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**Evaluation of the effects of BT-maize on non-target insects
using a demographic approach.**

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ABSTRACT

Greenhouse and laboratory bioassays have been performed on MON810 Bt-maize and its isoline for the aphid *Rhopalosiphum maidis* and the predatory coccinellid *Hippodamia variegata*.

An experiment on life-table of the aphid *Rhopalosiphum maidis* on Bt-maize and isoline was performed; endpoints were preoviposition period, fecundity and longevity of aphids, development time and survival to yield data for demographic analyses. In this assay, no significant differences were found between GM and isogenic maize (treatment). The most notable results in terms of aphid performance were the significantly lower adult and total longevity on the second generation compared to the first, and the opposite trend of the pre-reproductive time that significantly increased passing from the first to the second generation on isoline and decreased on Bt-maize. At population level, none of the demographic parameters analyzed showed significant differences even if a slightly increase of r_m and λ can be evidenced passing from the first to the second generation on Bt-maize. LTREs showed that this trend could be ascribed to a higher fecundity of young adult aphids of the second generation, probably connected to the lower pre-reproductive time. These differences could be the result of differences in chemical constituents that makes Bt-maize less defended from aphids. Analyses of secondary metabolites showed significant reduction of free (35.0 vs 71.1 mg/100g fresh leaf; $P < 0.001$) and total (61.4 vs 94.4 mg/100g fresh leaf; $P < 0.001$) polyphenols in Bt-maize respect to isogenic line.

The experiment with the coccinellid *H. variegata* were performed evaluating the effects due to eating Cry1Ab toxin in maize pollen; it was provided to adults *H. variegata* along with a low amount of *Myzus persicae*, to ensure oviposition. Measured endpoints were: pre-oviposition period, fecundity, fertility and longevity of females; offspring development time (egg-to-adult), survival and sex-ratio and weight of the adults emerged. Data on immature development and survival, sex-ratio of emerged adults along with daily schedules of adult mortality and fecundity were used to calculate the following demographic parameters: gross reproductive rate (GRR), net reproductive rate (R_0), intrinsic rate of increase (r_m), mean generation time (T), doubling time (DT), and finite rate of increase (λ). When *H. variegata* was fed with pollen, no significant differences were found between GM and near-isogenic maize in all the parameter studied. Even if no statistically significant, the reduced population growth rate (λ) of *H. variegata* feeding on Bt-maize pollen, caused a high population delay of 28 days. This value is just two days more than the generation time (T). LTRE decomposition showed a lower egg and larval survival in the offspring of Bt pollen fed *H. variegata*, all contributing most on λ reduction. Differences in fecundity appeared to influence λ in a lesser extent, and in the opposite manner.

A bioassays were carried out in the greenhouse to assess the population-level responses of the coccinellid *H. variegata*, feeding on aphids (*Rhopalosiphon maidis*) reared on Bt-Maize and near-isogenic plants in a more realistic condition respect to highly controlled laboratory trials. Potted corn plants (3 plants/pot) were greenhouse-reared and when plants reached ≈ 1 m in height they were inoculated with *R. maidis*. When the aphids had settled on the plants, pots were singularly enclosed in sleeve cages and then a fixed population of coccinellids (eggs, larvae of mixed ages, pupae and adults) were added. After a week and on the three subsequent weeks, the plants in the sleeve cages were sampled and the number of specimens of each coccinellid stage present counted. The data obtained, consisting of population time series (population vectors which contains information on the stage distribution of the studied population), were used to generate a stage-classified projection matrix with the aim of modelling the impact that exposure to a prey reared on GM-plant would have on a population of coccinellids. The model consisted of a matrix including survival probabilities (P_i , the probability of moving to the next stage and G_i , the probability of remaining in the same stage) and fertilities (F_i) of a population. Population growth across time can be then found via repeated matrix multiplications. The demographic parameters, intrinsic rate of increase (r_m) and finite rate of increase (λ) were calculated from the matrix. Demography is used to evaluate the total effects, lethal and sublethal, of GM-plant-exposed population by means of a Life Table Response Experiments (LTREs). There were no significant differences in the mean number of eggs, larvae, pupae, and adults of *H. variegata* between Bt-maize and near-isogenic line. On the contrary a significant reduction were observed among sampling dates for egg, larval and pupal stage. For the latter the interaction of treatment and sampling date was also significant with the higher number of pupae found a week early in Bt-maize treatment respect to near-isogenic line. No significant differences were observed in *H. variegata* population growth rates (λ and r_m) between Bt-maize and near-isogenic line. Using LTRE decomposition, the effect of Bt-maize exposition on λ have been decomposed into contributions arising from the effect on each stage-specific parameter. Respect to near-isogenic line, exposition to Bt-maize had little effect on preimaginal stages survival and no significant effect on λ . A more pronounced effect of Bt-maize treatment can be found for the adult stage. Both survival of adult within adult stage and fecundity resulted reduced, even if either difference or contribution values resulted not significantly different from zero

Chapter
1

Effects of *Bt* Maize on the non-target pest *Rhopalosiphum maidis*

Effects of *Bt* Maize on the non-target pest *Rhopalosiphum maidis*

INTRODUCTION

Due to worldwide broader cultivation of genetically modified (GM) plants an increased availability of information on their possible impact on non-target organisms is highly required. In the available literature studies on the effect of GM plants on non-target organisms have mainly converged on beneficial insects such as predators and parasitoids that play a fundamental role in pest regulation (see Lövei and Arpaia, 2005; Romeis et al., 2006; Wolfenbarger et al., 2008, for reviews). However little attention has been paid to non-target pests. The corn leaf aphid *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae) is a common pest species on maize crops (Head et al., 2001) and some studies reported that this aphid performs better on Bt maize lines respect to their non-transgenic counterparts (Faria et al., 2007; Stephens et al., 2012) even if causes remain, as yet, not fully acknowledged. Since the targets of Bt toxin are chewing insects, mainly Lepidoptera and Coleoptera, and the toxin is not translocated into phloem sap in maize plants (Raps et al., 2001) the sap-sucking insects are probably not affected by the Bt toxin. Hence other unintended changes in plant characteristics tied to Bt gene insertion could account for the differences in aphid performances. Consequently, the aims of the current study is to evaluate the effects of Bt maize on the performance of *R. maidis* in comparison with its corresponding near-isogenic line, both in the short and long term. In doing so both standardized laboratory bioassays and demographic approach by means of an age-structured matrix population model (Caswell, 2001) were performed. Furthermore to gain insight into the impact of Bt maize on *R. maidis* free and bound phenolics content on transgenic and near-isogenic plants were measured.

MATERIALS AND METHODS

Plants

A hybrid transgenic maize (Bt-maize), event MON 810 (DKC442YG), expressing a gene encoding a truncated form of the Cry1Ab toxin from the *B. thuringiensis* var. *kurstaki*, active against the European Corn Borer, *Ostrinia nubilalis*, and its correspondent non-transformed near isoline (DK440) (near-isogenic) were used in the experiments. Plants were grown from seed in 18-litre plastic pots (two plants per pot) with the same soil mixture (60% universal potting soil 40% sand) in a greenhouse (min. T 19 °C, max. T 29 °C; min. r.h. 55%, max. r.h. 85% and 16L:8D). Plants were watered using a timer driven

automatic drip watering system. Seedlings bearing eight true leaves were used to start aphid first generation.

Corn leaf aphid rearing

A mass culture of *Rhopalosiphum maidis* was established, starting from field collected specimens. The corn leaf aphid was reared on wheat seedlings in a climatic chamber at 25 ± 1 °C, $70\pm 10\%$ r.h., and 16L:8D photoperiod.

Aphid performance

In this bioassay, two consecutive aphid generations were studied. Transgenic and near-isogenic maize plants (5 plants per treatment) were transferred to a climatic chamber (25 ± 1 °C, $70\pm 10\%$ r.h., and 16L:8D) and ordered randomly. Then plants were infested with the aphid as described below. Other 5 plants per treatment were subsequently transferred to the climatic chamber to hold the second aphid generation. Before transfer, all plants were visually checked for unintended aphid infestation. The experiment was composed of three independent replicates each with a different set of plants and a total of 15 plants per treatment.

Single wingless adult aphids were transferred to clipcages (1.5 cm in diameter) attached to the 6th leaf of Bt-maize and near-isogenic plants (three clipcages per plant). Clipcages were equipped with a hole sealed with fine-mesh netting to ensure air circulation.

After 24 hours all aphids were removed except for one first instar nymph per each clipcages. In each treatment, about 10 to 15 first instar nymphs were kept to start a first generation's cohort. Aphids were then daily monitored through their life cycle for development and mortality. The presence of an exuviae in the clipcages indicated passage to the next instar and it was removed upon discovery.

When aphids molted to adult stage all newborn aphids were daily counted and removed from the clipcage until adult aphid death.

For each treatment, about 5-7 days after reproduction onset, 10 to 15 second instar (L2) nymphs coming from the first generation were transferred individually to new clipcages on new maize plants (three clipcages per plant), of the same planting date of that of the first generation, to build up a second generation's cohort. In each generation there were 3 cohorts as replication on each treatment.

With the sole exception of survival analysis, all aphids which were lost during the experiments were not considered.

The confined aphids were used to determine the following biological traits: (1) development time, time

from birth to adult appearance , (2) adult longevity, (3) total longevity, (4) total fecundity per female, (5) fecundity rate, fecundity per female per day, (6) pre-reproductive time, time from birth to reproduction onset, (7) proportion of alate offspring.

Demographic analyses

Data on nymphal development and survival, along with daily schedules of adult mortality and fecundity, were used to calculate age-specific survival rate (l_x) and age-specific fecundity (m_x). In each treatment, Bt-maize, near-isogenic, first and second generation, the life table parameters, including gross reproductive rate (GRR), net reproductive rate (R_0), mean generation time (T), doubling time (DT), and finite rate of increase (λ) were calculated as follow (Carey 1993):

$$GRR = \sum_{x=0}^{\sigma} m_x \quad (1)$$

$$R_0 = \sum_{x=0}^{\infty} l_x m_x \quad (2)$$

$$T = \frac{\sum_{x=0}^{\sigma} x l_x m_x}{\sum_{x=0}^{\sigma} l_x m_x} \quad (3)$$

$$DT = \frac{\ln 2}{r_m} \quad (4)$$

$$\lambda = e^{r_m} \quad (5)$$

The precise value of intrinsic rate of increase (r_m) was estimated iteratively solving the Euler equation:

$$\sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1 \quad (6)$$

Age-specific survivorship (l_x) and fertility (m_x) schedules were also used to construct a $z \times z$ age-classified population projection matrices \mathbf{A} (standard Leslie matrix) with a projection interval of one day.

$$\mathbf{A} = \begin{pmatrix} F_1 & F_2 & F_3 & \dots & F_z \\ P_1 & 0 & 0 & \dots & 0 \\ 0 & P_2 & 0 & \dots & 0 \\ \vdots & \dots & \dots & \dots & \vdots \\ 0 & 0 & 0 & P_{z-1} & 0 \end{pmatrix} \quad (7)$$

The only nonzero entries of the matrix, survival probability (P_i) appearing on the subdiagonal and fertilities (F_i) appearing in the first row, were calculated by means of the birth-flow formulas (Caswell, 2001) as follow:

$$P_i \approx \frac{l(i) + l(i+1)}{l(i-1) + l(i)} \quad (8)$$

$$F_i = \frac{l(0) + l(1)}{2} \left(\frac{m_i + P_i m_{i+1}}{2} \right) \quad (9)$$

where $l(i)$ is the survivorship from birth to age i and m_i is the mean number of female offspring per female in age class i .

The asymptotic population growth rate (λ) was calculated as the dominant eigenvalue of the matrix.

The life expectancy (e_x), i.e. the mean age at death, and the expected life time reproduction, i.e. the expected number of reproductive events during a female's lifetime, have been calculated treating the transition matrix as an absorbing Markov chain with death as absorbing state (Caswell, 2001).

We determined how much the observed differences in population growth rates (λ) between near-isogenic and Bt-maize and between first and second aphid generations were determined by the differences in each vital rate by means of a factorial life table response experiment (LTRE) analysis (Caswell, 2001, Walls et al., 1991). Factorial LTREs permit the examination of the interactions between factors. With this technique, $\alpha^{(i)}$ the main effect of maize genotype level i ($i =$ Bt-maize, near-isogenic), $\beta^{(j)}$ the main effect of generation level j ($j =$ first generation, second generation) and $(\alpha\beta)^{(ij)}$ the effect of the interaction on λ , measured respect to the overall mean, were estimated and decomposed into contributions from each age-specific fertility and survival probability terms. Following Caswell (2001), the linear model used for this experiment was:

$$\lambda^{(ij)} = \lambda^{(\cdot\cdot)} + \alpha^{(i)} + \beta^{(j)} + (\alpha\beta)^{(ij)} \quad (10)$$

where $\lambda^{(ij)}$ and $\lambda^{(\cdot\cdot)}$ are the dominant eigenvalue of the matrix $\mathbf{A}^{(ij)}$, resulting from treatment combination, and of the overall mean matrix $\mathbf{A}^{(\cdot\cdot)}$, used as reference matrix, respectively.

The treatment effects were estimate using the following formulas:

$$\hat{\alpha}^{(i)} = \lambda^{(i\cdot)} - \lambda^{(\cdot\cdot)} \quad (11)$$

$$\hat{\beta}^{(j)} = \lambda^{(\cdot j)} - \lambda^{(\cdot\cdot)} \quad (12)$$

$$(\alpha\beta)^{(j)} = \lambda^{(ij)} - \hat{\alpha}^{(i)} - \hat{\beta}^{(j)} - \lambda^{(\cdot\cdot)} \quad (13)$$

and decomposed into contributions from each matrix entries this way:

$$\tilde{\alpha}^{(i)} = \sum_{k,l} (a_{kl}^{(i,\cdot)} - a_{kl}^{(\cdot,\cdot)}) \frac{\partial \lambda}{\partial a_{kl}} \Big|_{\frac{1}{2}(\mathbf{A}^{(i,\cdot)} + \mathbf{A}^{(\cdot,\cdot)})} \quad (14)$$

$$\tilde{\beta}^{(i)} = \sum_{k,l} (a_{kl}^{(\cdot,j)} - a_{kl}^{(\cdot,\cdot)}) \frac{\partial \lambda}{\partial a_{kl}} \Big|_{\frac{1}{2}(\mathbf{A}^{(\cdot,j)} + \mathbf{A}^{(\cdot,\cdot)})} \quad (15)$$

$$\alpha \tilde{\beta}^{(ij)} = \sum_{k,l} (a_{kl}^{(i,j)} - a_{kl}^{(\cdot,\cdot)}) \frac{\partial \lambda}{\partial a_{kl}} \Big|_{\frac{1}{2}(\mathbf{A}^{(i,j)} + \mathbf{A}^{(\cdot,\cdot)})} - \tilde{\alpha}^{(i)} - \tilde{\beta}^{(j)} \quad (16)$$

Each of these equation approximate an observed change in λ respect to $\lambda^{(\cdot,\cdot)}$ and each term in the summation represents the contribution of the difference in the matrix element a_{kl} (age-specific fertility or survival probability) to the effect of treatment, on population growth rate. That contribution is the product of the difference between overall mean and treatment specific vital rate and the sensitivity of that vital rate calculated from a matrix halfway between the matrices being compared (Logofet and Lesnaya, 1997). The interaction term in equation 16 represents the deviation between the observed contribution of a_{kl} to $\lambda^{(ij)}$ of the treatment combination and that predicted on the basis of an additive model (Caswell, 2001).

Finally, an application of matrix models, the Delay in Population Growth Index (Wennergren and Stark, 2000; Stark et al., 2004; Stark et al., 2007a;b), that measure population growth delay after a stressful event, was calculated to compare the time required to the four populations studied to reach a predetermined number of individuals. In this study population delay was determined by choosing a population size of 100,000 individuals (Stark et al., 2004). In each treatment, population growth was simulated by multiplying the matrix \mathbf{A} by an initial population vector $\mathbf{n}(t) = [n_1 \ n_2 \ n_3 \ \dots \ n_z]^T$, containing information on the age distribution of the population. The population projection was started with a vector consisting of 100 individuals distributed according to the stable age distribution and ended when at least 100,000 individuals had been reached. In each treatment, the stable age distribution (\mathbf{w}) was calculated as the corresponding right eigenvector of the dominant eigenvalue of the matrix \mathbf{A} (Caswell, 2001).

All demographic calculations were performed with the software PopTools version 2.7.5 (Hood, 2006).

Secondary metabolite analyses

Free and bound phenolics were extracted according to Hartmann et al. (2008) protocol slightly modified. Briefly, 5 g of leaves were minced (Ultra Turrax T 25) with 40 ml of methanol/distilled

water/acetone (60+30+10; v/v/v) and shaken for 10 min. After centrifugation at 10000 rpm for 10 min, the supernatant containing the free soluble compounds was recovered and extraction was repeated once. Supernatants were pooled, evaporated to dryness and reconstituted in 10 ml of 80% methanol. The residue from the free phenolic extraction was subjected to alkaline and acid hydrolysis to recover the bound phenolic compounds, as reported by Mattila et al. (2005) with some modifications. After 1 h shaking with 10 ml of 2M NaOH at room temperature, extracts were acidified adding concentrated HCl. Samples were successively centrifuged (10000 rpm for 10 min) and supernatants collected and extracted 3 times with 15 ml of ethyl acetate. The ethyl acetate layers were pooled, evaporated to dryness and reconstituted in 10 ml of methanol. Free and bound phenolic extracts were filtered through a Watman4 filter paper and stored at -20°C until use for the subsequent analyses of polyphenol and content. Free and bound polyphenol content of each sample was determined using the Folin–Ciocalteu procedure described by Singleton et al. (1999). Gallic acid was used as standard and polyphenol content expressed as milligrams of gallic acid equivalent (GAE) per 100 g of dry weight. The experiment was composed of three independent replicates each with a different set of plants.

Statistical analysis

Data on aphid biological traits were analyzed by a factorial ANOVA, using maize genotype, generation, and replicate as main factors. Data which violated the ANOVA assumptions (homoscedasticity and normality) were log or rank transformed. Data on life table parameters were analyzed by a factorial ANOVA, using maize genotype and generation as main factors. Kaplan-Meier survival analysis was used to estimate survival over time among treatments. The log-linear rank test followed by the Holm-Sidak pairwise multiple comparison procedures was performed to detect significant differences ($P < 0.05$) among survival curves. Individuals, which were lost during the experiments were accounted as censored. Data on secondary metabolites were tested with factorial ANOVA using maize genotype and replicate as main factors. Statistical analyses were performed using STATISTICA software version 10 (StatSoft Inc.) and SigmaStat 3.0 (Systat Software Inc.).

RESULTS

Aphid performance

The survival analysis of two generations of *R. maidis* reared on near-isogenic and Bt-maize indicated significant differences among treatments (Log-rank test, $\chi^2 = 28.64$, d.f. = 3, $P < 0.001$) (Fig. 1). First generation's aphids survived significantly longer compared with second

generation's both on near-isogenic (Holm-Sidak test, $P < 0.001$) and Bt-maize (Holm-Sidak test, $P < 0.001$). In contrast no significant difference were found between maize genotypes in both generations (Holm-Sidak test, $P = 0.517$ and $P = 0.873$, for first and second generation, respectively).

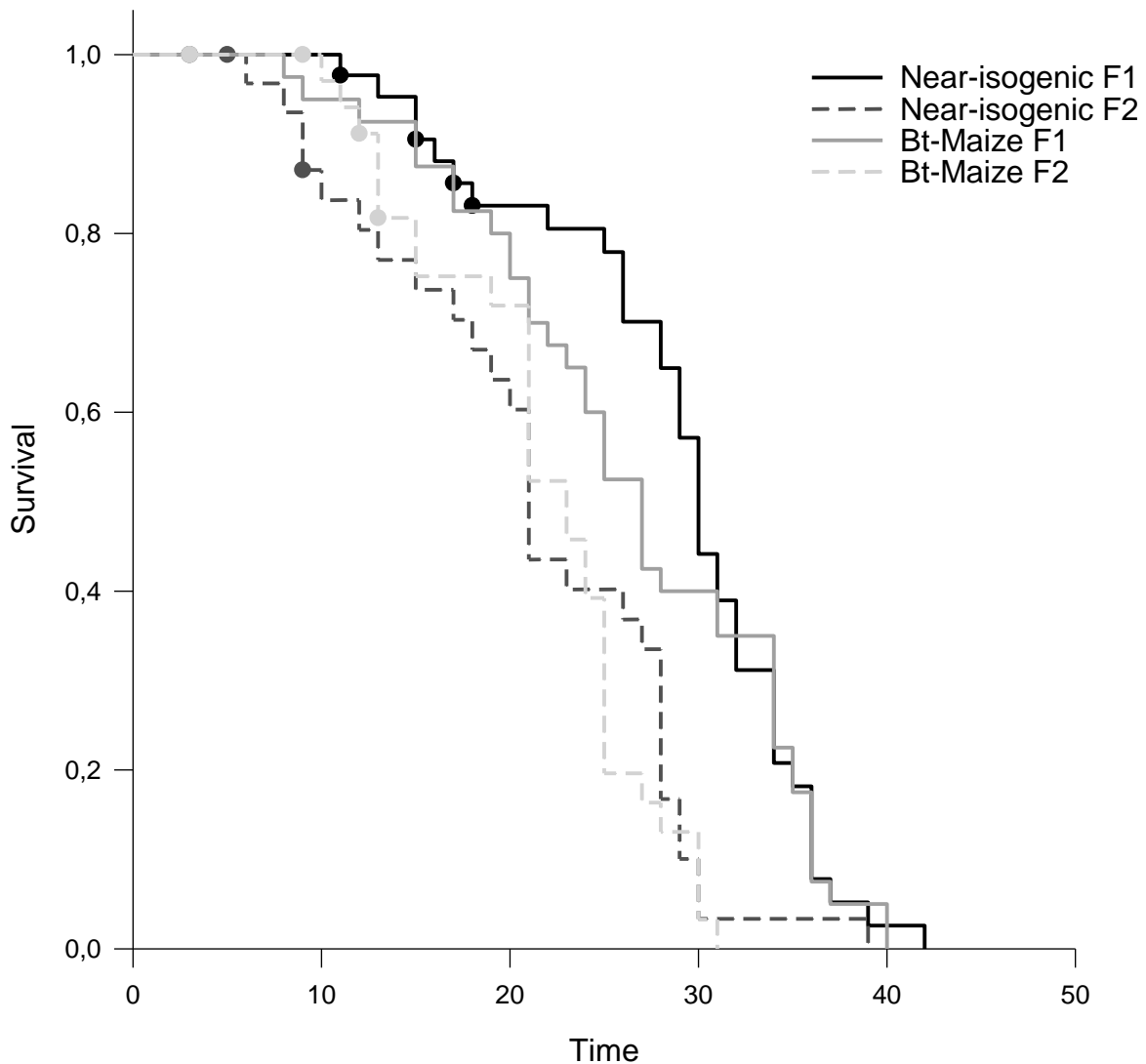


Figure 1. Survival curves of two generations of *Rhopalosiphum maidis* on near-isogenic and Bt-maize plants.

None of the aphid biological traits studied differed significantly between maize genotypes and replicates (Table 1). No significant differences in the development time was also observed within

Table 1. Biological traits (mean \pm SE) of two generations of *Rhopalosiphum maidis* on near-isogenic and Bt-maize plants.

	Near-isogenic		Bt-Maize		Source of variation						
	F1	F2	F1	F2	Maize genotype	Generation	Replicate	Maize genotype x Generation	Maize genotype x Replicate	Generation x Replicate	Maize genotype x Generation x Replicate
n	39	30	40	31							
Development time (Days)	6.9 \pm 0.3	7.6 \pm 0.5	7.8 \pm 0.5	6.4 \pm 0.3	F _(1, 120) = 1.443 P = 0.2321	F _(1, 120) = 3.339 P = 0.0701	F _(2, 120) = 2.991 P = 0.0540	F _(1, 120) = 8.750 P = 0.0037	F _(2, 120) = 2.783 P = 0.0659	F _(2, 120) = 24.879 P < 0.00001	F _(2, 120) = 6.541 P = 0.0020
Adult longevity (Days)	21.6 \pm 1.3	15.4 \pm 1.3	19.4 \pm 1.4	15.4 \pm 1.1	F _(1, 120) = 1.7238 P = 0.1917	F _(1, 120) = 10.545 P = 0.0015	F _(2, 120) = 0.5429 P = 0.5825	F _(1, 120) = 0.7534 P = 0.3871	F _(2, 120) = 0.3225 P = 0.7250	F _(2, 120) = 3.1065 P = 0.0484	F _(2, 120) = 0.3356 P = 0.7156
Longevity (Days)	28.6 \pm 1.2	21.1 \pm 1.5	26.6 \pm 1.4	21.5 \pm 1.1	F _(1, 128) = 0.468 P = 0.4949	F _(1, 128) = 20.174 P < 0.0001	F _(1, 128) = 0.971 P = 0.3816	F _(1, 128) = 1.167 P = 0.2821	F _(2, 128) = 0.001 P = 0.9990	F _(2, 128) = 1.376 P = 0.2564	F _(2, 128) = 0.380 P = 0.6845
Fecundity (Neanids/female)	30.5 \pm 2.9	23.3 \pm 3.2	24.5 \pm 3.2	24.9 \pm 2.2	F _(1, 120) = 2.4303 P = 0.1216	F _(1, 120) = 0.0015 P = 0.9691	F _(2, 120) = 1.2337 P = 0.2949	F _(1, 120) = 2.1378 P = 0.1463	F _(2, 120) = 0.5497 P = 0.5786	F _(2, 120) = 13.876 P < 0.0001	F _(2, 120) = 1.1717 P = 0.3134
Fecundity Rate (Neanids/female/day)	1.5 \pm 0.2	1.7 \pm 0.2	1.3 \pm 0.2	1.8 \pm 0.2	F _(1, 120) = 0.5329 P = 0.4668	F _(1, 120) = 5.1508 P = 0.0250	F _(2, 120) = 0.0413 P = 0.9595	F _(1, 128) = 0.0893 P = 0.7656	F _(2, 120) = 0.7125 P = 0.4925	F _(2, 120) = 6.5123 P = 0.0021	F _(2, 120) = 2.5029 P = 0.0861
Pre-reproductive time (Days)	7.8 \pm 0.3	8.2 \pm 0.6	9.1 \pm 0.6	6.7 \pm 0.3	F _(1, 115) = 0.1628 P = 0.6873	F _(1, 115) = 7.9671 P = 0.0056	F _(2, 115) = 1.5024 P = 0.2269	F _(1, 115) = 4.9446 P = 0.0281	F _(2, 115) = 0.0251 P = 0.9753	F _(2, 115) = 15.675 P < 0.0001	F _(2, 115) = 3.7004 P = 0.0277

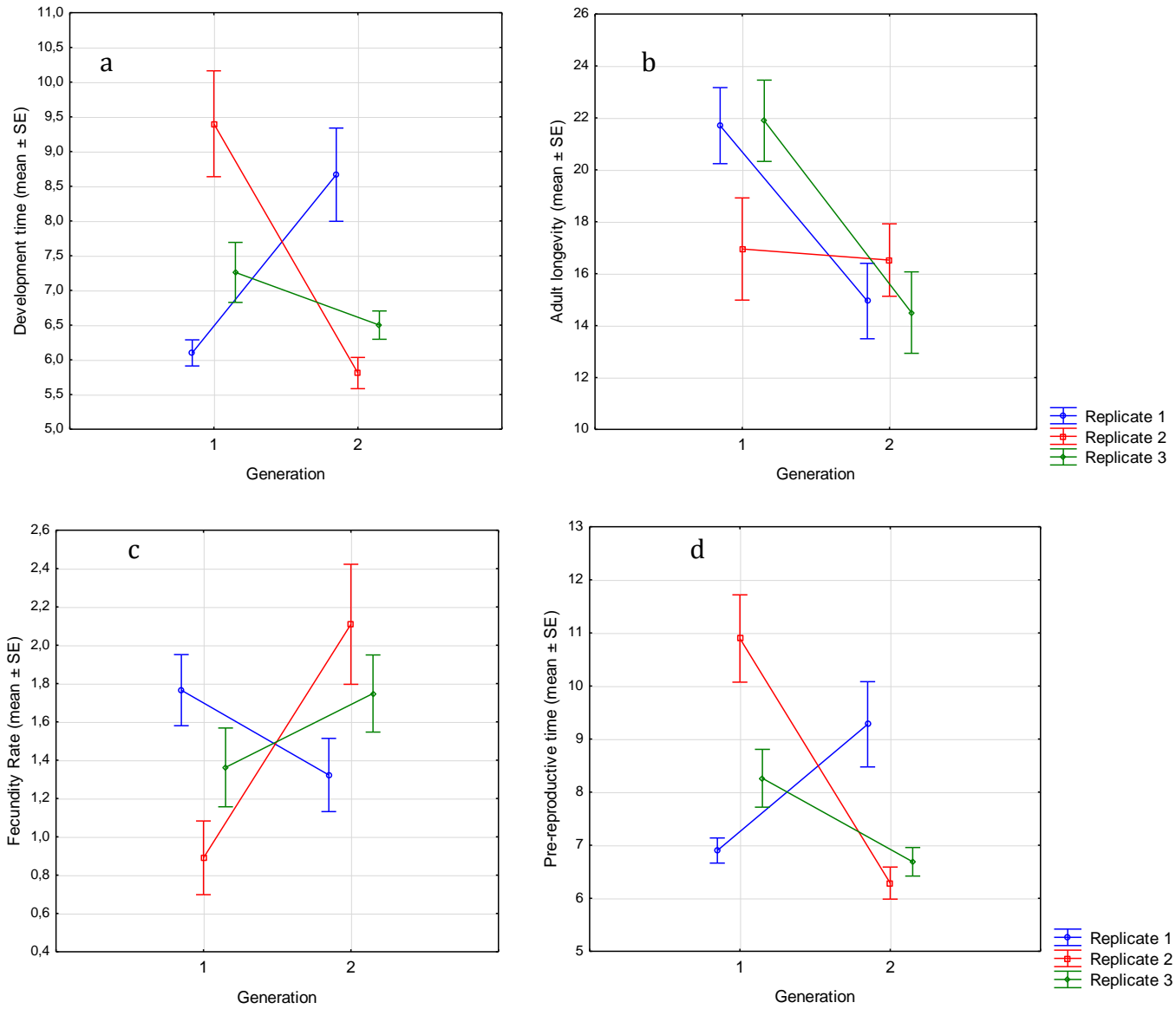


Figure 2. Plotted generation \times replicate interaction for the biological traits of *Rhopalosiphum maidis* reared on near-isogenic and Bt-maize plants.

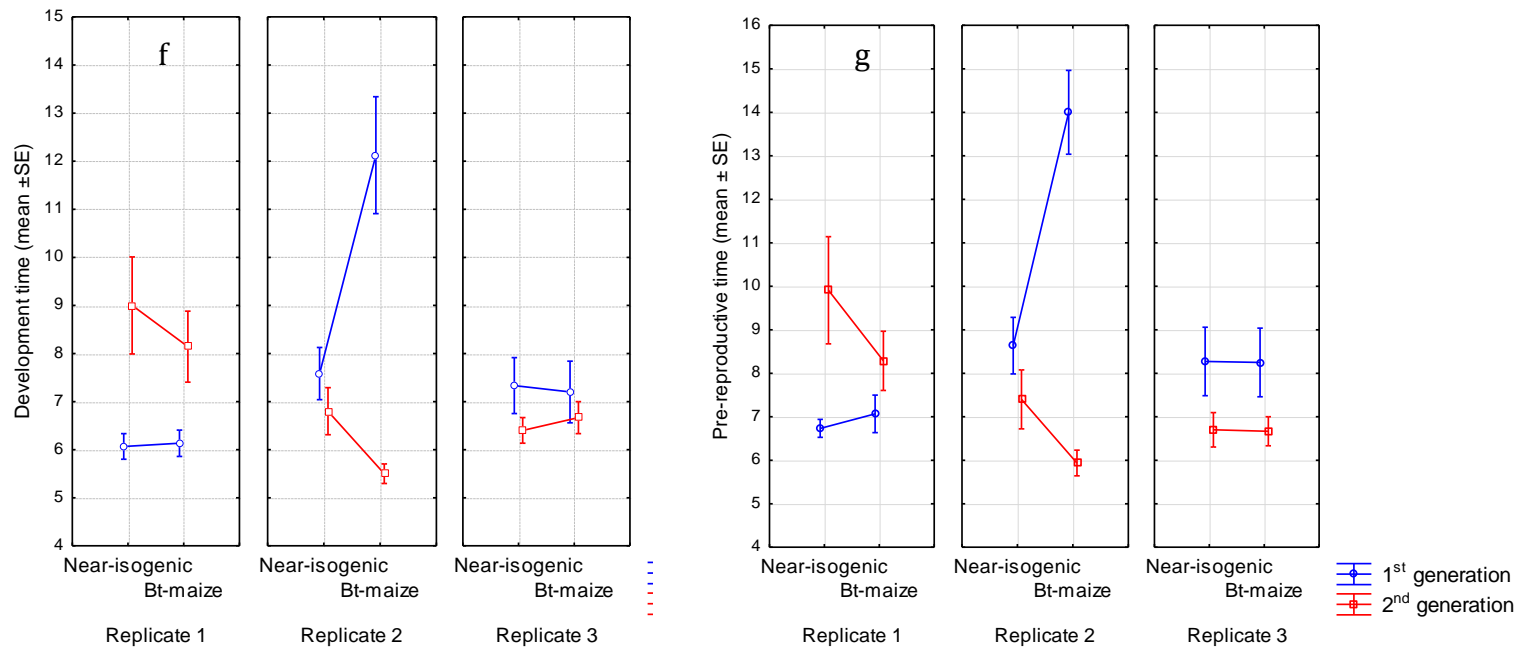
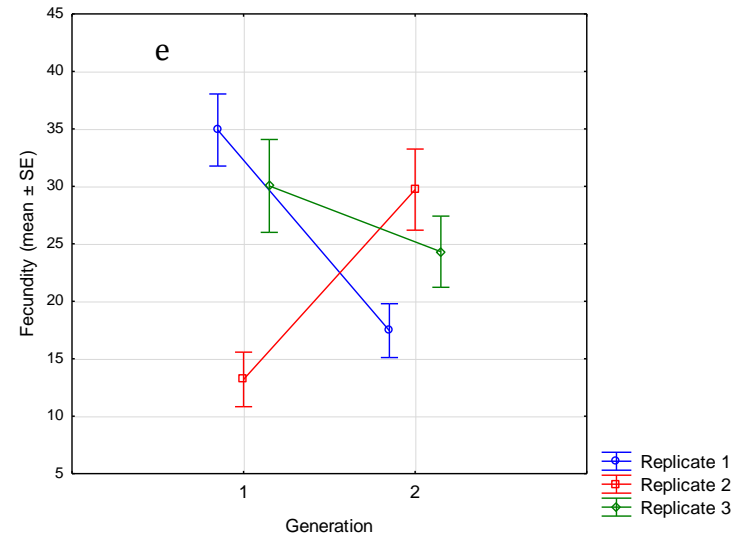


Figure 2. continue, showing maize genotype × generation × replicate interaction

generations, even if a significant interaction among maize genotypes and generations occurred (Table 1). Moreover a significant reduction of development time was observed in the second generation in replicates 2 and 3, but not in replicate 1 (Fig. 2a). Differently, the total longevity, the adult longevity, the fecundity rate and the pre-reproductive time varied significantly between generations. The first two parameters shortened and the fecundity rate increased in the second generation either on transgenic or near isogenic lines. On the contrary the pre-reproductive time became significantly longer in the second generation on isogenic line, and shorter on Bt-maize (Table 1). Moreover with the sole exception of longevity a significant generation \times replicate interaction was found for these parameters. In particular the adult longevity was higher in the first generation on replicate 1 and 3 (Fig. 2b) instead fecundity rate increased and pre-reproductive time shortened in the second generation in replicates 2 and 3 but not in replicate 1 (Fig. 2c and 2d, respectively).

There were no significant differences in the fecundity within generations but a significant interaction among generation and replicate was found (Fig. 2e). In this case replicate 2 showed a different trend between 1st and 2nd generation respect to the other two. The percentage of alate production on near-isogenic and Bt-maize was not significantly different but it decreased significantly between generations (Table 2).

Demographic analyses

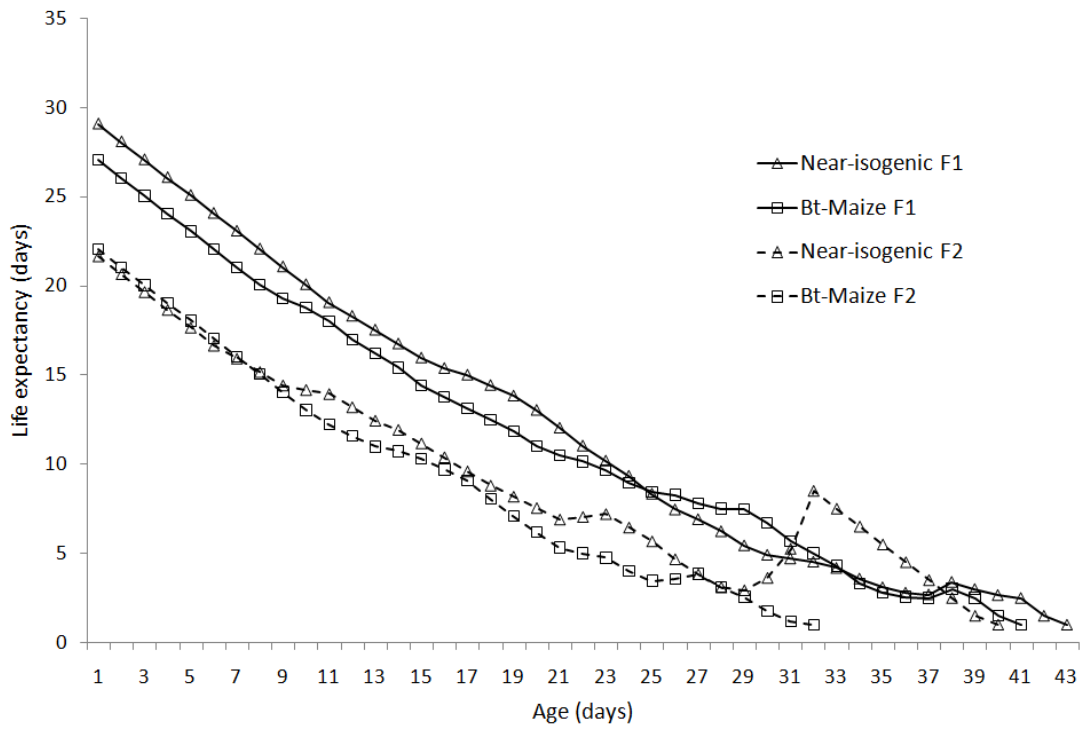
The demographic parameters analyzed showed no significant differences either between maize genotypes or generations (Table 2).

The life expectancy (e_x) represent the mean lifespan that an individual aged x is expected to live if the conditions whose is exposed were maintained (Fig. 3a). The estimated value of this parameter at birth for near-isogenic F1, near-isogenic F2, Bt-maize F1 and Bt-maize F2 was 29.1, 21.6, 27.0 and 22.0 days, respectively. Overall the life expectancy for aphids of the 2nd generation was lower than that of the 1st independently from maize genotypes with the sole exception of the aphids of the 2nd generation older than 30 days. In this case the aphids grown on non-transgenic plants showed a peak indicating a higher value of e_x . At this age however the expected lifetime reproduction tends to zero. This parameter, the expected number of reproductive event during an aphid lifetime, is reported in Fig. 3b. A newborn F1 aphid can expect to produce 30.8 and 23.5 offspring on near-isogenic and Bt-Maize, respectively, while a newborn F2 aphid 20.5 and 23.6 offspring. During its lifetime an aphid of the 1st generation could expect to reproduce more than that of the 2nd generation if reared on near-isogenic maize. This difference cannot be observed if aphids are reared on Bt-maize (Fig. 3b).

Table 2. Proportion of alate offspring and life-table parameters (mean \pm SE) of two generations of *Rhopalosiphum maidis* on near-isogenic and Bt-maize plants.

	Near-isogenic		Bt-Maize		Source of variation		
	F1	F2	F1	F2	Maize genotype	Generation	Maize genotype x Generation
n	39	30	40	31			
Proportion of alate offspring (%)	12.8 \pm 7.2	6.7 \pm 6.7	15.3 \pm 2.4	0.0 \pm 0.0	F _(1, 8) = 1.0595 P = 0.8135	F _(1, 8) = 6.0235 P = 0.0397	F _(1, 8) = 1.3229 P = 0.2833
Gross reproductive rate (GRR)	33.1 \pm 6.2	28.0 \pm 5.6	24.9 \pm 6.9	24.7 \pm 3.2	F _(1, 8) = 1.0500 P = 0.3355	F _(1, 8) = 0.2162 P = 0.6544	F _(1, 8) = 0.1942 P = 0.6712
Net reproductive rate (R₀)	29.8 \pm 7.2	21.3 \pm 4.3	21.5 \pm 7.1	22.1 \pm 2.7	F _(1, 8) = 0.4395 P = 0.5260	F _(1, 8) = 0.4797 P = 0.5081	F _(1, 8) = 0.6377 P = 0.4476
Intrinsic rate of increase (r_m)	0.2793 \pm 0.0357	0.2894 \pm 0.0380	0.2356 \pm 0.0621	0.2893 \pm 0.0292	F _(1, 8) = 0.2584 P = 0.6250	F _(1, 8) = 0.5475 P = 0.4805	F _(1, 8) = 0.2548 P = 0.6273
Finite rate of increase (λ)	1.3238 \pm 0.0466	1.3375 \pm 0.0501	1.2704 \pm 0.0764	1.3366 \pm 0.0396	F _(1, 8) = 0.244 P = 0.6344	F _(1, 8) = 0.527 P = 0.4885	F _(1, 8) = 0.227 P = 0.6463
Mean generation time (T)	14.8 \pm 0.9	13.0 \pm 1.3	15.2 \pm 1.8	12.4 \pm 0.5	F _(1, 8) = 0.0107 P = 0.9200	F _(1, 8) = 3.7907 P = 0.0874	F _(1, 8) = 0.1369 P = 0.7210
Doubling time (DT)	2.6 \pm 0.4	2.5 \pm 0.4	3.6 \pm 1.3	2.4 \pm 0.2	F _(1, 8) = 0.4986 P = 0.5001	F _(1, 8) = 0.8053 P = 0.3957	F _(1, 8) = 0.6047 P = 0.4592

a)



b)

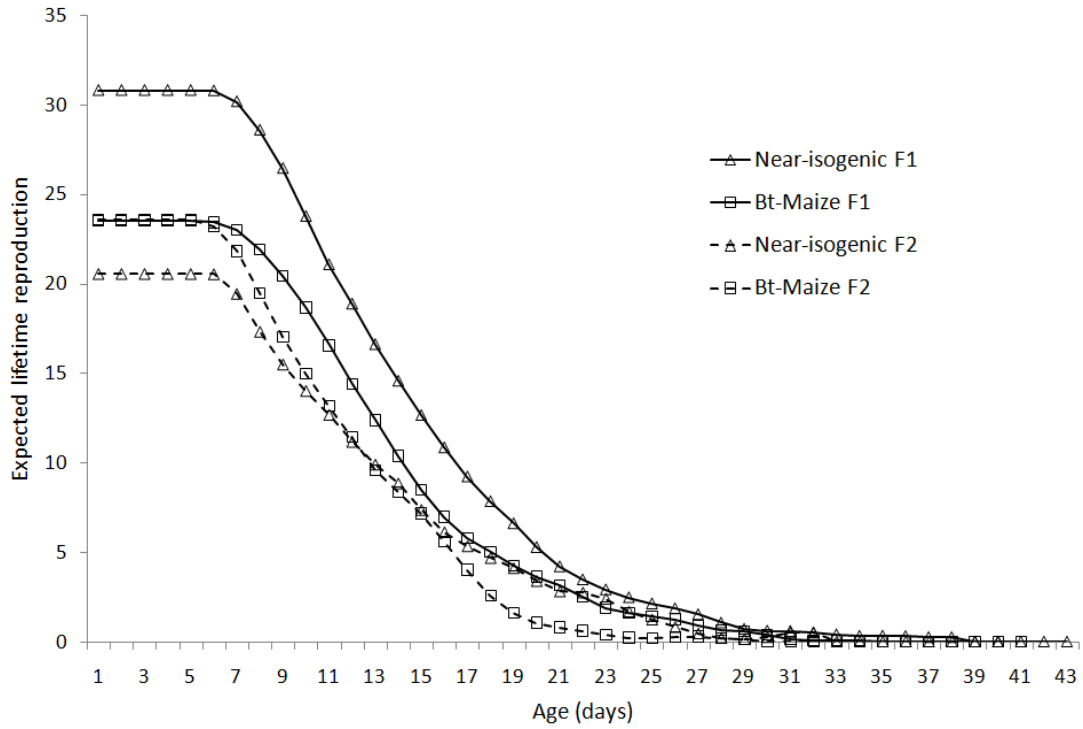


Figure 3. Life expectancy (a) and mean number of lifetime reproductive episodes (b) of two generations of *Rhopalosiphum maidis* on near-isogenic and Bt-maize plants.

The value of delay in population growth index showed that Bt-maize delayed population growth of the 1st generation of 2 days respect to near-isogenic line (Fig. 4). On the 2nd generation the effect reversed with the population growth on near-isogenic line delayed of just 1 day respect to Bt-maize. Furthermore the delay in population growth among generations exhibit a different behavior for the aphids reared on near-isogenic and Bt-maize (Fig. 4). The population reared on near-isogenic line exhibited the same population growth with no differences between generations. On the contrary on Bt-maize the 2nd generation exhibited an anticipation of 3 days respect to the 1st generation to reach 100,000 individuals.

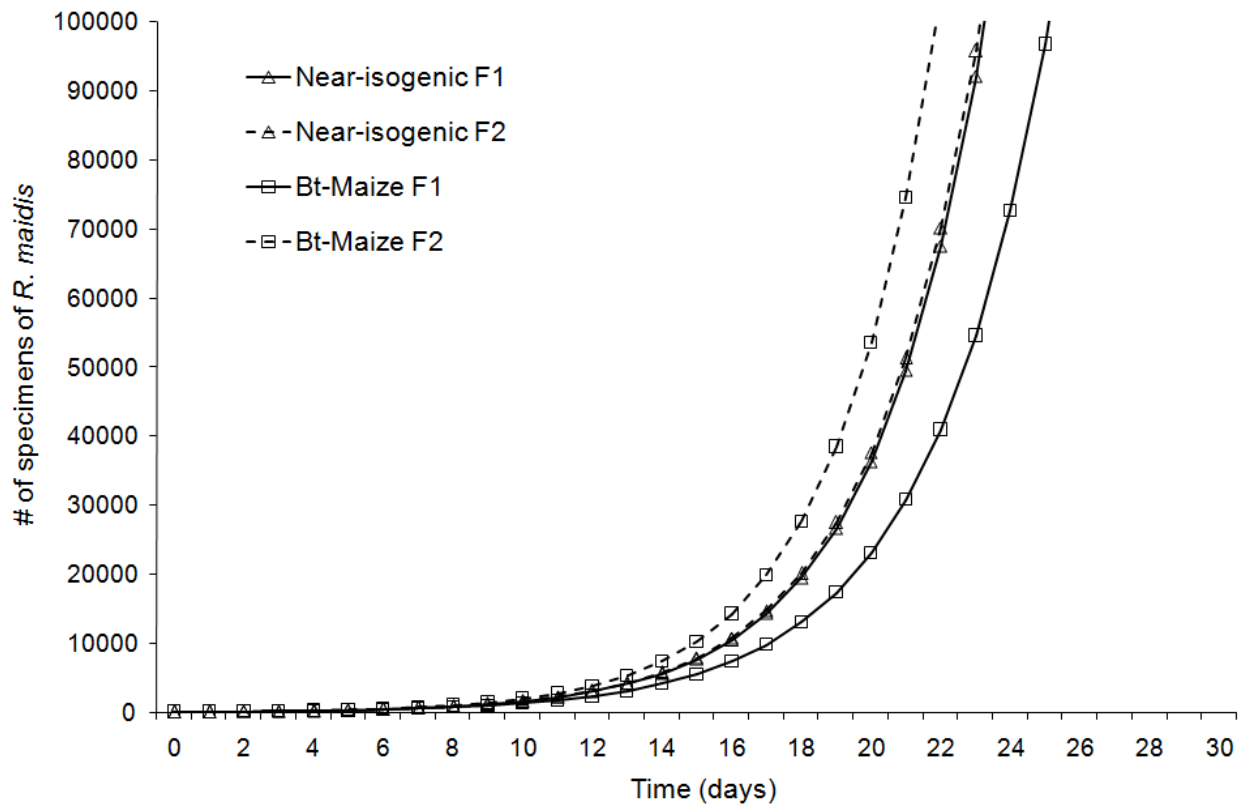


Figure 4. Matrix model projections and comparison of the delay in population growth to reach 100,000 specimens for two generations of *Rhopalosiphum maidis* on near-isogenic and Bt-maize plants. Simulation started with 100 specimens at a stable age distribution.

Using LTRE decomposition the effect of treatment on λ have been decomposed into contributions arising from the effect on each age-specific vital rate. Overall the treatment effects on λ are shown in Fig. 5. In this figure—are reported the observed and calculated values of λ , the latter predicted by the model either including the interaction terms or not. The observed and calculated values are very close.

The additive model shows a clear positive effect of the second generation on λ . The discrepancy of the additive model predictions from the observed values shows the character of the interaction. On near-isogenic treatment the difference of λ between generations results balanced, instead on Bt-maize the difference results enhanced.

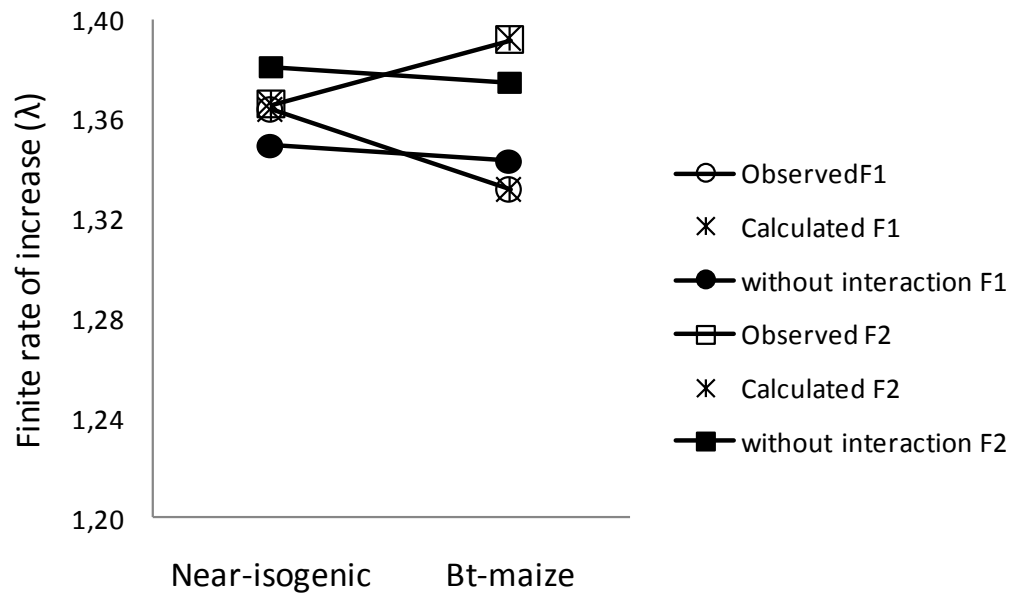


Figure 5. Finite rate of increase (λ) of *Rhopalosiphum maidis* as a function of maize genotypes and generation. Open symbol represent the observed values, filled symbol the values calculated with the additive model without the interaction terms, and the asterisks the values calculated using the model considering the interaction terms.

The effects of maize genotypes on survival is greatest at later ages (21 - 42 days) but these effects make negligible contribution to λ (Fig. 6). Fecundity is reduced by Bt-maize and increased by near-isogenic exposition (Fig. 7). Fecundity differences after ages 11-13, however, have essentially no impact on λ . Most of the fecundity mediated impact of maize genotypes on λ occurs between ages 6 - 11. On age 5 there is a different behavior with a reduction for the near-isogenic and an increase for the Bt-maize probably due to a shorter pre-reproductive time in the latter (Fig. 7).

As for maize genotypes, the effect of generation on survival is greatest at later ages (20 - 43 days) with negligible contribution of these effects to λ (Fig. 8). First generation have reduced fecundity from 5 to 8 days of age and increased fecundity at later ages (9 - 38 days); the opposite happens for the second generation (Fig. 9). Moreover later ages (9-38 days) make negligible or no contribution to population

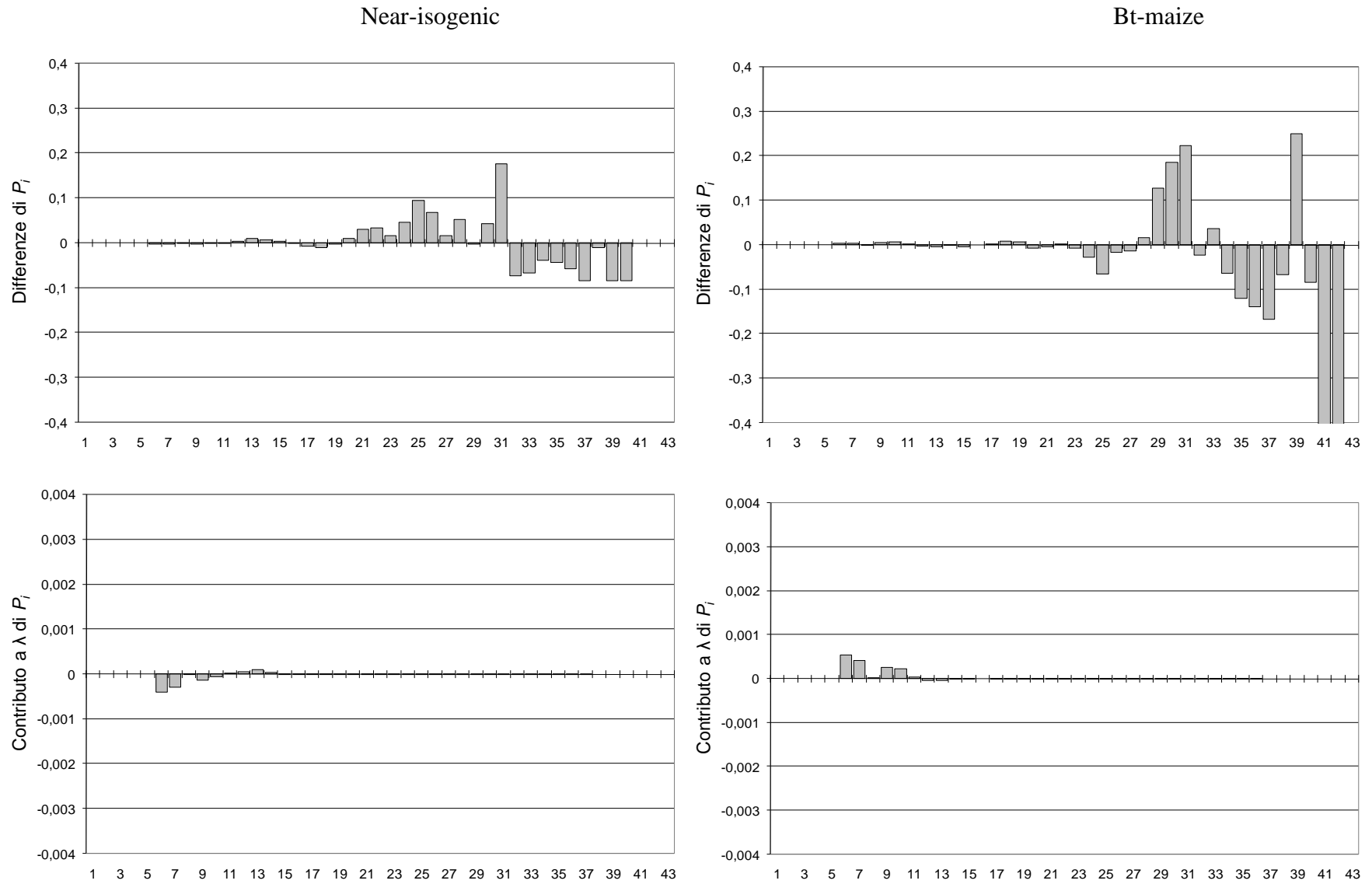


Figure 6. Effects of maize genotypes on age-specific survival of *Rhopalosiphum maidis* measured relative to the mean (Top) and contributions of those effects to population growth rate (λ) (Bottom).

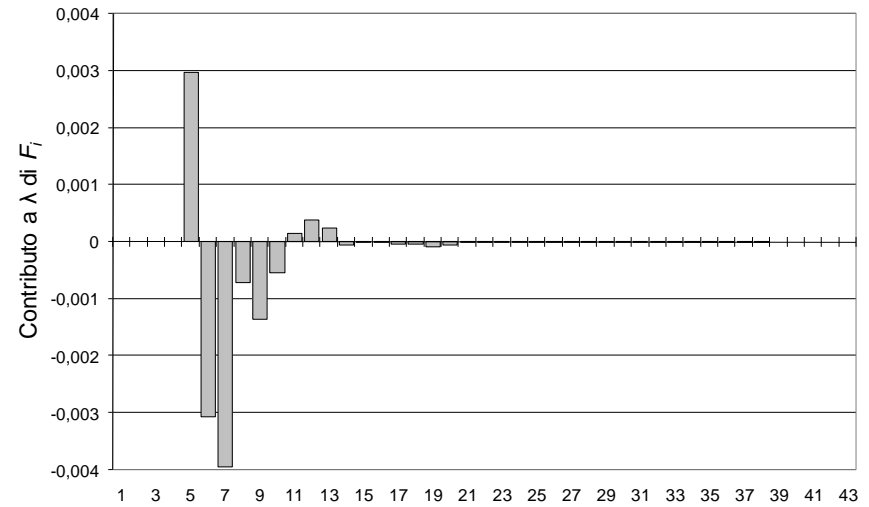
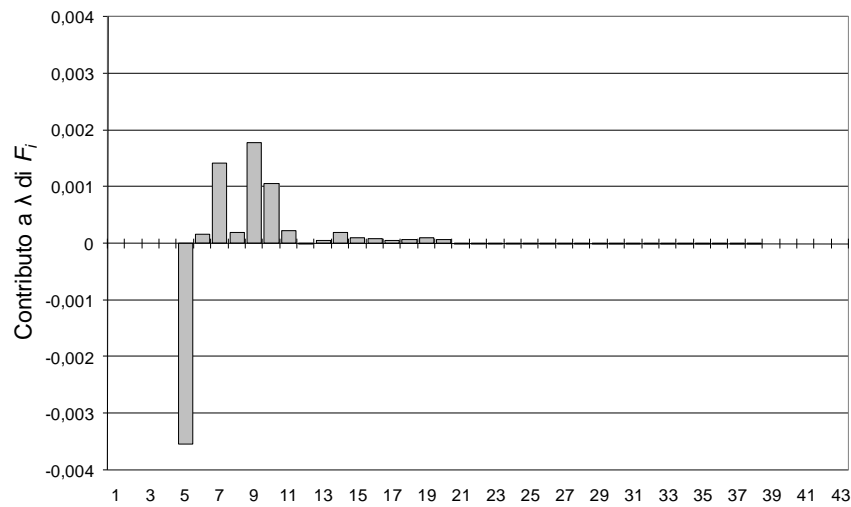
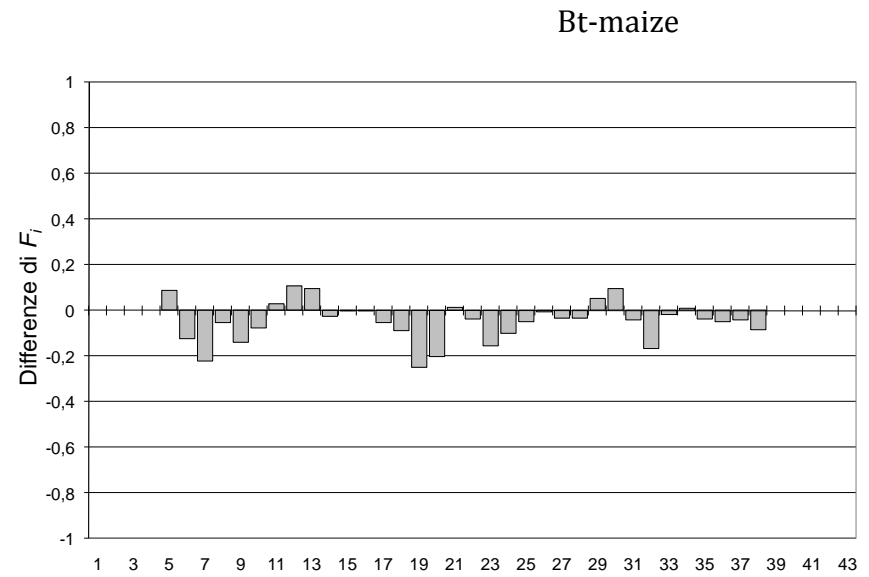
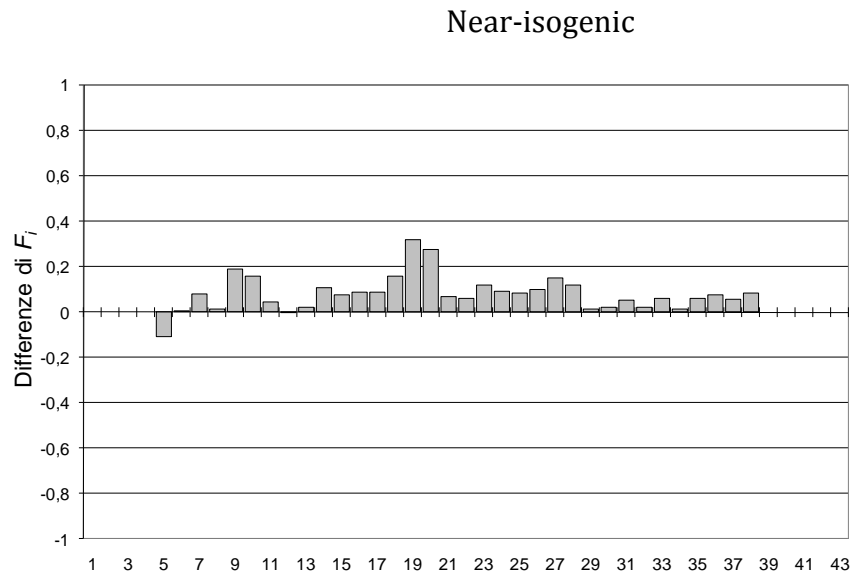


Figure 7. Effects of maize genotypes on age-specific fecundity of *Rhopalosiphum maidis* measured relative to the mean (Top) and contributions of those effects to population growth rate (λ) (Bottom).

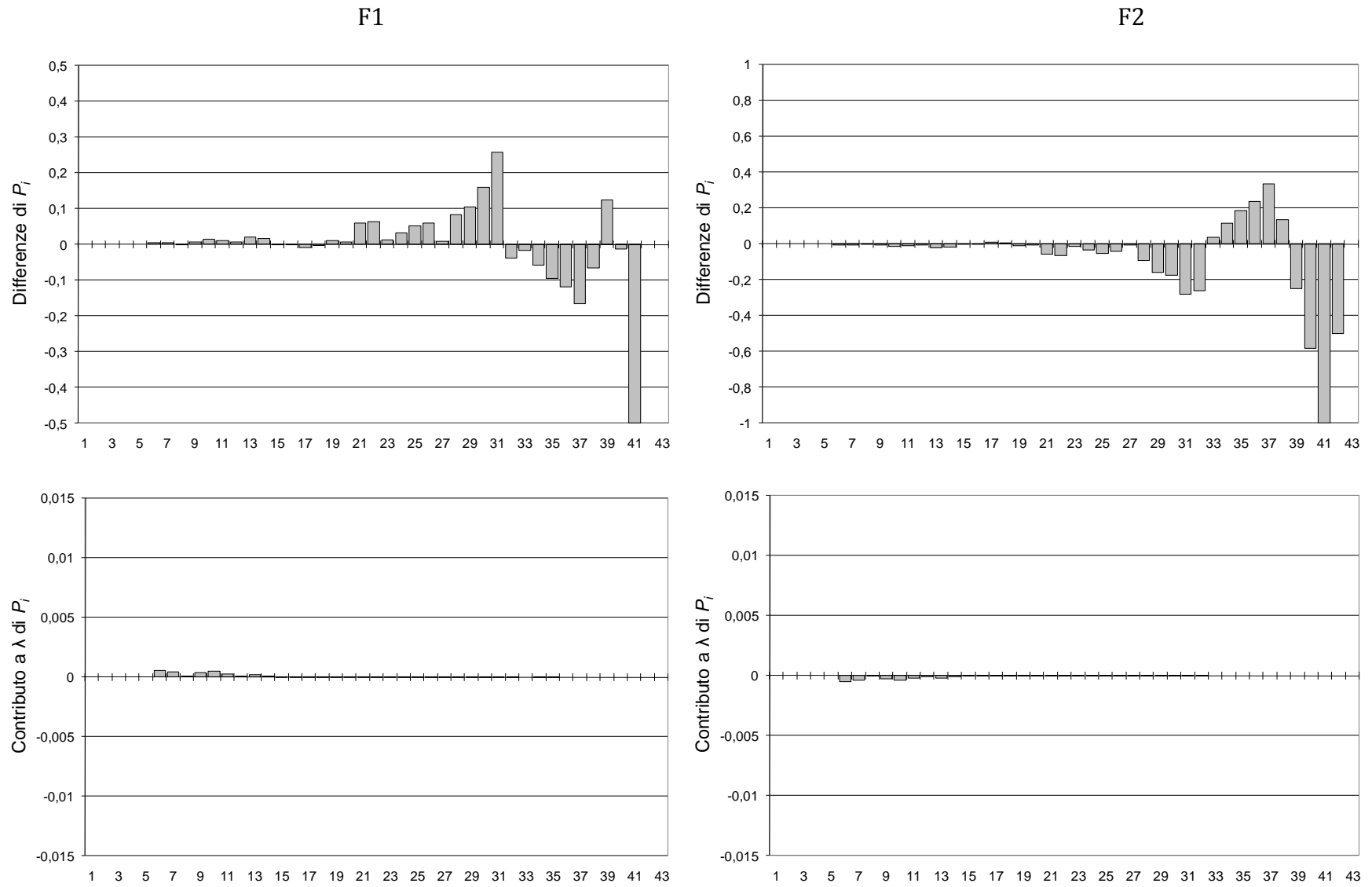


Figure 8. Effects of generation on age-specific survival of *Rhopalosiphum maidis* measured relative to the mean (Top) and contributions of those effects to population growth rate (λ) (Bottom).

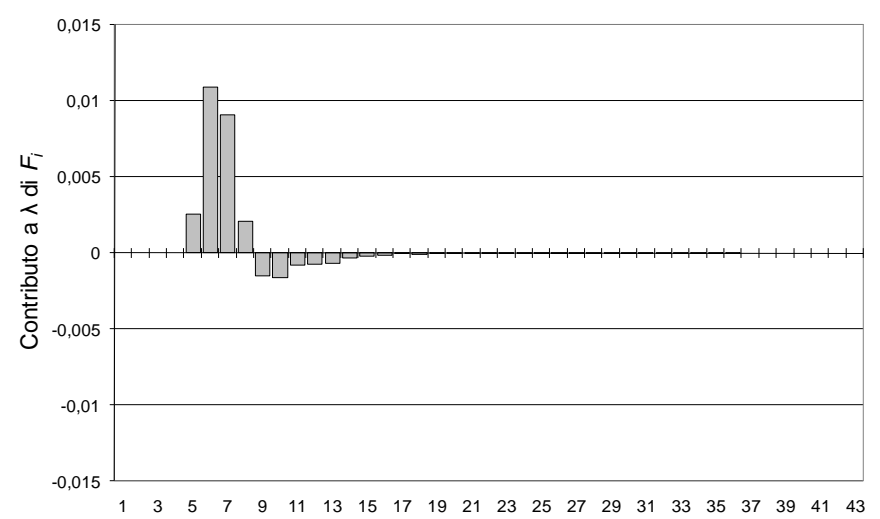
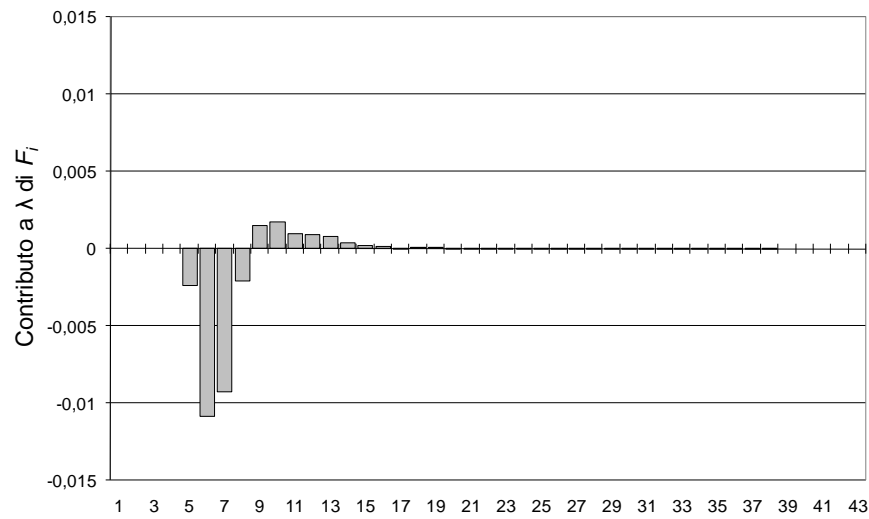
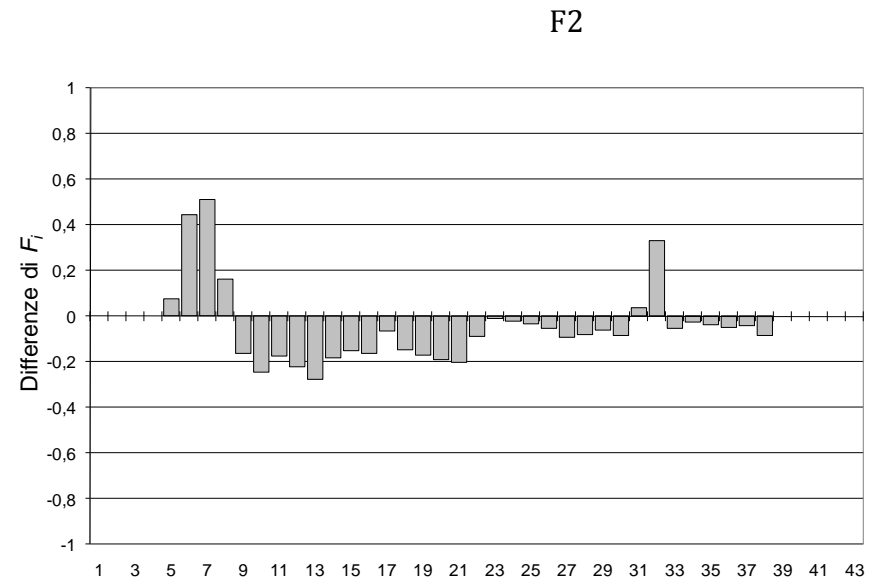
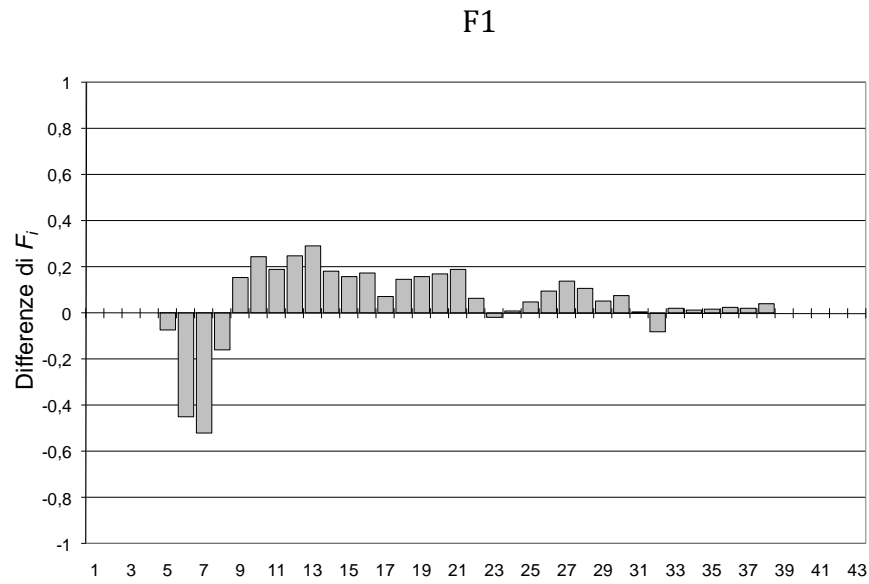


Figure 9. Effects of generation on age-specific fecundity of *Rhopalosiphum maidis* measured relative to the mean (Top) and contributions of those effects to population growth rate (λ) (Bottom).

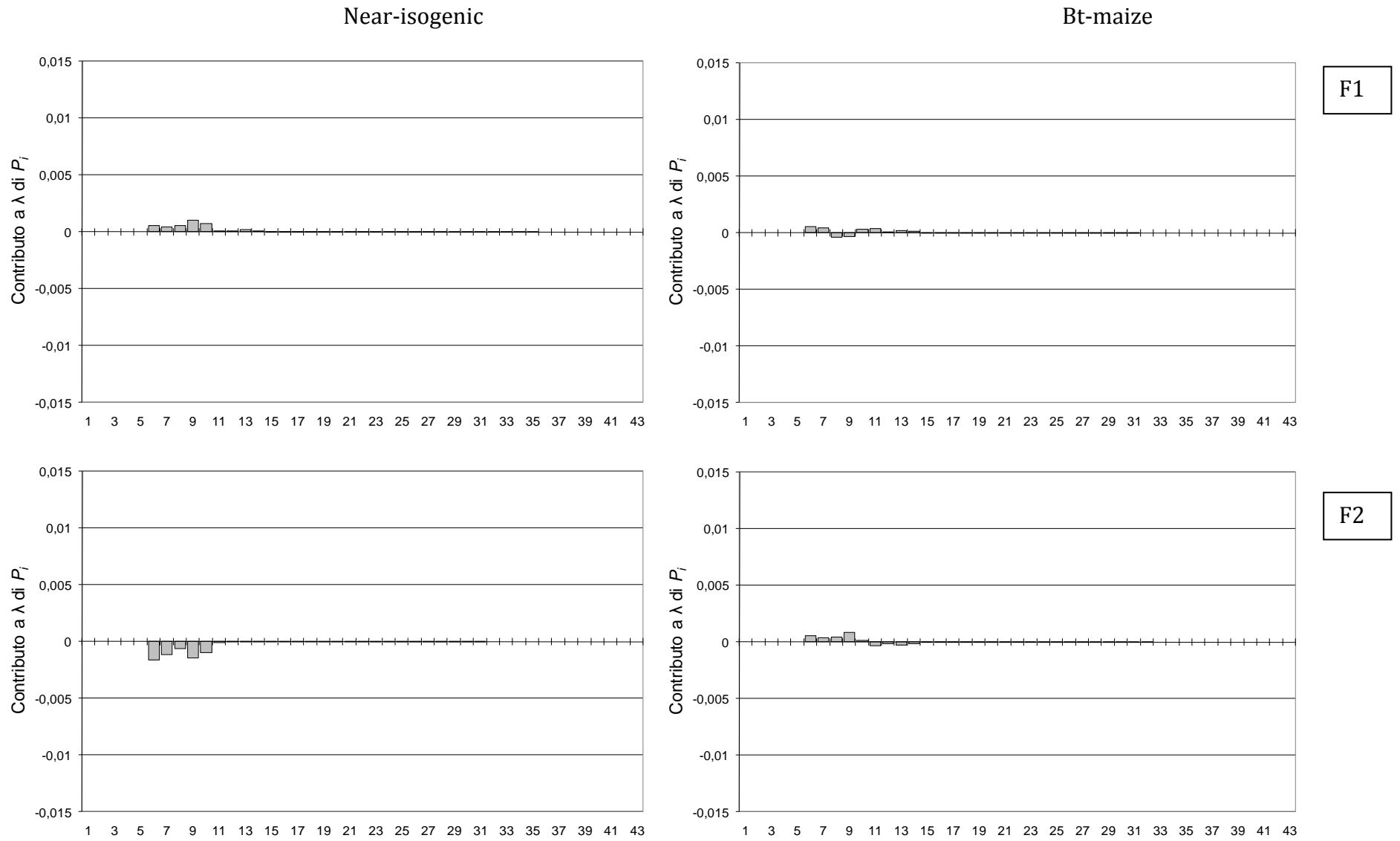


Figure 10. Contributions of age-specific survival to the interaction effect of maize genotypes and generation to *Rhopalosiphum maidis* population growth rate (λ)

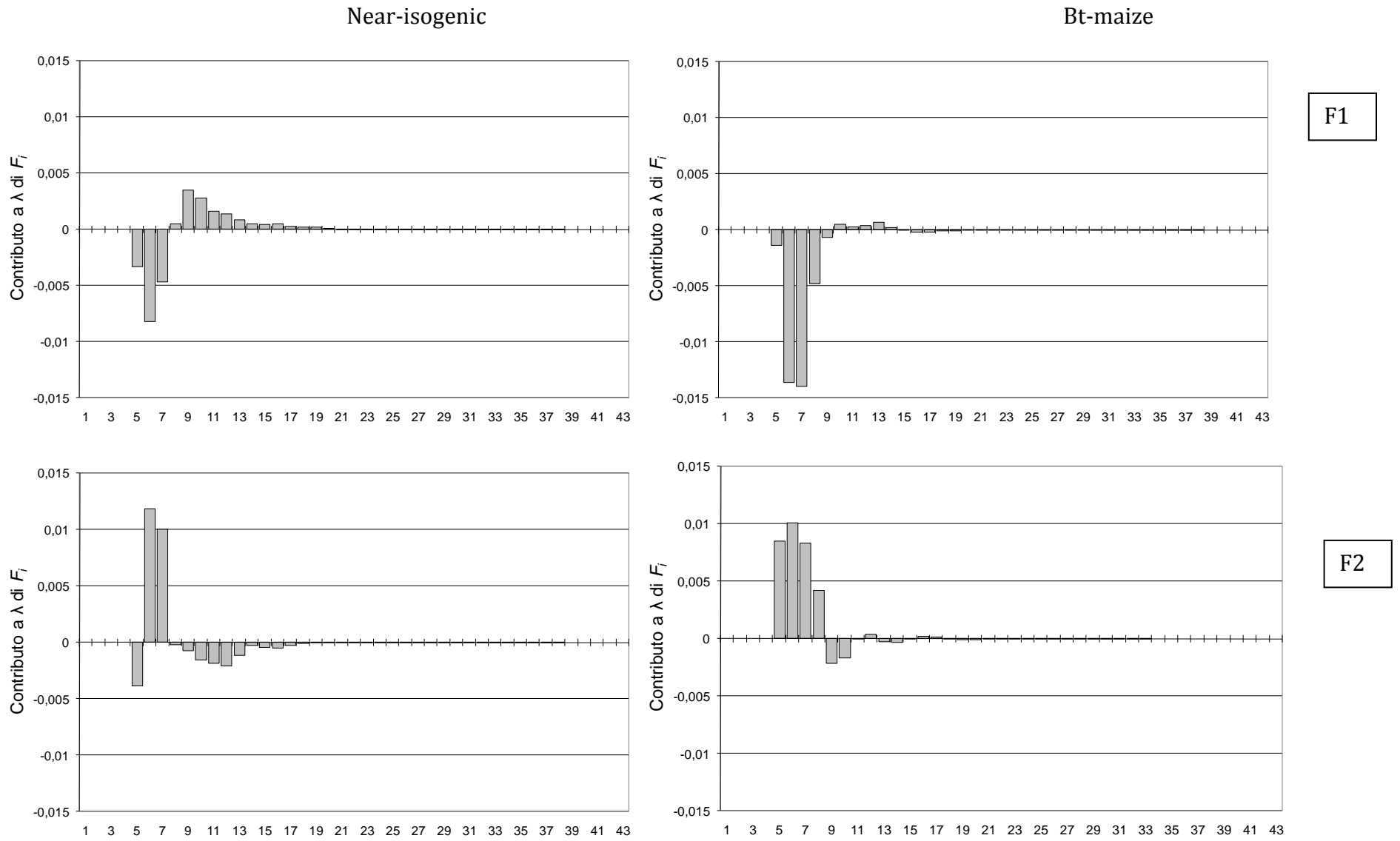


Figure 11. Contributions of age-specific fecundity to the interaction effect of maize genotypes and generation to *Rhopalosiphum maidis* population growth rate (λ).

growth rate on both generations. The contributions of generation to λ are limited to the age classes from 5 to 8 and are probably to be ascribed to significant differences in the pre-reproductive time.

As with the main effects, contribution of survival to the interaction terms $(\alpha\beta)^{(ij)}$ are negligible (Fig. 10). On near-isogenic treatment the contributions of fecundity effects to the interaction terms for the first generation are negative from 5 to 7 days of age and positive at later ages (8-20 days); the pattern of these effects reverse for the second generation keeping λ very similar among the two generations (Fig. 11). For Bt-maize the contributions of fecundity effects to the interaction terms for the first generation are negative, indicating that Bt-maize does worse than would be expected from an additive model. On the second generation, instead, the effect reverse indicating a better effect than would be expected from an additive model (Fig. 11). Thus near-isogenic treatment balance, whereas Bt-maize enhance the fecundity effects among the two generations.

Secondary metabolite analyses

To test if differences in aphid performance were due to differences in secondary metabolites we analyze the content of free and bound phenolics in the leaves of near-isogenic and Bt-maize plants. Phenolic content appeared to differ between near-isogenic and Bt-maize plants as well as among replicates (Fig. 12). Free phenolics were significantly lower in Bt-maize (Fig. 12a) whereas the difference was not significant for the bound phenolics (Fig. 12b). Moreover for free phenolics the interaction between maize genotype and replicate was significant with a lower content of free phenolics in the Bt-maize in replicate 2 and 3 (Fig. 12e).

DISCUSSION

The corn leaf aphid was found to perform better on Bt-maize respect to the correspondent near-isogenic line. This founding appeared however connected to and affected by generations. A previous study on *Aphis gossypii* Glover on Bt and non-Bt cotton plants showed differences in some biological traits, survival rate and fecundity, among three consecutive generations (Liu et al., 2005). In particular we found that the developmental and pre-reproductive times became significantly longer in the second aphid generation on isogenic line, and shorter on Bt-maize. Similar beneficial effects on these parameters due to Bt maize were reported on the offspring of alate of *Rhopalosiphum padi* (Lumbierres et al., 2004). However the opposite occurred for the offspring of apterous aphids. Moreover a reduction of aphid survival and adult and total longevity and an increase of fecundity rate between generations was found in this study independently from maize genotypes.

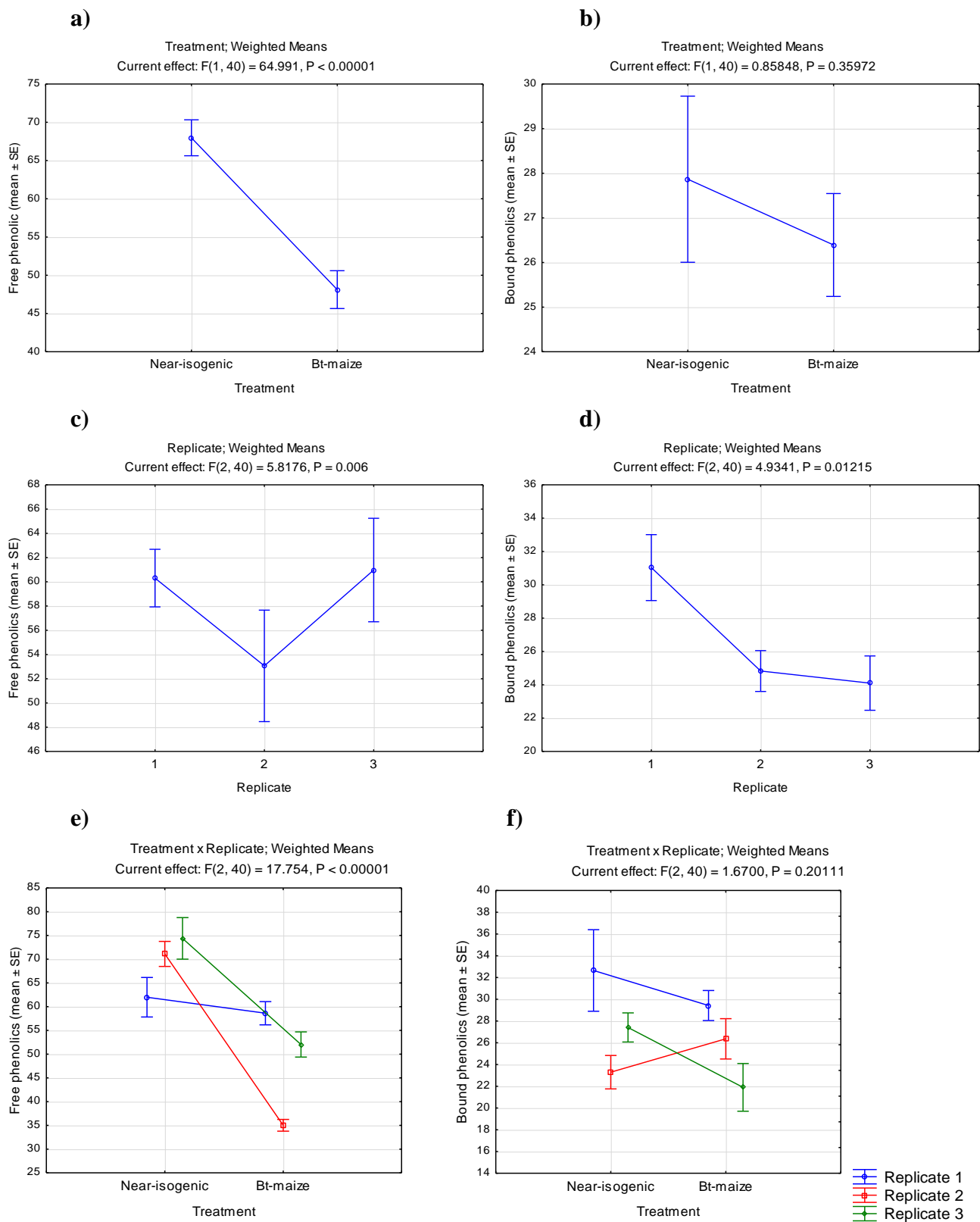


Figure 12. Comparison of free and bound phenolics content expressed as milligrams of gallic acid equivalent (GAE) per 100 g of dry weight among maize genotypes (a, b), replicates (c, d) and their interactions (e, f).

The life table parameters, gross reproductive rate (GRR), net reproductive rate (R_0), mean generation time (T), doubling time (DT), and finite rate of increase (λ) did not differ between near-isogenic and Bt-maize as well as generations. A reduction of life expectancy was found between generations without regard for maize genotypes. On the contrary the aphids of the second generation reared on near-isogenic line suffered a reduction of expected lifetime reproduction respect to that of the first. This was not the case for aphids on Bt-maize. A similar pattern was found in the delay of population growth index. The aphids reared on near-isogenic line exhibited the same population growth with no differences between generations. In contrast on Bt-maize the second generation exhibited an anticipation of 3 days respect to the first to reach 100,000 individuals.

The results of LTRE decomposition show that even if treatments did not significantly influence λ , Bt-maize acted by reducing fecundity of young aphids and by anticipating reproduction by shortening the pre-reproductive time. Bt-maize had substantial effects also on later fecundity and overall survival, but these effects resulted in an irrelevant impact on λ . Younger aphids of the first generation showed reduced fecundity and aphids of the second generation had increased fecundity. Large effects of generations on fecundity of older specimens and on overall survival had negligible influence on λ . The interaction between maize-genotype and generations resulted mediated principally by effects on fecundity of younger aphids. Bt-maize exacerbated the fecundity effects among the two generations instead near-isogenic line balanced it.

Since Cry toxin was not detected in the phloem sap of Bt maize plant and in apterous adults of *R. padi* (Raps et al., 2001; Dutton et al., 2002; 2004), and *R. maidis* (Head et al., 2001) some pleiotropic effects are expected to be the cause of the observed differences in aphid performances. Several factors can affect the capacity of aphids to exploit host plants such as changes in nutritional quality (Faria et al., 2007; Lawo et al., 2009) and secondary plant metabolites (Niemeyer, 1988; Givovich et al., 1994). The latter compounds play a fundamental role in defence against pests and pathogens. Among other the hydroxamic acid DIMBOA and free and cell wall-bound phenolics are common secondary metabolites of gramineous plants and have implications for the resistance of these plants specie against phytophagous pests (Bennett and Wallsgrave, 1994). The comparison of Bt-maize and its corresponding near-isogenic line in this study suggested that the transformation could have induced adverse effects on the biosynthesis and accumulation of free phenolics. In particular this reduction was evident on two out of three replicates the same for which was evident an effect on aphid biological traits chiefly developmental and pre-reproductive times. Indeed for these parameters a significant maize genotype \times generation \times replicate interaction was found. These findings could be an explanation

for the susceptibility to *R. maidis* of the Bt-maize in this study. An analogous reduction in phenolic acids was reported for four transgenic Bt maize compared to their non transgenic counterparts (Nie et al., 2005). A susceptibility to *R. maidis* of six pairs of maize hybrid belonging to three transformation events was also found by Faria et al. (2007) even if a relation with secondary metabolites in their study can be only speculative.

Even if *R. maidis* pest status varies in different parts of the world and usually do not cause economical damage to maize crop when non transgenic hybrid are cultivated, some level of risk could exist when using Bt maize (Stephens et al., 2012). Indeed these plants can be easier exploited by *R. maidis* likely due to lower level of secondary metabolites present in their leaves such as DIMBOA (Nie et al., 2005) and free phenolics (this study), allowing for aphid outbreaks.

**Dietary effects of transgenic Bt-maize pollen expressing Cry1Ab
toxin on fitness of *Hippodamia variegata* Goeze.**

Dietary effects of transgenic Bt-maize pollen expressing Cry1Ab toxin on fitness of *Hippodamia variegata* Goeze.

INTRODUCTION

Predaceous coccinellids play fundamental role in conservation biological control of pest insects. Adverse effect of genetically modified plant expressing Cry1Ab toxin derived from *Bacillus thuringiensis* Berliner on coccinellids may occur as a result of several factors including feeding on plant pollen. Indeed non-prey food represents part of the diet of many predaceous coccinellids that can be exploited when prey is scarce as a supplemental food source (Lundgren, 2009).

The Variegated Lady Beetle, *Hippodamia variegata* Goeze (Coleoptera: Coccinellidae) is a very common and important aphid predator in Mediterranean area in many herbaceous crops including maize (Burgio et al., 2004; 2006; Lami et al., 2016). Both larvae and adult feed preferably on aphids but they can use plant pollen as a supplemental food source (Lundgren, 2009). Consequently *H. variegata* can be exposed to Cry proteins when foraging on insect-resistant Bt-maize. However, since aphids contains no or very low amounts of Cry1Ab toxin when feeding on Bt-maize (Raps et al., 2001; Dutton et al., 2002; 2004; Head et al., 2001), *H. variegata* larvae and adults have little possibility to be exposed to insecticidal proteins by this way. On the other hand, they can feed on pollen, and thus being exposed directly to Cry1Ab in Bt-maize during the anthesis period. Indeed, significant quantity of Cry1Ab toxin was found in different coccinellid species in a quantitative evaluation of Bt toxin within non-target phitophagous insects and higher order arthropods in a field of transgenic Bt maize (Harwood et al., 2005).

In the current study an experimental methodology to provide pollen to the coccinellids and quantify the amount of pollen eaten was developed. Subsequently the effects of the consumption of Bt maize pollen on the performance of *H. variegata* in comparison with its corresponding near-isogenic line, both in the short and long term, were evaluated. Both standardized laboratory bioassays and demographic approach by means of an age-structured matrix population model (Caswell, 2001) were performed. Moreover the concentration of the Cry1Ab toxin in Bt-maize pollen and adults of *H. variegata* was analyzed.

MATERIALS AND METHODS

Plants

A hybrid transgenic maize (Bt-maize), event MON 810 (DKC442YG), and its correspondent non-transformed near isoline (DK440) (near-isogenic) were used in the experiments. Bt-maize plants express a gene encoding for a truncated form of the Cry1Ab toxin from the *B. thuringiensis* var. *kurstaki*, active against the European Corn Borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Plants were grown from seed in 18-litre plastic pots (two plants per pot) with the same soil mixture (60% universal potting soil 40% sand) in a greenhouse (min. T 19 °C, max. T 29 °C; min. r.h. 55%, max. r.h. 85% and 16L:8D). Plants were watered using a timer driven automatic drip watering system.

Insect rearing

Culture of *H. variegata* was established from adult specimens collected at the experimental field station of the Department of Agricultural sciences in Cadriano, Bologna province, Northern Italy (44°32'51"N, 11°24'37"E) in 2012. Adult coccinellids were maintained in Plexiglas cages (40 × 30 × 45 cm) and larvae in plastic cages (33 × 20 × 13 cm). Larvae and adults were fed with an excess of green peach aphids, *Myzus persicae* Sulzer, that had been reared on green pea (*Pisum sativum* L.) sprouts. Both adults and larvae were kept in a climatic chamber at 25±1 °C with a r.h. of 60-80 %, L:D = 16:8. Newly emerged (< 12 h after emergence) *H. variegata* adults were used in the experiments.

Pollen collection and provision to *H. variegata*

Maize pollen was collected by hand-shaking the maize tassel in a paper bag during the anthesis period. Pollen collected from different maize plants of each maize line was pooled together. Great attention was paid to avoid contamination among transgenic and non-transgenic pollen. Collected pollen was then sifted to eliminate anthers and any other impurity poured into 2-ml Eppendorf tubes and stored at -80 °C prior to use in the study. The concentration of Cry1Ab toxin in pollen was measured by ELISA. With the aim of providing pollen to *H. variegata*, the pollen was glued on tissue paper using a mixture of water and honey (50% water 50% honey in volume). On a piece of tissue paper (3 × 3 cm) a square of 5 × 5 mm was wetted with the water/honey mixture and immediately weighted using an electronic balance. Then the pollen was poured over the tissue paper. The excess of pollen was removed by shaking the tissue paper to obtain a single layer of pollen grains of 5 × 5 mm of surface. The layer of pollen was examined under a stereomicroscope and squares that were not composed by a single layer

were not used in the study. The tissue paper were then weighted again to quantify the amount of pollen in the square by difference.

Performance of *H. variegata* feeding on maize pollen

The sex of newly emerged *H. variegata* adults was determined and individuals were randomly paired. Sixteen pairs for each of the two pollen treatment (Bt-maize and near-isogenic) were isolated. Each pair was then kept in a cylindrical cage ($\text{Ø} = 5.5$ cm, $h = 7$ cm) that was covered with a screened lid. Each cage was lined on the inside with an air bubble plastic film to operate as oviposition substrate. Containers were daily replaced. Since *H. variegata* could not survive and oviposit solely on maize pollen (Lundgren, 2009) the adults received a diet treatment as follow: i) feeding *ad libitum* with *M. persicae* for the first four days after emergence, then ii) two days of maize pollen and two days of *M. persicae* alternating until the experiment ending. In each pollen supply a single 5×5 mm square was provided to each pair. Aphids were replaced every days instead the pollen square remained for both days. To increase the probability that *H. variegata* pairs would feed on pollen aphids were not added during pollen supply. After 21 days the experiment was stopped. This time allowed, at most, four two-day supplies of pollen for each pair. No pair depleted the pollen supply in none of the two-day periods. The suitability of this diet treatment to sustain survival and oviposition of *H. variegata* adults was assessed in a preliminary bioassay.

Every day the number of eggs laid by each female, including the cannibalised ones, was recorded. All the eggs laid by each female were collected and every day the number of larvae emerged was checked, in order to evaluate fertility. To prevent cannibalism, the eggs were provided with *E. kuehniella* frozen eggs before hatching. The pre-oviposition and oviposition periods and the survival of each female was also recorded based on daily observations. Any deceased males during the experimental period were replaced. Females that died and those which remained until the experiment was stopped, were stored at -80 °C. The concentration of Cry1Ab toxin in the female bodies was then measured by ELISA.

A sample of 49 newly emerged larvae (L1) was collected for each pollen treatment. Larvae were individually reared on *M. persicae* till adult emergence, with the aim of evaluating larval mortality, development time, sex ratio ($\frac{\text{♀}}{(\text{♂}+\text{♀})}$) and adult weight of the offspring generated by the pollen fed females. Trials were conducted under controlled conditions in a climatic chamber (25 ± 1 °C, 60-80% r.h., L:D = 16:8).

Demographic analyses

In each treatment age-specific survivorship (l_x) was estimated from data on immature development and survival and daily schedules of adult mortality whereas age-specific fecundity (m_x) was derived from the number of eggs laid by each female. The life table parameters, including gross reproductive rate (GRR), net reproductive rate (R_0), mean generation time (T), doubling time (DT), and finite rate of increase (λ) were calculated as follow (Carey 1993):

$$GRR = \sum_{x=0}^{\overline{w}} m_x \quad (1)$$

$$R_0 = \sum_{x=0}^{\infty} l_x m_x \quad (2)$$

$$T = \frac{\sum_{x=0}^{\overline{w}} x l_x m_x}{\sum_{x=0}^{\overline{w}} l_x m_x} \quad (3)$$

$$DT = \frac{\log 2}{r_m} \quad (4)$$

$$\lambda = e^{r_m} \quad (5)$$

The precise value of intrinsic rate of increase (r_m) was estimated iteratively solving the Euler equation:

$$\sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1 \quad (6)$$

Jackknife re-sampling technique was used to calculate the mean and standard error associated with population parameters (Maia et al., 2000).

Age-specific survivorship (l_x) and fecundity (m_x) schedules were also used to construct a $z \times z$ age-classified population projection matrices \mathbf{A} (standard Leslie matrix) with a projection interval of one day.

$$\mathbf{A} = \begin{pmatrix} 0 & F_2 & F_3 & \dots & F_z \\ P_1 & 0 & 0 & \dots & 0 \\ 0 & P_2 & 0 & \dots & 0 \\ \vdots & \dots & \dots & \dots & \vdots \\ 0 & 0 & 0 & P_{z-1} & G_z \end{pmatrix} \quad (7)$$

The only nonzero entries of the matrix, survival probability (P_i) appearing on the subdiagonal and fecundity (F_i) appearing in the first row, were calculated by means of the birth-flow formulas (Caswell, 2001) as follow:

$$P_i \approx \frac{l(i) + l(i+1)}{l(i-1) + l(i)} \quad (8)$$

$$F_i = \frac{l(0) + l(1)}{2} \left(\frac{m_i + P_i m_{i+1}}{2} \right) \quad (9)$$

where $l(i)$ is the survivorship from birth to age i and m_i is the mean number of female offspring per female in age class i . Since adults performance were observed for no more than 21 days and *H. variegata* can live longer and keep on reproducing (Lanzoni et al., 2004) the term G_z was added to the diagonal of the matrix. In each treatment the most realistic value of G_z was estimate from the characteristic equation of \mathbf{A} (Caswell, 2001).

The asymptotic population growth rate (λ) was calculated as the dominant eigenvalue of the matrix.

The discrete equivalent of the other stable population parameters (R_0 , r_m , T) were also calculated from the age-classified matrix as follow (Caswell, 2001):

$$R_0 = \sum F_i \prod_{j=1}^{i-1} P_j \quad (10)$$

$$r_m = \log \lambda_1 \quad (11)$$

$$T = \frac{\sum_i i (\prod_{j=1}^{i-1} P_j) F_i}{\sum_i (\prod_{j=1}^{i-1} P_j) F_i} \quad (12)$$

The life expectancy (e_x), i.e. the mean age at death, and the expected life time reproduction, i.e. the expected number of reproductive events during a female's lifetime, have been calculated treating the transition matrix as an absorbing Markov chain with death as absorbing state (Caswell, 2001).

We determined how much the differences in population growth rates (λ) between near-isogenic line and Bt-maize groups were determined by the differences in each vital rate by means of a life table response experiment (LTRE) analysis (Caswell, 2001; Hamda et al., 2012). With this technique, the effects of Bt-maize pollen on λ , measured respect to the near-isogenic line, was decomposed into contributions from each age-specific fecundity and survival probability terms as follow:

$$\lambda^{(Bt)} \approx \lambda^{(Iso)} + \sum_{ij} (a_{ij}^{(Bt)} - a_{ij}^{(Iso)}) \frac{\partial \lambda}{\partial a_{ij}} \Big|_{\frac{1}{2}(\mathbf{A}^{(Bt)} + \mathbf{A}^{(Iso)})} \quad (13)$$

In eq. 13 $\lambda^{(Bt)}$ and $\lambda^{(Iso)}$ denote the values of λ for Bt-maize and near-isogenic groups, respectively, and each term in the summation represents the contribution of the difference in the matrix element a_{ij} (age-specific fecundity or survival probability) to the global effect of eating Bt-maize pollen on population growth rate. That contribution is the product of the difference between Bt-maize and near-isogenic vital

rates and the sensitivity of that vital rate calculated from a matrix halfway between the Bt-maize matrix $\mathbf{A}^{(Bt)}$ and the near-isogenic line matrix $\mathbf{A}^{(Iso)}$ being compared (Logofet and Lesnaya, 1997).

Finally, an application of matrix models, the Delay in Population Growth Index (Wennergren and Stark, 2000; Stark et al., 2004; Stark et al., 2007a;b), a measure of population recovery, was calculated to compare the time required to a population supplied with near-isogenic line pollen and a population exposed to Bt-maize pollen to reach a predetermined number of individuals. In this study the population delay was determined by choosing a population size of 100,000 individuals (Stark et al., 2004). In each treatment, population growth was simulated by multiplying the matrix \mathbf{A} by an initial population vector $\mathbf{n}(t) = [n_1 \ n_2 \ n_3 \ \dots \ n_z]^T$, containing information on the age distribution of the population. The population projection was started with a vector consisting of 100 individuals distributed according to the stable age distribution and ended when at least 100,000 individuals had been reached. In each treatment, the stable age distribution (\mathbf{w}) was calculated as the corresponding right eigenvector of the dominant eigenvalue of the matrix \mathbf{A} (Caswell, 2001).

All demographic calculations were performed with the software PopTools version 2.7.5 (Hood, 2006).

ELISA measurements

The concentration of Cry1Ab toxin in pollen and *H. variegata* females was measured using a commercially available DAS-ELISA kit (QuantiPlate kit Cry1Ab/Cry1Ac, EnviroLogix Inc. Portland, Maine, USA). Before analyses, coccinellids were washed with the extracting buffer provided with the kit in order to remove any pollen grains that could be present on their surface.

Insect specimens and Bt-maize pollen, were weighted and homogenised in an adequate quantity of extraction buffer to obtain 1:10 and 1:25 dilution (mg sample: μl buffer) respectively. The homogenized samples were centrifuged for 5 min at 13000 rpm and analysed as reported in the manufacturer's instructions. Spectrophotometric measurements were conducted with a microtiter plate reader (Labsystem Multiscan, Dasit, Italy) at 650 nm. *Bt*-toxin concentrations were expressed in μg Cry1Ab g^{-1} of fresh weight.

Pollen consumption measurements

To calculate the weight of pollen eaten by each pair high resolution pictures of each of the 5×5 mm pollen squares were taken before and after being offered to the coccinellids. The two images are compared to evaluate the percent of pollen surface in the square that has been eaten using a box counting technique. By using fractal theory this technique allows the fractal dimension of the pollen

square surface represented in the pictures to be obtained along with the fraction (%) of surface that has been eaten (Posadas et al., 2003). This analyses were performed using the program `PSP_boxCounting` written in Python (Bittelli et al., 2015). For each 5× 5 mm pollen square, once the percent of pollen surface eaten has been obtained, it was multiplied by the weight of pollen to calculate the mg of pollen eaten. The amount of pollen eaten by each pair resulted by the summation of the mg of pollen consumed in each of the squares provided to the pair. Based on the quantity of pollen eaten and the mean concentration of Cry1Ab toxin in pollen, the amount of Cry1Ab toxin ingested by each pair in the Bt-maize treatment was calculated.

Statistical analysis

Data on *H. variegata* biological traits, life table parameters and pollen consumption were analyzed using one-way ANOVA. Data in percentage were arcsine transformed before analysis. Chi-square test was carried out to compare sex-ratio. Statistical analyses were performed using STATISTICA software version 10 (StatSoft Inc.).

RESULTS

The ELISA measurements showed that the concentration of Cry1Ab toxin (mean ± SE) in maize pollen was 0.014 ± 0.002 µg/g fresh weight (n = 7). No Cry1Ab toxin was found in any sample of the near-isogenic line pollen. After 21 days, when the experiment was stopped, the calculated amount of pollen that was consumed by the pairs did not differed significantly between treatments ($F_{1, 30} = 0.017$, $P = 0.897$) (Fig. 1).

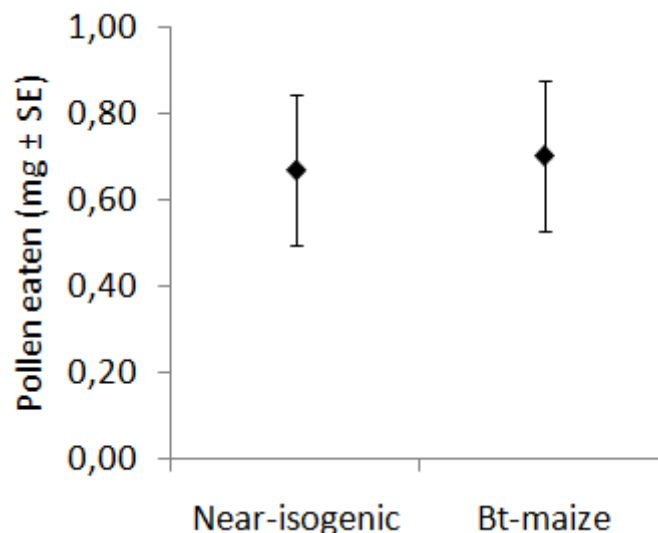


Figure 1. Consumption (mean ± SE) of maize pollen by pairs of *Hippodamia variegata*.

According to our measurements the mean (\pm SE) weight of Cry1Ab toxin eaten by *H. variegata* pairs in the Bt-maize treatment was 0.01 ± 0.003 ng. Notwithstanding, no Cry protein was detected in adults fed with Bt-maize pollen.

When the experiment was stopped, 37.5% of *H. variegata* females survived in the Bt-maize pollen treatment (Table 1). Percent survival and adult longevity did not differ between the Bt-maize and near-isogenic treatments. Similarly, the other biological parameter studied, fecundity, oviposition rate, preoviposition and oviposition periods, fertility, and the percent of ovipositing females were not affected by the Bt pollen treatment (Table 1).

Table 1. Biological traits (mean \pm SE) of females of *H. variegata* feeding on near-isogenic and Bt-maize pollen.

Parameters	Near-isogenic	Bt-Maize	Statistics
Fecundity (total eggs/female \pm SE) ^a	81.8 \pm 18.6	77.4 \pm 17.3	F _(1, 30) = 0.029 P = 0.867
Oviposition rate (eggs/female/day \pm SE) ^a	5.5 \pm 1.1	4.7 \pm 0.9	F _(1, 30) = 0.262 P = 0.613
Preoviposition period (days \pm SE) ^a	2.1 \pm 0.4	3.1 \pm 1.0	F _(1, 26) = 0.854 P = 0.364
Oviposition period (days \pm SE) ^a	5.4 \pm 0.7	5.4 \pm 0.8	F _(1, 26) = 0.005 P = 0.947
Female survival (%) ^b	25.0	37.5	$\chi^2_{(1)}$ = 0.580 P = 0.446
Adult longevity (days \pm SE) ^a	13.8 \pm 1.2	14.4 \pm 1.4	F _(1, 30) = 0.095 P = 0.760
Fertility (% hatched eggs/female \pm SE) ^a	61.0 \pm 6.8	46.3 \pm 6.7	F _(1, 26) = 1.928 P = 0.177
Ovipositing female % (O/T)	87.5 (14/16)	87.5 (14/16)	

^a ANOVA

^b Chi-square test

No significant differences were detected between the two treatments for development time, immature survival (larvae to adult) and sex ratio of the offspring generated by the *H. variegata* pollen fed females (Table 2). Likewise no significant difference was found for adult fresh weight. However the male in the Bt pollen treatment had a significant higher fresh weight than those in the non-Bt pollen treatment (Table 2).

All the demographic parameters analyzed showed no significant differences between the Bt-maize and near-isogenic treatments (Table 3). The life expectancy (e_x) represent the mean lifespan that an individual aged x is expected to live if the conditions whose is exposed were maintained (Fig. 2a). At birth and during immature stages the life expectancy of *H. variegata* in the Bt pollen treatment was lower by more than 20% respect to that in the non-Bt pollen treatment. This difference cannot be

observed for coccinellids older than 11 days. On the contrary the expected number of reproductive event during a female's lifetime was higher in the Bt pollen treatment (Fig. 2b).

Table 2. Biological traits (mean \pm SE) relative to the offspring of females of *H. variegata* fed with near-isogenic and Bt-maize pollen.

Parameters	Near-isogenic	Bt-Maize	Statistics
Development time (Days \pm SE) ^a	15.0 \pm 0.3	14.3 \pm 0.4	F _(1, 27) = 1.890 P = 0.181
Survival (larvae to adult) (%) ^a	33.2 \pm 5.2	24.4 \pm 10.9	F _(1, 6) = 0.832 P = 0.397
Sex ratio (% of female) ^b	35.3	50.0	$\chi^2_{(1)}$ = 0.630 P = 0.428
Adult weight (mg \pm SE) ^a	7.0 \pm 0.4	8.0 \pm 0.4	F _(1, 27) = 2.584 P = 0.120
Male weight (mg \pm SE) ^a	6.2 \pm 0.3	7.2 \pm 0.2	F _(1, 15) = 4.755 P = 0.046
Female weight (mg \pm SE) ^a	8.5 \pm 0.7	8.8 \pm 0.6	F _(1, 15) = 0.090 P = 0.770

^a ANOVA

^b Chi-square test

The number of eggs, larvae, pupae, and adults obtained using population projection matrix is plotted in Fig. 3. The population projection relative to the Bt-maize pollen consumption results in a lower increase of *H. variegata* population. The value of delay in population growth index showed that consuming Bt pollen delayed population growth of *H. variegata* of 28 days respect to eating non-Bt pollen (Fig. 4). This delay corresponds to 1.2 times the value of generation time (T) calculated for *H. variegata* (see Table 3).

Using LTRE decomposition the effect of Bt pollen exposition on λ have been decomposed into contributions arising from the effect on each age-specific vital rate. Even if statistically significant difference between λ was not found, demographically important effect of Bt pollen exposition on *H. variegata* can be highlighted (Fig. 5). The Bt-pollen-fed females showed a fecundity advantage especially from ages 29-31, instead a survival disadvantage occurred early in life (ages 2-3, corresponding to egg stage, and 9 to 11), on their offspring (Fig. 5). Summing the fecundity contributions, which total 0.0058 and the survival contributions which total - 0.0293 show that, since fecundity differences contributed less to λ than survival differences, reduction of survival on offspring seem only partially outweighed by increased female fecundity.

Table 3. Life-table parameters (mean \pm SE) of females of *H. variegata* feeding on near-isogenic and Bt-maize pollen.

Parameters	Gross reproductive rate	Net reproductive rate		Mean generation time		Doubling time	Intrinsic rate of increase		Finite rate of increase	
	(GRR) (♀♀ offspring)	(R_0) (♀♀ offspring)		(T) (days)		(DT) (days)	(r_m) (days^{-1})		(λ) (days^{-1})	
	LT ^a	LT	LM	LT	LM	LT	LT	LM	LT	LM
Near-isogenic	54.28 \pm 13.21	6.10 \pm 1.39	6.14	23.40 \pm 1.78	22.80	8.12 \pm 1.11	0.0839 \pm 0.0107	0.0852	1.0875 \pm 0.0116	1.0889
Bt-Maize	71.05 \pm 8.84	4.39 \pm 0.98	4.43	25.35 \pm 1.22	24.79	10.82 \pm 1.77	0.0625 \pm 0.0094	0.0633	1.0644 \pm 0.0100	1.0653
Statistics	$F_{(1, 30)} = 1.113$	$F_{(1, 30)} = 1.015$		$F_{(1, 30)} = 0.809$		$F_{(1, 30)} = 1.680$	$F_{(1, 30)} = 2.269$		$F_{(1, 30)} = 1.270$	
	P = 0.300	P = 0.322		P = 0.376		P = 0.205	P = 0.142		P = 0.143	

^a LT = Life-table, LM = Leslie matrix.

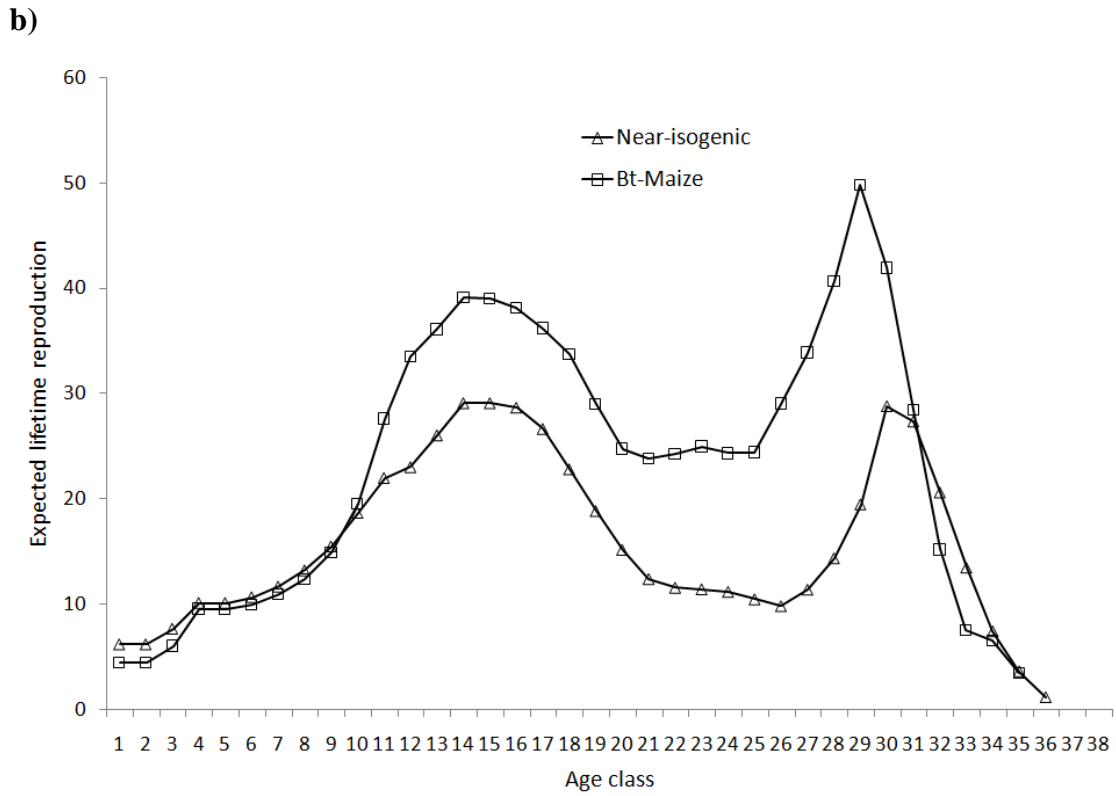


Figure 2. Life expectancy (a) and mean number of lifetime reproductive episodes (b) of Bt-maize pollen-fed and near-isogenic pollen-fed females of *H. variegata*.

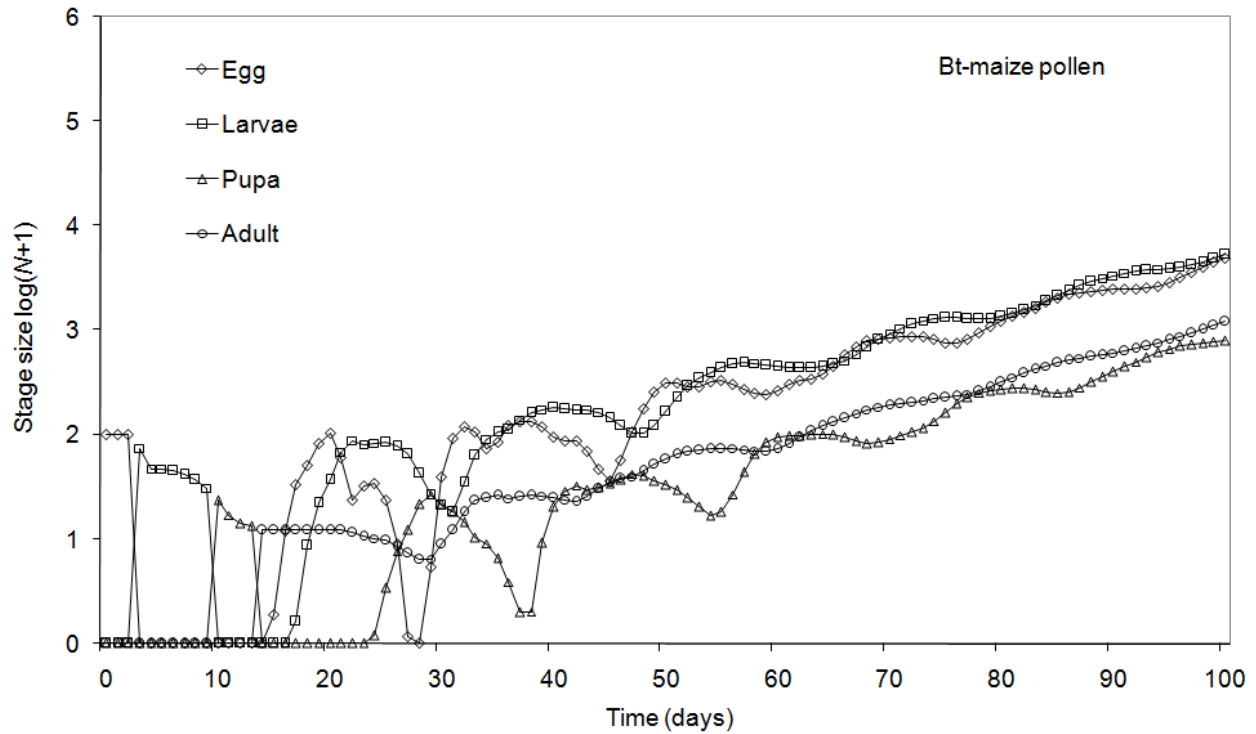
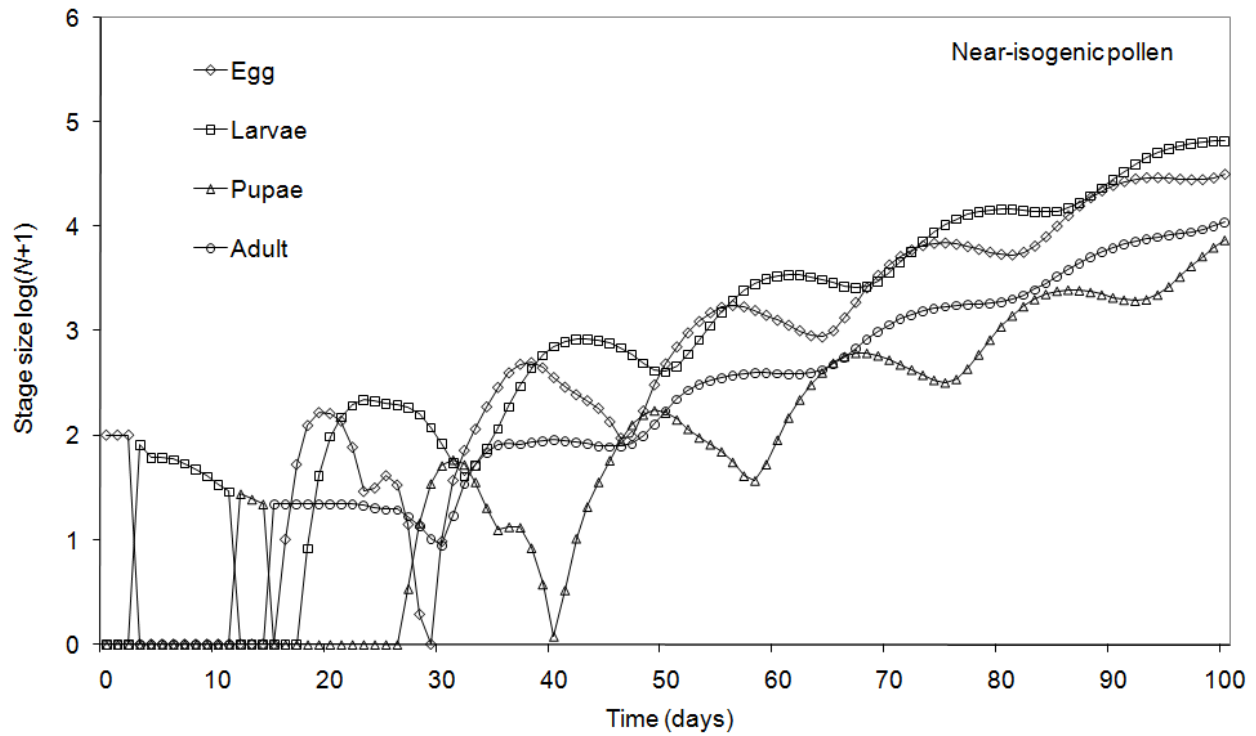


Figure 3. Population projection of *Hippodamia variegata* starting from an initial population of 100 eggs. Stage size (egg, larval, pupal and adult) was obtained summing the number of specimens in the corresponding ages.

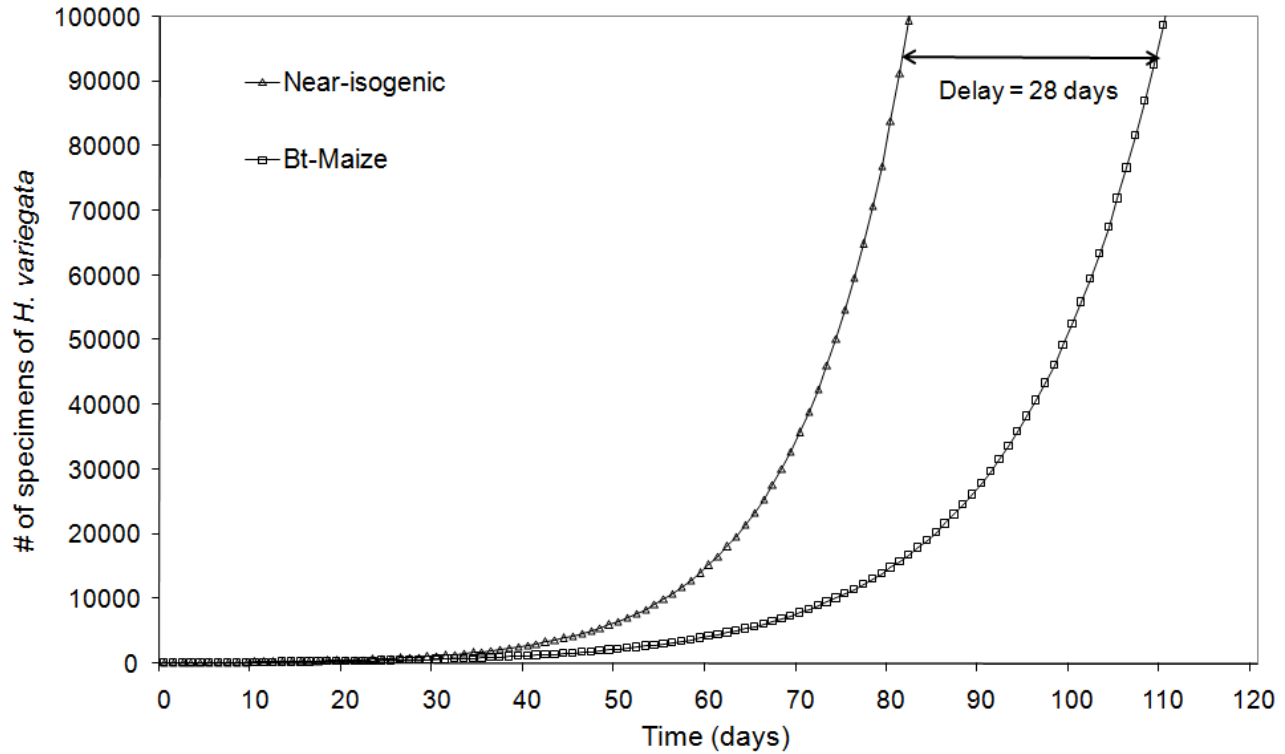


Figure 4. Matrix model projections and comparison of the delay in population growth to reach 100,000 specimens for Bt-maize pollen-fed and near-isogenic pollen-fed females of *H. variegata*. Simulation started with 100 specimens at a stable age distribution.

Discussion

Hippodamia variegata larvae and adult are probably exposed to very low level of insecticidal proteins in Bt maize expressing Cry1Ab toxin when feeding on aphids in this crop. Indeed aphids ingest no or very little amount of Cry1Ab protein in transgenic maize (Raps et al., 2001). However coccinellids can also feed various pollens (Lundgren, 2009) and then, by this way, being directly exposed to Cry toxin on Bt maize crops.

The methodology proposed in this study provides a sound exposure system to supply pollen to the coccinellids and appeared functional to quantify maize pollen consumption by *H. variegata* adults. The methodology allowed assessing that the *H. variegata* pairs consumed the same amount of pollen in both treatments during the 21-day experiment.

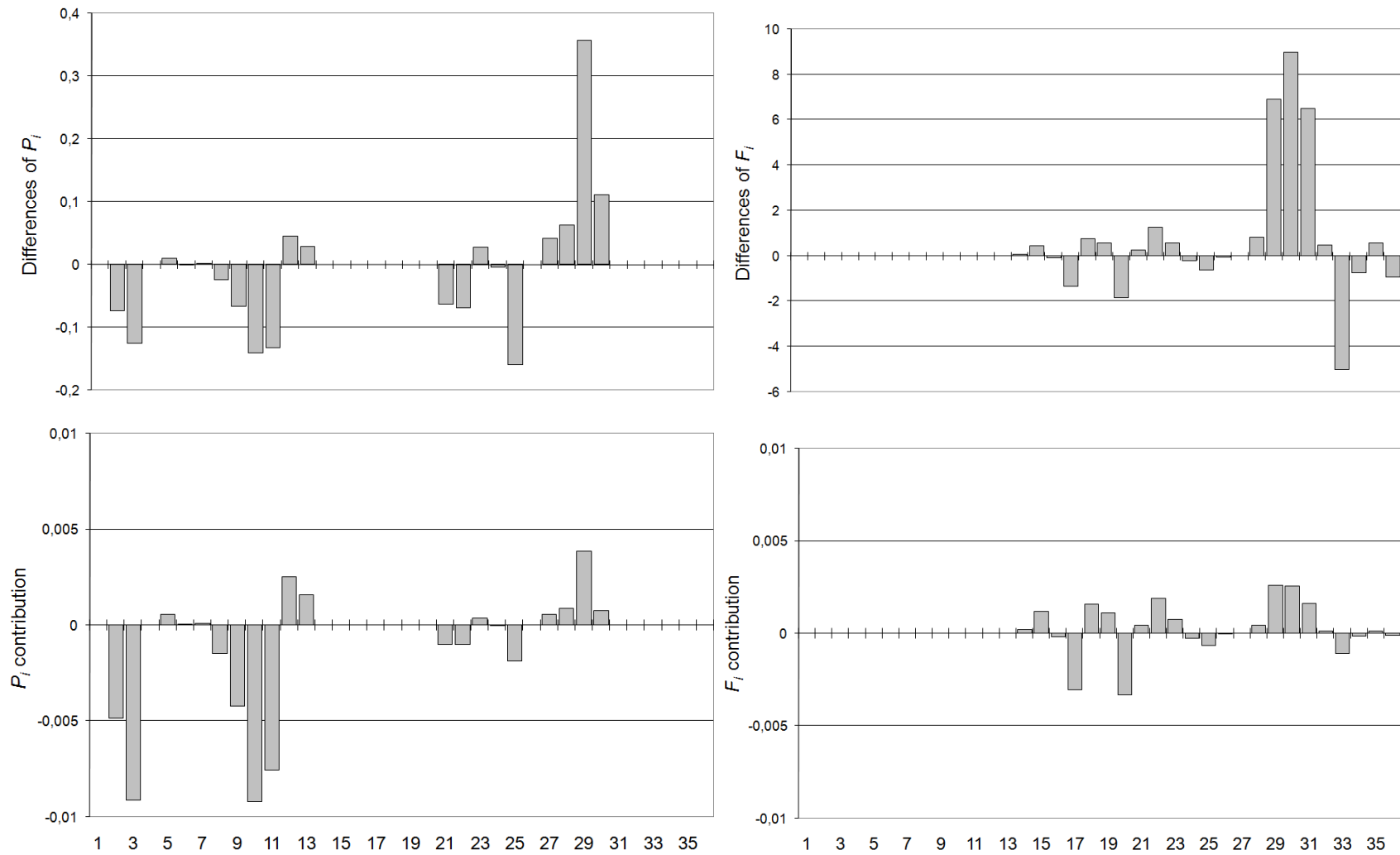


Figure 5. LTRE decomposition analysis of treatment effects using equation 13. (Top) Differences in age-specific survival (P_i) and fecundity (F_i) between Bt-maize pollen-fed and near-isogenic pollen-fed females of *H. variegata*. (Bottom) Contributions of those differences on population growth rate (λ). Negative values indicate disadvantages or negative contributions to λ of Bt-maize relative to near isogenic line.

Bioassay showed no adverse effects on the performance of female of *H. variegata* and on biological traits of their offspring after ingestion of Bt-maize pollen in comparison with pollen from the corresponding near-isogenic line. The sole exception was a higher weight on the adult male offspring in the Bt-maize treatment. The results were consistent with a previous study where adult *Propylea japonica* (Thunberg) (Coleoptera: Coccinellidae) suffered no evident negative effects eating rice pollen expressing Cry1Ab toxin (Bai et al., 2005). A similar scenario was also reported for *P. japonica* larvae (Bai et al., 2005; Zhang et al., 2014) and larvae and adults of *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae), the latter fed with a diet containing 50% in weight of maize pollen expressing Cry3Bb1 toxin (event MON 863) active against *Diabrotica* spp. (Coleoptera: Chrysomelidae) (Duan et al., 2002).

In our study the concentration of Cry1Ab toxin in pollen resulted very low, $\ll 100$ times lower than that reported for MON810 maize by Schmidt et al. (2009), but in line with Sears et al. (2001) and Hellmich et al (2001) that reported only Cry1Ab traces in MON810 maize pollen. The low level of Bt toxin in pollen could explain why no Cry1Ab protein was found in *H. variegata* adults after the 21-days bioassay. Realistically the concentration of toxin in adults could be inferior to the lower limit of quantification for the ELISA procedures.

Even if the exposure level reported in this study could not be sufficient to reduce *H. variegata* fitness in the short-term an extended exposure period have to be considered since long-term exposure to insecticidal Bt toxins can occur in the field (Harwood et al., 2005).

Our result revealed no detrimental effects on the life table parameters, gross reproductive rate (GRR), net reproductive rate (R_0), mean generation time (T), doubling time (DT), and finite rate of increase (λ) of *H. variegata* when fed on Bt-maize pollen. On the contrary, the expected life time reproduction of female of *H. variegata* fed with Bt pollen resulted enhanced, and the life expectancy of their offspring reduced. The tradeoff between these two parameters resulted in a reduced population growth in the Bt-maize treatment. This reduction was quantified by the delay in population growth index in 28 days to reach 100,000 individuals. Allowing 28 days for *H. variegata* to recover would give *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae), its aphid prey on maize, enough time to complete ≈ 2 generations, based on the value of generation time of the aphid (see chapter 1).

The results of LTRE decomposition show that even if Bt pollen exposition did not significantly influenced λ , it acted primarily by reducing fertility and survival of the offspring and only secondarily by elevated fecundity late in life. Negative survival contributions in the offspring were only partially outweighed by positive fecundity.

Overall this study show that the consumption of Bt-maize pollen expressing Cry1Ab by adults of *H. variegata* does not affect seriously their fitness, but it shows also that this outcome is the results of a tradeoff among vital rate such as age-specific fecundity or survival probability that resulted positively or negatively influenced. The result evidenced by the demographic analyses demonstrate that this experimental approach can provide the most complete representation of the population-level consequences of individual responses to stressors in the long-term.

**A semi-field approach for modelling *Hippodamia variegata*
Goeze exposition to Cry1Ab toxin at population level in a
tritrophic contest.**

A semi-field approach for modelling *Hippodamia variegata* Goeze exposition to Cry1Ab toxin at population level in a tritrophic contest.

INTRODUCTION

Most of the commercially available Bt maize hybrid, and the sole variety with insecticidal activity by now cultivable in EU, express Cry1Ab toxin for the control of the steamborer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Event MON810). In the case of insecticidal genetically modified plants one of the objectives of the risk assessment is represented by the potential adverse impact on non-target arthropods (Romeis et al., 2006). In particular it has been directed on beneficial species especially on those contributing to biological control of pests, such as predatory lady beetles (Coleoptera: Coccinellidae).

Since the ecological and economic importance of predaceous lady beetle, different study have been conducted to evaluate the potential toxicity of Cry1Ab toxin, the same used in this paper, to several coccinellid species. The majority of laboratory studies on lady beetle revealed no adverse effects of Cry1Ab even if contradictory results have been reported for some species (Álvarez-Alfageme et al., 2011; Zhang et al., 2014). Moreover, significant quantity of Cry1Ab toxin was found in different coccinellid species in a quantitative evaluation of Bt toxin within non-target phitophagous insects and higher order arthropods in a field of transgenic Bt maize indicating that some long-term exposure to Cry toxin do occurs in maize agroecosystems (Harwood et al., 2005). Such an exposure should therefore be addressed in risk assessment studies.

Hence, with the aim of modelling the impact that long-term exposure to a prey reared on GM-plant would have on a population of coccinellids, bioassays were carried out in the greenhouse to assess the population-level responses of *Hippodamia variegata* Goeze (Coleoptera: Coccinellidae), feeding on aphids (*Rhopalosiphum maidis*) reared on Bt-Maize and near-isogenic plants in a more realistic condition respect to highly controlled laboratory trials.

MATERIALS AND METHODS

Plants

A hybrid transgenic maize (Bt-maize), event MON 810 (DKC442YG), and its correspondent non-transformed near isoline (DK440) (near-isogenic) were used in the experiments. Bt-maize plants express a gene encoding for a truncated form of the Cry1Ab toxin from the *B. thuringiensis* var. *kurstaki*, active against the European Corn Borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Plants were grown from seed in 18-litre plastic pots (three plants per pot) with the

same soil mixture (60% universal potting soil 40% sand) in a greenhouse (min. T 19 °C, max. T 29 °C; min. r.h. 55%, max. r.h. 85% and 16L:8D). Plants were watered using a timer driven automatic drip watering system.

Insect rearing

A mass culture of Corn leaf aphid, *Rhopalosiphum maidis* (Fitch) was established, starting from field collected specimens. The corn leaf aphid was reared on wheat seedlings in a climatic chamber at 25 ± 1 °C, $70\pm 10\%$ r.h., and 16L:8D photoperiod.

Culture of *H. variegata* was established from adult specimens collected at the experimental field station of the Department of Agricultural sciences in Cadriano, Bologna province, Northern Italy (44°32'51"N, 11°24'37"E) in 2012. Adult coccinellids were maintained in Plexiglas cages (40 × 30 × 45 cm) and larvae in plastic cages (33 × 20 × 13 cm). Larvae and adults were fed with an excess of green peach aphids, *Myzus persicae* Sulzer, that had been reared on green pea (*Pisum sativum* L.) sprouts. Both adults and larvae were kept in a climatic chamber at 25 ± 1 °C with a r.h. of 60-80 %, L:D = 16:8. To obtain the amount of 3rd and 4th instar larvae of *H. Variegata* needed in the experiment egg masses from the rearing were singularly kept in plastic cylinders ($\varnothing = 5.5$ cm, h = 7 cm) containing green pea sprouts infested with *M. persicae*. The sprouts were replaced every other day to ensure *ad libitum* food for the growing larvae. Newly emerged (< 36 h after emergence) *H. variegata* adults were used in the experiments.

Experimental design

The experiment was conducted in a greenhouse at T 19 °C min. - 29 °C max., r.h. 55% min - 85% max., and 16L:8D photoperiod. When potted corn plants reached ≈ 1 m in height they were inoculated with *R. maidis*. When the aphids have been settled on the plants, pots were singularly enclosed in sleeve cages made of non-woven fabric ($\varnothing = 0.6$ m, h = 2.5 m). Then a fixed population of coccinellids, ~20 eggs, 4 larvae (3rd and 4th instar), 2 pupae and 6 adults (3♀ and 3♂) were added in each sleeve cage (T_0). After a week (T_1) and on the three subsequent weeks ($T_2 - T_4$), the plants in the sleeve cages were sampled and the number of specimens of each coccinellid stage present, were counted. There were a total of 24 sleeve cages, i.e. 2 treatment (Bt-maize and Near-isogenic line) × 4 sampling dates ($T_1 - T_4$) × 3 replicates. The samplings were destructive as the sleeve cages sampled were dismantled.

Matrix model

The data obtained, consisting of population time series (population vectors which contains information on the stage distribution of the studied population), were used to generate a $z \times z$ stage-classified projection matrix \mathbf{A} (Caswell, 2001).

$$\mathbf{A} = \begin{pmatrix} P_1 & F_2 & F_3 & \dots & F_z \\ G_1 & P_2 & 0 & \dots & 0 \\ 0 & G_2 & \dots & \dots & 0 \\ \vdots & \dots & \dots & \dots & \vdots \\ 0 & 0 & \dots & G_{z-1} & P_z \end{pmatrix} \quad (1)$$

The general model consists of a matrix containing survival probabilities (G_i , the probability of moving to the next stage on the subdiagonal, and P_i , the probability of remaining in the same stage, in the main diagonal) and fecundities (F_i , in the first row) of a population.

Hippodamia variegata life cycle has four stages (egg, larvae, pupae, and adult) and the correspondent life cycle graph is presented in Fig. 1.

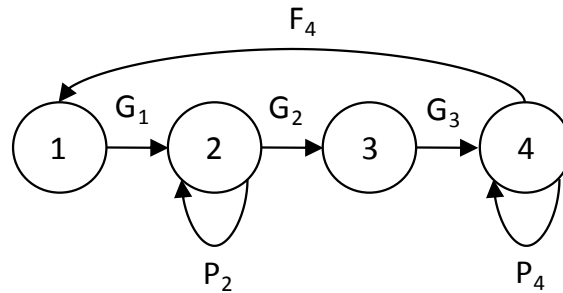


Figure 1. Life cycle graph of *H. variegata* corresponding to the stage-classified matrix \mathbf{A} (eq. 2). The number in the nodes correspond to stages: 1 = eggs, 2 = larvae, 3 = pupae, and 4 = adults. G_1 , survival of eggs into larval stage; G_2 , survival of larval stage into pupal stage; G_3 , survival of pupal stage into adult stage; P_2 , survival of larvae within larval stage; P_4 , survival of adult within adult stage; F_4 , fecundity of adult.

The relative stage-classified population projection matrix \mathbf{A} results

$$\mathbf{A} = \begin{pmatrix} P_1 & 0 & 0 & F_4 \\ G_1 & P_2 & 0 & 0 \\ 0 & G_2 & P_3 & 0 \\ 0 & 0 & G_3 & P_4 \end{pmatrix} \quad (2),$$

and the basic structure of the full model is represented by

$$\begin{pmatrix} n_1 \\ n_2 \\ n_3 \\ n_4 \end{pmatrix} (t+1) = \begin{pmatrix} P_1 & 0 & 0 & F_4 \\ G_1 & P_2 & 0 & 0 \\ 0 & G_2 & P_3 & 0 \\ 0 & 0 & G_3 & P_4 \end{pmatrix} \begin{pmatrix} n_1 \\ n_2 \\ n_3 \\ n_4 \end{pmatrix} (t) \quad (3)$$

where the vectors $\mathbf{n}(t)$ and $\mathbf{n}(t + 1)$ give the state of the population, i.e. the number of individuals in each of the four stages, at time t and $t + 1$, respectively.

Parameter estimation technique

Most of the methods utilized for estimate the parameters of age or stage-classified models rely on following cohorts of identified individuals (Caswell, 2001; see also the previous studies in this thesis). However in this study the observed data consisted of a time-series of population vectors $\mathbf{n}(t)$ for $t = T_0, T_1, \dots, T_4$, where individuals are not distinguished. The relationship between the observed data and the values of the parameters (G_i , for $i = 1, 2, 3$; P_i , for $i = 1, \dots, 4$; and F_4) that produced the series involves an estimation process called *inverse problem*. The set of parameters that minimize the residual between the collected data and the model output was estimated using the quadratic programming method (Wood, 1994, 1997; Caswell, 2001). The method was implemented using the routine qp, a constrained optimization function, in GNU Octave, version 3.6.4. Since egg and pupal development was shorter than the time between two samplings (Lanzoni et al., 2004), the value of the parameters P_1 and P_3 were set to 0. This also reduced the number of parameters to be estimated with a rather limited number of data. The estimated parameter for each treatment and replicate are summarized in Table 1.

Table 1. Set of matrix parameters estimated using the quadratic programming method.

Parameter	Near-isogenic			Bt-maize		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
G1	0.13211	0.82547	0.00000	0.07716	0.00000	0.38918
G2	0.07676	0.39327	0.27907	0.36060	0.01436	0.28362
G3	0.00000	1.00000	0.48020	0.71735	0.00000	0.46196
P2	0.00000	0.00000	0.53630	0.00000	0.14733	0.00000
P4	1.00000	1.00000	1.00000	1.00000	0.32609	1.00000
F4	13.82488	5.94444	6.80992	6.90402	1.96739	3.98068

Demographic analyses.

The asymptotic population growth rate (λ_1) was calculated as the dominant eigenvalue of the matrix. The corresponding continuous time rate, r_m was calculated as follow:

$$r_m = \log \lambda_1 \quad (4)$$

In each treatment, the stable stage distribution (\mathbf{w}), the abundance of each stage class relative to the total abundance, and the stage specific reproductive values (\mathbf{v}), were calculated as the corresponding right and left eigenvector of the dominant eigenvalue of the matrix \mathbf{A} , respectively (Caswell, 2001).

With the aim to explore the functional dependence of λ to changes in the matrix parameters (G_i , P_i , and F_i) the elasticity of \mathbf{A} in both treatment was calculated as follow (Caswell, 2001):

$$\mathbf{E} = \begin{pmatrix} a_{ij} & \frac{\partial \lambda}{\partial a_{ij}} \\ \lambda & \frac{\partial \lambda}{\partial \lambda} \end{pmatrix} \quad (5)$$

Moreover we determined how much the differences in population growth rates (λ) between near-isogenic line and Bt-maize groups were determined by the differences in each parameter (G_i , P_i , and F_i) by means of a life table response experiment (LTRE) analysis (Caswell, 2001). With this technique, the effects of Bt-maize on λ , measured respect to the near-isogenic line, was decomposed into contributions from each stage-specific survival probability and fecundity terms as follow:

$$\lambda^{(Bt)} \approx \lambda^{(Iso)} + \frac{\partial \lambda}{\partial G_1} \Delta G_1 + \frac{\partial \lambda}{\partial G_2} \Delta G_2 + \frac{\partial \lambda}{\partial G_3} \Delta G_3 + \frac{\partial \lambda}{\partial P_2} \Delta P_2 + \frac{\partial \lambda}{\partial P_4} \Delta P_4 + \frac{\partial \lambda}{\partial F_4} \Delta F_4 \quad (6)$$

where $\lambda^{(Bt)}$ and $\lambda^{(Iso)}$ denote the values of λ for Bt-maize and near-isogenic groups, respectively, ΔG_1 , ΔG_2 , ΔG_3 , ΔP_2 , ΔP_4 , and ΔF_4 are, for each parameter, the differences between Bt-maize and near-isogenic values, and $\partial \lambda / \partial G$, $\partial \lambda / \partial P$, and $\partial \lambda / \partial F$ are the sensitivities to λ calculated halfway between the two parameter sets (Bt-maize and near-isogenic) being compared (Logofet and Lesnaya, 1997). All demographic calculations were performed with the software PopTools version 2.7.5 (Hood, 2006).

Statistical analysis

Data on densities of *H. variegata* stages were analyzed by a factorial ANOVA, using treatment (Bt-maize and near-isogenic line) and sampling date ($T_1 - T_4$) as main factors. Data which violated the ANOVA assumptions (homoscedasticity and normality) were rank transformed. Data on demographic parameters (λ , r_m , \mathbf{w} , and \mathbf{v}) were tested with one-way ANOVA. Population growth indices and LTRE decomposition analysis outputs (parameter differences and contributions) were tested against 0 (1 in the case of λ) by means of one sample *t*-test. Statistical analyses were performed using STATISTICA software version 10 (StatSoft Inc.).

RESULTS

There were no significant differences in the mean number of eggs, larvae, pupae, and adults of *H. variegata* between Bt-maize and near-isogenic line (Fig. 2 and Table 2). On the contrary a significant reduction were observed among sampling dates for egg, larval and pupal stage (Fig. 2a-c and Table 2). For the latter the interaction of treatment and sampling date was also significant with the higher number of pupae found a week early in Bt-maize treatment respect to near-isogenic line. As a tendency it was found also for larvae even if in this case the interaction term was not significant. In contrast, a significant increase was found among sampling dates in the number of adults (Fig. 2d and Table 2).

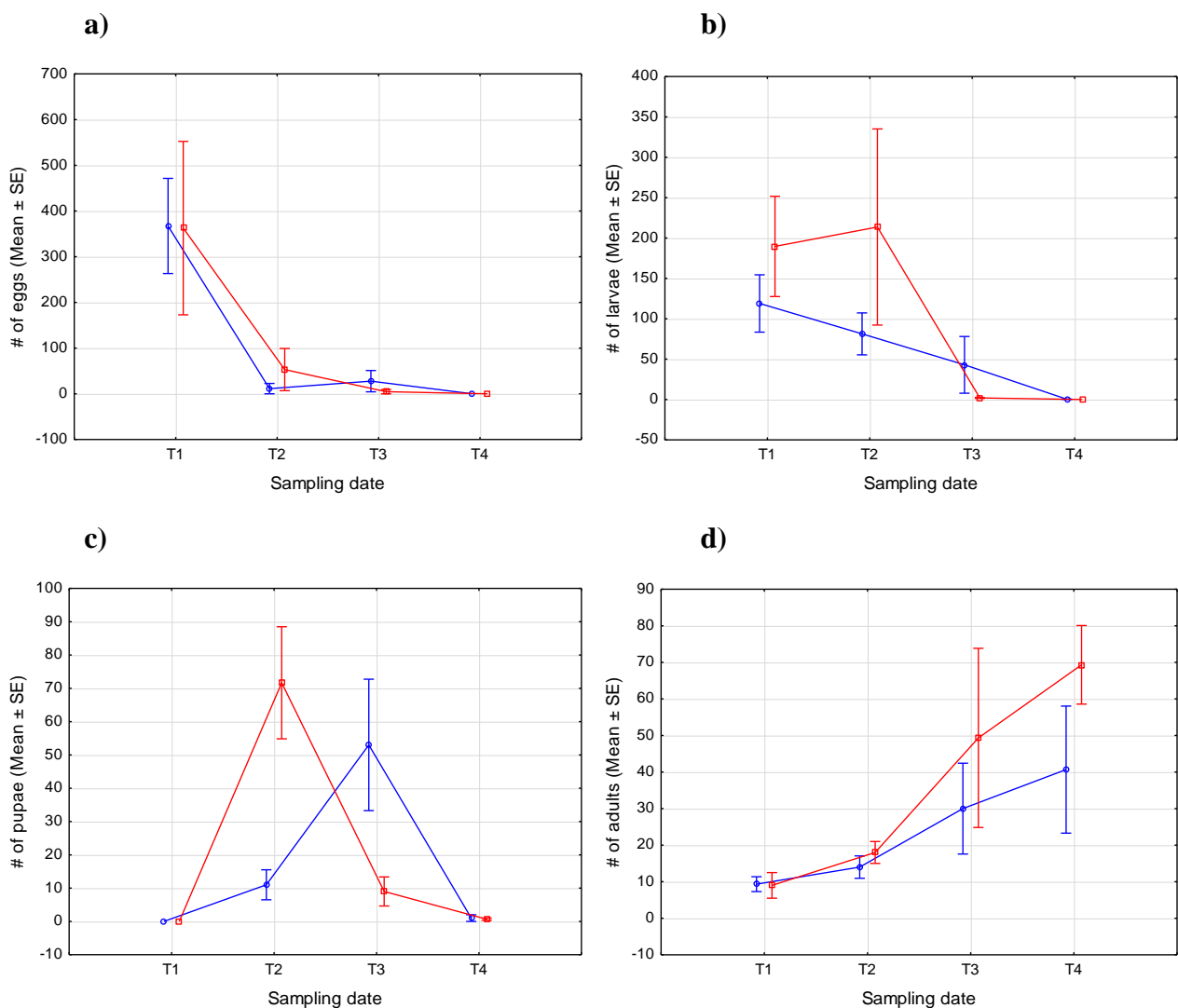


Figure 2. Mean number of *H. variegata* eggs (a), larvae (b), pupae (c), and adults (d) sampled in the sleeve cages ($n = 3$) in four consecutive sampling (T₁ – T₄).

Table 2. ANOVA results for the factors treatment (Bt-maize and Near-isogenic line) and sampling date (T1-T4), and their interaction.

<i>H. variegata</i> stage	Source of variation		
	Treatment	Sampling date	Treatment × sampling date
Egg	0.861	0.0006	0.611
Larvae	0.908	<0.0001	0.274
Pupae	0.659	<0.0001	0.013
Adult	0.287	0.033	0.931
Degrees of freedom	1, 16	3, 16	3, 16

In each treatment the matrix \mathbf{A} was multiplied by the starting (T_0) population vector $\mathbf{n}(t_0) = [20, 4, 2, 6]^T$. Population growth across time can be then found via repeated matrix multiplications. Each population was projected to $t=4$ and the resulting model fits are shown in Fig. 3.

No significant differences were observed in *H. variegata* population growth rates (λ and r_m) between Bt-maize and near-isogenic line (Fig. 4). Moreover, in both treatment, the value of finite rate of increase was not significantly different from 1 (near-isogenic, $t = 1.0$, $df = 2$, $P = 0.423$; Bt-maize $t = -0.545$, $df = 2$, $P = 0.640$) and the value of intrinsic rate of increase from 0 (near-isogenic, $t = 1.0$, $df = 2$, $P = 1.0$; Bt-maize $t = -0.724$, $df = 2$, $P = 0.544$), indicating stable populations. In both treatment, when the population asymptotic state is reached, egg stage represented over 70% of the population size (Fig. 5a), whereas adult stage mainly contributes to the asymptotic population dynamics with the highest reproductive values (Fig. 5b). Bt- maize exposition did not significantly affect both stable stage distribution and reproductive values of *H. variegata* (Fig. 5).

The elasticities calculated for each parameter (G_i , P_i , and F_i) in both treatments are reported in the following matrices \mathbf{E} :

$$\mathbf{E}_{Iso} = \begin{pmatrix} 0 & 0 & 0 & 0.1155 \\ 0.1155 & 0.0198 & 0 & 0 \\ 0 & 0.1155 & 0 & 0 \\ 0 & 0 & 0.1155 & 0.5180 \end{pmatrix} \quad \mathbf{E}_{Bt} = \begin{pmatrix} 0 & 0 & 0 & 0.0807 \\ 0.0807 & 0.0048 & 0 & 0 \\ 0 & 0.0807 & 0 & 0 \\ 0 & 0 & 0.0807 & 0.6725 \end{pmatrix}$$

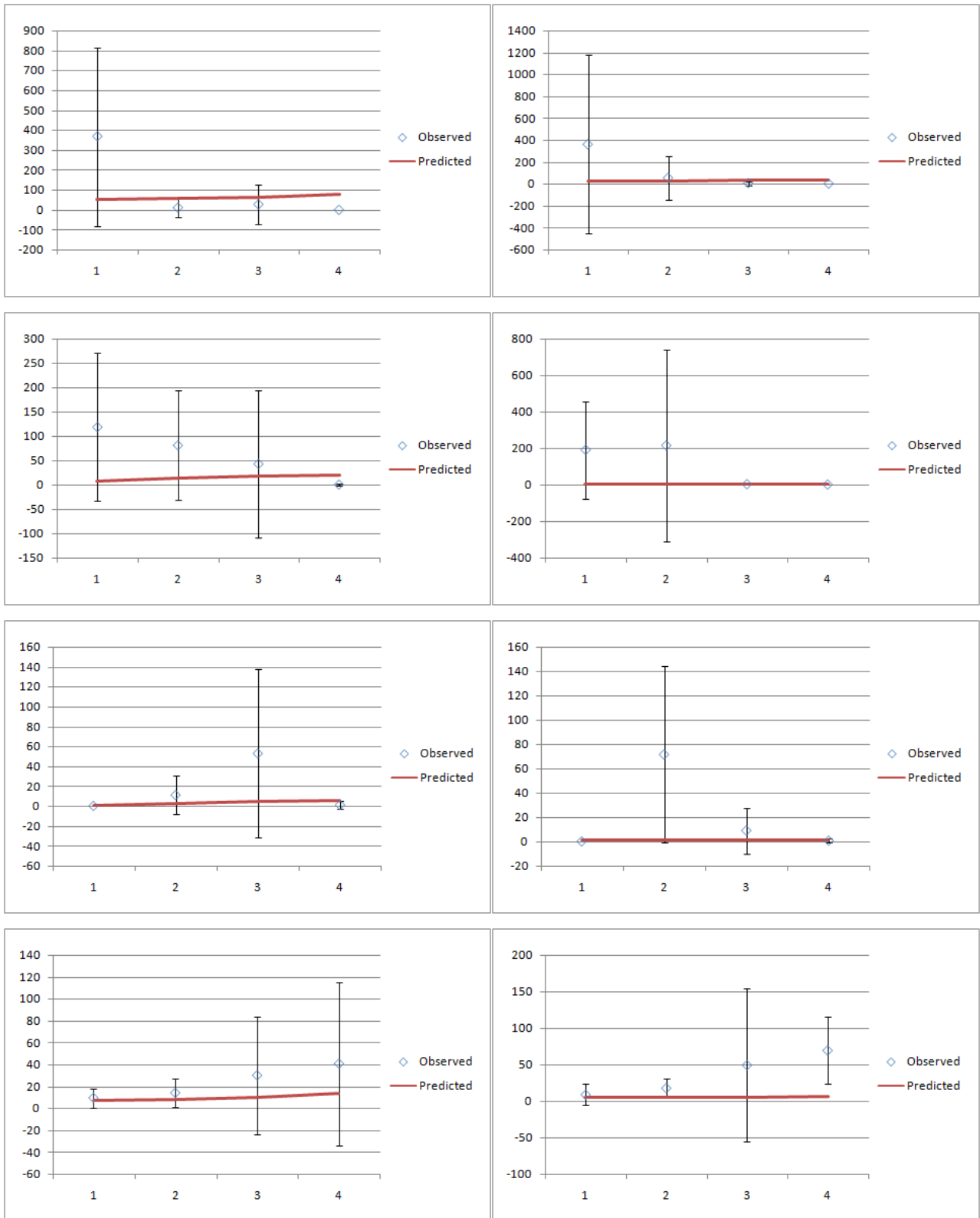


Figure 3. Model fit versus data. Comparison of observed (diamond) and predicted (line) number of eggs, larvae, pupae and adults of *H. variegata*. The observed values are means of three replicates; error bars represent 95% confidence intervals. Predicted values are based on matrix \mathbf{A} (eq. 2) estimated with the quadratic programming method.

The elasticity of λ to P_4 (survival of adult within adult stage) showed the highest value for both treatments. Roughly 52% and 67% of the value of λ would be determined by survival at that stage for near-isogenic and Bt-maize exposed *H. variegata* populations, respectively.

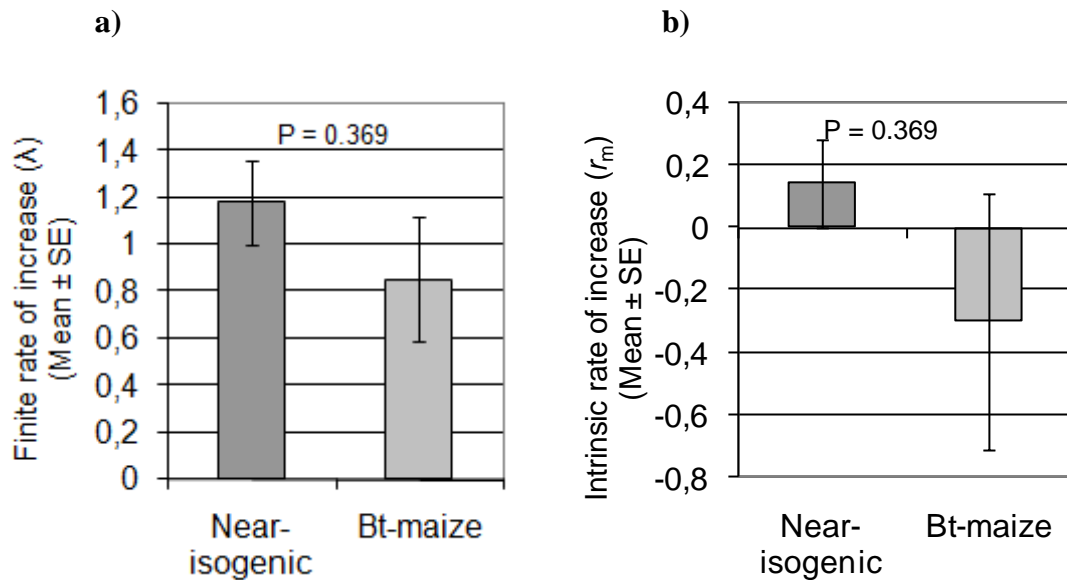


Figure 4. Values of finite rate of increase (a) and intrinsic rate of increase (b) for populations of *H. variegata* reared on near-isogenic line or Bt-maize.

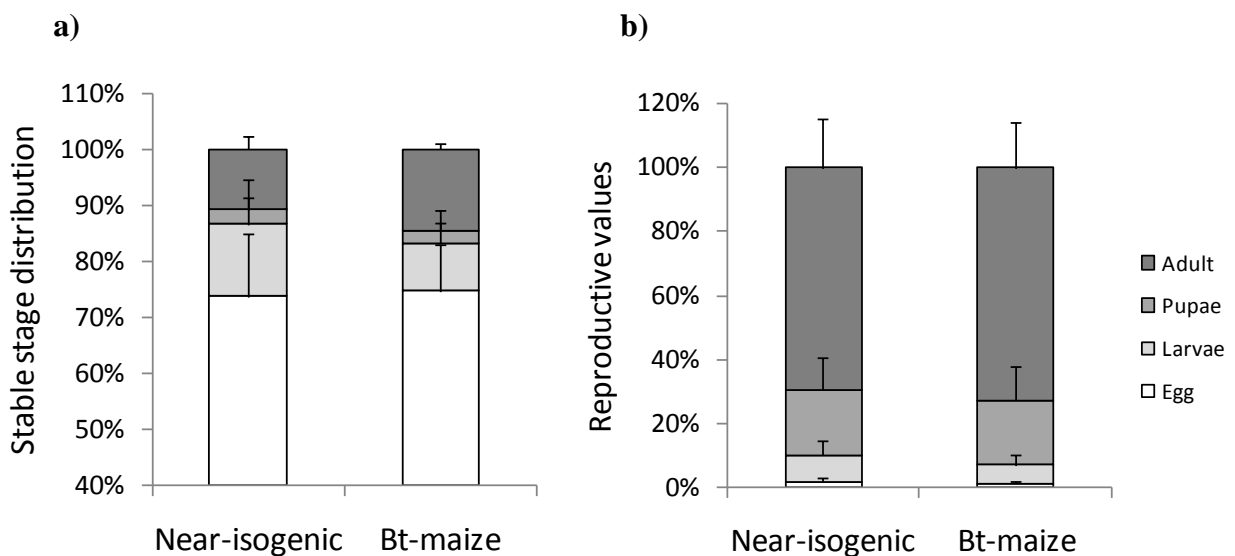


Figure 5. Stable stage distribution (a) and stage-specific reproductive values (b) of populations of *H. variegata* reared on near-isogenic line or Bt-maize. All data are mean + SE (n = 3).

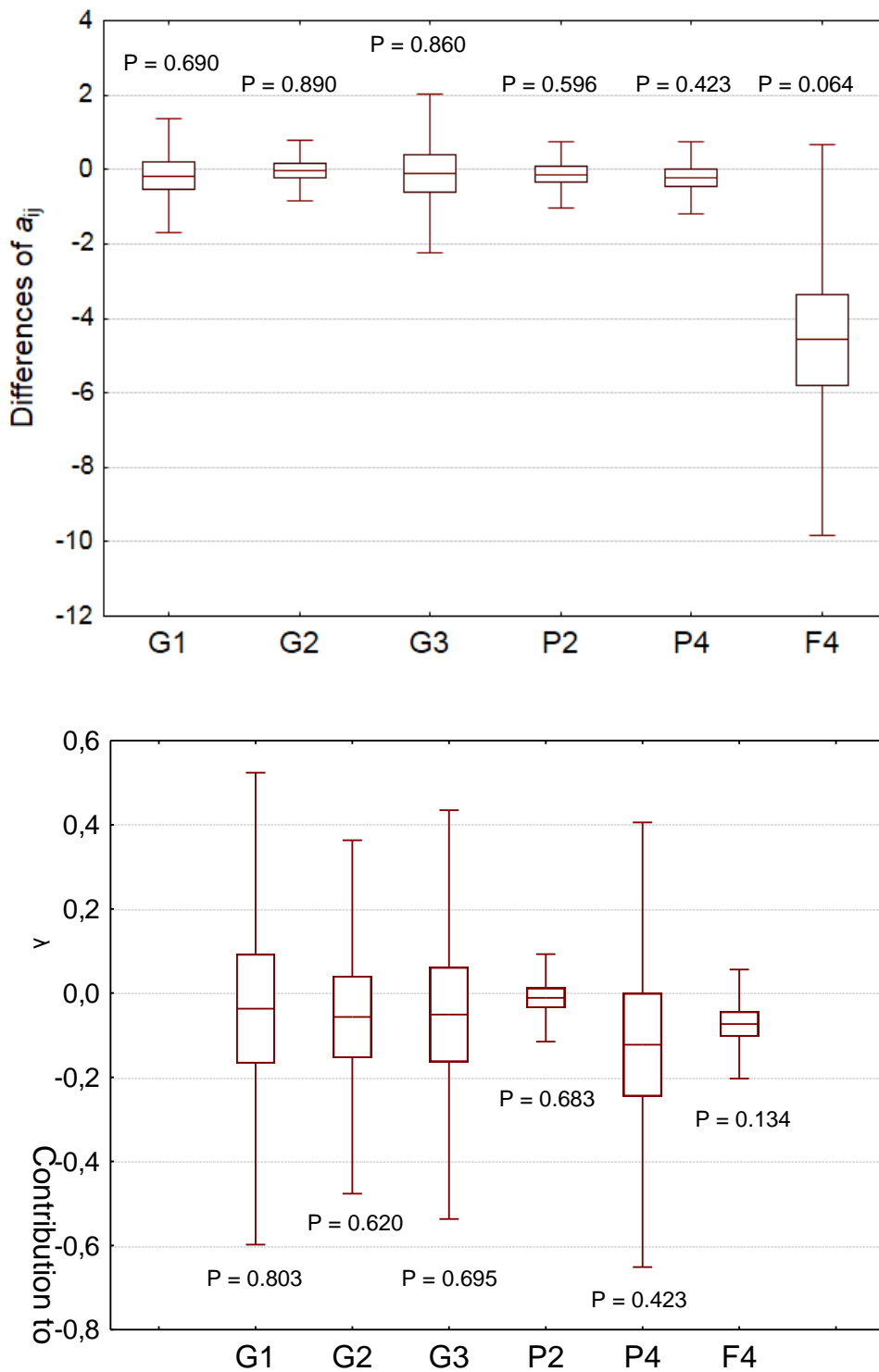


Figure 6. LTRE decomposition analysis of treatment effects using equation 6. (Top) Differences in parameters G_1 , G_2 , G_3 , P_2 , P_4 , and F_4 between Bt-maize and near-isogenic line. (Bottom) Contributions of those differences on population growth rate (λ). Negative values indicate disadvantages or negative contributions to λ of Bt-maize relative to near isogenic line. Lines represent mean ($n = 3$), boxes represent \pm SE and whiskers \pm 95% confidence interval. Statistical analysis: one sample t -test against zero.

Using LTRE decomposition, the effect of Bt-maize exposition on λ have been decomposed into contributions arising from the effect on each stage-specific parameter (Fig. 6). Respect to near-isogenic line, exposition to Bt-maize had little effect on preimaginal stages survival and no significant effect on λ . A more pronounced effect of Bt-maize treatment can be found for the adult stage. Both survival of adult within adult stage and fecundity resulted reduced, even if either difference or contribution values resulted not significantly different from zero (Fig. 6).

DISCUSSION

The solution of the demographic inverse problem utilized in this study seems to provide a consistent and reasonable estimate of stage-specific vital rates of *H. variegata* population exposed to Bt-maize in a tri-trophic system. The great advantage of semi-field based approach is that vital rate can be estimated under more realistic and comprehensive condition which cannot be achieved in the laboratory. Indeed both *H. variegata* larvae and adults were exposed to Cry toxin following most of the possible route of exposition that can be present into the field, i.e. preys, honeydew, and pollen.

The increase of the number of *H. variegata* adults inside the sleeve cages showed that the methodology proposed can be utilized for the evaluation of the response of *H. variegata* exposition to Bt-maize at population level. However a significant decrease of the number of eggs, larvae, and pupae among sampling date, indicate some difficulties of the close system represented by the sleeve cages to support fully population development. The primary cause was the heavy predation that caused the depletion of the aphid colonies inside the cages. A reduction of the sampling interval to 2 or 3 days could improve the methodology other than better fulfil the model assumptions (Caswell, 2001).

The early presence of a high number of pupae inside the sleeve cages in the Bt-maize treatment could be due to a higher aphid population respect to near-isogenic line, since *R. maidis* appears to perform better on Bt-maize respect to non-transgenic maize plants (Faria et al., 2007; Chapter 1 in this thesis). Other that, our study revealed no significant effects of Bt-maize on *H. variegata*. Indeed no or only trace of Cry1Ab toxin can be found in aphids and honeydew (Raps et al., 2001) and a very low amount of toxin was expressed in pollen (Chapter 2 in this thesis). However it cannot be excluded that without the aphid shortcoming inside the sleeve cages some effects due to other causes such as pleiotropic effects should have been evidenced.

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