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Susceptibility variation to new and established herbicides: examples of interpopulation sensitivity of grass weeds

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Creeping resistance

Base line sensitivity

Resistance detection

Abstract

The objectives of this study were to describe the intra-specific variation in herbicide response of weed populations when subjected to new vs. well-established herbicides, and to assess distributions of logLD₅₀- and logGR₅₀-estimates as a potential indicator for early resistance

detection. Seeds of two grass weeds (*Alopecurus myosuroides*, *Apera spica-venti*) were collected in southern Sweden, mainly in 2002. In line with the objectives of the study, the collections sites were not chosen for noted herbicide failures nor for detected herbicide resistance, but solely for the presence of the target species. For each species, seedlings were subjected to two herbicides in dose-response experiments in a greenhouse. One herbicide per species was recently introduced and the other had been on the market for control of the species for a decade, with several reports of resistance in the literature. Fresh weight of plants and a visual vigour score were used to estimate GR_{50} and LD_{50} , respectively. Resistance to fenoxaprop-P-ethyl in *A. myosuroides* was indicated by the LD_{50} -estimates to be present in frequencies sufficient to affect the population-level response in 9 of 29 samples, and was correlated to response to flupyrsulfuron, while low susceptibility to isoproturon in *A. spica-venti*-populations was not linked to the response to sulfosulfuron. In the study as a whole, the magnitude of the estimated herbicide susceptibility ranges differed irrespective of previous exposure. No consistent differences were found in the distribution of LD_{50} -estimates for new and “old” herbicides, and normality in the distribution of estimates could not be assumed for a non-exposed sample, even in the absence of an indication of cross-resistance.

1. Introduction

The occurrence of “creeping resistance”, i.e. a small stepwise, quantitative reduction in herbicide efficacy following repeated applications of herbicides selecting for the same resistance trait, is disputed and debated. Its existence is theoretically uncontroversial (Bull and Wichman, 2001), and has been documented in experimental populations (e.g. Ellis and Kay, 1975c; Vila-Aiub and Ghersa, 2005; Kniss et al. 2007). Hence, the controversy is more a matter of its incidence in production systems: is it a rare phenomenon or a common one occurring anywhere herbicides are used repeatedly? The first scenario suggests that, even on a

longer time-frame, it can be largely ignored (at least in the light of the more urgent cases of quickly emerging resistance). In contrast, the second scenario suggests that it is high time for producers to take various precautionary steps in herbicide usage to slow this process. A resistance case involving mechanisms that convey very high tolerance to normal field doses, as is often the case with target site resistance, may quickly become apparent in the field.. Furthermore, its existence can easily be established in laboratory or greenhouse experiments. In contrast, we would expect creeping resistance to go largely unnoticed in the field. This may be because the treatment efficacy always varies somewhat between years and farmer's as well as researchers perception of temporal trends is herbicide efficacy is not likely to be detailed enough. Also, if the shift in susceptibility is relatively slow, the statistical power of any scheme to detect it in the field has to be very high. Details here depend, of course, on the weed and herbicide involved and the prevailing production system, but some general points can be made.

If creeping resistance has occurred within an area, we can make two predictions about how this should be reflected in data collected from populations selected at random. First, we predict that the inter-populations differences would increase because fields differ in their history of herbicide usage (e.g. from none being exposed to some being exposed, or from a history of single exposures to varying numbers of multiple exposures). Hence, the range of LD_{50} (the dose that is lethal to 50% of treated plants) and GR_{50} (the dose causing 50% growth reduction in treated plants compared with untreated control) would increase. Second, we predict that their distribution would become more skewed towards higher values. The possibility to detect this depends not only on the rate of evolution, but also on the magnitude of variation in the data that cannot be accounted for. In the current context, it might be fruitful to distinguish between experimental error (variation due to sampling, experimental protocol, etc) and such that actually represent biological differences in susceptibility within and

between populations. Previous attempts have shown that weed populations can vary greatly in susceptibility to a herbicide (e.g. Bourdot et al., 1990; Cerdeira and Duke, 2006; DeGennaro and Weller, 1984; Ellis and Kay, 1975a; 1975b; Gillespie and Vitolo, 1993; Jacobsohn and Andersen, 1968; Patzoldt et al., 2002; Price et al., 1983; 1985; Somody et al., 1984; Tardif and Leroux, 1991a Thai et al., 1985; Tranel and Trucco, 2009). Hence, knowledge about the magnitude of such inter-population differences will be pivotal when assessing creeping resistance.

Repeated sampling of the same fields could increase the chance to detect resistance development that has occurred, but because GR_{50} and LD_{50} estimates are sensitive to experimental conditions, direct pair-wise comparisons seem less likely candidates for evaluation. Instead, range and skewness in GR_{50} and LD_{50} might be better candidates for analyses.

Valuable for future attempts to test whether creeping resistance has occurred is the availability of so called baseline data on herbicide efficacy, i.e. what is the natural susceptibility of populations without a history of treatment of a particular herbicide? And what is its variability among and within populations? Such baseline data is now required for target taxa in conjunction with registration of a new herbicide (e.g. Paterson et al., 2002; EPPO, 2004; Kalamarakis and Markellou, 2007), and can also give an indication of the range and skewness of GR_{50} or LD_{50} of unexposed populations. Knowledge about the natural variation may be used not only for a repeated sampling in a region but also for a sample of populations collected at one time from another region. One could then determine if the distribution of $\log(ED_{50})$ is more skewed than expected compared with extensive baseline data. The objectives of this study were to assess the variation in herbicide susceptibility between Swedish field samples of *A. myosuroides* and *A. spica-venti* when subjected to a new vs. a well-established herbicide. A better appreciation of the range of LD_{50} and GR_{50}

estimates expected in previously non-exposed populations might be valuable for future assessment of creeping resistance, as well as to assess the risk for treatment failures. We were also interested in comparing the distributions of logLD₅₀ and logGR₅₀ estimates, as a potential parameter to detect resistance development at an early stage. In the present study, conducted in southern Sweden, we sampled populations of two grass weed species and subjected each species to one newly released herbicide as well as one that had been in widespread use for a decade and for which there were reports in the literature about evolution of resistance. Hence, in two of the cases we had populations that had been subjected to no or to short term exposure, in the other two, candidates for the occurrence of reduced susceptibility. The *A. spica-venti* populations were sampled in 2002 and subjected to sulfosulfuron and isoproturon that had been introduced in 1999 and in the 1970s, respectively. For *A. myosuroides*, also sampled mainly in 2002, flupyrsulfuron and fenoxaprop-P-ethyl were used, herbicides that had been introduced in 2000 and 1992, respectively. Although the populations sampled cannot be claimed to never have been exposed to the new herbicide in question, it is unlikely that they have experienced repeated applications.

2. Materials and Methods

2.1. Seed collection

Sixty seed samples of *A. spica-venti* were collected during the summer of 2002 in agricultural fields in three regions in southern and mid-Sweden (Table 1). *A. myosuroides* seed samples were collected in the west part of the province Skåne only, which is the strong-hold of this species in Sweden, in 2001 (4 samples), 2002 (23 samples), and 2003 (2 samples) (Table 1).

The collection sites were in most cases at a minimum distance of 1.5 km apart. The sixty *A. spica-venti* samples were taken in separate cereal fields, mainly in winter wheat and

winter barley. The twenty-nine *A. myosuroides* seed samples were collected mainly in winter wheat (22 fields, of which 2 fields were sampled both in 2001 and 2002), but also in spring-sown crops (four fields) and fallow (one field). With the exception of a few cases of inadequate control of *A. myosuroides* by fenoxaprop-P-ethyl, there were no reports on failure of herbicide control on the collection sites (that could not be explained by treatment conditions).

In infestations well spread in the field, seed was collected from 40 -100 plants within a 50 x 100 m² square. In a few fields, smaller patches and fewer plants were sampled. Of *A. spica-venti*, entire panicles or parts of the panicles were picked and put into textile bags, while mature seeds of *A. myosuroides* were harvested from the panicles directly in the field.

2.2. Herbicide dose response experiments in greenhouse

Seeds were germinated on filter paper in glass-covered bowls in a greenhouse. Young seedlings were transplanted to plastic pots (18 cm diameter, 4 cm height) containing a sandy loam with organic matter content of about 1.5% total C. The target density was 11 plants per pot for *A. spica-venti* and 10 plants per pot for *A. myosuroides*. The pots were regularly watered from above (with a plant-nutrient solution containing macro- and micro-nutrients) and were kept in a greenhouse until herbicide treatments commenced. The diurnal greenhouse cycles were 14°C/10°C for 16 h/8 h during 22-25 days (from planting to treatment) for *A. spica-venti*, and 22°C/12°C for 17-22 days for *A. myosuroides*. At treatment, *A. spica-venti* plants had 3-5 leaves and *A. myosuroides* plants had 3-4 leaves.

Two pots per dose rate and seed sample were sprayed. The herbicide treatments, summarised in Table 2, were performed in a closed spray chamber (Experimental Pot Sprayer, 1992, Jens Kristensen, Ringsted, Denmark with Hardi ISO F-110, 025 Standard Flat Fan nozzles) set to resemble a field spray treatment (spray pressure 3 bars, spray boom speed 5.5 km per hour).

Commercial herbicide formulations were used throughout; Arelon FL(500 g a.i. L⁻¹ isoproturon, SC, CGNS Ltd), Monitor (sulfosulfuron 800 g a.i. kg⁻¹, MG, Monsanto Company), Event Super (fenoxaprop-P-ethyl 70 g a.i. L⁻¹, emulsion, oil in water, Bayer Crop Science) and Lexus 50WG (flupyr-sulfuron-methyl-Na 500 g a.i. kg⁻¹, water dispersible granulate, DuPont Sverige AB). Amount of spray liquid varied from 100-200 L ha⁻¹, and non-ionic wetting agents were used when applying sulfosulfuron (Biowet, alkyl alcohol 30-50%, Tergent AB, Sweden), fenoxaprop-P-ethyl and flupyr-sulfuron (Lissapol Bio, alcoholic ethoxylate >50% w/w, Syngenta Crop Protection A/S).

Following the herbicide treatments, the plants were placed in a green-house at 15C during day (16 h) and 10C at night (8 h) until plants were clearly affected.

2.3. Plant status evaluation and fresh weight harvest

When treated plants had become clearly affected, they were visually assessed and classified into five (0, 1, 2, 3, 4; *A. spica-venti*) or four (0, 1, 2, 3; *A. myosuroides*) vigour classes. The vigour classes were: dead (0), severely damaged (1), slightly affected or clearly affected but surviving (2) (for *A. spica-venti*, these were two separate classes, 2 and 3, as the range of symptoms allowed such separation especially for the isoproturon treatment), or unaffected (4 for *A. spica-venti*, 3 for *A. myosuroides*). Individual plant fresh weights were recorded. For *A. myosuroides*, data were collected 3 weeks after treatment while for *A. spica-venti*, corresponding time periods were 30 days and six weeks (isoproturon and sulfosulfuron, respectively). At harvest, there were no apparent effects of crowding or competition in pots.

2.4. Analyses

Each pot contained several plants (on average 8.3 and 9.9 for *A. spica-venti* and *A. myosuroides*, respectively). From the point of view of a strict experimental protocol, it would be justified to use the averages per pot in the dose-response analyses. However, if consistent

differences between pots are small or negligible, it might be justified to use the individual plants, ignoring the hierarchical structure in data, to achieve greater precision in estimates. To check for consistent between-pot differences, we calculated variance components for biomass, after eliminating population differences, of plants in the control treatment and in a herbicide treatment with intermediate effect. In one of eight cases there was a significant pot effect (*A. myosuroides*, flupyrsulfuron), while in the other the amount of variation accounted for was non-significant and low (2.6% or less). Hence, we decided to use individual plant data in the analyses in this paper.

The software GraphPad Prism 5.02 (GraphPad, 2008) was used to estimate GR₅₀. For one of the cases, biomass of *A. spica-venti* and isoproturon, there was an indication of hormesis so we selected a function [1] that allowed for a peak of biomass at low doses of the herbicide. For the other three cases, there was no indication of hormesis, so we selected a simpler model [2].

$$Y=(a+f*X)/(1+10^{((\log GR_{50}-X)*c)}) \quad [1]$$

$$Y=a/(1+10^{((\log GR_{50}-X)*c)}) \quad [2]$$

where “a” is the upper asymptote (the population-wise average of untreated control plants); “c” the HillSlope (that was set to be identical within one of the cases after a preliminary test), “f” a constant (that was set to be identical within the case) and “X” the log₁₀-transformed concentration of the herbicide in question. 95% confidence intervals (CI_{95%}) were calculated, and back-transformed for presentation.

The vigour classification was used to calculate a proxy of LD₅₀ (lethal dose), so that LD₅₀ meant that 50% of the plants scored in one the poor vigour classes. The model used [3] was:

$$Y=a/(1+10^{((\log LD_{50}-X)*c)}) \quad [3]$$

where “a” is the upper asymptote (4 or 3 for *A. spica-venti* and *A. myosuroides*, respectively); “c” the HillSlope and “X” the log₁₀-transformed concentration of the herbicide in question. Calculated CI_{95%} were back-transformed for presentation.

3. Results

3. 1. Range and shape of GR₅₀ and LD₅₀

For the four herbicides included in the present study, GR₅₀ (Fig. 1) and LD₅₀ (Fig. 2) estimates ranged 229-753 and 100-450 g a.i. ha⁻¹ (*A. spica-venti*/isoproturon); 1.8-8.9 and 1.6-12.2 g a.i. ha⁻¹ (*A. spica-venti*/sulfosulfuron); 7.8-10865 and 15-102031 g a.i. ha⁻¹ (*A. myosuroides*/fenoxaprop-P-ethyl); 1.66-6.43 and 3.0-87 g a.i. ha⁻¹ (*A. myosuroides*/flupyrsulfuron).

In three of the eight cases, there were indications that the sample of populations deviated from normality (Table 3; logGR₅₀ *A. spica-venti*/sulfosulfuron and both logGR₅₀ and logLD₅₀ for *A. myosuroides*/fenoxaprop-P-ethyl). When considering skewness, i.e. degree of asymmetry (if clearly different from 0, then asymmetrical) and degree of kurtosis i.e. peakiness of data (if clearly different than 0, then the distribution is either flatter or more peaked than expected from a normal distribution), these three cases also stand out as non-normal, together with logLD₅₀ for *A. spica-venti*/sulfosulfuron. Hence, one of the old and one of the new herbicides generated non-normal distributions of logGR₅₀ and logLD₅₀.

3.2. Plant size and GR_{50}

An interesting relationship noted were the two cases of significant correlation between GR_{50} and the biomass of control plants (Table 3, *A. spica-venti*/isoproturon and *A. myosuroides*/flupyrsulfuron).

3.3. Cross-correlation

For *A. myosuroides*, there was a positive correlation between the log GR_{50} and log LD_{50} for fenoxaprop-P-ethyl and flupyrsulfuron (Table 4, $P=0.00026$ and 0.0664 , respectively).

4. Discussion

There are four conclusions from this study, and we discuss them under separate headings below.

4.1. Magnitude of among-populations differences

Although it is a well-established fact that herbicide susceptibility can vary between genotypes, it is worth highlighting the magnitude of such differences between a near random selection of populations from arable fields. It appears that these ranges may differ among herbicides, irrespective of previous exposure to a herbicide (the old herbicide had both the largest and the smallest range; Table 3). There are at least two more reasons while range is a poor candidate for a variable to analyse for creeping resistance. First, “range” is always sensitive to the influence of sample size (the chance to pick up populations that reside on the outskirts of the distributions of GR_{50} or LD_{50} values increase with number of populations). Second, a screening of populations using a dose-response approach is unlikely to use test doses optimal for all populations. Consequently, populations at one or both ends of the range

of GR₅₀ or LD₅₀ will be poorly estimated. The latter is apparent for two of our cases where CI_{95%} varied greatly over the range (*A. spica-venti*/sulfosulfuron and *A. myosuroides*/fenoxaprop-P-ethyl; Fig. 1 and Fig. 2).

The differing ranges to be expected among herbicides also influence dose recommendations. Greater “safety margins” might be justified for species with a wide range, as is extra caution when using reduced doses.

4.2. Differential growth rate explain populations differences in susceptibility

In two of the GR₅₀ cases (*A. spica-venti*/isoproturon and *A. myosuroides*/flupyr-sulfuron), differential growth rate among populations seemed to influence their herbicide susceptibility, with a negative correlation between weight of untreated plants and GR₅₀ (Table 3). Hence, populations that grew slowly tolerated larger doses of herbicides better.

The functioning of many herbicides is linked to growth. While the relative importance of growth and growth stages will vary between combinations of species and herbicides (Kudsk, 2002; Reade and Cobb, 2002), growth should in general favour uptake and translocation to targeted tissues. Especially for systemic herbicides (as those tested here), lower growth could thus potentially have negative effects on efficacy (though there can be counter-acting factors).

In our studies we found a negative correlation between efficacy and growth only in two of four cases. For *A. myosuroides*/fenoxaprop, efficacy differences caused by higher growth were probably too small in relation to resistance effects to influence results. If the same resistance mechanism was responsible for the correlation with flupyr-sulfuron results, it is apparently still not blocking flupyr-sulfuron effects as effectively. Comparing responses to the two ALS-inhibitors, sulfosulfuron estimates were most uncertain in the lowest range,

which may have obscured an influence of growth for the most susceptible populations, while for flupyrsulfuron, the uncertainties of estimation were larger at the other end of the range.

It is worth noting that, if this is a widespread phenomenon, two very different selection pressures could be expected in a herbicide treated field: one promoting quickly growing genotypes (more competitive) the other slow growing ones (lower herbicide susceptibility). Or, phrased different, under the hypothesis of creeping resistance, the recruits could be likely to descend from individuals with slower growth.

4.3. No easy short-cuts

The assumption, that was the starting point for our analyses, was that $\log LD_{50}$ and $\log GR_{50}$ could be assumed to be normally distributed. If so, one might test for a shift in distribution over time, and might even infer creeping resistance based on a one-time sampling. Under this assumption, we expected the two new herbicides to yield normally distributed $\log LD_{50}$ and $\log GR_{50}$, while the old herbicides, being strong candidates for creeping resistance in these two species, might have displayed non-normal distributions. However, there is no justification for the assumption of normality in our data. One of the new herbicides had an even more deviating distribution compared with the old herbicides (Table 3, Figs 1 and 2).

The caveat mentioned above, that LD_{50} and GR_{50} might be poorly estimated at one end of the spectrum because the experimental protocol was sub-optimal, still hold here, of course. Hence, the estimates for populations contributing most to skewness might be biased. Nevertheless, from the point of view of a simple protocol to detect creeping resistance, non-normality in data should be expected.

Furthermore, it is difficult to justify any other “base-line” distribution of LD_{50} or GR_{50} for a one-time assessment: patterns were not consistent among the herbicides and especially so the new ones (Fig. 1 and Fig. 2). A more fruitful attempt might be to compare distributions from populations sampled at two points in time.

4.4. The elusive nature of Base Line Data

The conceptual nature of Base Line Data is that they would describe the natural state of susceptibility of a sample of previously unexposed populations towards a new herbicide and hence also the magnitude of between-population differences in susceptibility. Hence, based on these assumptions, data would have high external validity and say something about a particular combination of weed species and herbicide. This view of Base Line Data is, however, not appropriate because of the prevalence of cross-resistance between various combinations of herbicides. It has therefore been suggested that for species with widespread resistance, the aim of data collection for a new active ingredient must instead be to establish whether the new one is affected by existing resistance mechanisms, including determining already existing ranges of cross resistance due to enhanced metabolism. For this type of cross resistance with low predictability, empirical testing is considered to be essential (Moss, 2001). The view of the necessity of testing for cross resistance has been adopted in the resistance risk analysis standards of the European and Mediterranean Plant Protection Organization (EPPO (2003).

In our data, such a cross-resistance pattern was apparent in one of the two pairs of herbicides compared (Table 4). The cross-resistance between fenoxaprop-P-ethyl and flupyrsulfuron, and the lack thereof between isoproturon and sulfosulfuron, was to be expected, based on the literature (Heap, 2010).

Nevertheless, the existence of cross-resistance narrows down the meaning of Base Line Data: these do not describe the status of truly unexposed populations, in the sense of never exposed to herbicides, but the susceptibility status in the area sampled prior to the introduction of a new herbicide. Hence, depending on the history of herbicide usage, conclusions from Base Line Data might not be transferable to other situations. From the practical point of view, this has implications for transferability of recommended dose.

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Table 1

Number and origin of populations included in the study.

	Skåne	Öland	Östergötland
Latitude (°)	55.4-56.6	56.2-57.1	58.2-58.3
<i>Apera spica-venti</i>			
Old herbicide (isoproturon)	32	18	10
New herbicide (sulfosulfuron)	28	9	11
<i>Alopecurus myosuroides</i>			
Old herbicide (fenoxaprop-P-ethyl)	29		
New herbicide (flupyr-sulfuron)	29		

Table 2

Herbicide dose-response treatments in the greenhouse assay.

Species	<i>Apera spica-venti</i>		<i>Alopecurus myosuroides</i>	
Herbicide	“Old”: Isoproturon	“New”: sulfosulfuron	“Old”: fenoxaprop	“New”: Flupyr-sulfuron
Tested rates (g a.i./ha)	0 28 69 138 345 690 1380	0 2.2 3.8, 7.5, 11.2 15.0 22.5 30.0	0 22.4 56 140 224 364	0 1.5 4.0 10.0 25.0 40.0
Recommended rate (g a.i./ha)	1250-1500	20-25	56 – 70	10

1 **Table 3**

2 Characteristics of the dose-response analyses and outcomes from analyses (range of estimates, Resistance Factors; RF, the distribution of the

3 logLD₅₀ or logGR₅₀ estimates (deviation from normality, skewness and kurtosis), and the correlation with growth rate of populations. Numbers in

4 bold indicate deviations from normality, or null hypotheses.

	N (lack of dose- response solution)	Slope in dose- response	Hormesis in dose- response	Range of logGR ₅₀ or logLD ₅₀	RF _{average} (RF _{min})	Deviation from normality (KS test; d)	Skewness (SE)	Kurtosis (SE)	Correlation (r) between biomass of control and logGR ₅₀ or logED ₅₀
GR₅₀									
<i>Apera spica-venti</i>									
Old herb. (isoproturon)	56(4)	Individual	Yes	0.643	1.57 (4.39)	0.104 (NS)	-0.61 (0.319)	0.17 (0.628)	-0.352 (P=0.008)
New herb. (sulfosulfuron)	48	Joint	No	0.958	6.14 (7.78)	0.416 (P<0.01)	6.29 (0.343)	41.6 (0.67)	-0.117 (P=0.429)
<i>Alopecurus myosuroides</i>									
Old herb. (fenoxaprop-P-ethyl)	25 (4)	Individual	No	3.83	19.1 (6753)	0.342 (P<0.01)	0.98 (0.464)	2.21 (0.901)	0.026 (P=0.897)
New herb. (flupyr-sulfuron)	29	Individual	No	1.466	4.67 (29.2)	0.0872 (NS)	0.062 (0.433)	0.54 (0.845)	-0.385 (P=0.039)
LD₅₀									
<i>Apera spica-venti</i>									
Old herb. (isoproturon)	60	Individual	-	0.518	1.67 (3.30)	0.0977 (NS)	-0.16 (0.309)	-1.13 (0.608)	-0.1737 (P=0.185)
New herb. (sulfosulfuron)	48	Individual	-	0.697	3.10 (4.98)	0.109 (NS)	1.94 (0.343)	8.53 (0.674)	-0.179 (P=0.223)
<i>Alopecurus myosuroides</i>									
Old herb. (fenoxaprop-P-ethyl)	29	Individual	-	3.142	11.9 (1388)	0.253 (P<0.05)	1.40 (0.433)	1.64 (0.845)	-0.0807 (P=0.677)
New herb. (flupyr-sulfuron)	29	Individual	-	0.588	2.02 (3.88)	0.0907 (NS)	0.28 (0.433)	0.051 (0.845)	-0.022 (P=0.908)

5 **Table 4**

6 Correlation between herbicides tested, in dose-response experiments, on either *Apera spica-*
 7 *venti* or *Alopecurus myosuroides*.

	logGR ₅₀			logLD ₅₀		
	N	r	P	N	r	P
<i>Apera spica-venti</i>						
Old herbicide vs. new herbicide (isoproturon vs sulfosulfuron)	34	-0.0808	0.6498	34	0.2198	0.2116
<i>Alopecurus myosuroides</i>						
Old herbicide vs. new herbicide (fenoxaprop-P-ethyl vs. flupyr-sulfuron)	25	0.6679	0.00026	29	0.3455	0.0664

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Fig. 1. GR₅₀-estimates for four cases involving either an established herbicide, or one recently introduced on the market. Bars indicate 95% CI for estimate. The four cases were: 1a) *Apera spica-venti* old herbicide (isoproturon); 1b) *A. spica-venti* new herbicide (sulfosulfuron); 1c) *Alopecurus myosuroides* old herbicide (fenoxaprop-P-ethyl); 1d) *A. myosuroides* new herbicide (flupyr-sulfuron). Within each graph, populations are ordered according to increasing GR₅₀.

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 18 **Fig. 2.** LD₅₀-estimates for four cases involving either an established herbicide, or one recently
 19 introduced on the market. Bars indicate 95% CI for estimate. The four cases were: 1a) *Apera*
 20 *spica-venti* old herbicide (isoproturon); 1b) *A. spica-venti* new herbicide (sulfosulfuron); 1c)
 21 *Alopecurus myosuroides* old herbicide (fenoxaprop-P-ethyl); 1d) *A. myosuroides* new
 22 herbicide (flupyrsulfuron). Within each graph, populations are ordered according to
 23 increasing LD₅₀.