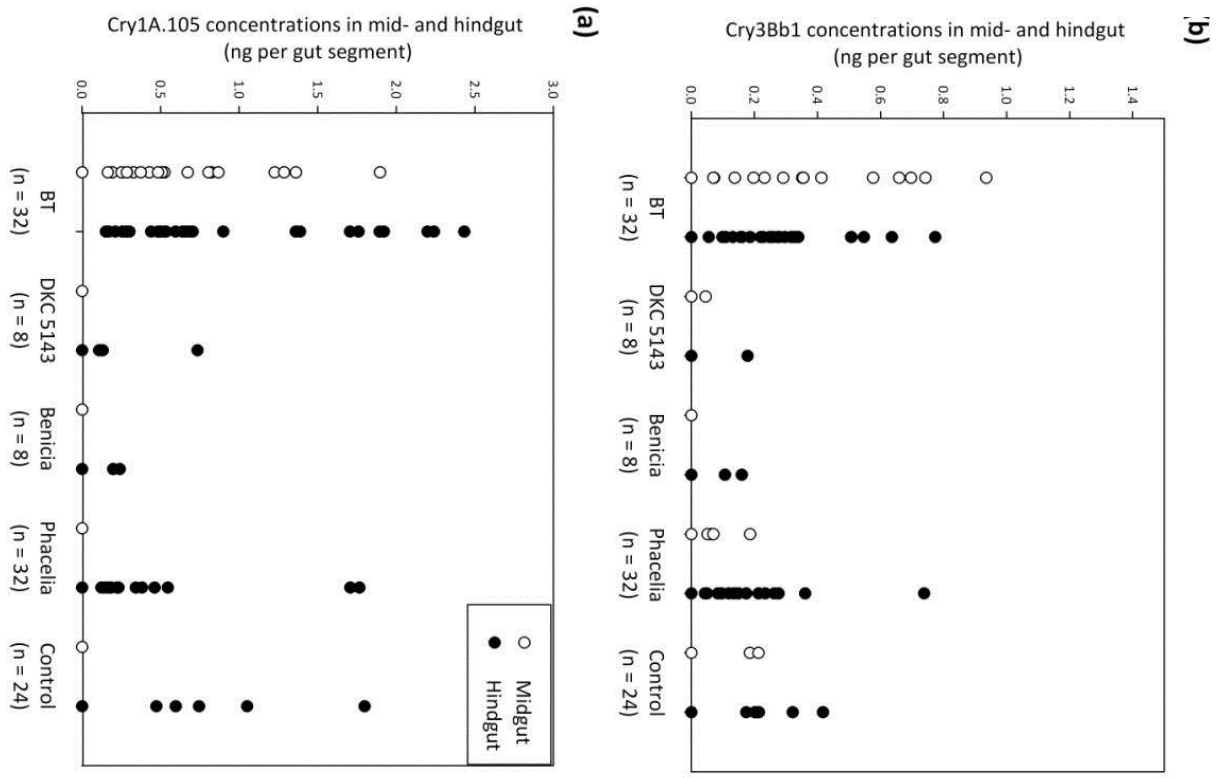


Fig. 2: Quantification of Cry-proteins (Panel (a), Cry1A.105; Panel (b), Cry3Bb1) in the mid- and hindgut of nurse bees collected from the different field plots of this study. Samples below DTC were set to zero.

The signal intensities of the ELISA indicated that the five Cry1A.105 positive bees from the control group contained amounts between 0.5 to 1.8 ng "Cry1A.105" in their hindgut. Since presence of the synthetic Cry1A.105 could be excluded in this control group, it was likely that the ELISA was also responsive for natural Cry-proteins, which can be explained by the fact that Cry1A.105 molecule comprises protein domains of the bacterial Cry1Ab, Cry1F and Cry1Ac, respectively (see Materials and Methods).

The specificity of the Cry1A.105 targeted ELISA to also detect native, bacterial Cry1A proteins was evaluated with sporulated pure cultures of four strains of *B. thuringiensis*, two belonging to the ssp. *kurstaki* and two to ssp. *aizawai*. All four strains generated in fact positive Cry1A.105 ELISA signals (Fig. S1) while no signal was detected with the negative control, *Bacillus subtilis* 168. Based on the correlation between spore/cell numbers and Cry1A.105 signal intensities it was possible to determine the spore/cell numbers needed to exceed the DTC for each particular strains and thus to calculate total *B. thuringiensis* spore/cell numbers required in a bees gut sample for positive detection by ELISA. The results with these pure cultures indicated huge differences depending on the strain selected (Table 1). While only 50 cells of *B. thuringiensis* ssp. *kurstaki* HD-73 were sufficient to cause an ELISA signal equivalent to 1 ng Cry1A.105 in a bees gut, 914 cells of the other *kurstaki* strain were required for the same response. Spore/cell numbers of above 10^7 were necessary to get a corresponding signal from the two *B. thuringiensis* ssp. *aizawai s*trains, suggesting that either expression of Cry1A related proteins were low or antibodies were not specific for their Cry proteins produced.

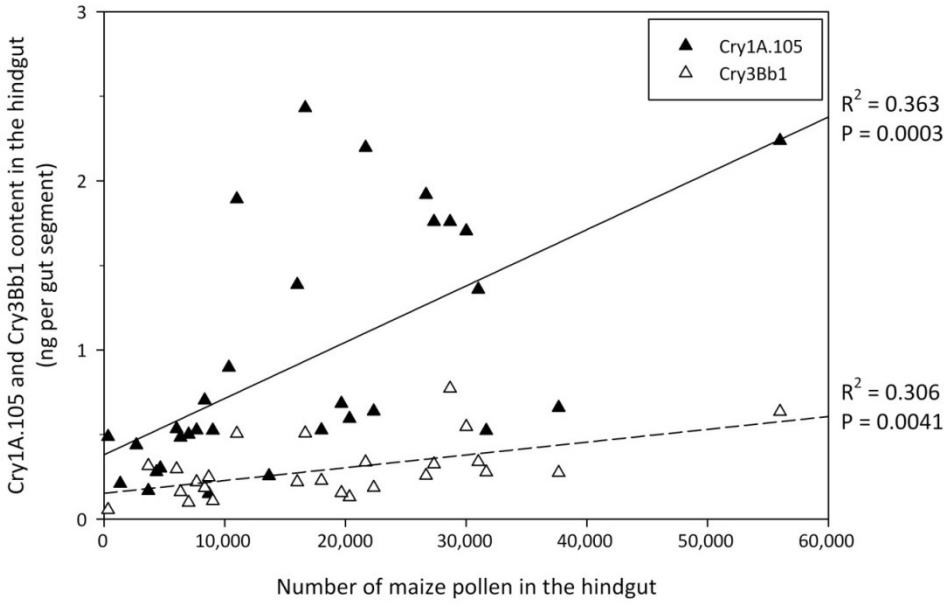


Fig. 3: Correlation between the contents of Cry-proteins (Cry1A.105 and Cry3Bb1) and maize pollen detected in the hindgut of nurse bees kept on field plots with flowering Bt (maize Mon89034xMon88017). Correlation data in the graph excluded nondetects.

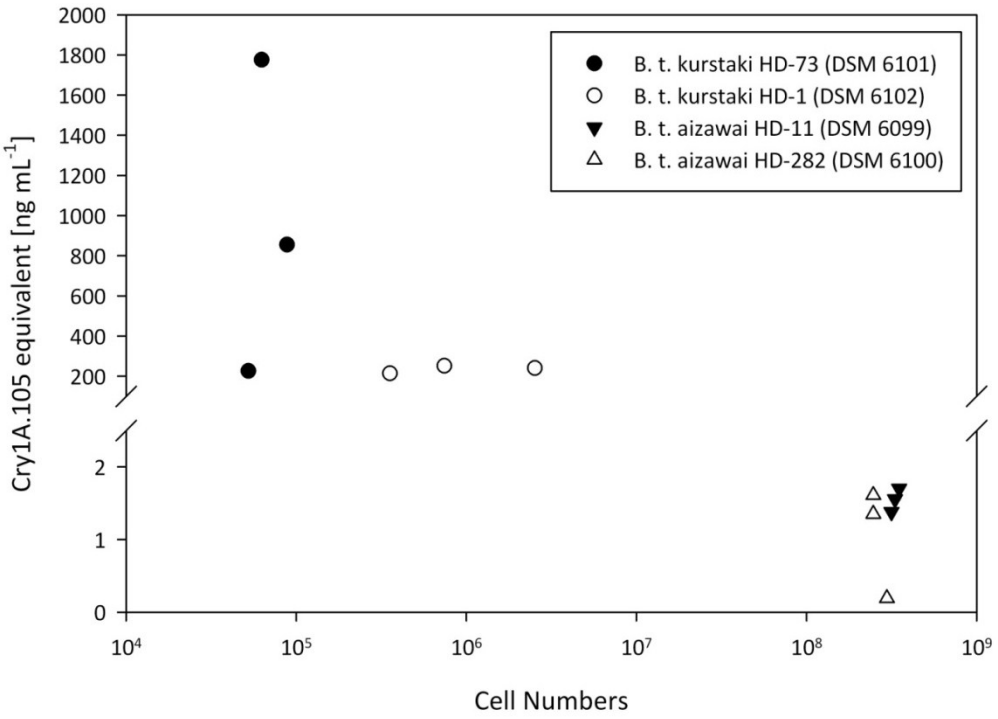


Figure S1: Response of selected *B. thuringiensis* strains, known to express natural Cry-proteins, to an ELISA targeting the synthetic Cry1A.105. Cell numbers and Cry1A.105 equivalents of the highest diluted cell suspension with a signal above the respective DTC are shown.

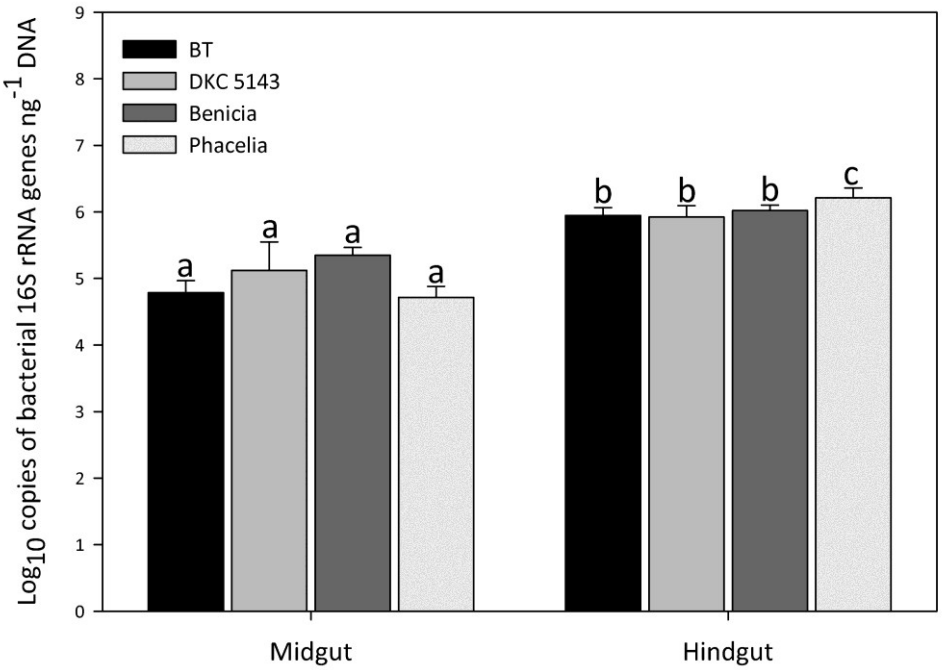


Fig. 4: Copy numbers of bacterial 16S rRNA genes in gut material from nurse bees quantified by PCR. Different letters on top of each column indicate significant differences. For explanation of treatments (Bt, Dkc5143, Benicia, Phacelia) see legend of Figure 1.

Bacterial diversity in response to pollen exposure

Bacteria in the mid- and hindgut of the nurse bees were quantified by qPCR. Copy numbers of the 16S rRNA genes ranged from 1 x 10^3 to 7 x 10^5 per ng total DNA for the midgut, and 2 x 10^5 to 3 x 10^6 per ng DNA for the hindgut samples (Fig. 4). Assuming an average bacterial genome size of 5 Mbp and four 16S rRNA gene operons, the expected maximal copy number per one ng total DNA would be 7 x 10^5. This indicates that the majority of the DNA extracted from the gut material was in fact of bacterial origin. Considering 3 x 10^6 rRNA gene copies per ng of these average bacteria, 100 µl of DNA-extract from the hindgut with a concentration of 9.4 ng μL^{-1} DNA (see Materials and methods) indicated a maximum of 7 x 10^8 bacteria in the bees' hindgut.

Overall, the bacterial abundance in the gut samples from bees was not significantly affected by the maize variety. This lack of response applied to both the mid- and hindgut samples (Fig. 4). For the hindgut, but not for the midgut, slightly but significantly higher 16S rRNA gene copy numbers were found in the free flying bees from the colonies next to the *P. tanacetifolia* field.

Species or, more accurately, phylotype richness was determined by T-RFLP, with each phylotype represented by a single T-RF. Profiles revealed for midgut of bees kept in the maize fields 1 to 9 T-RFs with an average of 3.4 ± 1.6. For hindgut, the range of T-RFs was similar with 2 to 7 and an average slightly but not significantly higher with 4.7 ± 1.2. The number of T-RFs from bees from the colonies with free flying bees was not significantly different. Clear differences, however, could be seen between the diversity of bacteria from midgut and hindgut ($P < 0.05$) (Fig. 5). Using clone libraries and DNA sequencing, the consistently occurring T-RFs could be assigned to different bacterial taxa (Fig. 5; Table S3). The profiles of the midgut were mainly composed of *Alpha*-, *Beta*-, and *Gammaproteobacteria*, while those of the hindgut were dominated by *Lactobacillus* (*Firmicutes*) and *Bifidobacterium* (*Actinobacteria*). Most of the identified T-RFs in this study showed high similarities (97 – 99 %) to 16S rRNA gene DNA sequences from other studies on bees (Table S3). Interestingly, *B. thuringiensis* like 16S rRNA genes sequences were not detected. An *in silico* analyses predicted a characteristic T-RF of 147 bp for *B. thuringiensis* which was not seen in any of the community profiles, indicating that *B. thuringiensis* did not belong to the dominant gut bacteria.

In order to statistically evaluate the differences between the T-RFLP profiles of the gut bacterial communities obtained from the different maize varieties, and, thus, search for indications of adverse effects caused by consumption of pollen from Bt, the profiles were statistically analyzed. For each of the four plant varieties, midgut and hindgut samples of 24 individual bee replicates were included. The similarity of profiles from replicate samples suggested less variability between individual bees in the

hindgut than in the midgut (Fig. 6a). For both mid- and hindgut, the similarity of the T-RFLP profiles was significantly higher for the free flying bees than for the bees from the maize field plots. When any two treatments (three maize varieties and free flying bees from *P. tanacetifolia*) were compared to each other, the similarities of profiles were in the same range as those found within each treatment (Fig. 6b), indicating no specific effect of Bt. Strong differences between varieties would have resulted in lower similarities than those found between replicates. Permutation analysis however revealed significant differences for all comparisons of hindgut samples and for four of six comparisons of the bacterial communities from the midgut, which demonstrated that each variety selected for its particular bacterial community in the gut. No single T-RFs could be identified that would have been indicative for the different maize varieties or the bees from the free flying colonies.

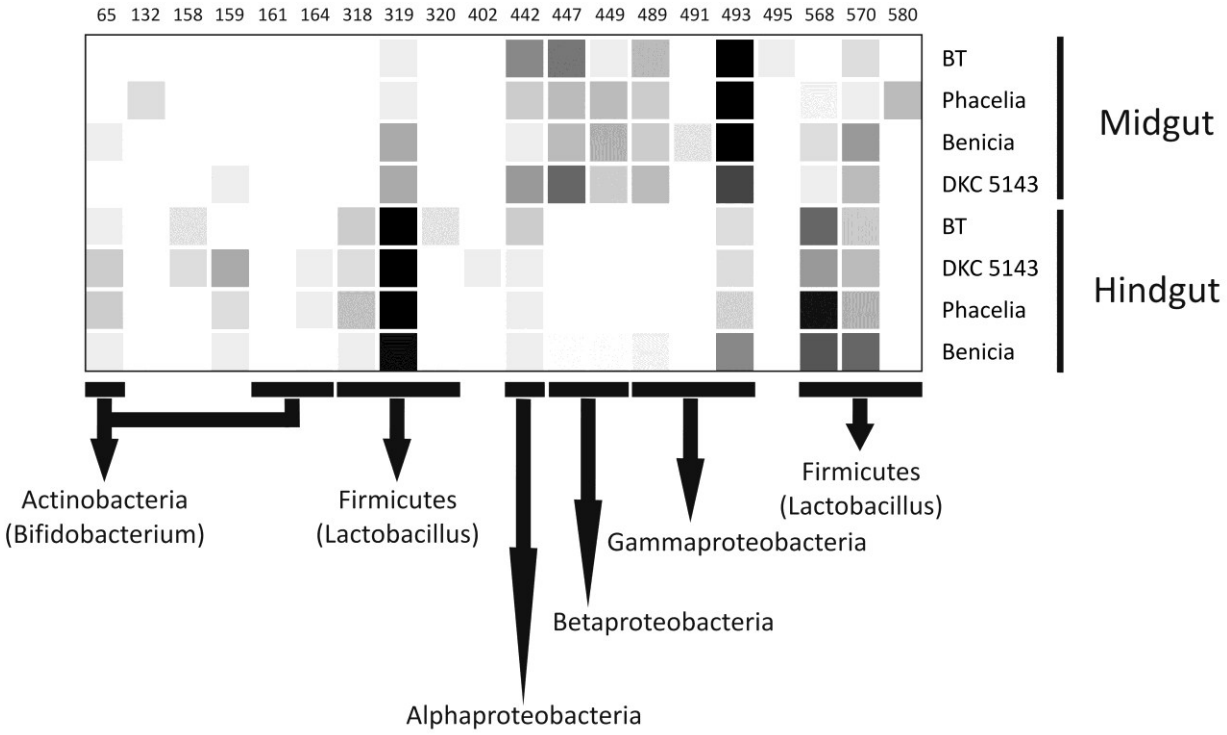


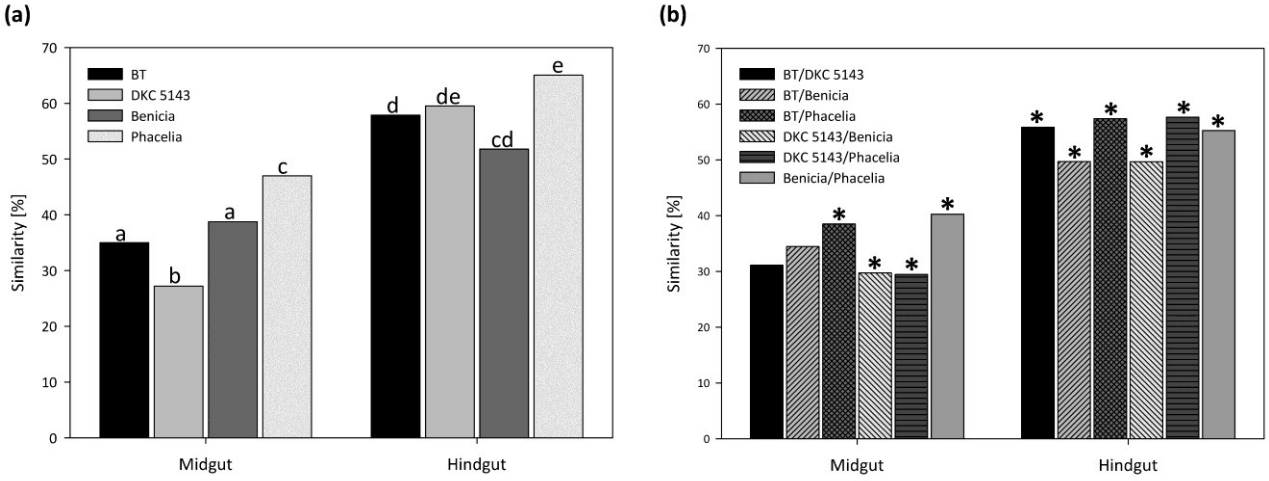
Fig. 5: Genetic profiles (T-RFLP technique) for bacterial community analysis of PCR-amplified 16S rRNA DNA sequences. The figure shows the diversity of T-RFs (terminal restriction fragments) and their corresponding bacterial phylotypes. This figure shows average profiles from each variety and from midgut and hindgut. The T-RF pattern is based on the relative abundance of all 20 T-RFs detected in this study. Abundances are indicated by squares and correlate with the grey scale. T-RFs were identified by a separate cloning and sequencing procedure. For more details see Tab. S3 in Supplemental Material.

Table 1: Hypothetical numbers of sporulated cells of *B. thuringiensis* strains expressing natural Cry-proteins required to cause detection of a Cry1A.105 equivalent by ELISA.

Bacillus thuringiensis ssp. strain	Number of cells necessary in a bees gut samples to give an ELISA above DTC[a]
kurstaki HD-73 (DSM 6101)	$2.50 \times 10^{1} \pm 1.20 \times 10^{1}$
kurstaki HD-1 (DSM 6102)	$9.14 \times 10^{2} \pm 5.86 \times 10^{2}$
aizawai HD-11 (DSM 6099)	$3.26 \times 10^{7} \pm 0.14 \times 10^{7}$
aizawai HD-282 (DSM 6100)	$1.30 \times 10^{8} \pm 0.98 \times 10^{8}$

[a] refers to an extraction volume of 300 µl; DTC, detection threshold was 0.5 mg mL

Figure 6: Similarities of T-RFLP profiles of gut bacterial communities in four different treatments.



(a): Similarity between 24 replicates within each treatment. Different letters above each column indicate significant differences.

(b): Similarities of pair wise comparisons of treatments. Stars on top of each column indicate significant differences between the particular treatments determined by Permutation analysis ($P < 0.05$).

Discussion

Honey bees normally have access to a number of different pollen sources within their foraging range (Seeley 1995). A different situation is observed in agricultural ecosystem due to the cultivation of large-scale monocultures. Under such restricted pollen availability, pollen foragers can be very opportunistic to cover the protein demands of the colony. Even maize as strictly wind pollinated crop can achieve enormous importance as mass flowering pollen source (Keller et al. 2005). In order to reflect worst case situations, as observed in intense agricultural landscapes, mono-floral pollen availability was secured by using flight cages to prevent bees from collecting alternative pollen sources. Thereby our experimental approach increased the exposure of bees to selected maize varieties, including the stacked Bt-maize Mon89034xMon88017 (Bt)." Microscopic analyses confirmed that this system was efficient to secure exposure to Bt pollen, since 96 to 100 % of all bees analyzed contained maize pollen in their gut. The detection rate for bees from colonies with free flying individuals was only 20 %, which underlines that exposure to maize was higher for the caged bee colonies, but also confirmed that bees within agricultural landscapes did include maize pollen into their diet. We found that nurse bees consumed up to 56,000 Bt-maize pollen grains under semi-field exposure conditions (Fig. 3), which reflects survival under a worst case maize pollen exposure, of up to 49.4 mg pollen. In comparison, European butterfly larvae fed with pollen grains from the transgenic maize variety Bt-176 were lethally affected at a more than a thousand times lower exposure dose: LD50 value of only 8 pollen grains (7.1µg) per Diamond-back moth larva, and 32-39 pollen grains (28-34µg) for Small tortoiseshells, Peacocks, European corn borers and Cabbage white larvae (Felke and Langenbruch 2005). In contrast, no indication of lethality was observed for the mean 14.1 mg stacked Bt-pollen (42ng Cry1 and 69ng Cry3). These observations with the stacked variety are in line with laboratory and field studies reporting the lack of adverse effects on bees which had fed on maize pollen with single Cry-proteins, i.e., Cry1Ab or Cry1 F 1Ab (summarized in Duan et al. 2008; Malone and Burgess, 2009), or the recently analyzed effect of Cry proteins of stacked Bt-pollen on *in vitro* reared honey bee larvae (Hendriksma et al. 2011b).

The intention of applying ELISA-based detection systems, targeting Cry1A.105 and Cry3Bb1, was to quantify the amount of Cry proteins from Bt maize pollen present inside the mid- and hindgut and, in fact, all bees collected from Bt-exposed colonies were found positive for Cry1A.105 and 80 % for Cry3Bb1 in the hindgut. Frequently both Cry-proteins were also detected at similar concentrations in the midgut, indicating that they were released here during the digestive process. The high variability in Cry protein concentrations inside the gut is correlated to different amounts of ingested Bt maize pollen.

Surprisingly, considerable concentrations of Cry-proteins were also indicated with both ELISA systems for bees from colonies of field plots with conventional cultivars. Theoretically, these detections could have been caused by pollen of Mon89034xMon88017 transported by wind from the Bt plots into the neighboring field plots, which is however negligible considered the limitated maize pollen flight radius (Jarosz et al. 2003) and the extremly low contamination in relation to the amounts of direct anther harvested pollen by the forager bees. Alternatively, Cry-proteins produced by bacteria from the natural environment are a more likely cause, provided that the antibodies of the ELISA would also react with them. The latter was confirmed with bacterial pure cultures in this study. While there was a good correlation between pollen numbers and Cry-concentrations for nurse bees from the Bt exposed colonies ($R^2 > 0.3$), there was no correlation for the bees from the conventional maize field plots or for those free-flying bees from outside. For the colonies outside of the experimental cages, pollen were not detected in 48 % of bees with Cry in their gut. Thus, there was ample evidence that the Cry proteins detected in bees from outside of the Bt plots must have mainly been caused by Cry-proteins of environmental bacteria. Considering that this natural background also existed in bees from the Bt-exposed colonies, this means that the actual instability of Cry Cry1A.105 and Cry3Bb1 from Mon89034xMon88017 in the gut was in fact above the values of 98.1 % and 99.5 % calculated by comparing the amount of ingested pollen and their concentration of Cry proteins to the amounts detected in the hindgut.

All known bacteria with the capacity to produce parasporal insecticidal δ-endotoxins (Cry-proteins) are phylogenetically closely related to each other and jointly named as *Bacillus thuringiensis*. However, their 16S rRNA genes do not allow to unequivocally distinguish them from members of the *Bacillus cereus* group which do not produce Cry-proteins (Bavykin et al. 2004; Chen and Tsen 2002) and consequently, the assignment of PCR-amplified 16S rRNA genes can only give an indication but not proof about their presence (Mohr and Tebbe 2007). The simultaneous detection of the Cry-proteins in this study, however, confirms presence of *B. thuringiensis* rather than *B. cereus*. Several studies have demonstrated that *B. thuringiensis* are widely abundant in aquatic and terrestrial environments (Martinez and Caballero 2002), including soils (Chilcott and Wigley 1993; Gao et al. 2008; Quesada-Moraga et al. 2004), plant leave surfaces including those of maize (Accinelli et al. 2008; Jara et al. 2006), and dead insects (Schnepf et al. 1998). Typically, *B. thuringiensis* has been detected from environmental samples by cultivation methods but the presence of the Cry-proteins in this study demonstrates its prevalence in the gut of bees independent of cultivation. The TRFLP-profiles of this study could also not detect *B. thuringiensis* (or *B. cereus*) even though their obvious presence because of Cry proteins detected. For the most responsive strain used as a control for detection of Cry1A proteins using the ELISA targeting

Cry1A.105, i.e., *B. thuringiensis* HD-73, only 50 cells were necessary for detection. However, the total bacterial population size of the gut was in the range of 10^8 to 10^9 cells and therefore such a small amount of cells would not have been detected since such profiling techniques represent only the most abundant members of a community (Marsh 1999; Schutte et al. 2008; Smalla et al. 2007). Thus the lack of detection of *B. cereus* / *B. thuringiensis* by TRFLPs does not challenge the explanation for the occurrence of natural Cry-proteins in the bees' GI.

The TRFLP-profiles of the PCR-amplified 16S rRNA genes revealed that the dominant bacterial community in the bees' gut tract was represented by 12 characteristic TRFs of which 11 could be assigned to 16S rRNA sequences using a separate clone library. While the midgut material showed a clear dominance of *Proteobacteria*, the hindgut bacterial community was mainly composed of members of the genus *Lactobacillus* (Firmicutes) and *Bifidobacterium* (Actinobacteria). The diversity detected here is in fully agreement with previous studies based on cultivation independent analyses (Babendreier et al. 2007; Mohr and Tebbe 2006). In a recent survey the main contributors of the bees' gut tract bacterial community was narrowed down to eight phylotypes (Martinson et al. 2011) and, in fact, seven of them, with the exception of a *Bartonella* (Alphaproteobacteria) were also detected in this study.

Even though the bacterial abundance and the presence of the dominant bacterial community members was not different between the treatments of this study (Bt, Dkc5143, Benicia, Phacelia), statistical analyses of their TRFLP-profiles revealed differences when comparing any of two treatments. Surprisingly, the similarities of the bacterial community found in the gut of nurse bees from the free-flying colonies to those from colonies caged in maize plots was not lower than for the maize varieties among each other, suggesting that the mono-floral quality and quantity of the pollen diet had no selective effect on the dominant bacteria. The data-set of the TRFLP profiles did not allow a direct linkage to the occurrence of certain bacterial phylotypes or their particular abundances (as indicated by peak heights in the TRFLP profiles) to these significant differences. One explanation for this lack of direct correlation can be the extremely high variability between TRFLPs from the different individual bees and also the fact that the nurse bees were confined in their particular colony, developing slightly individual characteristics until and during the period of this study. The multivariate RDA revealed that the treatments (maize vs. mixed pollen from the free-flying colonies), which were considered a main factor in the experimental design of this study for evaluation, only had a minor impact on the bacterial community structure. This confirms results of other studies in laboratory set-ups with bees where the treatments (different pollen amended or not with Cry1Ab proteins) explained 7 % of the overall differences between TRFLPs (Babendreier et al. 2007).

In conclusion, this study demonstrates that nurse bees receive a considerable amount of Cry-proteins of GM Bt-maize pollen which are then released into their GI during digestion. As a result of the gut passage, Cry-protein concentrations decline by approx. two orders of magnitude. The size of the GI inhabiting bacterial community was not affected in comparison to other conventionally bred maize varieties and their diversity is composed of the same dominant members. No nutritional difference was recorded for bees receiving pollen of the selected Bt maize or two conventional varieties. Thus, this study found no adverse effects of pollen of the Bt-maize Mon89034xMon88017 on bees and their gut bacteria, suggesting that a combined expression of the three Cry proteins in Bt maize is not harmful for bees.

Table S3. Comparison of DNA sequence of 16S rRNA genes to sequences in public databases and their taxonomic affiliation. The corresponding terminal restriction fragment sizes of the sequences to this shown in Figure 5 are indicated.

T-RF (bp) in T-RFLP profiles*	T-RF** (bp)		Closest relative (Gene bank accession no.)	Similarity (%)	Taxonomic class ***	Variety	MG	HG	No. of clones
Actinobacteria									
65	66		*Uncult. Bifidobact. sp.* (HM113200)	99	Actino-bacteria	Phacelia		x	1
			Uncult. Bifidobact. sp. (HM113220)	96-99		Dkc5143		x	5 (1***)
161/164	165		*Uncult. Bifidobact. sp.* (HM113243)	99		Phacelia		x	1
			Uncult. Bifidobact. sp. (HM113099)	99		Benicia		x	1
Firmicutes									
318-320	322		*Uncult. Lactobacillus sp.* (HM111911)	99	Firmicutes	Phacelia		x	1
			Uncult. Lactobacillus sp. (HM113313)	98-99		Phacelia		x	3
			Uncult. Lactobacillus sp. (HM113193)	98		Dkc5143		x	2
			Uncult. Lactobacillus sp. (HM111880)	99		Dkc5143		x	2
			Uncult. Lactobacillus sp. (HM113252)	98		Dkc5143		x	1
Proteobacteria									
442	441		*Uncult. Acetobacteraceae bact.* (HM112426)	96	Alpha-proteobacteria	Bt	x		4 (3***)
449	447		*Uncult. Neisseriaceae bact.* (HM113219)	98	Beta-proteobacteria	Bt	x		5 (3***)
	448		*Uncult. Neisseriaceae bact.* (HM113170)	98-99		Phacelia	x		2
489	488		*Uncult. bact.* (HM112036)	98	Gamma-proteobacteria	Benicia		x	1
491/493	492		*Uncult. bact.* (HM111973)	98		Bt	x		1
			Uncult. bact. (HM113151)	98		Phacelia	x		1
			Uncult. bact. (HM112085)	99		Phacelia	x		1
Firmicutes									
568	567		*Uncult. Lactobacillus sp.* (HM112042)	99	Firmicutes	Phacelia		x	1
	569		*Uncult. Lactobacillus sp.* (HM112126)	98		Phacelia		x	2 (1***)
570	570		*Uncult. Lactobacillus sp.*(HM113344)	98		Phacelia		x	1
			Uncult. Lactobacillus sp.(HM113214)	99		Phacelia	x		2 (1***)
			Uncult. Lactobacillus sp.(HM113344)	99		Phacelia	x		1
580	579		*Uncult. Lactobacillus sp.* (HM112788)	98		Phacelia	x		1

*see Figure 5; **T-RF as determined by *in silico* analyses of cloned DNA sequences; ***PCR fragments were cloned in reverse orientation, so that the determination of T-RFs was not possible. The assignment of these clones was done according to DNA sequence similarities (>97 %) to clones with known terminal sequences.

VIII. General Discussion

New assessment methods and biosafety data on GM-crops

The concern associated with genetically modified (GM) crops is a potential adverse effect on the environment, in particular risks for beneficial insects, wildlife and people. Hence, robust test-protocols are needed, with standardized and sensitive methods to monitor for potential adverse effects (Romeis et al. 2011). In this respect, the honey bee is an important test species within the environmental risk assessment (ERA) of GM crops. A main contribution of this dissertation is the development and application of practical tools to assess the safety of GM-crops for honey bees. The presented methodological approaches include field applications (Ch. II, III, VII) and laboratory studies (Ch. III, IV, V, VI, VII). They help to rapidly identify potential risks of GM-pollen for honey bee adults and brood. It attributes to secure pollination as vital ecosystem service, sustaining the natural biodiversity and securing the production of many agricultural crops.

Agricultural developments in the field of GM crops will intensify in the coming decades. The presented studies have a good prospect to contribute to the improvement of risk assessments strategies on all kinds of chemical and transgenic plant protection products. In cooperation with other German universities and research institutes, we combined our strengths and report on valuable eco-toxicological data on a stacked GM maize variety. The data are taken into account by international regulatory agencies on legislative GM-crop decisions. On the other side, also beekeepers have already shown a great interest in our honey bee studies, as well as critical consumers, the media and environmental activists.

Colony experiments

The semi-field experiments of 2008 and 2009 (Ch. III, VII) have been conducted at a very large scale. The applied "full life cycle tests" indicated that Bt-pollen neither affected the survival, nor the weight of colony-hatched individuals, in comparison to pollen of the near-isogenic line (III). In the 2009 field season natural Cry-proteins in the digestive tract of bees were found, but no adverse effects of pollen from Bt-maize on the gut function or their gut bacteria was indicated, suggesting that a combined expression of transgenic Bt-proteins is not harmful to the digestive tract of honey bees (VII). The field data on honey bee colonies give a strong indication that this particular Bt-maize pollen is no cause of harm to honey bees.

A high-tier (semi-)field test is demanding in terms of skills and resources necessary for their design, execution, and analysis. Field conditions are often difficult to control experimentally, resulting in data that are difficult to interpret, which does not contribute to the confidence in the conclusions of risk assessments (Romeis et al. 2011). Nonetheless, our field experiments went well, though having encountered a phenotypic difference between the tested Bt-maize and its near-isogenic control. The field data nevertheless corroborated the laboratory data considering that neither a lethal effect on survival, nor a sublethal effect on the weight of test-individuals was found.

A recent meta-analysis of published studies on non-target effects of Bt-crops confirmed that laboratory studies are either conservative or consistent with effects that are measured in the field, hence being good predictors of *toxicological* effects (Duan et al. 2010). However, from an *ecological* perspective, the data on colonies within our semi-field environments show that phenotypic plant differences can cause differences in colony development (as potentially caused by different pollen amounts or a difference in flowering period). Herewith it illustrates a valid argument against laboratory level studies, as they do not cover the eusocial factor of the complex honey bee behavior at colony level and the interaction with the environment.

Although some may suspect adverse Bt-effects from the reported field results, it should be stressed that such differences should not be called sublethal, as indications of toxic effects are not present. The differences, like on colony development (III), or the development of a distinct treatment related bacterial flora within the gut (VII), are likely a descriptive "crop-variety difference". Irrespective of being bred conventionally or transgenic, a variety difference between 2 maize lines is no risk to bees, when applied "active ingredients" are not causing harm (e.g. sprayed pesticides or transgenic insecticidal proteins). Nevertheless, from a nature conservation point of view, it would be positive to propagate crop-varieties that are a relative rich pollen source for pollinating insects.

For social insects, the colony is the principle level of reproduction, survival and homeostasis (Hölldobler and Wilson 2009). In the case of honey bee colonies, all workers are offspring of a single mother queen. In addition to the high degree of relatedness, colony members also share the same environment. They are exposed to identical biotic and abiotic factors such as bacterial, fungal and viral pathogens, macroparasites (e.g. *Varroa* mites, hive beetles), agricultural chemicals, and nutritional as well as climatic conditions. From a statistical perspective, worker bee data must be considered nested when multiple observations are taken from the same colony (Zuur et al. 2009). Without considering tested colony mates as a group, eco-toxicological data analysis would fail to take into account a fundamental assumption of standard statistical models, i.e. the independence of errors (Crawley 2007). By using linear mixed effects models and Cox proportional hazard survival analyses (III, IV, V, VI, VII), I implemented the nested data structure of test workers to eco-toxicological studies on honey bees.

Laboratory experiments

The standardized laboratory studies described in this dissertation establish several new test bioassays to improve the robustness and sensitivity of environmental risk assessments studies for honey bees. The studies clearly illustrate that the *in vitro* rearing methodology for larval tests is very suitable for first-Tier ERA bioassays. We introduced and describe general procedures including test system description, test diets, experimental design as well as suitable measurement endpoints and quality criteria such as low control mortality (Ch. IV). The direct exposure of Bt-pollen on the potentially most sensitive life stage considers and tests the natural Bt-toxin exposure route under controlled laboratory conditions (Ch. V). The methods are not only applicable to GM crop pollen, but are also highly suitable to test risks related to pesticide contaminated or poisonous pollen.

In addition, a premier event of mixed toxicity testing of multiple purified transgenic proteins on larvae *in vitro* is shown, applying worst-case exposure conditions to test for single and combined toxicity effects (Ch. VI). To assess the biosafety of stacked Bt-crops, the Bt-maize variety "Mon89034xMon88017" was taken as reference model. This maize variety is already approved for commercial cultivation by several countries (ISAAA 2011). Target pest insects of maize are reported to be synergistically affected by Cry1Ab and Cry1Ac (Sharma et al. 2010), and by Cry1Ac and Cry2Ab2 or Cry1F (Lee et al. 1996, Chakrabarti et al. 1998, Stewart et al. 2001, Khasdan et al. 2007). The stacked maize variety Mon89034xMon88017 expresses a total 12.4 µg Cry-protein/g pollen (Sauer and Jehle pers. comm.), which is 100 to 10,000 times more Cry-protein than expressed within pollen of the single Bt-variety Mon810 (0.001-0.097 µg Cry1Ab/g; Nguyen and Jehle 2007).

However, using *in vitro* tests, we demonstrated that the single proteins Cry1A.105, Cry2Ab2 and Cry3Bb1 were not lethal to developing honey bee larvae, using test concentrations exceeding the expected environmental exposure concentrations by 186 times (Ch. VI). Also, neither the CP4-EPSPS protein (for herbicide resistance), nor the mix of the four transgenic proteins was found to cause lethality or sub-lethal effects on the weight of prepupae (Ch. VI). Additional first-Tier tests were performed by monitoring the acute oral toxicity at high doses of single purified proteins and the mixed Cry-proteins in individuals and groups of adult bees under laboratory conditions. Even at worst-case exposure concentrations of 50 times EEC, adult worker bees showed no lethal effects (Kästner 2010, unpublished data). These case studies on Mon89034xMon88017 maize indicate an insusceptibility of honeybee larvae and adults for individual Cry-proteins, and their combination (at the tested concentrations). Such non-toxicity results are toxicologically indicated as 'inertism' (Greco et al. 2005).

The controlled ecotoxicological approach of laboratory testing enables a comprehensive monitoring for potential mixed toxicity effects under worst case scenarios (Ch. IV, V, VI). In addition, the studies at the level of colonies allow realistic environmental influences and the normal feeding interaction between attending nurse bees and larvae (Ch. III, VII). Within my dissertation, I bridge the gap between testing effects on honey bees in a colony setting (*in vivo*) and the more fundamental approach of *in vitro* testing of purified proteins. Principally the larvae phases of herbivorous pest insects are targeted by transgenic entomotoxic proteins. Therefore I consider especially honey bee larvae stages to be needing evaluations for GM-crop ERAs.

Multiple stressors and effects on the bacterial gut flora

Though not available in a peer reviewed publication, *Nosema apis* infected honey bee colonies might be found to decline quicker when exposed to Mon810 Bt-maize pollen, as compared to exposure to conventional maize pollen (Kaatz 2005). It was stated that these gut parasites might act as a stress factor, facilitating a toxicity effect by the expressed Cry1Ab proteins. This data forced experts to acknowledge the need to investigate unexpected synergism effects; an opinion which was shared by European Food Safety Authority (EFSA 2008).

At the semi field experiment in 2008 (Ch. III), all colonies of the Bt-maize and near-isogene maize treatment were found to be infected with *N. apis*. Having constructed all test-colonies standardized from the same mix of young bees at the Celle bee-institute, I could successively compare the fate of infection after the field experiment. I found that almost all colonies demonstrated *N. apis* spore presence, with similar infection rates and number of counted *N. apis* spores similar between the two treatment

groups (Bt-*versus* near-isogene maize) (Hendriksma et al. in prep., unpublished data). No indication of a different worker bee mortality rate was found (Ch. III). Although Mon89034xMon88017 expresses up to 10,000 times more Cry-protein than expressed within Mon810 pollen (Nguyen and Jehle 2007, Sauer and Jehle pers. comm.), the data do not corroborate the findings by Kaatz (2005). It is therefore not unlikely that the Kaatz findings may have been based on artefact observations, considering the difficulties of controlling *Nosema* infections when the test-colonies are not appropriately standardized. In this respect, and also in general, more honey bee colony studies on multiple stressor responses are required.

A previous study reported that neither Mon810 Bt-maize pollen nor high concentrations of Cry1Ab significantly affected bacterial communities in honeybee intestines (Babendreier et al. 2007). Kaatz (2005) stated: "*Wir haben in dem ersten Jahr gefunden, dass in mehreren Bienen solche gentechnisch veränderten Mikroorganismen vorhanden waren, die offenbar das Gen aufgenommen haben*", by which he stressed that genes from Mon810 Bt-maize pollen were incorporated in the genome of gut microorganisms (i.e. bacteria and yeasts). In contrast, other studies did not corroborate the horizontal gene transfer within bees (Mohr and Tebbe 2007). Moreover, results for the sampled worker bee guts of the 2009 field experiment also showed Cry-protein presence, also without a direct exposure to Bt-maize (Ch. VII). Furthermore, bees without maize pollen in the gut show Cry protein presence. Our data suggest that *Bacillus thuringiensis*, as typical insect affiliated bacterium, is native within the bee gut (Ch. VII). In absence of a conclusive evaluation, the studies are commendable for further research.

GM-crop risks to pollinators within a landscape context

To enable good assessments of risks of GM-crops, it is important to know the actual exposure conditions within the environment. For honeybees, few quantitative data on exposure rates are available. In addition to exposure data for honey bee larvae (2,000 maize pollen, Babendreier et al. 2004), we could add data on exposure to adult bees under semi-field conditions (56,000 Bt-maize pollen, Ch. VIII). Additional studies were implemented to monitor how honeybees forage in an open landscape, considering a gradient of low to high amounts of flowering maize fields available (Danner 2010, unpublished data). It was found that the collected amounts of maize pollen were generally low (<2%), with exceptions of some colonies collecting a considerable amount of maize pollen (>10%), as compared to pollen of other plants. The data are valuable, as they enable to better estimate the practical exposure risk for genetically modified maize.

A current general lack of data on transgenic protein expression levels in GM-crop pollen limits drawing conclusions on biosafety. It is a constraint on rational experimental laboratory designs for verifying realistic exposure conditions within the field (Malone et al. 2001). Clearly more empirical data

on the expression of transgenic products in pollen are needed. In addition, if pollen is the principle source of exposure to pollinators, then it is preferred that GM-constructs are expressed principally in plant tissues other than pollen. This would be an effective way to reduce exposure to pollinators down to a negligible level (Malone et al. 2002).

GM crop interaction with bees should not be restricted to the plant level alone. The crops are grown in large scale monocultures and farmers commonly apply multiple chemicals within an integrated pest management scheme accompanying the GM-crops. For instance, glyphosate-based herbicides are among the most ubiquitous pesticides, yet little is known about whether and how they might affect the environment (Evans et al. 2010). Glyphosate might not be directly toxic to bees, but it does affect bacteria and fungi (e.g. Ermakova et al. 2010), and thus may be an indirect disturbance within the bee gut, or reduce the fermentation of bee bread stocks within colonies.

In addition, as herbicide applications actively suppress weeds, a year round pollinator presence is very difficult to sustain (e.g. for honey bee colonies). The total complex of GM-crop management practices is amenable for evaluation upon attempting to protect beneficial insects within the agricultural environment.

Future GM-crop applications

Humans have been farming for about 600 generations, with the last 3 generations seeing a rapid change in agricultural intensification. Recently, the world's population has reached 7 billion people, and will surpass 9 billion by 2050 (UN 2011). To meet global food demands there is a considerable need to further intensify agricultural production. A challenge is that weeds, pests, and diseases reduce agricultural production, for example, 31% of losses due to pests are reported for maize (Birch et al. 2011). A currently widely applied strategy to combat weeds and pests within agricultural systems is by making crops resistant to herbicides and/or pest insects by means of genetic modification (GM).

It remains important to ascertain the non-toxicity of new Bt-crops to bees, despite that the Bt-studies in this dissertation found no indication of a significant harm. Few biosafety assessments to date are covered by bioassays on the honey bee larvae stages. In addition, since the initial commercialization of Bt-crops (i.e. maize variety "Mon810"), the development of Bt-applications is progressing by the quantitative and qualitative increases of traits within crops. Fundamental research is lacking on whether receptors for Cry-proteins are present within honey bee larvae or adults, and whether an actual binding of Cry-proteins takes place or not. Even to date, the mode of action of Cry-proteins is not fully understood and controversially debated (Pigott and Ellar 2007).

Biotech crop opportunities for insect pest control are not restricted to Cry-proteins. A growing multitude of arthropod-active proteins are available (reviewed by Sanchis 2011 and DeVilliers 2011). There are other *Bacillus thuringiensis* derived insecticidal proteins, such as cytolitic proteins (Cyt) and vegetative insecticidal proteins (Vips) (Gatehouse 2008, Schnepf et al. 1998). Plant lectins (sugar-binding proteins) can also be used for pest insect protection, for example those derived from snowdrop (*Galanthus nivalis* agglutinin; GNA), for which we found a lethal capacity for honey bee larvae and adults (Ch. V, and Kästner 2010, unpublished data). The ricin-protein from the castor oil plant (*Ricinus communis*) is also broadly insecticidal, and also a variety of protease inhibitors and neuropeptides like spider neurotoxins (reviewed by DeVilliers 2011). Another novel approach is exploiting insecticidal proteins produced by *Photorhabdus luminescens*, a nematode symbiotic bacterium (French-Constant et al. 2007).

The many GM-applications under development will need non-target biosafety assessments. The novel applications can have very different modes of action. Neurotoxic active products might cause paralysis or an affected learning or coordinative capacity. Others may cause cell lyses or a feeding inhibition when protein, sugar or biosynthesis pathways are blocked. In addition to our principle measured endpoints mortality and weight of larvae and bees, multiple other monitoring methods are available for biosafety assays (Desneux et al. 2007, Brodschneider et al. 2009).

Pollinators face threats in absence of legislative authorities. For instance, Chinese executive authorities were apparently unaware or unconcerned of a potential risk by the GM-cotton variety "CCRI41", expressing a cowpea trypsin inhibitor CpTI and the Bt-protein Cry1Ac. It was recently reported that this cotton variety affected adult bees by a lowered amount of pollen consumption (Han et al. 2010). This GM-crop was approved for commercialization, despite literature that clearly warned against the effects of transgenic protease inhibitors on bees (Belzunces 1994, Burgess et al. 1996, Jouanin et al. 1998, Malone et al. 1995;1998;1999;2001, Pham-Delègue et al. 2000, Sagili et al. 2005, Babendreier et al. 2005;2008). This example underlines the worldwide need to continually monitor new transgenic plant applications.

In 2010, 41% of a total 67 million hectares GM crops in the USA consisted of crops with stacked traits, including 78% of the planted Bt-maize (James 2010). Especially this combination of multiple traits within crops is an increasingly important GM crop feature. It is astonishing that so far no studies have been conducted to assess the risk of simultaneously expressed Bt-proteins on honey bees.

Conclusions

An increasing global food demand drives a worldwide progressive application of GM-crops. For assessing the risks and impacts of new advances in GM crop engineering, the honey bee holds an outstanding position. Honey bees are the prime pollinators within the agricultural environment, and as nontarget beneficial insect they play an important role within the biosafety testing of GM crops. Honey bee larvae and adults risk a direct exposure to transgenic products by the consumption of GM crop pollen. This theme currently attracts high interest by both the public and the research community. In light of worldwide reports on honey bee colony losses and a genuine concern about possible risks to the ecosystem as a whole, there is a pressuring demand for efficient regulatory study methods. Novel transgenes and new combinations of insecticidal traits within GM crops need biosafety monitoring.

The earlier science-based consensus, that Cry-proteins are in general safe for honey bees, is in line with my findings on the stacked Bt-maize variety Mon89034xMon88017 which expresses the Bt-proteins Cry1A.105, Cry2Ab2 and Cry3Bb1. In addition, considering a number of advancements in laboratory and semi-field study methods, I am sure of the prospect that the presented studies can contribute to an improvement of risk assessments strategies.

To sustain the vital ecosystem service of pollination, GM crop impacts on *A. mellifera* should always be a crucial part of regulatory biosafety assessments. In addition to the agricultural challenges and efforts of securing human food security the coming decades, I hope my dissertation will contribute in securing the pollinator safety within our unique environment.

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Chapter II

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Proceedings of the *4th Workshop on Post Market Environmental Monitoring of Genetically Modified Plants Challenges* meeting on *multiple/stacked events and long-term effects* PMEM, 03-04.05.2010, Quedlinburg, Germany. Poster abstract, page 7. Harmen P. Hendriksma, Stephan Härtel, Werner von der Ohe, Ingolf Steffan-Dewenter. Multiple insect resistant Bt maize pollen consumption in honeybee workers under semi-field conditions.

Programme of *the 4th Ecological Impact of Genetically Modified Organisms* EIGMO 14-16.05.2010, Rostock, Germany. Talk abstract, page 36: Stephan Härtel, Harmen P. Hendriksma, Werner von der Ohe, Ingolf Steffan-Dewenter. Effects of Bt maize with multiple insect resistance on lifespan and hatching weight of honeybee workers. Poster abstract, page 73: Evaluation of multiple insect resistant Bt maize pollen consumption in honeybee workers under semi-field conditions. Harmen P. Hendriksma, Stephan Härtel, Werner von der Ohe, Ingolf Steffan-Dewenter.

Proceedings of the DGaaE *German Society for General and Applied Entomology* meeting, 21-24.3.2011, Berlin, Germany. Abstracts, page 120. Stephan Härtel, Harmen P. Hendriksma, Ingolf Steffan-Dewenter. Assessing potential effects of multiple insect resistant maize on *in vitro* reared honey bee larvae.

11th Conference on Bacterial Genetics and Ecology BAGECO11; 31 may 2011. Effects of genetically modified maize expressing Bt-toxins on natural microbial communities in plants, rhizospheres and honey bees – Do new methods reveal new risks? *Meike Küting, Anja Dohrmann, Claudia Wiese, Harmen P. Hendriksma, Stephan Härtel, Ingolf Steffan-Dewenter, Sebastian Jaenicke, Rafael Szczepanowski, Andreas Schlüter and Christoph C. Tebbe*. Corfu, Greece.

Presentations and posters

March 2009 Presentation: Effects of stacked Bt maize on longevity and hatching weight of honeybee workers. 56th AG Tagung, 24-26.03.2009, Schwerin, Germany.

March 2009 Poster: Multiple insect resistant Bt maize pollen consumption in honeybee workers under semi-field conditions. 56th AG Tagung, 24-26.03.2009, Schwerin, Germany.

April 2009 Poster (Prized): Multiple insect resistant Bt maize pollen consumption in honeybee workers under semi-field conditions. BayCEER workshop, 02.04.2009, Bayreuth, Germany.

May 2009 Poster: Evaluation of multiple insect resistant Bt maize pollen consumption in honeybee workers under semi-field conditions. 4th EIGMO meeting 14-16.05.2009, Rostock, Germany.

February 2010 Presentation: Transgenic corn pollen, transgenic proteins and honeybee larvae; A laboratory method testing potential toxicity. Project meeting BMBF 0315215E Influence of stacked transgenic Bt corn on honeybees, 02-03.02.2010, Kitzingen, Germany.

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May 2010 Poster: Multiple insect resistant Bt maize pollen consumption in honeybee workers under semi-field conditions. Post Market Environmental Monitoring of Genetically Modified Plants Challenges. PMEM 03-04.05.2010, Quedlinburg, Germany.

September 2010 Presentation: *Effect of transgenic maize pollen on survival and weight of in vitro reared honey bees.* 4th EurBee congress, Middle East Technical University, METU, EURBEE 2010 07-09.09.2010, Ankara, Turkey.

November 2010 Poster: A new laboratory method to test direct GM crop effects on *in vitro* reared honeybee larvae. 11th Int. Symposium on the Biosafety of Genetically Modified Organisms (ISBGMO) Buenos Aires, Argentina. Abstract p.164

March 2011 Presentation: Digestion and bacterial community structures in the digestive tract of honey bees show no adverse effects by stacked Bt-maize pollen. 58th AG Tagung, 29-31.03.2011, Berlin, Germany. In cooperation with the Institute of Biodiversity (vTI), Braunschweig.

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Multiple insect resistant Bt maize pollen consumption in honeybee workers under semi-field conditions

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Honeybees are a key non-target species in ecological risk assessment of genetically modified (GM) insect resistant crops. Under laboratory conditions, possible adverse effects on bees are well studied. However, transgenic protein exposition data are lacking so far for functional colonies. We evaluate exposure to transgenic pollen in semi-field colonies.

Material

64 standardized colonies with 1000 workers were kept in 32 cages with 500 maize plants each. Our semi-field experiment had a randomized plot design of four maize varieties: multiple insect resistant Bt maize, isogenic line and two conventional maize varieties (Fig. 1,2). The Bt maize carried three insecticidal *Cry*-proteins against lepidopteran and coleopteran pest insects.

Method

From 12 workers out of each colony the rectums were dissected and maize pollen content was estimated using a haemocytometer. In addition, digestion degree of maize pollen was categorized as totally (T), partly (P) and not (N) digested (Fig. 4). During the experiment, mortality of honeybee workers was monitored by collecting dead bees with a trap (Fig. 3).

Figure 1: *Field experiment with flight cages*

Figure 2: *Honeybee collecting maize pollen*

Figure 3: *Bee hive with a mortality trap*

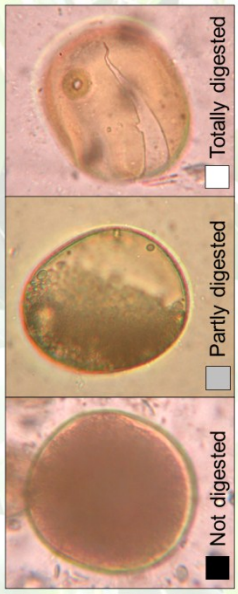

Figure 4: *Categories*

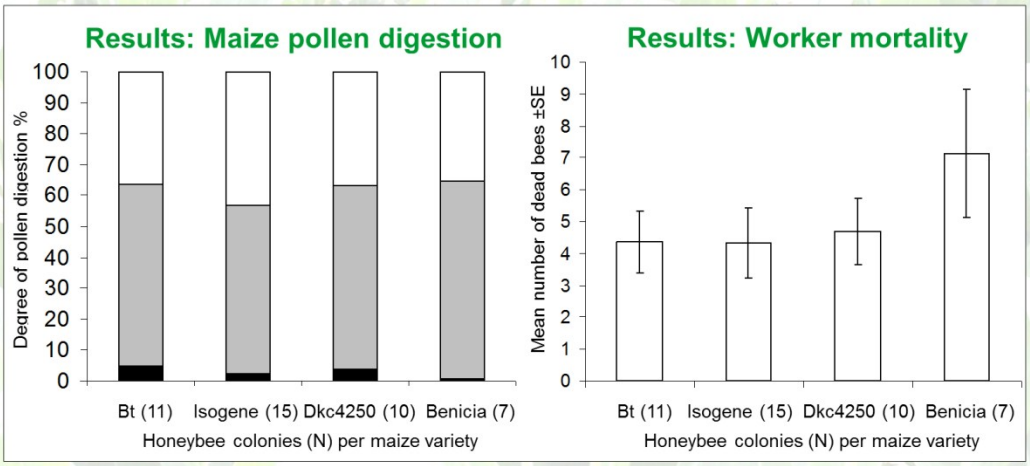

Figure 5: *Digestion of maize pollen in worker bee rectums. N=43 colonies, with totally 1157 pollen categorized. Analysis only on the colonies with more than 5 pollen categorized. No differences between the treatments: 1-way ANOVA on the weighted average of the digestion categories F(3,39)=1.32 p=0.281*

Figure 6: *Mortality of worker bees in colonies (N=43) with a total of 210 dead bees counted in 21 days. No difference between treatments: 1-way ANOVA on the mean number of dead bees F(3,39)=0.90 p=0.448*

Discussion

Pollen of different maize varieties did not differ in the degree of digestion (Fig. 5). The uniform digestibility of the maize grains indicate that intestinal functioning of adult honeybees was not affected by multiple insect resistant Bt maize pollen. Furthermore, no difference in worker mortality was observed (Fig. 6). Our data indicate differences between the ingested and the absorbed amount of Bt protein by honeybee workers. Since undigested Bt maize pollen do not contribute to exposure in worker bees, data on digestion is needed to calculate quantitative exposure estimates of Bt proteins to single worker bees.

Conclusions

Analysis of transgene pollen in honeybee rectums should be taken into account for GMO risk assessments.
Multiple insect resistant Bt pollen did not affect intestinal digestion and mortality of adult honeybee workers.

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A new laboratory method to test direct GM crop effects on *in vitro* reared honeybee larvae

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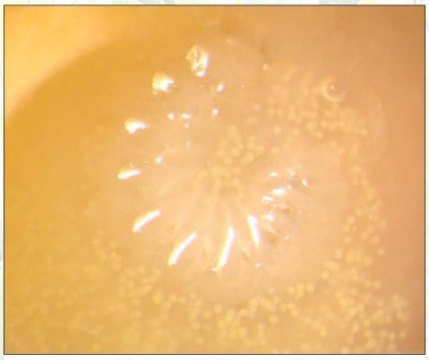
The honeybee (*Apis mellifera*) is a key non-target arthropod in environmental risk assessment of genetically modified insect resistant crops. Transgenic insecticidal proteins primarily affect the larval phases of target organisms and older honeybee larvae are directly exposed to GM pollen during their development. Therefore robust and highly standardized testing methods such as *in vitro* rearing of honeybee larvae are required to minimize the potential risks for this important pollinator.

Method

In the laboratory, we applied a new GM plant risk assessment method for honeybee larvae by adding transgenic pollen directly to the larval diet. We tested pollen of two transgenic and three conventional maize varieties. Assessment endpoints were survival and pre-pupae weights of the tested individuals. As positive control we fed a toxic pollen to the larvae (see table).

pollen	plant variety	n larvae	pollen/2mg
transgenic maize	*stacked Bt:* Mon89034xMon88017	20	1701
	single Bt: DKc7565 (event Mon810)	20	1750
control maize	*isogenic line of stacked:* DKc5340	19	1784
	more distant related: DKc4250	20	1753
	not related: Benicia	20	1722
no pollen control	-	12	0
positive toxic control	*Heliconia rostrata*	10	1600
pooled *Bt* maize	1 and 2	40	1726
pooled control maize	3, 4 and 5	59	1753



In vitro reared larva, with dietary application of maize pollen. Picture by Harmen P. Hendriksma

In vitro reared pupae. Picture by Harmen P. Hendriksma

Results

All *Bt*-maize pollen fed larvae survived upon the prepupae phase, day 11 (T: 40 out of 40 larvae; 100% survival). This survival rate did not differ from the control-maize pollen fed larvae (C: 56 out of 59; 95%) (Chi^2 = 0.724, *Df* = 1, *P* = 0.395). Also, neither the survival rates upon hatchment (Fig. 1), nor the weight op prepupae (Fig. 2) indicated an adverse effect of the transgenic maize. Our positive control (H), the feeding of *Heliconia* pollen to larvae, clearly caused mortality (Fig. 1).

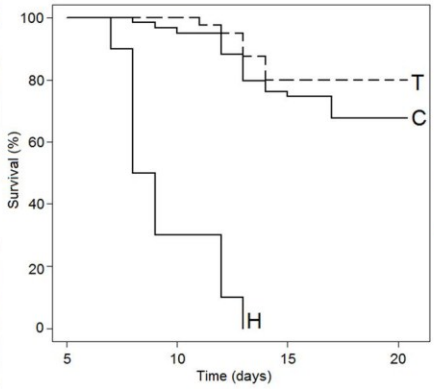


Figure 1: Survival of *in vitro* reared larvae, with dietary application of transgenic maize pollen (T), conventional maize pollen (C) and toxic pollen of *Heliconia* (H). All pollen treatments were applied to the larvae at day 6. The prepupae (day 11) indicated the end of the larval phase.

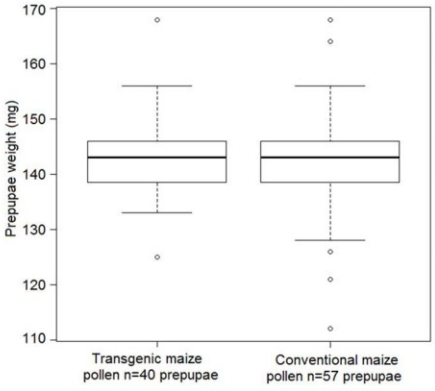


Figure 2: Boxplot if prepupae weights of *in vitro* reared larvae, after dietary application of transgenic pollen (pooled) and conventional maize pollen (pooled). No significant difference was found. *t*-Test: *t* = 0.164, *Df* = 94, *P*-value = 0.87

Discussion and conclusions

The tested transgenic *Bt*-maize pollen varieties are not found to be toxic to honeybee larvae *in vitro*. We recommend that laboratory risk assessments on non-target organisms should follow existent GM plant exposure patterns. The direct exposure to the plant produced insecticidal proteins via pollen feeding should improve the strength of GM plant risk assessments on honeybees. We believe that our novel method has the potential to become a standard method in honeybee environmental risk assessments.

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