DESIGNING EXPERIMENTAL PROTOCOLS TO INVESTIGATE THE IMPACT OF GM CROPS ON NON-TARGET ARTHROPODS

A literature-based study, proposing ecologically relevant experimental protocols to investigate the impact of GM crops on non-target organisms.

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EXECUTIVE SUMMARY

EU legislation requires risk-assessment studies to be carried out before approval can be granted to applications for field tests and commercial cultivation of genetically modified (GM) crops. Although risk-assessment studies are necessary it has not been specified which laboratory experiments should be carried out. This report describes detailed protocols for such experiments after giving the rationale for the type of experiments proposed. A previous study identified 19 non-target arthropod species for use in laboratory tests. This report continues the research and presents experimental guidelines for investigating the impact of GM crops on non-target arthropods.

Most research investigating the impact of GM crops on non-target arthropods has focussed on using protocols based on studies connected with synthetic pesticides, where the main parameter measured is that of mortality. However, mortality measurements fail to take sub-lethal effects into account, and changes in development time or fecundity can have important consequences for non-target arthropod populations. For instance, if organisms are sterilized but not killed, a mortality test will result in no effect whereas the population size would be strongly affected. The goal of assessing effects on non-target arthropods is to see whether populations of non-target organisms are negatively affected and if so, to what extent. This encompasses more than only lethal effects and sub-lethal effects can play an important role.

This report highlights the need to standardise and improve experimental techniques. Population growth measurements are suggested as an alternative to simple mortality tests because they combine both lethal and sub-lethal effects into one meaningful measure. An easy to carry out assessment of population growth is to address the instantaneous rate of increase (r_i): a census of the population is taken at time t0, followed by a census at a time after which substantial reproduction has occurred (such as time of maximum growth). Incorporating these measures into a simple equation generates an actual measure of the population growth rate. This technique can be used in either laboratory or field studies and enables researchers to obtain important data for a minimum of effort and expense.

The report presents detailed experimental protocols which can be used to investigate the impact of GM crops on non-target arthropods, including predators, parasitoids, pollinators and soil-dwelling organisms by making use of population growth measurements, such as the instantaneous rate of increase, to determine the impact on population fitness.

CHAPTER 1

INTRODUCTION

BACKGROUND

Ever since the publication of *Silent spring* (Carson, 1962) the public has been alarmed by chemical contamination of the environment. With a growing global population and industrial and technological progress pollutants continue to increase. Transgenic plants expressing insecticidal proteins from the bacterium *Bacillus thuringiensis* (*Bt*) are revolutionising agriculture and the introduction of genetically modified (GM) crops provide an opportunity to reduce the use of synthetic pesticides and ease environmental pollution. However, GM crops may also have the potential to pose risks to non-target organisms and as such require monitoring and assessment.

Environmental policy in the Netherlands advocates intensification of agriculture through environmentally sound means, including non-chemical techniques. To be granted permission to grow GM crops in the European Union (EU) an application has to be made to regulatory bodies. In this application information on the potential environmental effects should be provided. However, details on what information should be presented, and what experiments should be carried out, has not been clearly identified by regulators. In response to this The Netherlands Committee on Genetic Modification (COGEM) initiated a research program to develop a risk-assessment strategy for the release of GM crops in the Netherlands based on scientific data and reasoning.

Since the mid-1990's, when the first GM crops were commercialised a reasonably comprehensive set of testing methods was devised for assessing the likely impacts of GM plants on non-target organisms. A tiered toxicity approach, which has its origins in chemical pesticide testing, is generally taken. These tests begin in controlled laboratory conditions and then progress to more realistic environments. With some adaptation this approach is still suitable for use in evaluating GM plants (see figure 1 below).

Most risk-assessment studies on GM plants in current applications draw heavily on the testing protocols for pesticides, and experiments are carried out using "standard non-target arthropods", like lady bird beetles, green lacewings, parasitoids and honey bees, even though these species may not be appropriate within the crop ecosystem being studied. The EU considers transgenic plants as new entities, which by definition will require new adjusted risk-assessment strategies. As a result, the approach used in the research for COGEM has focussed on ecological interactions and the potential ecological effects of GM plants in multitrophic environments (Groot & Dicke, 2001; Knols & Dicke, 2003). Ecological food webs were constructed for three GM crops, and based on these multitrophic food webs 19 North-western European non-target arthropods were identified from which a subset should be selected for future testing (Scholte & Dicke, 2005). The non-target arthropods include: 4 pollinators, 7 predators, 4 parasitoids, 3 soil-dwelling organisms and 1 protected Lepidoptera species (Scholte & Dicke, 2005).

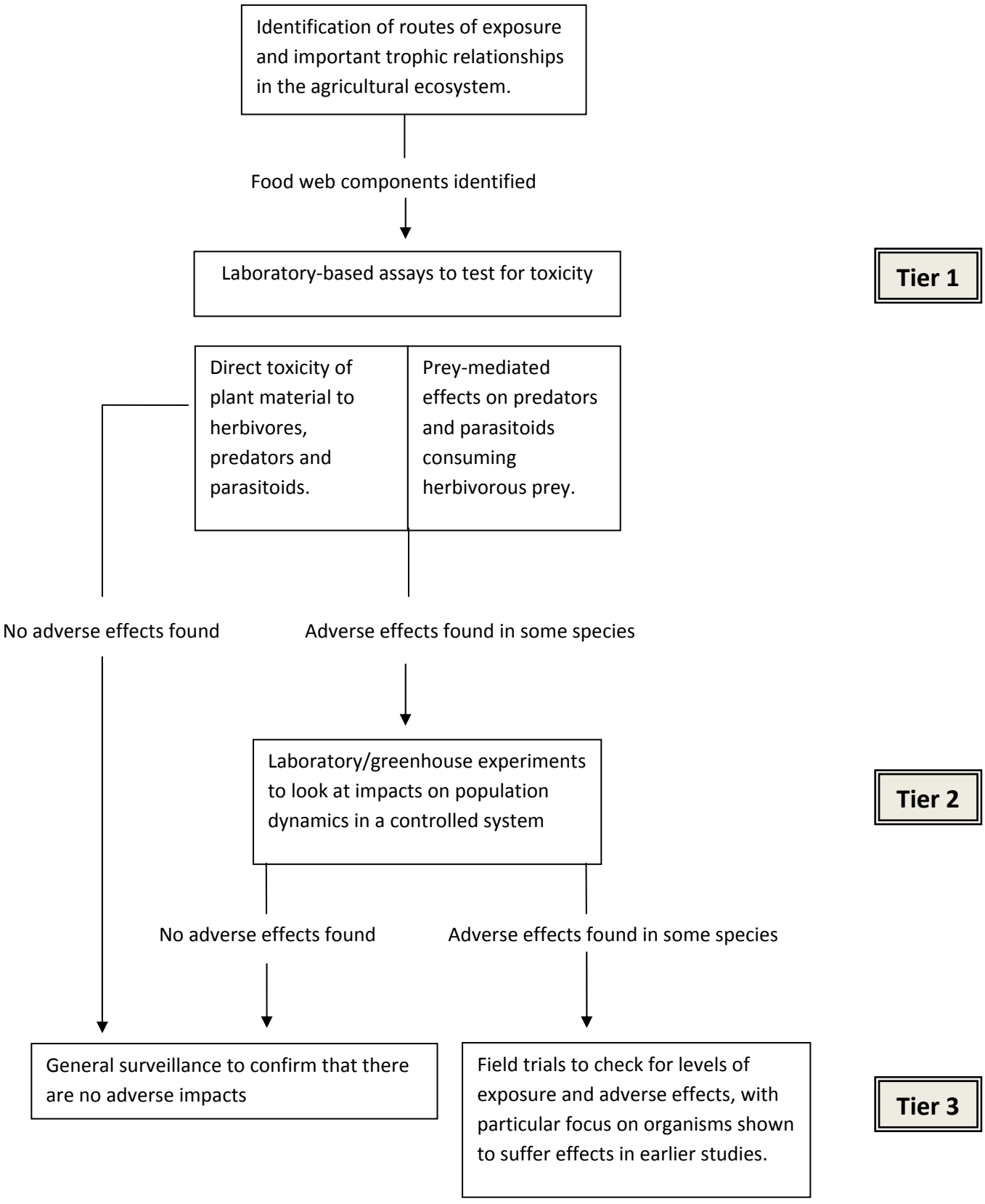


Figure 1. Example of a three-tiered risk-assessment procedure (after: GM science review, 2003)

This study continues the research on the impact of insect-resistant transgenic crops on non-target arthropods. We investigate the appropriate steps that should be taken in order to assess the effect of transgenic crops on these organisms. EU legislation requires that risk-assessment be carried out before GM crops can be introduced, however, it has not been specified which laboratory tests should be performed. This study will focus on determining appropriate experimental protocols for laboratory testing (i.e. tier 1 assessment) of the 19 non-target arthropods selected in the previous study by Scholte and Dicke (2005).

INSECT-PLANT INTERACTIONS

Insects and plants are linked by intricate relationships, and insects are thought to be one of the main forces responsible for shaping the plant world (Marquis, 2004). However, despite their large numbers and diversity herbivorous insects cause relatively little visible damage to plants in natural ecosystems. Plants appear to be well protected and make use of a variety of defence mechanisms to reduce insect attack. These defence mechanisms are passed to future generations and genetic resistance, i.e. the heritable characteristics that influence the degree of damage done by herbivores, is one of the oldest recognised bases of host plant resistance and herbivore control (Panda & Khush, 1995). In 1782 a wheat variety resistant to the Hessian fly, *Mayetiola destructor*, was discovered, and in 1831 an apple variety was found to be resistant to the woolly apple aphid, *Eriosoma lanigerum* (Panda & Khush, 1995). However, the development of active plant breeding for resistance to insects was postponed until the mid-1960's, when problems associated with synthetic insecticides necessitated exploration of alternative control strategies. With the development of plant engineering technology, a whole new array of possibilities was made available to develop pest-resistant plants, for example the introduction of genetic material from any species into crop plants (Duck & Evola, 1997). In the context of insect-pest control this technology has been applied by e.g. inserting genes that code for toxins. The δ-endotoxins (Cry proteins) produced by the bacterium *Bacillus thuringiensis* (*Bt*) are the best known examples, and genes coding for these endotoxins have been transferred to a number of major crops (Merrit, 1998; Jenkins, 1999), including maize, rice, potato, cotton, tomato, tobacco, soybean and *Brassica* species (cabbage, oilseed rape). GM plants can be considered as a specific form of plant resistance, since a toxin is produced in the plant for defence against herbivores.

Bt-genes in crops are generally used to control specific target pest insects; depending on the type of protein (Cry1-5) the target insects belong to one of three insect orders: Lepidoptera, Coleoptera or Diptera. Target organisms die as the δ-endotoxins bind to the midgut, form pores in the membrane and cause cellular disruption (Liang *et al*., 1995; Schnepf *et al*., 1998; De Maagd *et al*., 1999, 2001; Jenkins *et al*., 2000; Broderick *et al*., 2006). However, there are a number of other, non-target, organisms associated with these plants and the impact on their life history parameters may be varied. There are three routes of exposure to GM plants for non-target organisms 1) by feeding on the plant or plant products (i.e. pollen, nectar or plant sap); 2) by feeding on or parasitizing contaminated prey/hosts and 3) by feeding on honeydew excreted by sap sucking insects (see figure 2 below). Many species belonging to the Coleoptera and Diptera are not herbivores, but are in fact carnivores or detritivores. Only about 35% of coleopteran species are herbivorous, the remaining 65% are carnivorous or

saprophagous, while only 30% of Diptera are herbivorous and 70% are carnivorous or saprophagous (Strong *et al*., 1984). Most species within the Lepidoptera are herbivores (99%), however there are a number of species within the order that are important in terms of conservation, such as the Monarch butterfly (*Danaus plexippus*) and within the Netherlands: the dusky large blue (donker pimpernelblauwtje) *Maculinea nausithous*, the small blue (dwergblauwtje) *Cupido minimus*, the lulworth skipper (dwergdikkopje) *Thymelicus acteon* and many others (see: http://nl.wikipedia.org/wiki/Nederlandse_Rode_Lijst_%28dagvlinders%29). Any research will need to consider the impact of GM plants on both the herbivores and their associated natural enemies.

LIFE-HISTORY

Alterations in plant resistance, brought about by the introduction of insect resistance genes, can substantially affect components of the life history of organisms associated with these plants and consequently alter the growth of insect populations. Life history lies at the heart of biology and has a direct influence on the reproduction and survival of organisms. Life history studies address the diversity of life cycles, from the familiar cycle of birth, reproduction and death to the more complex life cycles of alternating sexual and asexual generations. Life history theory explains the broad features of a life cycle; i.e. how fast the organism will grow, when it will mature, how long it will live, how many times it will give birth, how many offspring it will have, etc. (Stearns, 1992).

Principal life history traits are:
Size at birth
Growth pattern
Development rate
Age at maturity
Size at maturity
Number, size and sex-ratio of offspring
Age- and size-specific reproduction
Age- and size-specific mortality
Length of life

Organisms have evolved many different ways of combining life history traits to promote fitness. Life history theory analyses how variation in life history traits leads to variation in fitness among individuals. Fitness is a composite, relative measure of birth and death rates and is a short-term measure of the numerical dominance of an organism (Stearns, 1992). It can be measured by population growth rates. Population growth can be determined from the life-history components of the organism such as developmental rate, ovipositional rate, survival rate and the sex-ratio of the offspring. Population growth can be determined in a number of different ways, for illustration purposes we have made use of the intrinsic rate of increase of populations or r_m which characterises the potential or innate capacity of increase for idealised populations in idealised environments, i.e. constant climate, unlimited food, no interference and a stable age distribution. Standard proportional increases in developmental rate, survival and oviposition rates will increase r_m, but their relative effects on population

growth can be very different, as illustrated in figures 3a-e (Lewontin, 1965). Using figure 3a as a basis we demonstrate in figures 3b-e, how changes in life history traits influence populations.

Adaptation to novel evolutionary challenges such as pesticides or new habitats often imposes a fitness cost on organisms. The characteristics of host plants can influence the performance and fitness of an insect by a multitude of developmental, physiological and behavioural mechanisms. The introduction of a GM plant provides a variety of new challenges to all those organisms associated with the plant, not only the target organism but also associated non-target herbivores, predators, parasitoids, pollinators and soil dwelling organisms. The GM plant may influence life history traits of all these organisms accordingly.

Impact of delayed development on insect populations (figures 3b & c)

The age at which an organism reaches sexual maturity is pivotal, because fitness is often more sensitive to changes in this trait than in any other (Stearns, 1992). Early maturity has a number of benefits, organisms spend less time as juveniles and therefore have a higher probability of surviving to maturity (Bell, 1980), and organisms have a higher fitness because their offspring are born earlier and start reproducing sooner (Cole, 1954; Lewontin 1965; Hamilton, 1966). Compared with other life history traits changes in age at maturity and juvenile survival have a proportionally greater effect on population growth than other factors, particularly in short-lived species with high reproductive rates, as exemplified by many insect species. There are however, costs associated with early maturity: a smaller adult size may result in lower initial fecundity, the quality of offspring produced may be lower and the juvenile mortality rate of offspring may be higher, and finally the organism may have a shorter life and lose in lifetime reproductive success, if these costs are higher than the benefits for early maturity, then development will be delayed.

In the context of evolution it can be predicted that the higher the intrinsic rate of increase of the population (r_m) the more it will pay to increase r_m further by increasing developmental rate. However, there will be costs associated with this, and at some stage the physiology of the organism will simply not permit any further reduction in development time. In studies looking at tetranychid mite species (Carey & Bradley, 1982; Gutierrez, 1976; Coates, 1974) the interspecific variation in developmental time is no more than 1 or 2 hours. Showing that development rate in these species has been driven to its physiological maximum, in this case other life history traits may be subjected to greater selection pressure.

If development is prolonged, as illustrated in figures 3b & c, this can have a significant impact on the population growth of the affected species. A seemingly small change in r_m value (e.g. from 0.300 in figure 3a to 0.258 in figure 3c) can have an unexpectedly large impact on population growth, this is further illustrated in table 1 below: If development is delayed by 20%, within 17 days the population density is reduced by 50%, and within 1 month the population density is 72% lower than the original population (Table 1). Developmental delay will have similar impacts on population densities of herbivores, parasitoids, predators, pollinators and soil-dwelling organisms.

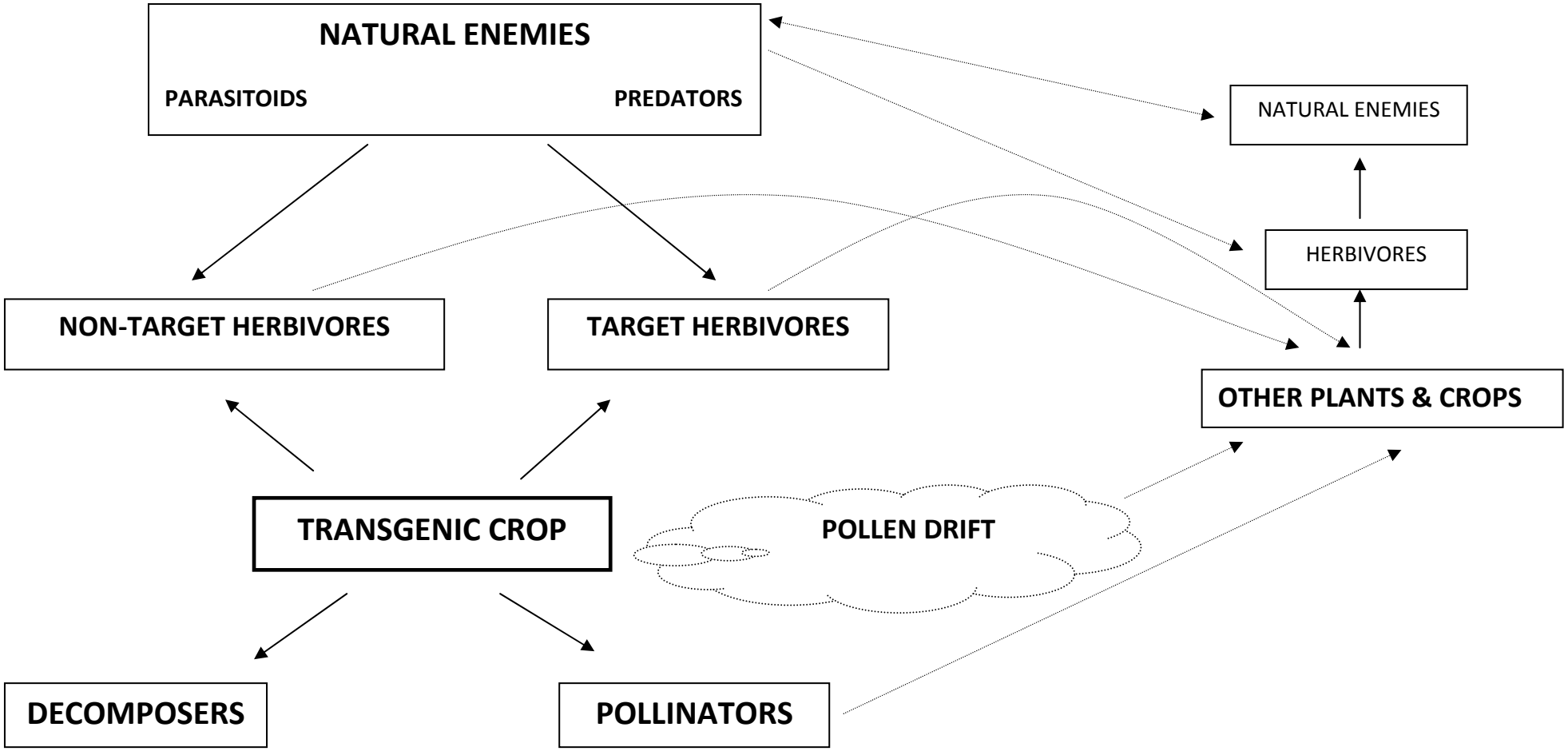
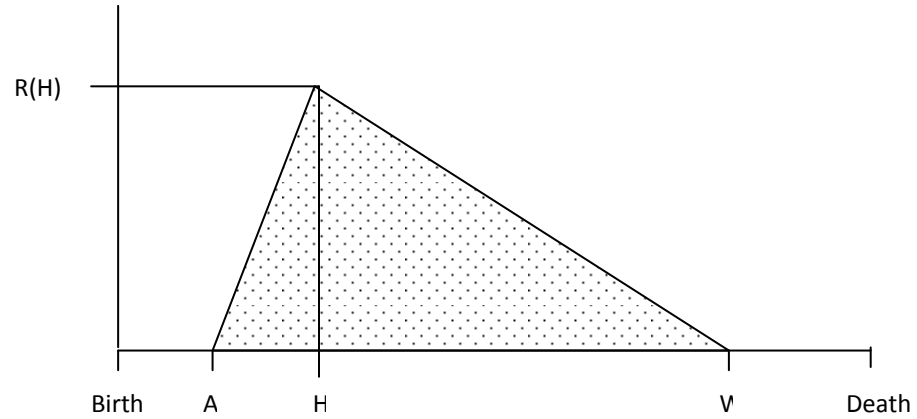


Figure 2. Possible ecological interactions with transgenic crops.



A= Age of first reproduction (= 12 days*)

H= Age at which reproduction peaks (= 23 days*)

R(H)= Peak reproductive rate (= 38.14 eggs per day*)

W= Age at which reproduction stops (= 55 days*)

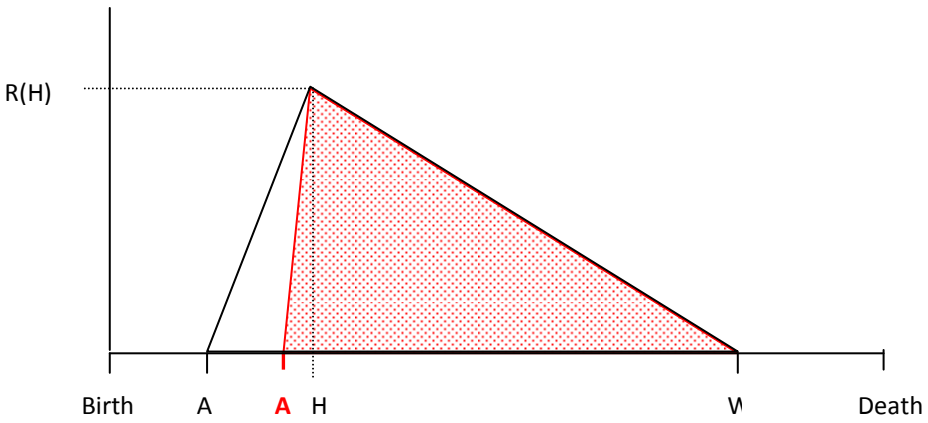
△ = Ro= Life time reproduction (= 820 eggs*)

r_m = Relative rate of increase (Population growth) (= 0.3 per day*)

It takes 2.31 days for the population to double.

Figure 3a. The influence of life history parameters on population growth.

* Figures taken from the Lewontin model (http://www.dpw.wageningen-ur.nl/cwe/popeco/). Example for *Drosophila* sp.



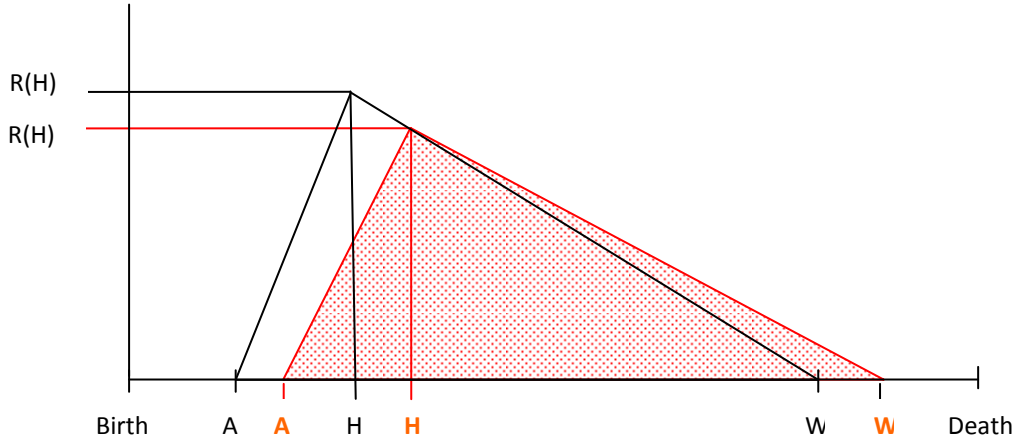
A = 14.4 days

Ro = 774.23

r_m = 0.274

It now takes 2.57 days for the population to double.

Figure 3b. The influence of a change in the age of sexual maturity on r_m. Age of first reproduction (A) is increased by 20%.



A = 14.4 days

H = 27.6 days

W = 66 days

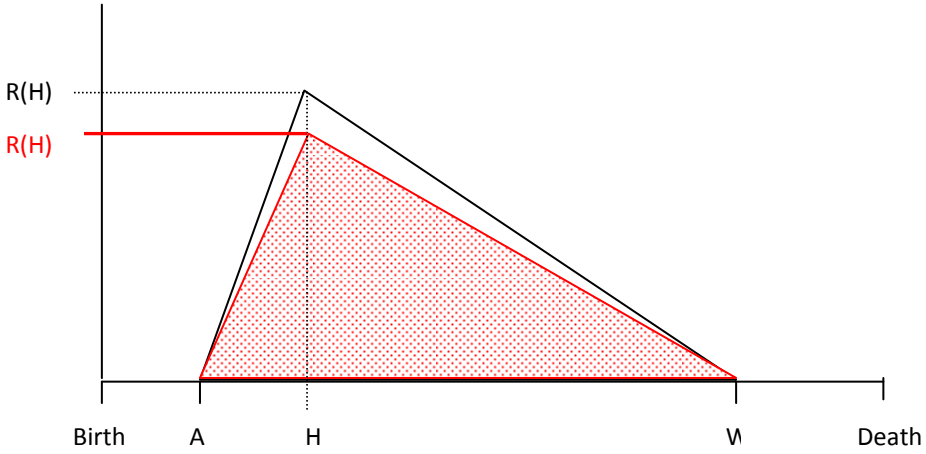
Ro = 984

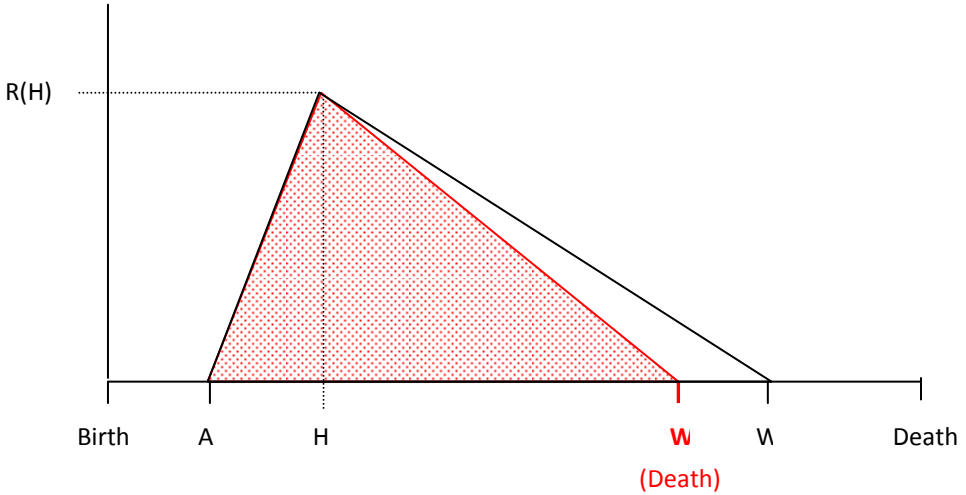
r_m = 0.258

It now takes 2.69 days for the population to double.

Figure 3c. The influence of a change in the age of sexual maturity on r_m. Development is delayed by 20%

* Figures taken from the Lewontin model (http://www.dpw.wageningen-ur.nl/cwe/popeco/)





Ro = 656

R(H) = 30.51

r_m = 0.288

It now takes 2.39 days for the population to double.

Figure 3d. The influence of a change in life-time reproduction (Ro) on R_m. at which Lifetime reproduction (Ro) is decreased by 20%

W = 44

Ro = 610.233

r_m = 0.299

It now takes 2.32 days for the population to double.

Figure 3e. The influence of increased mortality on R_m. Age reproduction stops (W) is decreased by 20%.

* Figures taken from the Lewontin model (http://www.dpw.wageningen-ur.nl/cwe/popeco/

Table 1. Illustrating changes in population growth if development is delayed by 20%. For illustration the original population is considered as 100 individuals on day 0.

Days	Population: $r_m = 0.300$ per day	Population: $r_m = 0.258$ per day	% reduction in population density if development is delayed by 20%
Day 0	100.00	100.00	---------
Day 1	134.99	129.43	4.11
Day 2	182.21	167.53	8.06
Day 3	245.96	216.84	11.84
Day 4	332.01	280.67	15.46
Day 5	448.17	363.28	18.94
Day 6	604.96	470.21	22.28
Day 7	816.62	608.61	25.47
Day 8	1102.32	787.74	28.54
Day 9	1487.97	1019.61	31.48
Day 10	2008.55	1319.71	34.30
Day 11	2711.26	1708.16	37.00
Day 12	3659.82	2210.93	39.59
Day 13	4940.24	2861.70	42.07
Day 14	6668.63	3704.00	44.46
Day 15	9001.71	4794.24	46.74
Day 16	12151.04	6205.37	48.93
Day 17	16402.19	8031.85	51.03
Day 18	22140.64	10395.94	53.05
Day 19	29886.74	13455.86	54.98
Day 20	40342.88	17416.45	56.83
Day 21	54457.19	22542.78	58.60
Day 22	73509.52	29178.00	60.31
Day 23	99227.47	37766.21	61.94
Day 24	133943.08	48882.28	63.51
Day 25	180804.24	63270.23	65.01
Day 26	244060.20	81893.11	66.45
Day 27	329446.81	105997.44	67.83
Day 28	444706.67	137196.60	69.15
Day 29	600291.22	177578.88	70.42
Day 30	810308.39	229847.24	71.63

A resistant plant may affect herbivores directly by prolonging their development. For example, caterpillars feeding on more resistant leaves have prolonged development and their size at maturity is reduced (Feeny, 1968, Slansky & Feeny 1977). If larval development is delayed it may also have an impact on the synchrony between male and female emergence, and this may decrease mating success (Tikkanen *et al*., 2000). Prolonged herbivore development results in a longer exposure to natural enemies (Price *et al*., 1980; Price, 1986), which may result in higher mortality for the effected herbivore.

Parasitoid development may be prolonged if exposed to herbivores that have been feeding on resistant plants. When developing in smaller suboptimal hosts early destructive feeding by the parasitoid results in a smaller adult parasitoid. However, allowing the host to grow larger increases parasitoid size at the potential cost of extending development time (Hemerik & Harvey, 1999). Prolonged development has been shown for *Cotesia plutellae* feeding on larvae of the diamondback moth *Plutella xylostella* that have been exposed to plants treated with a botanical pesticide (Charleston *et al*., 2005) and also for *Parallorhogas pyralophagus* parasitizing *Eoreuma loftini* stemborers fed *Bt*-maize (Bernal *et al*., 2001). The parasitoid *Hyposoter exiguae* suffers prolonged development if feeding on hosts that have been feeding on tomato plants (Campbell & Duffey, 1979). Green lacewing predators have also experienced developmental delay when feeding on caterpillars that have been exposed to *Bt*-maize (Dutton *et al*., 2002; Raps *et al*., 2001). These developmental delays are expected to have an impact on population growth and density of the affected species.

Impact of reduced fecundity on insect populations (figure 3d)

Relative to the development rate, the total number of eggs produced is expected to be subject to much less severe selection (Lewontin, 1965), as indicated in figure 3d. However, once development rate has been increased to a physiological maximum ('development time will certainly never be reduced to zero') then ovipositional rates may play a more important role in population growth (Sabelis 1985). Caswell and Hastings (1980) developed a model and found that for r_m values above 0.1 per day an increase in development rate is more important in increasing population growth than an equivalent increase in ovipositional rate. However, for r_m values below 0.1 per day the reverse is true, oviposition will then be subjected to the most severe selection, relative to developmental time.

If fecundity is reduced, as illustrated in figure 3d, this can have some effect on population growth, but the impact is much less than the impact of a prolonged development. Reproductive investment responds to a variety of factors, including food supply, population density, and temperature. For example, fruit flies lay more eggs when kept at higher temperatures with adequate food and at lower population densities (Stearns, 1992). The grain aphid, *Sitobion avenae*, is more fecund on the ears of cereal plants than on other parts of the plant (Watt, 1979). The bird cherry aphid, *Rhopalosiphum padi*, is more fecund on younger plants than older plants (Dixon, 1971; Leather & Dixon, 1981). The cabbage aphid, *Brevicoryne brassicae*, is also strongly affected by host quality, and is most fecund on plants of good quality with high nitrogen content (Raworth *et al*., 1984; van Emden & Bashford, 1971). In more resistant plants herbivores will need to allocate more energy to overcoming plant defences and detoxifying secondary compounds, and therefore fecundity is likely to be reduced.

Aspects of parasitoid life history (e.g. growth, survival and sex ratio) are expected to respond directly to host properties such as body size (Godfray, 1994; Visser, 1994), and the effect of prolonged development and smaller size of herbivores feeding on resistant plants has also been found to alter the fecundity of certain parasitoids (Sznajder & Harvey, 2003; Harvey *et al*., 2005).

Impact of reduced survival on insect populations (figure 3e)

If mortality is increased then the impact on the population will depend on the age of mortality, juvenile mortality occurring before maturity will have a significant impact on populations, whereas adult mortality occurring after peak reproduction has a much less significant impact on the population growth, as illustrated in figure 3e. If fecundity is high and maturity is early, then fitness of the organism will be sensitive to further changes in age at maturity and fecundity early in life, but changes in life history after the first reproductive event have little impact on population growth.

In the case of non-target herbivores exposed to GM plants, provided mortality occurs after peak reproduction, then populations will not be greatly affected; however, late mortality of the herbivore may have an impact on natural enemies associated with this organism. In an experiment conducted by Orr and Boethel (1985), the high mortality of *Pseudoplusia includens* (Lepidoptera: Noctuidae) feeding on resistant varieties of soybean had a negative impact on the parasitoid *Copidosoma truncatellum* (Hymenoptera: Encyrtidae), as the parasitoid was unable to complete development before the death of its host.

It has also been shown that some herbivores are able to sequester toxins and alter their suitability to natural enemies. For example *Heliothis zea* (Lepidoptera: Noctuidae) is not affected by tomatine an alkaloid defence chemical found in tomato plants, however the parasitoid *Hyposoter exiguae* (Hymneoptera: Ichneumonidae) suffers prolonged development, reduced survival, fecundity and size if exposed to *H. zea* that has been feeding on tomato (Campbell & Duffey, 1979). Similar effects are found for the parasitoid *Cotesia congregata* (Hymenoptera: Braconidae) exposed to *Manduca sexta* (Lepidoptera: Sphingidae) larvae that have been feeding on tobacco plants with high nicotine content (Thorpe & Barbosa, 1986). It is possible that exposure to GM plants may also result in similar knock-on effects to higher trophic levels.

OTHER MEASURES OF POPULATION GROWTH RATES

Despite the high ecological relevance of the r_m endpoint in ecotoxicological studies, it has been applied less widely than mortality-based, standard toxicity tests. This is due in part to the time labour, and expense associated with generating schedules of survivorship and fecundity necessary to generate the measurement (Walthall & Stark, 1997a). The risk assessment of GM plants based on population studies only becomes feasible when research can be performed cheaply and quickly. What is needed is a population measure that integrates both survivorship and fecundity as r_m does, yet is not as time consuming and labour intensive. The instantaneous rate of increase or r_i (discussed in chapter 3) has been suggested as a suitable alternative. The instantaneous rate of increase measures the ability of a population to increase exponentially over time (Hall, 1964). Research has shown that r_i produces results that are comparable with r_m in less time and at lower expense (Walthall & Stark, 1997a), therefore providing a suitable alternative technique for estimating the impact of GM plants on the fitness of non-target organisms.

WHERE TO NEXT?

We recommend that experimental procedures studying the impact of GM plants on non-target organisms investigate the impact of these plants on basic life history traits such as development rate, mortality and fecundity. By fitting life-history parameters to a model, one can compare population dynamics and investigate whether the impact of a GM plant is sufficient to significantly affect population growth of a non-target organism. Lewontin's (1965) triangular reproductive function (illustrated in figure 3), provides a basic tool for comparing population development. The instantaneous rate of increase (r_i) is comparable to this technique (Walthall & Stark, 1997a), and is also less labour intensive, therefore providing a suitable alternative technique. In this report we discuss the use of population growth rates in measuring the impact of environmental stress on organisms with particular focus on the use of the instantaneous rate of increase (r_i).

A previous comprehensive investigation (Scholte & Dicke, 2005) has identified 19 North-western European non-target arthropods that are expected to play an important role in the ecology surrounding three GM crops. This report continues the work and provides a number of standardised experimental procedures that can be used to determine whether the GM crop is likely to have a negative impact on ecological processes. The experimental procedures include effect on mortality as well as sub-lethal effects.

CHAPTER 2

THE APPLICATION PROCESS AND EXAMPLES OF PREVIOUS APPLICATIONS SUBMITTED TO COGEM

One of the key concerns related to ecological risks of GM crops are potential ecological consequences on non-target organisms, including a range of arthropod species that fulfil important ecological functions such as biological control, pollination and decomposition. Most regulatory systems have adopted a comparative risk assessment approach in which the transgenic crop is compared with the corresponding non-transgenic crop, taking into account non-transgenic agricultural practice, including conventional pest control. However, no instructions are provided on how to evaluate effects on non-target organisms. We address laboratory tests and in this chapter we will give an impression of the types of tests used in applications that were submitted to COGEM.

Legislation in the Netherlands covering the work with genetically modified organisms (GMOs) is based on two European directives, 90/219/EC as amended by 98/81/EC on the contained use of GMOs, and 2001/18/EC on deliberate release into the environment. A third law, 1829/2003/EC, covers the use of GMO's in food and feed. Any applications to cultivate GM crops, or to place food or animal feed derived from them onto the market, are reviewed by scientists serving on advisory committees. The scientific evidence is submitted, and a process of iteration often follows between the committee and applicant, with the applicant providing further information before the committee formulates its advice. Some of the evidence in the application dossier is based on peer-reviewed published papers, while other material is based on specific laboratory tests which might not have been published. Scientific advisory committees must then evaluate applications on a case-by-case basis.

Requests for the approval of 88 different GM plants have been received within the EU (GMO database, 2007). The Dutch government has issued over 30 licenses for field trials of GM crops in the Netherlands, however, in 2006, only seven of these licenses were being used: five for field experiments with GM potatoes, one with GM apples, and one with GM flowers (GAIN report, 2006).

APPLICATIONS REGARDING GMOS RECEIVED BY COGEM

The Netherlands Commission on Genetic Modification (COGEM) serves as the scientific advisory committee for the government, and provides advice on the potential risks of genetic modification to human health and the environment. In addition to scientific advice on risk assessment, COGEM brings ethical and social issues related to genetic modification to the attention of the relevant ministers. The scope of the material as reviewed by COGEM covers all fields, ranging from agriculture to medicine; and from contained use to deliberate release of GMO's. COGEM does not, however, advise on food safety. In 2006 COGEM issued 58 advisory documents/reports, with a significant increase in the number of applications for

laboratory experiments with GMOs. COGEM provides advice and information to the Ministry of Housing, Spatial Planning and the Environment (VROM), who must then make the final decision.

Despite legislation there are no standard protocols for testing the effects of GM plants on non-target organisms, and in many cases the non-target organisms that are tested are not ecologically relevant to the cropping system. As an illustration we show three examples of applications for the approval of GM crops that have been received by COGEM (summarised below in Table 2). All applications were for GM maize. Among other things the applications included the testing of non-target arthropods. In all applications, laboratory testing was conducted in compliance with Good Laboratory Practice (GLP) standards as published by the U.S. Environmental Protection Agency (EPA).

Application 1 (CRY 1A(b) maize - received in 1999/2003)
This application presented evidence from laboratory testing of two non-target organisms, both of which are soil-dwelling detritivores (Table 2).

For the laboratory experiment on the Collembola (*Folsomia candida*) 10 individuals were added to 4 replicate vessels containing artificial soil and varying concentrations of *Bt*-maize leaf protein. Two control groups were also tested, one group was exposed to artificial soil only, and one group was exposed to artificial soil and non-transgenic maize leaf protein, the concentration of non-transgenic leaf protein in the control sample was the same as that of the highest concentration of *Bt*-maize protein. When these two control groups were analysed there were no significant differences between the plain artificial soil and the artificial soil mixed with non-transgenic leaf protein, therefore the two control groups were pooled for comparison with the treated group. Mortality and lethargic behaviour were measured at test termination on day 28. Mortality of Collembola exposed to the highest two concentrations of *Bt*-maize leaf protein was significantly greater that those exposed to the control, indicating that this species is sensitive to *Bt*-maize. Although lethargic behaviour was to be noted there is no record of these observations. The number of offspring produced in each replicate vessel was also calculated at test termination and again the two highest concentrations of *Bt*-maize protein had a significant impact on collembolan reproduction.

The procedure for the laboratory experiment on the earthworms (*Eisenia foetida*) was the same as that described above, except for the added observation of burrowing time which was recorded at test initiation. Mortality and lethargic behaviour were recorded on day 7 and at test termination on day 14. Body weight was also measured at test initiation and test termination. There were no significant differences between the control groups and the treatment for any of the parameters measured, although the results for the observation of lethargic behaviour are again not presented.

This application ignored organisms that are involved in a number of important ecological functions such pollination, predation and parasitism. The main life history parameter measured was mortality, although reproduction in Collembola was also measured and some

additional aspects were considered in the investigation on earthworms, such as burrowing time and body weight. The number of replicates in these experiments was low and sub-lethal effects were not considered.

Application 2 (CRY 1F maize - received in August 2003)

This application presented evidence from laboratory testing of 5 non-target organisms, covering a diverse range of ecological functions, including predators (2 species), a parasitoid, a detritivore and a pollinator (Table 2).

For the experiment on lacewing larvae (*Chrysoperla carnea*) 30 individuals were housed separately and exposed to a diet containing a mixture of moth eggs (*Sitotroga* sp.) and a single concentration of a *Bt*-endotoxin which represented up to 30x the expression of the *Bt*-endotoxin present in pollen. The control diet consisted of the moth egg meal without the addition of any test substance. Observations of mortality and pupation were made daily. The test had to be terminated at day 13 when mortality in the control group reached 24%, at this stage pupation was only 14% (4 individuals). Mortality of the lacewing larvae in the treated group was 30%, and pupation in this group was at 17% (5 individuals). The conclusion was that the lacewing larvae were not affected by the treatment, however due to the low replication and the high mortality in the control group after such a short experimental period, it is difficult to confirm this.

For the experiment with the ladybird beetle (*Hippodamia convergens*) three replicate test chambers were maintained in each treatment and control group, with 25 individuals in each chamber. The beetles were exposed to honey containing a single concentration of a *Bt*-endotoxin which represented up to 30x the expression of the *Bt*-endotoxin present in pollen. The control honey diet did not contain any *Bt*. Mortality in the control group reached 21% on day 29 at which point the test was terminated. Mortality in the control group was higher than the treatment, and was also extremely variable with 4% mortality in one replicate and 48% mortality in another replicate. There were also observations of lethargic behaviour within the control group. Replication in this experiment is too low (n = 3) to draw any meaningful conclusions, particularly with the added problems in the control group. Despite these shortcomings the report concludes that the treatment has no impact on the ladybird beetles.

The laboratory experiment with the parasitoid species (*Nasonia vitripennis*) was conducted as explained above for the ladybird beetle. After 11 days mortality in the control group reached 23% and the experiment was terminated. There was large variability in the results from the control groups with mortality in one replicate of 52% and another replicate with no mortality. Mortality in the treatment group was high at 47%. The experiment was repeated using a lower dose of the treatment (20x the expression of the *Bt*-endotoxin present in pollen). For this experiment mortality in the control group reached 39% after 12 days, when the experiment was terminated. Mortality in the treatment group was 47%. Although mortality between replicates in the control group was not as variable (36%, 37% and 44%), the number of replicates (n = 3) was still too low to make any meaningful conclusions. In addition to this, a number of wasps got stuck in the honey, which was not accounted for in mortality

calculations. Despite these problems the report concludes that the treatment has no impact on parasitoids.

The test substance used in the laboratory experiment conducted with the earthworms (*Eisenia foetida*) was a single concentration of a *Bt*-endotoxin which represented up to 100x the expression of the *Bt*-endotoxin present in the top 6 inches of a acre of soil following the incorporation of 25 000 senescent maize plants. Four replicate chambers were maintained for the treatment and control with 10 individuals in each chamber. Earthworms were exposed to artificial soil mixed with the test-substance, while the control group was exposed to artificial soil only. Mortality and lethargic behaviour were recorded on day 7 and at test termination on day 14. Body weight was also measured at test initiation and test termination. There were no significant differences between the control groups and the treatment for any of the parameters measured. Despite the low replication (n = 4) the conclusion was that the *Bt*-treatment does not affect earthworms.

The application includes an experiment conducted using larval honey bees (*Apis mellifera*). The larvae were exposed to pollen and sucrose solutions. The control group was exposed to unmodified pollen moistened with sucrose solution. Three other treatments were used: one treatment consisted of *Bt*-pollen moistened with sucrose solution, one treatment consisted of *Bt*-endotoxin suspended in sucrose solution and one treatment consisted of unmodified pollen and sucrose solution mixed with potassium arsenate. An additional untreated control group was included, in which the larvae were simply mapped and received no pollen or liquid additives. Four replicates with 20 individuals in each replicate were used. Mortality was measured at 6 days after treatment, 12 days after treatment and then daily until test termination 16 days after treatment. The potassium arsenate treatment caused significant mortality, but none of the other treatments had an impact on survival, leading to the conclusion that *Bt*-pollen does not have an impact on honey bees.

The laboratory experiments presented in this application include species incorporating a variety of ecological niches however the species that were chosen are not necessarily those that play an ecologically relevant role in the crop ecosystem. For example the parasitoid chosen for the non-target testing is a parasitoid of blowfly pupae and therefore is unlikely to play an ecological role in the maize crop ecosystem. The pesticide used as positive control in the experiment with the honey bees (potassium arsenate) is not registered for use in agriculture in Europe. In all cases the main life-history parameter measured was mortality. High and variable mortality in some control groups makes the results difficult to interpret and puts doubt on the expertise of the experimental capacities of group that carried out the tests.

Table 2. Non-target organisms tested in a selected set of applications received in the Netherlands. Testing was carried out by laboratories following GLP.

Application no.	Non-target organism	Ecological role	Aspects studied	Effect	Additional notes*
1.	Collembola: *Folsomia candida*	Detritivore	Mortality, no. of offspring, Visual observation for abnormalities.	Yes	No record for visual observations Low replication (n = 4)
1.	Earthworm: *Eisenia foetida*	Detritivore	Mortality, Burrowing time, Body weight, Visual observation for abnormalities.	No	No record for visual observations Low replication (n = 4)
2.	Lacewing: *Chrysoperla carnea*	Predator	Mortality, Visual observation for abnormalities	No	Used artificial diet Low replication (n=30) Short duration (13 days) resulting in low pupation: i.e. control: n = 4 Treatment: n = 5
2.	Ladybird beetle: *Hippodamia convergens*	Predator	Mortality, Visual observation for abnormalities.	No	Used honey Variable mortality in control: i.e. one replicate 4% mortality another replicate 48% mortality. Low replication (n = 3)
2.	Hymenoptera: *Nasonia vitripennis*	Parasitoid	Mortality, Visual observation for abnormalities.	No	Used honey Parasitoid of blowfly pupae and therefore unlikely to be found in crop ecosystem. Variable mortality in control: i.e. one replicate 0% mortality another replicate 52% mortality. Mortality due to wasps sticking in honey reduces statistical power and conclusions drawn. Low replication (n = 3)

2.	Honey bee: *Apis mellifera*	Pollinator	Mortality	No	Only tested larval stage Used arsenic as a positive control Low replication (n = 4)
2.	Earthworm: *Eisenia foetida*	Detritivore	Mortality, Body weight	No	Low replication (n = 4)
3.	Ladybird beetle: *Coleomegilla maculate*	Predator	Mortality, Body weight	No	This species is not found in Europe Used pollen Low replication (n = 30)
3.	Hymenoptera: *Nasonia vitripennis*	Parasitoid	Mortality, Visual observation for abnormalities	No	Parasitoid of blow fly pupae and therefore unlikely to be found in crop ecosystem Mortality in control group due to drowning, reduces statistical power for comparison Low Replication (n = 3)
3.	Honey bee: *Apis mellifera*	Pollinator	Mortality	No	Only tested larval stage Used arsenic as a positive control Variable mortality in treatments, i.e. Treatment 4: mortality was 75% in one replicate and 15% in another. Low replication (n = 4)

***see below – "Problems highlighted by above examples" - for further explanation**

Application 3 (cry34Ab1, cry35Ab1 maize - received in May 2007)
This application presented evidence from testing of a predator, a parasitoid and a pollinator (Table 2).

For the laboratory investigation using ladybird beetles (*Coleomegilla maculate*) two experiments were conducted. The first experiment had 3 treatments with 40 replicates per treatment, in this experiment beetle larvae were exposed to an artificial diet containing 10x the expected environmental concentration of *Bt*-endotoxins. The second experiment consisted of 5 treatments with 30 replicates in each, in this experiment beetle larvae were exposed to pollen containing 1.5x the expected environmental concentration of *Bt*-endotoxins. The three treatments in first experiment consisted of 1) artificial diet containing *Bt*-protein, 2) artificial diet containing heat-inactivated *Bt*-protein, 3) artificial diet with no *Bt*-protein. Mortality and bodyweight were measured after 7 days. The treatment did not have a significant impact on survival; however, there was a significant impact on the larval weight, and those beetles exposed to the treatment weighed significantly less than those on the control treatments. The second experiment used pollen mixed with ground corn earworm eggs. Pollen in 4 of the 5 treatments consisted of pollen from different maize lines genetically modified to express *Bt*-proteins, the control treatment consisted of pollen from unmodified maize. Larvae were monitored daily to record mortality and adults were weighed within 24 hours after emergence. Results indicated that the treatment had no significant impact on survival or adult weight, and the conclusion was that the *Bt*-treatment had no significant impact on the ladybird beetle. However, the reduced larval weight found in the first experiment appears to have been ignored. In addition to this, the relatively low replication in the second experiment (30 individuals), makes it difficult to draw meaningful conclusions; in this experiment one extra death in the treated group would have resulted in a significant difference between the treatment and control.

For the experiment on the parasitoid (*Nasonia vitripennis*) three replicate test chambers were maintained with 25 individuals in each replicate. Parasitoids were exposed to sugar water containing a single concentration of *Bt*-endotoxin representing 10x the amount expressed in *Bt*-pollen. The control diet consisted of sugar water without any test-substance. Mortality was calculated daily and the test was terminated on day 11 when control mortality reached 27%. Results led to the conclusion that *Bt*-treatment did not have a significant impact on the parasitoid. However, there were problems in the control experiment with wasps drowning in water from the vials, which is not accounted for in mortality calculations, and again the low replication (n = 3) makes results difficult to interpret.

The laboratory experiment investigating the impact of GM maize on the pollinator made use of larval honey bees (*Apis mellifera*). The larvae were exposed to pollen and sucrose solutions. One (control) group was exposed to unmodified pollen moistened with sucrose solution. Five other treatments were used: one treatment consisted of *Bt*-pollen moistened with sucrose solution, one treatment consisted of *Bt*-endotoxin mixed in a sucrose solution, two treatments consisted of different *Bt*-proteins mixed in a sucrose solution, and one treatment consisted of unmodified pollen and sucrose solution mixed with potassium arsenate. An untreated control

group was left undisturbed, and only mapped for mortality and emergence. Four replicates with 20 individuals in each replicate were used. Mortality was measured at 6 days after treatment, 12 days after treatment and then daily until test termination 26 days after treatment. The potassium arsenate treatment caused significant mortality, but none of the other treatments had an impact on survival. Again the replication in this experiment was low (n = 4).

While this application included the testing of species from different ecological niches, the choice of non-target organisms was again questionable, the parasitoid species selected attacks blowfly pupae and is not likely to play an important role in the crop ecosystem, and the ladybird beetle selected as a non-target organism does not occur in Europe (Dutton *et al*., 2003). As noted above (in application 2), potassium arsenate is not registered for use in agriculture in Europe and the pesticide treatments used should be representative of the maize insecticides registered for use in the EU. Mortality was again the main life-history parameter measured. Low replication means that the results lack statistical power. High mortality in the control groups caused by drowning, and the variable mortality between replicates, when replication is already low, makes results difficult to interpret.

PROBLEMS HIGHLIGHTED BY THE ABOVE EXAMPLES

Each of the applications share a number of common problems, listed below.

1. Sub-lethal effects are ignored, mortality is the main life history factor measured.

   Sub-lethal effects can drive a population to extinction (Hallam *et al*., 1993), and by ignoring these factors an underestimation of the impact of the GM plant on the population of the non-target organism may occur. Organisms that do not die after exposure to GM plants may still suffer significant damage and this may have an impact on important life history parameters such as development and reproduction. Studies have revealed that toxins can affect populations well below the traditional concentration-response curve, resulting in population decline and extinction at levels previously assumed to have no effect based on mortality using LC_{50} endpoints (Walton *et al*., 1982; Bechmann, 1994). Allan and Daniels (1982) calculated the LC_{50} for the estuarine copepod *Eurytemora affinis* exposed to the organochlorine pesticide Kepone. The acute LC_{50} value was found to be $40\mu g.l^{-1}$, however a reduction in population growth rates was observed for all values over $5\mu g.l^{-1}$ and populations actually reached extinction if exposed to concentrations as low as $20\mu g.l^{-1}$. Clearly the LC_{50} value (based on mortality) does not provide an accurate assessment of the impact of this pesticide on the copepod. Developmental delay, reduced fecundity and decreased survivorship all played an important role in the population decline, which cannot be predicted from mortality measurements alone.

   Population growth rates combine both lethal and sub-lethal effects and are therefore superior to mortality measurements alone. By including sub-lethal effects more precise measurements of population level risks are obtained (Banks & Stark, 1998). It is important for future studies to take these sub-lethal effects into consideration, and

the use of population growth rates instead of mortality as a measured endpoint provides a good solution.

2. Selection of inappropriate non-target organisms

   In two of the applications the parasitoid species selected for testing does not play any role in the crop ecosystem being studied, and the ladybird beetle selected for testing in application 3 is not found in Europe. Ecology has been ignored and testing has been carried out on the commercially available species. In application 1 ecologically important roles played by natural enemies and pollinators have been ignored and the focus has been entirely on the soil ecosystem.

   In order to get a realistic idea of the impact that a GM plant can have on non-target organisms a number of different ecological niches should be considered and the appropriate species selected. Scholte & Dicke (2005) have provided suggestions to solve this dilemma by producing a comprehensive list of 19 non-target organisms appropriate to north-western Europe and ecologically significant within GM crop ecosystems.

3. Other problems associated with the selection of test organisms

   While some ecologically relevant species have been chosen the life stage selected may not have been entirely appropriate. In applications 2 and 3 honey bee larvae are the preferred life stage for tests, and while larvae do consume pollen, the adult life stage is expected to play the main role in pollination and has been ignored. Different life stages of an organism may exhibit different susceptibility to GM plants. This has been indicated in aphid populations exposed to insecticides, where populations exposed from birth were much more susceptible than populations exposed as adults (Stark & Wennergren, 1995), similar results were found when comparing differently structured populations of two-spotted spider mites and pea aphids, the population growth rate after exposure to pesticides was significantly influenced by the starting population structure (Stark & Banken, 1999). Differential susceptibility among life stages can significantly alter the age distribution and demographic characteristics of the exposed population and it is important that this factor is taken into consideration when planning experiments.

4. Replication is low, thus reducing statistical power.

   The functions of experimental replication are: 1) to provide an estimate of experimental error, 2) to improve the precision of the experiment by reducing the standard deviation of the treatment mean, 3) to increase the scope of inference of the experiment by selection and appropriate use of more variable experimental units, and 4) to effect control of the error variance (Steel & Torrie, 1981). An estimate of experimental error is required for statistical tests of significance and confidence interval estimation. If replication is low then estimation of experimental error is imprecise and there is no way to determine whether observed differences indicate real differences or are due to inherent variation. As the number of replicates increase, the

estimation of the population means become more precise and the power of the statistical test increases.

In the applications submitted to COGEM replication was never done with more than 30 individuals. Sometimes the difference of one more death would have made the test result significant. Other problems associated with low replication are also highlighted in the applications: variable mortality between replicates, especially when the replication is low makes it even harder to interpret results and there is an even greater need for replication to be increased. Applications 2 and 3 had particular problems with variable mortality between replicates. It should be noted that the number of independent replicates is often low. For instance if 3 containers, each with 25 test animals are tested, the number of independent replicates for which the percent mortality is calculated is only 3 and not 75. It is not uncommon that the replicate individuals are tested as one group, getting exactly the same food and thus one may argue that replication is reduced to 1 (i.e. there is pseudoreplication).

CONCLUDING COMMENTS

As evident from the above examples, there are a variety of problems associated with these applications. It is clear that a standardised set of guidelines is required. An initial study requested by COGEM has solved some of these problems by identifying relevant non-target species as test organisms (Scholte & Dicke, 2005). Now that guidelines for the selection of non-target arthropods has been provided a standardised approach to non-target testing is required. In this study we aim to develop a series of guidelines for the testing of these non-target arthropods.

In the examples above the main life-history parameter measured was mortality. Experimental protocols used in these applications were those set out by the U.S Environmental Protection Agency guidelines for microbial pesticide testing, which places an emphasis on lethal effects and mortality. However, low mortality rates do not imply that there is no effect on the non-target population as sub-lethal effects can have important consequences for insect populations. Robertson and Womer (1990) recommended monitoring the population response, rather than the response of individuals, as this provides a more realistic picture of responses in the field. Population growth assays have been recommended as superior laboratory bioassay endpoints to that of acute lethal toxicity levels (LC_{50}) because they combine lethal and sub-lethal effects into one meaningful measure (Stark *et al*., 1997; Kammenga & Laskowski, 2000). Studies addressing population growth rather than individual survival after exposure to environmental hazards would help to elucidate relationships among different levels of biological organisation. In chapter 3 we provide some examples of studies that have taken this approach, and in chapter 4 we investigate how they can be adapted for testing the effects of GM crops on non-target arthropods.

CHAPTER 3

POPULATION STUDIES

Regulatory bodies require a detailed environmental risk assessment for the release of GM plants, and although present regulations provide general guidelines for evaluating the risks associated with GM plants; there is still a need for detailed descriptions for non-target risk assessment procedures and the establishment of standardised test protocols. Non-target organisms can be affected by GM plants in variety of ways, either directly via exposure to the toxin, indirectly via a reduction in host/prey quantity or quality, or indirectly via unintended changes in plant properties (i.e. chemical or physical). Although this complexity can make assessment difficult, uncertainty can be minimised by selecting the correct non-target species and conducting suitable tests to provide meaningful crop-specific results (Dutton *et al*., 2003). EU regulations require evaluation of both direct and indirect effects before commercial release (Directive 2001/18/EC). However there is no indication which organisms should be selected or what type of tests should be conducted.

Procedures and guidelines are well established for the assessment of pesticides. The International Organisation for Biological and Integrated Control of Noxious Animals and Plants (IOBC) classifies pesticides into one of four categories depending on the degree at which plant protection products reduce the beneficial capacity of natural enemies. However, the use of pesticide protocols for assessing GM plants is deficient in several aspects. First, pesticide release is controlled by the applicator, who determines timing, point location, concentration, frequency, etc. Spray coverage of the crop plant is rarely ever complete, and thus unsprayed refuges remain where non-target and target arthropods can survive. Second, pesticide degradation begins immediately after application. And third, the mode of action for most synthetic pesticides is typically acute and immediate. In contrast, toxin-producing GM plants release the toxin continuously and in almost all plant parts. The tissue-specific toxin production varies over time and in different environments, and the mode of action is not immediate and not necessarily acute. Sub-lethal, chronic effects become more important for non-target arthropods. The resulting dynamics and types of non-target effects in GM crops therefore differ from those caused by pesticides (Hilbeck, 2002). Since exposure of non-target arthropods to GM plants differs from exposure to sprayed pesticides we suggest that life history parameters including development, reproduction and survival of the selected non-target arthropods are assessed, and that population growth rates are used to investigate the impact of GM plants on insect fitness.

MEASURING MORTALITY

GM crops are commonly evaluated according to guidelines set out for the testing of microbial pesticides, and as such, most studies on non-target organisms have focussed on survival and mortality factors, with an overwhelming use of lethal dose/concentration estimates (LD_{50} / LC_{50}) and mortality as measured end-points (see also examples of tests reported in applications received by COGEM as mentioned in chapter 2). For the LD_{50}, lethal doses are

based on either (*a*) a known amount of toxin per amount of body weight (e.g. milligrams toxin/kilogram bodyweight) or (*b*) amount of toxin per animal (e.g. milligrams toxin/animal). In both cases, the amount of toxin the organism receives is known precisely (Stark & Banks, 2003). In contrast, the LC_{50} is based on the amount of toxin in an environmental medium such as water, soil, or air (e.g. milligrams toxin/litre of water), and the amount of toxin that enters the organism is not known (Stark & Banks, 2003). In 1990, the use of mortality and LD_{50} / LC_{50} as toxicological endpoints comprised 95% of published studies in the SELCTV database (Croft, 1990), emphasizing how entrenched this methodology had become. However, organisms that do not die after exposure to a toxin may nevertheless incur severe damage. Modelling efforts have in fact predicted that population extinction may occur entirely as a result of sub-lethal effects (Hallam *et al*., 1993). Precisely because exposure to a toxin can result in mortality as well as multiple sub-lethal effects, the use of simple toxicity measures, such as LD_{50} and LC_{50}, often results in an underestimation of the total effect of the toxin. To really understand the impact of toxins on non-target organisms both lethal and sub-lethal effects should be measured.

SUB-LETHAL EFFECTS

Despite the singular focus of most toxicological studies on survival/mortality estimates, there is an increasing awareness of the impact of sub-lethal effects on populations. Since 1990 publications investigating the impact of toxins on population dynamics of insects have increased four-fold, while research investigating the impact of toxins on insect mortality has remained the same (CAB Abstracts). Analyses based on population growth rates would result in more accurate assessments of the impacts of toxins because measures of population growth rate combine lethal and sub-lethal effects, which lethal dose/concentration estimates (LD/LC_{50}) cannot do. The inclusion of sub-lethal effects provides a more precise estimate of population-level risks. More ecologically relevant end-points are necessary to better estimate the impact of toxins. Population growth rates have been suggested to be superior endpoints compared to mortality based on LD/LC_{50} because they combine lethal and sub-lethal effects into one meaningful measure (Allan & Daniels, 1982; Gentile *et al*., 1982; Day & Kaushik, 1987; Romanow *et al*., 1991; Bechmann, 1994; Stark & Wennergren, 1995).

GM PLANTS

Under EU legislation GM plants are to be considered as new entities. This necessitates new testing procedures, and the need to move away from standard experimental protocols used in the testing of microbial pesticides. Plants play a central role in insect populations and have an influence on aspects of an insect's life history. Genetic modification of a plant may have an impact on longevity, fecundity, behaviour and developmental time of insects associated with that plant. The timing of onset, peak, and cessation of reproduction may also be affected by GM plants. Whether these alterations reduce insect population growth significantly depends on how responsive population development is to the respective changes (see chapter 1). By fitting life-history data to a model, one can compare population dynamics on different host plants, and investigate whether the range of variability available is sufficient to significantly affect population dynamics. Studies investigating population responses have a number of

advantages over traditional mortality estimates and can easily be adapted to consider the impacts of GM plants on non-target arthropods.

LIFE TABLE RESPONSE EXPERIMENTS (LTRE) AND THE INTRINSIC RATE OF INCREASE (r_m).
Experiments examining life table responses to stressors are known as Life Table Response Experiments (LTREs) (Levin *et al*. 1987). To date most LTREs have been carried out in the context of evaluating the population effects of chemicals (Sibly 1996), however this does not preclude their use in the evaluation of the population effects of GM plants. Life table response experiments are conducted by exposing individuals or groups to increasing doses or concentrations of a toxin over their life span. Daily mortality and reproduction are recorded, and these data are then used to generate life table parameters. The measure that is obtained, the intrinsic rate of increase (r_m), has been shown to be a more accurate measure of toxic effect than lethal concentration estimates (Forbes & Calow, 1999). The intrinsic rate of increase (r_m) is a measure of the ability of a population to increase logarithmically in an unlimited environment (Lewontin, 1965). The calculation of the population growth rate requires knowledge of a population's survivorship and fecundity schedule, which is usually recorded from studies of individuals or groups in LTREs (Carey, 1994). The value of r_m is then calculated from the following equation:

$$1 = \sum l_x m_x e^{-r_m x}$$

where x is the age of the cohort, l_x is the proportion of individuals surviving to age x and m_x is the number of female offspring produced at age x (Stark & Banks, 2003). However, this equation is an 'implicit' equation, which cannot be solved directly, only by iteration on a computer (Begon *et al*., 1990), therefore it is customary to make use instead of an approximation to this equation as follows:

$$r_m \approx \ln R_o / T$$

where R_o is total reproduction and T is generation time.

Positive values of r_m indicate exponential population increase, r_m equal to zero indicates that the population is stable, and negative values of r_m indicate that the population is declining exponentially and heading toward extinction (Stark & Banks, 2003). The intrinsic rate of increase is an ecologically meaningful bioassay parameter as it measures both survival (l_x) and fecundity (m_x).

Advantages of r_m as an endpoint compared to mortality measurements and LD/LC_{50}
Results from several studies have indicated that sub-lethal effects can be subtle and affect populations at concentrations lower than the traditional concentration response curve (LD/LC_{50}). For example; 96-h acute toxicity tests detected no effects of acidic water on *Daphnia pulex* populations, but important negative effects were found when evaluating the

intrinsic rate of increase (Walton *et al*., 1982); and in a later study Bechmann (1994) discovered that some toxins can affect populations well below the traditional concentration-response curve, resulting in population decline and extinction at levels previously assumed to have no effect based on mortality using LC_{50} endpoints. In contrast the LC_{50} endpoint may occasionally overestimate the effects of toxins, in a study investigating the impact of imidacloprid on the aphid *Acyrthosiphon pisum,* LC_{50} and r_m were compared as endpoints (Walthall & Stark, 1997b). It was found that populations exposed to levels exceeding the LC_{50} (i.e.72-hr LC_{60}) were still able to maintain rates of population increase (r_m = 0.224 per day). The ability of surviving individuals to maintain high reproductive rates allowed them to compensate for losses and act as reservoirs for future reproduction. It is not possible, using LC_{50} estimates alone, to predict this ‘‘reservoir effect,’’ and therefore not possible to predict how a population’s growth rate will respond or change based on this endpoint (Walthall & Stark, 1997b).

As a population endpoint, r_m has been successfully utilized to determine the effect of several different pollutants such as pesticides (Daniels & Allan, 1981; Allan & Daniels, 1982; Day & Kaushik, 1987; Walthall & Stark, 1997b; Kim *et al*., 2004), metals (Winner & Farrell, 1976; Bertram & Hart, 1979; Gentile *et al.,* 1982, van Straalen *et al*., 1989; Bechmann, 1994; Kammenga *et al*., 1996), and radiation (Marshall, 1962). More recently r_m has been used to investigate the impact of plant resistance breeding on whiteflies (Romanow *et al*., 1991) and plant morphology on spider mites (Gotoh & Gomi, 2003).

INSTANTANEOUS / REALISED GROWTH RATES (r_i)

The development of life table data is time-intensive; therefore several researchers have been investigating techniques to gather more complete data regarding effects at population levels without having to resort to life table development. Hall (1964) suggested a simplified population growth estimate – the instantaneous growth rate – r_i which has proved very robust (Stark *et al*. 1997; Walthall & Stark 1997a; Stark & Banken 1999; Stark & Banks 2003). The instantaneous or realized growth rate (r_i) gives a close approximation to the more commonly used intrinsic rate of growth (r_m). The advantage of this technique is that it is easy to use: a census of the population is taken at time t0, followed by a census at a time after which substantial reproduction has occurred (such as time of maximum growth). Incorporating these measures into a simple equation generates an actual measure of the population growth rate. This technique can be used in either laboratory or field studies and enables researchers to obtain important data for a minimum of effort and expense. The instantaneous rate of increase (r_i) is calculated as follows:

$$r_i = \ln (Nf / No) / \Delta t$$

where *Nf* is the final number of animals, *No* is the initial number of animals, and Δt is the change in time (number of days the experiment was run). Solving for r_i yields a rate of population increase or decline similar to that obtained by the intrinsic rate of increase (r_m). Positive values of r_i indicate a growing population and r_i = 0 indicates a stable population,

while a negative r_i value indicates a population in decline and headed toward extinction. This technique does not take the age structure of the population into account, but provided the experiment runs for a reasonable amount of time during the peak reproductive phase, results obtained from this technique are similar to those found using r_m as an endpoint (Stark *et al*., 1997; Walthall & Stark, 1997a; Sibly 1999).

Advantages of r_i as an endpoint compared to mortality measurements and LD/LC_{50}

In an experiment with two mite species, i.e. *Tetranychus urticae* (a herbivore) and *Iphiseius degenerans* (a predator), acute lethal concentration estimates (72-hr LC_{50}) and population growth rates (7-day instantaneous rate of increase) were developed after exposure to two pesticides, dicofol and Neemix. For each pesticide, LC_{50} estimates for both species were similar, yet the two species exhibited completely different susceptibility when r_i was the endpoint evaluated; *I. degenerans* was much more susceptible than *T. urticae* to both pesticides (Stark *et al*., 1997). Populations of *T. urticae* were able to withstand exposure to high concentrations of pesticides, sometimes higher than the LC_{50}, and continue to grow. In contrast, populations of *I. degenerans* exposed to low pesticide concentrations (as low as the LC_{13} in one case) were in decline (Stark *et al*., 1997).

The use of r_i as a measurement of population growth provides a simple rapid method of comparison and has been used in a number of studies, for example: in an experiment looking at the impact of plant breeding and predation on populations of pea aphids, *Acyrthosiphon pisum* (Kareiva & Sahakian, 1990); in a study investigating the impact of pesticides on pea aphids, *Acyrthosiphon pisum* (Walthall & Stark, 1997a); in a study investigating the impact of heavy metals on the collembolan *Paronychiurus kimi* (Son *et al*., 2007); in a study investigating the impact of botanical pesticides (NeemAzal) on the coffee red mite, *Oligonychus ilicis* (Venzon *et al*., 2005); and to investigate the impact of acaricides on the phytophagous southern red mite, *Oligonychus ilicis* and its predator *Iphiseiodes zuluagai* (Teodoro *et al*., 2005).

COMPARING INTRINSIC (r_m) AND INSTANTANEOUS (r_i) RATES OF INCREASE.

The main obstacle to using LTREs and subsequently r_m is the time and labour involved, r_i has been suggested as a faster and more efficient technique for measuring population growth. Walthall and Stark (1997a) investigated the impact of the pesticide imidacloprid on pea aphids (*Acyrthosiphon pisum*) and compared the results obtained from calculating r_m and r_i (see below). The final results were comparable, showing that r_i provides a suitable alternative to the more time consuming and expensive techniques that require LTREs. When using r_i as an end-point, it is important to determine the correct length of the experiment. If population level end-points are supposed to find the contaminant levels at which population growth is inhibited, then it is important to discover the point at which an untreated population reaches its maximum rate of increase and test the impact of the pollutant up to this point (Walthall & Stark, 1997a). Walthall and Stark (1997a) report that the required length of the experiment can be calculated without resorting to LTREs. They generated a curve fit using the r_i values calculated from the censused population (see below).

Performing population studies

In the study by Walthall & Stark (1997a) the impact of 8 different concentrations of the pesticide imidacloprid on populations of pea aphids (*Acyrthosiphon pisum*) was investigated comparing r_i and r_m as measured end points.

Method for calculating r_m

1) On untreated plants: Broad bean plants were reared in pots until reaching 25 cm in height, at which the plants were thinned so that 5 plants remained in each pot. Four pots were randomly selected and 10 adults were placed individually into clip cages and fastened on the underside of randomly selected leaves in each pot (n = 40). Twenty four hours later all aphids were removed from each clip cage apart from one neonate, thus ensuring exposure to the plant from birth. Mortality and reproduction were recorded at 24 hour intervals throughout the lifespan of each aphid (up to 40 days). A life table was constructed from daily schedules of mortality and fecundity, and mean daily values for r_m were calculated.
2) On treated plants: Broad bean plants were reared in pots until 25 cm high and then thinned to five plants per pot. Pots were then sprayed with one of eight different concentrations of imidacloprid or the control (distilled water), with four replicates for each treatment/control (36 pots). Once plants were dry 10 adults were placed individually in leaf clip cages and fastened on the underside of randomly selected leaves in each pot, and the experiment continued as explained above.

Method for calculating r_i

1) On untreated plants: Twenty one pots of broad bean plants were reared at a density of 8-10 plants per pot. When reaching 25 cm in height they were thinned to 6 plants per pot. Mylar sleeves were placed over the pots and batches of 10 neonates were transferred onto plants inside the sleeve using a fine camel-hair paintbrush. Mylar sleeves were covered with a nylon mesh screen, and neonates were allowed to migrate and reproduce freely on their new host. Populations from three randomly selected pots were then counted at 8, 11, 13, 15, 17, 19 and 21 days after introduction. The instantaneous rate of increase was then calculated for each time interval.
2) On treated plants: Broad bean plants were treated with one of eight different concentrations of imidacloprid or the control (distilled water), with three replicates for each treatment/control (27 pots). After drying a Mylar sleeve was placed over the pot and 10 neonates were introduced. The Mylar sleeve was covered with a nylon mesh screen and aphid populations were left undisturbed for 11 days after which the total aphid population in each pot was counted and r_i was calculated.

Statistics

Significant differences in the day-to-day r_m values were calculated using one-way analysis of variance (ANOVA) on ranks; and mean separation was calculated using Student-Newman-Keuls method. The same technique was used to compare the r_i values obtained at each census. A t-test ($\alpha = 0.05$) was used in order to compare the r_m and r_i values at each census date. To determine the point at which the untreated population reaches its maximum rate of

increase (and thus the minimum required experimental period) a curve fit of r_i values over time for the censused population was calculated. The peaks of this function represent the mean maximum rate of growth for these populations. To investigate the relationship between increasing imidacloprid concentrations and decline in r_i a linear regression model was used, and the correlation between r_m and r_i values following 11 day exposure to the different concentrations of the pesticide was found using the Pearson product moment correlation.

Results

Mean daily rates of r_m on untreated plants increased rapidly following the onset of reproduction (at day 6) and peaked at 0.331 per day on day 12. After day 12 r_m declined and stabilised at 0.293 per day. Comparing r_m and r_i values for days in which sampling dates overlapped there was a significant difference between r_m and r_i on days 8 and 15, but not on days 11, 13, 17, 19 and 21, suggesting that 11 days would be the minimum experimental time at which accurate results for toxic impacts on this population could be determined if r_i is used as the measured end-point. By fitting a curve to the results obtained from determining r_i values this was confirmed as a suitable test period. Once these results were obtained using an untreated population the experimental period for the aphids exposed to imidacloprid, in the study using r_i as an endpoint, were only censused once, after 11 days. The r_m and r_i values for populations exposed to imidacloprid showed a decline in a concentration dependent manner. These r_m and r_i values were highly correlated and statistically significant, indicating that r_i can be applied as a substitute measure for r_m in monitoring population responses to pollutants.

Advantages of r_i as an end-point compared to r_m

In the study described above the calculation of r_m required daily observation of 40 separate leaf clip cages for each treatment/control over an entire lifespan (up to 40 days). By comparison the calculation of r_i required 10 individuals to be placed on each of three replicate plants, and only one population census at 11 days. So, while the r_i end-point does not provide the detailed demographic information provided by life tables it appears to have some advantages over r_m because it evaluates real populations with density-dependent feedback mechanisms and can be generated in less time, with less expense than the traditional life table method (Walthall & Stark, 1997a).

One problem with life tables and the estimation of r_m is that they may not consider the impact of the pollutant on oogenesis or embryogenesis (Van Leeuwen *et al*., 1985; 1987). The impact of pollutants may only become apparent after several generations of exposure. Experiments based on r_i as a measured end-point are low maintenance compared to those required in the generation of life tables and calculation of r_m values, and could be carried out over several succeeding generations, thereby providing a more practical method for determining safe concentrations at the population or community level (Walthall & Stark, 1997a).

In the study described above the results yielded a linear relationship between the r_i value and the pesticide concentration to which the population was exposed. The integration of pesticide concentration and population growth rates into a linear regression creates a concentration-

response function for population-level exposure, which may significantly improve the ability to predict the effect of pesticides in an ecosystem (Walthall & Stark, 1997a). Using linear regression models it may also be possible to integrate a population's response to more than one environmental stress (Gentile *et al*., 1982). The ability to monitor the effect of multiple stressors on population growth rates will benefit risk assessment, which can make use of this information to model and predict a population response to a combination of biotic and abiotic factors.

CONCLUDING COMMENTS

Population growth assessments provide a valuable technique for measuring the impact of stress on a population. As emphasised by the examples above these measurements have a distinct advantage over mortality measurements as they take important sub-lethal effects into account. In a previous study 19 non-target arthropods were identified (Scholte & Dicke, 2005). These organisms fell into one of four categories: detritivores, pollinators, predators and parasitoids, and also included one protected species. Population studies have been conducted on a variety of organisms falling into these categories (Table 3).

Although the studies that make use of population growth rates are mostly focussed on the impact of toxins and pollutants there is no reason why similar studies cannot be used to investigate the impacts of GM plants on insect populations. Traditionally studies investigating GM crops have focussed on lethal concentration/lethal dose and have used mortality as the measured endpoint. The main disadvantage with this technique is the failure to take sub-lethal effects into account. The list of studies provided above is by no means exhaustive, it does however provide a good example showing the benefits to using population growth studies and we feel that future research investigating the impact of GM crops on non-target organisms should make use of similar techniques. In Chapter 4 we provide clear detailed guidelines on experimental procedures that should be followed in the testing of GM plants on the 19 species of non-target arthropods selected in the previous study.

Table 3. Examples of population studies conducted on non-target organisms

Species studied	Ecological role	Type of study	Population measure used	Reference
Orchesella cincta	Detritivore	Impact of cadmium (toxin)	Intrinsic rate of increase (r_m)	van Straalen *et al*., 1989
Platynothrus peltifer	Detritivore	Impact of cadmium (toxin)	Intrinsic rate of increase (r_m)	As above
Paronychiurus kimi	Detritivore	Impact of cadmium, Mercury, Lead (toxin)	Instantaneous rate of increase (r_i)	Son *et al*., 2007
Euseius stipulates	Pollinator	Comparison of pollen types	Intrinsic rate of increase (r_m)	Bouras & Pappadulis, 2005
Kampimodromus aberrans	Pollinator	Comparison of pollen types	Intrinsic rate of increase (r_m)	Kasap, 2005
Apis mellifera	Pollinator	Impact of *Varroa jacobsoni* (parasite)	Intrinsic rate of increase (r_m)	Martin, 1998
Orius niger	Predator	Impact of temperature	Intrinsic rate of increase (r_m)	Baniameri *et al*., 2005
Harmonia axyridis *Propylea quatuordecimpunctata* *Coleomegilla maculate*	Predators	Comparison of prey suitability	Intrinsic rate of increase (r_m)	Mignault *et al*., 2006
Rhynocoris marginatus	Predator	Impact of insecticides (toxin)	Intrinsic rate of increase (r_m)	George & Ambrose, 2006
Orius insidiosus	Predator	Suitability of prey	Intrinsic rate of increase (r_m)	Butler & O'Neil, 2007
Haplothrips brevitubus	Predator	Impact of prey density	Intrinsic rate of increase (r_m)	Kakimoto *et al*., 2006
Iphiseiodes zuluagai	Predator	Impact of acaricides (toxin)	Instantaneous rate of increase (r_i)	Teodoro *et al*., 2005
Iphiseiodes zuluagai	Predator	Impact of botanical pesticide (toxin)	Instantaneous rate of increase (r_i)	Venzon *et al*., 2005
Aphidius colemani *Lysiphlebus testaceipes* *Aphelinus varipes*	Parasitoids	Impact of temperature	Intrinsic rate of increase (r_m)	Van Steenis 1993 Van Steenis 1994 van Steenis & El-Khawass 1995
Habrobracon hebetor	Parasitoid	Comparison of host suitability	Intrinsic rate of increase (r_m)	Amir-maafi & Chi, 2006
Tetrastichus giffardii	Parasitoid	Comparison of host suitability	Intrinsic rate of increase (r_m)	Mohamed *et al*., 2006
Bracon hebetor	Parasitoid	Impact of host diet	Intrinsic rate of increase (r_m)	Singh *et al*., 2006
Acarophenax lacunatus	Parasitoid	Impact of insecticide (toxin)	Instantaneous rate of increase (r_i)	Goncalves *et al*., 2002
Aphidius ervi	Parasitoid	Impact of insecticide and cadmium (toxin)	Instantaneous rate of increase (r_i)	Kramarz & Stark, 2003

CHAPTER 4

EXPERIMENTAL PROCEDURES FOR LABORATORY TESTING OF NON-TARGET ARTHROPODS

The use of population growth models provides an excellent technique for measuring the impact of environmental stress on insect fitness. As such we will be making use of these techniques to investigate the impact of GM crops on non-target arthropods. A tiered risk assessment is recognized as being the most appropriate and rigorous approach to assess non-target effects from both scientific and regulatory standpoints (see Chapter 1). Both hazard and exposure can be evaluated within different levels or "tiers" that progress from "worst-case" hazard and exposure to more realistic scenarios in the field. In this chapter we focus on lower tier tests which serve to identify potential hazards. These tests are conducted in the laboratory to provide high levels of replication and study control which increase the statistical power to test hypotheses.

Before testing can commence it is necessary to select representative non-target organisms from a proposed set of species that capture key ecological functions. Criteria such as amenability to testing, availability of test methods that respect the standards of Good Laboratory Practices (GLP) and unambiguous taxonomic recognition are crucial for non-target testing. Based on these criteria, a list of 19 non-target arthropods was compiled (Scholte & Dicke, 2005); these species represent those living in the crop and adjacent non-crop habitats. The next step is the development of test protocols. The non-target arthropods need to be evaluated in properly designed tests that fulfil established quality control standards, (e.g. GLP). Experience has shown that early tier tests conducted under "worst-case" conditions in the laboratory can be well standardized. This is important to assure study repeatability, interpretability and quality, and thus to ensure a high level of confidence in the reported data.

Many of the experiments used to test the environmental safety of GM crops are poorly replicated, of short duration and/or test only a few of the response variables (see Chapters 2 & 3). Protocols developed to assess the impact of pesticides have historically formed the basis for the standard protocols used for the assessment of the potential effects of GM crops on non-target organisms (e.g. Candolfi *et al*., 2000; see http://www.epa.gov/opptsfrs/home/guidelin.htm). Testing in the USA often proceeds as follows: organisms are exposed to the pure insecticidal protein at a range of doses and concentrations that range from 10 to 100 times higher than those expected in the environment. These tests are often followed by field studies (Rose, 2007). In the EU a more extensive assessment is required in which the potential immediate and delayed effects of the GM plant resulting from both direct and indirect interactions with non-target organisms is required. Despite these requirements there are no guidelines on how to proceed with laboratory testing. As a result most tests submitted in the EU follow the same guidelines as those set out by the USA for the testing of microbial pesticides.

Before entering into testing, the objectives of the individual studies need to be defined, and specific measurement endpoints described. This is where the proposed testing system described in this chapter differs from those based on pesticide testing protocols. As explained in previous chapters, protocols based on the testing of the impact of pesticides on non-target arthropods focus on mortality, and often fail to consider sub-lethal effects, whereas the use of population growth as a measured endpoint takes both lethal and sub-lethal effects into account. For this reason, the experimental protocols described in this chapter will make use of population growth as a measured endpoint.

Early tier laboratory tests usually entail a simple, well-defined test system designed to measure a specific endpoint (in this case population growth) using concentrations that are several times higher than those that will be seen in the field. Elevated doses are applied since these tests use a small number of surrogate arthropods and because higher dose limit tests can add additional certainty to the safety assessment. All tests should adopt quality control parameters that help validate the test system which may include: (i) low negative control mortality, (ii) use of a positive control, (iii) homogeneity of test material, (iv) stability of the insecticidal compound, and (v) sufficient statistical power.

Insecticidal proteins (e.g. Cry proteins) used in GM plants have no dermal contact toxicity and must be ingested by a susceptible organism to be effective. Therefore, non-target arthropods need to have direct dietary exposure in order to evaluate toxicity. In a typical laboratory test a known concentration of the insecticidal protein is incorporated into a diet substrate and fed to the non-target arthropods. For higher trophic levels, such as predators and parasitoids, the route of exposure to insecticidal proteins in nature is via consumption of host or prey species that have fed on transgenic plant tissues or via consumption of GM plant products such as pollen or nectar. Initial laboratory studies should be conducted using "worst-case" scenarios by direct exposure to pure insecticidal protein in artificial diets at excessive concentrations, or, if this is not possible, by natural routes of exposure through contact with hosts/prey feeding on the GM plant, or exposure to GM plant products such as pollen.

The experimental protocols outlined below were determined from a variety of different studies that investigate population growth of invertebrates (see Table 4 below), and where possible the guidelines set out under the European and Mediterranean Plant Protection Organisation (EPPO) and by the US Environmental Protection Agency (EPA) have been followed.

Table 4. Selected references to experiments investigating population growth of invertebrates that have been incorporated into the experimental protocols outlined below.

Ecological Role	Species	Reference
Predator (Acari: Phytoseiidae)	*Amblyseius finlandicus* (Oudemans) *Amblyseius potentillae* (Garman) *Typhlodromus pyri* Scheuten	Dicke *et al*., 1990
Predator (Acari: Phytoseiidae)	*Iphiseiodes zuluagai* Denmark & Muma	Teodoro *et al*., 2005
Predator (Acari: Phytoseiidae)	*Iphiseius degenerans* Berlese	Stark *et al*., 1997
Predator (Hemiptera: Reduviidae)	*Rhynocoris marginatus* (Fabricius)	George & Ambrose, 2006
Predator (Neuroptera: Chrysopidae)	*Chrysoperla carnea* (Stephens)	Romeis *et al*., 2004
Predator (Neuroptera: Chrysopidae)	*Chrysoperla carnea* (Stephens)	Dutton *et al*., 2002
Predator – General		EPA Guidelines: OPPTS: 885:4340
Parasitoid (Hymenoptera: Aphidiidae)	*Aphidius ervi* Haliday	Kramarz & Stark, 2003
Parasitoid (Prostigmata: Acarophenacidae)	*Acarophenax lacunatus* (Cross & Krantz)	Gonçalves *et al*., 2002
Parasitoid (Hymenoptera: Encyrtidae)	*Syrphophagus aphidivorus* (Mayr)	Buitenhuis *et al*., 2004
Parasitoid (Hymenoptera: Platygasteridae)	*Amitus fuscipennis* MacGown & Nebeker	Manzano *et al*., 2002
Parasitoid – General		EPA Guidelines: OPPTS: 885:4340
Soil organism (Collembola: Onychiuridae)	*Paronychiurus kimi* (Lee)	Son *et al*., 2007
Soil organism (Collembola: Entomobryidae) (Acari: Camisiidae)	*Orchesella cincta* (L.) *Platynothrus peltifer* (Koch)	van Straalen *et al*., 1989
Soil organism (Collembola: Isotomidae)	*Folsomia candida* Willem	Fountain & Hopkin, 2005 ISO 11267: 1999
Pollinator (Hymenoptera: Apiidae)	*Apis mellifera* L.	EPPO Guidelines: PP 1/170 EPA Guidelines: OPPTS 885.4380

DETERMINING EXPERIMENTAL PERIOD

As has been noted in chapter 3 the main difficulty in using the instantaneous rate of increase to detect changes in population growth is determining the length of time that the experiment should run for. Insecticidal proteins target the digestive system and symptoms of toxicity can usually be seen within 3-5 days, and this would be a suitable period if mortality is the end-point to be measured. However, in the proposed experimental protocols we aim at investigating population growth rates of non-target organisms, so as to incorporate both lethal and sub-lethal effects. In this case the experimental period will be determined by the biology of the non-target organism, the nature of the insecticidal protein and the test concentration selected. Therefore, the duration of the experiment will need to be adjusted accordingly, and the biology and life-stage of each organism being tested will need to be considered. If the time of peak reproduction is already known then the experimental period can be determined from this information. If this information is unknown then it should be determined prior to the experiment. The experimental period must be long enough to incorporate the period of peak of reproduction and should be terminated shortly thereafter.

DETERMINING TEST CONCENTRATION

Before population studies can be conducted it is necessary to determine the concentration of the test substance. The Maximum Hazard Dose (MHD) can be used. This concentration is usually 10 - 100x the maximum expected exposure concentration in the field. The level of exposure should be based on a detailed quantification of the Estimated Environmental Concentration (EEC) in the field. The test concentration should be designed to reasonably achieve in excess of the anticipated maximum (EEC) while accounting for variation in species sensitivity and inter-plant variation in insecticidal protein expression level. The MHD could also be represented as the High End Exposure Estimate (HEEE) of the plant material to which the organism may be exposed, which is based on an estimate of insecticidal protein expression levels in plant tissue at the upper end of exposure, preferably the 90th percentile (Rose, 2007). For the examination of sub-lethal effects it may be sufficient to use a lower concentration, for example 5x the EEC.

GENERAL MATERIALS AND METHODS COMMON TO ALL EXPERIMENTS

- Non-target arthropods may be obtained from commercial external sources, from university insect cultures or reared within the laboratory of the company performing the experiments.
- To avoid variation among the test organisms they should be of similar age and obtained from the same, identified, source.
- Preferably the history of the culture should be well-known and the culture available to third parties that wish to validate data with organisms from the same source.
- Experiments should be carried out in controlled environment rooms, with continuous monitoring of temperature and humidity. Lighting should be controlled on a 16:8 L:D cycle when testing for temperate regions.
- Plants should be grown under controlled environmental conditions that relate to those found in the field with respect to light, temperature, relative humidity, nutrition and substrate.

- Transgenic plants used in experiments should be tested to determine the presence and concentration of the insecticidal protein.

1. PREDATORS: DEVELOPMENT OF ASSAY PROTOCOL

Various laboratory tests have been conducted on predators. Results vary according to how the tests were conducted. Predators may be exposed to the insecticidal protein in GM plants by feeding on pollen, which is often used to prolong their life span when prey is scarce, or by consuming prey which has been feeding on the GM plant.

It is possible to maintain some predator species on artificial diet, pollen, or sucrose solutions. When these options are available, then the experimental protocol 1.1 outlined below should be followed. However some species cannot be reared in this manner and must feed on prey organisms. In this case the experimental protocol 1.2 outlined below should be followed.

1.1 Experimental protocol suitable for testing predators that can be successfully reared on artificial diet

This test is developed to assess the impact of transgenic products on the population growth of predators that can be successfully reared on artificial diet or pollen.

Test substance

- Using a pre-determined concentration the pure insecticidal protein should be mixed with artificial diet / sucrose solution (hereafter referred to as the "treatment").
- If the predator can be successfully reared on pollen, and the pollen is shown to contain the insecticidal protein, then pollen should be collected from transgenic plants and presented to adults or larvae (hereafter referred to as "transgenic pollen"). Before making use of pollen it is necessary to also determine whether the pollen is of sufficient quality to provide a suitable diet, to avoid using an inadequate pollen source as food.
- A control diet should be prepared in the same manner without the addition of the toxin (hereafter referred to as the "control diet").
- For those predators reared on pollen a control diet of pollen collected from untransformed plants should be provided to the predator (hereafter referred to as "untransformed pollen").

Experimental procedure

- Female predators must be offered a suitable surface on which to lay eggs. This can be provided by untransformed plants, or leaves from them, or by the use of a suitable artificial surface.
- The untransformed plants that are to be used for egg-laying must be planted and grown under controlled environmental conditions.
- Leaf discs of equal size can be cut from the untransformed plants and used in the test arenas.
- Predators should be contained in a suitable arena preventing escape, but still allowing free movement and access to diet and to the egg-laying area (e.g. mites can be placed in glass Petri dishes fixed in a water bath (Teodoro *et al*., 2005), or on leaves/leaf discs on top of

moist filter paper / cotton wool in glass Petri dishes (Kasap, 2005; Kim & Yoo, 2002; Kim *et al*., 2004). Paper cups have been used to test ladybird beetles and predatory bugs (Butler & O'Niel, 2007). Insect cages could also be used.

- A suitable shelter should be constructed for the mite predators within the arena (e.g. tent-shaped structures composed from paper or plastic sheeting (Dicke *et al*., 1990)
- At least 40 suitable test arenas should be prepared for each treatment / control
- Food should be placed into each arena prior to predator introduction so that predators have immediate access to the treatment / control diet or to the transgenic / untransformed pollen.
- 10 newly emerged neonate predators should be transferred to each test arena.
- The food source needs to be replenished regularly so that the predators have continuous access to food. If pollen is used then the pollen needs to be replaced regularly so as to prevent fungal infection.
- After a suitable experimental period the number of individuals should be counted, and the instantaneous rate of increase (r_i) calculated.

1.2 Experimental protocol suitable for testing predators that cannot be reared on artificial diet

Predators may be exposed to the GM plant via their prey, and it possible to expose suitable prey species that have been feeding on the GM plant to the predator rather than making use of artificial diet or pollen. However, if offering prey it is important that suitable species are offered, and that the prey is of sufficient quality. Experiments have shown that *Chrysoperla carnea* experienced delayed development and reduced survival if feeding on susceptible prey, but were not affected if feeding on prey that were not susceptible to *Bt* (Dutton *et al*., 2002). The negative effects appeared to be prey-mediated (Romeis *et al*., 2004), and the sub-optimal quality of the susceptible prey appeared to be a more important factor than the *Bt* toxin itself. Therefore it is important that a suitable prey species is selected.

This experiment will assess the effects of the transgenic trait on predators that have been feeding on prey that have been reared on artificial diet or GM plants. In this experiment the effect of transgenic versus non-transgenic plants on predator population growth rate will be evaluated.

Test organisms

1. Prey organisms reared on artificial diet

- If the predators cannot be reared on artificial diet then they should be provided with prey organisms that have been reared on artificial diet.
- Using a pre-determined concentration the pure insecticidal protein should be mixed with artificial diet and fed to prey organisms (hereafter referred to as "treated" prey).
- A control diet should be prepared in the same manner without the addition of the toxin (hereafter referred to as the "control" prey).
- The artificial diet should be provided just prior to hatching so that the prey is exposed to the treatment / control from the first day.

2. Prey organisms reared on plants

- If the prey organisms cannot be reared on artificial diet, then they should be reared on GM plants containing the insecticidal protein (hereafter referred to as "treated" prey).
- For the control treatment the prey organisms should be reared on untransformed plants (hereafter referred to as "control" prey).
- The GM or untransformed plants must be grown under controlled environmental conditions. Plants should be enclosed within insect cages or muslin sleeves so as to prevent escape of the prey. Adult prey organisms should be introduced and left to lay eggs on the plants, so that the prey has been exposed to the GM / untransformed plant from birth.

Experimental procedure

The experimental procedure described in section 1.1 should be followed with some alterations.

- Instead of artificial diet, the predators are provided with prey organisms that have been feeding on treated / control artificial diet or on GM / untransformed plants.
- The prey organisms must be of the correct age for the stage of the predator and should be provided at high density and renewed or supplemented at suitable intervals to ensure a continuous ample supply of food and to avoid effects of suboptimal gut filling of the predators which may affect the development and oviposition rate (Sabelis, 1990).

Note: for predators that are cannibalistic or unsuitable for containment in enclosures with more than 1 individual.

The experimental protocols described in section 1.1 should be followed, with some alterations.

- The enclosures should be designed to contain only one individual (e.g. Plexiglass experimental cages manufactured by Isoplex AG (Regensdorf, Switzerland), or small insect cages, glass Petri dishes are also suitable for smaller predators).
- At least 40 individuals need to be used for the experiment.
- Predator eggs must be placed into the enclosure and provided with the treatment / control (as described above).
- The results should be pooled with 5 individuals in each group and r_i values calculated for each pooled group (resulting in 8 replicates).

2. PARASITOIDS: DEVELOPMENT OF ASSAY PROTOCOL

Adult parasitoids may be exposed to the insecticidal protein via the consumption of nectar from GM plants or honeydew from aphids that fed on the GM plant (Hogervorst 2006). Most laboratory experiments have investigated this by exposing adult parasitoids to honey/sucrose solutions containing the toxin and determining the impact on survival. Population studies necessitate that this is taken one step further and that parasitoids are allowed to reproduce.

Parasitoid fitness is closely related to host quality, and in turn host quality is partly determined by the host plant, resulting in a complex relationship between host diet and

parasitoid fitness. For this reason it is useful to also test the impact of GM plants on the larval stage of the parasitoid, necessitating the exposure of the herbivore host to GM plants.

2.1 Experimental protocol suitable for testing adult parasitoids.

This test is developed to assess whether adult-parasitoid exposure to the insecticidal protein affects the rate of parasitisation of herbivores and the development of parasitoid offspring, and thus parasitoid population growth rate.

Test substance

- Using a pre-determined concentration the pure insecticidal protein should be mixed with sucrose solution and presented to adult parasitoids on a cotton swab (hereafter referred to as the "treatment").
- A control treatment should be prepared in the same manner using the same concentration of sucrose, but without the addition of the toxin (hereafter referred to as the "control" treatment).

Experimental procedure

Provision of herbivore hosts for oviposition

- In order to determine population growth it is necessary to provide the adult parasitoid with host organisms in which to lay eggs.
- The herbivore host will require a plant on which to feed.
- Suitable untransformed plants should be planted and grown in pots under controlled environmental conditions.
- Suitable herbivore host species should be reared in culture, or obtained from a commercial insectary.
- Once plants have reached a suitable height they should be exposed to the herbivore.
- Prior to herbivore introduction plants should be covered with muslin sleeves or placed into insect cages to prevent escape of the herbivore (hereafter referred to as "enclosures").
- The herbivores need to be treated with the utmost care and should be transferred onto the plant using a soft paintbrush. Any herbivores dropped or injured during transfer should be discarded.
- Depending on the instar that is naturally used for parasitisation, the most suitable herbivore instar should be presented to the adult parasitoids.
- Each plant/group of plants should be infested with sufficient hosts (of the correct stage) to satisfy the egg-laying potential of the parasitoid species, assuming that for each replicate 5 female parasitoids will be introduced to the infested plants.
- At least 40 enclosures will be required for each treatment / control (hereafter referred to as "treatment" / "control" enclosures).
- Each enclosure should contain the treated / control sucrose solution.

Exposure of parasitoids

- Parasitoid cocoons or mummies of the same age should be placed into two insect cages prior to emergence. One of these "emergence cages" will be provided with the treatment, whilst the other will be provided with the control.
- Once parasitoids start to emerge they should be left to move, feed and mate freely within the emergence cage allowing sufficient time for fertilization of the females.
- Five female parasitoids should be randomly selected from the emergence cages for each of the 40 treatment / control enclosures.
- The parasitoids should be handled with the utmost care, and can be transferred into the enclosures containing hosts by using an oral aspirator, or by trapping individuals in a small vial, and then releasing them into the enclosure. Any parasitoids dropped or injured during transfer should be discarded.
- The parasitoids should be allowed free access to the plant/host complex and the treatment / control diet.
- The experimental period should allow for sufficient time to see emergence of new parasitoids.
- Once the experiment is terminated the number of individuals in each cage should be counted and the instantaneous rate of increase (r_i) calculated.

2.2 Experimental protocol suitable for testing juvenile parasitoids.

In order to investigate the impact of GM plants on larval stages of the parasitoid the parasitoid larva will need to develop within a herbivore host that has been feeding on a GM plant. This experiment will assess the effects of the transgenic trait on parasitoids while they develop in hosts that feed on the transgenic plant, with comparison to the proper control on non-transgenic plants. In this experiment the effect on population growth rate on transgenic versus non-transgenic plants will be assessed.

Test plants

- Transgenic plants should be planted and grown in pots under controlled environmental conditions (hereafter referred to as "GM" plants).
- Untransformed control plants should be grown in the same manner (hereafter referred to as "control" plants).

Experimental procedure

Provision of herbivore hosts for oviposition

- Suitable herbivore host species should be reared in culture, or obtained from a commercial insectary.
- Once plants have reached a suitable height they should be exposed to the pupal stage of the herbivore.
- Prior to herbivore introduction plants should be placed within a suitable enclosure (e.g. insect cages or enclosed within muslin sleeves) to prevent escape of the herbivore.

- The same number of pupae of the same age, and close to hatching, should be carefully transferred into the enclosures containing the plants. Any pupae dropped or injured during transfer should be discarded.
- Once the adult herbivores have emerged they should be left in the enclosures allowing for sufficient time for fertilisation of females. Once oviposition has started the adults should be left to lay eggs for 24 hours and then removed.
- The herbivore's offspring should be left to develop naturally on the GM/control plants.
- Once the herbivores reach the instar that is used for parasitisation, they should be counted and any excess individuals randomly chosen and removed so that an equal number remains in each enclosure.
- Depending on the instar that is used for parasitisation, the most suitable herbivore instar should then be presented to the adult parasitoids.
- Each plant/group of plants should be infested with sufficient hosts (of the correct stage) to satisfy the egg-laying potential of the parasitoid species, assuming that for each replicate 5 female parasitoids will be introduced to the infested plants.
- At least 40 enclosures will be required for each treatment/control. Each enclosure must provide the adult parasitoids with sufficient food/water in the form of a sucrose solution.

Exposure of parasitoids

- Parasitoid cocoons or mummies of the same age should be placed into insect cages prior to emergence. Once parasitoids start to emerge they should be left to move, feed and mate freely within the emergence cage allowing sufficient time for fertilisation of females.
- Five female parasitoids should be randomly selected from the emergence cages for each of the 40 treatment / control enclosures.
- The parasitoids should be handled with the utmost care, and can be transferred by using an oral aspirator, or by trapping individuals in a small vial, and then releasing them into the enclosure. Any parasitoids dropped or injured during transfer should be discarded. The parasitoids should be allowed free access to the plant/host complex.
- Parasitoids should be left for 24 hours and then removed from the enclosure.
- The experimental period should allow for sufficient time to see emergence of new parasitoids.
- Once the experiment is terminated the number of parasitoid individuals in each cage should be counted and the instantaneous rate of increase (r_i) calculated.

3. POLLINATORS: DEVELOPMENT OF ASSAY PROTOCOL

Pollinators are expected to be exposed to the toxins in GM plants via the ingestion of pollen and nectar. Lepidopteran pollinators are exposed to the GM plant as adults, and while larval stages may feed on GM plants this is not a consideration when investigating the role of Lepidoptera as pollinators. It is possible to test the impact of GM plants on adult Lepidoptera by exposing the adults to purified forms of the insecticidal protein in the form of a sucrose solution, the experimental protocol is described in section 3.1 below.

The social organisation of Hymenopteran pollinators means that the investigation of population growth in honey and bumble bee colonies requires the study of numerous hives

under field conditions, and this makes it an impractical technique for initial investigations of the impact of GM plants on these species. Under these circumstances it is suggested that standard procedures recommended under EPPO guidelines (PP 1/170) and EPA assessment guidelines (OPPTS 885.4380) for the evaluation of side-effects of plant protection products on honey bees be followed as summarised in section 3.2 below.

3.1 Experimental protocol suitable for testing Lepidopteran pollinators and protected species

This test is developed to assess the impact of transgenic products on the population growth rate of adult Lepidopteran pollinators and protected species.

Test substance

- Using a pre-determined concentration the pure insecticidal protein should be mixed with sucrose solution and presented to adult Lepidoptera on a cotton swab (hereafter referred to as the "treatment").
- A control treatment should be prepared in the same manner using the same concentration of sucrose, but without the addition of the toxin (hereafter referred to as the "control" treatment).

Experimental procedure

- Lepidoptera must be provided with a suitable surface on which to lay eggs this can be provided by untransformed plants, or by the use of a suitable artificial surface.
- The untransformed plants that are to be used for egg-laying must be planted and grown under controlled environmental conditions.
- Once plants reach a suitable height, they should be placed in a suitable enclosure, preventing the escape of the adult Lepidoptera, but still allowing them free movement and access to diet and to the egg-laying area (e.g. insect cages – hereafter referred to as enclosures).
- The treatment / control sucrose solutions should be placed into the enclosures and replenished as necessary.
- 40 suitable test enclosures should be prepared for each treatment / control
- Prior to emergence, the pupal stage of the Lepidoptera should be transferred to two insect cages. One of these "emergence cages" should contain the treatment, and the other the control.
- Pupae should be transferred with the utmost care and any individuals damaged during transfer should be discarded.
- Once the Lepidoptera adults start to emerge they should be allowed free access to the treatment / control and left to move and mate freely within the emergence cages.
- Five females should be randomly selected and carefully removed from the emergence cages and placed into each of the 40 treatment/ control enclosures, ensuring that they have access to the treatment/ control diet and a suitable surface on which to lay eggs.
- Once the adult Lepidoptera have died they should be removed from the enclosure.

- The experimental period should allow for sufficient time to see the hatching of the eggs and thus the emergence of the larval stage of the Lepidoptera.
- Once the experiment is terminated, the number of individuals should be counted, and the instantaneous rate of increase (r_i) calculated.

3.2 Experimental protocol suitable for testing honey and bumble bees

Both adults and larvae of Hymenopteran pollinators may be exposed to toxins in the GM plant. Therefore it is important that both life stages are tested. The majority of studies investigating the impact of GM plants on these insects have made use of purified transgene products (i.e. insecticidal proteins). However it is also important to test the impact of pollen from GM plants as the insecticidal proteins may also be expressed in the GM pollen. It is possible to test the impact of both types of products by making use of purified insecticidal protein mixed in a sucrose solution and exposed to adults, as explained in section 3.2.1 below; and by making use of pollen collected from GM plants and exposing this to larval stages as described in section 3.2.2 below.

3.2.1 Experimental protocol suitable for testing adult honey and bumble bees

The social organisation of Hymenopteran pollinators makes it difficult to assess the impact of transgenic products on population growth in the laboratory; therefore standard toxicity tests looking at mortality are recommended (EPPO guideline: PP 1/170). The test below assesses the impact of transgenic products on adult honey and bumble bees.

Test substance

- Using a pre-determined concentration the pure insecticidal protein should be mixed with sucrose solution and presented to adult worker bees through feeders (hereafter referred to as the "treatment").
- A control treatment should be prepared in the same manner using the same concentration of sucrose, but without the addition of the toxin (hereafter referred to as the "control" treatment).

Experimental procedure

- Bees should be adequately fed and from a healthy and queen-right colony.
- Use young adult worker bees of the same age (5-10 days old).
- Bees should be collected in a standardised way, either from frames without brood, or from the flight board at the hive entrance.
- Bees may also be kept in an incubator after emergence from cells and fed with fresh pollen and sucrose solution, until required for the experiment.
- Bees should be kept in darkness during the whole trial period, except during assessments.
- Keep bees in holding cages that are well ventilated and easily cleaned.
- Use at least 10 groups of 10 bees for each treatment / control.
- Provide the treated sucrose solution to one of the groups and the control solution to the other group.
- Count the number of dead bees at 24 hour intervals for a suitable length of time.

3.2.2 Experimental protocol suitable for testing larval honey and bumble bees

Larval stages of the honey and bumble bees are also exposed to transgenic products and the test below is used to assess the impact on the larval stage. Once again a standard toxicity test based on mortality is used (EPA guideline: OPPTS 885.4380), although additional measurements of pupal and adult weights are taken.

Test substance

- Pollen should be collected from the GM plant, provided the pollen is shown to contain the insecticidal protein, and moistened with a sucrose solution to form a paste (hereafter referred to as the "treatment")
- Pollen should be collected from untransformed plants and moistened with a sucrose solution to form a paste (hereafter referred to as the "control")
- An additional group of cells which will not receive any treatment should be mapped and development / mortality in these cells noted (hereafter referred to as "mapped" cells).

Experimental procedure

- Bees should be adequately fed and from a healthy and queen-right colony.
- Brood frames should be selected from the hives to contain large, uniformly sized populations of larval brood.
- Worker larvae should be between 3-5 days old.
- A brood patch should be selected from each brood frame.
- Brood frames should be transported to the laboratory inside a clean insulated container.
- Use at least 10 brood patches, each containing 25 larvae, for each treatment / control.
- The brood frames should be kept in a controlled environment (Temperature: 20-35^{o}C; RH: 20-60%) while the treatment is administered.
- Before the experiment, those cells receiving the treatment / control should be mapped on an acetate sheet laid over the brood frame.
- Approximately 2mg of pollen should be placed into each treatment / control cell, after placement into the cell 10µl of the sucrose solution should be pipetted into the cell allowing the pollen to liquefy and flow down to the mouth of the larva.
- After treatment brood frames should be covered with a moistened towel to maintain humid conditions and left for at least 30 minutes before being transported back to the hive.
- Six days after treatment the brood frames should be evaluated for mortality, using the acetate sheet to locate the treated / control / mapped cells.
- Larvae are considered to be alive if the cell is capped and dead if the cell is empty.
- Twelve days after treatment the brood frames should be removed from the hives and re-evaluated for mortality, and placed into an incubator.
- At this stage an emergence cage is placed over the treated / control / mapped brood cells.
- The emergence cage can be constructed from 3.2mm wire mesh constructed over a metal frame approximately 1.5 cm tall which is bent down at the edge and pushed into the wax of the honey comb in order to cover the area of the treated / control / mapped cells. Any cells which were not part of the treatment / control / mapped cells that happen to fall into

this area should be removed, therefore allowing only treated / control / mapped bees to emerge into the enclosed space.
- Once pupae reach the black-eye stage, 5 pupae per replicate should be removed from the control / treatment / mapped cells, and their weights noted.
- The emergence cages should be checked twice daily for adult emergence, adults should be counted, removed and weighed, before being euthanized by freezing.
- Evaluation should continue until 48 hours after the last bee emerges from the mapped cells.

4. SOIL DWELLING ORGANISMS: DEVELOPMENT OF ASSAY PROTOCOL

The potential impact of GM plants on soil-dwelling organisms depends, in part, on the persistence of the transgene-derived protein and its biological activity within the soil ecosystem (O'Callaghan *et al*., 2005), which may be affected by soil type. Soil-dwelling organisms may be exposed to the transgene-derived proteins via exudates from roots of GM plants or via the ingestion of GM plant residues.

Testing the effects of GM plants on non-target soil organisms has a number of difficulties, the heterogeneity of the soil environment, the complexity of communities present in the soil, and the aggregation and patterns of movement of soil fauna and flora all present a significant challenge to the design of ecologically relevant test methods (O'Callaghan *et al*., 2005). However initial studies can be performed under laboratory conditions making use of artificial soil and guidelines have been developed by the International Organisation for standardisation (ISO 11267: 1999), the Organisation for Economic Co-operation and Development (OECD – Guidelines for testing chemicals) and by EPPO (PP 3/7).

4.1 Experimental protocol suitable for testing soil dwelling organisms

The experimental protocol outlined below assesses the impact of transgenic products on soil organisms and is taken from standard guidelines for testing the impact of toxins (ISO 11267: 1999; Fountain & Hopkin, 2005), although the end-point measured in the protocol below is population growth and not mortality.

Test substance

- Using a pre-determined concentration pure insecticidal protein should be mixed with artificial soil (hereafter referred to as the "treatment"). The protein can be extracted from the leaves of transgenic plants and then lyophilised.
- A control substance should be prepared in the same manner, using untransformed leaf protein mixed with artificial soil (hereafter referred to as the "control 1" treatment).
- An additional control substance should be used consisting of artificial soil without any added protein (hereafter referred to as the "control 2" treatment).
- An additional replicate vessel should be prepared for each treatment/control to which no test organisms will be added. This vessel is used to monitor pH, temperature and percent moisture at test initiation and termination (hereafter referred to as the "blank control" treatment).

- Artificial soil can be prepared by mixing 70% industrial sand, 20% kaolin colloidal powder and 10% sphagnum peat moss. Reagent grade water (1286.1 ml) should be added to the artificial soil during mixing to yield approximately 40% of the total water holding capacity. $CaCO_3$ should be added to adjust the soil pH to 6.0 ± 0.5.
- The test substance should be added to the moistened artificial soil at the appropriate concentration, and mixed for at least 10 minutes using a mechanical/electrical mixer. All treatments / controls must be mixed equally.

Experimental procedure

- Test vessels composed from glass (e.g. vials / Petri dishes) with tightly fitting lids should be used. Each vessel should contain an appropriate amount of soil for the organisms that are to be tested. The test vessels should be maintained under controlled environmental conditions (e.g. in a temperature-controlled water bath).
- At least 40 test vessels per treatment / control should be used.
- Newly hatched juveniles should be selected from the culture/commercial sample and placed into each vessel, with 10 individuals added to each vessel except the blank control.
- Transfer should be done with the utmost care using a soft paintbrush. Any organisms dropped or injured during transfer should be discarded.
- Each vessel should be carefully sealed to ensure that the organisms cannot escape.
- Organisms should be provided with sufficient food (e.g. dry granulated yeast) at test initiation and again half-way through the experimental period.
- At test initiation a sample should be removed from the blank control and a measurement taken of soil pH, temperature and percent moisture.
- At test termination another sample should be taken from the blank control and these measurements taken again.
- The test should be terminated after the appropriate experimental period.
- At test termination the number of organisms present in each vessel should be counted and the instantaneous rate of increase (r_i) calculated.

ANALYSING RESULTS

Instantaneous rate of increase (r_i)

In order to calculate the instantaneous rate of increase it is necessary to count the number of individuals at the beginning of each experiment and then again when the experiment is terminated. The instantaneous rate of increase can then be calculated from the following equation:

$$r_i = \ln (Nf / No) / \Delta t$$

where Nf is the final number of animals, No is the initial number of animals, and Δt is the change in time (i.e. number of days the experiment was run). Positive values of r_i indicate a growing population and $r_i = 0$ indicates a stable population, while a negative r_i value indicates a population in decline and headed toward extinction (Walthall & Stark, 1997).

The r_i values for the control should be compared with the r_i values obtained from the treatment and these values should not be considered in isolation from one another.

Statistical analysis

Once mortality or r_i values are determined then statistical analysis can be used to detect differences between the treatments / controls. Any test used in statistical analysis must be sufficiently sensitive to detect treatment-related effects. The sensitivity of a test will be a function of the test system and its inherent variability, experimental design, and the level of replication (Rose, 2007).

Statistical error and the importance of replication

There are two types of potential error that should be considered in selecting a statistical design. The first type of error, known as the type I error or α, occurs when treatments are found to be different when in fact they are not (reject the null hypothesis of no differences, H_o, in favour of the alternative hypothesis, H_a, when H_o is true). The second type of error, known as the type II error or β, occurs when results show treatments to be the same when in fact they are different (fail to reject H_o when H_a is true) (Steel & Torrie, 1981). The ability to detect effects accurately, when they are present (i.e., not making a type II error), is referred to as the power of the experiment, and is improved when variability is reduced and/or replication is increased (Steel & Torrie, 1981). By using approaches that increase the statistical power of non-target invertebrate tests, the chance of detecting a treatment-related effect is improved. Therefore it is important that the sample size is large enough to provide sufficient statistical power to detect differences that may exist. Investigations looking at population growth usually make use of 40 replicates or more (Walthall & Stark 1997a; Stark *et al*., 1997) and this is taken as a suggested guideline in this report.

Statistical tests to determine differences

Analysis of variance, ANOVA (or *t*-test), is often used to test if the mean response within an experimental treatment is significantly different from that in a negative (e.g., untreated) control. H_o in this case is that the mean response within both groups is equal. Type I error (α) in these experiments is commonly set to 0.05, indicating that the probability of the observed effect size (e.g., difference in mean response) is considered significant if it is less than 5%. When using ANOVA, the level of replication that is needed to achieve a given level of power (to detect a specified magnitude of difference with a given probability) depends on variability within treatment groups. In order to make use of ANOVA, the data must be normally distributed and the variability among observations within each of the treatment groups must be equal (i.e. homogeneity of variance). However, there are statistical methods to transform the data so that they fulfil these requirements, or if this is not feasible then non-parametric test alternatives such as Kruskal-Wallis,or Wilcoxon's signed-ranks tests can be used. The ANOVA approach can be done using software, such as the SAS procedures PROC GLM or MIXED, which determines whether or not differences in mean response among treatments are greater than expected by chance (Rose, 2007). Multiple comparison tests (e.g. Least Significant Difference (LSD), Dunnett's *t*-test, Student-Newman-Keuls method) can then be used to determine where these differences lie.

A second approach is to apply a proportions test (e.g., z test or SAS procedures PROC CATMOD or GLIMMIX) to binary responses, such as mortality, number of affected individuals, or number of individuals that pupated, to determine if the proportion of individuals exhibiting a response is significantly different from some pre-determined, hypothetical proportion (e.g., 0.5) or from some proportion exhibited by the control individuals (Rose, 2007). This type of analysis is suitable for detecting differences in mortality and could be used in the analysis of results gained from the experiments on honey and bumble bees. An endpoint of 50% mortality is often used therefore it follows that the observed proportion of responding individuals can be compared to a 50% effect to determine if the observed proportion is significantly lower than 50%. A one-sided test is appropriate (Rose, 2007).

Other considerations

It is important that mortality in the control groups is low; generally it should be less than 15% (EPPO guidelines: PP 1/170). If control mortality is higher than this then the test must be repeated and the experimental methods checked to ensure correct handling of the test organisms and other experimental conditions. If a positive control is to be used then it should be a pesticide that is registered for use within the EU. Non-target test organisms must be correctly identified, and the source of identification noted. The non-target test organisms must be well satiated, and sufficient food should always be available. All raw data should be available on request. All experimental conditions must be carefully recorded and reported.

CONCLUDING COMMENTS

It has long been debated on the best method to determine the impact of environmental stress on organisms. Much of the research in the past has focussed on investigating the impact of toxins such as pesticides, and many of the methods focussed on measuring individual mortality (LD / LC_{50}). However, under normal conditions the health of a population is dependent upon life span, time to reproductive maturity, and reproductive rate; and as such, these are important parameters that should be included in toxicological studies. Despite this, acute lethal concentration estimates, such as the LD and LC_{50}, are the most common end-points used to assess toxicity. Unfortunately lethal concentration estimates are an incomplete measure of the impact of environmental stress as they only include one end-point (i.e. mortality) and are of short duration (often only 1-4 days) (Walthall & Stark 1997b). A solution to this problem lays in the use of population growth measurements as end-points. Population growth assessments integrate survivorship and fecundity into a single parameter making it a more realistic measurement of population responses. Population growth models provide more ecologically relevant endpoints than mortality-based, standard toxicity measurements. There have been studies comparing population growth models to LD / LC_{50} values (Bechmann, 1994; Walthall & Stark, 1997b; Stark *et al*., 1997), and results from these studies indicate that one measurement cannot be used to replace the other. While mortality measurements are valuable, it is now accepted that population growth rates provide a more realistic and comprehensive measurement by combining both lethal and sub-lethal effects into a single parameter (Stark & Banks, 2003). These previous studies have focussed on measuring the impacts of synthetic pesticides on populations, and therefore it may be useful

to conduct additional experiments comparing LD / LC_{50} values and population growth rates on GM crops.

This report set out to provide experimental protocols for investigating the impact of GM plants on non-target arthropods. Evaluating the impact of GM plants on the environment necessitates assessment that goes beyond the traditional lethal concentration estimates (i.e. LD and LC_{50}), and the potential effects of GM plants at the population, community and ecosystem level should be considered. Population growth assessments provide more ecologically relevant endpoints than mortality-based, standard toxicity measurements (Stark *et al*., 1997), and we recommend that assessment of the impact of GM plants on non-target arthropods be based, as far as possible, on measures of population growth. Two measures of population growth are considered, the instantaneous rate of increase (r_i) and the intrinsic rate of increase (r_m). Measurement of r_m requires that individuals within the population are observed daily, throughout their lifespan, for mortality and development, this technique is time-consuming and labour intensive. Measurements of r_i require less time and are less labour intensive, and therefore it is suggested that this population measurement be used whenever possible, provided the non-target arthropods are suited to the technique. This report presents guidelines on the laboratory experiments that can be used in assessing the impact of GM plants on non-target arthropods, and suggests a technique to identify negative impacts on invertebrate populations at an early stage.

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