Weed Science 2014 62:29–37

Impact of Genetic Background in Fitness Cost Studies: An Example from Glyphosate-Resistant Palmer Amaranth

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Since its discovery in 2005, glyphosate-resistant Palmer amaranth has become a major problem for many farmers in the southern United States. One mechanism of resistance found in a Georgia population of glyphosate-resistant Palmer amaranth is amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene throughout the genome, with some resistant plants containing and expressing more than 100 *EPSPS* genes. Such high numbers of *EPSPS* genes and protein production could result in a fitness cost to resistant plants due to (1) metabolic cost of overproduction of this enzyme and (2) disruption of other genes after insertion of the *EPSPS* gene. A greenhouse experiment was set up to investigate differences in growth and reproduction between glyphosate-susceptible and -resistant Palmer amaranth plants. Measurements included growth rate, plant height/volume ratio, final biomass, photosynthetic rate, inflorescence length, pollen viability, and seed set. This study found no significant fitness costs for plants with the resistance trait. This study also provided a clear example of how controlling for genetic background is important in fitness cost studies and how potentially misleading results can be obtained if only a few fitness traits are measured. These results indicate that glyphosate-resistant Palmer amaranth plants with high *EPSPS* gene copy numbers are likely to persist in field populations, even in the absence of glyphosate, potentially leading to long-term loss of glyphosate as a control option for Palmer amaranth.

Nomenclature: Glyphosate; Palmer amaranth, *Amaranthus palmeri* S. Wats AMAPA.

Key words: 5-Enolpyruvylshikimate-3-phosphate synthase, *EPSPS* amplification, herbicide resistance.

Palmer amaranth is a dicotyledonous weed species native to Southern California and now prevalent across most of the southern United States (Steckel 2007). It is highly competitive, capable of growing 2 to 3 m in height, and producing upward of 600,000 seeds per female plant when left uncontrolled (Ward et al. 2013). This results in significant yield losses for several crop species (Ward et al. 2013).

Adding to the difficulty of Palmer amaranth control is the accumulation by this weed of resistance to several herbicides since the late 1980s. Resistance to dinitroaniline herbicides was reported first (Gossett et al. 1992), followed soon after by acetolactate synthase (ALS) inhibitors and photosystem II inhibitors (Heap 2012; Horak and Peterson 1995). In 2006, Culpepper et al. confirmed the first appearance of glyphosate-resistant Palmer amaranth in Macon County, GA. Glyphosate-resistant Palmer amaranth has since spread to 13 U.S. states (Heap 2012). Control of glyphosate-resistant Palmer amaranth is difficult, especially in cotton fields where the problem first originated. Many cotton farmers are turning to hand-hoeing to rid their fields of Palmer amaranth: between 2000 and 2005, only 17% of growers hand-hoed their cotton fields, but between 2006 and 2010, this number rose to 92% of cotton growers (Sosnoskie et al. 2012).

A variety of mechanisms confers resistance to glyphosate in plants, including 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) target site mutations, altered translocation/vacuolar sequestration, and *EPSPS* gene amplification (Shaner et al. 2012). The Palmer amaranth population from Georgia used in this study is resistant to glyphosate via *EPSPS* gene amplification (Gaines et al. 2010). In susceptible plants, glyphosate binds to the active site of EPSPS, inhibiting its normal function as a key enzyme in the shikimate pathway, necessary for the production of aromatic amino acids (Steinrucken and Amrhein 1980). When a glyphosate-resistant Palmer amaranth plant amplifies the number of *EPSPS* genes, it produces a proportionally increased amount of EPSPS, sufficient to maintain the shikimate pathway in the presence of glyphosate applied at field rates (Gaines et al. 2010). Gene amplification as a mechanism of herbicide

DOI: 10.1614/WS-D-13-00066.1

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resistance has now been reported in two other weed species, kochia (*Kochia scoparia* (L.) Schrad.; Wiersma 2012) and Italian ryegrass (*Lolium perenne* spp. *multiflorum* (Lam.) Husnot; Salas et al. 2012). Fitness costs associated with glyphosate resistance have been detected in rigid ryegrass (*Lolium rigidum* Gaudin; Pederson et al. 2007; Preston and Wakelin 2008) and tall morningglory (*Ipomoea purpurea* (L.) Roth; Baucom and Mauricio 2004). However, no published studies have examined the relative fitness of Palmer amaranth plants with *EPSPS* amplification-mediated glyphosate resistance compared with glyphosate-susceptible plants.

In Palmer amaranth with *EPSPS* gene amplification, mutated copies of the gene have not been found (Gaines et al. 2010). Nevertheless, fitness costs may be associated with this form of glyphosate resistance for other reasons. Some glyphosate-resistant Palmer amaranth plants with gene amplification have more than 90 copies of the *EPSPS* gene with a corresponding increase in production of EPSPS protein (Gaines et al. 2010). This level of gene expression potentially diverts resources from other metabolic processes. In addition, the amplified copies of the *EPSPS* gene are scattered throughout the Palmer amaranth genome, possibly via a mobile genetic element (Gaines et al. 2010). If these additional *EPSPS* gene copies are inserted into functional genes, they may disrupt their activity.

When quantifying the relative fitness of a resistant population vs. a susceptible population, a comparison between S and R biotypes will not accurately estimate fitness costs associated with the resistance trait. This comparison assumes that if the resistance allele was removed, the resistant biotype would behave exactly like the susceptible. However, there is a large amount of natural genetic variation in most weed species (Clements et al. 2004), often resulting in a wide spectrum of variation in fitness-related traits, even among individuals collected from the same field population. Differential fitness between R and S biotypes could therefore be due to segregation at fitness-related loci unrelated to the resistance trait.

The best measure of fitness costs associated with resistance would be to determine the change in the frequency of the resistance allele for several generations (Vila-Aiub et al. 2009). This would integrate all the components of fitness across all life-history stages. However, tracking allele frequencies for several generations is both time-consuming and expensive, so very few published herbicide resistance fitness studies have attempted this method. Instead, it is common for researchers to use metrics that contribute to and therefore function as surrogates for fitness, such as final plant biomass or total seed production (Bagavathiannan et al. 2011; Menalled and Smith 2007; Vila-Auib et al. 2005). This study uses an F2 population that is segregating for the resistance trait across diverse genetic backgrounds to study the impact of *EPSPS* amplification on fitness.

This research was conducted to compare relative fitness in glyphosate-susceptible and -resistant Palmer amaranth with *EPSPS* gene amplification. The objectives were (1) to measure vegetative and reproductive growth parameters in open-pollinating pseudo-F_2 progeny segregating for the resistance trait in a nonselective controlled greenhouse environment; and (2) to measure the vegetative and reproductive growth parameters of glyphosate-resistant and -susceptible Palmer amaranth females crossed to the same male under controlled conditions.

Materials and Methods

Plant Material. Plants grown from Palmer amaranth seed collected from Macon County, GA in 2009 were characterized as either glyphosate resistant or susceptible on the basis of a leaf-disc shikimate assay (Shaner et al. 2005) and estimation of *EPSPS* gene copy number via quantitative polymerase chain reaction (PCR) (details given below). Confirmed glyphosate-susceptible and glyphosate-resistant plants were used as parents in controlled crosses to develop an F_1 population. No emasculation was necessary because Palmer amaranth is a dioecious species and all crosses were produced in the greenhouse under pollination bags. All parent pairs were comprised of different plants and each pair was bagged separately throughout anthesis.

Levels of glyphosate resistance in individual F_1 progeny were evaluated using a shikimate leaf-disc assay and *EPSPS* copy number was determined using a quantitative (q)PCR-based assay. Confirmed susceptible and resistant F_1 individuals were then crossed to produce the segregating F_2 families used in this study (Table 1). These F_2 families were actually "pseudo-F_2s" because Palmer cannot self to produce true F_2 offspring. This study used four F_2 populations segregating for the resistance trait across a range of genetic backgrounds because, as stated earlier, simply comparing a glyphosate-susceptible population with a glyphosate-resistant population may detect fitness differences due to other background fitness traits that differ between the two populations.

https://doi.org/10.1614/WS-D-13-00066.1 Published online by Cambridge University Press

Table 1. List of F_2 populations generated and their respective parental 5-enolpyruvylshikimic acid-3-phosphate synthase (*EPSPS*) copy numbers. All *EPSPS* gene number estimates were obtained by quantitative polymerase chain reaction (qPCR), measured against a low-copy internal reference gene (acetolactate synthase, *ALS*).

Generation	Sample size	Maternal *EPSPS* copy number	Paternal *EPSPS* copy number
F2-1	31	1	11
F2-2	31	1	1
F2-3	31	6	39
F2-4	31	47	68

Fifty pseudo-F_2 seeds from four crosses were plated onto sterile 1% agar in 100 mm by 15 mm petri plates (25 seeds/plate) and placed into a germination chamber set to 35 C light/30 C dark (12-h photoperiod). Once germinating seeds had produced at least 1 cm of shoot, they were transferred to 9 by 9 by 9 cm^3 square pots filled with potting soil (Fafard #2 SV, Conrad Fafard Inc., Agawam, MA 01001) and 1 teaspoon of Osmocote (Osmocote Smart-Release Plant Food, 19-6-12, Scotts Miracle-Gro, Marysville, OH 43041) and placed in a growth chamber set to 30 C, 12-h days and 75% humidity. Once plants reached the two- to four-leaf stage, 31 plants were randomly selected from each family, transplanted into 1-L pots with 1 tablespoon of Osmocote, and grown under greenhouse conditions. Greenhouse conditions consisted of natural light conditions supplemented with 400-W sodium halide lamps to provide a 14-h day length. Daytime temperature was 24 C and nighttime temperature was 18 C. Plants were spaced at least 35 cm apart to ensure adequate room for growth and randomized on a weekly basis to average out any position-specific effects. Of the 124 plants, 100 were randomly selected for the primary fitness costs study and the extra 24 were set aside for a reproductive study. This additional study was required because some measurements of growth, including cumulative height and final biomass, could not be measured on plants that were used for controlled crosses because of the growth limitations placed on the plants by enclosing the inflorescences in pollination bags.

Experimental Design. All 124 F_2 plants were arranged on a greenhouse bench in a completely randomized design and placed 36 cm apart to eliminate competition between plants for light or space. Plants were hand-watered daily and rerandomized on the bench weekly. Additional fertilization was supplied twice during the experiment (Miracle-Gro Plant Food Spikes, two spikes per pot, Scotts Miracle-Gro). The 100 plants used in the main fitness study were left uncovered for the entire period of the experiment, which allowed open pollination to occur. The 24 plants used for the reproductive fitness investigation were individually covered with a microperforated pollination bag as soon as the first flowers appeared and then bagged together in sets of three for controlled cross-pollination, as described below.

Estimation of *EPSPS* Gene Copy Number. Young leaf tissue was collected from each F_2 plant and genomic DNA was extracted using DNeasy Plant Mini Kits (Qiagen) for estimation of *EPSPS* gene copy number. Concentration and purity of DNA were determined with a ND-1000 Nanodrop spectrophotometer (Thermo Scientific), and only DNA samples with low protein contamination (260/280 ratio of ≥ 1.8) and low salts/phenol contamination (260/230 ratio of ≥ 2.0) were used for qPCR.

For the qPCR assay, DNA was diluted to 2 ng μL^{-1} in highly purified water. Using PerfeCTa SYBR Green Supermix with ROX (Quanta), and following the supplied protocol, qPCR reactions were set up containing 2.5 µL of genomic (g)DNA template, 1× Perfecta SYBR Green Supermix (containing AccustartTaq DNA polymerase, deoxyribonucleotides, SYBR Green I dye, ROX reference dye, $MgCl_2$, and stabilizers), and 250 nM each of forward and reverse primers for a final reaction volume of 12.5 µL. Primer sequences were identical to those in Gaines et al. (2010).

Primer efficiency curves were created for each primer set using a 1/10× dilution series of gDNA from a resistant plant. The *EPSPS* primers (EPSF1: 5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′ and EPSR8: 5′-TGAATTTCCTCCAGCAAC GGCAA-3′) had an efficiency of 95.2% and the *ALS* primers (ALSF2: 5′-GCTGCTGAAGGCT ACGCT-3′ and ALSFR2: 5′-GCGGGACTGAG TCAAGAAGTG-3′) had an efficiency of 95.6%. *ALS* was used as a reference gene because it is known to occur at one locus in the genome (Gaines et al. 2010). These efficiencies were very similar and thus directly comparable in subsequent calculations. The gDNA templates were run with each primer set in triplicate in 12.5-µL reactions on a 96-well PCR plate. Amplification was performed using the ABI Prism 7000 Real-Time PCR Detection System with the following thermoprofile: 15 min at 95 C, 40

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cycles of 95 C for 30 s and 60 C for 1 min, and finally a melt-curve analysis to check for primer-dimers. No-template reaction mixes, consisting of 10 μL of Master Mix (1× Perfecta SYBR Green Supermix and 250 nM primers) and 2.5 μL of water, served as the negative controls for this procedure. No primer-dimers and no amplification products were seen in the melt-curve analysis and the controls, respectively.

Threshold cycles (*C*t) were calculated by the ABI Prism 7000 program and relative copy number was determined by using a modified version of the $2^{-\Delta\Delta Ct}$ method (Gaines et al. 2010). The *ALS* gene was used a reference gene present in the genome at a copy number of 1. *EPSPS* gene copy number was estimated by finding $\Delta Ct = (Ct, ALS - Ct, EPSPS)$ and calculating $2^{\Delta Ct}$ to obtain a relative *EPSPS* copy number count.

Plant Growth Measurements. Starting at the two- to four-leaf stage, plant height was measured weekly as the distance in centimeters from the soil surface to the tip of the apical meristem. Plant volume was also measured weekly by taking two width measurements: one measuring the width of the canopy at its widest point and the other measuring the width at 90° to the first measurement at that same height. Volume was calculated as $\pi r^2 h$, with the average of the two widths equal to r and plant height equal to h. These measurements provide a nondestructive estimate of plant biomass accumulation over the growing season (Bussler et al. 1995). When plants had reached maturity, total aboveground biomass was harvested, separated into vegetative and floral components, and air-dried in large paper bags for 2 wk. Vegetative and floral biomass was then weighed and reweighed 5 d later to confirm that all plant tissue was fully dried to constant weight. These measurements were used to calculate total biomass (floral plus vegetative tissue) and harvest index (floral biomass divided by total biomass). Harvest index is normally calculated as the mass of the harvested product divided by the total biomass, but total floral biomass was used as a surrogate measure in this case so both male and female Palmer amaranth could be included.

Measurements of carbon fixation and transpiration rates were taken on a randomly selected subset of 40 plants from the 100 main study plants three times between 10:00 A.M. and 2:00 P.M. on July 21, 2011 and repeated on July 22, 2011, when vigorously growing plants were just beginning floral initiation. The youngest fully expanded leaf was removed from the top third of each plant and the distal half of the detached leaf was immediately clamped with the palisade layer upward in the chamber of a photosynthesis measurement system (LI-6400XT portable photosynthesis system, LI-COR, Lincoln, NE 68504). System settings were reference CO_2 at 400 ppm, flow rate 400 μmol s^{-1}, photosynthetically active radiation 1,400 and 10% blue. Each individual measurement was recorded as the mean of five readings taken at 3-s intervals. Carbon fixation rate for each plant was estimated as the mean of three separate measurements (15 total readings) taken over 2 d.

Fecundity Measurements. The 100 pseudo-F2 plants were visually examined twice a week for floral production and the number of days until the first flower emerged was noted. For females, time of first flowering was recorded as the date on which stigmas could first be seen, whereas for males, first flowering was the date upon which unopened pollen sacs were first visible. Once the anthers had dehisced, pollen was collected from 12 randomly selected male plants, and pollen viability was assessed via a fluorochromatic assay (Heslop-Harrison and Heslop-Harrision 1970), which uses fluorescein diacetate to check for an intact pollen membrane and the presence of functioning hydrolytic enzymes. Pollen grains with both intact membranes and working enzymes are considered viable and will generate a fluorescence that is detectable under a fluorescence microscope. For this assay, two drops of the fluorescence/growth mix (1.75 M sucrose, 3.23 mM boric acid, 3.05 mM calcium nitrate, 3.33 mM magnesium sulfate heptahydrate, 1.98 mM potassium nitrate, and 7.21 mM fluorescein diacetate dissolved in acetone) were pipetted onto a glass slide, and male flowers from the selected plants were gently tapped three to four times over the slide to deposit pollen in contact with the liquid. Three separate slides of pollen were collected per plant and immediately examined under a Zeiss Axioskope fluorescence microscope. Using the blue 450- to 490-nm excitation filter, two images of fluorescing pollen grains were captured per slide. Within the viewing field of each image, the number of fluorescing pollen grains and the number of total pollen grains were counted. The total number of fluorescing pollen grains was then divided by the number of total pollen grains to get an average viability ratio per slide, and ratios across all three slides were averaged per plant to obtain mean percent pollen viability.

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Additional fecundity-related measurements recorded immediately before total aboveground biomass harvest were length of the longest inflorescence of both males and females and the amount of seed produced by each female. The longest inflorescence length was used as an estimator of both male and female floral production and was measured as the entire length of the apical flowering structure. Seed production was measured by hand-threshing seed from each female and weighing the total seed per plant. Palmer amaranth is normally wind-pollinated, so conditions in the greenhouse were not optimal for pollen movement. As a result, this measure of seed production may underestimate plant reproductive potential.

Controlled Crossing Fecundity Measurements. Pollination could not be controlled in the 100 pseudo-F2 plants used for the main fitness study because any controlled crosses would require covering the plants with pollination bags that would restrict growth. Therefore, to estimate reproductive potential of resistant and susceptible plants, controlled crosses were carried out using an additional 24 pseudo-F2 plants as previously described, grown in the same greenhouse environment. Immediately before flowering, each of the 24 plants was bagged with a microperforated pollination bag to prevent pollen movement. Once all plants had started flowering, crosses between two females and one male were assigned according to individual *EPSPS* copy number. Each cross consisted of one high-copy-number maternal parent and one low-copy maternal parent competing for the same pollen. Bags were shaken every other day to promote pollination and groups of pots were randomized across the greenhouse bench on a weekly basis during this period. After pollination had occurred and seeds had matured, each maternal plant was hand-threshed and seed was weighed to determine total seed production. Seed viability tests were carried out by plating 40 seeds from each maternal plant onto 1% agar in 100 mm by 15 mm petri plates and counting the number of seedlings with cotyledons produced after 14 d in a germination chamber set at 35 C light/30 C dark with a 12-h photoperiod. Seed viability tests were replicated twice.

Male : Female Ratio. Anecdotal evidence has suggested a prevalence of female plants over male plants in Palmer amaranth populations. For this reason, the gender of each plant was recorded and a chi-square analysis was conducted to determine if the population of plants used in this experiment deviated from a 50 : 50 male : female ratio. A two-sample *t* test was also conducted to test for any difference in *EPSPS* copy number between males and females, treating gender as the groups and copy number as the independent samples within each group.

Statistical Analysis. All data analysis was conducted using the JMP statistical package (JMP, SAS Institute Inc., Cary, NC). QQ plots and normality tests were conducted on the growth and reproductive data to check for normal distributions. Final plant height, final plant volume, and longest inflorescence were log transformed to meet the normality and equal variance assumptions of regressions. The initial analyses carried out on these data were regression tests to examine the potential effects of *EPSPS* gene copy number and associated glyphosate resistance on fitness. Each of the growth/fecundity measurements was regressed against individual plant copy number to assess any significant relationships between the two variables.

To differentiate between fitness differences due to *EPSPS* copy number and fitness differences due to family, an analysis of covariance (ANCOVA) was also performed with family as the independent variable, growth and fecundity measurements as the dependent variables, and *EPSPS* copy number as the covariate. The assumptions for ANCOVA (normality, equal variance, independence, equal regression slopes) were met, as tested for by QQ plots and Levene's test. The assumption of equal regression slopes was tested and supported by confirming no interaction between family and *EPSPS* copy number for each dependent variable.

Separate regression analyses between *EPSPS* copy number and growth measures were conducted for each family. The gender data were tested for a 1 : 1 male : female ratio using a chi-square analysis for goodness-of-fit.

Results and Discussion

Plant Growth and Fecundity Measurements. There was no statistically significant ($\alpha = 0.05$) relationship between final biomass and *EPSPS* copy number (Figure 1A) for pooled progeny from all four families. There was also no relationship between seed production and *EPSPS* copy number (Figure 1B).

Results from initial pooled-family analysis indicated differences in relationships between *EPSPS* copy number and six of the fitness-related variables.

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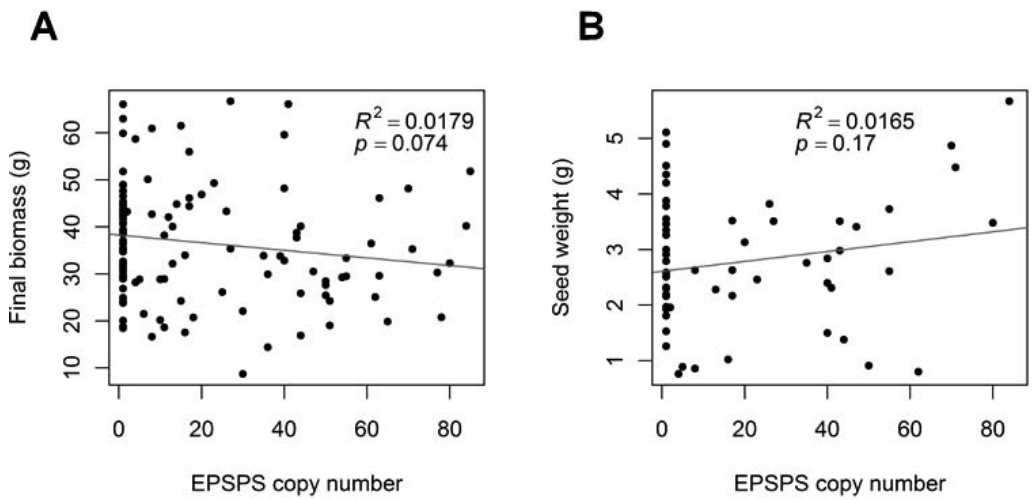

Figure 1. (A) Regression of final plant biomass (g) on 5-enolpyruvylshikimic acid-3-phosphate synthase (*EPSPS*) copy number. (B) Regression of total seed weight per plant (g) on *EPSPS* copy number.

These six variables were seedling plant height, mature plant height and volume, days to first flower, harvest index, and inflorescence length (Figure 2). Plant growth-related variables (harvest index, height, and volume) showed a positive correlation with *EPSPS* copy number, suggesting a fitness benefit for resistant individuals. The fecundity-related measures of inflorescence length and days to first flower, on the other hand, suggested fitness costs for the resistant plants as a result of later flowering and shorter inflorescences. However, results of ANCOVA indicated that all apparent relationships between fitness-related traits and EPSPS copy number in the pooled data were due to differences between families (Table 2). That is, any relationships revealed in the initial regressions on pooled individuals from all families were not due to fitness effects associated with *EPSPS* copy

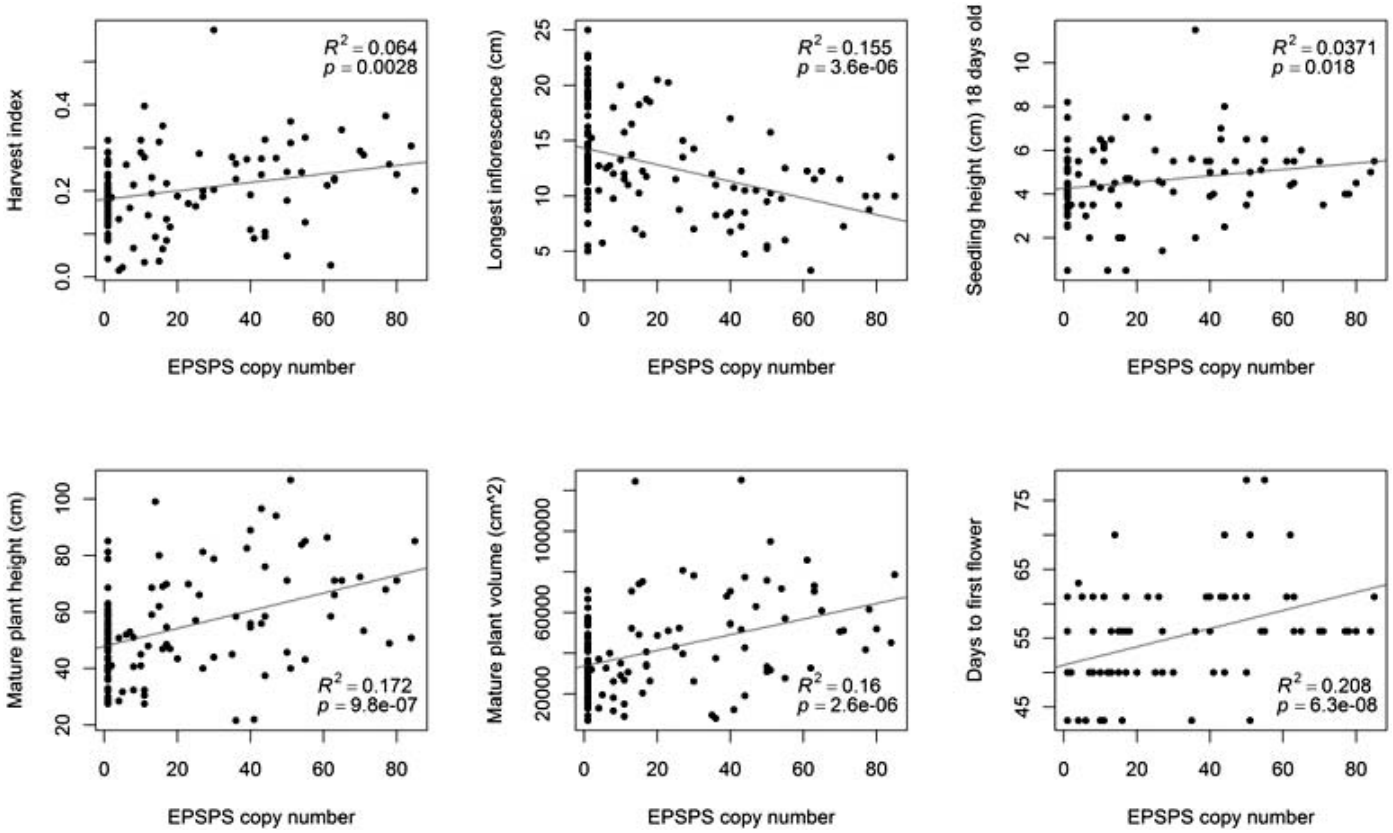

Figure 2. Regression of a subset of the fitness measures on 5-enolpyruvylshikimic acid-3-phosphate synthase (*EPSPS*) copy number.

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Table 2. Significance of 5-enolpyruvylshikimic acid-3-phosphate synthase (*EPSPS*) copy number and family designation on growth and reproductive measurements. An analysis of covariance (ANCOVA) was run on all measurements, using *EPSPS* copy number and family as the two main effects.

	P-value (*EPSPS* copy number)	P-value (family)
Biomass (g)	NS[a]	NS
Harvest index	NS	0.0174*
Average weekly height increase (cm)	NS	NS
Average weekly volume increase (cm^2)	NS	0.0351*
Inflorescence length (cm)	NS	0.0003*
Seedling plant height (cm)	NS	0.0246*
Mature plant volume (cm^2)	NS	0.0019*
Mature plant height (cm)	NS	0.0008*
Days to anthesis	NS	0.0009*
Photosynthesis rate	NS	NS
Transpiration rate	NS	0.0055*
Seed production	NS	0.0243*
Seed germination	NS	NS
Pollen viability	NS	NS

* Significance level set at $\alpha = 0.05$.
[a] Abbreviation: NS, nonsignificant result.

number, but to among-family differences in segregation at other fitness-related loci.

This effect was further confirmed when regression analyses were conducted separately on individual families to examine *EPSPS* copy number effects on fitness (Table 3). There was a relationship between *EPSPS* copy number and (1) seedling plant height and days to first flower in familyF_2-1, (2) percent seed germination in family F_2-2, and (3) seed production in family F_2-4, but no relationships were consistent across all families. This is an important point to consider for future fitness studies. In an outcrossing species like Palmer amaranth, high genetic diversity across all loci can be seen (Chandi et al. 2013), making it difficult to pinpoint the true causative sources of fitness differences. Even in the population of plants from this study, where segregating F2 families were used to average out the genetic background, some statistically significant relationships were still observed. Without examining several families, it would be easy to conclude that increased *EPSPS* copy number directly affected one or more fitness-related traits. However, comparison among different families with different genetic backgrounds showed that no trends were consistent across families and no real relationships were observed.

We can conclude from these results that no fitness costs associated with glyphosate resistance due to *EPSPS* gene amplification were evident for this Palmer amaranth population when grown under controlled conditions in the greenhouse. As fitness-related measures were taken from plants grown in a greenhouse environment, these results may not directly translate to field conditions.

Controlled Crossing Fecundity Measures. Results of regression analyses conducted on pollen source determined that there was no relationship between *EPSPS* copy number and seed production or seed viability (data not shown). The crossing of both glyphosate-resistant and glyphosate-susceptible female plants with one male plant ensured exposure to the same pollen source, but no differences in seed production or seed viability between resistant and susceptible mother plants were found. This suggests that there is no maternal reproductive fitness penalty associated with the *EPSPS* gene amplification

Table 3. Effect of 5-enolpyruvylshikimic acid-3-phosphate synthase (*EPSPS*) copy number on growth and reproductive measurements within each family ($N = 31$ per family).

	F_2-1		F_2-2		F_2-3		F_2-4	
	R^2	P	R^2	P	R^2	P	R^2	P
Biomass (g)	0.0209	NS[a]	0.45529	NS	0.00188	NS	0.001802	NS
Harvest index	0.0435	NS	0.007619	NS	0.020129	NS	0.049097	NS
Inflorescence length (cm)	0.0174	NS	0.128755	NS	0.0688	NS	0.083663	NS
Seedling plant height (cm)	0.3117	0.0022*	0.00417	NS	0.0220	NS	0.0070	NS
Mature plant volume (cm^2)	0.0077	NS	0.009527	NS	0.05733	NS	0.043495	NS
Mature plant height (cm)	0.0238	NS	0.0009	NS	0.15387	NS	0.046908	NS
Days to anthesis	0.1534	0.0301*	0.045191	NS	0.01365	NS	0.019886	NS
Photosynthesis rate	0.0000	NS	0.040232	NS	0.128737	NS	0.017598	NS
Transpiration rate	0.0000	NS	0.170281	NS	0.010381	NS	0.009437	NS
Seed production	0.1090	NS	0.042551	NS	0.00054	NS	0.367299	0.0367*
Seed germination	0.0641	NS	0.240679	0.0387*	0.075336	NS	0.037444	NS
Pollen viability	0.0000	NS	0.6452	NS	0.5037	NS	0.0275	NS

* Significance level set at $\alpha = 0.05$.
[a] Abbreviation: NS, nonsignificant result.

https://doi.org/10.1614/WS-D-13-00066.1 Published online by Cambridge University Press

mechanism of glyphosate resistance in Palmer amaranth. Relative fitness is defined as the relative ability of different genotypes to pass on their alleles to future generations (Hedrick 2005). Although they are still surrogate measurements for fitness, seed production and germination are direct estimates of the number of potential offspring produced by an individual. The lack of a difference in seed production and seed viability between high and low *EPSPS* copy-number female plants in controlled crosses is therefore one of the most notable results indicating the lack of any fitness cost associated with the glyphosate resistance trait.

It is important to note that plants function within a larger ecosystem and the relative fitness of herbicide-resistant and -susceptible weeds therefore also depends on interactions with other organisms in the agricultural environment. These interactions mean that the magnitude of a fitness penalty is dependent on how it behaves in a competitive environment, and these penalties are often more easily measured when competition exists for resources (Reboud and Till-Bottraud 1991). Additionally, it has been documented that fitness costs may vary across an environmental gradient, usually increasing with increased environmental stress such as low nutrient availability (Paris et al. 2008; Raymond et al. 2010). Future studies of fitness costs should test for growth and reproduction differences both in competition studies and across diverse environmental gradients.

Gender Ratio. A chi-square test of goodness of fit revealed no significant difference in the proportion of males to females, $\chi^2(1, N = 100) = 1.02$, P = 0.312. There was an observed difference in the emergence time of flowers between the sexes, however, perhaps contributing to the previous observations suggesting disequilibrium. The *t* test for *EPSPS* copy number differences between male and female plants found no difference between the two genders, $t(54) = -0.05$, P = 0.96.

Implications. The lack of fitness penalty associated with the glyphosate resistance mediated by *EPSPS* amplification in Palmer amaranth could have serious repercussions for growers attempting to control this weed. A fitness cost would theoretically favor herbicide-susceptible genotypes and shift the weed population back toward increased herbicide susceptibility once herbicide selection was removed (Vila-Aiub et al. 2009). In the case of Palmer amaranth, the lack of fitness costs will likely result in the persistence of glyphosate resistance in the population regardless of selection pressure, leading to the long-term loss of glyphosate as a weed control tool for fields heavily infested with Palmer amaranth.

Results from this study also illustrate the importance of controlling for genetic background when estimating fitness costs, and the need to base fitness estimates on multiple growth, fecundity, and phonological measurements. As this study showed, even full-sib plants from the same family exhibited a high amount of variation in fitness-related measurements, regardless of the level of herbicide resistance. In addition, within-family trends for one fitness-related trait were not duplicated for other traits. These data show that basing estimates of herbicide resistance fitness costs on simple comparisons between resistant and susceptible biotypes for one or two growth-related traits can be highly misleading. Such fitness estimates should be based on multiple growth and fecundity measurements, and must consider the effect of diverse genetic backgrounds.

The results of this study will be also useful in the development of resistance evolution simulation models to predict the trajectory of herbicide resistance and its impact on population dynamics (e.g., Gressel and Segel 1990; Richter et al. 2002). A paper modeling glyphosate resistance in Palmer amaranth demonstrated the potential for this type of tool in testing different weed management strategies (Neve et al. 2010). Including estimates of relative fitness of resistance in future models would lead to more accurate predictions of the impact of different management approaches.

Acknowledgment

The authors acknowledge Monsanto for partial support of this research.

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Received April 25, 2013, and approved July 15, 2013.