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Low toxin doses change plant size distribution in dense populations – Glyphosate exposed *Hordeum vulgare* as a greenhouse case study

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ABSTRACT

Numerous intentionally released toxins persist in agricultural or natural environments at low concentrations. Such low toxin doses are regularly associated with hormesis, *i.e.*, growth stimulation, and they are suspected to affect mortality and within-population plant size distribution in dense plant stands. However, it is not known whether all these low-dose effects exist when plants grow in soil. We exposed barley to a range of low glyphosate doses and let the plants grow in dense stands for several weeks in soil. Six experiments were done that contained altogether 10,260 seedlings in 572 pots. We evaluated if the changes in average biomass and shoot length occur at the same concentrations as do the effects on slow- and fast-growing individuals, if seed size or early vigor explains variation in the response to glyphosate, and if low toxin doses change within-population mortality.

Plant biomass, length and survival of subpopulations changed at doses that did not affect mean biomass. Effects of early vigor faded early, but differences in seed size and particularly vegetative growth had impacts: fast-growing plants hardly showed hormesis, whereas hormesis was particularly strong among slow-growing individuals. Compared to the population mean, glyphosate effects started at lower doses among slow-growing individuals and at higher doses among fast-growing individuals. Several times higher doses were needed before the fast-growing individuals showed the same toxicity as most of the population. Low toxin doses regularly enhanced the growth of the smallest individuals, which reduced size variation within populations and was associated with a higher number of surviving plants. Indeed, in one experiment self-thinning was not observed at low doses that stimulated the growth of slow-growing plants.

As glyphosate levels in this study match those observed in agricultural fields and natural environments, we conclude that even low-levels of agro-environmental contamination are likely to shape phenotypic response, which might lead to adaptation and cascading ecological impacts.

1. Introduction

In almost all environments, plants are exposed to a variety of compounds that are present either unintentionally (Tao et al., 2011; Klaschka et al., 2013) or after being applied, for example, to agricultural fields for pest control (Kjær et al., 2005; Kauppi et al., 2012). Many chemical exposures found in the environment are within the range of subtoxic low-dose exposures (Hansi et al., 2014). In case of agrochemicals, subtoxic low-dose exposures of plants following regular applications for pest control can occur on treated fields (*e.g.*, errors in application, leaf contact of treated and untreated plants, protection by taller plants or mulch, herbicide resistance, soil degradation/immobilization) or in surrounding environments *via* spray drift deposition or run-off (Belz and Duke, 2014, 2017; Velini et al., 2017). Several recent studies suggest that such subtoxic low-dose exposures are not without effect but can lead to alterations in plant growth, *i.e.,* hormesis (Duke et al., 2006; Cedergreen et al., 2007), and/or selective growth effects on individual plants within a population. The latter effects may change the size of fast- or slowly-growing subpopulations, without changing the mean plant size in the overall population and, thus, without a visible effect at the population level (Sinkkonen et al., 2008, 2009, 2011; Belz and Sinkkonen, 2016a, 2016b; Belz et al., 2018a). Selective growth effects are likely to directly change the size distribution of a population and in the long-term affect the survival of individual phenotypes within a plant population (Sinkkonen et al., 2011). It is thus indicated that low toxicant doses can select for and against certain subpopulations due to variable responses of individual plants of the same population (Belz et al., 2018a). The immediate effect of this

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selection pressure is unlikely to change the overall population response. However, the long term effect from such a shift in phenotypes may cause genotypic adaptation and changes in the overall population response followed by cascading impacts on biodiversity and ecosystem processes (Silva et al., 2016; Belz and Sinkkonen, 2016a; Velini et al., 2017). Moreover, a recent study indicates this phenomenon is widespread among plant toxicants (Belz et al., 2018a). While the occurrence of plant hormesis with toxins under greenhouse or field conditions is documented (*e.g.*, Velini et al., 2008; Cedergreen et al., 2009), most previous studies confirming selective low-dose effects have been conducted only under non-natural laboratory conditions (Sinkkonen et al., 2008, 2009, 2011; Belz and Sinkkonen, 2016a, 2016b; Belz et al., 2018a). The potential occurrence under more natural conditions or at a broader ecological scale has not been investigated. Therefore, the transferability of previous laboratory findings of selective low-dose effects to soil-grown plant populations represents a critical step towards understanding the significance of selective low-dose effects in toxin-exposed environments.

Within a dose-response continuum the low-dose range is located at subtoxic doses, *i.e.*, at doses lower than those causing an increasing adverse response with increasing dose up to a maximum effect at infinitely high doses (reviewed in Belz et al., 2018a). In the low-dose range, divergent responses can occur depending on the dose at action. A "*first (ultra-)low-dose part*" leaves most of a population unaffected and shows no significant changes in mean responses. If the ultra-low-dose part is preceding the toxic dose range, we speak of a *monophasic* or *monotonic* dose-response curve. A *biphasic* or *hormetic* pattern results if the ultra-low-dose part is followed by a "*second low-dose part*" characterized by an increase in mean responses (hormesis peak) at the population level before high-dose inhibition. The quantitative features defining these dose zones are effective doses (*ED*) causing a certain level of response, *e.g.,* the ED_{110} causing 10% stimulation over control to mark the end of the first ultra-low-dose part and the beginning of the second low-dose part and the ED_{10} causing 10% inhibition to mark the end of the low-dose part and the beginning of high-dose inhibition (Belz et al., 2018a). Since the response of a population to a toxin depends on its sensitivity, the *ED* values and, thus, the boundaries of the low-dose part are not static, but population-dependent. For example, a herbicide resistant weed population can still show hormesis at herbicide doses that are lethal to a sensitive population of the same weed species (Belz et al., 2018b). Hence, a certain dose can induce low-dose responses in one population, while another suffers high-dose toxicity at the same dose. Within a population, the same phenomenon can occur between individuals of this population due to differences in individual sensitivity, *e.g.*, sensitive and resistant plants (Belz et al., 2018b) or slow- and fast-growing plants, being the basic principle for selective low-dose effects.

Selective toxin effects on the most fast- and slow-growing individuals within a plant population have been observed in the form of "selective low-dose toxicity" and "selective low-dose stimulation" occurring at ultra-low doses, but also in the formation of hormesis (Sinkkonen et al., 2008, 2009, 2011; Belz and Sinkkonen, 2016a, 2016b). Previous studies observed that selective low-dose toxicity, selective low-dose stimulation and hormesis can occur in parallel within a dose-response continuum (Belz and Sinkkonen, 2016a; Belz et al., 2018a). Selective low-dose toxicity implies that even though a low-dose exposure seems ineffective on the overall population, a certain percentile within the population (*e.g.*, plants below the 5th or beyond the 95th percentile) can suffer deterioration (Aina et al., 2006; Sinkkonen et al., 2011). At doses close to the onset of toxicity at the overall population, the slow-growing subpopulations can already selectively suffer substantial inhibition due to a higher sensitivity (Belz and Sinkkonen, 2016a, 2016b; Belz et al., 2018a). In contrast, since the fast-growing subpopulations can be considerably less sensitive than most of the population, they can still show selective hormesis at doses that do already harm most of the population (Belz and Sinkkonen, 2016a, 2016b; Belz et al., 2018a). Hence, selective effects on individual plants can be observed at doses exceeding the low-dose range of a population due to an individual shift of the low-dose range towards higher doses at fast-growing individuals. Resulting changes in size distribution of crowded plant populations have been confirmed for several toxins in Petri dish bioassays on aqueous media with wild and cultivated plant species (Sinkkonen et al., 2008, 2009, 2011; Belz and Sinkkonen, 2016b; Belz et al., 2018a; Patama et al., 2019).

This study fills the crucial gap in knowledge on the significance of selective low-dose toxin effects under more natural conditions and addresses the low-dose mediated shifts in size distribution of crowded plant populations in a greenhouse pot trial. The main objective was to investigate selective low-dose effects for the most used herbicide worldwide glyphosate [N-(phosphonomethyl)glycine] on the most fast- and slow-growing individuals in high-density populations of the common crop plant *Hordeum vulgare* L. (barley). Moreover, the mechanistic basis for selectivity was investigated. Glyphosate was selected for being agriculturally and ecotoxicologically relevant and for showing selective low-dose effects (Belz et al., 2018a) and biphasic responses towards barley (Cedergreen, 2008; Cedergreen et al., 2009). Non-target terrestrial plants can be exposed to glyphosate *via* unintended drift during spraying in neighboring fields or in environments surrounding the treated fields (Cederlund, 2017; Lucadamo et al., 2018; Ravier et al., 2019). The resulting effects can be complex and include adverse effects as well as stimulatory, hormetic effects depending on *e.g.* the level of spray drift, the endpoint measured, or the plant species exposed (Cederlund, 2017).

Based on this, we exposed barley in three high-density trials (2880–3120 individuals per trial) and three mechanistic experiments *via* spray application to glyphosate at doses that do and do not change the mean response of a barley population. The effects were evaluated for three different endpoints, namely shoot length, shoot dry weight as well as plant survival. The following hypotheses were studied in detail: (1) low-dose effects on size distribution are relevant for glyphosate under greenhouse conditions; (2) a biphasic response at the population level may be accompanied by a monophasic response at low or high percentiles, and *vice versa*; (3) responses of fast-growing individuals are least sensitive and stimulatory and the opposite is found among slow-growing individuals; (4) low-dose stimulation at the population level has the potential to reduce plant mortality in crowded stands; and (5) seed size and early germination vigor contribute to the variation in glyphosate sensitivity between individual plants.

2. Materials and methods

2.1. Herbicide

Glyphosate was used as formulated product [Glyfos Supreme with 450 g active ingredient (a.i.) per liter (607 g/l isopropylamine salt), Cheminova Deutschland GmbH] that was mixed in demineralized water to give various test solutions for spray application. Depending on the individual experiment, 11–16 treatments were tested besides an untreated control (Table 1) at a concentration range of between 0.02 and 2000 g a.i./ha. Herbicide application was carried out using a laboratory sprayer equipped with a flat-fan nozzle (type TP 8002 EVS, TeeJet-Spraying Systems, USA) at 300 kPa pressure, at a constant spray volume of 200 l/ha, at a speed of 800 mm/s, and at 50 cm distance between sprayed surface and nozzle. In order to achieve a most uniform exposure of plants, pots were consistently placed in the middle of the sprayed area. The majority of approved field rates for glyphosate treatments in Germany vary between 1080 and 1800 g ai/ha.

2.2. Greenhouse pot trials

2.2.1. Cultivation

Seeds from spring barley var. RGT Planet (source: University of

Table 1
Individual dose-response experiments conducted to investigate the effect of glyphosate on *Hordeum vulgare* var. RGT planet.

Exp.	Trial period (month-year)	Plant density (plants/replicate)	Number of treatments/replicates	Greenhouse temperature and relative humidity	Endpoints
1	07/08-2018	30	13/8 (total 3120 plants)	Day 31.9 ± 6.7 °C, 41 ± 16% Night 21.5 ± 2.9 °C, 66 ± 12%	Shoot dry weight Survivors
2	09/10-2018	30	12/8 (total 2880 plants)	Day 27.2 ± 6.4 °C, 38 ± 10% Night 17.5 ± 2.0 °C, 58 ± 7%	Shoot dry weight Shoot length
3	10/11-2018	30	12/8 (total 2880 plants)	Day 26.0 ± 5.5 °C, 44 ± 11% Night 17.4 ± 1.7 °C, 61 ± 6%	Survivors
4	10/11-2018	5	12/6	Day 22.1 ± 2.5 °C, 50 ± 7% Night 15.7 ± 1.1 °C, 63 ± 6%	Shoot dry weight Shoot length
5	12/01-2018/19	5	17/6	Day 23.9 ± 3.1 °C, 50 ± 7%	
6	12/01-2018/19	5	17/6	Night 15.7 ± 1.7 °C, 65 ± 7%	

Hohenheim, Ihinger Hof 2018, Germany) were placed into plastic boxes (20 × 20 × 6 cm) onto one layer of filter paper soaked with 20 ml of demineralized water and allowed to germinate depending on the individual experiment (exp.) for one, two or three days in a growth chamber (24/18 °C, 12 h light). The boxes were closed with a lid and watered as required with demineralized water.

Germinated seeds (seeds with an emerged radicle of 0.5–2.0 cm with or without a coleoptile) were then transplanted into polypropylene pots (10 cm diameter, 7.5 cm height, 0.43 l volume) filled with a soil substrate (high loamy sand with pH 6.9 and 5.1% C_{org}) and covered with a thin layer of vermiculite (Agrivermiculite 2–3 mm, Floragard Vertriebs GmbH, Germany) to keep seeds moist. Seedlings were cultivated in a greenhouse for four to five days and then treated with glyphosate at a growth stage of one shoot [BBCH 10 (Meier, 1997)].

After the application, pots were put on water tight trays (31 × 53 × 1 cm) and trays were placed in a completely randomized design that was changed three times a week. Plants were watered with tap water as required and exposed to additional light (300 μE/m^2s) from 6 to 11 am and 4–8 pm. The average day (6 am to 8 pm) and night (8 pm to 6 am) temperatures and the relative humidity prevailing during the respective trial periods are given in Table 1. After a growth period of 21 days after treatment (DAT) with glyphosate, each individual plant of the 30 plants per replicate was cut at soil surface, dried for three days at 80 °C, and the number of dead plants per replicate (survival) as well as the dry weight and shoot length of each individual plant were measured (Table 1).

2.2.2. Individual experiments

Three separate experiments (exp. 1–3; Table 1) were conducted to investigate selective low- and high-dose effects. In these experiments, a high plant density of 30 plants/pot was used (one pot represented one experimental unit or one replicate). Initially, 32 germinated seeds were transferred to each replicate pot and thinned to 30 plants prior to glyphosate application in exp. 1 and 2. In exp. 3, pre-soaked seeds (one day on moist filter paper) were transferred at a density of 40 seeds/replicate and thinned to 30 plants prior to glyphosate application. The emergence of residual seeds after application was checked once at 3 DAT.

Three additional experiments (exp. 4–6; Table 1) were conducted to investigate the underlying mechanisms of response inequality at a plant density of 5 plants/replicate. Therefore, six germinated seeds were initially transferred to each replicate pot and thinned to 5 plants prior to glyphosate application. Exp. 4 and 5 considered an inequality in thousand seed weight (TSW; weight of 1000 seeds) by comparing glyphosate responses of the smallest seeds with those of the largest seeds within the barley seed batch used. For that reason, particularly small and large seeds were visually selected from the entire seed batch and their TSW estimated by weighing three aliquots of 100 seeds. The TSW was significantly different [analysis of variance (ANOVA) with *Tukey* test, $\alpha = 0.05$] each time with 29.3 ± 0.2 g (exp. 4) and 31.1 ± 0.4 g (exp. 5) for small seeds compared to 63.8 ± 0.8 g (exp. 4) and 62.1 ± 0.5 g (exp. 5) for large seeds. Exp. 4 was conducted with 12 glyphosate doses and exp. 5 with additional doses in the low dose range to give 17 doses. Exp. 6 investigated the impact of the inequality in early development speed within the barley variety used by comparing the most fast germinating seeds with the slowest germinating seeds. For that reason, seeds that had developed a radicle and a coleoptile after two days of germination were used to establish the fast germinating variant. All seeds without visible emergence after two days were further cultivated and used the next day to establish the slow germinating variant (seeds with emerged radicle and coleoptile after three days). To determine differences in the above ground-biomass at application, 15 plants per variant (3 replicate pots) were cut at the soil level on the day of glyphosate application, and the shoot dry weight and length was measured. These values were compared for significant differences by ANOVA with *Tukey* test ($\alpha = 0.05$).

2.3. Statistical analysis

2.3.1. Data preparation

The mean values of the overall population for shoot dry weight and length per replicate were first calculated (mean of the 5 or 30 plants/replicate). These six or eight replicate values per treatment, along with the survivor data, were used to model dose-response relations at all experiments conducted.

In order to evaluate selective low/high-dose effects in high-density populations (exp. 1–3), the absolute shoot dry weight and length values per replicate (30 plants) were used to calculate percentile (%ile) values for each replicate. At the left tail of the size distribution (*i.e.*, the most short-grown individuals referred to as the slow-growing subpopulations), the 5 and 10%iles were calculated. At the right tail of the size distribution (*i.e.*, the longest individuals referred to as the fast-growing subpopulations), the 90 and 95%iles were calculated. These percentile values per replicate and treatment were used to analyse selective low-dose effects and to model selective dose-response relations at the subpopulation level. The number of seedlings per percentile replicate out of 30 that fell into the respective percentile in the various treatments and endpoints is given in supplementary tables (Supplement Tables A.3–A.5). On average across all calculated percentiles, 2.9 ± 0.8 seedlings fell into a percentile replicate. All analyses were done with SAS® 9.4.

2.3.2. Selective low-dose effects (exp. 1–3)

The statistical analysis applied was adapted from previous publications (*e.g.*, Sinkkonen et al., 2009, 2011; Belz and Sinkkonen, 2016a, 2016b). Prior to significance testing, all datasets were tested for normality (*Shapiro-Wilk's* test, $p > 0.05$). Datasets that violated the assumption of normality were transformed *via* Box-Cox power transformation. The transformation was done after estimating the optimal value of the transformation parameter λ from the data by the maximum likelihood method ($-3 < \lambda < 3$) using the TRANSREG procedure of SAS (Piepho, 2009; Osborne, 2010; Perla, 2016; Damesa et al., 2018).

Transformation of data was necessary for 15 datasets out of 25. The application of the Box-Cox transformation fixed the problem of violating normality for all but three datasets. Thereafter, a *Mann-Whitney U* test sorted out treatments with significantly different values in mean shoot dry weight and length per dose from control treatments ($\alpha = 0.05$). For glyphosate treatments showing no difference in mean values per dose, the percentile values per replicate (5, 10, 90, 95%ile) were compared with those of the control treatment with *Mann-Whitney U* tests (Sinkkonen et al., 2009, 2011). In order to account for the multiple testing, the obtained *p*-values were adjusted with *Bonferroni* correction using the MULTTEST procedure of SAS.

2.3.3. Dose-response modeling

Dose-response curves were modeled based on the mean values per replicate. Moreover, in high-density populations (exp. 1–3), shoot dry weight and length responses were also modeled at two 'percentile-dependent' subpopulation levels, namely the most slow-growing (5%ile) and the most fast-growing individuals (95%ile). Three equations with biologically meaningful parameters were used in order to have a high flexibility in modeling. The NLMIXED procedure of SAS was used to fit response values per dose (y) as a nonlinear function of dose (x) to either the monophasic function of Streibig (1988) (Eq. (1)), the biphasic function of Brain and Cousens (1989) (Eq. (2)), or the hormetic dose-response model of Cedergreen et al. (2005) (Eq. (3)):

$$y = c + ((d - c)/(1 + \exp(b*\ln(x/ED_{50})))), \quad (1)$$

$$y = c + (((d - c) + f*x)/(1 + \exp(b*\ln(x/e)))), \quad (2)$$

$$y = c + (((d - c) + (f*\exp(-1/x^a))/(1 + \exp(b*\ln(x/e)))) \quad (3)$$

where c denotes the mean response at infinitely high doses, d denotes the mean response of the untreated control, f denotes the degree of hormetic increase, b determines the slope of the decreasing curve part, the size of a determines the steepness of the increasing curve part, and ED_{50} the dose causing 50% inhibition while parameter e has no straightforward biological meaning (Cedergreen et al., 2005).

The hormetic models were fitted to all datasets that displayed an estimate of f with a 95% confidence interval (CI_{95}) that did not cover the value zero ($f > 0$) and, thus, indicated a significant biphasic response (Schabenberger et al., 1999; Belz and Piepho, 2012). Parameter a (Eq. (3)) was partly fixed according to the smallest residual sum of squares in order to achieve significance in the parameter f. The choice of the specific hormetic model fitted was primarily based on the significance of f. If both or none of the hormetic models fulfilled $f > 0$, a pairwise likelihood ratio test with the monophasic model (Eq. (1)) as the reduced model was performed as goodness-of-fit test with the p-value of the test statistic being approximated by the chi-square distribution χ^2 (Seber and Wild, 1989; Belz and Piepho, 2017). If the monophasic model was rejected ($p < 0.05$), the hormetic model with the lower p-value was fitted.

Besides the directly estimated parameters of the original model functions, further quantitative features were deduced using reparameterizations (Schabenberger et al., 1999; Belz and Piepho, 2012, 2013), namely the maximum stimulatory response y_{max} at dose M, as well as ED_{110} (dose causing 10% stimulation), ED_{10} (dose causing 10% inhibition) and ED_{50} doses. Moreover, a sensitivity factor (SF) was calculated to quantify the dose distance (difference between doses) between two variants as the quotient of estimated effective doses (*e.g.*, $ED_{50\ 5\%ile}/ED_{50\ population\ mean}$) by the ESTIMATE statement within the NLMIXED procedure of SAS.

Starting values for the regression parameters where selected based on the graph of the data for response y *versus* dose x. Response variance heterogeneity was accounted for by using the inverse variance of replicates at each dose as weight. Significant differences between dose-response curves were evaluated by comparing regression parameters using the CONTRAST statement within the NLMIXED procedure of SAS.

2.3.4. Correlation analysis

Interdependencies between survival data and shoot biomass accumulation were evaluated over all three high-density experiments (exp. 1–3). This was done by calculating *Pearson* correlation coefficients and performing a two-sided significance test at $\alpha = 0.05$ using the CORR procedure of SAS.

3. Results

The results are clustered for experiments addressing high-density populations (exp. 1–3) and experiments addressing mechanistic response inequality (exp. 4–6). A detailed listing of estimated dose-response parameters with their standard error can be found in two supplementary tables (Supplement Tables A.1 and A.2).

3.1. Selective dose-response effects in high-density populations (exp. 1–3)

3.1.1. Dose-response patterns at the population level (exp. 1–3)

Six dose-response relations out of eight for the overall population were biphasic (75%) and reached up to 24% stimulation above control within a hormetic dose zone ($ED_{110} \leq x \leq ED_{10}$) of 10- to 272-fold equalling < 1% up to 15% of the approved field rates for glyphosate treatments in Germany.

3.1.1.1. Survival. The number of survivors showed a biphasic response in two experiments (exp. 1 and 3) and a monophasic response in exp. 2. (Fig. 1; Supplement Table A.1). The monophasic curve showed an upper asymptote d of 29.8 surviving plants/replicate, while d values of the two biphasic curves were significantly lower with 23.1 (exp. 1) and 26.0 surviving plants/replicate (exp. 3). Correlating the number of survivors in control treatments with shoot biomass over all three experiments resulted in a significant positive correlation (*Pearson* correlation coefficient of 0.59; $p = 0.003$). Hence, the more biomass was accumulated in an untreated crowded barley population, the higher the rate of survival of individual plants and, thus, the lower the probability for self-thinning. Moreover, analysing the same correlation for slow- (5%ile) and fast-growing (95%ile) control plants resulted only for slow-growing plants in a significant positive correlation (0.56; $p = 0.005$). This indicated that especially the biomass accumulation by the slow-growing subpopulation was decisive for the occurrence of self-thinning. In accordance, the difference in dry weight of slow- (5%ile) and fast-growing (95%ile) control plants was negatively correlated with the rate of survivors (−0.67; $p < 0.001$). Hence, the smaller the variation in biomass

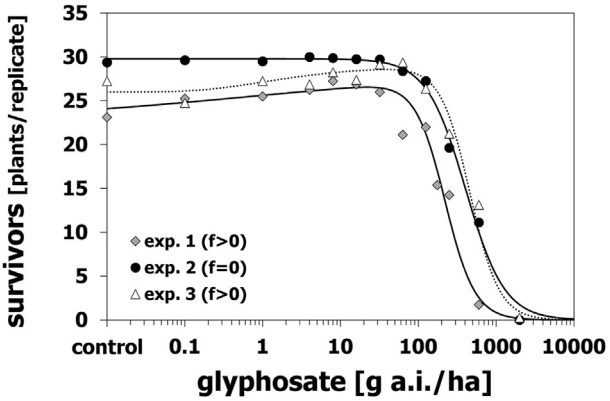

Fig. 1. Effect of foliar applied glyphosate at 21 days after treatment on the survival rate of *Hordeum vulgare* (cv. RGT Planet) planted at an initial density of 30 plants/replicate in three independent greenhouse trials (exp. 1–3). Parameter f denotes significant hormesis (f > 0) or a significant monophasic relation (f = 0). Data give means of eight replicates per treatment and ≤30 plants per replicate.

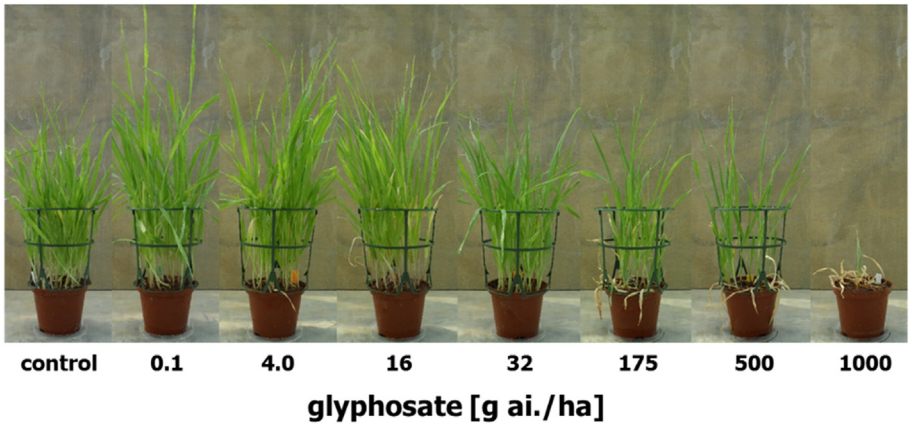

Fig. 2. Dose responses of a spring barley cultivar (*Hordeum vulgare* cv. RGT Planet; 30 plants/replicate) to different doses of glyphosate at 21 days after spray treatment. Plants showed significant hormesis in growth traits at this life stage across similar dose ranges in three independent experiments.

between individual plants within the population, the higher the rate of survival. Furthermore, correlating the survival data of the untreated controls with the actual mean temperature in each experiment (Table 1), resulted in a significantly negative correlation [−0.53 ($p = 0.010$) for day temperature; −0.57 ($p = 0.004$) for night temperature]. This indicated that temperature stress may have contributed to the observed lower survival rates in exp. 1 and 3.

With increasing glyphosate doses, the number of surviving plants increased to a maximum of 26.5 plants/replicate (15% increase) at $M = 21.7$ g ai/ha in exp. 1 and to 28.6 plants/replicate (10% increase) at $M = 37.0$ g ai/ha in exp. 3. Hence, in both experiments low-dose glyphosate treatments were able to increase plant survival. The ED_{50} value was lowest in exp. 1 with 249.5 g ai/ha, 1.6-fold higher in exp. 2 and 2.0-fold higher in exp. 3.

3.1.1.2. Shoot dry weight responses. Visually, hormesis was evident at the population level in all high-density experiments conducted (Fig. 2). Hormesis was significant in exp. 2 and 3 and best fit by a biphasic model in exp. 1 (Fig. 3; Supplement Table A.1). The hormesis peak at the population level was quite similar between experiments with an average relative y_{max} of 122% of control appearing at M doses between 26.7 g ai/ha (exp. 1) and 55.7 g ai/ha (exp. 3). The ED_{50} value was lowest in exp. 1 with 228.9 g ai/ha and highest in exp. 3 with 329.9 g ai/ha.

3.1.1.3. Shoot length responses. Shoot length responses were not evaluated in exp. 1, but included thereafter to see if this endpoint results in greater low-dose variability than observed for shoot weight in exp. 1. Responses were monophasic in exp. 2 and best fit by a biphasic model in exp. 3 with a relative y_{max} of 106% of control at $M = 55.0$ g ai/ha (Fig. 4; Supplement Table A.1). The upper asymptote d was higher in exp. 2 with 44.1 cm compared to 38.1 cm in exp. 3 where hormesis occurred. The ED_{50} value was 306.8 g ai/ha in exp. 2 and 1.2-fold lower in exp. 3.

3.1.2. Selective low-dose effects (exp. 1–3)

Selective low-dose effects were observed for all percentiles evaluated (≤10%ile and ≥90%ile) and for all of the five dose-response relations modeled at the population level for dry weight and shoot length responses.

3.1.2.1. Shoot dry weight responses. The mean weight of eight (exp. 2 and 3) and ten doses (exp. 1) were not significantly different from control (Table 2). Of these doses, 3–4 were located each time in the ultra-low-dose range below the ED_{110}, 3–4 doses each time in the low-dose range between ED_{110} and ED_{10}, and 1–3 doses each time were in the high-dose range above the ED_{10}. In the ultra-low-dose range, no

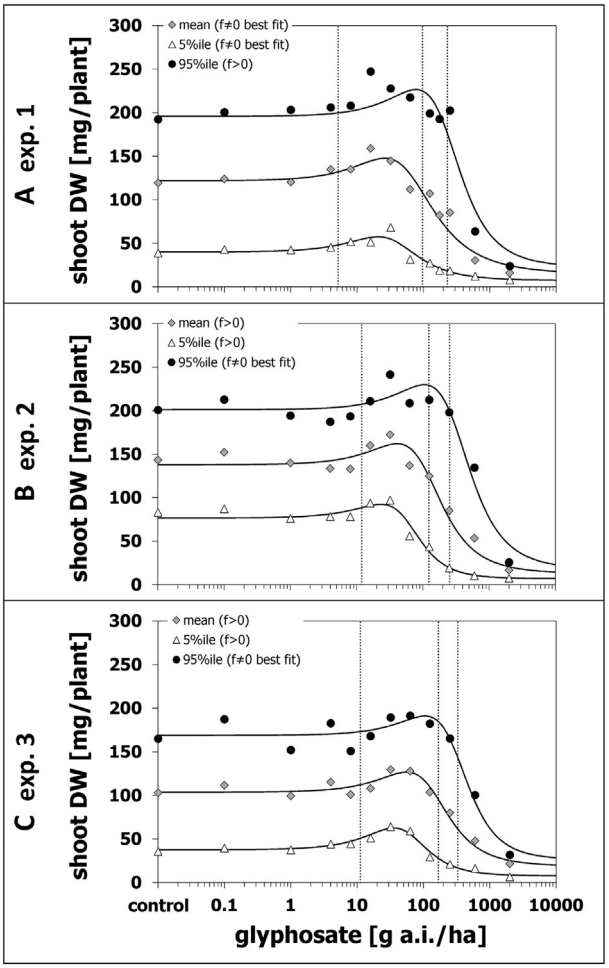

Fig. 3. Dose-response effects of glyphosate (21 days after spray treatment) on shoot dry weight (DW) of *Hordeum vulgare* (cv. RGT Planet) at the population level and the 5 or 95% percentile (%ile) in three independent greenhouse trials (exp. 1–3). Parameter f denotes significant hormesis (f > 0) or a biphasic relation as best fit (f ≠ 0 best fit). The vertical lines represent the ED_{110} (left; effective dose giving 10% stimulation), the ED_{10} (middle; effective dose giving 10% inhibition), and the ED_{50} (right; effective dose giving 50% inhibition) for the overall population. Data give means of eight replicates per treatment and ≥2 to 30 plants per replicate.

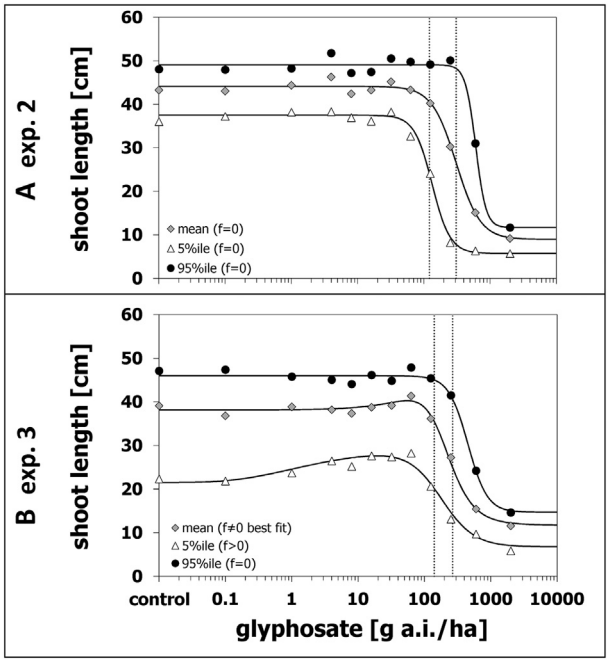

Fig. 4. Dose-response effects of glyphosate (21 days after spray treatment) on shoot length of *Hordeum vulgare* (cv. RGT Planet) at the population level and the 5 or 95% percentile (%ile) in two independent greenhouse trials (exp. 2–3). Parameter f denotes significant hormesis (f > 0), a significant monophasic relation (f = 0) or a biphasic relation as best fit (f ≠ 0 best fit). The vertical lines represent the ED_{10} (left; effective dose giving 10% inhibition) and the ED_{50} (right; effective dose giving 50% inhibition) for the overall population. ED_{110} values (effective dose giving 10% stimulation) lack due to a monophasic mean response (A) or a mean stimulation < 10% (B). Data give means of eight replicates per treatment and ≤30 plants per replicate.

treatment showed a significant selective difference from the control treatment. In the low-dose range, selective stimulation was observed in all three experiments. In exp. 1, fast-growing plants were significantly stimulated at a dose of 16 g ai/ha (both percentiles). In exp. 2 and 3, both slow- and fast-growing plants were significantly stimulated at a dose of 32 g ai/ha (three percentiles). The high dose range was in each experiment characterized by selective inhibition of slow-growing plants at both percentiles tested (125 g a.i./ha in exp. 2; 250 g a.i./ha in exp. 1 and 3).

3.1.2.2. Shoot length responses. In exp. 2 and 3, mean shoot length was not significantly different from control at eight doses tested in each case (Table 3). In exp. 2, seven of these doses were within the low-dose range of the monophasic curve (ED_{10} = 121.1 g ai/ha) and one dose was located within the high-dose range. Two low-dose treatments significantly stimulated shoot length of fast-growing plants (4 and 32 g ai/ha; both percentiles). The highest dose of 125 g ai/ha caused a significant reduction of shoot length at both percentiles representing slow-growing plants. In exp. 3, the hormetic increase at the population level remained below 10%, therefore, the ED_{10} (140.1 g ai/ha) was set as the end of the low-dose range. All doses showing no change in population mean were located within this low-dose range. One dose (8 g ai/ha) caused significant selective low-dose toxicity towards fast-growing plants (90th percentile).

Summing up all possible cases of selective low-dose effects (168 cases; Tables 2 and 3), 19 of these cases (11%) showed selective low-dose effects. The phenomenon occurred less often among fast-growing individuals (42% of all 19 cases) and more often among slow-growing individuals (58%). Low-dose stimulation (53% of all 19 cases) was as prevalent as selective low-dose toxicity (47%). Most of the 19 cases were located within the second low-dose part (58%) and 42% of all cases were located in the 'high-dose' zone just below the application rate that caused a toxic effect on mean size. Since all cases of selective effects within the 'high-dose' zone were low-dose toxicity among slow-growing plants, they most likely represent the beginning of high-dose inhibition. Selective low-dose stimulation occurred primarily among fast-growing individuals (7 of all 10 cases), while selective low-dose toxicity occurred primarily among slow-growing individuals (8 of all 9 cases). Regarding the endpoint measured, the phenomenon was somewhat more prevalent for shoot dry weight with 13% of the 104 possible cases (Table 2) compared to shoot length with 9% of the 64 possible cases (Table 3).

3.2. Dose-response patterns at the subpopulation level (exp. 1–3)

Among the 10 'percentile-dependent' dose-response curves modeled for the 5%ile and the 95%ile, seven were biphasic (70%). The stimulation ranged between 9 and 67% above control and appeared within a hormetic dose zone of 5- to 286-fold equalling < 1% up to 18% of the approved field rates for glyphosate treatments in Germany.

3.2.1. Shoot dry weight responses

In all experiments, shoot dry weight responses of both percentiles were significantly biphasic or better fit by a biphasic model, as observed at the population level (Fig. 3; Supplement Table A.1). The absolute values for *c*, *d*, and y_{max} were consistently different between subpopulations at all experiments and increased in the order 5%ile < population mean < 95%ile. In contrast, the relative y_{max} at the 5%ile ranged between 121 and 167% of control and was thus always higher as compared to the overall population and the 95%ile (109–113% of control). This increase in hormesis magnitude with slow-growing plants was significant in exp. 3, where slow-growing seedlings showed a 1.4-fold relative increase in amplitude as compared to the rest of the population. Hormesis was always least pronounced at the 95%ile, but this decrease in amplitude was only significant as compared to the slow-growing seedlings.

The hormetic dose range at the 5%ile was always partially located within the ultra-low-dose range of the overall population and, thus, shifted to lower doses leaving most of the population unaffected (1.2- to 1.8-fold at *M*). As a consequence, the ED_{10} doses of the overall population (Fig. 3) equalled a 21–50% inhibition at the 5%ile and indicated selective low-dose toxicity among slow-growing plants. The hormetic dose range at the 95%ile was partially located within the high-dose range of the overall population and, thus, shifted to higher doses by 1.9- to 2.7-fold (exp. 1) at the *M* dose level. As a consequence, the ED_{10} doses of the overall population were close to the *M* doses causing maximum stimulation at the 95%iles (Fig. 3). This indicated selective hormesis among fast-growing individuals at doses causing no or beginning high-dose inhibition on most of the population. The difference between *M* doses of the percentiles was 2.9-fold (exp. 3) to 4.5-fold (exp. 2). ED_{50} values were significantly lower than at the population level for the 5%ile (1.5- to 2.2-fold) and significantly higher for the 95%ile (1.7- to 2.5-fold). The difference between ED_{50} doses of the percentiles was 2.6-fold (exp. 3) to 5.7-fold (exp. 2).

3.2.2. Shoot length responses

In exp. 2, shoot length responses of both percentiles were monophasic as at the population level (Fig. 4A; Supplement Table A.1). ED_{50} values were significantly lower than at the population level for the 5%ile (2.3-fold) and significantly higher for the 95%ile (2.0-fold). The difference between ED_{50} doses of the percentiles was 4.5-fold. The absolute values for *c* and *d* were consistently different between subpopulations and increased in the order 5%ile < population mean < 95%ile. The same was true for exp. 3 where responses were significantly biphasic at the 5%ile as at the population level, but monophasic at the 95%ile (Fig. 4B). The relative y_{max} at the 5%ile was 128% of control at *M* = 19.4 g ai/ha. Hence, compared to the overall

Table 2
Statistical significance of effects of glyphosate at the population mean and the 5th, 10th, 90th or 95th percentiles on shoot dry weight of *Hordeum vulgare*. Displayed are only concentrations for which mean dry weight at the population level was not significantly different from control (*Mann-Whitney U* test with *Bonferroni* correction of *p*-values; α = 0.05). Data given as mean of 8 replicates per concentration ± standard deviation.

Exp.	Dose	Mean	Dose	Percentiles for shoot dry weight [mg]			
	[g a.i./ha]		Range	5%	10%	90%	95%
1	Control	119.4 ± 37.1	Ultra-low	38.9 ± 21.8	57.8 ± 31.0	178.5 ± 17.6	192.3 ± 22.5
	0.1	123.9 ± 30.4		43.0 ± 23.8	54.0 ± 26.1	185.9 ± 34.8	200.6 ± 32.0
	1	120.4 ± 23.4		42.6 ± 24.8	53.6 ± 24.1	186.8 ± 38.4	203.2 ± 38.6
	4	135.0 ± 32.8		45.6 ± 37.1	61.5 ± 42.0	191.6 ± 32.5	206.1 ± 33.9
	8	135.0 ± 17.6	Low	51.7 ± 20.6	65.7 ± 23.2	188.8 ± 20.4	207.9 ± 13.2
	16	158.9 ± 27.6		51.4 ± 34.6	66.1 ± 36.7	232.0 ± 35.9**	247.3 ± 35.6**
	32	144.8 ± 39.6		68.3 ± 41.3	81.6 ± 42.4	212.1 ± 54.5	227.8 ± 55.5
	63	112.1 ± 26.2		31.6 ± 25.4	41.6 ± 22.5	195.1 ± 31.2	217.5 ± 38.7
	125	107.2 ± 17.8	High	27.3 ± 2.5	33.5 ± 5.2	181.7 ± 30.9	199.1 ± 31.5
	175	82.2 ± 19.7		19.0 ± 6.1	23.3 ± 6.6	164.7 ± 42.0	192.7 ± 36.0
	250	85.3 ± 26.8		18.4 ± 5.9*	23.2 ± 7.0*	179.0 ± 36.9	202.6 ± 31.8
2	Control	143.3 ± 18.2	Ultra-low	83.1 ± 24.5	94.0 ± 28.2	186.6 ± 24.1	200.9 ± 33.7
	0.1	152.4 ± 19.9		87.3 ± 21.9	99.8 ± 18.2	201.6 ± 24.7	212.8 ± 23.4
	1	140.0 ± 17.8		76.1 ± 11.2	98.8 ± 14.5	181.5 ± 25.4	194.4 ± 27.7
	4	133.7 ± 9.6		78.4 ± 10.9	93.1 ± 10.0	171.3 ± 13.4	187.3 ± 15.5
	8	133.2 ± 9.5		78.1 ± 10.7	89.0 ± 11.8	182.3 ± 19.4	193.6 ± 24.6
	16	160.1 ± 12.6	Low	94.0 ± 20.0	111.3 ± 20.1	202.0 ± 17.3	211.0 ± 15.6
	32	172.5 ± 22.7		97.4 ± 11.3	122.5 ± 16.4*	225.8 ± 29.2*	241.7 ± 35.7
	63	137.2 ± 13.8		56.1 ± 18.8	68.2 ± 23.3	189.9 ± 20.7	208.6 ± 32.0
	125	125.0 ± 6.7	High	43.6 ± 10.2**	52.8 ± 10.6**	191.9 ± 20.6	212.5 ± 37.7
3	Control	103.0 ± 12.5	Ultra-low	35.7 ± 10.4	45.7 ± 8.7	153.2 ± 18.2	165.1 ± 22.9
	0.1	111.7 ± 23.7		39.6 ± 26.4	46.4 ± 30.8	175.5 ± 23.3	187.3 ± 24.8
	1	99.4 ± 19.3		37.5 ± 21.0	50.0 ± 18.6	139.4 ± 27.7	152.2 ± 25.9
	4	115.4 ± 20.3		44.1 ± 24.5	58.5 ± 26.8	171.7 ± 25.0	182.9 ± 24.5
	8	101.0 ± 20.8		44.5 ± 15.9	51.1 ± 15.1	144.5 ± 28.4	151.0 ± 31.1
	16	108.1 ± 26.9	Low	51.1 ± 27.8	59.1 ± 27.9	160.7 ± 44.9	168.0 ± 46.5
	32	129.7 ± 20.3		64.1 ± 22.9*	79.7 ± 21.8**	178.4 ± 20.2*	189.4 ± 22.9
	125	103.7 ± 17.2		29.2 ± 10.2	41.9 ± 13.8	164.0 ± 45.2	182.4 ± 47.1
	250	80.2 ± 24.2	High	21.0 ± 9.5**	29.8 ± 13.8*	140.9 ± 40.0	165.2 ± 39.0

Significantly different from control at '**' α = 0.05 or '*' α = 0.1 according to *Mann-Whitney U* test with *Bonferroni*correction of *p*-values.

population, hormesis was significantly more pronounced (1.2-fold) and showed a maximum stimulation at a 2.8-fold lower dose. Moreover, the hormetic dose range was entirely located within the low-dose range of the overall population (Fig. 4B). Hence, at doses leaving most of the population unaffected, slow-growing seedlings showed selective glyphosate hormesis. ED_{50} values were not significantly lower than at the population level for the 5%ile, but significantly higher for the 95%ile (1.6-fold). The difference between ED_{50} doses of the percentiles was 1.6-fold.

3.3. Mechanisms of response inequality (exp. 4–6)

3.3.1. Seed size (exp. 4–5)

In both experiments, shoot dry weight and shoot length varied with

Table 3
Statistical significance of effects of glyphosate at the population mean and the 5th, 10th, 90th or 95th percentiles on shoot length of *Hordeum vulgare*. Displayed are only concentrations for which mean dry weight at the population level was not significantly different from control (*Mann-Whitney U* test with *Bonferroni*correction of *p*-values; α = 0.05). Data given as mean of 8 replicates per concentration ± standard deviation.

Exp.	Dose	Mean	Dose	Percentiles for shoot length [cm]			
	[g a.i./ha]		Range	5%	10%	90%	95%
2	Control	43.3 ± 2.0	Low	36.0 ± 6.1	38.1 ± 5.4	47.4 ± 1.5	48.1 ± 1.8
	0.1	43.1 ± 2.3		37.2 ± 3.3	39.1 ± 2.2	47.0 ± 2.3	48.0 ± 2.8
	1	44.4 ± 2.8		38.2 ± 2.4	40.4 ± 2.8	47.8 ± 3.4	48.3 ± 3.7
	4	46.3 ± 2.5		38.3 ± 6.4	40.7 ± 6.3	50.9 ± 2.5**	51.8 ± 2.1**
	8	42.4 ± 2.7		36.9 ± 3.3	38.5 ± 3.1	46.3 ± 2.8	47.2 ± 2.8
	16	43.3 ± 3.0		36.1 ± 5.7	38.9 ± 3.6	47.0 ± 2.8	47.5 ± 2.8
	32	45.2 ± 1.3		38.2 ± 2.0	40.6 ± 1.9	49.7 ± 1.5**	50.6 ± 1.8
	63	43.3 ± 4.1		32.7 ± 8.0	36.1 ± 6.4	48.9 ± 3.5	49.7 ± 3.5
	125	40.3 ± 1.8	High	24.1 ± 7.1**	29.3 ± 5.8**	47.6 ± 2.5	49.2 ± 3.5
3	Control	39.1 ± 3.5	Low	22.3 ± 7.8	25.8 ± 8.6	46.7 ± 3.5	47.1 ± 3.5
	0.1	36.8 ± 6.8		21.9 ± 12.4	24.0 ± 13.2	46.5 ± 3.3	47.4 ± 3.6
	1	38.9 ± 2.3		23.7 ± 9.1	28.4 ± 10.2	45.0 ± 5.5	45.8 ± 5.7
	4	38.2 ± 5.9		26.5 ± 9.8	29.5 ± 10.2	44.5 ± 5.5	45.1 ± 5.7
	8	37.4 ± 4.5		25.2 ± 8.5	28.7 ± 8.7	43.3 ± 2.8*	44.1 ± 2.5
	16	38.8 ± 4.4		27.6 ± 10.6	29.8 ± 11.1	45.3 ± 2.6	46.2 ± 3.1
	32	39.2 ± 2.9		27.4 ± 6.6	31.9 ± 6.8	44.2 ± 3.3	44.9 ± 3.3
	63	41.4 ± 2.7		28.2 ± 6.0	32.7 ± 6.0	46.5 ± 2.8	47.9 ± 3.1
	125	36.2 ± 2.9		20.6 ± 5.3	24.3 ± 4.7	44.6 ± 3.4	45.5 ± 3.7

Significantly different from control at '**' α = 0.05 or '*' α = 0.1 according to *Mann-Whitney U* test with *Bonferroni*correction of *p*-values.

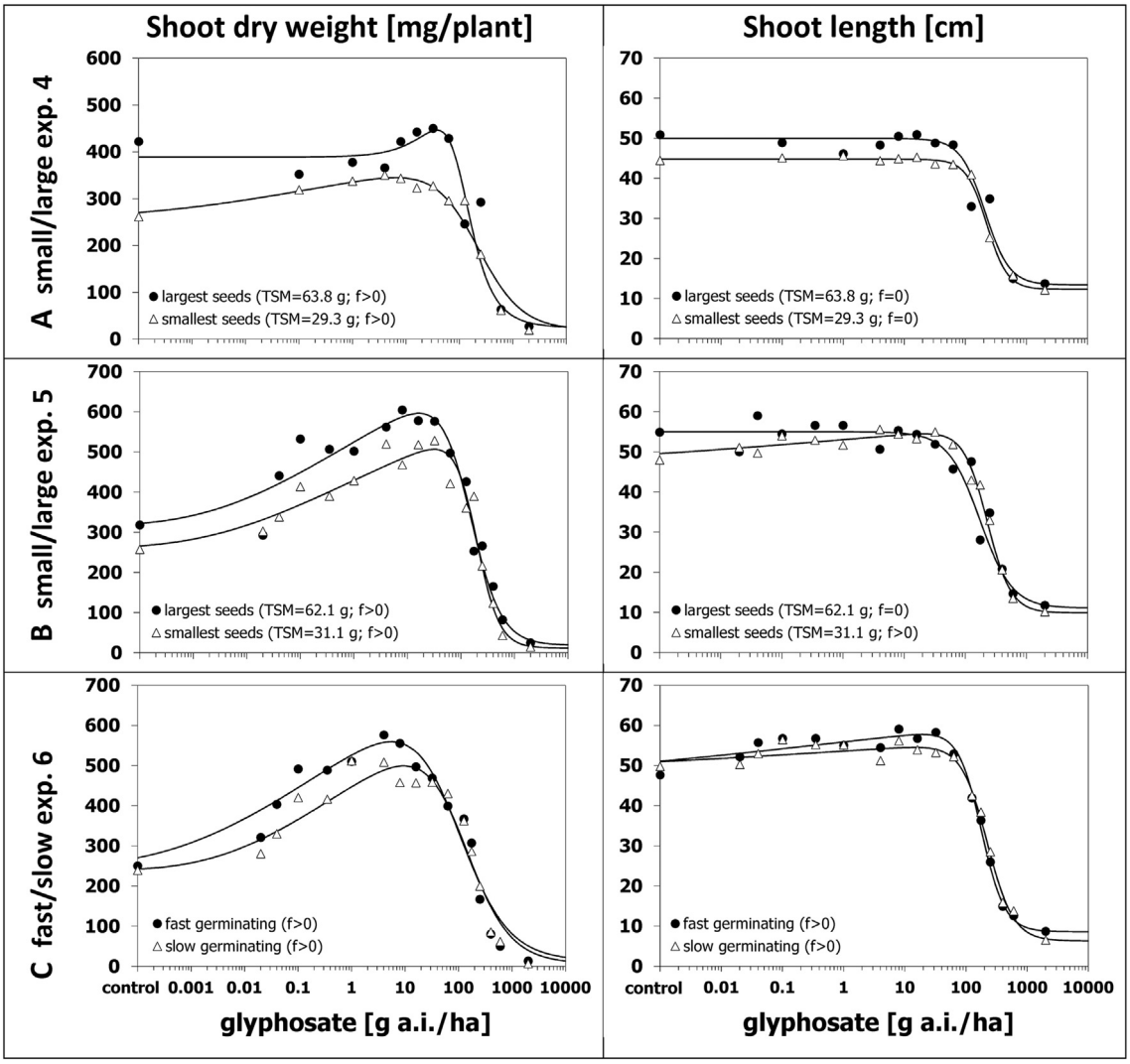

Fig. 5. Dose-response effects of glyphosate (21 days after spray treatment) on shoot dry weight and shoot length of *Hordeum vulgare* (cv. RGT Planet) in three independent experiments (exp. 4–6). Exp. 4 (A) and 5 (B) compare plants grown from the smallest and the largest seeds within the population. Exp. 6 (C) compares plants grown from fast germinating seeds and slow germinating seeds (1 day delayed) within the population. Parameter f denotes significant hormesis ($f > 0$) or a significant monophasic relation ($f = 0$). Data give means of six replicates per treatment and ≤5 plants per replicate.

seed size at the day of application (up to 1.5-fold) and at the end of the experiment (up to 1.6-fold for untreated controls and up to 1.7-fold for plants treated with the highest dose) (Fig. 5; Supplement Table A.2). In accordance, estimated *d* values of curves modeled for plants from small seeds were always significantly lower than those of large seeds (up to 1.6-fold). Hence, plants grown from small seeds were continuously shorter and of lower biomass than plants grown from large seeds.

In exp. 4, there was a tendency for low-dose toxicity in plants grown from large seeds (Fig. 5A), but this decrease was neither significant (*Tukey* test, $\alpha = 0.05$) nor modellable by a triphasic pattern. Based on this, exp. 5 included additional ultra-low doses to better capture this phenomenon. An ultra-low-dose decrease (8–9%) appeared in exp. 5 also among plants grown from large seeds (Fig. 5B) but was likewise not significant.

The curves modeled for plants grown from small and large seeds were consistently biphasic for shoot dry weight responses and predominantly monophasic for shoot length responses, with the exception of the biphasic response of plants from small seeds in exp. 5 (Fig. 5; Supplement Table A.2). The absolute y_{max} value was always significantly higher for plants from large seeds (1.2- to 1.3-fold), while plants from small seeds showed a consistently higher hormesis amplitude with a relative y_{max} of 15–96% stimulation over control, compared to 0–89% for plants from large seeds. This increase in the relative y_{max} was significant for dry weight responses in exp. 4 and for shoot length in exp. 5 where plants from large seeds lacked hormesis. Significant differences were also found in the dose *M* leading to maximum stimulation. The observed difference was 1.8- to 2.3-fold. In exp. 4, plants from small seeds were more sensitive and in exp. 5 plants from large seeds. Regarding high-dose effects, there was a tendency of lower ED_{50} values and, thus, a higher sensitivity for plants from large seeds at both endpoints measured. This higher sensitivity was significant for dry weight responses in exp. 4 and shoot length responses in exp. 5. Here, the ED_{50} doses for large seeds were 1.5-fold lower each time.

Based on this, differences in seed size may partially explain the observed response inequality within the overall population. The overall variation between plants from small and large seeds was up to 1.7-fold for growth parameters and up to 1.3-fold for relative y_{max}. Compared to the variation observed within the overall population (up to 5.0-fold for growth parameters; up to 1.5-fold for relative y_{max}), the variability between plants from small and large seeds was somewhat smaller. The same applied for effective doses with up to 2.3-fold difference in *M* and ED_{50} doses based on seed size compared to up to 5.7-fold difference within the overall population.

3.3.2. Germination speed (exp. 6)

The absolute values for shoot dry weight and shoot length between plants developed from fast and slow germinating seeds were significantly different at the day of application (untreated plants; 1.5- to 1.8-fold difference). However, at the end of the experiment at 21 DAT a significant difference between variants was only apparent for plants treated with the highest dose (1.3- to 2.0-fold lower for slow germinating seeds), while untreated controls plants did not significantly differ between variants (Fig. 5C). In accordance, estimated *c* values of curves modeled for slow germinating plants were significantly lower (1.3- to 2.0-fold), but *d* values were equal to those of fast germinating plants (Fig. 5C; Supplement Table A.2). Hence, plants grown from slow germinating seeds were only initially shorter and of lower weight than plants grown from fast germinating seeds, while growth differences disappeared thereafter.

The curves modeled for fast and slow germinating plants were consistently biphasic for both endpoints with a hormesis amplitude of 10–126% stimulation over control (Fig. 5C; Supplement Table A.2). Both endpoints showed no differences between variants in low- and high-dose effects as indicated by insignificant differences between *M* and ED_{50} values. Moreover, observed differences in absolute and relative y_{max} responses were partly significant, but very minor (Supplement Table A.2). Based on this, the initial speed of germination shows no major impact on the variability of responses within the overall population.

4. Discussion

This study tested selective glyphosate effects on two growth parameters of a high-density model population of *H. vulgare* under greenhouse conditions to assess effects on individual plants at doses considered protective, hormetic or adverse to a plant population. At the population level, observed glyphosate hormesis showed 6–24% stimulation over control being widely in line with the general quantitative features of hormesis reported in the literature (10–50% stimulation for > 60% of the reports) (Calabrese and Blain, 2009). Such a moderate stimulation by glyphosate was previously reported for barley and several other plant species (reviewed in Belz and Duke, 2014, 2017; Brito et al., 2017), but some annual and woody plants (*e.g.*, *Conyza sumatrensis*, *Zea mays*, *Glycine max*, *Eucalyptus grandis*) have been reported to show > 100% stimulation over control plants (Velini et al., 2008; Brito et al., 2017). Although hormesis can be easily missed if a study does not actively look for such a response or does not test sufficiently low doses (Duke et al., 2006), reports of glyphosate hormesis are accumulating and indicate that glyphosate causes hormesis in plants (Cedergreen et al., 2009; Brito et al., 2017; Nadeem et al., 2017). Nevertheless, in the case of high-density populations, the situation is more complex than with individual plants as the effects of low-levels of glyphosate can be quite variable among the individuals within a population. This variability was previously shown in microplate assays for several plant toxins (Sinkkonen et al., 2008, 2009, 2011; Belz and Sinkkonen, 2016a, 2016b; Belz et al., 2018a) and the current study indicates that selective low-dose effects persist in a more natural experimental setting.

4.1. Selective low-dose effects

Selective low-dose effects on percentiles were observed in all three experiments at doses having no impact on the population mean. Selective effects occurred for shoot length responses at glyphosate doses ≤8 g a.i./ha. Selectivity occurred in more than one endpoint per experiment and with a frequency of 11%. A higher frequency of 25% was reported by Belz et al. (2018a) studying six different toxins in microplate assays. Selective ultra-low-dose effects were widely absent in all the experiments of this study. A partial explanation for this may be that in the current study 240 plants/dose were exposed, while in previous microplate assays 1200 plants/dose could be managed (Belz and Sinkkonen, 2016a, 2016b; Belz et al., 2018a). The lower number of plants and replicates in this study may contribute to a high proportion to missing ultra-low-dose effects, compared to previous microplate studies. Another aspect to consider is the more variable environmental conditions in the greenhouse. Belz and Cedergreen (2010) showed that growth conditions can significantly influence the expression of low-dose stimulation. This may apply for selective ultra-low-dose effects as well and hints that ultra-low-dose effects may become mixed with other environmental factors shaping plant growth in soil. However, the influence of environmental conditions has yet not been addressed in corresponding studies.

In contrast to the ultra-low-dose range, the occurrence of selective low-dose effects was much more pronounced at higher, but still subtoxic glyphosate doses. This clearly supports the first study hypothesis that size distribution changes are relevant for subtoxic doses of glyphosate under more natural growth conditions. Both phenomena, low-dose stimulation and toxicity, were found in parallel, but at different doses within the same subpopulation or at different subpopulations. Selective stimulation was the most prevalent phenomenon among fast-growing individuals and occurred only in the low-dose range. Selective toxicity was widely restricted to the slow-growing part of the population and occurred only at doses exceeding the low-dose range. Such a reversed effect on slow- and fast-growing individuals within the population was previously reported by Belz et al. (2018a) and substantiates that 'no effect' toxin exposures at the population level can lead to size heterogenization in plant populations (see also Chu et al., 2009).

4.2. Dose-response patterns at the subpopulation level

In this study, response patterns at the population level were in most cases synchronized with the response at both subpopulation levels (*i.e.*, high and low percentiles). An exception was the shoot length responses in exp. 3 where fast-growing individuals showed a monophasic response despite hormesis on most of the population. Previous findings led to the assumption that the response at the population level is strongly governed by the response of the fast-growing individuals (Belz and Sinkkonen, 2016a, 2016b; Belz et al., 2018a). Results of exp. 3 indicate that this governing of population responses may be less pronounced and somewhat different than expected from previous studies (Sinkkonen et al., 2011; Belz and Sinkkonen, 2016b; Belz et al., 2018a) when plants are growing in soil, instead of microplates. Nevertheless, results confirm study hypothesis two that mono- or biphasic responses observed for the overall population do not necessarily represent the response of plants below or beyond a certain percentile within this population.

In comparison to the population mean, in all experiments slow-growing seedlings were significantly more sensitive and fast-growing seedlings were more resilient. Doses up to 3-fold higher were needed before the fast-growing part showed the same effect as the overall population, and doses up to 6-fold higher were needed before the fast-growing part showed the same effect as slow-growing individuals. This variance in herbicide responses was significant and, thus, may even allow the lower sensitivity of the fast-growing part to be categorized as a low-level herbicide resistance (Heap, 2005). As we studied a commercial, relatively homogeneous population of cultivated barley, the level of variance may be higher in natural plant populations due to a more pronounced genetic and phenotypic variation (Sinkkonen et al., 2012; Belz and Sinkkonen, 2016b).

Our results confirm previous findings (Belz and Sinkkonen, 2016a, 2016b; Belz et al., 2018a) of a more pronounced stimulation with the slow-growing subpopulation (y_{max} ≤ 67% stimulation) as compared to the overall population and especially the fast-growing subpopulation showing a consistent low expression of hormesis (y_{max} ≤ 13% stimulation). Previous studies (Belz and Cedergreen, 2010; Belz and Sinkkonen, 2016a, 2016b) ascribed this to an already high growth rate

in the absence of a stimulating treatment leaving hardly any capacity for enhanced growth. Observed *d* values support this assumption by a consistent increase from the 5%ile to the 95%ile. In accordance, in exp. 3 the slow-growing plants showed the lowest growth rate of all experiments, but developed the most pronounced hormesis. This indicated that particularly the slow-growing part of the population benefitted from glyphosate hormesis. Slow-growing plants showed selective glyphosate hormesis at doses leaving most of the population unaffected, followed by selective toxicity when most of the population still showed enhanced growth. The fast-growing plants were still selectively stimulated when most of the population suffered already under toxicity. This demonstrates that fast- and slow-growing plants within a population differed in their responses and sensitivity to glyphosate, in support of the third study hypothesis. As a consequence, alterations in size distribution will result leading to a more homogeneous or heterogeneous population depending on the dose at action (Belz and Sinkkonen, 2016b; Belz et al., 2018a). This segregating effect of low toxin doses seems general, but the extent of the segregation and the putative ecological implications are believed to differ depending on the toxin and dose at action, the plant trait considered, and environmental conditions that change the growth rate and, thus, a plant's capacity to develop hormesis (Belz et al., 2018a).

4.3. Plant mortality

The phenomenon of self-thinning, *i.e.*, plant mortality because of competition in crowded even-aged stands (Westoby, 1984), took place in this study in exp. 1 and 3. The fact that the rate of biomass accumulation drives mortality (Westoby, 1984), could also be observed in this study since the number of survivors in control treatments was positively correlated with biomass accumulation. Self-thinning was especially reduced if the slow-growing part of the population accumulated more biomass leading to a more homogeneous biomass distribution within the overall population. Moreover, it is indicated that plants were at an accumulating disadvantage in exp. 1 and 3 depending on the temperature dynamics during the trials, which designates temperature as one of the factors governing the occurrence of self-thinning in this study.

When self-thinning occurred, plant mortality was reduced at low glyphosate doses resulting in a biphasic dose-response pattern. Doses causing maximum stimulation (*M*) in the overall rate of survivors matched *M* doses for dry weight of slow-growing plants. Based on this, correlating the individual accumulation of biomass within the low-dose range of each experiment with the rate of survivors resulted only for slow-growing plants in a significant positive correlation (0.63; $p < 0.001$). Moreover, the difference in dry weight of slow- and fast-growing plants was significantly negative correlated (-0.50; $p > 0.001$). Hence, the increase in plant survival was associated with an increase in biomass accumulation of slow-growing plants and the resulting homogenization of the biomass distribution within the population. Therefore, it is indicated that low-doses of glyphosate are able to compensate plant mortality within crowded plant stands by selective hormesis among slow-growing plants and the associated homogenizing effect on size distribution of the exposed population. Or in other words, within crowded stands fast-growing plants are less competitive at glyphosate doses that selectively promote slow-growing plants.

An intriguing question is now how long the hormesis induced change in self-thinning processes prevails, and whether the same phenomenon can be found in nature. Since hormesis is known to be a transient phenomenon (Cedergreen, 2008), it may be possible that self-thinning is just delayed in toxin stressed populations. A mere delay in self-thinning was for example reported for water-stressed populations (Liu et al., 2006). Moreover, hormesis can result in trade-offs over time (Duke et al., 2006; Cedergreen, 2008), so that hormetically stimulated plants may suffer an even greater accumulating disadvantage over time. In nature, the outcome of hormesis and self-thinning is further challenged by multiple- or successive-stressor situations that may lead to a complex interplay of environmental conditions favoring or hampering a hormetic response (Belz and Cedergreen, 2010; Belz and Duke, 2014) and also self-thinning processes (Westoby, 1984; Liu et al., 2006). The progression of this interplay may be hard to predict. Nevertheless, this study indicates that low-dose toxin stress can change self-thinning processes within crowded populations due to selective stimulation of smaller plants.

4.4. Mechanisms of response inequality

Several aspects have been discussed as putative mechanistic reason for differences in dose responses expressed by subpopulations within an overall population. These include genetic and phenotypic differences as well as exposure-related dose differences.

Genetic and phenotypic aspects span from differences in toxin uptake and/or relative toxin concentration per biomass, differences in individual capacity for enhanced growth, natural variation in seed vigor, within-population genetic differences, to genotoxic or gene regulation effects that depend on plant growth rate (Aina et al., 2006; Quaggiotti et al., 2007; Sinkkonen et al., 2009, 2011; Hansi et al., 2014; Belz and Sinkkonen, 2016b; Belz et al., 2018a). This study focused on natural variation in seed vigor as manifested in germination speed or seed size. With many plants, the physiological status and the size of the seeds produced can vary depending for example on when the seed was formed and on what part of the plant it developed (*e.g.*, Thompson and Pellmyr, 1989). In our study it was however indicated that variation in germination speed leading to differences in biomass at the day of application did not significantly impact resulting dose responses and played a minor or no role. In contrast, seed size variation caused significant differences in dose responses although the observed variation was less pronounced, compared to the high-density experiments. Seed size may be just one of several factors governing population inequality. The observed trend in low-dose toxicity among plants from large seeds would support the assumption that fast-growing individuals are especially prone to selective low-dose toxicity (Sinkkonen et al., 2008, 2009, 2011). However, a considerably larger dataset is necessary to really substantiate these hypotheses. Nevertheless, natural variation in seed size most likely contributes to response variation and may have an even greater impact in a natural plant population where seed size inequality may be higher than for the commercial, more uniformly-sized barley cultivar tested in this study.

Besides geno- and phenotypic mechanisms for selective responses within a population, exposure-related dose differences are another aspect to consider tracing the source of variation. Under field conditions, the pesticide doses that reach the plants are not uniform on a treated field or the areas adjacent to the treatment (Velini et al., 2017). The reasons can include, for example, movements of the spray boom, protection by mulch or taller plants, or lowering of individual doses in dense stands (Velini et al., 2017). This dose variability is inevitably associated with response inequality within a population and may act in concert with or amplify selective low-dose effects under field conditions. As this study used a laboratory sprayer providing a high uniformity in spray flow and exposed barley plants at an early growth stage with low competition for spray droplets, differences in the individual dose should have been reduced to a minimum.

4.5. Practical implications

Low levels of glyphosate were shown to have abundant selective toxin effects modifying plant growth in populations growing beyond the germinating seedling stage under more natural conditions that mimic spray (drift) exposure in the field. It is possible that this selectivity and the associated change in size structuring of a population can induce cascading ecosystem effects in the long-term, especially under extreme environmental conditions. A change in size distribution within a

population is related to the survival of individual phenotypes and, hence, ultimately to reproduction (Chu et al., 2008, 2009; Sinkkonen et al., 2009; Belz and Sinkkonen, 2016a, 2016b). This selection for and against certain phenotypes may change population dynamics and lead to genotypic adaptations and/or ecotype formation in the longer term and, thus, affect the ecosystem or biodiversity (Chu et al., 2009; Sinkkonen et al., 2009). Moreover, it is indicated that a repeated spray drift exposure of a non-target population to sub-lethal glyphosate doses may lead to reduced sensitivity or even contribute towards resistance due to a hormetic response pattern and/or selection (Busi and Powles, 2009; Silva et al., 2016; Cederlund, 2017; Velini et al., 2017). Changes in size distribution towards fast-growing, less sensitive subpopulations as observed in this study, may likewise threaten the sensitivity towards glyphosate. Therefore, changes in size distribution without a visible effect on population mean in short-term poses a clear long-term risk to have ecosystem effects in toxin-exposed environments. The question if this risk is a realistic scenario for a field situation is however beyond current understanding. Selective low-dose effects have been observed for plant length and dry weight at the germinating seedling stage or at vegetative growth stages, but it is uncertain if the general outcomes observed for these endpoints and growth stages are representative for reproductive endpoints, generative growth stages, or plant communities. Moreover, while selective low-dose effects have been observed for single stress events, short-term periods, and controlled environmental conditions; it is uncertain if these short-term effects prevail, if there is a carryover effect to the next exposure or generation, and how size structuring changes under repeated or changing stress events and environmental conditions.

The dose range of herbicide spray drift on plants growing outside field edges was estimated at 1–10% of the applied field rate (Asman et al., 2003; Cedergreen, 2008). Approved field rates for glyphosate treatments in Germany vary between 1080 and 1800 g ai/ha, corresponding to spray drift doses of 10–180 g a.i./ha. These values clearly cover the low-dose range estimated for barley in this study and others (Cedergreen, 2008; Cedergreen et al., 2009). A recent review on effects of spray drift of glyphosate on terrestrial plants concluded that a level below 5 g a.i./ha would be widely protective against minor adverse effects (Cederlund, 2017). Moreover, an EFSA (2013) report considered the risk posed from a drift rate below 9.7 g a.i./ha acceptable (Cederlund, 2017). The results of this study support these assumptions towards adverse effects, as glyphosate doses up to 63 g a.i./ha did not cause major low-dose toxicity in barley. On the other hand, the estimated dose range for glyphosate hormesis in barley started, especially for slow-growing individuals, at ED_{110} values well below doses considered protective or acceptable and ended at ED_{10} doses widely lower than estimated realistic spray drift doses of up to 180 g a.i./ha. Hence, the likelihood that a plant population in the field is exposed to glyphosate doses causing selective low-dose effects seems realistic. Glyphosate may however just be a case study as low-dose effects and segregating effects were widespread among toxins (Belz et al., 2018a). Therefore, in view of the adequacy of current threshold values for environmental concentrations of active substances of a wide range of plant protection products and their transformation products (EFSA, 2017), there is a need to find out if selective low-dose effects function as a factor governing population behavior in toxin-exposed environments.

5. Conclusions

This study has shown that glyphosate changes plant size distribution within a population and affects plant mortality in dense plant stands at concentrations that are currently considered protective or acceptable. Importantly, as the fast-growing part of the population needed several times higher doses than the average population before toxic effects were measured, the apparent lower sensitivity among fast-growing plants may reduce the efficacy of glyphosate treatments. Further, as low glyphosate doses enhanced growth and reduced mortality among slow-growing plants, glyphosate is likely to affect size distribution within plant populations. As the effects were measured three weeks after glyphosate treatments, it should be investigated whether they stay until maturity and affect reproduction, also among wild plant populations, such as weeds. Moreover, since wild plant populations are likely to show a greater genetic and phenotypic variation than crops, the variation in low-dose effects and, thus, the ecological impacts may be even greater with many wild species than indicated for barley in this study. Nevertheless, the reported results are in line with previous shorter-term experiments. Therefore, if plant responses to toxins generally follow the pattern observed in this study, the effects of low-toxin doses on fast- and slow-growing individuals should be considered when the safety of toxins used in agriculture or elsewhere are evaluated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105072.

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