Modelling evolution and management of glyphosate resistance in *Amaranthus palmeri*

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Received:
Revised version accepted:

Running head: Modelling glyphosate-resistant *Amaranthus palmeri*

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Word count = 9493

Summary

A population-based model was developed to simulate the evolution of glyphosate resistance in populations of *Amaranthus palmeri*. Model parameters were derived from published and unpublished sources and the model was implemented using previously established principles and methods. Sensitivity analyses indicated that the model was sensitive to variations in population size, mutation rate and seed bank dynamics. A distribution was assigned to these parameters and Monte Carlo type simulations were performed. Simulation results are therefore derived from a range of possible input parameters, enabling the risk of resistance evolution to be assessed when parameter values were unknown, uncertain or variable. In the 'worst-case' of five annual glyphosate applications in continuous glyphosate resistant cotton, evolution of glyphosate resistance was predicted in 39% of populations after five years and in approximately 60% of populations after 10 years. These results are consistent with observations of the timescale for evolution of glyphosate resistance in *A. palmeri* in the field. The main drivers for glyphosate resistance evolution were selection pressure and population size, the greatest risks being associated with the largest *A. palmeri* populations. Risks of resistance were reduced when one of the five glyphosate applications was replaced by another mode of action with identical efficacy. However, not all glyphosate application exerted the same selection pressure. Application of a soil residual herbicide at the time of crop sowing can provide control of *A. palmeri* well into the growing season and significantly reduced the rate and risk of glyphosate resistance evolution.

Keywords: GM crops, herbicide resistance, resistance management, population dynamics, simulation model

Introduction

Evolved glyphosate resistance was first reported in *Lolium rigidum* Gaudin biotypes from Australia in the mid- to late 1990s (Powles *et al*., 1998). Since then, glyphosate resistance has been documented in an additional 18 species across six continents (Heap, 2010). The scale of the glyphosate-resistance problem, in terms of both geographical distribution and area infested, is greater in the United States than in other areas of the world, a phenomenon that has coincided with the widespread adoption of glyphosate-resistant crops.

The first glyphosate-resistant weed species from an arable production system in the United States was *Conyza canadensis* (L.) Cronq. reported in Delaware in 2000 (VanGessel, 2001). Since 2000, glyphosate-resistant *C. canadensis* populations have been reported in an additional 17 states (Davis & Johnson, 2007; Hanson *et al*., 2007). Glyphosate resistance has also been confirmed in *Amaranthus rudis* Sauer, *Ambrosia artemisiifolia* L., *Ambrosia trifida* L., *Lolium rigidum*, *Lolium perenne* L. spp. *multiflorum* (Lam.) Husnot, *Conyza bonariensis* (L.) Cronq., *A. palmeri*, *Sorghum halepense* (L.) Pers. and *Kochia scoparia* (L.) Roth (Heap, 2010). To date, glyphosate resistance in these weed species remains more localized than for *C. canadensis*. Nevertheless, many of these glyphosate-resistant species are more competitive than *C. canadensis* and thus pose a substantial threat to the sustainable use of glyphosate. This is particularly true in the case of *A. palmeri* (Horak & Loughin, 2000; Morgan *et al*., 2001).

Since 2005, glyphosate-resistant *A. palmeri* populations have evolved throughout the south-eastern United States, making it the most troublesome weed of cotton in Arkansas, Georgia, Missouri, North Carolina, South Carolina, and Tennessee (Webster, 2005; Culpepper *et al.*, 2006; Norsworthy *et al*., 2007). As of 2009, glyphosate-resistant *A. palmeri* was estimated to infest more than 675,000 hectares of land planted to soybean and cotton (Nichols *et al.*, 2009). *A. palmeri* populations have also evolved resistance to acetolactate synthase (ALS)-inhibiting, dinitroaniline, and triazine herbicides (Gossett *et al*., 1992; Burgos *et al*., 2001; Bond *et al*., 2006), and this, together with the species preference for widely-adopted conservation-tillage systems (Price *et al.*, 2009) have resulted in its increased success. *A. palmeri* exhibits a higher dry weight per plant, leaf area, and growth rate than other *Amaranthus* species (Horak and Loughin, 2000). It can grow at rates exceeding 3.5 cm d^{-1} and reach heights in excess of 2 m (Norsworthy *et al*., 2008), quickly overtopping a slower growing crop. *A. palmeri* is also dioecious with a single female plant producing up to 600,000 seeds $plant^{-1}$ (Keeley *et al*., 1987). These characteristics, together with its ability to

germinate and emerge over an extended period during the growing season, make *A. palmeri* a very effective weed species, capable of large crop yield reductions and rapid seed bank replenishment (Klingaman and Oliver, 1994; Rowland *et al*., 1999; Jha and Norsworthy, 2009). The continuing widespread evolution of glyphosate resistance in *A. palmeri* requires the design and adoption of proactive resistance management strategies that will reduce glyphosate use in cropping systems where it occurs as a weed.

Glyphosate-resistant soybean was released in the United States in 1996 and glyphosate-resistant cotton in 1997. This new technology provided soybean and cotton producers with broad-spectrum weed control and flexibility in application timings. Both glyphosate-resistant crops were rapidly adopted, and by 2006, 92% of the soybean hectares were treated with glyphosate (USDA, 2006) and 85% of the cotton hectares by 2007 (USDA, 2008). Often, cotton is grown as a continuous monoculture, but where rotation is practiced the most common rotational crops are corn and, less commonly, soybean, of which a high percentage is glyphosate-resistant. Currently, as many as five glyphosate applications are used for weed management in glyphosate-resistant cotton, which comprises approximately 98% of the cotton hectares in Arkansas (Norsworthy *et al*., 2007). As glyphosate use has increased, tillage has decreased (Young, 2006), exerting very high selection pressure for evolution of glyphosate resistance in *A. palmeri*.

In response to the evolution of glyphosate-resistant *L. rigidum* in Australia, computer models were developed to simulate evolution of glyphosate resistance under a number of cropping scenarios. In this way, glyphosate use patterns and cropping practices that increased the risks of glyphosate resistance and those that minimized selection pressure for resistance were identified and evaluated (Diggle *et al*., 2003; Neve *et al*., 2003a,b). More recently, modelling approaches have also been used to address risks of weed resistance to glyphosate in cropping systems with intensive use of glyphosate-resistant crop technology in the United States (Gustafson, 2008; Neve, 2008) and in Australia (Stanton *et al*., 2008; Werth *et al*., 2008).

Simulation studies have the advantage of providing rapid results without the need for time-consuming and costly large-scale field trials. Simulations can run over time-scales that are not practical in the field. Simulations may also consider stochastic demographic and genetic parameters and, in doing so, provide an indication of the risk of resistance evolution for a particular management practice or cropping system. Models are not, however, panaceas for resistance management studies and they require a good deal of quantifiable knowledge of the biology of the species in which resistance is being simulated, of the genetics and

inheritance of the resistance trait, and of cropping system parameters that influence the evolution of herbicide resistance (Jasieniuk *et al*., 1996; Diggle and Neve, 2001). Where data are available, simulation studies can provide insight into the species, herbicide and cropping systems characteristics that predispose towards evolution of resistance. They can provide an excellent comparison of resistance management strategies. They can also highlight important areas where data and knowledge are missing, and in this way, direct efforts towards future research priorities. In this paper, we describe the development and application of a model for simulating evolution and management of glyphosate resistance in *A. palmeri* growing in cotton-based agroecosystems in the southern United States. In particular, we assess risks of glyphosate resistance evolution under current 'worst-case' scenarios and explore management principles for mitigating these risks.

Materials and Methods

Model Overview.

The simulation model is implemented in the STELLA modelling software (STELLA version 9.0, isee systems[1]). A copy of the model can be made available to use on request from the corresponding author. The core structure of the model is based on the life cycle of *A. palmeri* (Figure 1). The initial *A. palmeri* population is the seed bank of viable, non-germinated seeds present in a single agricultural field. Within this seed bank, glyphosate-susceptible (S) and -resistant (R) individuals exist in numbers determined by the initial frequency of resistant alleles. Seeds germinate and emerge from the seed bank to produce a number of emergence cohorts. The relative survival of emerged S and R seedlings is determined by the weed management strategies that are deployed during each iteration (growing season) of the model. Surviving mature R and S adult plants produce seed according to a competition sub-model. The proportion of new seed of each of the three glyphosate resistance genotypes (SS, RS, and RR) is determined by a population genetics sub-model that describes the *A. palmeri* breeding system and the mode of inheritance for glyphosate resistance. At the end of the growing season, newly-produced seed is added to the soil seed bank. The model runs over 20 growing seasons, during which the population size and proportion of SS, RS, and RR genotypes are accounted.

Parameter values used in the model, together with sources are described in detail below. Generally, a default value is specified for each parameter and where parameters may

be region-specific they are based on Arkansas production areas. However, where there is uncertainty associated with the parameter estimate or where there is likely to be seasonal variation in the value, a range and distribution of values is specified. Due to the stochastic nature of the model, multiple iterations are run for any simulation. Data from multiple model iterations provides an indication of the risk of resistance evolution associated with different management scenarios.

Figure 1 near here

Model development I: A. palmeri Biology and Life Cycle.

Initial Seed Bank Population. The initial *A. palmeri* population size is the product of the initial seed bank density (seeds m^{-2}) and the field size (m^2). Seed bank densities will likely vary quite widely, depending on local conditions and management practices. The default value used in our analyses is 500 seeds m^{-2} and is based on local expert opinion The default field size is 60 ha (600,000 m^2), representing a typical field in cotton production in Arkansas. Default parameter values are summarized in Table 1 for ease of reference.

Annual germination proportion. Keeley et al. (1987) reported close to 50% annual recruitment of *A. palmeri* sown at an optimum depth of 1cm. However, these results do not agree with observations in South Carolina (Jha & Norsworthy, unpublished data) where 1% annual recruitment of *A. palmeri* was observed when seed was sown at 0-10cm depths. Recruitment studies for the closely related weedy amaranth, *A. rudis* conducted in Iowa have reported annual emergence to range from 1 to 7% of the seed bank (Buhler and Hartzler, 2001; Leon and Owen, 2004). Another Iowa study documented between <1 and 22% *A. rudis* recruitment per year over 4 years (Hartzler *et al*. 1999). Local experts in the southern central states believed that the proportion of recruitment of *A. palmeri* is similar to these values (L. Steckel, personal communication) and a default value of 0.05 was used.

Seedling recruitment. Seasonal patterns of weed recruitment determine the proportion of the population that is exposed to various herbicide applications and management practices. Recruitment patterns also determine the age structure of the population so that the efficacy of herbicide applications against different cohorts can be specified. Differences in the relative emergence times of weed cohorts determine their seed production potential.

A data set for *A. palmeri* recruitment under field conditions over three consecutive seasons (2004 through 2006) at Pendleton, South Carolina, USA (Jha, 2008) was used to derive a regression model to describe *A. palmeri* recruitment. The three years of data were pooled, and a three-parameter sigmoidal model (Eqn. 1) was found to adequately describe the data,

$$y = \frac{a}{1 + e^{-(\frac{x - x_0}{b})}} \quad [1]$$

where y = cumulative percentage recruitment at day x, a is the upper asymptote (final percentage cumulative recruitment), x_0 is days to 50% total emergence, and b is the slope of the curve around x_0. The fitted model ($R^2 = 0.797$) had parameter values of $a = 100$, $b = 10.25$ and $x_0 = 38.37$. The first day of seedling emergence ($x = 0$) for *A. palmeri* growing under field conditions in Arkansas was set as April 20.

In the simulation model, seven *A. palmeri* recruitment cohorts are defined based on the timing of crop and weed management practices. For example, cohort one is all individuals that emerge before crop planting (the default date for crop planting is set at May 1). Further details of the cohort structure are given in later sections that discuss weed management options.

Seedling Survival. Following recruitment, the probability of a seedling surviving to become a mature reproductive plant depends on (i) the efficacy of herbicide and other weed control practices and (ii) the probability of natural mortality. Herbicide efficacy depends on time of application, mode of action, seedling size, and glyphosate-resistance genotype. Full susceptibility to all herbicides other than glyphosate was assumed. Herbicide efficacy does not vary among years. It is possible to specify control efficacies for a potentially unlimited number of herbicides, and details of herbicide control efficacies are provided for all simulations presented. Natural mortality is independent of weed management and plant density and is assumed to increase from 5% mortality of cohort 1 plants to 50% mortality of cohort 6 plants (Jha *et al*., 2008) (Table 1).

When the number of herbicide-resistant individuals within the population is small, it is possible to predict the recruitment and/or survival of fractional plants. For example, if a total of 10 RS plants emerge and there is 95% herbicidal control, the model will predict that 0.5 of these RS plants will survive. This of course is not what happens in practice, where in reality there is a 50% chance that the single resistant plant survives. If this one-half of a plant

is entered into the competition sub-model it will produce thousands of seeds (an individual female *A. palmeri* plant can produce in excess of 600,000 seeds), and the number of R alleles in the population will have increased substantially. To overcome this, when the model predicts the survival of less than 10 plants of any genotype, an integer is drawn from a poisson distribution with a mean equal to the number of predicted surviving plants. Without this demographic stochasticity, the model may considerably over-estimate risks and rates of resistance evolution.

Table 1 near here

Competition and Seed Production. A competition sub-model predicts *A. palmeri* seed production per square meter. The competition model was adapted from Massinga *et al*., (2001) who fitted a nonlinear hyperbolic model (Eqn. 2) to their data which examined *A. palmeri* seed production in a corn crop,

$$S = \frac{gd}{1 + gd / B} \qquad [2]$$

where: S = number of seeds produced per square meter, d is *A. palmeri* population density (plants m row^{-1}), g is the number of seeds per plant as d approaches zero, and B is the maximum number of seeds that can be produced per square meter. Fitted parameter values were g = 421,000 and B = 582,300.

A number of modifications were made to the competition model to account for (i) the less competitive nature of a cotton compared to a corn crop, (ii) the different emergence times of various *A. palmeri* cohorts and (iii) the sub-lethal effects of glyphosate applications on surviving RS and RR plants.

It is assumed that a cotton crop is 20% less competitive than a corn crop. To account for this, when a cotton crop was simulated *A. palmeri* seed set potential is increased by increasing values of g and B by 20% (i above). *A. palmeri* cohorts 1 and 2 emerge before the crop and with the crop, respectively, and are assumed to achieve the seed production described by the competition model (Eqn. 2). The seed production potential of later emerging cohorts is lessened due to increased crop and weed competition. The crop emerges synchronously 10 d after planting, and the mean emergence date for each of the *A. palmeri* cohorts is calculated from the recruitment sub-model. From this, the number of days between emergence of the crop and *A. palmeri* cohorts can be calculated. For each day that an *A. palmeri* cohort emerges after the crop, there is assumed to be an exponential 7% reduction in

final biomass. This means that, for example, in a cotton crop, where cohort 3 emerges 13 days after the crop, each individual of cohort 3 will be equivalent to 0.39 (39%) of a cohort 1 plant. When seed production is calculated, each cohort 3 plant per square meter would be considered as 0.39 plants in the competition model (ii above).

Glyphosate-resistant *A. palmeri* plants often display phytotoxic effects following glyphosate application (Steckel *et al*., 2008). Injured plants resume growth and produce seeds, although plant size and seed production are reduced. The size, and therefore seed production, of glyphosate-resistant genotypes is modified to account for this effect by considering glyphosate-treated RS and RR plants to be 0.25 and 0.75 of untreated plants, respectively (iii above).

Once overall survival of *A. palmeri* plants has been calculated and effective plant population density has been modified to account for relative recruitment dates and sub-lethal glyphosate effects, total seed production is calculated according to Eqn. 2. We assume that 10% of freshly-produced seeds will be non-viable (Jason Norsworthy, unpublished data).

Seed mortality. Each year a proportion of non-germinated seeds will lose viability. Seed bank depletion studies have documented this process in *A. retroflexus*. Egley and Chandler (1983) found 1% viable seeds after 5.5 years burial, and Schweizer and Zimdahl (1984) found a similar level after 6 years. These seed bank depletion rates suggest an exponential decline in seed viability in *A. retroflexus* of approximately 70% per year and this value is assumed for *A. palmeri* also.

Seed predation. Post-dispersal seed predation, predominantly by invertebrates, results in removal of large quantities of newly produced seed from the soil surface before it is incorporated into the seed bank. In field experiments, Gallandt *et al*. (2005) have measured up to 58% seed predation and O'Rourke *et al*. (2006) between 80 and 90% during late summer. Both of these studies included *Amaranthus* species. Based on these studies, a conservative estimate of 50% removal by predation of freshly produced *A. palmeri* seeds is assumed in the model.

Seed importation. Weed populations are rarely closed systems and seed is regularly imported into fields as contaminants of crop seed, or via other vectors including farm machinery and animals. The model allows for annual importation of seed and the glyphosate R frequency of this seed can be specified. A default assumption of annual importation from surrounding

populations of 0.1 glyphosate susceptible seeds m^{-2} is made. As a result, extinction of the population never occurs.

Model development II: Genetics and inheritance of glyphosate resistance.

Inheritance of resistance. There have been no published studies of the inheritance of glyphosate resistance in *A. palmeri*, though a study by Gaines *et al*. (2010) has reported an increase in copy number of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) target enzyme for glyphosate in populations from Georgia, USA. Studies in other weed species have shown that glyphosate resistance is predominantly endowed by a single, nuclear, incompletely dominant gene, inherited in Mendelian fashion (Lorraine-Colwill *et al*., 2001; Ng *et al*., 2004; Wakelin and Preston, 2006; Zelaya *et al*., 2004). The model assumes that glyphosate resistance in *A. palmeri* is inherited in the same way.

Initial Frequency of R alleles. Empirical estimates of the frequency of resistant genotypes in populations with no history of herbicide exposure are almost impossible to obtain. Mutation-selection equilibrium theory suggests that equilibrium is reached between the continuous generation of new resistant alleles by mutation and the selective disadvantage of these alleles in the absence of the herbicide (Jasieniuk *et al*., 1996). Mean rates of mutation per locus per generation have been estimated to be between 10^{-6} and 10^{-7} (Maynard Smith, 1989). The rate of generation of functional resistant alleles is likely to be orders of magnitude lower, though this is somewhat balanced by the possibility of resistance being endowed by mutations at more than a single loci. There is evidence from ethylmethanesulfonate (EMS) mutagenizied *A. thaliana* lines that mutation rates for glyphosate resistance are lower than for other herbicides (Jander *et al*., 2003). Given these considerations, a default susceptible-to-resistant mutation rate of 5×10^{-9} is assumed within the model. If we assume there is no selective disadvantage for the R allele in the absence of selection, this will result in an equilibrium (initial) frequency of the R allele of 5×10^{-8} (see Jasieniuk *et al*., 1996). It is assumed that the initial population is in Hardy-Weinberg equilibrium and initial genotype (SS, RS, and RR) frequencies are calculated based on this assumption.

Reproductive System. A. palmeri is a dioecious species (Keeley *et al*., 1987). A survey conducted in Arkansas cropping fields during 2007 found the typical sex ratio to be three male plants to one female plant (Ken Smith and Jason Norsworthy, unpublished data). Ova

are produced on female plants in proportion to predicted seed production (for the purposes of the model an ovum is considered as a non-fertilized seed), and male plants produce pollen, which fertilizes these ova. Haploid ova and pollen carrying R or S alleles are produced in proportion to the number of reproductively mature plants of each genotype. Mutation of gametes from S to R and vice versa occurs at this stage according to the mutation rate. Gametes are recombined in a random fashion (panmixis), and mature seeds of the three resistance genotypes (SS, RS, and RR) replenish the seed bank.

Resistance Phenotypes. The glyphosate-resistance phenotype is the probability of survival of the resistance genotypes when glyphosate is applied to *A. palmeri* at recommended field rates. In inheritance studies with other species, glyphosate resistance has been expressed as an incompletely dominant trait and the percentage survival of SS, RS, and RR genotypes in the model is based on dose response curves from these studies (Lorraine-Colwill *et al*., 2001; Ng *et al*., 2004; Wakelin and Preston, 2006; Zelaya *et al*., 2004). When glyphosate is applied at the relevant field rate for *A. palmeri* control in cotton, it is assumed to have 99.9, 20, and 5% efficacy against SS, RS, and RR genotypes, respectively. In the model, these control percentages may vary according to the timing and method of glyphosate application.

We assume that glyphosate resistance does not confer cross-resistance to any other herbicide modes of action, and that all three glyphosate-resistance genotypes will be equally controlled by other herbicides used in this model. The model does not consider the potential for evolution of resistance to other herbicides as a result of repeated use, though pre-existing resistance to other modes of action can be assumed by altering control efficacies.

Model development III: Crop and weed management

For each simulation year, the crop grown and a weed management program are specified. Each management practice (e.g. crop planting, herbicide application) is carried out on specific dates to reflect best practice in Arkansas cotton-based systems (Table 2). Seven *A. palmeri* recruitment cohorts are defined in relation to these dates. Cohort 1 is all *A. palmeri* seedlings that emerge before crop planting (between April 20 and April 30). Cohort 2 emerges between crop planting and the first post-emergence herbicide application, cohort 3 between the first and second post-emergence application, cohort 4 between the second and third post-emergence application, and cohort 5 between the third post-emergence and final post-emergence applications. Cohort 6 emerges for 14 d after the final post-emergence application, and cohort 7 encompasses all plants that emerge after cohort 6. It is assumed that

cohort 7 plants will not set seed as they will not reach maturity prior to harvest. The relative contribution of each of these cohorts to total annual recruitment is shown in Table 3.

Table 2 near here

Table 3 near here

Model simulations

An initial series of 1000 model runs were completed to determine predicted risks of glyphosate resistance evolution under a 'worst-case scenario' of five annual glyphosate applications in continuous cotton cultivation. The timing and efficacy of these applications are in Table 4. For this initial analysis all parameters were fixed at default values (Table 1). Following this, a sensitivity analysis was performed on five key parameters that were judged to be associated with high levels on uncertainty due to difficulty in their estimation or likely season-to-season or demographic stochasticity. These parameters were the initial seed bank density, the initial frequency of the R allele, the annual emergence fraction, the annual proportion of seed bank mortality and the daily exponential decline in plant size for plants emerging after the crop. Each of these parameters was varied systematically while all other parameters were maintained at default values. The parameter ranges for sensitivity analyses reflected the likely range of parameter uncertainty. These likely ranges were up 1000-fold variation for initial frequency of the R allele and much less for other parameters (see Tables 1). One thousand model runs were completed for each analysis.

In the following simulations, based on the results of sensitivity analyses, the mean values for these five parameters were maintained, but a distribution of values around this mean was specified. For initial seed density, a random number generator was used to select from 11 densities (100, 200, 300, 400, 500, 750, 1000, 1250, 1500, 1750 and 2000 seeds m^{-2}) so that the probability of each of these densities was 5, 10, 10, 10, 25, 10, 10, 5, 5, 5 and 5%, respectively. The initial frequency of R alleles and mutation rate were log-normally distributed with standard deviations of 1×10^{-7} and 1×10^{-8}, respectively. Annual seed germination percentage, annual seed bank mortality and daily exponential decline in plant size were normally distributed with standard deviations of 0.1, 0.1 and 0.01, respectively. At every iteration of the model, a value was independently drawn from each of these distributions. The 'worst-case' scenario simulation was repeated with the modified model and 10,000 runs were performed to enable predicted outcomes to be simulated from across this input parameter space.

In order to assess the impacts of reducing reliance on glyphosate for *A. palmeri* control, a series of simulations were performed where one of the five glyphosate applications was substituted for an alternative herbicide with identical efficacy to glyphosate. Finally, the impacts of replacing the first glyphosate application at the time of crop sowing with a residual herbicide that provided 99.9, 95, 80 and 40 percent control of *A. palmeri* cohorts one to four, respectively, was assessed.

For each run of the model, data were saved for proportion of SS, RS and RR genotypes in the seed bank, the number of surviving *A. palmeri* plants per m^2 and the total *A. palmeri* seed bank population size. A population was deemed to have evolved resistance to glyphosate when in excess of 20% of the population was phenotypically resistant (RS + RS genotypes). Each run was considered to represent a discrete *A. palmeri* population and results were analysed and presented to indicate in what proportion of populations and over what timescale glyphosate resistance was predicted to evolve.

Table 4 near here

Results

Glyphosate resistance evolution in a 'worst-case' scenario

Simulation with default parameters. With five annual glyphosate applications in continuous glyphosate-resistant cotton, resistance is predicted to evolve in 32% of *A. palmeri* populations after four years and in a further 19% of populations after five years (Figure 2). After year seven, there are only rare occurrences of resistance evolution and over the 20-year simulation period, resistance is predicted in 58% of populations. Hence, generally, where resistance does not evolve within seven years, the predicted risk of subsequent evolution of resistance is low. These dynamics result from the particular biological characteristics of *A. palmeri* (low annual germination fraction and high seed bank mortality) and the highly effective nature of the glyphosate-dominated weed management strategy when resistance does not evolve. Simply, if none of the glyphosate-resistant genotypes initially present in the population survives to replenish the seed bank, then it is likely that by year seven the population will have been reduced to such a degree that new mutants are unlikely to arise (low population size and low mutation rate).

Figure 2 near here

Sensitivity analysis. Model output is particularly sensitive to the initial seed bank density (*A. palmeri* population size), the initial frequency of R alleles and the annual recruitment proportion and less so to variations in seed bank mortality and the daily reduction in final plant size for later emerging cohorts (Figure 3). There can be no 'correct' value for initial seed density as this will vary from field to field. These results clearly demonstrate that risks of resistance evolution are greater in larger populations. The initial frequency of R alleles is the most difficult parameter to estimate and the model is highly sensitive to this parameter over a narrow range of realistic values. As for initial population size, this parameter is likely to vary between populations. Sensitivity to annual recruitment proportion over the range 0.01 to 0.1 reflects the importance of this parameter for determining the likelihood that resistant phenotypes will germinate, survive and produce new seed versus the likelihood that they will lose viability in the soil seed bank prior to germination. Where the recruitment proportion is higher, survival and production of new seed becomes more likely resulting in increases in the probability of resistance evolution. Although the model is sensitive to varying degrees to all the parameters included in this analysis, we take the view that there is no correct value for any of these parameters and that they will vary from population-to-population and from season-to-season. Hence, for remaining analyses, values for these five parameters and for the mutation rate have been allowed to vary stochastically according to specified distributions (see Methods).

Figure 3 near here

Glyphosate resistance in demographically and genetically stochastic A. palmeri populations. Where glyphosate resistance was not able to evolve (initial frequency of R alleles and the mutation rate were set at zero), *A. palmeri* population densities generally declined over the 20 year simulation period, though there was considerable season-to-season and run-to-run variation in population dynamics (Figure 4). Although we do not have access to validation datasets for *A. palmeri* population dynamics in continuous glyphosate-resistant cotton, we are satisfied that simulated datasets are not inconsistent with realistic population trajectories in the absence of evolved resistance.

In the modified stochastic model, resistance is predicted in 18 and 20 percent of populations after 4 and 5 years, respectively, and in 73% of populations over the 20 year simulation (Figure 5). These results are not substantively different to those predicted with the default model (Figure 2), though predicted risks of resistance are lower during the first five years, but higher between years 5 and 20 of the simulation. The increased probability of

resistance after 5 years is a result of simulated annual fluctuations in population size and mutation rate, and therefore, the *de novo* generation of R alleles within the population. Generally, with default model parameters there is a more deterministic decline in population size which means that the population more rapidly declines to a level where *de novo* generation of R alleles does not occur.

Figure 4 near here

Figure 5 near here

Reducing selection for glyphosate resistance in continuous cotton.

Simply reducing the number of annual glyphosate applications is not an effective means for mitigating risks of glyphosate resistance evolution. This strategy results in incomplete control of *A. palmeri* and an increase in the proportion of predicted resistant populations as novel resistant mutants are rapidly accumulated and selected in large populations (data not shown). To maintain *A. palmeri* control and reduce selection for glyphosate resistance it is necessary to replace glyphosate applications with alternative herbicides with identical efficacy. However, when this is done, predicted reductions in both the rate and risk of resistance evolution vary according to which of the five glyphosate applications is substituted (Figure 6). Replacing the burndown glyphosate application has no impact on predicted glyphosate resistance evolution. An alternative first post-emergence application delays the evolution of resistance by a year, but does little to reduce predicted risks of resistance over the longer term. The greatest impacts are observed when the second or third post-emergence glyphosate applications are substituted. In particular, substitution of the third post-emergence application delays predicted evolution of resistance by two years and reduces the proportion of resistant *A. palmeri* populations at year 10 from 59% to 34%. These results are a clear demonstration that not all glyphosate applications exert the same selection pressure for resistance evolution and this insight is valuable for the design of potential management strategies for mitigating resistance risks in glyphosate-resistant cotton.

Replacement of glyphosate applied at the time of crop sowing with an alternative non-residual herbicide was ineffective for reducing resistance risks. However, when this glyphosate application was replaced with a residual herbicide that provided some control of *A. palmeri* cohorts one through four, predicted resistance risks were substantially reduced (Figure 7).

Figure 6 near here

Figure 7 near here

Discussion

The worst-case scenario simulated here is a reality in some areas of the southern USA cropping region. A survey conducted in 2006 found that two-thirds of cotton producers in this region had grown glyphosate-resistant cotton continuously for 3-5 years. Of all cotton producers surveyed, 21% used glyphosate only, 21% used glyphosate in a tank mixture with another herbicide mode of action and 52% used a pre-emergence residual herbicide in advance of glyphosate-only for post-emergence weed control (Foresman & Glasgow, 2008). Increases in glyphosate use have coincided with a reduction is soil cultivation (Young, 2006). Concerns that these practices are unsustainable have been heightened by the recent, widespread evolution of resistance to glyphosate in *A. palmeri* (Nichols *et al*., 2009.). The stochastic population-based model for *A. palmeri* simulates realistic population trajectories for this species under glyphosate-only management systems in cotton and, where resistance does not evolve, the utility of this system for controlling *A. palmeri* populations is clear. In our 'worst-case' simulations, resistance was predicted in 4-5 years in approximately 40% of populations. This result, in combination with the producer survey results (Foresman & Glasgow, 2008), suggests that within 5 years of adopting glyphosate-resistant cotton, approximately 5% of cotton producers will have glyphosate-resistant *A. palmeri* populations (40% risk amongst producers using glyphosate-only in glyphosate-resistant cotton monoculture). These results are corroborated by field observations where glyphosate resistance was first reported in 2004, 4 or 5 years after the widespread adoption of this technology (Culpepper *et al*., 2006). It is probable that glyphosate resistance will only be readily apparent in the field a year or two after its evolution and thus, the rapid and widespread reports across many regions of the southern USA of glyphosate resistant *A. palmeri* populations during 2005-2007 (Nichols *et al.*, 2009) suggest that these simulations are realistic. The absence of field management histories at many locations where glyphosate resistance has evolved and a lack of detailed knowledge of the precise number of independent evolutions of glyphosate resistance make model validation problematic. Detailed model validation can only really be achieved with multiple large-scale and long-term field experiments and these are impractical. In light of these facts, we believe the corroboration between model predictions and field observations provides good evidence to support the validity of the *A. palmeri* model.

Sensitivity analyses have been important for highlighting those demographic and genetic parameters that have the largest impact on risks of resistance evolution. Population

size is paramount and resistance risks are far greater in large compared to small populations. There are two key considerations here: if populations are small at the time that glyphosate-resistant technologies are adopted then risks can be minimised; also, if weed management efficacy is low, causing the population size to increase, risks of glyphosate resistance are exacerbated, even where selection pressure is reduced. Put simply, evolution of resistance is a numbers game – the larger the population, the more likely that resistant mutants will pre-exist or will arise by spontaneous mutation. These results are contrary to previous suggestions that risks of resistance evolution are lower when herbicide efficacy is low (Jasieniuk & Maxwell, 1994; Diggle & Neve, 2001). However, these conclusions arose from models with an infinite population size, meaning that the relationship between population size and mutation rate was obscured.

The model is most sensitive to the initial frequency of R alleles and to the mutation rate and these are the most difficult parameters to estimate empirically. Population demography is also important and here the interplay of seed bank processes that result in the proliferation of genotypes (germination and recruitment) versus those processes that remove genotypes from the population (seed mortality and/or predation) is key. Where annual recruitment is high, the probability that initially rare glyphosate-resistant genotypes will survive and proliferate is increased. Conversely, when the rate of seed loss from the seed bank increases, it becomes more likely that rare mutant types will be lost from the population by random genetic drift.

The model is highly sensitive to input parameter values. However, for all parameters included in sensitivity analyses, variation from year-to-year and from population-to-population is a reality. Initial population sizes clearly vary from field-to-field. Spontaneous mutation is a stochastic process and demographic parameters such as annual recruitment rate and seed mortality are clearly influenced by variable climatic and other environmental variables. Without demographic stochasticity the population trajectory is fixed and if resistance does not evolve within a certain period then initial R alleles go extinct and the population size becomes small enough that *de novo* generation of R alleles by spontaneous mutations becomes highly unlikely. To reflect this we adopted a Monte Carlo type approach to simulations, so that for any scenario, population dynamics and resistance evolution were simulated in thousands to tens of thousands of 20 year model runs. Each model run represents a different *A. palmeri* population and the risk of glyphosate resistance evolution is presented by performing simulations across all of the input parameter space.

559 Many proposed strategies for mitigating risks of evolution of herbicide resistance
560 advocate the use of herbicide rotations, sequences and mixtures and the efficacy of these
561 strategies has been investigated theoretically (Wrubel & Gressel, 1994; Diggle *et al*., 2003;
562 Jacquemin *et al*., 2009) and empirically (Beckie & Reboud, 2009). Our modelling analysis
563 has shown that replacing a single glyphosate application with an alternative mode of action
564 can reduce the rate and risk of glyphosate resistance evolution. However, it is particularly
565 noteworthy that not all glyphosate applications exert the same selection pressure. Glyphosate
566 applied at crop planting, controls early emerging *A. palmeri* seedlings (cohort one) and this
567 cohort represents only 6.5% of total annual emergence. The greatest selection pressure for
568 resistance is exerted by the third post-emergence glyphosate application. This application
569 controls *A. palmeri* emergence cohorts three and four, the two largest cohorts. Hence, this
570 application provides the greatest percentage control of the population and, by extension,
571 exerts the most selection pressure. Similar results have been demonstrated previously for *L.*
572 *rigidum* in Australian cropping systems (Neve *et al*., 2003a). It should be recognised that any
573 alternative herbicide will also be prone to evolution of resistance. The continued use of an
574 alternative mode of action in combination with glyphosate should reduce risks of resistance
575 to both herbicides (Diggle et al., 2003). However, the greater the diversity of alternative
576 modes of action used, the more these risks can be reduced.
577 Application of a soil residual herbicide around the time of crop sowing provides mode
578 of action diversity for control of *A. palmeri* cohorts one to four and, in effect, this single
579 herbicide application means that each of these cohorts is exposed to a sequence of the
580 residual herbicide and glyphosate (albeit with reducing efficacy of the residual herbicide
581 against the later cohorts). This relatively simple and inexpensive strategy, practiced by many
582 cotton growers (Foresman & Glasgow, 2008) is effective in delaying resistance and
583 approximately halves the number of *A. palmeri* populations in which resistance is predicted.
584 In future, it will be important to continue to investigate the genetic basis of glyphosate
585 resistance. In most cases, where patterns of inheritance have been established, glyphosate
586 resistance has been endowed by single gene traits (Preston *et al*., 2009). Nevertheless, there is
587 mounting evidence that this may not always be the case (Busi & Powles, 2009; Gaines *et al*.,
588 2010) and it will be interesting to contrast selection for single gene versus polygenic traits in
589 future empirical and modelling-based studies. Similarly, the implications of costs of
590 resistance for glyphosate have not been investigated here, though a number of studies have
591 begun to demonstrate small costs in the absence of glyphosate selection (Baucom &
592 Mauricio, 2004; Pedersen *et al*., 2007; Preston *et al*., 2009). Finally, our model does not

consider pollen-mediated gene flow between adjacent populations and this will be an important consideration in the design and implementation of resistance management strategies (Dauer *et al*., 2009).

The utility of the developed model, both for understanding evolution and management of glyphosate resistance in *A. palmeri* and for demonstrating wider principles of resistance management has been demonstrated. *Amaranthus palmeri* presents a particularly severe risk of glyphosate resistance evolution for a number of reasons, including its preference for no-tillage cropping systems and its season-long germination. However, the species prolific seed production capacity poses the greatest threat, meaning that survival and reproduction by a single resistant plant contributes potentially hundreds of thousands of seeds to the seed bank. Given this, there is an urgent need to design and evaluate new resistance management strategies in glyphosate-resistant cotton that will deliver cost-effective, yet sustainable control of *A. palmeri* whilst maintaining other benefits associated with glyphosate-resistant crop technology. The principles on which these strategies can be founded have been demonstrated here and future studies are planned that will evaluate how these principles can be put into practice in novel weed management strategies.

Acknowledgements

Support for this work was provided to PN by Syngenta LTD. Partial support of this research to JKN and KLS by Cotton Incorporated is greatly appreciated.

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762
763 **Table 1.** Summary of biological parameters for *A. palmeri* model.

764

Parameter	Value
Field size	60 ha
Initial seed bank density†	500 seeds m^{-2} *(100 – 2000)*
Initial frequency of R allele†	5×10^{-8} *(5×10^{-10} - 5×10^{-7})*
Mutation rate†	5×10^{-9}
Annual germination proportion†	0.05 *(0.01 – 0.2)*
Proportion natural mortality	
Cohort 1	0.05
Cohort 2	0.1
Cohort 3	0.2
Cohort 4	0.3
Cohort 5	0.4
Cohort 6	0.5
Number of seeds produced per plant as density approaches zero	505,200
Maximum seed production m^{-2}	698,760
Exponential decline in plant size for each day cohort x emerges after the crop†	0.07 *(0.04 – 0.1)*
Relative size of glyphosate-treated RS plants	0.25
Relative size of glyphosate-treated RR plants	0.75
Proportion loss of seed viability in seed bank†	0.7 *(0.3 – 0.9)*
Proportion of new seed predated	0.5
Proportion viability of fresh seed	0.9
Annual immigration of glyphosate-susceptible seed	0.1 seeds m^{-2}

Male: female sex ratio	0.75

765

766 † For these parameters sensitivity analyses were performed and parameter ranges are shown in
767 parentheses below the default value. In the modified version of the model, these parameters
768 values are drawn from a distribution whose mean is the default value.

769

Table 2. Crop and weed management operations and default dates for Arkansas cotton production.

Management operation	Date
Pre-crop sowing residual herbicide	March 30
Crop sowing	May 1
Residual herbicide at sowing	May 1
Burndown herbicide at sowing	May 1
First post-emergence herbicide	May 15
Second post-emergence herbicide	May 30
Third post-emergence herbicide	June 15
Final post-emergence herbicide	July 1

775 Table 3. *A. palmeri* recruitment cohorts.

776

Cohort number	Dates	% of total annual recruitment
1	April 20 – April 31	6.5
2	May 1 – May 14	14.9
3	May 15 – May 29	32.6
4	May 30 – June 14	30.9
5	June 15 – June 30	11.6
6	July 1 – July 14	2.7
7	After July 14	1.0

777

Table 4. Glyphosate application timings for control of Palmer amaranth in glyphosate-resistant cotton crops. Control efficacies are specified on a cohort and a genotype basis.

Glyphosate application timing	Date	Palmer amaranth control																	
		Cohort 1			Cohort 2			Cohort 3			Cohort 4			Cohort 5			Cohort 6		
		SS	RS	RR	SS	RS	RR	SS	RS	RR	SS	RS	RR	SS	RS	RR	SS	RS	RR
		%																	
Burndown at planting	May 1	99.9	20	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
First post-emergence	May 15	99.9	20	5	99.9	20	5	0	0	0	0	0	0	0	0	0	0	0	0
Second post-emergence	May 30	99.9	20	5	99.9	20	5	99.9	20	5	0	0	0	0	0	0	0	0	0
Third post-emergence (directed[1])	June 15	0	0	0	0	0	0	95	20	5	99	20	5	0	0	0	0	0	0
Final post-emergence (directed)	July 1	0	0	0	0	0	0	0	0	0	95	20	5	99	20	5	0	0	0

[1] – Directed sprays are applied to base of cotton plants due to the insufficient tolerance of glyphosate-resistant cotton plants to glyphosate application past a certain growth stage. These directed sprays provide insufficient control of larger weeds (for example, cohort 1) due to insufficient coverage of foliage.

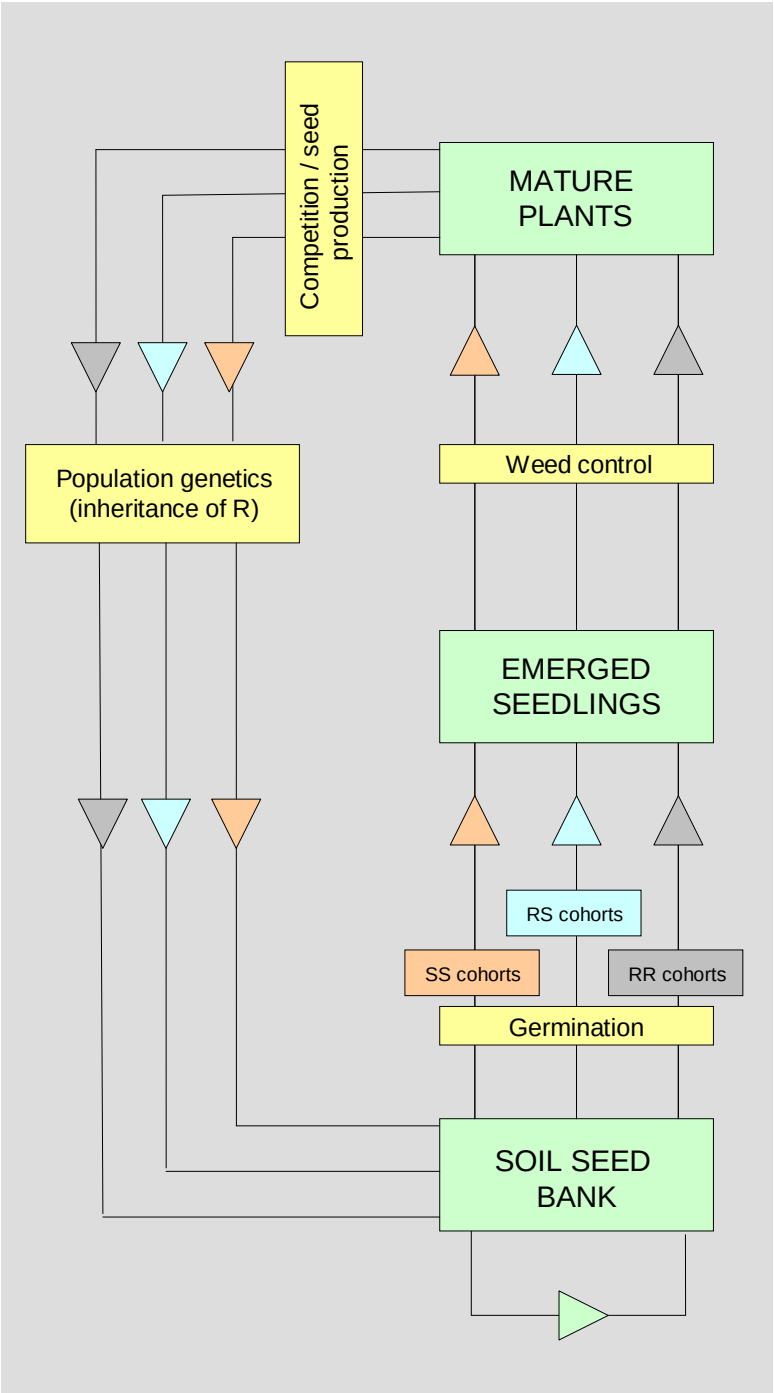


Figure 1. A simplified schematic representation of the *A. palmeri* glyphosate-resistance simulation model. Large green-shaded boxes represent the three major life history stages. The yellow boxes represent modelled processes that determine the numbers of individuals of each resistance genotype that move from one life history stage to the next. The RR (homozygous resistant), RS (heterozygous resistant), and SS (homozygous susceptible) genotypes are represented by the orange, blue, and gray triangles, respectively.

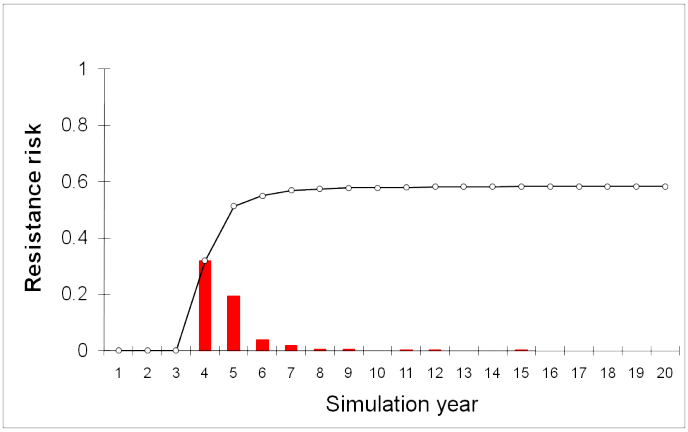
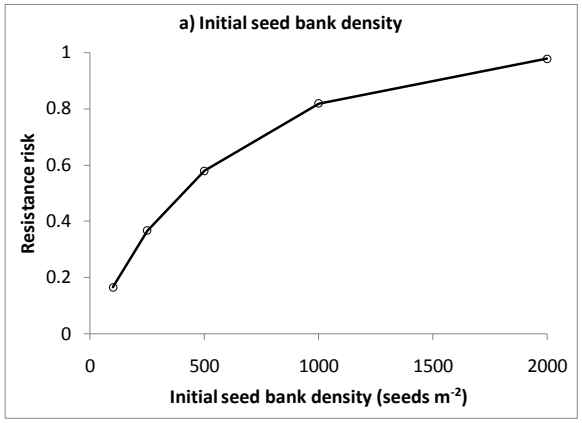
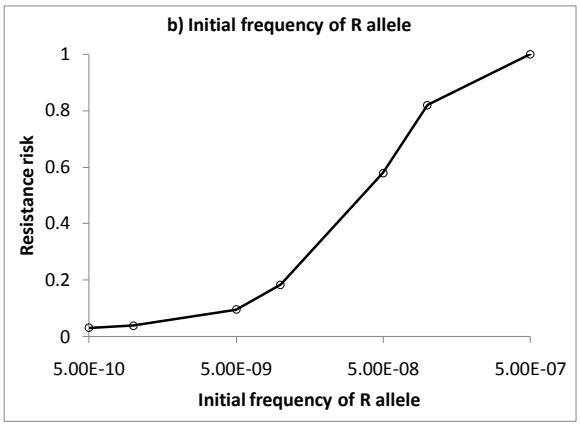
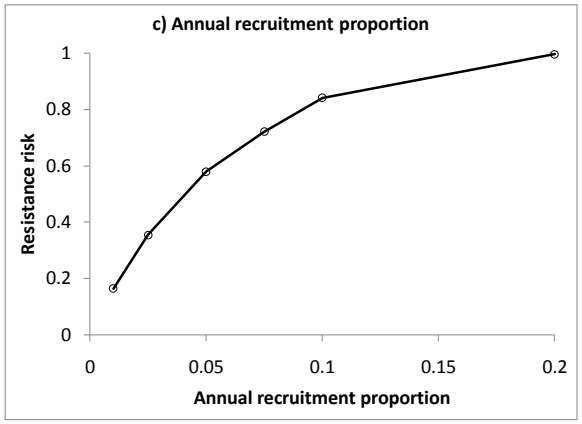
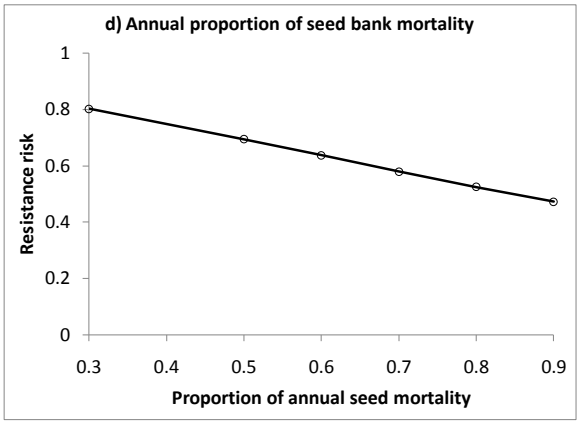


Figure 2. Simulated evolution of glyphosate resistance in *A. palmeri* populations under a worst-case scenario of five annual glyphosate applications in continuous glyphosate-resistant cotton where demographic and genetic parameters are fixed at default values. A population is deemed to be resistant when 20% of individuals are phenotypically resistant (RS or RR genotype). Resistance risk is a measure of the proportion of populations (of 1000 runs) in which resistance is predicted at simulation year *x*. Bars represent the proportion of populations becoming resistant in each of the 20 simulation years and the line plots show the cumulative probability of resistance over the 20 year simulation.









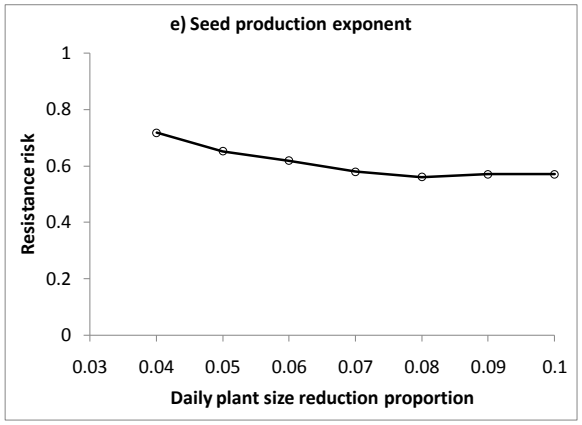


Figure 3. Sensitivity analyses showing impacts of parameter variability on the simulated evolution of glyphosate resistance in *A. palmeri* populations after ten years of continuous glyphosate-resistant cotton. Each data point represents the proportion of 1000 model runs in which glyphosate resistance was predicted.

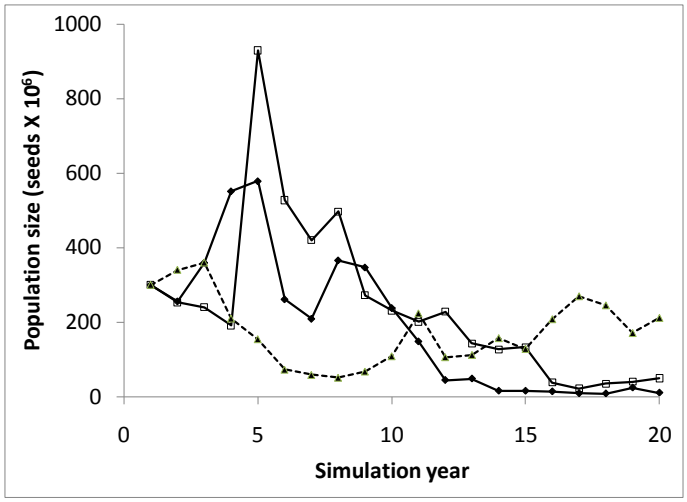


Figure 4. Simulated *A. palmeri* seed bank population densities in continuous glyphosate-resistant cotton monoculture where glyphosate resistance does not evolve. The three plots are from discrete model runs demonstrating the impacts of demographic stochasticity on variations in population size and trajectory from year-to-year and between model runs.

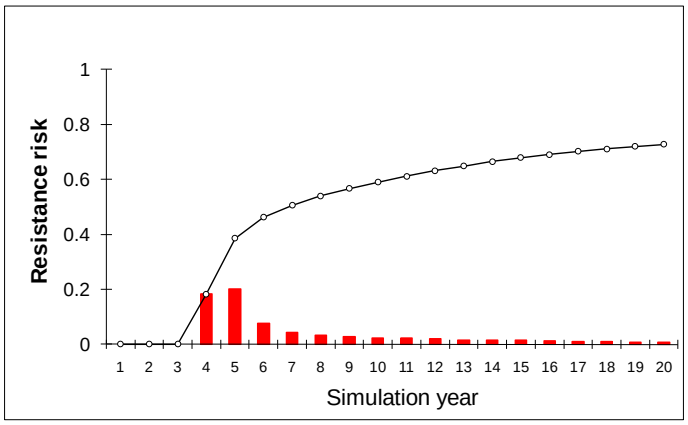


Figure 5. Simulated evolution of glyphosate resistance in *A. palmeri* populations in continuous glyphosate-resistant cotton with five annual glyphosate applications. In contrast to Figure 2, values for key demographic and genetic parameters vary stochastically from year-to-year and between model runs. A population is deemed to be resistant when 20% of individuals are phenotypically resistant (RS or RR genotype). Resistance risk is a measure of the proportion of populations (of 10,000 runs) in which resistance is predicted at simulation year *x*. Bars represent the proportion of populations becoming resistant in each of the 20 simulation years and the line plots show the cumulative probability of resistance over the 20 year simulation.

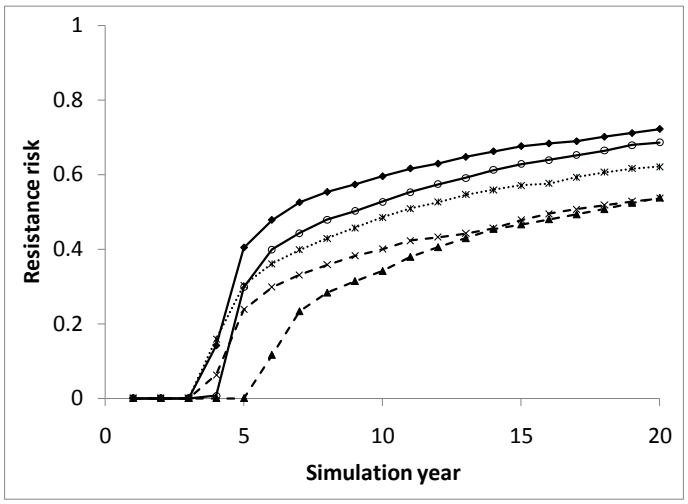


Figure 6. The Simulated evolution of glyphosate resistance in *A. palmeri* populations in continuous glyphosate-resistant cotton when burndown (solid line, black diamonds), first post-emergence (solid line, white circles), second post-emergence (dashed line, black triangles), third post-emergence (dashed line, black crosses) and final post-emergence (dotted line, white triangles) herbicides are substituted for notional alternative herbicide modes of action with identical efficacy. A population is deemed to be resistant when 20% of individuals are phenotypically resistant (RS or RR genotype). Resistance risk is a measure of the proportion of populations (of 1000 runs) in which resistance is predicted at simulation year *x*.

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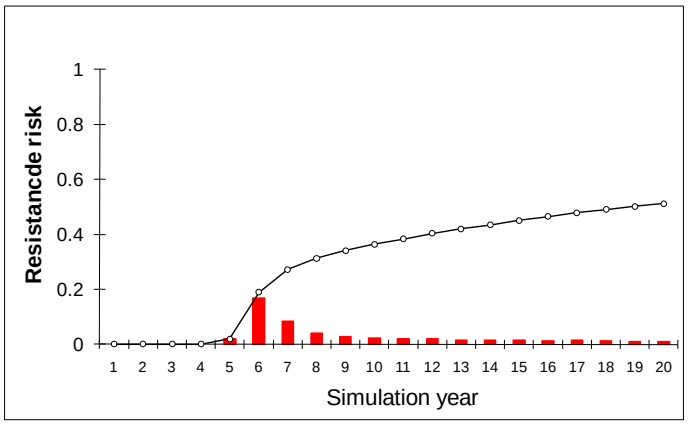

Figure 7. Simulated evolution of glyphosate resistance in *A. palmeri* populations in continuous glyphosate-resistant with application of a residual herbicide at crop sowing time that provides 99.9, 95, 80 and 40% control of cohorts one to four, respectively. A population is deemed to be resistant when 20% of individuals are phenotypically resistant (RS or RR genotype). Resistance risk is a measure of the proportion of populations (of 10,000 runs) in which resistance is predicted at simulation year *x*. Bars represent the proportion of populations becoming resistant in each of the 20 simulation years and the line plots show the cumulative probability of resistance over the 20 year simulation.