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Demographic responses of *Daphnia magna* fed transgenic *Bt*-maize

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Abstract The food/feed quality of a variety of genetically modified (GM) maize expressing Cry1Ab Bt-toxin was tested over the life-cycle of *Daphnia magna*, an arthropod commonly used as model organism in ecotoxicological studies. Demographic responses were compared between animals fed GM or unmodified (UM) near isogenic maize, with and without the addition of predator smell. Age-specific data on survival and birth rates were integrated and analysed using life tables and Leslie matrices. Survival, fecundity and population growth rate (PGR) data generally disfavoured transgenic Bt-maize as feed for *D. magna* compared to animals fed the unmodified (UM) near isogenic line of maize. Decomposition of age-specific effects revealed that the most important contributions to a reduced PGR in the GM-fed group came from both fecundity and survival differences early in life. We conclude that juvenile and young adult stages are the most sensitive experimental units and should be prioritized in future research. These stages are often omitted in toxicological/ecotoxicological studies and in feeding trials.

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Introduction

The overall quality of genetically modified (GM) plants, including their nutritional, antinutritional and toxicological properties, has great health and environmental relevance. Industry sources claim a global growth in the production of GM-plants, though concentrated on a handful of countries (James 2006). Thus both trans-boundary movements of GM plants may increase, and more GM material will flux through food chains. Current commercial GM plants have been claimed to be substantially equivalent to unmodified (UM) plants (Brake and Vlachos 1998; Clark and Ipharraguerre 2001; Sidhu et al. 2000). However, the Substantial Equivalence Principle is just a coarse characterisation of the biochemical components of the plant and may overlook important elements like post-translational modifications and immune effects (Prescott et al. 2005), insertional effects leading to up- or down regulation of endogeneous genes (Filipecki and Malepszy 2006), abberations in transgene-specific mRNAs (Rosati et al. 2008), or subtle changes in the proteome and metabolome (Manetti et al. 2006; Zolla et al. 2008).

Few studies have addressed the potential toxicity and health risks of GM plants to animals and humans (Domingo 2007). Feeding studies performed to assess the safety of Bt-maize have mainly focused on adult animals over a relatively short period of their life span (Pryme and Lembcke 2003). However, important effects of toxicants often occur early in life, often due to higher sensitivity of juvenile stages to nutritional inadequacies. Hence, it is

necessary to focus on juveniles and young adults in both laboratory and field testing (Levin et al. 1996).

Life-cycle experiments cover all life stages of an organism and can thereby detect a broader spectrum of effects on fitness components, being therefore preferable to short-term studies. However, for relatively long lived test organisms like mammals, the costs in resources and time of life-cycle experiments may often be prohibitive. Nevertheless, since GM plants are not expected to be directly toxic or lethal, but may have long-term effects, it is more relevant to perform thorough feeding tests over extensive periods of time, preferably for the whole life-cycle of the animal.

In model organisms with a limited generation time, life-cycle experiments are feasible. The aquatic crustacean *Daphnia magna* (phylum Arthropoda) has a generation time of about 2 weeks and is commonly used in toxicological and ecotoxicological research (Atienzar et al. 2001; Barry 1996; Kramer et al. 2004). The minimal genetic variation, ensured by its clonal reproduction, as well as easily measurable and plastic life history traits make it an ideal model organism. In short term (48 h) acute toxicity tests, *D. magna* has shown no treatment-related adverse effects to transgenic Cry1Ab-maize pollen (Mendelson et al. 2003), but negative, sub-lethal effects were detected by a life cycle feeding test (Bøhn et al. 2008). Life history data collected over the life cycle of test organisms can be further processed to estimate age- or stage-specific survival and fecundity rates. These demographic parameters (i.e. vital rates) are organized in life-tables (Metcalf and Pavard 2007), which make it possible to quantify integrated effects of toxicants on individuals (fitness consequences) and populations (growth rate consequences) (Kammenga and Riksen 1996). It is an explicit goal of ecotoxicology to link physiological effects of contaminants or toxins to their population- and community-level consequences (Moriarty 1988), i.e. ecotoxicological experiments can be analyzed at more than one level of biological organization (Levin et al. 1996). When demographic estimates are based on individual level data, a complete temporal portrait of toxicity (e.g. the age dependent sensitivity) can be obtained, increasing the resolution and scope of toxicological studies (Forbes and Calow 1999).

The link between sub-population level responses and population level consequences can be made using structured population models and Life-Table Response Experiments (LTREs) (Caswell 1989; Caswell 2001). Data generated within LTREs give a complete set of (st)age-specific vital rates (Levin et al. 1996). Thus, it is possible to determine which part of the life-cycle is responsible for an observed population-level response to a treatment. Also, the sensitivity (or elasticity) of each part of the life-cycle is easily obtained from LTRE data (Caswell 2001).

As organisms in the field face multiple challenges or stressors simultaneously, understanding mechanisms of interaction between factors, especially those acting synergistically, is crucial to understand environmental impact (Clarke and Harris 2003; Sih et al. 2004). For example, strong synergistic effects of pesticides and predation on aquatic animals show that two 'safe' effects combined caused 80–90% of amphibian larvae to die (Relyea 2003; Relyea and Mills 2001). In their most pronounced example of a synergistic effect, the pesticide carbaryl was made 46 times more deadly to bullfrogs in the presence of a predator (Relyea and Mills 2001; Sih et al. 2004). A typical adaptive response to the presence of a predator is to reproduce earlier (i.e. before being eaten) (Stearns 1992). The increased energetic investment in early reproduction has a cost in terms of reduced investment in maintenance, growth and future reproduction expected to amplify the fitness effects of toxic and low nutritional quality food early in life.

Bøhn and co-workers performed a life-cycle experiment with *D. magna* fed on transgenic Cry1Ab-maize and its near isogenic control, grown in the same environment. They demonstrated increased mortality, reduced growth and a lower number of eggs produced in *D. magna* feeding on Bt-transgenic maize, likely due to a toxic response to the Bt-maize (Bøhn et al. 2008). In the present study we perform a demographic analysis of those data combined with unpublished data from a predation risk treatment completing the results obtained from our multifactorial design. The demographic analysis allows calculating population growth rate and age-specific responses and sensitivity throughout the life cycle of the test organism. To our knowledge no previous studies have used demographic models to analyse toxicological experiments to test GM foods or feeds.

We tested the following hypotheses:

i) Cry1Ab-maize is not substantially equivalent to the near isogenic control maize with respect to effects on *D. magna* health
ii) Juveniles are more sensitive experimental units than adults to potential differences in food or predator treatment
iii) The presence of predator will trigger an allocation trade-off with increased early investment in reproduction at the cost of survival and/or late reproduction that amplifies the response to Cry1Ab-maize
iv) The potential effects of food and predator treatment in combination will act in synergy (as opposed to purely additive effects)

Materials and methods

Experimental conditions

All individuals of *D. magna* ($n = 80$) used in the experiments were born within 30 h from the third clutch of a

single clonal population. Twenty juvenile individuals were randomly chosen and assigned to separate glasses with 60 ml autoclaved ADAM medium. Ten animals received the GM feed (treatment) and ten the UM feed (control) under identical environmental conditions in a climate chamber at 20°C and 24 h daylight (resembling the summer conditions at our latitude). Every third day, all individuals were transferred to new glasses with new medium using a broad tipped pipette, and the position of the glasses was re-randomized. Each *D. magna* was fed daily and inspected for survival and number of eggs produced. The experiment lasted for 42 days. Care was taken to provide the same amount of food for all experimental units within and among GM and UM food recipients. Each individual was fed daily with 100 µl of maize feed, corresponding to 0.4 mg dry weight of maize or about 0.2 mg C per individual per day, i.e. within the range recommended for laboratory experiments with *Daphnia* (Sims et al. 1993). This experimental set-up was performed consecutively three times. In the first of these experiments, parallel experiments were run with and without the additional factor of predator smell, i.e. a 2×2 fully factorial set-up ($n = 40$ animals). With the repeated experiments two and three the total number of animals was $n = 80$. The present study uses previously published data (Bøhn et al. 2008) combined with unpublished data, i.e. the predator treatment, included in a multifactorial design. The demographic analysis allows calculating population growth rate and age-specific responses and sensitivity throughout the life cycle of the test organism.

Feed

The transgenic Cry1Ab-maize was of the variety Dekalb 818 YG (a hybrid of MON 810 and a Philippine, local variety of maize called Dekalb 818). Both varieties (Dekalb 818 YG and the near isogenic control maize Dekalb 818) were grown side by side in adjacent fields, divided by a small river, in Elizabeth Cruzara, near Iloilo City in 2003. Maize had been grown on these fields for many years. This was the very first year of GM-maize cultivation. The neighboring farmers delivering the GM and UM-maize have stated that there were no external pesticides used in the fields. We inspected the fields and confirmed their GM and UM status by PCR analyses of field-collected samples before buying the adequate maize material from the local farmer. The transgenic status of the MON810 event was further verified by DNA nucleotide sequencing, employing an Applied Biosystems 3130xl genetic analyzer (data not shown).

On average, the GM-maize expressed 67 (±27 SD) ng Cry1Ab toxin per gram of dried grain tissue ($n = 15$ samples analysed by ELISA, diluted 1:20–1:33, using a commercially available Abraxis kit). All negative controls and the UM-maize showed negative results.

Sub-samples of GM and UM-maize were drawn from 50 kg bags, and 35 g of dried kernels were ground with separate coffee-grinders (Petra Espresso), first on the coarsest grinding and then five times repeatedly on the finest setting. The resulting flour was sieved through a filter with 250 µm mesh size. 800 mg of the filtered maize flour were added to 250 ml of Aarchnia Daphnien Medium (ADAM) (Kluttgen et al. 1994), homogenized and frozen in 10 ml test tubes. This is later referred to as the feed. All steps of the feed production were the same for GM and UM-maize.

Measurements

Body length was measured 17, 29 and 42 days after the experiment was initiated. Individual *D. magna* from both feeding groups were measured for body length (distance from the top of the head to the base of the caudal spine) using a 40× binocular microscope. While performing the length measurements, the observer did not know to which treatment group the measured individual belonged, to protect from biased measurements.

Predator smell

The predator smell was produced by feeding three spined sticklebacks *Gasterosteus aculeatus* with *D. magna* for 3 days. Stickleback lived in aquarium in the same autoclaved ADAM medium as the daphnids. After feeding on *Daphnia* spp., fish release 'alarm substances' that trigger predator responses in other daphnids (Brodin et al. 2006; Stabell et al. 2003). Water containing predator smell was filtered and frozen, to obtain ice-cubes of fixed volume (16 ml). The ice cubes containing 'predator smell' were melted and a subsample were added to the medium of the predator smell group, following the protocol of Stabell and colleagues (Stabell et al. 2003).

Demographic analysis

Age-specific survivorship and fecundity data were obtained for both GM and UM-fed *D. magna* by daily inspection and egg counts. These data were used to build life-tables and to parameterize the Leslie projection matrix of an age-structured population model (Caswell 2001) using a projection interval of 3 days, i.e. 14 stages covering the 42 days of the experiments (Tables 1 and 2). We included a stage 15 for the animals that were still alive at the end of the experiments. Survival and fecundity for this stage were estimated from stages 12–14.

The Leslie matrices were used to estimate the main demographic statistics for each food treatment, with and without predator. To obtain confidence limits (95% CI) for our parameter estimates, we used a bootstrap procedure,

Table 1 Leslie matrices showing fecundity (first row) and survival (diagonal) for 15 stages of *D. magna* fed on UM (top) or GM (bottom) maize feed. Data from all experiments, with and without predator ($n = 80$)

0	0	0	0	0.09	0.81	0.67	0.62	0.75	0.65	0.64	0.45	0.87	0.73	0.34
1.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	**0.97**	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	**0.95**	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	**0.97**	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	**1.00**	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	**0.91**	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	**0.97**	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	**0.87**	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	**0.92**	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	**0.96**	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	**0.96**	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	**0.91**	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	**0.75**	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	**0.73**	**0.79**
0	0	0	0	0.10	0.45	0.67	0.48	0.74	1.06	0.40	0.92	1.43	1.00	0.56
0.97	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	**0.90**	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	**1.00**	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	**0.88**	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	**0.96**	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	**1.00**	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	**0.77**	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	**0.82**	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	**0.86**	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	**0.83**	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	**0.90**	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	**0.89**	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	**0.50**	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	**1.00**	**0.76**

performed in Matlab. The procedure re-sampled randomly, with replacement, the individual data, stratifying by treatment group, and calculated the number of eggs, the generation time, the average lifetime reproduction (R_0), and the population growth rate (λ) for each of 2000 iterations. The re-sampling was performed so that the proportion of mature animals in each group was constant.

Results

Survival (lx)

The GM-fed *D. magna* had lower survival (lx) than the UM-fed *D. magna* throughout the experiment (Fig. 1). In the 'no predator' group the difference in survival was significant ($p = 0.037$, coxph-test). With predator and for the total material, the differences were not consistent and not statistically significant ($p = 0.45$ and $p = 0.16$, respectively). For both groups the survival increased somewhat after the main reproductive stages. For the GM-fed groups the killing power (an additive parameter) was about twice as high in comparison to the UM-fed groups in the main reproducing stages (Adult II–VI) (Table 2).

Reproduction (mx)

Egg production

The total number of eggs produced by GM-fed *D. magna* was lower than that of those fed UM-maize, 109 versus 141 eggs. The main contributing life stages to egg production

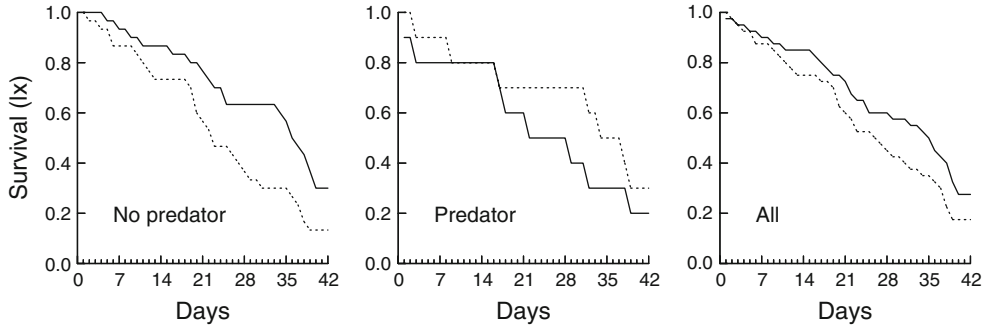

Fig. 1 Survival (*lx*) for *D. magna* fed GM-maize (*dashed lines*) and UM-maize (*solid lines*), without predator ($n = 60$), with predator ($n = 20$) and all samples combined ($n = 80$)

were animals aged 18–30 days in both groups. The number of offspring per surviving individual (*mx*) was somewhat higher in the UM-fed group in the first reproductive stages, but lower in adults older than 30 days (Fig. 2). The group with predator smell was an exception to this trend as the GM-fed animals consequently had a lower fecundity as compared to the UM-fed animals.

Growth (only with predator)

Daphnia magna fed UM maize were significantly larger in body size at day 29 and 42 as compared to *D.* magna fed GM maize (*t*-test; $p = 0.046$ and $p = 0.021$, respectively) (Fig. 3). Without predator a similar but weaker trend was observed (Bøhn et al. 2008).

Demographic parameters and population growth rates

Daphnia magna fed GM maize had consistently lower population growth rates as compared to the control groups fed UM maize, both without (21% higher) and with predator (87% higher) present, and thus also for the pooled data (35% higher) (Fig. 4). With predator and for the total material, the 95% confidence intervals for the population growth rate did not overlap between the groups (Fig. 4). The inclusion of predator smell stimulated the UM-fed animals, but not the GM fed animals. Age at first reproduction was the same for both groups (13 days) and the generation time was similar (23.9 versus 24.2 days for GM and UM-fed animal, respectively), but the number of eggs and the average lifetime reproduction (R_0) was lower in the GM-fed group as compared to the group fed UM maize ($R_0 = 2.73$ and 3.53, respectively). The bootstrapped confidence intervals did not overlap (Table 3).

Decomposition of food treatment effects on age-specific fecundity and survival

The decomposition of fecundity into (st)age-specific effects of food treatment ($n = 80$) showed that the higher fecundity of UM fed *D. magna* in the earliest adult groups was the dominant contributor to differences in the population growth rate (Fig. 5 upper panels). Conversely, the higher fecundity of GM fed animals in the latest (st)ages had a relatively small contribution to the population growth rate (Fig. 5 upper panels).

For the survival, (st)age-specific effects were more mixed with alternating curves for the two food treatments (Fig. 5 lower panels). However, it is noticeable that after stage 7 (at which the animals were 21 days old) even large absolute differences in survival did not contribute much to the population growth rate (Fig. 5 lower panels).

Decomposition of predator effects on age-specific fecundity and survival

The higher fecundity observed in early stages when the predator was present (Fig. 6, upper left panel) gave the most significant contribution to population growth rate compared to all later differences (Fig. 6, upper right panel).

For the survival, (st)age-specific effects were seemingly asynchronous and mixed between the predator and the non-predator treatment (Fig. 6, lower left panel). The two main peaks of contribution to population growth rate were early in life with a reduced survival for the predator treatment (Fig. 6, lower right panel).

Discussion

Daphnia magna fed Bt-transgenic maize showed a reduced fitness performance as compared to animals fed the near isogenic unmodified control maize grown in the same environment. The addition of predator smell increased the differences between the feeding groups, indicating an interaction between food and predator treatment.

Hypothesis 1 Cry1Ab-maize is not substantially equivalent to the near isogenic control maize with respect to effects on *D. magna* health

Table 2 Life-tables based on three days intervals for *D. magna* fed UM and GM maize. Data from all experiments, with and without predator ($n = 80$). Agegroup 15+ represents the rest of the life span after the end of the experiment at day 42

Agegroup	Stage	Age (days)	Numbers dying during stage	Numbers observed at the end of each stage	Proportion of cohort dying during stage	Mortality rate per stage	Killing power Log(ax/ax + 1)	Proportion of original cohort surviving to end of stage	Survival rate per stage	Total number of eggs	Number of eggs per survivor	R(0)	Intrinsic rate of pop. growth r	Stable age structure
		x		ax	dx	qx	kx	lx			mx	lxmx	$\ln(R_0)$	
	***D. magna* fed unmodified (UM) maize**													
0	Newborn	0	0	40	0.000	0.000	0.022	1.000	1.000	0	0.000	0.000		
1	Juvenile I	3	2	38	0.050	0.000	0.012	0.950	1.000	0	0.000	0.000		1.000
2	Juvenile II	6	1	37	0.025	0.026	0.024	0.925	0.974	0	0.000	0.000		0.859
3	Juvenile III	9	2	35	0.050	0.054	0.013	0.875	0.946	0	0.000	0.000		0.713
4	Juvenile IV	12	1	34	0.025	0.029	0.000	0.850	0.971	0	0.000	0.000		0.571
5	Adult I	15	0	34	0.000	0.000	0.040	0.850	1.000	3	0.088	0.075		0.472
6	Adult II	18	3	31	0.075	0.088	0.014	0.775	0.912	25	0.806	0.625		0.405
7	Adult III	21	1	30	0.025	0.032	0.062	0.750	0.968	20	0.667	0.500		0.335
8	Adult IV	24	4	26	0.100	0.133	0.035	0.650	0.867	16	0.615	0.400		0.265
9	Adult V	27	2	24	0.050	0.077	0.018	0.600	0.923	18	0.750	0.450		0.208
10	Adult VI	30	1	23	0.025	0.042	0.019	0.575	0.958	15	0.652	0.375		0.161
11	Adult VII	33	1	22	0.025	0.043	0.041	0.550	0.957	14	0.636	0.350		0.139
12	Adult VIII	36	2	20	0.050	0.091	0.125	0.500	0.909	9	0.450	0.225		0.119
13	Adult IX	39	5	15	0.125	0.250	0.135	0.375	0.750	13	0.867	0.325		0.081
14	Adult X	42	4	11	0.100	0.267	–	0.275	0.733	8	0.727	0.200		0.051
15+	Adult XI	43+	11	0	0.275	1.000	–	0.000	0.000	13	0.559	0.320		0.108
Sum where relevant							0.561			141+13		3.525	1.260	
	***D. magna* fed Bt-transgenic (GM) maize**													
0	Newborn	0	0	40	0.000	0.000	0.022	1.000	1.000	0	0.000	0.000		
1	Juvenile I	3	2	38	0.050	0.033	0.036	0.950	0.967	0	0.000	0.000		1.000
2	Juvenile II	6	3	35	0.075	0.103	0.013	0.875	0.897	0	0.000	0.000		0.847
3	Juvenile III	9	1	34	0.025	0.000	0.040	0.850	1.000	0	0.000	0.000		0.665
4	Juvenile IV	12	3	31	0.075	0.115	0.014	0.775	0.885	0	0.000	0.000		0.583
5	Adult I	15	1	30	0.025	0.043	0.015	0.750	0.957	3	0.100	0.075		0.451
6	Adult II	18	1	29	0.025	0.000	0.082	0.725	1.000	13	0.448	0.325		0.378
7	Adult III	21	5	24	0.125	0.227	0.058	0.600	0.773	16	0.667	0.400		0.331
8	Adult IV	24	3	21	0.075	0.176	0.043	0.525	0.824	10	0.476	0.250		0.224
9	Adult V	27	2	19	0.050	0.143	0.048	0.475	0.857	14	0.737	0.350		0.162
10	Adult VI	30	2	17	0.050	0.167	0.054	0.425	0.833	18	1.059	0.450		0.121

Table 2 continued

Agegroup	Stage	Age (days)	Numbers dying during stage	Numbers observed at the end of each stage	Proportion of cohort dying during stage	Mortality rate per stage	Killing power Log(ax/ax + 1)	Proportion of original cohort surviving to end of stage	Survival rate per stage	Total number of eggs	Number of eggs per survivor	R(0)	Intrinsic rate of pop. growth r	Stable age structure
		x		ax	dx	qx	kx	lx			mx	lxmx	ln(R_0)	
11	Adult VII	33	2	15	0.050	0.100	0.062	0.375	0.900	6	0.400	0.150		0.089
12	Adult VIII	36	2	13	0.050	0.111	0.269	0.325	0.889	12	0.923	0.300		0.070
13	Adult IX	39	6	7	0.150	0.500	0.000	0.175	0.500	10	1.429	0.250		0.054
14	Adult X	42	0	7	0.000	0.000	–	0.175	1.000	7	1.000	0.175		0.024
15+	Adult XI	43+	4	0	0.175	1.000	–	0.000	0.000	11	0.341	0.270		0.063
Sum where relevant							0.757			109+11		2.725	1.002	

The H_0-hypothesis (i.e. Cry1Ab-maize and near isogenic line are substantially equivalent) was rejected. On a biological level, as food sources for *D. magna*, the two maize varieties were not performing similarly: (1) the survival of the GM-fed animals were significantly lower than UM-fed, without predator ($p = 0.037$, *coxph*-test), but not significantly different for the group with predator or for the total material ($p = 0.45$ and $p = 0.16$, respectively, *coxph*-test); (2) the total number of eggs produced by GM-fed animals were lower as compared to animals fed on UM-maize; (3) the growth showed a trend towards larger body sizes in the UM-fed group without predator smell present (Bøhn et al. 2008). When the predator smell was included the UM-fed animals had a significantly larger body size at day 29 and 42 in the experiments; (4) the population growth rate, combining survival and fecundity data, showed a higher growth rate in the UM-fed animals with non-overlapping 95% confidence intervals for both the predator groups ($n = 20$) and for the total material ($n = 80$). These results sum up differences in repeated and fully randomized life-cycle experiments, using maize as the only source of food.

The most common definition of substantial equivalence has emphasized the biochemical components of the plant (Brake and Vlachos 1998; Clark and Ipharraguerre 2001; Sidhu et al. 2000), thus potentially overlooking important elements like post-translational modifications and immune effects (Prescott et al. 2005), insertional effects leading to up- or down regulation of endogeneous genes (Filipecki and Malepszy 2006), abberations in transgene-specific mRNAs (Rosati et al. 2008), or subtle changes in the proteome and metabolome (Manetti et al. 2006; Zolla et al. 2008). Animal feeding studies are performed to reveal subtler effects on survival, growth, histological indicators, and other characteristics of consumers. This means that effects can be picked up even without a clear understanding of the cause(s), e.g. the mode of action for a toxin.

Our data demonstrate negative effects in the non-target organism *D. magna* feeding insecticide maize, but we lack the mechanistic explanation for the effect. A number of other studies, mainly in terrestrial invertebrates, have also showed negative effects of Bt-transgenic plants on non-target species. Lövei and Arpaia (2005) found significantly negative effects on about 30% of the studied invertebrate predators and 57% of the studied parasitoids after feeding Bt-plants or pollen. Hilbeck and Schmidt (2006) report 27 out of 54 studies (50%) that showed negative effects after feeding Bt-plants or pollen. Ramirez-Romero and co-workers showed a tri-trophic effect where a parasitoid hymenopteran species was negatively affected (developmental time, adult size and fecundity) via host exposure to Cry1Ab proteins and Bt-plants (Ramirez-Romero et al. 2007). The same group also showed that honey bees were negatively affected in their feeding behaviour and that their

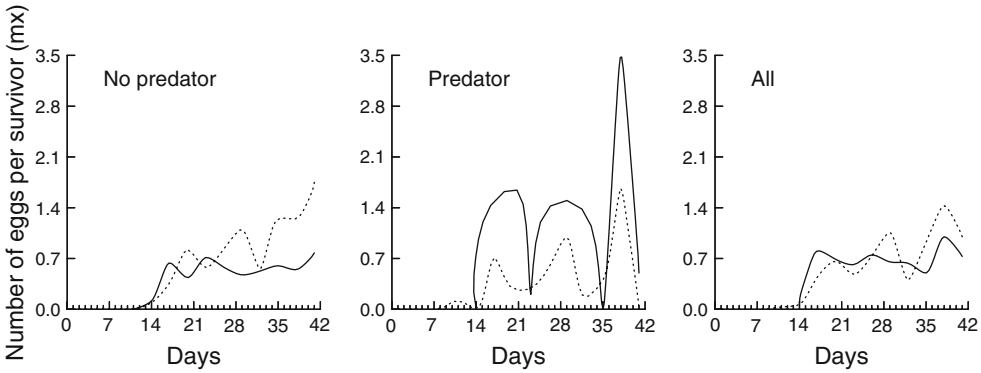

Fig. 2 Fecundity (*mx*) for *D. magna* fed GM-maize (*dashed lines*) and UM-maize (*solid lines*), without predator ($n = 60$), with predator ($n = 20$) and all samples combined ($n = 80$)

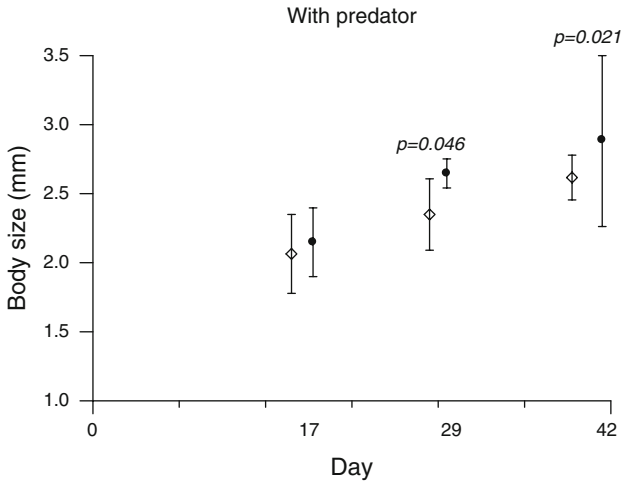

Fig. 3 Mean body size (whiskers, 95% CI) of *D. magna* fed on GM-maize (*open squares*) and UM-maize (*filled circles*) at days 17, 29 and 42. The *p*-values of significant differences are shown (*t*-test)

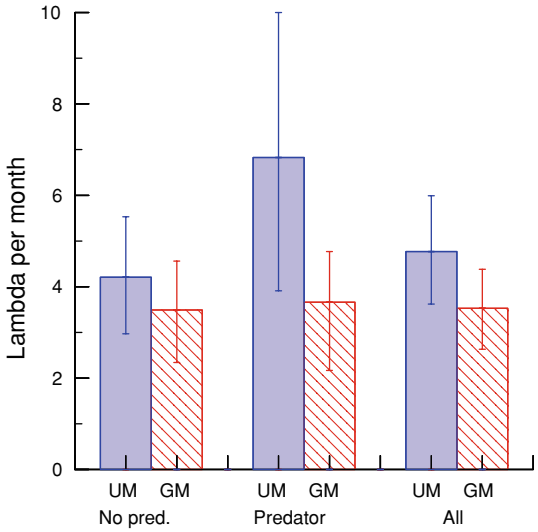

Fig. 4 Population growth rate per month (lambda—whiskers, 95% bootstrap CI) for *D. magna* fed unmodified (UM) and modified (GM) maize, without predator ($n = 60$), with predator ($n = 20$) and for all samples combined ($n = 80$)

Table 3 Demographic data on *D. magna* fed GM and UM maize

Stage-classified model	GM-fed	UM-fed
No. of females in experiments	40	40
Age at first reproduction (days)	13	13
Number of eggs	109	141
Upper 95% confidence interval	134	175
Lower 95% confidence interval	82	109
Average lifetime reproduction (R_0)	2.73	3.53
Upper 95% confidence interval	3.35	4.37
Lower 95% confidence interval	2.05	2.73
Generation time	23.9	24.2
Upper 95% confidence interval	24.6	24.7
Lower 95% confidence interval	22.3	23.3

Confidence intervals are calculated from bootstrapping. Data from all experiments, with and without predator ($n = 80$)

learning performance was reduced when exposed to 5,000 ppb of Cry1Ab protein (Ramirez-Romero et al. 2008). Moser and co-workers showed that the developmental time of *C. maculata* increased after Bt hybrid corn treatments compared with non-Bt corn treatments (Moser et al. 2008). In the aquatic environment, it has been demonstrated that toxin-containing crop byproducts from maize are dispersed, decomposed and consumed by stream insects (Rosi-Marshall et al. 2007). When fed Bt-maize in the laboratory, two non-target caddisflies showed higher mortality and reduced growth (Rosi-Marshall et al. 2007).

None of the mentioned studies were able to identify the cause(s) of the effect(s), e.g. the mode of action for the Bt-toxins. This leaves us with a knowledge gap and an urgent need for more detailed studies focusing on mechanism. New methodological techniques like functional genomics, transcriptomics, proteomics, metabolomics, etc. may prove helpful in order to improve the understanding about both the properties of the plant, and the properties of the test organism that feed on that plant. This will immediately bring the discussion well beyond the coarse assumptions made by the substantial equivalence principle.

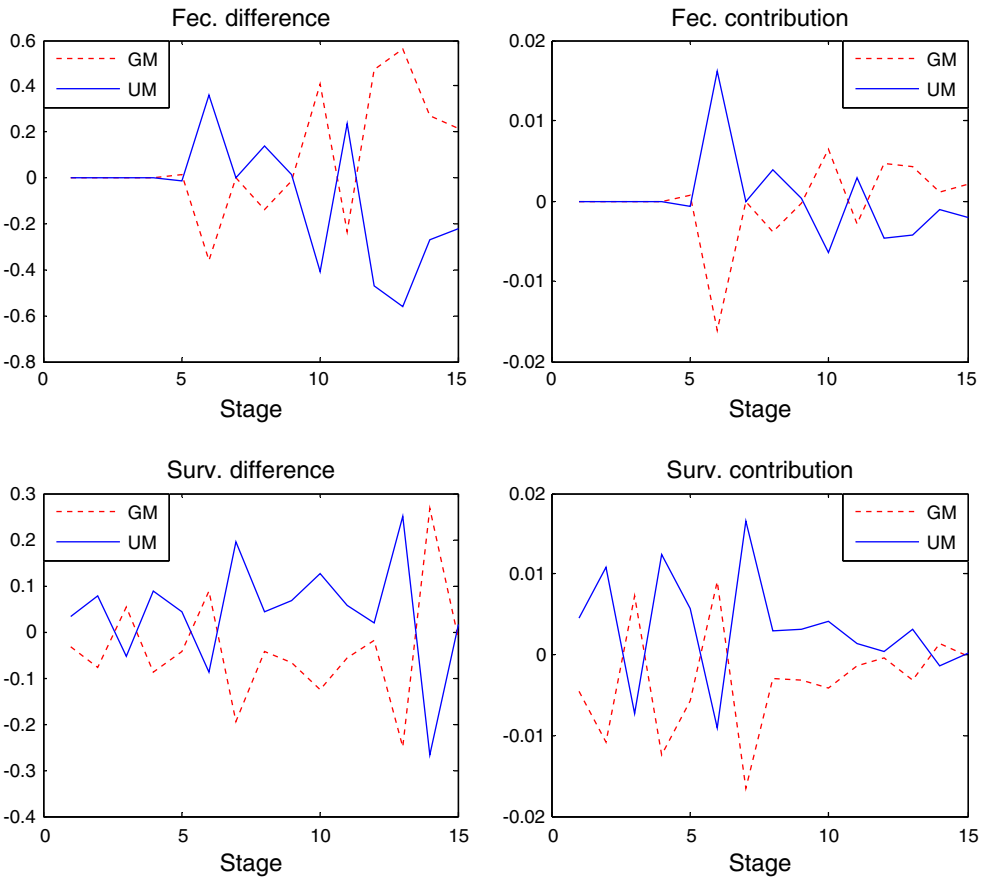

Fig. 5 (*Top*): decomposition of food treatment (*dashed line* = GM, *solid line* = UM) effects on the (st)age-specific fecundity for the total material ($n = 80$). *Left panel* shows absolute effects (relative to the mean) and *right panel* shows the contributions of those effects on the population growth rate. (*Bottom*): decomposition of food treatment effects on the (st)age-specific survival. *Left panel* shows absolute effects (relative to the mean) and *right panel* shows the contributions of those effects on the population growth rate

One promising research direction is ecotoxicogenomics where gene expression patterns are studied under the impact of different treatments, e.g. exposure to chemicals. Watanabe and co-workers showed that chemicals expected to impact oxidative stress, respiratory uncoupling, metabolic change and heavy metal exposure altered gene expression patterns in *D. magna* in recognizable ways (Watanabe 2007). Also, DNA microarrays for *D. magna* have been used to link gene responses to the impact of toxicants on life-history traits and population growth rate in *D. magna* (Connon et al. 2008). If, or when further studies are able to link a negatively affected phenotype after exposure to e.g. Bt-toxin, or after feeding on a transgenic plant, to up- or down-regulation of certain genes or pathways, a new and promising step towards finding modes of action would have been established. However, a functional genomics determinism, i.e. tightly relating changes in microarrays, changes in proteins and changes in the metabolome is far from established. Assuming such determinism could narrow our investigations and understanding of the phenotype.

In spite of the abovementioned, the basis for analysis of risks from transgenic plants still largely remains that of substantial equivalence. Following Millstone and co-workers that called the Substantial Equivalence Principle a regulatory principle without any biological relationship or theoretical validity (Millstone et al. 1999), we argue that the concept of substantial equivalence should have a definition based on its biological activity. It is the biological activity that is relevant in the agro-ecosystem, in relation to non-target effects and in a human health context. Otherwise, there would be no necessary link between substantial equivalence and safety.

Hypothesis 2 Juveniles are more sensitive experimental units than adults to potential differences in food or predator treatment

Hypothesis supported. Given the differences in the performance of *D. magna* fed GM versus UM maize and the detailed demographic analysis, we were able to evaluate which stages of the *D. magna* life cycle were the most sensitive to differences in food quality, and to predator smell. The survival curves of both feeding groups were relatively straight and the fecundity tended to increase with age. However, the sensitivity analysis revealed that what really mattered to the population growth rate came from differences in survival in juveniles and young adults and from fecundity differences in young adults. Thus, the structured population models offer results on sensitivity which may have important implications: juveniles and

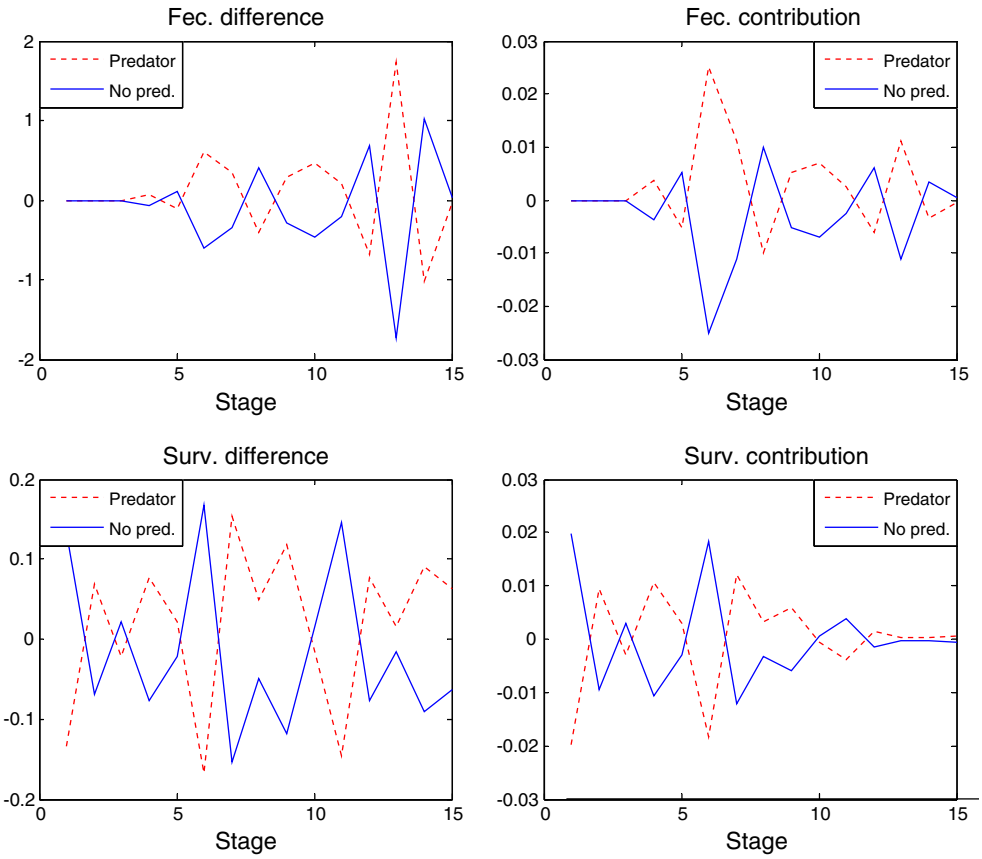

Fig. 6 (*Top*): effects of predator treatment (*dashed line* = predator, *solid line* = no predator) on (st)age-specific fecundity (*left*) and the contributions of those effects on population growth rate (*right*). (*Bottom*): effects of predator on (st)age-specific survival (*left*) and the contributions of those effects on population growth rate (*right*)

young adult stages are the most sensitive experimental units and should be prioritized in toxicological/ecotoxicological research (Connon et al. 2008; Forbes and Calow 1999). This is often contrary to established practice, where well fed adults are used for testing. For example, Cry1Ab pollen is tested in adult *D. magna*, short term with no treatment related effects found (Mendelson et al. 2003). In short-lived test organisms, we recommend the use of life cycle experiments to enable stage specific evaluation of toxicity and general life-history performance, including population level comparisons. When applicable, these methods are superior to traditional testing practices.

Hypothesis 3 The presence of predator will trigger an allocation trade-off with increased early investment in reproduction at the cost of survival and/or late reproduction that amplifies the response to Cry1Ab-maize

Hypothesis partly supported. A trade-off response was observed in animals exposed to predator, but only in the UM-fed animals. This group increased their population growth rate by increasing their early reproduction as anticipated by life-history theory (Hansen et al. 1999; Mauri et al. 2003; Twombly et al. 1998). However, the early boost in reproduction coincided with a relatively high mortality, indicating a cost of the response. When a predator is present, the expectation of a shorter life span may trigger early reproduction (Sakwinska and Dawidowicz 2005). Also other stress factors than predation may lead to high fecundity early in life (Coors et al. 2004) at the cost of future quantity or quality of offspring, e.g. due to a compromised immune system (Hanssen et al. 2005; Sheldon and Verhulst 1996). In general, a negative correlation between current and residual reproductive value, or between other life-histoty traits, has been used to show the cost of reproduction (Harshman and Zera 2007; Stearns 1992).

Interestingly, the GM-fed animals showed no apparent response in fecundity to the presence of the predator cue. We argue that the lack of the anticipated response and the relatively low reproductive output for this group was caused by a reduced health condition after feeding the Bt-transgenic maize. The appropriate life-history response may simple require a reasonably good health condition. The lack of response by early reproduction may, however, explain the somewhat higher survival in the animals fed Bt-transgenic maize and exposed to predator, compared to the early reproducing animals that were fed unmodified maize and exposed to predator.

Looking at the population level, the predator smell stimulated a higher population growth rate for the group fed UM-maize, which may seem paradoxical since a predator usually represents a stress factor that reduces growth and delays reproduction (Auld and Relyea 2008; Brodin et al. 2006). However, as the experiments did not last long enough to detect possible counteracting responses towards the end of life, i.e. a reduced reproductive output after the end of the experiment (day 42), the expected trade-off between early and late reproduction could not be evaluated completely. Also, the predator smell may have contributed with a small portion of nutrients, e.g. bacteria, to the sterile ADAM medium, thereby stimulating the test animals.

Hypothesis 4 The potential effects of food and predator treatment in combination will act in synergy (as opposed to purely additive effects)

Hypothesis supported. The differences between the GM-fed and UM-fed animals were larger in the presence of a predator: the population growth rate was markedly reduced in animals exposed to both GM maize and predator as compared to animals exposed to UM maize and predator. Also, the differences in body size for GM and UM-fed animals were much clearer in the groups with predator treatment, c.f. (Bøhn et al. 2008). Thus, the results indicate that the effects of food treatment and predator treatment were not additive, but interacted. As argued above, we emphasize that an organism under stress from two factors may be unable to respond adequately to several stressing conditions in the environment.

In conclusion, this study shows negative effects of a Bt-transgenic maize variety on a non-target model organism. The study thus rejects the substantial equivalence of the tested GM and UM-maize. Extended analysis using structured population models combine effects on survival and reproduction and enabled us to compare effects both on the population level, and on the sensitivity of different age-groups within food and predator treatments. Life-cycle experiments analysed with structured population models should be used, when applicable, to derive better estimates of the impact of novel food stuff, pesticides, toxicants and other stressors. Such demographic studies offer complete time-series portraits of toxicity and represents an improvement over traditional toxicological data and analysis. We urge more research on altered phenotypes and life-histories due to exposure from transgenic plants, ideally in combination with detailed—omics techniques, to follow the missing link of the specific causes and mechanisms involved in observed negative effects.

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