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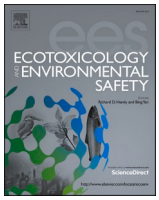

Performance of *Daphnia magna* on flour, leaves, and pollen from different maize lines: Implications for risk assessment of genetically engineered crops

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ABSTRACT

Non-target effects of genetically engineered (GE) plants on aquatic *Daphnia magna* have been studied by feeding the species with different maize materials containing insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*). The results of those studies were often difficult to interpret, because only one GE plant was compared to one related non-GE control. In such a setting, effects of the Cry proteins cannot be distinguished from plant background effects, in particular when the test species is nutritionally stressed. In the present study, we tested the suitability of three different maize materials, i.e., flour, leaves and pollen, from five diverse non-GE maize lines (including EXP 258, a breeding line that is closely related to a SmartStax *Bt* maize) as exclusive food sources for *D. magna*. The parameters recorded included survival, sublethal endpoints such as body size, number of moltings to first offspring, time to first offspring, number of individuals in first clutch, total number of clutches, total number of offspring, average number of offspring per clutch, and population measures such as net reproductive rate R_0, generation time T and intrinsic rate of increase r_m. The results showed that *D. magna* can survive, grow and reproduce when fed only maize materials, although the performance was poorer than when fed algae, which indicates nutritional stress. Large differences in life table and population parameters of *D. magna* were observed among the different maize lines. Our results suggest that confounding effects caused by nutritional stress and plant background might explain some of the conflicting results previously published on the effects of *Bt* crops on *D. magna*. Using 95% confidence intervals for the means of the five maize lines for all measured parameters of *D. magna* performance in our study, we captured the natural range of variation. This information is useful for the interpretation of observed differences in *D. magna* performance between a GE plant and its non-GE comparator as it helps judging whether observed effects are of biological relevance. If differences between a GE and comparator line are observed and their biological relevance needs to be assessed in future risk assessments of GE maize, 1) the data on natural variation of the different parameters generated by previous studies can be informative (e.g. data from our study for maize fed *D. magna*); 2) for additional experiments the inclusion of multiple unrelated non-GE comparators should be considered; In addition, it should be taken into account that nutritional stress can affect the outcome of the study.

1. Introduction

Aquatic and terrestrial environments are interlinked and influenced by human activity, such as agriculture, mining, landfills, industrial and urban wastewater, as well as natural geogenic releases (Schwarzenbach et al., 2010). Pollutants include heavy metals, hormonally active substances, microplastic, and chemicals. Agriculture, which releases several million tons of fertilizers and pesticides each year, is an important source of pollutants (Bockstaller et al., 2009). With the rapid development of gene technology, genetically engineered (GE) crops are grown on steadily increasing areas worldwide (ISAAA, 2018). GE crops can reduce the need for pesticides (Brookes and Barfoot, 2018). On the other hand, the currently grown insect-resistant GE crops produce high amounts of insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) that can pose a risk to non-target organisms when entering terrestrial and aquatic ecosystems (Carstens et al., 2012; Romeis et al., 2019; Tank et al., 2010; Viktorov, 2011). The Cry proteins that are produced by *Bt* crops have an oral mode of action. After ingestion and activation in the gut, they bind to specific gut receptors of sensitive insects, where they lead to pore formation, unbalanced ion fluxes and ultimately death (Bravo et al.,

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2012; Jurat-Fuentes and Crickmore, 2017).

Zooplankton is an essential part of the aquatic food chain. It is the main consumer of bacteria, small algae, and organic detritus, and at the same time, a major food source for higher trophic levels. Changes in abundance, diversity, and distribution of zooplankton may thus have cascading effects throughout a water ecosystem (Gannon and Stemberger, 1978). Moreover, zooplankton is very sensitive to many contaminants and thus used as an indicator to monitor changes in water quality (McNaught, 1992). The Cladocera species *Daphnia magna* (Diplostraca: Cladocera) is one representative of zooplankton, and widely used in environmental toxicology because of its rapid life cycle, a predominantly asexual mode of reproduction, minimal genetic variation, and high sensitivity to environmental contaminants (Brausch and Salice, 2011; Meyer et al., 2015). There are several standardized testing protocols for *D. magna* including short term tests (24 or 48 h) for the acute toxicity of chemicals, water samples, and sediments (ASTM (American Society for Testing and Materials), 2005; ISO (International Organization for Standardization), 2012; OECD Organization for Economic Co-operation and Development, 2004) and longer term tests for more subtle, chronic toxicity including effects on reproduction (ASTM (American Society for Testing and Materials), 2005; OECD Organization for Economic Co-operation and Development, 2012). Those ecotoxicological tests with *D. magna* have mainly been used for industry pollutants (Alkimin et al., 2020; Galhano et al., 2020; Liu et al., 2020; Zimmermann et al., 2020), medical pollutants (Pan et al., 2019; Grzesiuk et al., 2020; Sarapultseva et al., 2017), and agricultural pollutants (Aksakal and Arslan, 2020; Knapik and Ramsdorf, 2020; Wyn et al., 2007).

D. magna may be exposed to plant-produced Cry proteins through ingestion of pollen, plant residues or root exudates that enter aquatic environments (Carstens et al., 2012; Viktorov, 2011). Maize in particular has a high biomass and detritus, such as shredded plant remains after harvest, can enter small streams draining the fields. In addition, maize is open pollinated and releases high amounts of pollen, which can also enter waterbodies (Carstens et al., 2012; Douville et al., 2007; Jensen et al., 2010; Rosi-Marshall et al., 2007; Tank et al., 2010). Even though maize might not be a natural food for *D. magna*, exposure to maize material in agricultural landscapes is likely. For the environmental risk assessment of GE crops, *D. magna* has been used in non-target studies as one representative species for aquatic environments. Studies were conducted with *Bt* rice (Zhang et al., 2016) and *Bt* maize (summarized in Pott et al., 2018). For *Bt* maize, several studies have investigated the impact on *D. magna* using pollen (Mendelson et al., 2003), pulverized leaves (Holderbaum et al., 2015), or flour (Bøhn et al., 2008, 2010; Zhang et al., 2018) as test material. Maize flour is a less realistic route of exposure for *D. magna*, but can serve as a model material to expose the test animals to Cry proteins. The aim of such feeding studies is to create worst case exposure scenarios, where the test animals ingest large amounts of *Bt* maize (and the insecticidal Cry proteins contained therein). In contrast to chemicals in water or sediment, the GE plant material that contains the orally active test substances (e.g., insecticidal Cry protein) also serves as food for the test species (e.g., *D. magna*) and ideally there is no need for additional food supply (e.g., green algae). Consequently, *D. magna* has been fed exclusively with *Bt* maize material to achieve high exposure. For suitable test protocols, however, it is essential that the plant materials containing the insecticidal proteins can be ingested by *D. magna* (appropriate particle size) and that they supply enough nutrients for survival, growth and reproduction so that the organisms are not under nutritional stress. The standardized ASTM, ISO and OECD test protocols mentioned above include validity criteria for the tests. However, they are not designed for orally active substances (Bundschuh et al., 2019). Researchers thus had to adapt the protocols for assessing potential impacts of *Bt* plant materials, but those have usually not been validated or ring tested in different laboratories. Consequently, the published studies conducted with different maize materials resulted in unconfirmed and sometimes conflicting results on the effects of *Bt* crops on *D. magna* (Pott et al., 2018).

One problem with most previous studies with *Bt* maize is that only one *Bt* maize hybrid was compared to one non-*Bt* maize hybrid. Even if the non-*Bt* maize is the nearest comparator line to the *Bt* line, the transformation process and several breeding steps may lead to changes in plant composition and physiology, which may translate into differences in performance of organisms feeding on those plants (Ladics et al., 2015). There is the possibility that adverse effects seen in some of the *Bt* maize studies might have been caused by such plant background-related effects rather than the *Bt* protein itself (Romeis et al., 2011, 2019). Another problem of studies using maize materials to feed *D. magna* is the possibility that nutritional stress might have led to effects in addition to those caused by the plant background and the *Bt* proteins, which could further impede the interpretation of the study results.

It is evident that there is a large variation in compositional analytes including nutrients and antinutrients in conventional maize lines that are grown commercially and have a history of safe use (Cong et al., 2015; Hong et al., 2014), but it is difficult to link the content of those compounds to the performance of *D. magna.* As long as the mechanistic relationship between plant components and *D. magna* performance remains unknown, the relevance of differences between particular lines can be judged if the natural variation among different conventional maize lines is known.

In the present study, we tested three different maize materials, i.e., flour, leaves and pollen, as exclusive food sources for *D. magna*. We used those materials from five diverse non-GE maize lines, including one breeding line that is closely related to a SmartStax *Bt* maize. The following objectives were addressed:

1) How suitable are maize flour, leaves, and pollen as exclusive food sources for *D. magna* to sustain growth and reproduction compared to green algae?
2) How do life table and population parameters of *D. magna* differ among non-GE maize lines and what is the potential natural range of variation?

The data generated in our study with non-GE maize lines is useful for the interpretation of observed differences in *D. magna* performance between GE plants and their non-GE comparators in the context of future risk assessments of GE maize.

2. Materials and methods

2.1. Plant materials

Five lines of conventional maize were used for all experiments: Rheintaler, a Swiss landrace and population maize, Tasty Sweet, a sweet maize, ES-Eurojet and Planoxx, two commercial varieties used in Switzerland (ES-Eurojet is early maturing durum maize while Planoxx is late maturing dent maize), and EXP 258, the nearest conventional hybrid to one SmartStax *Bt* line. All maize lines were planted on May 14th, 2018 in two heated glasshouse cabins, set to 21 °C during the day and 17 °C at night and additional light to ensure a minimum day length of 16 h. Plants were grown individually in 12 L pots filled with soil. Ca. 40 g long-term fertilizer (Manna Cote 4M Wilhelm Haug GmbH, Ammerbuch, Germany) were added per pot. Pots were arranged in a block design (each block containing each maize line) to account for differences in light and climatic conditions within the glasshouse cabins. After plants were 4 weeks old, they were fertilized with liquid fertilizer (0.2%) Manna (Wilhelm Haug GmbH) once per week.

Seven weeks after planting (highest maize plants had 15–16 leaves), the 10th true leaf counted from the bottom of each plant was cut and the middle vein was removed. The leaves were cut into pieces and stored in paper envelopes at −70 °C. Later, leaf-pieces were lyophilized and ground with a coffee mill for 5 min. Subsequently, a finer powder was generated with a mixer mill (MM400, Retsch, Haan, Germany) set to a frequency of 25 Hz and a grinding time of 30 s, with a 20 mm diameter

tungsten carbide ball. Finally, the powder was sieved through a 75 μm metal sieve and stored at −70 °C.

Maize tassels were placed in air-permeable cellulose bags (Celloclair, Liestal, Switzerland) and pollen was collected every second day. The collected pollen was poured through a 200 μm gauze to remove anthers into a 12 cm glass Petri dish, where it was left for 24 h for drying at room temperature. After that, the pollen was stored in screw-cap glass tubes at −70 °C. Plants were discarded when pollen shedding stopped. Because pollen grains, which have a diameter of 80–90 μm (Meissle et al., 2014), are too large for *D. magna* as food (Burns, 1968), pollen was also ground with the mixer mill at 25 Hz for 30 s, sieved through a 75 μm mesh, and stored at −70 °C.

Finally, maize grains were also ground with the coffee mill (5 min) and mixer mill (30 Hz, 150 s), sieved through a 75 μm mesh, and stored at −70 °C. In contrast to leaves and pollen, which were collected from plants uniformly grown in our glasshouse, maize flour was produced from the original batch of (untreated) seeds obtained from the breeders. This implies that the plants from the different maize lines were raised in the field under different conditions.

For the feeding assays, the sieved maize materials were used to make suspensions with a concentration of 3 mg/mL using Aachener Daphnien Medium (ADAM) (Klüttgen et al., 1994, medium composition modified after Ebert et al., 1998), which were stored in 2 mL aliquots at −20 °C.

2.2. Algae and D. magna

Algae (*Acutodesmus obliquus*) that served as optimal food for *D. magna* and a monoclonal strain of *D. magna* (GB-EL75-69) were obtained from Dieter Ebert, Zoological Institute of Evolutionary Biology, University of Basel (Switzerland).

D. magna were cultured in ADAM medium in a climate chamber (20 °C, 70% RH, 16 h light / 8 h dark cycle). The medium was prepared and stirred at room temperature for at least 12 h before use. *D. magna* of the culture were transferred to new medium every two weeks, using Pasteur pipettes. The cultured *D. magna* showed no signs of stress, i.e., presence of males or ephippia, discolored animals or high mortality.

The culture medium for the green algae was prepared according to the description by D. Ebert (Web-guide to *Daphnia* parasites, http://www.evolution.unibas.ch/ebert/lab/algae.htm) and autoclaved in 1 L baffled flasks. When the medium cooled down, ca. 2 mL algae suspension were added and each flask was closed with a sterilized PTFE membrane cap. The bottles were incubated on a platform shaker in a climate cabinet (20 °C) with lights from three directions and a 23 h light / 1 h dark cycle. When the color of the algae suspension was dark green, the bottles were stored at 4 °C. Before feeding to *D. magna*, the algae were centrifuged (4500 × *g*, 15 min) in 50 mL centrifuge tubes, the supernatant was discarded and the pellet was resuspended in ca. 25 mL ADAM medium by shaking the tubes.

The carbon concentration of algae, measured in a Euro EA300 elemental analyser (HEKAtech GmbH, Wegberg, Germany) and calculated with CallidusH 2E3 (HEKAtech, Germany), was about 55% of the dry weight and 10 million algal cells had a dry weight of ca. 0.28 mg. Algae were counted in a Thoma chamber (http://www.evolution.unibas.ch/ebert/lab/counting.htm#4).

2.3. Effects of maize materials on D. magna

Newly hatched *D. magna* (within 6–24 h of hatching) from the culture were kept individually in 100 mL glass beakers containing 50 mL ADAM medium, and fed with 100 μL suspension of maize materials from one of the five maize lines per animal per day. According to guideline OECD211, the amount of supplied diet should be based on organic carbon and the recommended feeding ration per *D. magna* per day is between 0.1 and 0.2 mg C (OECD Organization for Economic Co-operation and Development, 2012). Assuming a carbon content of 50% in maize materials (Hart et al., 2007, unpublished raw data of Meissle et al., 2011), 100 μL of the 3 mg/mL suspension prepared for the different maize materials contained ca. 0.15 mg C. The suitability of this feeding dose had been confirmed in a preliminary experiment using a different clone of *D. magna* (see Table S4 in the supplementary online material).

The experiment had two repetitions and ten *D. magna* per maize material (flour, leaves, pollen) and maize line (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258) were tested in each repetition (in total 20 replicates). Thus the total number of *D. magna* fed with maize material in this experiment was 300.

As a control treatment, 10 additional *D. magna* in each experimental repetition were fed daily with 10 million algae, which equals ca. 0.15 mg C. *D. magna* were transferred to new medium every two days to ensure high medium quality throughout the experiment. The experiment was conducted in a climate chamber (20 °C, 70% RH) under a 16 h light / 8 h dark cycle. The number of *D. magna* surviving, the number of molts, and the number of released offspring were recorded daily until day 28, and then every two days. Food was provided daily throughout the experiment. All offspring were removed after counting, so it was not possible to determine the sex of the offspring. The body length (distance from the top of the head to the base of the caudal spine) and body width (distance between back and front) was measured on day 7, day 14, and then every 14 days. Individual *D. magna* were removed from the rearing containers, photographed with a photomicroscope (Keyence VHX 6000, Mechelen, Belgium), and returned to the medium as soon as possible. Body length and body width were subsequently measured with ImageJ (ImageJ-win64, version 1.8.0, National Institutes of Health, USA). Ingestion of the different food materials was evident by the color of the gut under the stereo-microscope (Fig. S1, supplementary online material). The experiment ended when all individuals had died.

2.4. Medium quality analyses

The quality of the ADAM medium was measured at different time points during the experiment described previously to make sure the values were within the recommended range of guideline OECD211 (OECD Organization for Economic Co-operation and Development, 2012):

W0: pure ADAM medium; W1: ADAM medium after adding food (flour, leaves, pollen or algae); W2: ADAM medium 24 h after adding food (including one *D. magna* per container); W3: W2 after adding another food dose for one day; W4: W3 after another 24 h.

For the first repetition of the feeding experiment, the medium quality W1–W4 was measured in one randomly chosen replicate of each treatment once within the first week, when *D. magna* were juveniles, and once when *D. magna* were adults. For the second experimental repetition, the medium quality of all treatments was checked randomly three times throughout the experiment.

The following parameters were analysed: pH value (FiveEasy™ pH meter FE20, Mettler-Toledo AG, Greifensee, Switzerland), total hardness (MColortest™ Total Hardness Test, Merck KGaA, Darmstadt, Germany) and dissolved oxygen concentration (DOC) (FiveGo™ F4 portable meter, Mettler-Toledo AG, Greifensee, Switzerland).

2.5. Data analysis

Data were analysed using R, version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria). All data are presented as mean ± standard error (SE), unless otherwise indicated. Data were compared among the different maize treatments (lines and materials). Data from the control treatment (*D. magna* fed exclusively algae) were not included in the analyses. The data used for statistical analysis are available in the supplementary online material.

The survival probability of *D. magna* was analysed for each food source separately using Kaplan–Meier estimates and log-rank test (survival package). Total offspring (individuals not producing any offspring

excluded) and offspring per clutch were analysed using full factorial linear mixed effects models (LMER) with maize (five lines) and food (flour, leaves, pollen) as fixed factors, and experimental repetition as random factor (lme4 package). The time when first offspring was released (days), the number of moltings to first offspring, the total number of clutches, and the number of individuals in the first clutch were analysed by generalized linear mixed effects models (GLMER) assuming Poisson distribution with the same factors (lme4 package). Comparisons among treatments were analysed with Anova function using type III sum of squares (car package). Body length and body width were analysed using full factorial LMER with the fixed factors maize, food and time (days when measurements were taken) and individual (each *D. magna*) as random factor. In all models, factor contrasts were set to orthogonal. Differences were considered significant at $p < 0.05$. When interactions between food and maize were significant in the overall analyses, we conducted separate analyses for each food type. The net reproductive rate (R_0), generation time (T) and intrinsic rate of increase (r_m) of *D. magna* were calculated based on the theory of age-stage, two-sex life table (Chi and Liu, 1985; Chi, 1988) using bootstrap method (Akca et al., 2015) with 10′000 bootstrap replicates. The differences among maize lines were analysed with paired bootstrap tests (Hesterberg et al., 2010; Smucker et al., 2007) for each food type separately. Those lifetable analysis were performed using the software TWOSEX-MSChart (TWOSEX-MSChart-B100000, version 2020.05.28, National Chung Hsing University; Taiwan, provided by Chi H).

To illustrate the variability among different maize lines, we calculated the 95% confidence interval (CI) for the mean of each parameter for each maize line. The range of variation was then defined as the interval from the highest upper to the lowest lower CI boundary of all maize lines. We also calculated the ratio between the highest and the lowest mean of each parameter and the ratio between the highest upper and the lowest lower CI boundary (highest / lowest).

3. Results

3.1. Medium quality

All pH values of the ADAM medium were between 7.7 and 8.1 (Table S1, Supplementary online material). The value was lowest 24 h after adding food (W2) and then increased slightly. All DOC values were between 4.0 mg/L and 6.3 mg/L. The hardness gradually increased with time, and all values were between 210 mg/L and 305 mg/L. All values for the water quality (pH, DOC, hardness) were within the range demanded in OECD211 (OECD Organization for Economic Co-operation and Development, 2012), i.e., pH 6–9, DOC > 3 mg/L, and hardness > 140 mg/L.

3.2. Performance of D. magna in the control treatment

After 21 days (the time when the test recommended by OECD211 ends), mortality in the control treatment (*D. magna* fed algae) was 0%, *D. magna* molted 5.1 ± 0.05 times to first offspring release, which occurred after 9.2 ± 0.09 days. The mean number of individuals in the first clutch was 15 ± 0.4. *D. magna* produced 4 clutches, the mean total number of offspring produced was 101 ± 2.0, and each clutch consisted of 25 ± 0.5 offspring.

D. magna in the control treatment survived for a maximum of 123 days. The first individuals died at day 32, and a mortality of 20% was reached at day 69. The mean longevity was 93 ± 5.6 days. In total, *D. magna* produced 23 ± 1.4 clutches and the mean total number of offspring produced during the whole life time was 665 ± 39. Each clutch consisted of 30 ± 0.60 offspring. The net reproductive rate R_0 was 665 ± 38, the generation time T was 18 ± 0.20 days, and the intrinsic rate of increase r_m was 0.35 ± 0.0024 day^{-1}. The body length and body width of *D. magna* in the control treatment increased from day 7 (n = 20) to day 112 (n = 4), from 2.7 ± 0.02 mm to 4.7 ± 0.04 mm length and 1.8 ± 0.02 mm to 3.1 ± 0.03 mm width.

3.3. Mortality

There was no statistically significant difference in *D. magna* survival among the five maize lines for each of the food sources (all $p \geq 0.1$) (Table 1, Fig. 1). When fed maize flour, *D. magna* lived longest, i.e., a mean of 54–77 days, depending on maize line (Table S2, Fig. S2, Supplementary online material). The ratio between the highest and the lowest mean was 1.4 (Table S3). In the maize leaf treatments, mean longevity was 27–38 days (ratio 1.4), and when fed maize pollen, mean longevity was 35–42 days (ratio 1.2). The last *D. magna* in the flour treatments died between day 99 (ES-Eurojet) and day 105 (Tasty Sweet); in the leaf treatments between day 60 (Rheintaler) and day 78 (Tasty Sweet); and in the pollen treatments between day 58 (ES-Eurojet) and day 87 (Tasty Sweet).

The 95% CI of the mean longevity of *D. magna* fed flour from any of the five maize lines ranged between 40 and 90 days (ratio 2.3), for leaves between 19 and 49 days (ratio 2.6) and for pollen between 28 and 52 days (ratio 1.9) (Tables S2 and S3, Fig. S2).

3.4. Growth parameters

The body length and body width of *D. magna* fed maize materials increased over time (body length: $\chi^2 = 753.6$, $p < 0.0001$; body width: $\chi^2 = 498.1$, $p < 0.0001$) (Fig. 2). Size differed significantly among the five maize lines (body length: $\chi^2 = 37.9$, $p < 0.0001$; body width: $\chi^2 = 24.3$, $p < 0.0001$) and the three food sources (body length: $\chi^2 = 7.5$, $p = 0.02$; body width: $\chi^2 = 7.9$, $p = 0.02$). Because the interaction of the factors time, food source and maize line was also significant (body length: $\chi^2 = 24.5$, $p = 0.002$; body width: $\chi^2 = 20.6$, $p = 0.008$), separate analyses were conducted for each food source. For maize flour treatments, *D. magna* fed Rheintaler had significantly lower length and width compared with those fed other maize lines, EXP 258 had lower length and width than Planoxx, Tasty Sweet, and ES-Eurojet, and individuals fed ES-Eurojet had higher length and width compared with those fed other lines (length: maize line: $\chi^2 = 33.4$, $p < 0.0001$; interaction maize line × time: $\chi^2 = 28.4$, $p < 0.0001$; width: maize line: $\chi^2 = 22.2$, $p = 0.0002$; interaction maize line × time: $\chi^2 = 18.2$, $p = 0.001$). When fed Rheintaler leaves, *D. magna* had significantly lower length (maize line: $\chi^2 = 8.9$, $p < 0.0001$; interaction maize line × time: $\chi^2 = 2.3$, $p = 0.7$) and width (maize line: $\chi^2 = 12.8$, $p < 0.0001$; interaction maize line × time: $\chi^2 = 2.7$, $p = 0.6$) than when fed maize from the other lines. For pollen treatments, there were no differences among maize lines in length (maize line: $\chi^2 = 10.1$, $p = 0.8$; interaction maize line × time: $\chi^2 = 15.6$, $p = 0.004$) and width (maize line: $\chi^2 = 7.6$, $p = 0.7$; interaction maize line × time: $\chi^2 = 15.5$, $p = 0.004$).

There were no significant differences in the number of moltings to first offspring release for *D. magna* feeding on the three food sources (food, maize lines, and interaction, all $p \geq 0.1$, Table 1, Fig. 3A). The ratios of the highest to the lowest means were 1.4, 1.2, and 1.1 for flour, leaves, and pollen, respectively. The 95% CI for the mean number of moltings to first offspring release ranged between 6.0 and 9.4 for flour (ratio 1.6), 4.9–7.4 for leaves (ratio 1.5) and 5.9–7.6 for pollen (ratio 1.3) (Fig. 3A, Tables S2 and S3).

3.5. Reproduction parameters

For the time to first offspring release, significant differences were identified among the three food sources ($p < 0.0001$) and the five maize lines ($p = 0.0005$) (Table 1). Since the interaction of food source and maize line was also significant ($p = 0.006$), separate analyses were conducted for each food source. *D. magna* fed Rheintaler flour needed longer to reproduce than those fed flour of the other four maize lines ($p < 0.0001$). For maize pollen or leaf treatments, there were no significant differences among maize lines (all $p \geq 0.3$, Table 1, Fig. 3B). The

Table 1
Statistics of life table parameters of *Daphnia magna* fed flour, leaves, or pollen from five maize lines during their whole life time. N = 20 per maize material and line.

Parameter	Statistics, main analysis[a]	Statistics, separate analyses for maize materials		
		Flour	Leaves	Pollen
Longevity(Kaplan–Meier with log rank)		$\chi^2 = 7.9$, $p = 0.1$	$\chi^2 = 3.9$, $p = 0.4$	$\chi^2 = 3.4$, $p = 0.5$
Moltings to first offspring(GLMER)	Food: $\chi^2 = 4.6$, $p = 0.1$ Plant: $\chi^2 = 4.5$, $p = 0.3$ F × P: $\chi^2 = 6.7$, $p = 0.6$			
First offspring time(GLMER)	Food: $\chi^2 = 36.1$, $p < 0.0001$ Plant: $\chi^2 = 20.1$, $p = 0.0005$ F × P: $\chi^2 = 21.7$, $p = 0.006$	$\chi^2 = 47.3$, $p < 0.0001$	$\chi^2 = 4.6$, $p = 0.3$	$\chi^2 = 0.9$, $p = 0.9$
Individuals in first clutch(GLMER)	Food: $\chi^2 = 34.8$, $p < 0.0001$ Plant: $\chi^2 = 4.9$, $p = 0.3$ F × P: $\chi^2 = 6.3$, $p = 0.6$			
Total clutches(GLMER)	Food: $\chi^2 = 137.5$, $p < 0.0001$ Plant: $\chi^2 = 33.7$, $p < 0.0001$ F × P: $\chi^2 = 38.2$, $p < 0.0001$	$\chi^2 = 33.6$, $p < 0.0001$	$\chi^2 = 33.6$, $p < 0.0001$	$\chi^2 = 4.3$, $p = 0.4$
Total offspring(LMER)	Food: $\chi^2 = 38.0$, $p < 0.0001$ Plant: $\chi^2 = 31.0$, $p < 0.0001$ F × P: $\chi^2 = 43.9$, $p < 0.0001$	$\chi^2 = 36.7$, $p < 0.0001$	$\chi^2 = 24.5$, $p < 0.0001$	$\chi^2 = 6.6$, $p = 0.2$
Offspring per clutch(LMER)	Food: $\chi^2 = 38.9$, $p < 0.0001$ Plant: $\chi^2 = 38.5$, $p < 0.0001$ F × P: $\chi^2 = 32.9$, $p < 0.0001$	$\chi^2 = 65.8$, $p < 0.0001$	$\chi^2 = 16.0$, $p = 0.003$	$\chi^2 = 10.3$, $p = 0.04$

[a] F × P stands for food × plant interaction. In case of significant interactions in the main analysis, separate analyses were conducted for each maize material.

ratios of the highest to the lowest means were 1.5, 1.2, and 1.1 for flour, leaves, and pollen, respectively. The 95% CI for the mean time of first offspring release ranged between 14 and 25 days for flour (ratio 1.8), 12–16 days for leaves (ratio 1.3), and 13–15 days for pollen (ratio 1.2) (Fig. 3B, Tables S2 and S3).

The number of offspring in the first clutch of *D. magna* was significantly different among the three food sources ($p < 0.0001$), but not among the five maize lines ($p = 0.3$), and no interaction between the two factors was present ($p = 0.6$) (Table 1). *D. magna* fed maize leaves produced more offspring in the first clutch than those fed pollen or flour, and *D. magna* fed pollen produced more than those fed flour ($p < 0.0001$, Table 1, Fig. 3C). The ratios of the highest to the lowest means were 1.4, 1.4, and 1.3 for flour, leaves, and pollen, respectively. The 95% CI for the mean number of individuals in the first clutch ranged between 1.9 and 3.9 for flour (ratio 2.1), 2.6–6.5 for leaves (ratio 2.5), and 2.6–5.0 for pollen (ratio 1.9) (Fig. 3C, Tables S2 and S3).

The total number of clutches of *D. magna* differed significantly among the three food sources and among the five maize lines with a significant interaction of food source and maize line (all $p < 0.0001$, Table 1). Thus, separate analyses were conducted for each food source. The total number of clutches of *D. magna* fed Rheintaler or EXP 258 flour was lower than for the other three maize lines ($p < 0.0001$). *D. magna* fed Rheintaler or ES-Eurojet leaves produced less clutches than individuals fed Tasty Sweet or Planoxx leaves and individuals fed EXP 258 had fewer clutches than those fed Tasty Sweet ($p < 0.0001$). There were no significant differences for clutch number among maize lines in the pollen treatments ($p = 0.4$, Table 1, Fig. 4A). The ratios of the highest to the lowest means were 1.6, 2.1, and 1.2 for flour, leaves, and pollen, respectively. The 95% CI for the mean number of clutches ranged between 6.5 and 17 for flour (ratio 2.5), 4.0–12 for leaves (ratio 3.0), and 5.0–9.5 for pollen (ratio 1.9) (Fig. 4A, Tables S2 and S3).

The total number of offspring of *D. magna* differed significantly among the three food sources and the five maize lines with a significant interaction (all $p < 0.0001$, Table 1). Separate analysis for each food source revealed that *D. magna* fed Rheintaler or EXP 258 flour produced less total offspring than those fed Tasty Sweet or ES-Eurojet flour ($p < 0.0001$). *D. magna* fed Rheintaler leaves produced significantly less total offspring than those fed Tasty Sweet or Planoxx leaves and those fed Eurojet produced less than those fed Tasty Sweet ($p < 0.0001$). There were no significant differences among maize lines in the pollen treatments ($p = 0.2$, Table 1, Fig. 4B). The ratios of the highest to the lowest means were 2.1, 2.5, and 1.4 for flour, leaves, and pollen,

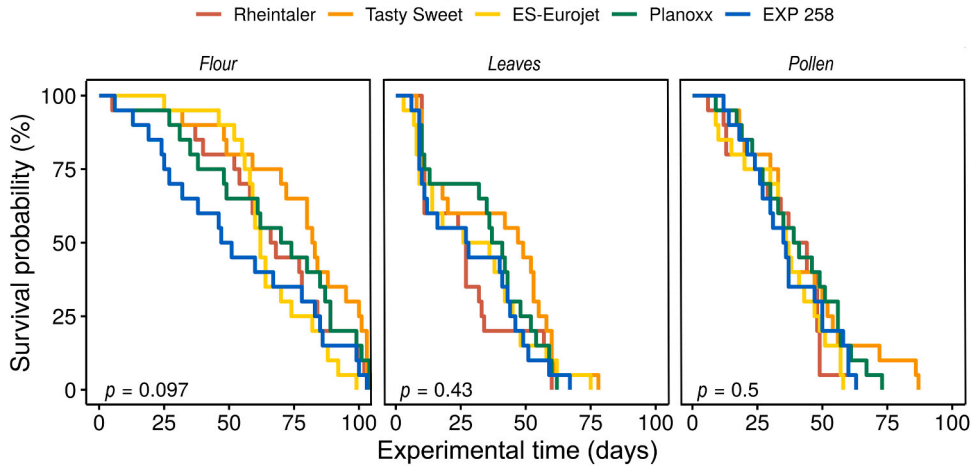

Fig. 1. Survival probability (%) of *Daphnia magna* fed flour, leaves, or pollen from five maize lines (n = 20). Data were analyzed for each food source separately using the Kaplan-Meier procedure with log-rank test.

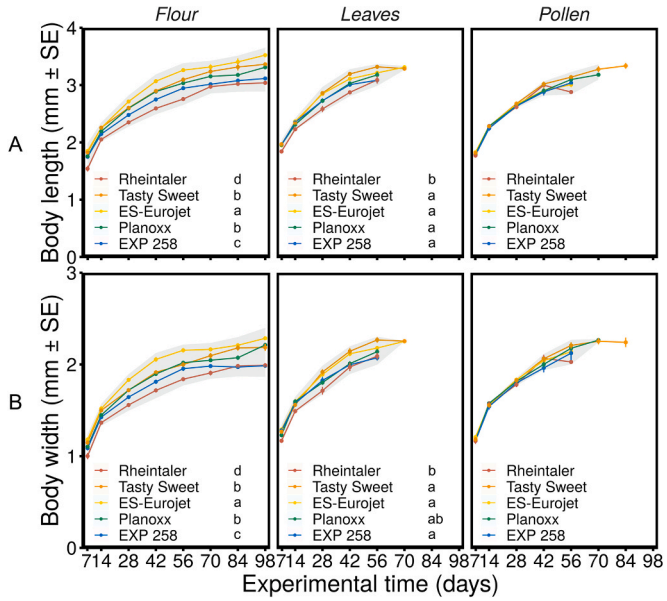

Fig. 2. Body length (A) and body width (B) of *Daphnia magna* fed flour, leaves, or pollen from five maize lines (n = 20). Measurements were taken at day 7, day 14, and then every 14 days. Data were analyzed using full factorial linear mixed effects models (LMER) with the fixed factors maize line, food and days of measurements, individual (each *D. magna*) as random factor. Different letters indicate significant differences ($p < 0.05$). Gray bands illustrate the highest and lowest value of the 95% confidence intervals over all maize lines.

respectively. The 95% CI for the mean total number of offspring ranged between 30 and 116 for flour (ratio 3.8), 26–99 for leaves (ratio 3.9), and 30–71 for pollen (ratio 2.3) (Fig. 4B, Tables S2 and S3).

Similar to the total number of offspring, also the number of offspring per clutch of *D. magna* differed for the three food sources and the five maize lines with a significant interaction (all $p < 0.0001$). *D. magna* fed EXP 258 flour produced less offspring per clutch than those fed other maize lines except for Rheintaler. Individuals fed Rheintaler produced less offspring per clutch than those fed Tasty Sweet or ES-Eurojet, and those fed Planoxx or Tasty Sweet produced less offspring per clutch than those fed ES-Eurojet ($p < 0.0001$). *D. magna* fed Planoxx or EXP 258 leaves produced less offspring per clutch than those fed ES-Eurojet leaves ($p = 0.003$). *D. magna* fed Rheintaler pollen had significantly more offspring per clutch than those fed EXP 258 pollen ($p = 0.04$, Table 1, Fig. 4C). The ratios of the highest to the lowest means were 1.6, 1.3, and 1.3 for flour, leaves, and pollen, respectively. The 95% CI for the mean number of offspring per clutch ranged between 4.0 and 8.0 for flour (ratio 2.0), 5.4–9.3 for leaves (ratio 1.7), and 5.1–8.2 for pollen (ratio 1.6) (Fig. 4C, Tables S2 and S3).

3.6. Age-stage life table parameters

The net reproductive rate (R_0) of *D. magna* fed flour of ES-Eurojet or Tasty Sweet was significantly higher than that of *D. magna* fed flour of Rheintaler and EXP 258 (ES-Eurojet with Rheintaler, $p < 0.0001$, adj. $\alpha = 0.005$; with EXP 258, $p < 0.0001$, adj. $\alpha = 0.005$; Tasty Sweet with Rheintaler, $p = 0.0008$, adj. $\alpha = 0.006$; with EXP 258, $p = 0.0009$, adj. $\alpha = 0.007$). R_0 of *D. magna* fed Tasty Sweet or Planoxx leaves was higher than that of *D. magna* fed Rheintaler leaves ($p = 0.002$, adj. $\alpha = 0.005$; $p = 0.0008$, adj. $\alpha = 0.006$, respectively). R_0 of *D. magna* fed pollen was not affected by the different maize lines (all $p >$ adj. $\alpha = 0.005$) (Fig. 5A). The ratios of the highest to the lowest means were 2.3, 2.9, and 1.3 for flour, leaves, and pollen, respectively. The 95% CI for R_0 ranged between 27 and 114 for flour (ratio 4.2), 11–74 for leaves (ratio 6.5), and 27–63 for pollen (ratio 2.3) (Fig. 5A, Tables S2 and S3).

The generation time (T) of *D. magna* fed Rheintaler flour was

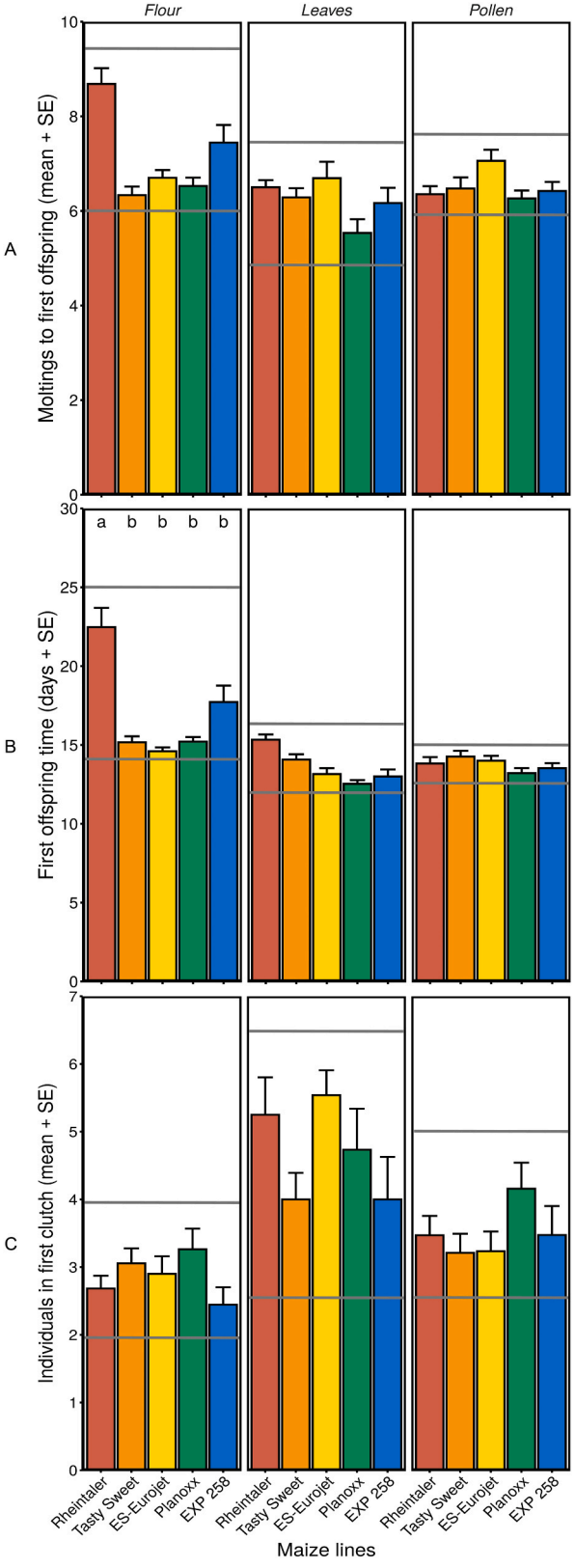

Fig. 3. Moltings to first offspring (A), time to first offspring (B), and individuals in first clutch (C) of *D. magna* fed flour, leaves, or pollen from five maize lines. Data were analysed using GLMER with maize (five lines) and food (flour, leaves, pollen) as fixed factors, experimental repetition as random factor. Different letters indicate significant differences ($p < 0.05$). Bars represent means + SE for each maize line (n = 20). Gray lines illustrate the highest and lowest value of the 95% confidence intervals over all maize lines.

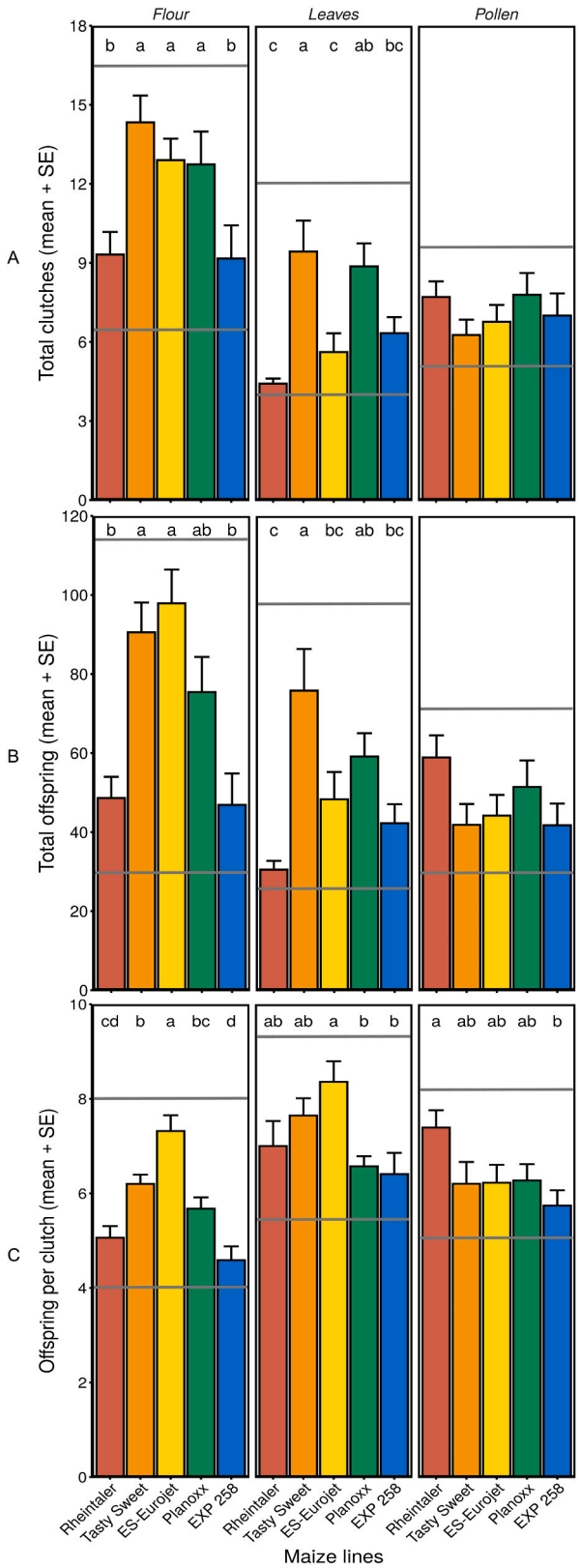

Fig. 4. Total number of clutches (A), total number of offspring (B), and number of offspring per clutch (C) of *D. magna* fed flour, leaves, or pollen from five maize lines. Data were analyzed using GLMER with maize (five lines) and food (flour, leaves, pollen) as fixed factor, experimental repetition as random factor. Different letters indicate significant differences ($p < 0.05$). Bars represent means + SE for each maize line (n = 20). Gray lines illustrate the highest and lowest value of the 95% confidence intervals over all maize lines.

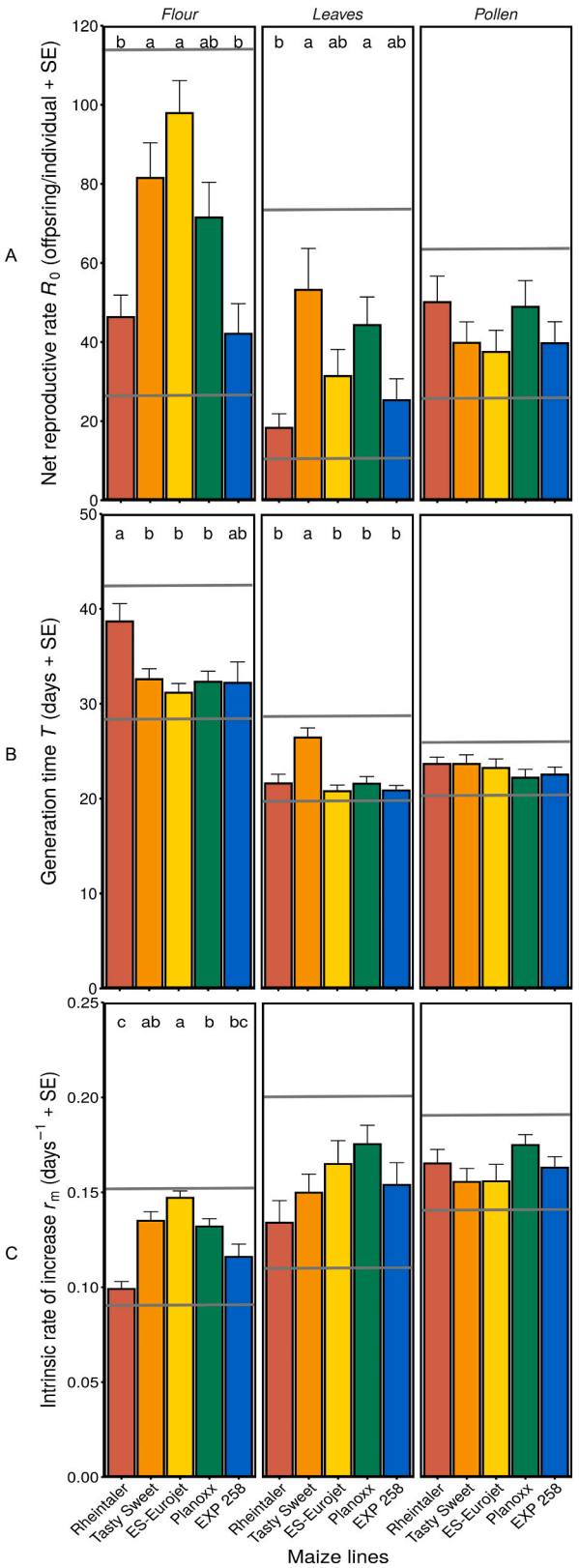

Fig. 5. Age-stage life table parameters of *D. magna* fed flour, leaves, or pollen from five maize lines: net reproductive rate R_0 (A), generation time T (B) and intrinsic rate of increase r_m (C). Data were analyzed by paired bootstrap test with the TWOSEX-MSChart software. Bars represent means + standard error (SE) calculated with 10′000 bootstrap replicates. Different letters within the same column indicate significant difference ($p <$ adj. α). Gray lines illustrate the highest and lowest value of the 95% confidence intervals over all maize lines.

significantly higher than of those fed flour from other maize lines except EXP 258 (differences with ES-Eurojet, $p = 0.0006$, adj. $\alpha = 0.005$; with Planoxx, $p = 0.002$, adj. $\alpha = 0.006$; with Tasty Sweet, $p = 0.005$, adj. $\alpha = 0.006$). T of *D. magna* fed Tasty Sweet leaves was higher than of those fed leaves from other maize lines (differences with EXP 258, $p = 0.0001$, adj. $\alpha = 0.005$; with ES-Eurojet, $p = 0.0001$, adj. $\alpha = 0.005$; with Planoxx, $p = 0.0004$, adj. $\alpha = 0.006$; with Rheintaler, $p = 0.001$, adj. $\alpha = 0.007$) and T of *D. magna* fed pollen was not affected by maize line (all $p >$ adj. $\alpha = 0.005$) (Fig. 5B). The ratios of the highest to the lowest means were 1.2, 1.3, and 1.1 for flour, leaves, and pollen, respectively. The 95% CI for T ranged between 28 and 42 days for flour (ratio 1.5), 20–28 days for leaves (ratio 1.5), and 21–26 days for pollen (ratio 1.2) (Fig. 5B, Tables S2 and S3).

The intrinsic rate of increase (r_m) of *D. magna* fed ES-Eurojet flour was significantly higher than that of *D. magna* fed flour from the other maize lines except for Tasty Sweet (differences with EXP 258, $p < 0.0001$, adj. $\alpha = 0.005$; with Rheintaler, $p < 0.0001$, adj. $\alpha = 0.005$; with Planoxx, $p = 0.007$, adj. $\alpha = 0.008$); r_m of *D. magna* fed Tasty Sweet or Planoxx flour was higher than of those fed Rheintaler flour (all $p < 0.0001$, adj. $\alpha = 0.005$). The r_m of *D. magna* fed pollen or leaves was not affected by maize line (all $p >$ adj. $\alpha = 0.005$) (Fig. 5C). The ratios of the highest to the lowest means were 1.5, 142, and 1.1 for flour, leaves, and pollen, respectively. The 95% CI for r_m ranged between 0.09 and 0.15 day^{-1} for flour (ratio 1.6), 0.11–0.20 day^{-1} for leaves (ratio 1.8), and 0.14–0.19 day^{-1} for pollen (ratio 1.4) (Fig. 5C, Tables S2 and S3).

4. Discussion

4.1. Experimental conditions

The experimental conditions in our study were adjusted according to the guideline OECD211 (OECD Organization for Economic Co-operation and Development, 2012), and all values for the quality of the ADAM medium (pH, DOC, hardness) were within the demanded range. According to the OECD guideline standardized M4 or M7 medium is recommended, but other media are accepted if the performance of *D. magna* is shown to meet the validity criteria of the test. Several studies used ADAM medium, which is well suited for culturing *D. magna* (Ebert et al., 1998; Ho et al., 2019; Martin-Creuzburg et al., 2019). We also conducted a preliminary experiment with *D. magna* fed algae for 21 days to compare ADAM medium with M4 medium. No individuals died in either medium and no significant differences were observed for growth or reproduction parameters (Table S4, supplementary online material). Therefore, we decided to use ADAM medium, which is less complex and easier to prepare. At day 21 of the present study, *D. magna* fed algae showed 0% mortality and the cumulative fecundity was 101, which is in accordance with the validity criteria of the OECD211 test, i.e., mortality after 21 days < 20% and mean number of living offspring produced per parent animal ≥ 60 (OECD Organization for Economic Co-operation and Development, 2012). This indicates that the specimens used for our experiment were healthy and the experimental conditions suitable. At day 69, the mortality of *D. magna* reached 20%, at which time the individuals had been measured for body size for five times. This indicates that the handling necessary for recording body measurements did not impair *D. magna* performance.

4.2. Suitability of maize materials as exclusive food for D. magna

D. magna can survive, grow and reproduce when fed only maize and all three tested materials proved suitable. At day 21, the mortality of *D. magna* fed flour was 0–15%, when fed leaves 30–45%, and when fed pollen 15–20%, depending on the maize line. Mortality was thus exceeding the maximum of 20% set as a validity criterion in the OECD standard in the leaf treatments. In addition, the mean total number of offspring produced by *D. magna* fed maize material within the first 21 days remained below the minimum of 60 offspring set by OECD for all maize materials and lines (varying between 3.3 and 19 depending on material and line).

For the full life-cycle, *D. magna* fed maize flour survived longer than those fed pollen or leaves, but had a higher generation time T and a reduced body size. In addition, *D. magna* fed flour produced more offspring and more clutches during their life time and had a higher net reproductive rate R_0, than those fed pollen or leaves, but they needed more time to release the first offspring, and they had a lower intrinsic rate of increase r_m. This demonstrates that the maize materials have a different nutritional quality for *D. magna* and also the allocation of nutrients to survival, growth and reproduction may differ. *D. magna* fed maize flour tended to allocate nutrients to survive first, followed by growth and reproduction. Compared with the algae treatment, however, *D. magna* fed maize flour, leaves or pollen showed smaller body size, lag in the first time of reproduction, a reduction in the total number of offspring, a reduction of the net reproductive rate R_0, an increase in generation time T, and a reduction of the intrinsic rate of increase r_m.

Previous studies have shown effects of low quality food on *D. magna* performance. Stige et al. (2004) reported that *D. magna* exposed to nutritional stress by reduced food (green algae *Selenastrum capricornutum*) quantity and/or quality (phosphorus-limitation) showed reduced growth and reproduction. Bouchnak and Steinberg (2010) reported that fertility was decreased in *D. magna* fed low quality food (baker's yeast compared to green algae *Pseudokirchneriella subcapitata*). In addition, food stress has also been reported to initiate diapause (Han et al., 2011) and increase the production of male offspring (Hobaek and Larson, 1990; Kleiven et al., 1992).

Some previous studies to assess GE plant effects on *D. magna* used maize materials as food. When Zhang et al. (2018) fed *D. magna* with maize flour for 28 days, the mean time of first offspring release was 12.5 days and similar results were reported by Bøhn et al. (2010) (13 days). Thus the values of both studies were lower than the range of the five maize lines of the present study (14–25 days). The reported mean body length in Zhang et al. (2018) at day 28 was 2.5 mm and in the studies of Bøhn et al. (2008, 2010) between 2.5 and 3.0 mm at day 42. The means of the five maize lines in our study cover those values with body length of *D. magna* at day 28 between 2.3 and 2.7 mm and at day 42 between 2.6 and 3.1 mm. Bøhn et al. (2008) fed *D. magna* with maize flour at a similar feeding dose as in our study for 42 days, and the mean number of offspring per clutch was 5.1, which was within the range of our results (4.0–8.0). While their study showed that not all individuals in the experiments reached maturation, in our experiments, all the individuals in both experimental repetitions reached maturation before 42 days. Holderbaum et al. (2015) fed *D. magna* with maize leaves at a similar dose than in our study for 42 days, and the median time of first offspring release was 12 days, which was within the range of the five maize lines in our study (12–16 days). However, Holderbaum et al. (2015) observed the production of ephippia (protective structures enclosing two dormant eggs), while no ephippia were produced in our study. These differences are likely due to the different *D. magna* clones and a different photoperiod used. Holderbaum et al. (2015) and Bøhn et al. (2008, 2010) used an arctic clone and a photoperiod of 24 h daylight. Photoperiod can change the life cycle of zooplankton and significantly affect the development and proliferation. Ferrari and Hebert (1982) found that arctic clones of *D. magna* with 24 h daylight tend to produce ephippia and males, which is part of the survivorship and reproductive behavior adapted to extreme conditions, i.e., populations must produce males and bisexual eggs to survive the periods when ponds are frozen. Furthermore, Gao et al. (2006) reported that *D. magna* has a reduced feeding rate under 24 h daylight. The clone we selected for our study produced only females and no ephippia under our experimental conditions.

In summary, *D. magna* can survive, grow, and reproduce on different maize materials, but performance is reduced compared to optimal food, such as green algae. This has also been acknowledged in previous studies (Bøhn et al., 2008; Holderbaum et al., 2015; Zhang et al., 2018). The fact that the OECD validity criteria for chronic exposure tests with *D. magna*

are not met indicates nutritional stress. This bears the risk of confounding effects, which may generally limit the reliability of studies.

4.3. Differences among maize lines

In this study, five very different non-GE maize lines were used. Rheintaler is a Swiss landrace and population maize (no hybrid), with different breeding goals and obvious phenotypical differences to commercial hybrid maize. Tasty Sweet is a sweet maize bred for human consumption with different grain composition than field maize (very little starch in the grains). ES-Eurojet and Planoxx are two commercial varieties used in Switzerland with different maturation times and different grain characteristics (dent maize and durum maize), and EXP 258 is a breeding line from the USA and the nearest non-GE hybrid to one SmartStax *Bt* line.

In the flour treatments of our study, *D. magna* fed Rheintaler showed smaller body size, longer time to first offspring release, less clutches, less total offspring, higher generation time *T*, lower net reproductive rate R_0, and lower intrinsic rate of increase r_m than those fed any of the other maize lines. Similarly, *D. magna* fed Rheintaler leaves had the smallest body size, least total clutches, least total offspring, least R_0 and least r_m. In contrast, in the pollen treatments, *D. magna* fed Rheintaler produced more offspring per clutch than those fed EXP 258 maize pollen. Differences of EXP 258 maize to the other hybrids were less pronounced. In the flour treatments, however, *D. magna* fed EXP 258 were smaller, had less offspring, and reduced R_0 and r_m than at least one of the three commercial hybrids. In addition, some differences between EXP 258 and other hybrids were also observed when fed leaves.

These results illustrate that different maize materials and lines differed in their nutritional quality for *D. magna*. In the maize flour and leaf treatments, more significant differences and higher variability for the life table parameters of *D. magna* were observed than in the pollen treatments. Reproductive parameters showed a relatively high variability among the different maize lines, such as the total number of clutches, total offspring, and R_0 for flour and leaf treatments and offspring per clutch for flour (ratios of highest to lowest mean values between 1.6 and 2.9). Other parameters in the flour and leaf treatments and all parameters in the pollen treatments were less variable with ratios between 1.1 and 1.5.

By calculating the 95% CI around each parameter mean for each maize material and line, we provide estimates in which ranges the true means would lie. We defined the interval between the highest value and the lowest value of those 95% CI boundaries over all maize lines as the natural range of variation and the ratio of the highest value divided by the lowest value provides an impression how variable the individual parameters can be among different maize lines. Naturally, those ratios of the highest and lowest confidence limits are higher than the ratios of the actual means. Once more, the highest ratios were evident for total number of clutches, total offspring, and R_0 (ratios between 1.9 and 6.5), while other parameters had lower ratios (1.2–1.9). When we take the total number of offspring as an example, those ratios indicate that the true mean of one maize line might be around 4 times higher than that of another maize line. This is relevant since the commercialized non-GE maize lines are generally considered to cause no unacceptable harm to the environment.

That life-table parameters or food consumption of non-target insects can strongly vary among different maize hybrids has previously been reported in laboratory feeding studies for terrestrial species, including *Porcellio scaber* (Isopoda: Oniscidea) (Wandeler et al., 2002), *Drosophila melanogaster* (Diptera: Drosophilidae) (Knecht and Nentwig, 2010), *Megaselia scalaris* (Diptera: Phoridae) (Knecht and Nentwig, 2010), *Coleomegilla maculata* (Coleoptera: Coccinellidae) (Pilorget et al., 2010), *Oulema melanopus* (Coleoptera: Chrysomelidae) (Meissle et al., 2012), and *Chrysoperla carnea* (Neuroptera: Chrysopidae) (Meissle et al., 2014).

4.4. Implications for risk assessment of GE plants

Previous scientific studies to assess the impact of *Bt* maize on *D. magna* compared tissue from one *Bt* maize line to that of a non-*Bt* line. This carries the risk that adverse effects seen in some studies might have been caused by differences in the plant background rather than the *Bt* protein itself (Romeis et al., 2011, 2013), especially since maize material is clearly a suboptimal food for *D. magna* causing nutritional stress. Even if the closest related non-GE counterpart to a given GE plant is chosen as a comparator, the transformation process, the production of the new GE trait, and the regeneration and breeding steps after the transformation may lead to differences in plant composition. It is thus very difficult to control for plant background effects, especially because knowledge about the effects of all the different nutrients and antinutrients in plant material on *D. magna* (and other species used for ecotoxicological testing) is limited. To address this, Chambers et al. (2010) selected *Bt* and non-*Bt* maize lines for testing different stream macroinvertebrates based on C:N ratios and lignin content. However, this selection seems arbitrary because there might be many other plant compounds that potentially influence invertebrate performance.

The natural variation among maize lines can be used to interpret statistical differences detected when comparing a particular GE line with its non-GE comparator and to define whether they might be of biological relevance. In the GE crop risk assessment this approach is commonly applied in the comparative food/feed safety assessment where substantial equivalence analyses are conducted to assess whether foods and feed derived from the GE crop are as safe as their conventional counterparts (Anderson et al., 2019, 2020; EFSA, 2010; Hong et al., 2014). In our study, the natural variation for our maize lines, based on the ratios of the highest to the lowest confidence limit, ranged between a factor of 1.2 (first offspring time of *D. magna* when fed pollen) to 6.5 (R_0 when fed leaves).

We acknowledge, however, that our subsample of five maize lines is unlikely to represent the population of all possible maize lines, so the natural range of variation for all potential maize lines is likely to be much broader.

For example, Bøhn et al. (2008) reported that *D. magna* fed flour of a *Bt* maize showed a 37% reduction in longevity compared to a non-*Bt* line (ratio 1.6). Despite the fact that this reduction was statistically significant, it might not be of high biological relevance given the fact that the maximum mean difference in longevity among the various non-GE maize lines in our study was also around 30% (ratio 1.4) and the potential difference based on the 95% CI was estimated to be 56% (ratio 2.3).

Better than interpreting the values of the current study would be if future studies with plant material from GE and non-GE maize would include multiple conventional lines to capture the natural range of variation in that particular context. This, however, would increase the complexity (and costs) of non-target studies and would only be helpful if differences between the GE and non-GE comparator would actually be detected. One solution would be to first conduct a study with only the GE and non-GE comparator and only if adverse effects of the GE line are observed, repeat the study with multiple conventional comparators 1) to confirm the observed effects between the GE and non-GE comparator, and 2) to interpret this effect in the context of natural variation of conventional lines.

In the case of *D. magna* even feeding studies with a range of maize lines as additional comparators need to be interpreted with caution given the fact that maize material overall is of low nutritional quality for *D. magna*. In the environment the organisms will have access to a range of different food items and maize material is likely to represent only a small fraction of their diet. One might thus question if *D. magna* is a suitable surrogate test organism for crop residues in aquatic ecosystems or if there are other species that perform better when fed maize materials. In fact, other aquatic species have been used for feeding assays with *Bt* maize, e.g. other crustaceans, such as isopods (Jensen et al.,

2010) or amphipods (Li et al., 2013; Chambers et al., 2010), caddisflies (Rosi-Marshall et al., 2007; Chambers et al., 2010; Jensen et al., 2010), or fly larvae, such as Tipulidae (Jensen et al., 2010) and Chironomidae (Prihoda and Coats, 2008; Li et al., 2013). Similar to *D. magna*, however, the nutritional quality of maize material as exclusive food for those species is also likely to be suboptimal and standardized test protocols for oral toxicity are also lacking.

5. Conclusions

To our knowledge, this is the first study, which compared different food types (flour, leaves and pollen) from a number of non-GE maize lines throughout the complete *D. magna* life cycle. The species can survive, grow, and reproduce on all three maize materials. Performance of *D. magna* fed maize, however, was reduced compared to high quality food (green algae) and some of the validity criteria formulated by the OECD standard (OECD Organization for Economic Co-operation and Development, 2012) were not met. It is thus apparent that *D. magna* provided only with maize as food are nutritionally stressed. This implies that confounding effects of poor food quality might have influenced previously published results on the effects of *Bt* maize on *D. magna*. In our study, large differences in life table and population parameters of *D. magna* were observed among the five different maize lines. The natural range of variation based on 95% CI showed that in particular reproductive parameters may vary up to a factor of 6, while other parameters, such as time to first offspring release, were less variable (factor 1.2–1.8).

If differences between a GE and comparator line are observed and their biological relevance needs to be assessed in future risk assessments of GE maize, 1) the data on natural variation of the different parameters generated by previous studies can be informative (e.g. data from our study for maize fed *D. magna*); 2) for additional experiments the inclusion of multiple unrelated non-GE comparators should be considered; In addition, it should be taken into account that nutritional stress can affect the outcome of the study.

CRediT authorship contribution statement

Yi Chen: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Jörg Romeis:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing. **Michael Meissle:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.111967.

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