

# ENVIRONMENTAL RISK ASSESSMENT OF TRANSGENIC PLANTS USING HONEY BEE LARVAE

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## Abstract

*An environmental concern regarding the cultivation of transgenic crop plants is their effect on non-target organisms. Honeybees are obvious non-target arthropods to be included in a risk assessment procedure but due to their complex social behaviour, testing on individual bees is not possible in bee colonies. Here we present results of a laboratory larval rearing technique that is new in testing impacts of a proteinase inhibitor, a source of insect resistance in transgenic plants, on honey bee larvae reared individually in the laboratory. Our results suggest that the proteinase inhibitor may have an impact on development and mortality of honeybees.*

## 1. Introduction

Gene technologies may prove to be a powerful tool for generating new plant cultivars that possess improved traits in relation to crop production (1-5). However, before releasing genetically modified (GM) cultivars, a thorough environmental risk assessment is required. Widely accepted risk assessment protocols do not exist but it is a general requirement that released GM plants do not cause adverse effects on the environment including non-target arthropods (6-7). Honey bees (*Apis mellifera* L.) (Hymenoptera: Apidae) are obvious non-target arthropods to be included in a risk assessment analysis; honey bees are of huge economic importance as the most important pollinator of insect-pollinated wild and cultivated plants (8). Honey bees also hold an important role of the public perception of biodiversity and a healthy environment. Bees, by feeding on nectar and pollen (also of wind-pollinated crops (8-9)) are potentially exposed to non-target effects of GM plants. Testing for non-target effects of GM plants (10) or pesticides (11) on bees have so far been restricted to adults. Hence, effects on larval development cannot be tested using these methods. However, investigation of the impact of larval food on individual larval development is not possible in bee colonies because the larvae are tended by nurse bees. These will detect and remove diseased larvae several days before symptoms are visible to the human eye (12). To follow possible influence of food composition on the individual level, it is, therefore, necessary to rear the larvae *in vitro* without the interference of nurse bees (13). We suggest that the *in vitro* rearing system can be used for testing non-target effects of GM plants. Furthermore, the system can be used as a pre-plant test evaluation system with artificial diets containing the concentration of the gene product that is expected to be expressed by the transgene plants. Here we report on the application of this *in vitro* system and show that honey bee larvae are sensitive to a plant based proteinase inhibitor (PI).

Genes coding for plant derived PIs can contribute to pest resistance in host plants (14). Though the full physiological effects of PIs on insects remain unknown (3), PIs have been reported to inhibit growth and development of a range of insect juveniles as well as to reduce adult longevity (15-17). A reduced adult longevity has also been reported for

honey bees (18-21) as has behavioural disturbances (20-21). Acute toxicity is seldom seen in insects (3) and not in honey bees (22). By 1998, at least 14 different PI genes had successfully been introduced into crop plants (3). One of these, the Kunitz Soybean Trypsin Inhibitor (SBTI), a serine proteinase inhibitor, is reported engineered into potato and tobacco (23). Honey bees have serine proteinases as digestive enzymes (24). SBTI may therefore have an impact on honey bee protein digestion and, thus, especially on larval development.

## **II. Materials and methods**

Expression levels of transgenes in GM plants vary according to plant tissue and species (3). We chose 0.1% and 1.0% SBTI as realistic low and high expression levels, respectively (4), and investigated the juvenile development, mortality, and adult body mass with larval diets containing 0.1% or 1.0% (w:w) SBTI of total protein. A control group was fed with a larval diet containing 1.0% (w:w) Bovine Serum Albumin (BSA). We did not include the fact that nectar is dehydrated when it is converted into honey in the bee hive and that this process increase the protein content in the honey that is fed to the larvae *in situ* compared to the protein content in secreted nectar.

The larvae were reared using the method by Brødsgaard *et al.* (13) with the modifications that the larvae were reared in sterile tissue culture multi-wells (Ø 16.2 mm) and grafted daily to new wells with food. Handling was hereby reduced to one time per day with no additional feeding (25). Pure SBTI and BSA were obtained from Sigma (St. Louis, USA) and mixed into the standard food (26) in concentrations of 0.1% and 1.0% (w:w) of total protein content in the food. The larvae and pupae were monitored once daily until adult emergence. Larval and pupal stage as well as survival were noted. Larval development time was calculated at the LS stage (27) (the larvae stop feeding and defecate in this stage to begin pupation) and at adult emergence. The newly emerged adults were weighed to investigate differences in body mass.

## **III. Results and discussion**

Comparison of the present study with previous experiments (13) and unpublished results suggests that the addition of BSA to the standard food does not change bee development time or mortality. Our experiments suggest that larval development will be affected in several ways if the larval food contain SBTI (Table 1). Significantly slower juvenile development was observed if the food contained 1.0% SBTI. The increased development time was evident both in the feeding stages (until LS) and the non-feeding stages (LS to adult). Development times were not significantly influenced by 0.1% SBTI. Juvenile mortality was significantly increased when larvae were fed 1.0% SBTI compared to the control fed 1.0% BSA. A dose-response relationship of SBTI has also been reported for adult longevity of both honey bees (17, 20) and bumble bees (28). The wet body mass of newly emerged adults was significantly lower when the larvae were fed 1.0% SBTI whereas 0.1% SBTI in the larval food did not have an effect on adult mass. Reduction of mass gain due to PIs in the food is also reported for other insect herbivores (15, 29). The consequences of smaller adult bees will probably be that these surviving bees will have reduced performance as adults regardless of their food intake as adults.

## **IV. Conclusion**

Adult worker honey bees mainly eat pollen as nurse bees, with a peak in pollen intake at day nine after emerging. The pollen intake and, thus, amount and type of digested protein, is correlated to the developmental status of the hypopharyngeal glands (30-31). The secretions of these glands are important components of the larval food. It is therefore likely that nurse bees that ingest PIs will be poorer producers of larval food both in terms of quantity and quality. Hence, not only will the longevity and learning ability of

adult bees be reduced (17-20) affecting their performance as forager bees if they are influenced by a SBTI containing pollen or nectar source, they will probably also be sub-optimal tenders of larvae as nurse bees. A crop expressing SBTI in a 1.0% concentration in pollen or nectar will, therefore, have both a direct impact on honey bee larvae through digestive inhibition (resulting in increased development time, increased juvenile mortality, and individuals surviving to adulthood being smaller) and an indirect impact through nourishment depletion through affected nurse bees. The *in vitro* rearing technique presented here makes it possible to monitor individual larval development and we suggest that this should be included in an environmental risk assessment procedure before releasing transgenic plants for field planting.

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Table 1: Development time from egg to LS stage and egg to adult, adult body mass, and juvenile mortality of *Apis mellifera ligustica* L. reared *in vitro* on diets with different proteinase inhibitor content.

Diet	Development time to LS stage <sup>*</sup> (days <sup>#</sup> ± SE (n) <sup>▫</sup> )	Development time to adult (days <sup>#</sup> ± SE (n) <sup>▫</sup> )	Adult body mass (g <sup>#</sup> ± SE (n) <sup>▫</sup> )	Juvenile excessive mortality (% <sup>~</sup> (N) <sup>=</sup> )
Standard <sup>▫</sup> + 1.0%BSA	9.62a ± 0.06 (188)	21.05a ± 0.16 (99)	0.138a ± 0.003 (99)	- (4)
Standard <sup>▫</sup> + 0.1%SBTI	9.79a ± 0.03 (312)	20.73a ± 0.17 (121)	0.139a ± 0.003 (120)	11.76 ns (4)
Standard <sup>▫</sup> + 1.0%SBTI	10.29b ± 0.03 (258)	22.06b ± 0.13 (74)	0.109b ± 0.002 (74)	26.39 * (4)

<sup>\*</sup> Larval stages L<sub>1</sub> to LS are feeding (29)

<sup>#</sup> Means not followed by the same letter are significantly different (pair wise T-tests,  $P < 0.0001$ )

<sup>▫</sup> Number of individuals

<sup>~</sup> Significant difference from the control group (pair wise T-tests on arcsine transformed data,  $P < 0.05$ ) (ns = not significant)

<sup>=</sup> Number of repetitions

<sup>▫</sup> Standard *in vitro* feed (26)

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## *Curriculum Vitae*

**Name:** Camilla J. Brødsgaard.

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**Present Employment:** Senior Scientist at Danish Institute of Agricultural Science, Department of Crop Protection, Research Group Entomology.

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### **Key Qualifications:**

My research experience is focused on the biology of *Paenibacillus larvae larvae* (causing AFB) and the ectoparasitic mite *Varroa destructor*, drug-free control of bee diseases and pollination, research management and planning. During my Ph. D. study, I developed a laboratory method for testing of larval and adult bee resistance towards AFB. The method is now used for testing different Danish bee strains' resistance levels to AFB and possible impacts of GMOs on larval development.

Furthermore, I have assisted in administration of the statutory control of bee diseases and bee disease diagnosis. I have participated in training of beekeepers in bee diseases and teaching beekeeping advisors and students.

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([http://www.agrsci.dk/plb/cjb/cjb\\_home.htm](http://www.agrsci.dk/plb/cjb/cjb_home.htm) )