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Author for correspondence:
Sarah B. Yakimowski
e-mail: sarah.yakimowski@queensu.ca

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Departures from Mendelian inheritance of *EPSPS* gene copies in a herbicide-resistant weed

Lisa H. Han[1,2], Kathryn F. Dungey[1,3] and Sarah B. Yakimowski[1]

[1]Department of Biology, Queen's University, Kingston, Ontario, Canada
[2]Institute of Forestry and Conservation, University of Toronto– St George Campus, Toronto, Ontario, Canada
[3]Department of Plant Sciences, University of Cambridge, Cambridge, UK

SBY, 0000-0002-2465-5314

Here, we report on inheritance and intra-individual variation in gene copy number associated with the rapid evolution of herbicide resistance in the dioecious, agricultural weed *Amaranthus palmeri*. Copy number variation for the glyphosate resistance gene *EPSPS* was quantified using digital droplet polymerase chain reaction within individuals and for parents and offspring of controlled crosses, with copy numbers ranging from 1 to approximately 160. The nonlinear pattern of gene copy number inheritance from parent to offspring exhibits shifting directionality. Below the threshold of approximately 33 copies, the slope of the parent–offspring relationship is high (1.60) because parents with fewer gene copies yield similar or increased mean offspring copy numbers, relative to parents. By contrast, above the threshold, the slope is low (0.21), and offspring of high copy number parents carry increasingly fewer *EPSPS* copies than parents. Intra-individual variance in copy number increases with mean individual copy number, probably contributing to variation in offspring copy number, but not sufficient to explain extreme transgressive copy number variants nor the loss of high copy number. In short, the copy number is less stable and transmitted to offspring at a lower rate in parents with more copies. This suggests a constraint on the evolution of herbicide resistance through increased *EPSPS* copy number.

1. Introduction

Modern biology is largely built around a Mendelian model of inheritance, as described by the modern synthesis [1,2]. However, this view is complicated by structural genomic re-arrangements among individuals [3], and genomic reorganization in response 'to stress or unusual challenge' as investigated by McClintock (e.g. transposable elements) in the early decades of genetics [4]. Variation in gene copy number represents polymorphism in genome structuring, and is observed in humans and many other species [3,5,6], including the model plant *Arabidopsis thaliana* for which copy number varies for approximately one-third of genes [7]. Copy number variation can affect key phenotypes including fitness. For example, tandem repeats of multiple genes in soyabean confer nematode resistance [8], and copy number variation of two genes is associated with flowering time variation in wheat (*Triticum aestivum* L.; [9]). More recent studies have demonstrated the occurrence of gene copies residing on 'extrachromosomal DNA' [10,11], distinct from the core genome. Yet, in the current era of high-throughput sequencing and bioinformatics, the methods required to resolve structural genomic variation are more complex [12–15] and thus understanding the dynamic nature of genomes has lagged behind the study of relatively stable genome sequence

[16]. In turn, understanding of the evolutionary dynamics [17] of multi-copy gene traits [18–20] remains limited.

Rapid evolution of herbicide resistance is a threat to food security and the sustainability of agricultural practices [21,22]. At least 60 plant species worldwide have reported resistance to the ubiquitous herbicide glyphosate [23] since its commercialization in 1974 [24,25]. A variety of genetic mechanisms of resistance have been detected; for example, point mutations and increased copy number [10,26] of the *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) gene have resulted in functional maintenance of the shikimate pathway under glyphosate stress [27–29]. The North American weed *Amaranthus palmeri* (Palmer's amaranth) has evolved extrachromosomal positioning of highly duplicated genes. This extrachromosomal DNA (ecDNA) is often circular (eccDNA) and putatively tethers to chromosomes, replicating and segregating during mitosis and meiosis [30,31]. The discovery of ec(c)DNA gene copies is relatively recent in plants [11,30,32], but well known in bacteria since plasmids were described in the 1960s (e.g. *penicillinase* plasmids in *staphylococcal* strains—*Staphylococcus aureus*; [33–35]). The potential involvement of ec(c)DNA in resistance gene copy number raises fundamental questions about its stability, pattern of inheritance and contribution to rapid evolution.

Extrachromosomal DNA is an example of genetic material defined by unique characteristics relative to chromosomal DNA, thus giving rise to questions about its pattern of variation through organismal growth/development and reproduction. ec(c)DNA was first described in 1965 [36] and is a general term for double-stranded DNA that resides external to the chromosomes, varying in size from smaller (0.2–100 kb) to larger structures (several hundred kb to several Mb). It has been identified in both healthy cells [11,37] and cancerous tumour tissue [38], and varies in content (e.g. repetitive and transcribed regions) [39,40]. ec(c)DNA was detected in plants 20 years later, but recent genomic and bioinformatic methods have revealed eccDNA in several model plants (e.g. *Arabidopsis* and *Poplar*) and agriculturally important species (e.g. rice, potato, carrot). The agricultural weed *palmeri* has the largest extrachromosomal structure (approx. 400 kb replicon) detected in plants to date [41]. In contrast to many small, non-coding eccDNAs, the *A. palmeri* replicon carries a copy of the glyphosate resistance gene (*EPSPS*) and more than 50 other genes, most of which are transcribed [31]. The well-characterized ec(c)DNA structure(s) occurring in *A. palmeri* populations presents an opportunity to investigate inheritance and stability of gene copy number variation involving extrachromosomal DNA. Gene copies carried by ec(c)DNA are predicted to be less stable than chromosome-integrated tandem repeats, owing to the dynamic association with chromosomes via tethering during mitosis and meiosis [30,42].

The pattern of inheritance can be estimated based on the resemblance between parents and offspring. This approach is often used to estimate the heritability of organismal phenotypic traits [43], but when applied to the copy number of an individual gene [44,45], this facilitates direct estimates [46] of gene transmission. Here, we study inheritance and stability of resistance gene copy number in *A. palmeri* (Palmer Amaranth), a crop weed with reported resistance to 10 herbicidal modes of action [23,47], including glyphosate. The glyphosate resistance phenotype in *A. palmeri* is strongly associated with *EPSPS* copy number variation [48], with copy numbers ranging from one to over 160 per genome [30,31]. Experimental crosses between single/low copy × high copy number individuals and fibre-FISH (fluorescence *in situ* hybridization) analysis of copy number variation established that ec(c)DNAs carrying the *EPSPS* gene can be transmitted from parent to offspring [30]. To investigate the stability of this resistance gene copy number polymorphism through sexual reproduction, we estimated the pattern of inheritance with 39 crosses (n = 1038 offspring total), spanning the dynamic range of *EPSPS* copy number observed in the multi-resistant agricultural weed *A. palmeri*.

Stability of *EPSPS* copy number through plant development was estimated by quantifying intra-individual variation for 117 plants (n = 749 total estimates). Specifically, we address the following questions: (i) does midparent copy number predict mean offspring copy number? If so, is the parent : offspring copy number relationship linear and 1 : 1, as predicted by the Mendelian expectation for the segregation and assortment of copy number variation? (figure 1A); (ii) are deviations of offspring from mean parental copy number randomly distributed around a constant mean (approx. 0), and independent of parental midpoint copy number, as expected for measurement error (figure 1C)? and is there evidence for copy number instability in the form of greater deviations between offspring and parents as copy number increases?; and (iii) how does intra-individual variation in *EPSPS* copy number compare with inter-individual and parent–offspring copy number variation? In particular, does genome instability affect copy number throughout growth and development, yielding directional loss or gain of copies?

2. Methods

(a) Inheritance of gene copy number

(i) Plant material and growth conditions

Amaranthus palmeri seeds used in this study were sourced from open-pollinated agricultural fields in Georgia, North Carolina, and Illinois, USA. Seeds from populations GA-IL (Gallatin Co. Illinois) and WO-GA (Worth Co. Georgia), in which individuals exhibit a wide distribution of *EPSPS* copy number and report high mean phenotypic glyphosate resistance and amplified *EPSPS* gene copy number [48], were used for the initial inheritance study. Seeds were germinated in 3.81 cm plug trays using Sungro Professional Growing Mix no. 1 in a controlled greenhouse environment (22–30°C, 15 L : 9 D h cycle) at Queen's University Phytotron. Seedlings were transplanted into 15.24 cm pots 3–5 days post-germination, and fertilized once with 20-20-20 nitrogen-phosphorous-potassium (50 ppm) one week after transplanting, and once more during inflorescence development. Note that specific history of glyphosate use in populations of origin is not known, although past glyphosate use is expected; during our experiments, no glyphosate was applied.

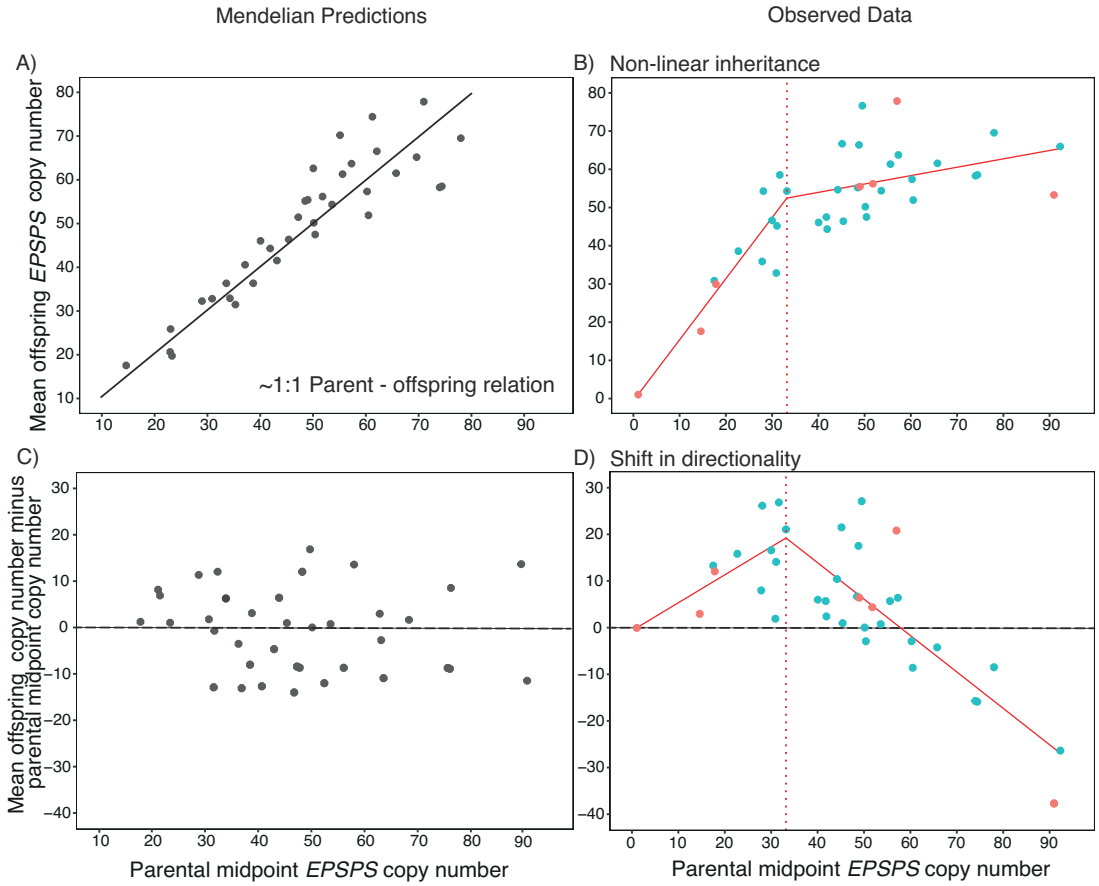

Figure 1. (A) Predicted linear and approximately 1 : 1 relationship between parental midpoint *EPSPS* copy number and mean offspring copy number under Mendelian inheritance. (B) Observed nonlinear relationship between the parental midpoint *EPSPS* copy number and mean offspring *EPSPS* copy number in *Amaranthus palmeri* ($n = 39$ crosses). (C) Predicted differences between mean offspring *EPSPS* copy number and parental midpoint *EPSPS* copy number under Mendelian inheritance. Differences are consistent with random sampling error (approx. equal differences above and below zero, dashed horizontal line) and independent of parental midpoint. (D) Observed differences between parental midpoint *EPSPS* copy number and mean offspring *EPSPS* copy number for *A. palmeri* ($n = 39$ crosses). In data panels (B) and (D), blue points = original set of controlled experimental crosses ($n = 30$), coral red points = highly isolated experimental crosses ($n = 9$; 3× data points at parental midpoint copy number = 1).

(ii) DNA extraction and droplet digital polymerase chain reaction estimation of *EPSPS* copy number

Fresh leaf tissues (2–3 cm in length) from the third and/or fourth leaves from the shoot apical meristem were collected two weeks post-transplant. All fresh samples were flash-frozen in liquid nitrogen and subsequently stored at −80 °C for DNA extraction. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide protocol [48]. DNA purity and concentration were assessed using a DeNovix DS-11 FX+ spectrophotometer, and samples were standardized to 0.7 ng μl^{-1}. *EPSPS* gene copy number was quantified using droplet digital polymerase chain reaction (ddPCR; Bio-Rad QX200). Each 20 µl reaction included 1× ddPCR Supermix for Probes (no dUTP), 450 nM forward and reverse primers for target *EPSPS* and single-copy reference gene *ALS* (acetolactate synthase), 250 nM target and reference probes (HEX (hexachlorofluorescein) and FAM (fluorescein)-labelled, respectively, primer and probe sequences in [48]), 0.2 µl of 2U HindIII and 5 µl of DNA template. Droplets were generated using the Bio-Rad DG8 cartridge, and amplified using a BioRad deep well thermocycler (C1000 Touch™; 95°C for 10 min; 94°C for 30 s, 56°C for 1 min, 72°C for 30 s, repeated 49×; 98°C for 10 min and 4°C for 10 min). Droplets were assayed with QX200 Droplet Reader (BioRad 1863001), and the ratio of positive droplet *EPSPS : ALS* amplifications was analysed using QuantaSoft/QX Manager software (BioRad). Samples with droplet counts of less than 10 or greater than 10 000 for either the target or reference genes were subsequently re-diluted (0.1 ng μl^{-1} to 2.0 ng μl^{-1}) and re-run (following [48]). Replicability of copy number estimates using this method is high (i.e. a set of 31 samples ranging in *EPSPS* copy number from one to approximately 93 run in duplicate at template concentration 0.7 ng μl^{-1}; correlation between duplicates was $r = 0.99$, $p < 0.0001$).

(iii) Controlled crosses by resistance gene copy number

To investigate *EPSPS* copy number inheritance, controlled crosses were performed between (full sibling) female and male plants based on *EPSPS* copy number estimates (above). Prior studies [30,44] focused on crossing single/low copy × high copy number individuals to establish whether high copy number could be transmitted to the next generation, and confirmed that offspring from such matings can carry many (up to 91) copies. Therefore, to better understand patterns of inheritance and to represent the wide variety in matings between copy number variants, we included crosses between females and males with both similar

and varying parental copy numbers. Crosses (n = 31) ranged in parental midpoint *EPSPS* copy number from 17.5 to 92.3 (see the electronic supplementary material, table S1), and crosses varied in the magnitude of difference (range 0.4 to 52.5) between maternal and paternal copy numbers. Similar numbers of crosses were performed in which maternal copy number was greater than paternal copy number (n = 12 crosses) and in which paternal copy number was greater than maternal copy number (n = 19 crosses). As plants approached flowering, female and male pairs were enclosed in custom-made pollination bags (PBS International, 3D.55), with an average pore size (15 µm) smaller than the estimated range of *A. palmeri* pollen diameter (21–38 µm; [49]). In cases where anthers dehisced prior to bagging, male plants were spatially isolated for 24 h, longer than the estimated length of pollen viability, prior to being paired with female plants to minimize pollen contamination among crosses [49]. Mature offspring seeds were harvested from each controlled cross. The seed count for five representative inflorescences and their corresponding inflorescence lengths (mm) were used to estimate total offspring seed count per cross. One cross (A2; see the electronic supplementary material, table S1) of moderate parental midpoint (51.8) was not included in the inheritance analysis because of low germination of offspring (i.e. 30 of the 31 original crosses included). Offspring (mean n = 27/cross; range 15–35) were germinated, grown and *EPSPS* copy number was estimated using the same methods as detailed above for parents.

Additional crosses were performed, motivated by the broad range of offspring copy number observed in the first experiment. Our goal was to assess whether pollen contamination between experimental crosses conducted concurrently is a likely contributor to observed variation in offspring copy number. Sex-specific markers [50,51] were used to identify females and males, allowing female–male pairs to be isolated in bags prior to plants becoming reproductive. We further eliminated the possibility of pollen contamination by restricting the copy number variation of reproductive *A. palmeri* grown in our experimental facilities: first, 'control' crosses were created (March to mid-April 2022) to confirm that crosses between single *EPSPS* copy number individuals yield single-copy offspring. We used seed from a population that is predominantly composed of single *EPSPS* copy number individuals (EF-IL; Effingham Illinois), confirmed via ddPCR that each parent carried a single copy *EPSPS* (electronic supplementary material, table S1), and then crossed three female–male pairs. Note that no other *A. palmeri* was reproductive in the growth facility during the reproductive period of these 1 × 1 crosses. Then, seed from GA-IL was again used to experimentally cross six additional pairs (late April to May, 2022), with *EPSPS* copy number parental midpoints ranging from 14.6 to 91.0: two lower copy crosses (parental midpoints 15 and 18) were isolated in one greenhouse, three higher copy crosses (parent midpoints 52–91) in another separate greenhouse and one mixed (parental copy numbers 21 × 77) in a third greenhouse to further minimize opportunity for contamination between lower and high copy number crosses. As in the original crossing experiment, mature seeds were collected, and an average of 27 offspring per cross were grown and assayed for *EPSPS* copy number.

(iv) Data analysis: *EPSPS* copy number inheritance

To investigate the pattern of inheritance of *EPSPS* copy number from parents to offspring, we first apply a classic approach from quantitative genetics to estimate h^2 (narrow-sense heritability), the proportion of phenotypic variance that is explained by (additive) genetic variance, V_A / V_T [43]. The slope of the linear regression of the 'midparent value'—mean of the maternal and paternal trait values—on the mean phenotype of the offspring estimates narrow-sense heritability (h^2). Here, because we investigate the heritability of a genomic trait rather than an organismal one—directly estimating the copy number of a specific resistance gene per diploid genome—we denote the slope of the relationship between parental midpoint and mean offspring copy number as h^2_G. Under a purely Mendelian model of segregation and independent assortment, parents that vary in chromosomal copy number would yield offspring with a copy number intermediate to the parents [52]. In contrast to morphological phenotypic traits for which environmental contributions can be substantial, when estimating the copy number of a specific gene in parents and offspring, high estimates of h^2_G (~1) are expected if gene copy number is stable within individuals and there are minimal environmental effects on gene copy number variation. In this experiment, plants were grown in a relatively benign glasshouse environment, and glyphosate stress was not directly applied, thus minimizing environmental effects on estimates of copy number. In the human genome, h^2_G was estimated for 34 genes with continuous gene copy number, and 23 of these h^2_G estimates ranged from 0.8–1.2, which the authors interpreted to be consistent with Mendelian inheritance [45]. Departures from high (~1) h^2_G for a copy number variant do not alone prove or identify a mechanism of non-Mendelian inheritance, but for a direct estimate of gene copy number variation, they raise a flag suggesting the possibility of a statistical deviation from Mendelian predictions worthy of further analysis (see below).

All data analysis here and in §2b(ii) was performed in R (v. 4.2.1) and RStudio (v. 2024.04.2 + 764). We examined the relationship between parental midpoint (mean) and offspring mean *EPSPS* copy number as follows. First, we applied a linear model (LM) to estimate the slope of the regression between parental midpoint and offspring mean. The residuals of this model did not deviate from normality (Shapiro–Wilk normality test: W = 0.98, p = 0.60), and thus a Gaussian distribution was used for the subsequent threshold analysis. Then, to test for nonlinear inheritance, we applied a linear quadratic model and a segmented threshold model (library 'chngpt'; [53]) to identify any potential variation in the slope of the relationship between parental midpoint and offspring mean copy number across the dynamic range of *EPSPS* copy number. The fit of these three models was compared with Akaike information criteria (AIC) and Bayesian information criterion (BIC). Note that modelling the relationship using a generalized linear model (GLM) with Poisson distribution yielded the worst fit; including both linear and quadratic terms improved the fit, but nonetheless, much worse fit than the models presented below (§3a). We ran each of these models using data from 39 crosses (30 original, nine isolated).

We also tested the Mendelian prediction that differences between offspring and parents should be approximately equally positive and negative by examining the relationship of difference between the parental midpoint and offspring mean copy number against the parental midpoint (figure 1C); this relationship was similarly examined with linear and nonlinear threshold

models. Also, we tested whether crosses with larger differences between parental copy numbers are associated with high s.d. of offspring copy number. Finally, we compared linear relationships between offspring copy number and each of maternal and paternal copy number to test whether offspring copy number is biased by either parent.

(b) Estimating intra-individual variation in *EPSPS* copy number

(i) Intra-individual sampling

Seeds were germinated from four *A. palmeri* populations (GA-IL, WO-GA as above, plus WA-NC (Wake Co. North Carolina) and RA-NC (Randolph Co. North Carolina); electronic supplementary material, table S1 in [48]). Two of these populations intentionally overlap with the populations used in the inheritance study above. We studied populations from three eastern states; although we had no *a priori* prediction that intra-individual variation in *EPSPS* would vary geographically, we included samples from three geographical regions to increase the generality of results. A total of 117 plants from four populations (22–39 plants per population) were studied. Seeds were germinated in the same conditions as above, but with 12 L : 12 D photoperiod, transplanted and grown as above.

From each individual plant (n = 117), a series of leaf tissue was collected from a standardized position (third or fourth leaf from the apical meristem), over time, to capture variation in copy number among leaf tissues, and any potential temporal variation in leaf tissue copy number as plants develop. The first leaf sample was collected from juvenile plants at 10–14 days post-transplant. Following the first sampling, leaf tissue was collected at 10 day intervals, for a total of five temporal replicates per plant (except for four plants, for which only four temporal replicates were successfully collected; a total of 573 temporal leaf tissue samples from the 117 plants). At each temporal sampling point, biological replicates were randomly chosen for approximately 20% of samples (109 leaf pairs, for 22–25 plants per sampling point) by dividing one leaf approximately in half. Inflorescence tissue was also sampled in biological replicates (1 cm per replicate) from the primary inflorescence once it reached a minimum length of 4–5 cm for 29 plants. All fresh tissues sampled were placed in 1.5 ml microcentrifuge tubes on ice during sample collection, and then immersed in liquid nitrogen to flash freeze samples before storage at −80°C. Each biological replicate underwent DNA extraction and analysis of copy number independently as detailed above (see §2a(iii)). A total of 749 tissue samples from 117 *A. palmeri* plants were assayed for *EPSPS* copy number.

(ii) Data analysis: intra-individual variation in *EPSPS* copy number

Using the temporal replicate estimates of *EPSPS* copy number, we first examined the relationship between mean individual *EPSPS* copy number and intra-individual variance relative to a Poisson expectation ($E(x) = \mathrm{Var}(x)$). Residuals were calculated as the difference between observed variance in intra-individual copy number and expected variance (individual mean copy number), and standardized by dividing by s.d.(residuals). We then examined the relationship between standardized residuals and mean copy number using a linear model. Owing to overdispersion of *EPSPS* copy number, we subsequently used quasi-Poisson GLMs, which fitted a dispersion parameter to account for additional variance, to examine variation in *EPSPS* copy number with the following models and explanatory effects: (i) overall copy number variation was tested with individual, population, temporal replicate (leaf tissue) nested within individual and biological replicate nested within temporal replicate and individual (leaf tissue); (ii) directionality of temporal replicates were tested using individual and temporal replicate nested within individual to allow for estimation of slope and significance for each individual; Bonferroni-corrected α = 0.05/117 = 0.0004; and (iii), tissue type (leaf, inflorescence) was tested for a small subset of individuals (n = 11 individuals, ranging in mean copy number from 1 to 81) for which both a pair of leaf and inflorescence biological replicates were assayed; tissue type * biological replicate nested within individual (Poisson was sufficient).

Finally, to investigate whether offspring from controlled crosses with copy number (§2a(iii) and §2a(iv) greater than the sum of parental copy numbers could be the result of intra-individual copy number variation, we applied the following analysis. For each parent, we estimated the maximum range of intra-individual variation by averaging across up to five of the closest mean copy number estimates to parental copy number; the maximum intra-individual range of the two parents was averaged. The maximum range divided by two (to capture the potential for positive bias) was added to each of the parents as an upper estimate of parental copy number. Parental sum copy number was recalculated based on these upper estimates, and the proportion of offspring (if any) above this more stringent limit was calculated.

3. Results

(a) Nonlinear inheritance of *EPSPS* copy number

Based on the linear regression between parental midpoint and mean offspring *EPSPS* copy number, we detected a significant positive relationship (LM: β/h^2_G (± s.e.) = 0.66 ± 0.09, t = 7.71, $p < 0.0001$; electronic supplementary material, figure S1). A significant quadratic relationship (LMquadratic: $F_{2,36}$ = 85.85, R^2_{adj} = 0.82, $p < 0.0001$; electronic supplementary material, figure S1) was also observed, reflecting a peak in offspring copy number at moderate parental midpoint copy number. A threshold model identified a changepoint in the slope of the parent–offspring relationship at 33.2 copies (95% confidence interval = 28.1 to 57.0; figure 1B; electronic supplementary material, figure S2A): inheritance of *EPSPS* copy number for crosses with lower parental copy number is associated with a high slope (segment 1, β (± s.e.) = 1.60 ± 0.25), p =< 0.0001), whereas for

crosses with higher parental copy number, the slope of the parent–offspring relation is much lower (segment 2, β (± s.e.) = 0.21 ± 0.20, $p < 0.0001$). Of the nonlinear models, the quadratic model is the best fit (AIC = 277.54, BIC = 284.20), although the nonlinear threshold model fit (AIC = 280.65, BIC = 288.96) is similar to the quadratic model, and both nonlinear models exhibit substantially better fit than the linear model (AIC = 306.53, BIC = 311.52).

The difference between mean offspring copy number and parental midpoint copy number exhibits a pattern of strong directionality across parental midpoint (figure 1D), shifting from positive to negative across the dynamic range of *EPSPS* copy number (figures 1D, 2A; electronic supplementary material, figure S2B). This shift in directionality was observed across the initial set of 30 crosses (figure 1D, blue points), and data points from highly isolated crosses (figure 1D, coral red points) are consistent with this pattern. Crosses with parental midpoint copy numbers below 33.2 copies (threshold model: segment 1, β = 0.60 ± 0.25, $p < 0.02$), all consistently yielded offspring with more copies on average than the parental midpoint copy number, with a maximum mean increase of 26.8 copies. Above 33.2 copies, the slope reverses from positive to negative (figure 1D), and the first mean decline in offspring copy number was observed at the parental midpoint of approximately 50.4 copies. All eight of the highest parental midpoint crosses (parental midpoints >60.3+) exhibited a mean decrease in *EPSPS* copy number for offspring relative to parental midpoint. Also, the decline in offspring copy number increased in magnitude with increasing parental midpoint copy number (threshold model: segment 2, $\beta = -0.79 \pm 0.20$, $p < 0.0001$), with a maximum mean decrease of 38.3 copies that was observed in a highly isolated cross.

A shift in directionality also applies to observations of offspring exhibiting extremely high and low copy numbers, relative to parents. Below the changepoint (up to 33.2 copies, dotted line on figure 2B), on average, 23% of offspring exhibited a copy number greater than the sum of both parental copy numbers. By contrast, above the changepoint, on average, 3% of offspring exhibited copy number greater than both parental copy numbers (figure 2B, dark purple bars). Also, below the changepoint, on average, 16% offspring exhibited a copy number lower than the lowest copy number parent, whereas above the changepoint, on average, 37% did (figure 2B, yellow bars).

We tested whether larger differences between individual parental copy numbers result in more dispersion in the distribution of resulting offspring copy numbers, and found no significant relationship between the difference in parental copy numbers and the standard deviation of offspring copy number (LM: $\beta = 0.12$, $t = 1.87$, $p = 0.07$; electronic supplementary material, figure S3). No evidence of large sex-specific effects was detected; the relationship between parent and offspring copy number is similar when based on maternal copy number ($\beta = 0.64$, $t = 7.11$, $p < 0.0001$) or paternal ($\beta = 0.49$, $t = 5.82$, $p < 0.0001$) copy number.

(b) *EPSPS* copy number variation: intra- and inter-individual sources

Intra-individual temporal replicates facilitated testing of the relationship between individual mean and individual variance for estimates of *EPSPS* copy number. Overall, we observed a clear increase in copy number variation among temporal replicates as individual mean copy number increases, with variation approximately symmetrical relative to 1 : 1 (figure 3A). The Poisson expectation for count data is that the variance of intra-individual samples will increase because of increasing mean count alone (electronic supplementary material, figure S4A, red line). As the mean copy number increases, individuals exhibited variance of temporal replicates far greater than expected because of the Poisson expectation alone (electronic supplementary material, figure S4A). A significant positive (LM: $R^2 = 0.33$, $t = 7.57$, $\beta = 0.03$ $p < 0.0001$) relationship between mean copy number and standardized residuals verified a significant increase in the deviation from the Poisson variance expectation as mean copy number increases, and indicated overdispersion relative to the Poisson expectation (electronic supplementary material, figure S4B), consistent with the overall estimate of dispersion $\varphi = 1.98$ (if $\varphi > 1$ Poisson underestimates variance).

Using a quasi-Poisson specified GLM model to account for the overdispersion of copy number, we found that the majority of variation in *EPSPS* copy number occurred among individual plants (GLM: $F_{66,155} = 70.93$, $p < 0.0001$), with lower magnitude variation among populations also detected (GLM: $F_{3,221} = 42.17$, $p < 0.0001$). However, two sources of intra-individual variation were also significant: biological replicate, although significant, explained the lowest amount of variation in *EPSPS* copy number (GLM: $F_{70,55} = 1.76$, $p = 0.02$), with temporal replicates explaining more variation (GLM: $F_{30,125} = 6.99$, $p < 0.0001$).

EPSPS copy number estimates of biological leaf tissue replicates ($n = 109$ pairs) differed by 5.83 (s.d. 6.97) copies on average, with differences ranging from 0 to 53 copies. The difference between biological leaf tissue replicates was only statistically significant for one individual from WO-GA following Bonferroni correction ($\alpha = 0.05/70 = 0.0007$). Also, tissue type (leaf versus inflorescence) did not significantly affect variation in biological replicates (GLM : tissue type × individual : biological replicate $z = 0.05$, $p = 0.95$). Temporal replicates of leaf tissue, on average, exhibited maximum pairwise difference in copy number of 20.64 copies (s.d. 14.50; range 0–87 copies). We tested whether variation observed across (five) temporal replicates per individual ($n = 117$) exhibits directionality (i.e. significant increase or decrease), and after Bonferroni correction ($\alpha = 0.0004$), no individuals exhibited significant directional change in copy number across temporal replicates (minimum p-value observed is 0.0012; figure 3B, blue lines).

4. Discussion

In this study, controlled experimental crossing revealed a nonlinear pattern of inheritance for resistance gene copies (figure 1). A threshold in the relationship between parent and offspring copy number suggests a shifting directionality of offspring *EPSPS* copy number relative to the parental midpoint. This shift was owing to the fact that offspring mean copy number was consistently maintained or increased in crosses between lower mean parental copy numbers (figure 2), but declined steadily across the highest parental copy numbers. Also, copy number variation within individuals increased with the mean plant copy

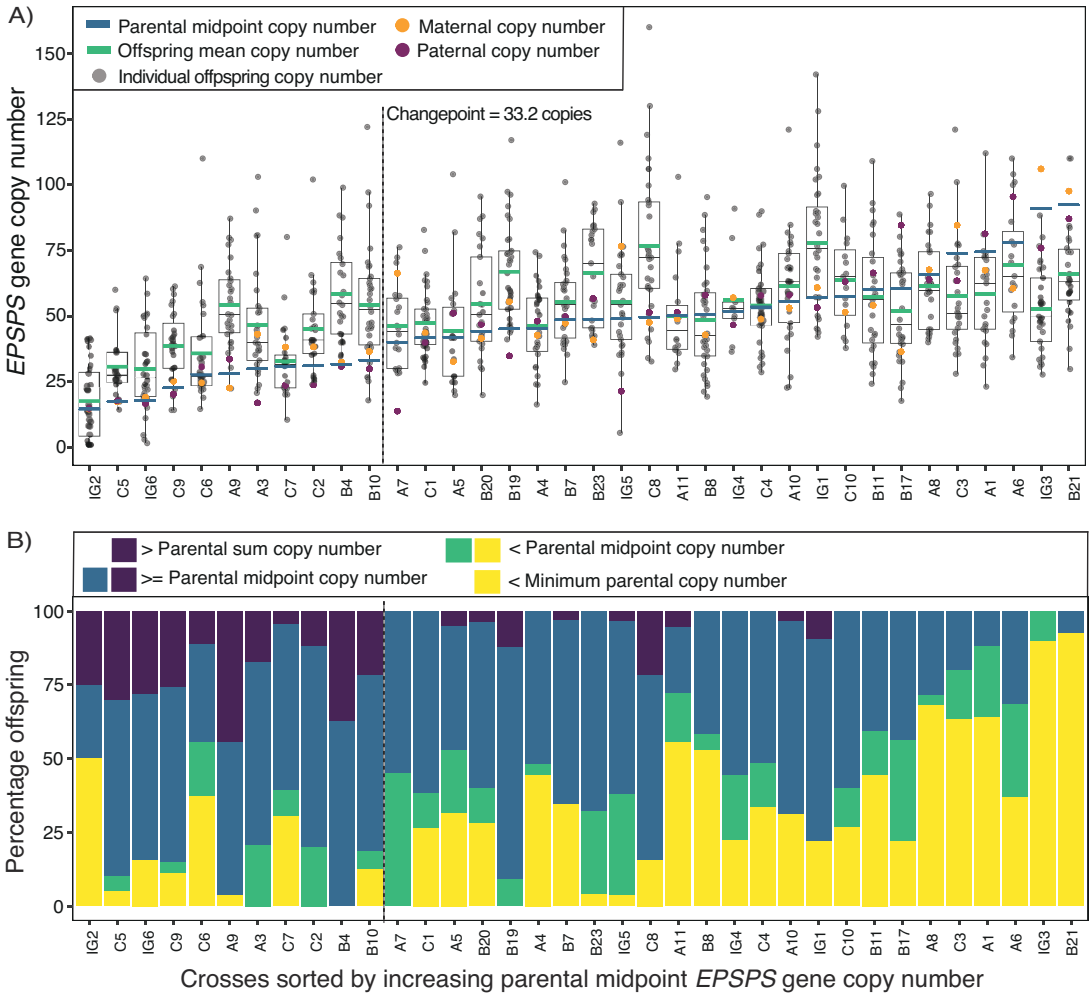

Figure 2. Variation in *A. palmeri* offspring *EPSPS* copy number relative to parental *EPSPS* copy number. (A) Box plots of *EPSPS* copy number variation for each experimental cross. Blue lines = parental midpoint copy number; green lines = offspring mean copy number; grey points = individual offspring copy number; yellow-orange circles = maternal copy number, purple circles = paternal copy number, black box plot median line = offspring median. (B) Bar plot of per cent of offspring exhibiting copy number less than parental midpoint copy number (green and yellow) and greater than or equal to parental midpoint copy number (blue and purple). Purple = per cent of offspring exhibiting copy number greater than the sum of parental copy numbers. Yellow = per cent of offspring exhibiting copy number less than the lowest parental copy number. Crosses (electronic supplementary material, table S1) on the *x*-axis of both (A) and (B) are sorted from left to right by increasing parental midpoint copy number. The occurrence of the changepoint (parental midpoint 33.2 gene copies) identified in figure 1B,D, relative to the sorted crosses, is shown with a vertical dotted line in both panels. Each stack of bars in panel (B) corresponds to the cross displayed directly above in box plot (A).

number (figure 3). Here, we discuss these deviations from stability of gene copies among tissues within individual plants, and through sexual reproduction in the context of the potential for these sources of gene copies to both enhance and limit evolutionary change in *A. palmeri* glyphosate resistance, across its lower and upper ranges of gene copy number variation, respectively.

(a) Inheritance of resistance gene copies: deviations from stability and Mendelian expectations

The concept of 'narrow-sense heritability', a function of additive genes and environmental influences, is typically applied to quantitative organismal phenotypic traits [43]. Owing to the quantitative nature of gene copy number variation, a genomic trait, the 'heritability' approach has been applied [44,45] to describe the pattern of gene copy inheritance. Such estimates of heritability are expected to vary among estimates [46]. Overall, our estimate of $h^2_G = 0.66$ is higher than a previous estimate $h^2_G = 0.29$ [44]; this difference may be owing to the previous study being based mostly on crosses between single/low copy × high copy individuals, whereas our study deliberately included crosses between parents with more similar copy numbers. Although investigating the linear relationship between parent and offspring copy number is a reasonable starting point, this approach is insufficient to fully capture the pattern of *EPSPS* gene copy number inheritance from parents to offspring.

The pattern of *EPSPS* gene copy number inheritance varies across the dynamic range of copy number. The slope of the regression between parental midpoint and mean offspring copy number is estimated to change at a threshold of approximately 33 gene copies. The slope is high (1.60) below the threshold, and substantially lower (0.21) above the threshold (figure 1B). That is, for parental midpoints ranging 1–33, for each parental copy of the *EPSPS* gene, on average, offspring carry 1.6× copies. By contrast, for parental midpoints 33+, the estimated slope of 0.21 means that for every approximately five parental *EPSPS* copies, only approximately one copy of *EPSPS* is transmitted to offspring. This shifting directionality of copy number inheritance is further evident when the direction (positive, negative) of differences between parental midpoints and offspring means is

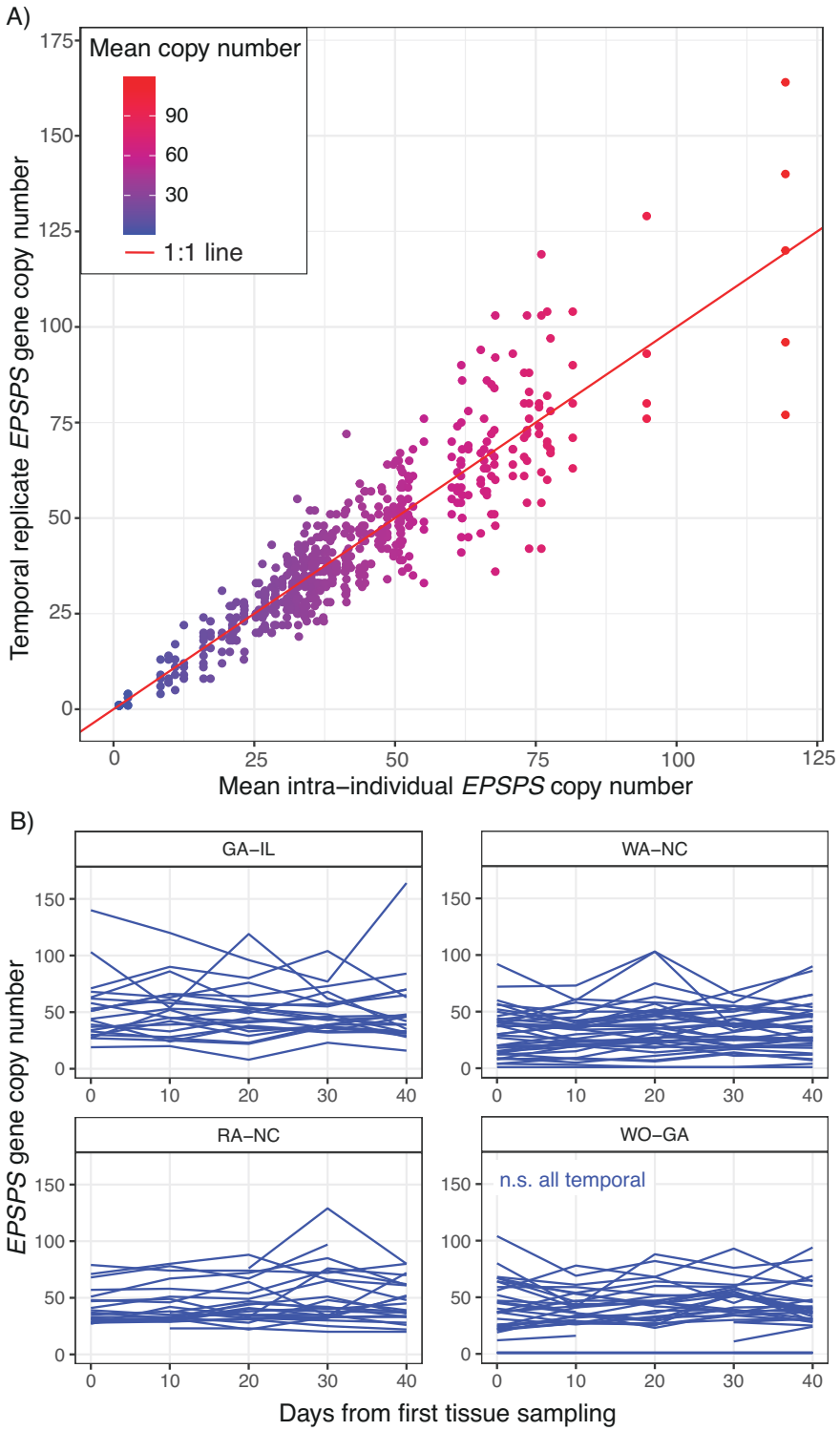

Figure 3. Intra-individual variation in *EPSPS* copy number in *Amaranthus palmeri*. (A) Scatterplot of individual (leaf tissue) estimates of *EPSPS* copy number by mean intra-individual copy number ($n = 117$ individual plants—each vertical stack of points corresponds to copy number estimates for an individual plant; $n = 583$ copy number estimates total); intra-individual variation increases with mean copy number. Red line = 1 : 1. (B) Temporal intra-individual variation based on five leaf tissue samples for four *A. palmeri* populations (GA-IL, RA-NC, WA-NC and WO-GA). Each blue line joins (up to) five estimates of *EPSPS* copy number from leaf tissue collected at 10 day intervals within an individual *A. palmeri* plant; all temporal relations were non-significant following Bonferroni correction.

examined. When differences between parents and offspring are owing to random measurement error, these differences should be symmetric around zero (figure 1C). Instead, we found a positive bias in differences between offspring and parents across the lower range of copy number, and a negative bias across the upper range (figure 1D). The consistency of offspring mean increases in copy number at lower copy number and offspring mean losses at higher copy number suggests that this directionality cannot be explained by sampling error alone (figure 1D). Pollen contamination could cause an increase in offspring copy number in low copy crosses, and a decrease in high copy crosses. However, the consistency of results from highly isolated crosses (figure 1D, coral red points) and the original crosses rules out this possibility.

The subsequent discussion of this observed pattern of shifting directionality in transmission of *EPSPS* gene copies from parents to offspring includes hypotheses involving the potential for some *EPSPS* copies to reside on ec(c)DNA. Although the

current study does not directly distinguish between a chromosomal versus ec(c)DNA location [54] for each of the *EPSPS* copies detected, the following suggests that there is an increased probability of ec(c)DNA involvement in *EPSPS* copy number with increasing mean individual *EPSPS* copy number: (i) Koo *et al.* [30], using FISH mapping, established that copies of *EPSPS* could be visualized as external to chromosomes, for individuals estimated to have 12+ copies of *EPSPS*; (ii) the first sequencing efforts of the *EPSPS* eccDNA replicon established its circular nature; *A. palmeri* accessions sequenced were estimated to have 80 and 34 *EPSPS* copies, respectively [30,31]; (iii) an independent study of six *A. palmeri* accessions (*EPSPS* copy numbers 42–213) used long-read sequencing, confirming the occurrence of the first-sequenced eccDNA replicon [31], and characterizing another variant of the eccDNA replicon that carries the *EPSPS* gene plus additional genes for resistance to the herbicide glufosinate [54]; and (iv) finally, using samples from the source populations used in the current study to quantify patterns of inheritance, we detect a strong correlation (GA-IL $r = 0.81$; WO-GA $r = 0.89$) between *EPSPS* (copy number range 1 to approx. 80 copies) and the copy number of another gene carried on the eccDNA replicon(s), *Hsp70* (range 1 to >100 copies; Ngo, C.X., L. Joshi and S.B. Yakimowski 2026 unpublished data). High correlation between copy numbers for two genes exhibiting high copy number that occur on the ec(c)DNA replicon(s) [31] is consistent with ec(c)DNA involvement in the occurrence of high *EPSPS* copy number.

Whether individuals exhibiting low *EPSPS* copy number could be associated with ec(c)DNA is less clear. Previous visualization of ec(c)DNA suggests that the lower part of the copy number range (1 to at least 12 copies) does not involve ec(c)DNA [30]. Also, a long-read sequencing/bioinformatic approach did not detect eccDNA in glyphosate 'susceptible' accessions [54]. If low copy number individuals tend not to involve ecDNA, predicted patterns of inheritance should be more consistent with Mendelian expectations. Our 1 × 1 copy crosses yielded all but one (see the electronic supplementary material, file SA) single copy offspring.

That mean offspring copy numbers for all crosses below the threshold of approximately 33 *EPSPS* copies (and up to parental midpoint 50) are all positive values relative to parents suggests that through this lower range, *EPSPS* copy number is maintained, and associated with a capacity for increase through meiosis and sexual reproduction. The difference between mean offspring and parental midpoint copy number consistently ranges between 2 and 27 copies across lower copy numbers (segment 1, figure 1D). A positive bias in offspring copy number is expected for the lowest range of copy numbers because of the constraint of requiring at least one copy of *EPSPS*, but this constraint lessens with increasing copy number. Instead, the positive bias persists up to (parental midpoint) approximately 50 copies—this is consistent with Koo *et al.*'s [30] observation that ec(c)DNA structures carrying *EPSPS* copies randomly associate with chromosomes through the pachytene, diplotene, diakinesis and metaphase stages of meiosis. Thus, there is a mechanism by which an offspring could inherit multiple (up to all) ec(c)DNAs/copies from each parent in addition to chromosomal gene copies. Up to the changepoint of 33 copies, 77% of individual offspring exhibited *EPSPS* copy number estimates greater than the parental midpoint and 23% of offspring exhibited copy number greater than the sum of both parents. In fact, the 33-copy changepoint coincides with the peak of the increase in copy number of offspring relative to parents: for cross B10 between parents with 37 and 30 copies, the maximum offspring value was approximately 122 copies, double the sum of both parents. By contrast, for a lower copy number cross between 14.5 and 14.7 copies, the maximum offspring value was approximately 12 copies greater than the parental sum. The large gains in copy number drop precipitously below the changepoint (mean offspring > parental sum above the changepoint is 3%).

In contrast to inherited copies, intra-individual temporal replicates suggest that there is no general positive increase in copy number through plant development (figure 3B). Intra-individual variation in copy number did not account for the range of variation observed in offspring (see §2b(ii) and the electronic supplementary material, file SB). For all but one cross, up to 19% of the highest copy number offspring are higher than the sum of estimated variation among tissues of the parents. Thus, overall, mechanisms responsible for intra-individual variation probably contribute to the range of copy number gains observed in offspring, but are insufficient to explain the consistently positive bias of offspring relative to parents, or the most extreme high copy number offspring that exceed the sum of both parents.

The consistent positive bias in offspring copy number, and the occurrence of individuals with copy numbers greater than the sum of their parents, are congruent with the previously proposed self-replication mechanism: the *A. palmeri EPSPS* eccDNA replicon carries several genetic elements putatively involved in autonomous DNA replication [31], and when these sequences are transformed into yeast, they are functional and facilitate DNA replication [55]. Autonomous self-replication has similarly been documented in *Drosophila* eccDNA (e.g. rolling circle replication [56]) and occurs in the majority of yeast eccDNAs [37]. Further work is needed in *A. palmeri* to test whether the self-replication mechanism of the eccDNA replicon functions through meiosis and sexual reproduction. Yet, regardless of the molecular mechanism(s) involved in the increases in resistance gene copy number observed here, this is equivalent to a rapid increase in the standing genetic variation of a quantitative trait, which increases the rate of adaptive evolutionary response to natural selection [43].

In contrast to the positive bias in offspring copy number at low copy number, a decline in inheritance involving a reversal from copy number gains to copy number loss was observed above approximately 33 copies of the *EPSPS* gene. The significant negative slope above the changepoint (segment 2, figure 1D) suggests that the magnitude of copy number loss in offspring increases with copy number. We do not currently know what mechanism(s) are responsible for the decline of inheritance of high copy number. As a starting point for future investigation of factors that may contribute to this instability of high copy number through sexual reproduction, we propose two hypotheses: one hypothesis for this decline in gene copy number transmission is that as the mean individual copy number increases, the number of extrachromosomal replicon structures increases. The stronger decline at high copy number suggests a potential for the influence of the spatial arrangement and density of ec(c)DNAs. For example, perhaps a high density of ec(c)DNAs contributes to a larger proportion of damage and lowers the transmission of gene copies during meiosis. The structural polymorphism of ec(c)DNAs characterized by Koo *et al.* is consistent with extrachromosomal structures being prone to structural damage (e.g. approx. 50% intact circular versus approx. 50% linear and irregular forms). ec(c)DNA polymorphism was assayed for an individual carrying approximately 80 gene copies

[30]; it would be of future interest to test whether the stability of circular forms plays a role in the stable inheritance and positive copy number bias observed here over a lower copy number range: is a greater proportion (>50%) of ec(c)DNAs in the range of 15 to 33 copies circular?

The magnitude of mosaicism [57,58] of *EPSPS* copy number [30,44] is shown here to increase, with intra-individual estimates of copy number becoming overdispersed as mean copy number increases (figure 3A; electronic supplementary material, figure S4). This suggests a second hypothesis for the loss of high gene copy number through sexual reproduction: the copy number of gametes could be associated with high variance in high copy number parents, yet we observe a striking negative bias of offspring copy number. This suggests that copy number could have direct genetic effects on gamete performance. Copy number can be associated with either positive or negative effects on fertility: female gametes in potato were found to benefit from increased copy number in a linkage group [59], whereas human male infertility has been associated with increased gene copy numbers on the Y-chromosome [60]. In plants, variation in pollen tube growth rate can be a significant component of gametic competition [61,62]; low inheritance of high copy number could be the result of high copy number pollen tubes being outcompeted by lower copy number pollen tubes. A first step towards testing this hypothesis would be to quantify the variance in copy number/ec(c)DNAs among pollen grains, followed by a comparison of pollen tube success by copy number/ec(c)DNAs.

(b) Evolution of resistance gene copy number

High copy number individuals exhibit the most consistently high phenotypic glyphosate resistance [48], despite the increased intra-individual copy number variation shown here (figure 3). Small gains in resistance per copy occur from 16 to 160 copies [48], consistent with the strong correlation between *EPSPS* copy number and transcript abundance [27]. Moreover, the signature of cost of resistance/high *EPSPS* copy number detected in other glyphosate-resistant plants [63–65]—a negative relationship between copy number and fitness-related traits in the absence of glyphosate—is often not detected in *A. palmeri* [66–68]. Here, we detect no relationship between seed count of crosses and parental midpoint copy number (LM: $t = 1.43$, $p = 0.16$). The only evidence of a cost was detected by a field assessment and found a fitness penalty (lower seed number and biomass) associated with *EPSPS* copy number ≥2 copies in *A. palmeri* [68]. Therefore, given the modest costs associated with high copy number but the most consistent highest resistance conferred, it has seemed paradoxical that high copy number individuals have only been detected in agricultural populations at very low frequencies [48]. One possibility is that more time is required and directional selection, if glyphosate selection continues, will further increase mean *EPSPS* copy number in these agricultural populations [27,66]. To date, the highest population mean *EPSPS* copy number detected is approximately 58 [48], which is near the upper 95% confidence interval (approx. 57 copies) of the threshold copies detected here. In addition, populations with higher mean copy number exhibited lower standard deviation [48], consistent with stabilizing selection and the possibility that population *EPSPS* copy number is already near a stable evolutionary peak. Our findings here suggest that declining inheritance could be the primary factor limiting the evolution and maintenance of high *EPSPS* copy number in *A. palmeri*.

5. Conclusion

Despite significant deviations from Mendelian expectations, the inheritance of resistance gene copy number, with previously documented involvement of ecDNA, follows a clear pattern characterized by a shift in directionality. The lack of temporal directionality among tissues of the same individual shows that copies are not generally lost during mitosis, but rather the decline in offspring copy number relative to high copy number parents points to loss occurring during meiosis and sexual reproduction. Future studies of intra-individual variation and inheritance of *EPSPS* copy number under glyphosate and other stresses would be valuable: does glyphosate stress increase the production of gene copies/ec(c)DNAs, furthering the positive bias in copy number observed in offspring here at lower copy numbers and/or ameliorating the consistent and increasing loss of copies towards the upper range of copy number? Or, is the genomic constraint on the maintenance of high copy number observed here observed under all environmental conditions? Overall, diminished transmission of high *EPSPS* copy number from *A. palmeri* parents to offspring suggests a potential genomic constraint on the evolution of herbicide resistance via increased *EPSPS* copy number.

Ethics. *Amaranthus palmeri* seed used in this study was imported from the USA to Canada, and used for research with strict containment protocol as outlined in CFIA (Canadian Food Inspection Agency) permit P-2016-02254.

Data accessibility. All data and associated R code are available at Dryad [69].

Supplementary material is available online [70].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. L.H.H.: data curation, formal analysis, investigation, methodology, supervision, validation, visualization, writing—original draft; K.F.D.: formal analysis, investigation, methodology, visualization; S.B.Y.: conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, visualization, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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