The factors driving evolved herbicide resistance at a national scale

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Abstract

Repeated use of xenobiotic chemicals has selected for the rapid evolution of resistance threatening health and food security at a global scale. Strategies for preventing the evolution of resistance include cycling and mixtures of chemicals and diversification of management. We currently lack large-scale studies that evaluate the efficacy of these different strategies for minimizing the evolution of resistance. Here we use a national scale dataset of occurrence of the weed *Alopecurus myosuroides* (Blackgrass) in the UK to address this. Weed densities are correlated with assays of evolved resistance, supporting the hypothesis that resistance is driving weed abundance at a national scale. Resistance was correlated with the frequency of historical herbicide applications suggesting that evolution of resistance is primarily driven by intensity of exposure to herbicides, but was unrelated directly to other cultural techniques. We find that populations resistant to one herbicide are likely to show resistance to multiple herbicide classes. Finally, we show that the economic costs of evolved resistance are considerable: loss of control through resistance can double the economic costs of weeds. This research highlights the importance of managing threats to food production and healthcare systems using an evolutionarily informed approach in a proactive not reactive manner.

Introduction

Xenobiotic chemicals including antibiotics, anti-cancer treatments, insecticides and herbicides, have brought enormous health benefits and increases in food production [1-3]. However, pathogens and pests are highly adaptable, and can rapidly evolve resistance to these chemicals rendering them ineffective. As a result, evolution of resistance is a major threat to public health and food security at a global scale [2-4].

The development of new xenobiotics plays an important role in the control of pathogens and pests. However, finding new chemical tools that are effective and meet regulatory safety standards involves significant time and cost [5]. The useful life of these chemicals can be very short, and in extreme cases resistance has evolved in just a few years [2, 5]. In the case of herbicides there have been no new modes of action developed in the past 30 years, and evolved resistance is reducing the range of management options available [5]. Slowing the evolution of resistance to current chemicals is thus a crucial priority [2, 3, 6]. Consequently, research on the evolution of resistance is carried out across a diverse range of applied disciplines [7, 8].

The primary approach to minimizing the rate of evolution of resistance is through using multiple xenobiotics with contrasting modes of action (MOAs: families of chemicals that target cellular machinery or metabolic processes in different ways). Four principal strategies exist for combining two or more chemical MOAs over space and time, with the objective of delaying the evolution of resistance to pesticides and drugs [9]: *Periodic application* and *Responsive alternation* (collectively referred to as 'temporal cycling') where treatments vary over time, but not space; *Mosaic* where treatments vary spatially but not temporally; and *Combination* where treatments vary over both space and time (with multiple MOAs administered at once). In medicine, drug *combination* therapies have slowed the evolution of resistance in HIV [10] and are recommended for treating tuberculosis [11] and malaria [12]. In agriculture both the scientific literature and industry advice suggest managing the evolution of resistance with *temporal cycling* and/or *combination* of different MOAs [8, 13-18]. The rate of evolution for herbicide resistance should be slowed more effectively by *combination* (simultaneous use of multiple MOAs) than by *responsive alternation* (annual rotation) of MOAs [13, 14, 16, 17], however this has yet to be tested at large scales and under the usual scenario where resistance has already evolved to some MOAs. Notwithstanding in broad terms current management is founded on the theoretical prediction that increasing the diversity of chemicals used can reduce the rate of evolution of resistance.

It is not inevitable that using a *combination* of MOAs will reduce the rate of evolution of resistance. The concept of *combination* treatment is based on the assumption that resistance to each MOA is driven by mutations at specific loci (target site resistance), each of which confers a large effect [7]. However, much resistance is driven by more general, non-specific non target site resistance [7]. This resistance may confer resistance to multiple MOAs, and thus *combination* and *temporal cycling* of products may have a reduced impact.

To date, most recommendations for managing the evolution of resistance are predicated on the assumption that there are multiple effective modes of action [9]. However, this may not always be the case, particularly in systems where xenobiotics have been in use for several decades. Historical use means that some resistance already exists to some MOAs available for inclusion in a *combination* or *temporal cycle*. For weed control in particular this problem is exacerbated because new MOAs are introduced very infrequently [5]. In addition, non-target site resistance mechanisms may be present in populations never exposed to xenobiotics, pre-adapting those populations to quickly evolve resistance [19].

In agriculture, resistance management is embedded within Integrated Management (IM), where pests are controlled by varying crops and management practices, including options beyond chemical control [20]. Significantly, mortality from non-chemical control is unaffected by the extent of evolved resistance and should not select for increased xenobiotic resistance. By reducing population sizes independently of chemical control, IM is argued to be effective at both delivering pest control as well as reducing the rate of evolution to xenobiotics [21]. However, it is generally unclear how effective such strategies are, as well as the extent to which managers proactively use these methods.

Understanding of the effectiveness of alternative strategies is limited by the availability of long-term management data that simultaneously records the abundance of pests, weeds or diseases and the extent of evolved resistance to xenobiotics. Here we report such a dataset and use it to analyze the factors driving herbicide resistance at a landscape scale. We use blackgrass (*Alopecurus myosuroides*), an arable weed in the UK, as an empirical system for investigating the evolution of resistance at scales relevant to national cropping and food production. Data from a national network of farms are used to investigate the role of historical management in the evolution of resistance. We collated field management histories for up to 10 years on each farm, which allow us to measure real-world management where herbicide applications are commonly used alongside integrated management control methods. We describe the national distributions of the weed, demonstrating a large-scale cline in occurrence and confirming the role of resistance in driving densities. By linking densities and resistance status to management we are able to demonstrate how different management strategies have affected the evolution of resistance. Finally, we explore the wider consequences of evolved resistance, measuring the costs of management and showing how resistant weeds are driving losses in crop production.

Results and Discussion

Distribution and spread. The distribution of *A. myosuroides* is now extensive, with eighty-eight percent of 24 824 quadrats surveyed containing at least one blackgrass plant. Thirty-two percent of quadrats contained high or very high densities. We found that weed density varies geographically (Figures 1a and 1b) with significantly higher densities found in the southern regions of the study

(F=93.48, df = 564, p <0.001). For example, we recorded high and very high densities in 75% of quadrats in Buckinghamshire (Southern England), compared to only 20% in Yorkshire (Northern England).

Changing herbicide usage suggests that *A. myosuroides* is becoming increasingly difficult to manage with chemicals: recent years have seen increases in the geographical range of *Alopecurus myosuroides* (Figure 1c) and concomitantly both the volume and diversity (Figure 1d) of herbicides used has increased with time as successive products become ineffective. Particularly evident is a dramatic increase in the use of Glyphosate (Figure 1d/e), a broad-spectrum herbicide that is used to manage problematic outbreaks.

Is resistance driving high weed densities? Herbicide resistance was first reported in the 1950's [19] and, as of March 2017, is confirmed in 252 weed species globally, covering a broad range of herbicides [23]. Resistance is widespread in populations of *A. myosuroides* in the UK. The three herbicides tested caused <40% mortality (very high resistance) in 96% (FEN), 82% (ATL) and 57% (CYC) of the 138 blackgrass populations, when applied at recommended field rates (see Experimental Procedures for details). Most populations were resistant to multiple herbicides (Figure 2): 79% of populations had high levels of resistance (defined as <80% mortality after exposure) to all three herbicides. This suggests two possibilities: firstly, that target-site resistance combined with extensive gene flow has led to the evolution of resistance to all three MOAs independently, or alternatively, evolution of resistance to one MOA confers cross resistance to the other MOAs (i.e. one that the plant is yet to meet), potentially through metabolic mechanisms.

Our data indicate that resistance appears to be a key factor driving the abundance of *A. myosuroides*: we find a positive relationship between blackgrass density and herbicide resistance across all three herbicides tested (Figure 3a). The fraction of plants surviving herbicide treatment increased with blackgrass density in the source population, but the relationship differs between herbicides (χ^2 (3) = 128.13, p<0.001. Corrected R^2=0.34; Figures 3a/b). The dry weight of blackgrass (per plant) after treatment with herbicides also increases with blackgrass density, and the relationship between weed density and biomass differs between herbicides (χ^2 (3) =98.154, p<0.001. Corrected R^2=0.52; SI: Figure S1).

To further explore the relationship between herbicide resistance and black grass density we analysed the relationship between resistance and densities in successive winter wheat crops. The significant relationship between herbicide resistance and density can be seen in Figure 4a, where fields with higher levels of resistance tended to have a higher mean density state in 2014 ($F_{1,43}$ = 12.9, P = 0.0009) and 2016 ($F_{1,43}$ = 11.1, P = 0.0017). As shown in Figure 4b, the relationship between resistance and density drives weed levels in the subsequent crops: there is a close relationship between densities in successive crops, correlated with resistance. Although there is slight evidence for increases in density between 2014 and 2016 (30 out of 45 populations increased in density, sign test P = 0.036) the closeness of the relationship between densities in 2014 and 2016 (*r* =0.81, $F_{1,43}$ = 83.1, P < 0.0001) emphasizes the importance of previous density and, hence historical resistance, in generating long-term infestations.

How does previous management affect levels of resistance? From healthcare to agriculture a major objective of resistance management is to preserve the efficacy of existing chemicals by limiting or optimizing their use [2, 24]. Evidence suggests that resistance can evolve after as few as three years of consecutive use of a single xenobiotic [5] and that repeated application of chemicals with the same MOA has the greatest risk for evolution of herbicide resistance [25, 26]. Reducing the rate of evolution of resistance requires the minimization of both the survival and reproduction of resistant individuals. Integrated weed management (IWM), where herbicide strategies [18] are combined with cultural control methods such as crop rotation and soil cultivation [27] are the most common approach to achieve this. These strategies impose mortality or reduce rates of population increase through mechanisms unconnected with susceptibility or resistance to xenobiotics.

Contrary to previous literature, industry recommendations and common agricultural sector practice [9, 28, 29], we found that herbicide diversity does not appear to reduce the likelihood of herbicide resistance evolving (Table 1). Note that in our farm management data high herbicide diversity could be achieved through *combinations* (different MOA applied together on the same date) or *temporal cycling* (different MOA applied on different dates within a year), and both strategies were frequently employed simultaneously. Instead, we found that higher levels of herbicide resistance are associated with greater intensity (*frequency*) of herbicide applications. We split the management data into two time periods to allow us disentangle the effects of earlier management (2004-2009), from those of more recent management (2010 – 2014). The results were essentially the same for both, although herbicide intensity only had a significant effect on survival (and not dry-weight) for the more recent time period (Table 1).

Herbicide diversity (mean number of MOA applied within a crop year) is correlated with herbicide intensity (mean number of herbicide application dates within a crop year) (2004 – 2009: rho= 0.874; 2010 – 2014: rho= 0.827). To assess the effect of this correlation we fit models with either herbicide diversity or herbicide intensity. Although there was a relationship between herbicide diversity and resistance, when compared in the same model herbicide diversity was always a *weaker* predictor of resistance than herbicide intensity, and so was not retained in any of the final models. The intensity of herbicide applications (number of applications within a growing season), irrespective of the type of herbicide, is thus the most important management variable correlated with the evolution of resistance.

We considered the directionality of the relationship between herbicide usage and resistance. One possibility is that the relationship between volume of herbicide applied and resistance could reflect recent increases in herbicide use in response to high weed densities resulting from resistance. Crucially three findings render this interpretation unlikely. First, as shown in Table 1, the relationships are robust whether we consider management in the past (2004-2009) or recently (2010-2014). Second, these relationships remained when we analysed data on resistance to the most recently introduced product to the market, Atlantis, separately (Supplemental Information: Table S2). Atlantis was only introduced in 2005, however the correlates of resistance remain the same. Thirdly, we found no relationship between weed density and volume of herbicide used either recently (2010-2014) or in the past (2004-2009) indicating that weed density is not a driver of herbicide usage, notwithstanding the correlation of both volume of herbicide and weed density with resistance (See Supplemental Information: Table S3). Taken both individually and together these three results do not support the interpretation that resistance is driving herbicide usage rather than vice versa.

Our results suggest that using multiple MOAs (either in *combination* or *cycles*) may be ineffective as a reactive strategy for managing resistance that has already evolved. In addition, our analysis that focused solely on Atlantis suggests that use of multiple MOAs may also fail when new products appear on the market and are introduced to a *combination* or *cycle* comprised of older MOAs where resistance has already evolved. Given how infrequently herbicides with novel MOAs are introduced [5] this is likely to be a common scenario in weed control.

A recent study in Germany found no relationship between number of MOA used and resistance status of *A. myosuroides* [30]. Alongside our finding that the intensity of herbicide application was a stronger predictor, we found the widespread occurrence of resistance to multiple herbicides in our dataset (Figure 2). This suggests a significant role for multiple herbicide resistance driven by metabolic mechanisms. Multiple herbicide resistance driven by metabolic mechanisms is a significant threat to the sustainability of chemical management because evolution or resistance under selection by one herbicide can lead to resistance to others, including those that populations have not yet been exposed to. Thus, future options for management are constrained if multiple herbicide resistance is widespread.

Another study to find that volume (intensity) of applications is a very important factor in the evolution of resistance, did, however, also find that combining MOAs may delay the evolution or resistance in systems with no evidence of metabolic resistance [31]. This highlights that the best management strategy may often be context dependent in terms of the previous history of herbicide management. The authors note that the major challenge for the future of crop production is identifying effective mixes against weeds that have already evolved resistance to many of the previously effective herbicide options [31]. This will remain to be the case even when crops are genetically engineered to contain traits conferring tolerance to multiple herbicides.

Despite widely repeated recommendations that diversity of crop rotation, changes in cultivation and ploughing regimes should be adopted to reduce *A. myosuroides* infestations [32, 33], our results fail to detect an effect of cultivation intensity, frequency of ploughing or crop type (PCA axis 1: combining frequency of winter wheat, cereal and autumn sown cropping) on the evolution of herbicide resistance (Table 1). Thus, although such techniques are expected to have demonstrable impacts on population sizes [33], at least in the medium-term, impacts on resistance are undetectable in our dataset.

Measuring the impacts of evolved resistance and its management. Since its widespread emergence, herbicide resistance has become a major threat to global food security [34]. Herbicide resistant weeds are one of the biggest threats to crop yields. Weeds cause average yield losses of 35%, worldwide [35], this figure could be much higher without effective herbicides [10]. Yield losses incurred by *A. myosurorides* infestations are thought to make it the most economically important weed in Western Europe [32]; our dataset offers a unique resource to estimate these costs from field to regional scales.

At the field scale, our data show total yield losses to range from 0.2% to 12.8% and overall yield decreased significantly with increased weed density ($F_{1,8}$=5.643, p=0.045). Within fields, *A. myosuroides* only begins to impact wheat yields when it occurs at high densities (Figure 5a). Herbicide treatments targeted at control of *A. myosuroides* cost between £105/ha to £176/ha, but there is no relationship between costs of herbicides applied/ha and weed density ($F_{1,8}$=1.061,p=0.33) (Figure 5b). This suggests that farmers do not vary their management approaches with respect to weed density. Combined costs (herbicides + yield loss) ranged from £115/ha to £320/ha, accounting for profit losses of between 4% and 12% (see SI: Table S5). Total cost of *A. myosuroides* (herbicide costs/ha + yield loss) increased significantly with weed density ($F_{1,8}$ =6.631, p=0.033) (Figure 5c), where an increase in average *A. myosuroides* density, at the field level, to the next density state results in a 2.5% loss in profit. The distribution of *A. myosuroides* within a field tends to be clumped, and so average densities were often increased by a larger area of a field developing high density infestations, and yield losses in those areas could be very high (Fig 5). Increasing blackgrass density state explained 34% of the reduction in yield and 39% of the increase in total management cost.

Conclusions. Resistance to herbicides, pesticides and antibiotics creates enormous costs in terms of reduced health and lost food production worldwide. We demonstrate a case using a spatially extensive dataset where there is no evidence that using a diversity of MOAs reduces selection for resistance, contrary to current industry advice and scientific literature [13, 14, 16, 17]. These findings raise a strong caution that temporal cycling, or combinations of MOAs might not be enough to combat resistance at landscape scales, particularly where resistance to some MOAs has already evolved. This could equally be the case in pesticide and antibiotic resistance. It is a matter of urgency to test this hypothesis in these important systems.

We also find that populations of *A. myosuroides* only have substantial economic impacts when they reach high densities. This, combined with our finding that it is the number of applications that drives the evolution of herbicide resistance, suggest that in the long-term balancing herbicide usage and economic impacts against the likelihood of selecting for resistance will be a possible route for developing sustainable management regimes. Previous papers that have promoted similar ideas, for instance based on thresholds [36, 37], have made similar arguments. The results we present here are an empirical demonstration that reliance on herbicides has led to wide-scale evolution of resistance. Managing to reduce weed density is not the same objective as minimizing resistance. Future management should more explicitly address the question of how to minimize resistance and maximize the efficacy of herbicides.

There is a belief that new compounds will continue to become available in the future [38, 39], and so there is no need to change the way we use these valuable chemical tools. The lessons learned from case studies such as this are vital to ensure that the value of any new product is maximized. With resistance evolving over short timescales [4, 5] it is inevitable that any new products will become ineffective if application strategies do not change. A major imminent threat to food production is the growing reliance on glyphosate as a weed management tool (Figure 1d/e). Resistance to glyphosate is already present in eight different countries [40]. How long until resistance to glyphosate becomes near universal is uncertain, but in evolutionary terms it is inevitable unless standard management practices change.

Author contributions

Conceptualization, HH, RPF, PN, DZC, KN; Methodology, RPF, HLH; Formal analysis, HLH, RPF, SRC, DC; Investigation, HLH, DC, LC, RH; Writing - Original Draft, HLH, RPF; Writing - Review & Editing, HLH, RPF, DZC, SRC, DC, PN, KN; Funding Acquisition, RPF, DZC, PN, KN.

Competing financial interests

RF, DZC, LC, HH, SC, PH and DC have no competing financial interests. PN supervises a PhD student co-funded by Bayer (not part of this project).

Data availability statement

Data that support the findings of this study have been deposited in the University of Sheffield Online Research data archive (ORDA) and can be accessed from the following URL: https://figshare.com/s/eb21f4d1862741d50ceb.

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Figure legends

Figure 1 a, Field level density *of A. myosuroides* in fields surveyed in 2014. Colours relate to mean weed density measured on ordinal scale from 0 (absent) to 4 (very high); green colours represent low weed densities, red colours represent high weed densities. **b**, Relationship between blackgrass density and latitude captured through the 2015 rapid assessment survey data (see Supplemental Experimental Procedures: *Rapid Assessments* for methodology). **c**, Historical distribution of *Alopecurus myosuroides* in the UK derived from Botanical Society of Britain and Ireland atlas data. Green dots represent records appearing in the 1960s atlas [41]. Orange dots represent new records appearing in the 1990s atlas [42]. Red dots represent new records from 2015/16 surveys. **d**, Herbicide usage records for Great Britain for three target-site herbicides and one broad-spectrum herbicide (Glyphosate), lines represent total area treated (ha) across all crops, data extracted from the Pesticide Usage Survey (https://secure.fera.defra.gov.uk/pusstats/) **e**, Total herbicide usage for Great Britain, line represents total area treated (ha) across all crops, data extracted from the Pesticide Usage Survey.

Figure 2 Percentage of fields tested for resistance to three herbicides, where resistance has been confirmed and is highly likely to reduce herbicide effectiveness. 79% of fields were resistant to all three herbicides; 1% of fields were not resistant to any of the herbicides tested. Resistance refers to <80% mortality when herbicide applied at recommended field rate – see Experimental Procedures for details.

Figure 3 a, Relationship between mean blackgrass density state measured on ordinal scale from 0 (absent) to 4 (very high) and percentage survival of plants after treatment with each herbicide. Plotted lines represent predicted survival of weeds after treatment with herbicide for differing blackgrass densities; models are mixed effect models with mean blackgrass density state and herbicide as fixed effects and farm name as a random effect. **b**, Heat maps showing percentage survival of plants (as a measure of herbicide resistance) to each of three herbicides. Red colours show high survival rates (i.e. low herbicide effectiveness), green colours show low survival rates (i.e. high herbicide effectiveness).

Figure 4 Blackgrass density measured on ordinal scale from 0 (absent) to 4 (very high) and resistance status of each field that was in winter wheat in both 2015 and 2016. **a,** The relationship between density of blackgrass and resistance. Lines connect the same field across years. **b,** Relationship between densities in successive years. Point color indicates resistance to the most effective herbicide tested. The dashed line indicates equality in both years.

Figure 5 Farm management impacts of blackgrass. **a**, The effect of density state on the yield for each 20m by 20m grid square (gray points), for 10 fields where high resolution yield data was available. Black points show the average effect of blackgrass density on yield, controlling for differences between fields. Black lines show 95% parametric bootstrapped confidence intervals. Relationship between; **b**, Costs of herbicides (£/ha), and **c**, total costs of blackgrass (yield loss + herbicide costs, £/ha), and mean density state of blackgrass for each field (each point represents one field). Costs were calculated at a wheat price of £115.10/t (source: Agriculture and Horticulture Development Board, Corn Returns). All costings were calculated at 2014 prices.

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Table 1 Final models of herbicide resistance. Generalized linear mixed effects models (GLMM) were used to determine the effect of farm management histories on two measures of herbicide resistance (survival and dry weight) across two timeframes (old: 2004-2009 and recent: 2010 – 2014). Mean black-grass density state, herbicide, soil type and herbicide parameters (mean number of herbicide application days per harvest year (herbicide intensity), mean number of herbicide MOAs applied within a harvest year (herbicide diversity)) were fitted as fixed effects in the models, and farm name was fitted as a random effect to describe the structure of the data. Observation-level random effects were used to account for over dispersion in the models. Here we present only the final models with significant predictor terms. A set of secondary analyses investigated the additional effect of crop type (derived from the proportion of years the field was in winter wheat/ an autumn sown crop/ a cereal crop), the proportion of years the field was ploughed and a mean cultivation intensity score. R-square values were calculated using MuMIN [39] and parametric bootstrap using Kenward Roger methods [40] (using the 'pbkrtest' package in R) were used for model comparison and calculation of p-values.

OLD

SURVIVAL			**Model fit**	
Model structure	**Effect size (Sum Sq)**	**P value**	**R2 GLMM (m)**	**R2 GLMM (c)**
Black-grass Density	24.311	0.001	0.281	0.353
Herbicide	126.364	0.001		
Soil type	9.907	0.006		
Herbicide intensity	17.099	0.002		
+ Crop type (PCA axis 1)	2.244	0.168		
+ Plough frequency	0.149	0.718		
+ Cultivation score	0.100	0.808		

RECENT

SURVIVAL			**Model fit**	
Model structure	**Effect size (Sum Sq)**	**P value**	**R2 GLMM (m)**	**R2 GLMM (c)**
Black-grass Density	23.380	0.001	0.275	0.351
Herbicide	124.661	0.001		
Soil type	9.634	0.006		
Herbicide intensity	13.188	0.003		
+ Crop type (PCA axis 1)	0.757	0.447		
+ Plough frequency	1.168	0.357		
+ Cultivation score	0.736	0.465		

OLD

DRY WEIGHT			**Model fit**	
Model structure	**Effect size (Sum Sq)**	**P value**	**R2 GLMM (m)**	**R2 GLMM (c)**
Black-grass Density	7.263	0.001	0.289	0.525
Herbicide	49.117	0.001		
Soil type	2.992	0.023		
Herbicide intensity	2.863	0.013		
+ Crop type (PCA axis 1)	0.221	0.513		
+ Plough frequency	0.127	0.622		
+ Cultivation score	1.197	0.100		

RECENT

DRY WEIGHT			**Model fit**	
Model structure	**Effect size (Sum Sq)**	**P value**	**R2 GLMM (m)**	**R2 GLMM (c)**
Black-grass Density	7.192	0.001	0.258	0.508
Herbicide	49.117	0.001		
Soil type	2.923	0.023		
+ Crop type (PCA axis 1)	0.433	0.394		
+ Plough frequency	0.087	0.647		
+ Cultivation score	0.003	1.000		

Methods

We surveyed 138 fields on 71 farms across England. Study sites were selected to cover a large geographic range, and to include a variety of farm sizes, crop rotations and management strategies within each region. Two fields were selected on each farm, one known to have large black-grass populations and one with a smaller weed population. For accurate comparison, all fields selected were cropped with winter wheat for harvest in 2014.

Weed population surveys

138 Fields with black-grass present were censused in a six week period from 1st of July 2014. Fields were divided into contiguous 20 x 20m grid squares and weed density was estimated in each grid square. The surveys followed a density-structured approach, recording density state of black-grass rather than numerical abundance. Each grid square was assigned to one of 5 density states that correspond to the number of plants per 20x20m square; 0 (absent), 1 (1-160 plants), 2 (160-450 plants), 3 (450-1450 plants) and state 4 (1450+ plants). These density states have been shown to accurately capture the variation within field populations and the 20 x 20m grid size sufficient to be representative of 1m^2 subplots where blackgrass plants were physically counted [45]. Areas within fields that were sprayed off or cut early were classified as state 4, to reflect management for very high levels of black-grass infestation.

Resistance testing

We quantified resistance to three herbicides that have been commonly used for grass weed control in arable crops: fenoxaprop ('FEN': inhibitor of ACCase; Aryloxyphenoxypropionates (FOPs), introduced to Europe in 1989), cycloxydim ('CYC': inhibitor of ACCase; Cyclohexanediones (DIMs) introduced to Europe in 1989) and mesosulfuron-methyl, henceforth referred by its UK trade name Atlantis ('ATL': inhibitor of acetolactate synthase [ALS] introduced to Europe 2001).

We quantify resistance in two ways: a) survival and b) dry weight of biomass, three weeks after exposure to herbicide.

Black-grass seeds were collected from ten different locations within each field surveyed in 2014, using a semi-random seed collection strategy (See Supplemental Experimental Procedures: *Seed Collection* for further details). *A. myosuroides* seedlings were germinated and allowed to grow for 18-21 days until reaching the three leaf stage before spraying with herbicide. We tested for resistance to three herbicides at the following rates: 'Atlantis' (Mesosulfuron + Iodosulfuron at 300 g ha^{-1}), 'Cheetah' (Fenoxaprop at 1.25 L ha^{-1}), and 'Laser' (Cycloxydim at 0.75 L ha^{-1}). These application rates were chosen as previous experimentation has shown them to provide the best approximation of field rate doses under glasshouse conditions and were applied with a track sprayer. Plants remained in the glasshouse for three weeks following herbicide treatment, at which point plant mortality was recorded before harvesting aboveground biomass from each pot. Plant material was dried at 80°C for 48 hours before weighing (See Supplemental Experimental Procedures: *Resistance Testing* for more details).

Field Management Data

Historical field management data was requested for each of the 138 fields that we surveyed for weed density. Data were available for 96 fields and up to 10 years data were collated for each field. For each year we recorded the following: crop, first cultivation type and herbicide applications (product name and date of application). From this we derived herbicide intensity (average number of herbicide application days per year) and herbicide diversity (average number of modes of action applied per year). We also derived cropping patterns (e.g. autumn or spring sown, cereal or non-cereal). Cultivation types were recorded and scored on a scale of intensity from 0-4 (where direct drilling = 0, to ploughing = 4) (See Supplemental Experimental Procedures: *Cultivation Intensity Scores* for more detail). Soil type for each field was extracted from the National Soil Resources Institute, NATMAP1000 database and classified into two groups (clays, non-clays) after [46, 47]. Where available, yield maps were obtained for fields that we surveyed to enable direct comparison of within field black-grass density and crop yield. See Supplemental Information: Table S1 for outlines of chemical/ cultural control techniques and corresponding model input variables.

Statistical analyses

Does resistance drive weed abundance and the role of diversity of management in the evolution of resistance?

We used R (v 3.2.2) and *lme4* [48] to perform linear mixed effects analyses of the relationship between herbicide resistance, black-grass density and farm management parameters. Herbicide resistance was classified in two ways; firstly, as a binary parameter of plant survival three weeks after herbicide application (number that survived and number that died), and secondly, as dry weight of above ground plant material three weeks after herbicide application. We modeled the survival measure of resistance using a binomial error term and the dry weight measure of resistance using a normal error distribution.

Models were created for both measures of resistance using both older (2004 to 2009) and more recent (2010 to 2014) management records, so that a total of four models were built (Table 1). Field management histories were split into two time-frames to assess whether management had changed over the preceding 10 years. In all models mean weed density state and herbicide were entered as fixed effects, along with management predictors; herbicide intensity (mean number of herbicide application days per harvest year), herbicide diversity (mean number of herbicide MOAs applied within a harvest year), a measure of crop rotation (PCA axis 1 that describes crop choice, Table S1), proportion of years the field was ploughed, and mean cultivation intensity score. Soil type was also included in the models (Table 1, SI: Tables S2 and S3).

Farm was used as a random effect to account for multiple fields within a farm. We used a hierarchical approach, putting the most important terms into the model first (i.e. black-grass density state and herbicide). Observation-level random effects were used to account for over dispersion in the survival model [49]. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality.

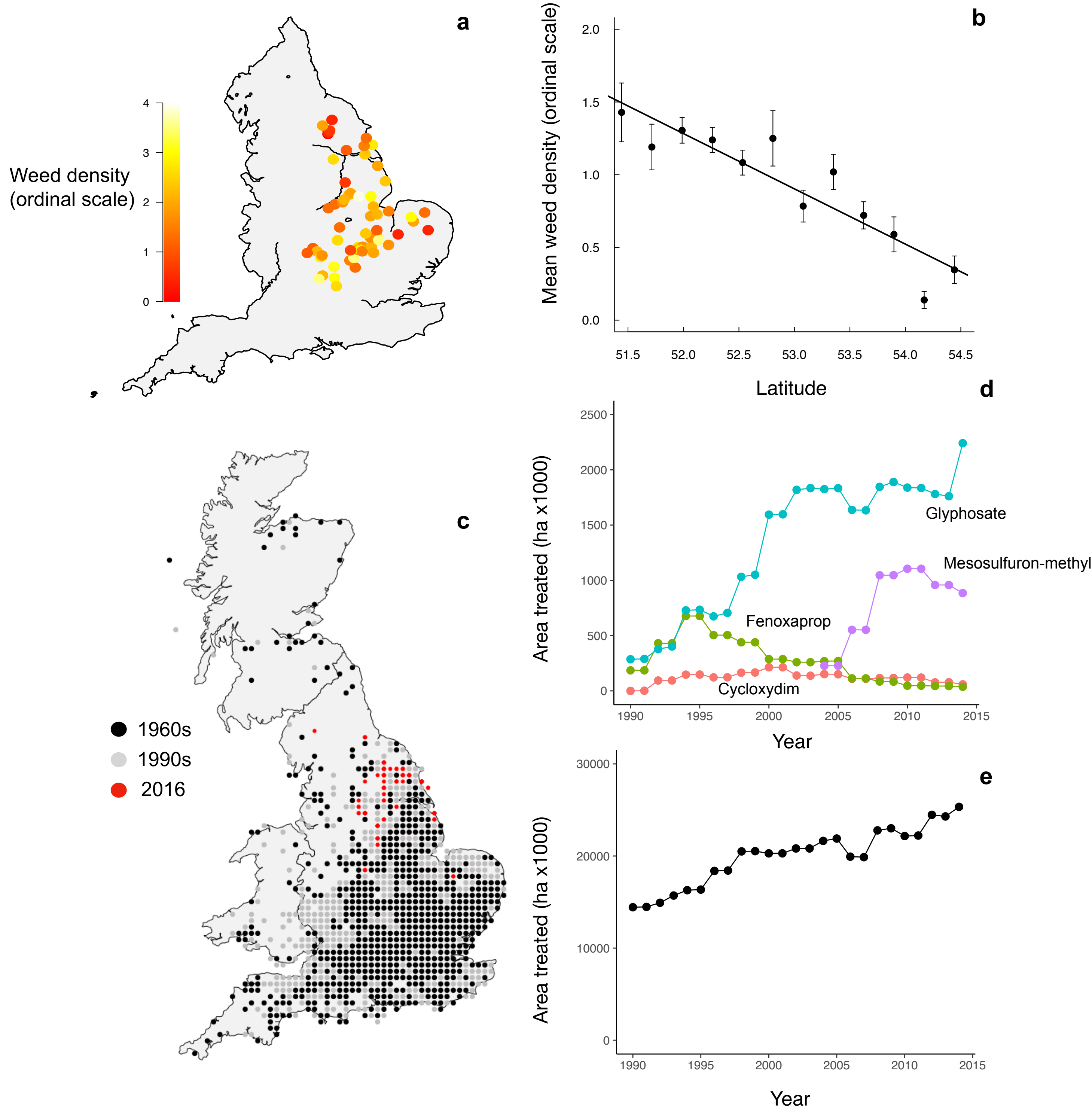
Marginal and conditional R-squared values were calculated for resulting models using the 'MuMIN' package [39]. Parametric bootstrapping was used for mixed model comparison and to calculate p-values for each predictor in the final models (using the 'pbkrtest' package [42]). Model residuals were plotted against farm name. Moran's I (using R package 'lctools' [50]) was used to test for spatial autocorrelation.

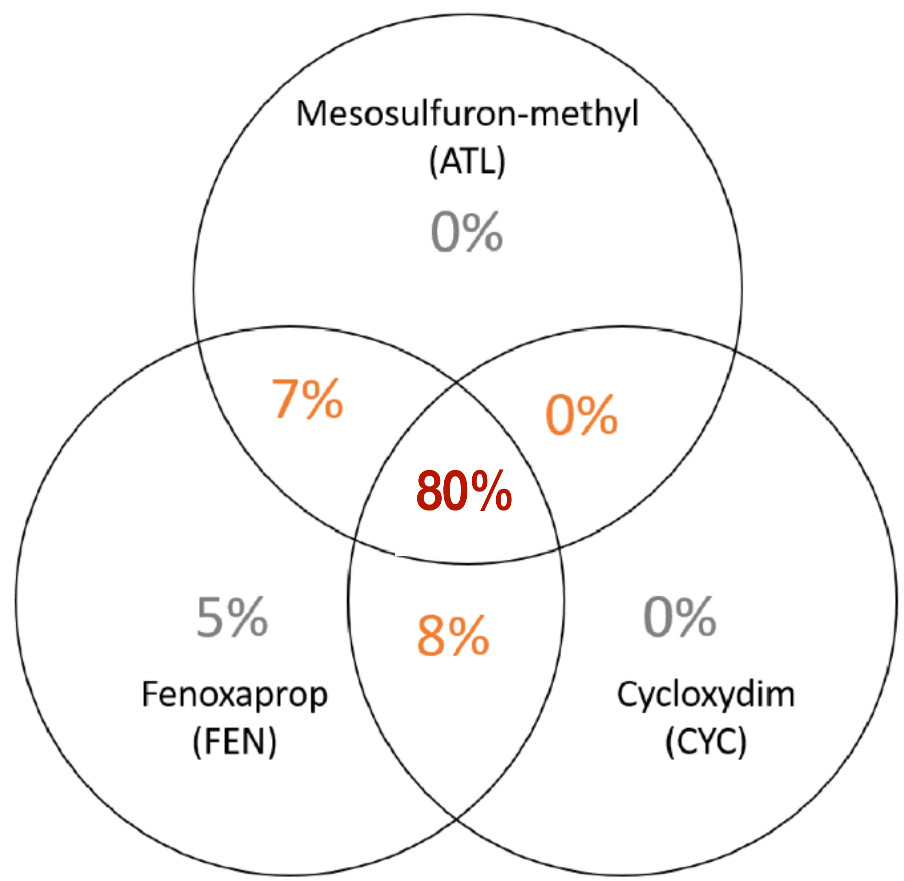
To test the relationship between resistance and black grass density we used a linear model to predict Ln(mean density state) for each field in winter wheat. We use resistance to the most effective herbicide as a measure of resistance because most farmers applied multiple herbicides and resistance was correlated across herbicides (Figure 2). Under these conditions the efficacy of the most effective herbicide will determine overall efficacy. Densities in successive years were compared with resistance and with each other using simple linear models.

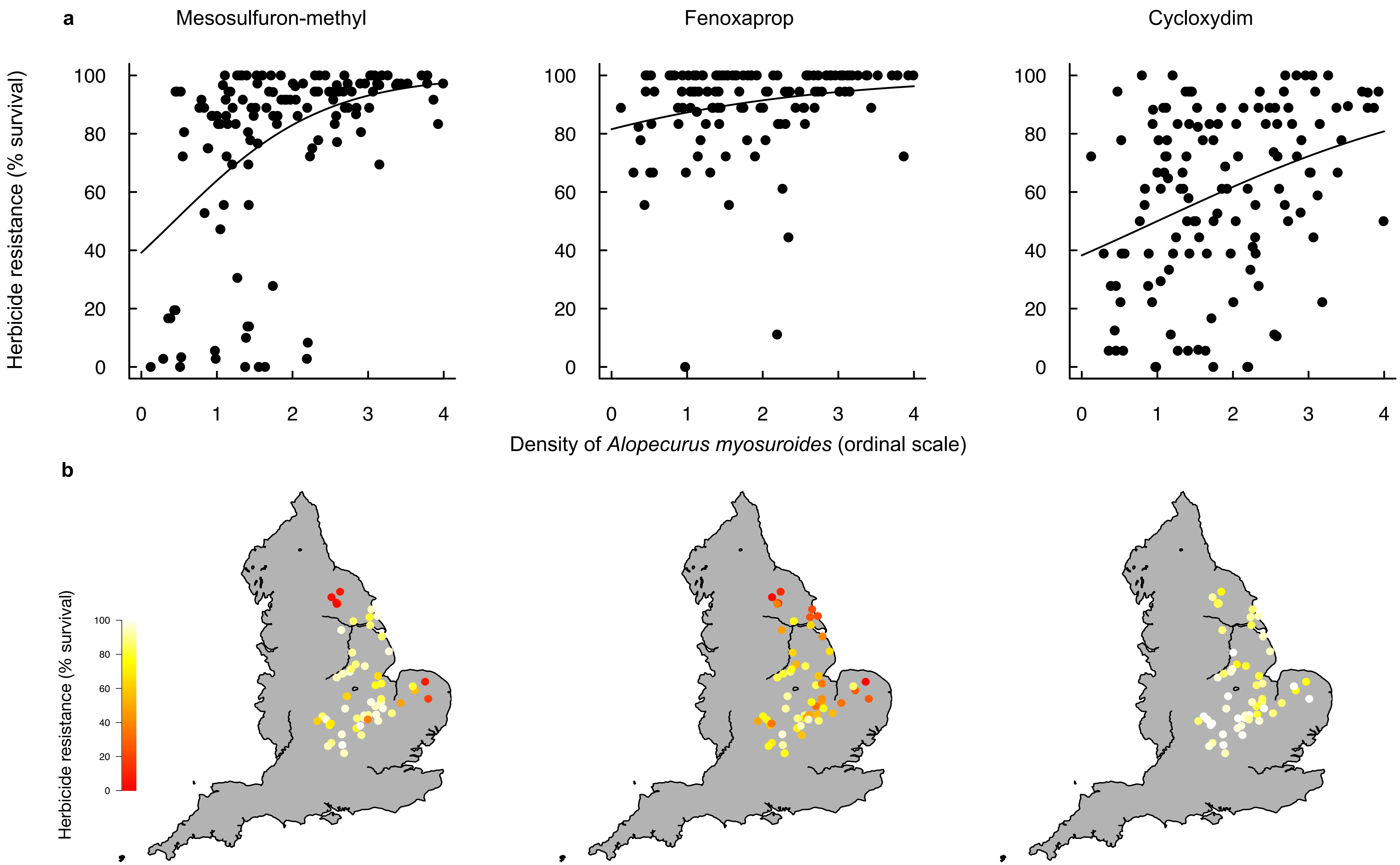
What impact does a black-grass infestation have on yield?

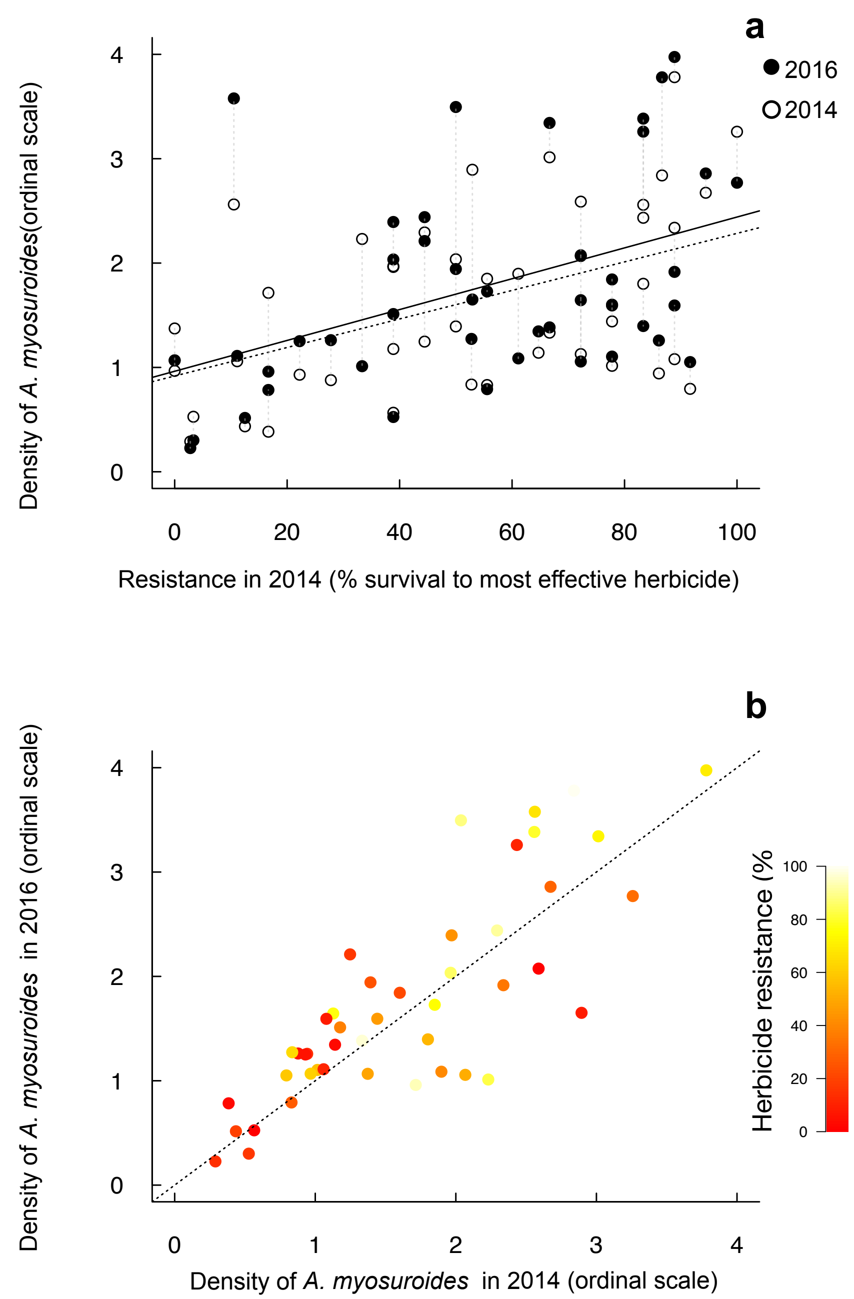
For ten fields where high resolution wheat yield data were available black-grass density data were overlaid onto yield maps (in ArcGIS 10.1). Mean yield (t/ha) was extracted for each 20x20m grid square in which black-grass density had been estimated. For each field, details of products applied for control of *A. myosuroides* were obtained within that crop year (product name, date applied, rate applied). Herbicide product prices were obtained from industry sources and prices per hectare were calculated for the application of each herbicide. We assume a wheat price of £115.10/t (source: Agriculture and Horticulture Development Board, Corn Returns). All costings were calculated at 2014 prices, in line with the time of data collection and weed surveys.

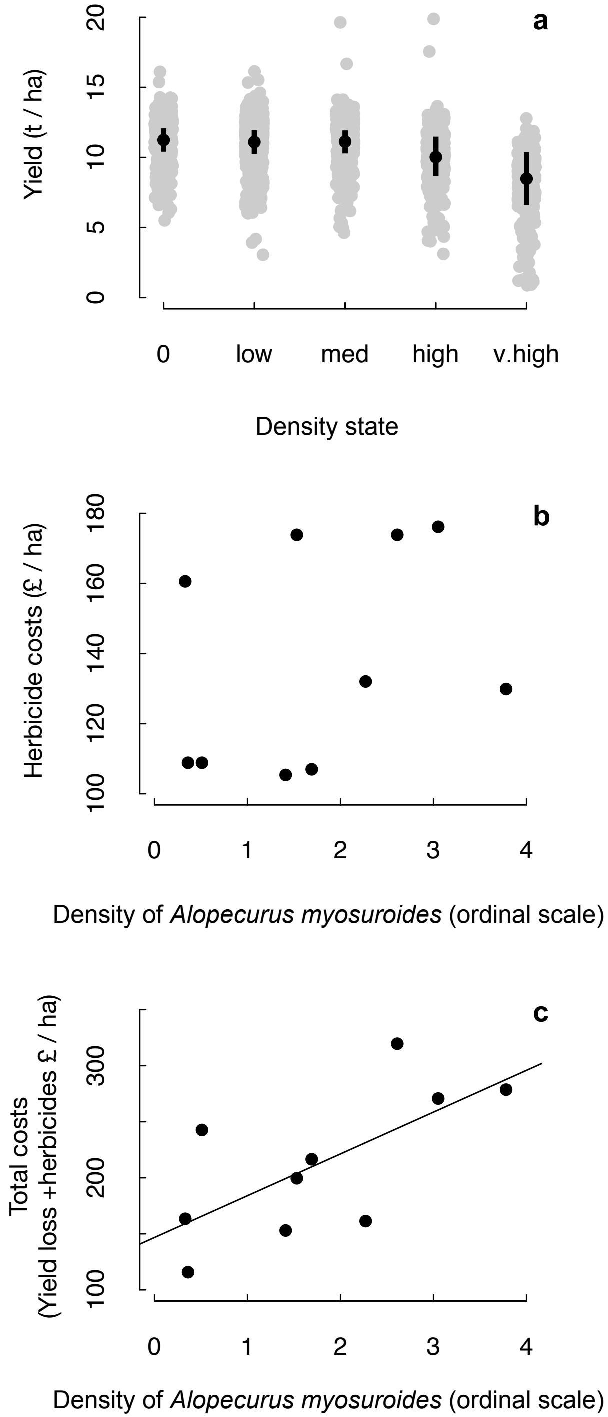
We used the linear model yield ~ density state + (density state | field) to predict yield at the 20m by 20m grid square level (fit using lmer() in the 'lme4' package) for the ten fields with high resolution yield data. Density state was treated as categorical to allow a non-linear effect of density on yield, and field was used as a random effect to control for differences between fields. Linear regressions were performed on field scale relationships between weed density and herbicide costs/ha, and weed density and total costs of *A. myosuroides* (herbicide costs + yield loss) for these same ten fields.


a
Weed density
(ordinal scale)
4
3
2
1
0
b
Mean weed density (ordinal scale)
2.0
1.5
1.0
0.5
0.0
51.5
52.0
52.5
53.0
53.5
54.0
54.5
Latitude
c
1960s
1990s
2016
d
Area treated (ha x1000)
2500
2000
1500
1000
500
0
Glyphosate
Mesosulfuron-methyl
Fenoxaprop
Cycloxydim
1990
1995
2000
2005
2010
2015
Year
e
Area treated (ha x1000)
30000
20000
10000
0
1990
1995
2000
2005
2010
2015
Year


Mesosulfuron-methyl
(ATL)
0%
7%
0%
80%
5%
8%
0%
Fenoxaprop
(FEN)
Cycloxydim
(CYC)

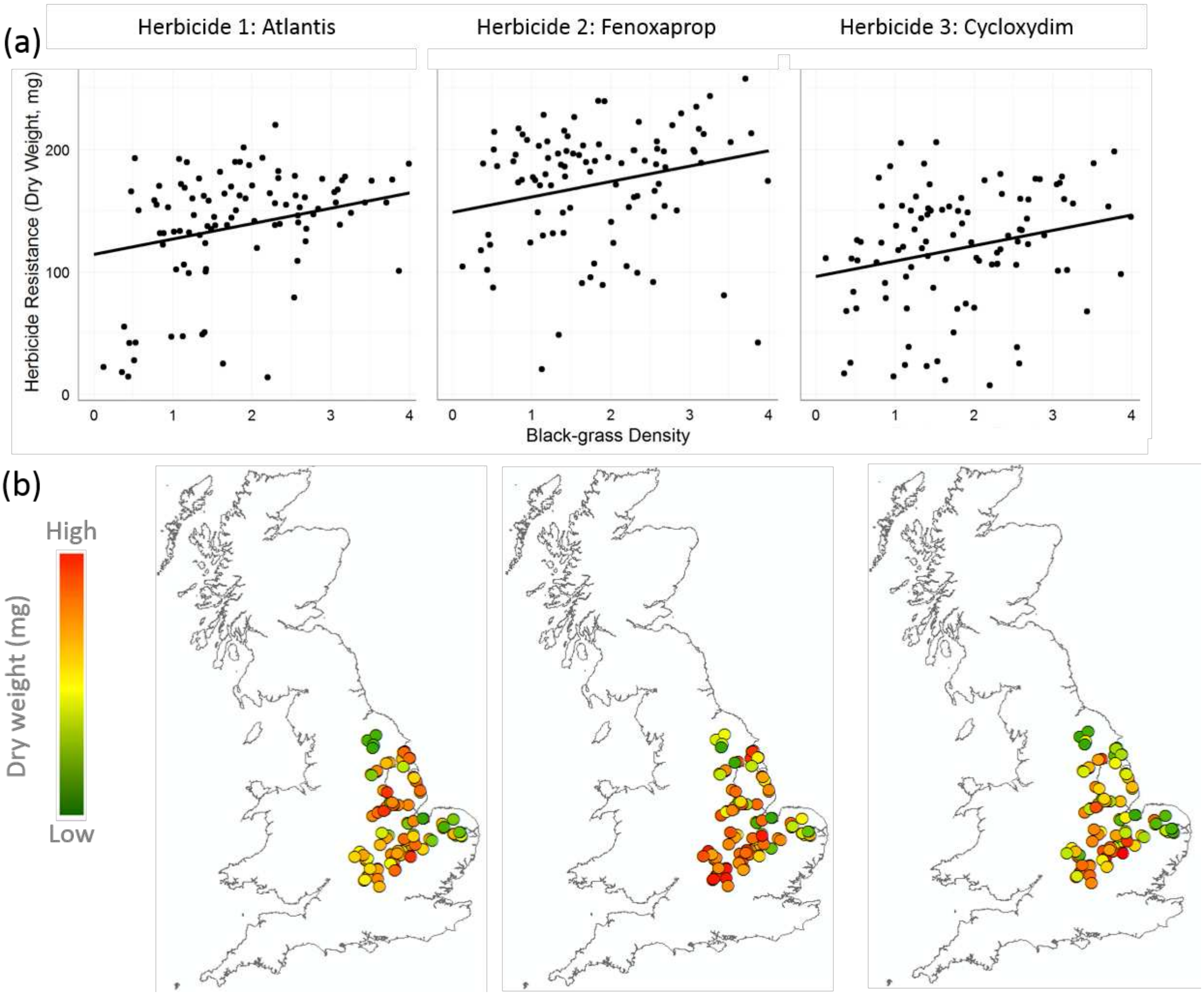

a
Mesosulfuron-methyl
Fenoxaprop
Cycloxydim
Herbicide resistance (% survival)
100
80
60
40
20
0
0
1
2
3
4
Density of *Alopecurus myosuroides* (ordinal scale)
b
Herbicide resistance (% survival)
100
80
60
40
20
0


a
2016
2014
Density of A. myosuroides(ordinal scale)
Resistance in 2014 (% survival to most effective herbicide)
0
1
2
3
4
0
20
40
60
80
100
b
Density of A. myosuroides in 2016 (ordinal scale)
Density of A. myosuroides in 2014 (ordinal scale)
Herbicide resistance (%
0
20
40
60
80
100


a
Yield (t / ha)
0
5
10
15
20
0
low
med
high
v.high
Density state
b
Herbicide costs (£ / ha)
100
120
140
160
180
0
1
2
3
4
Density of *Alopecurus myosuroides* (ordinal scale)
c
Total costs
(Yield loss +herbicides £ / ha)
100
200
300
0
1
2
3
4
Density of *Alopecurus myosuroides* (ordinal scale)

The factors driving evolved herbicide resistance at a national scale: Supplementary Information

Helen L. Hicks[1], David Comont[2], Shaun R. Coutts[1], Laura Crook[2], Richard Hull[2], Ken Norris[3], Paul Neve[2], Dylan Z. Childs[1], Robert P. Freckleton[1*]



Supplementary Figure 1 a) Relationship between mean black-grass density state and dry weight of plant material after treatment with each herbicide. Plotted lines represent predicted dry weight of weeds after treatment with herbicide for differing black-grass densities; models are mixed effect models with mean black-grass density state and herbicide as fixed effects and farm name as a random effect. **(b)** Heat maps showing dry weight of plant material (as a measure of herbicide resistance) after treatment with each of three herbicides. Red colours show larger amounts of plant material (i.e. low herbicide effectiveness), green colours show lower amounts of plant material (i.e. high herbicide effectiveness).

Supplementary Table 1 Outline of chemical and cultural control measures for managing resistance. Both herbicide intensity and herbicide diversity were higher in more recent years, while cultivation intensity decreased alongside the proportion of years in which a field was ploughed. Nineteen percent of fields were not ploughed at all in the period of study, seven percent were ploughed every year Two fields had been in continuous winter wheat for at least 10 years prior to the survey, the remainder had a rotation of crops (an average of 4 crops in a rotation, up to a maximum of eight crops). A third of fields had been in continuous autumn crops for the 10 years prior to the survey, but all remaining fields had some variation in autumn and spring cropping. Seven fields had been in cereals for the 10 years preceding the study.

Mechanisms to reduce resistance	**Management measures**	**Farm management Predictor variable(s) included in models**	**Prediction**	**Summary statistics**
Chemical	Temporal cycling (treatments vary over time and not space)	**Herbicide diversity**: # MOAs applied in single harvest year **Herbicide intensity**: # herbicide application days in a single harvest year	Negative correlation with resistance	**Herbicide diversity** 2004 – 2009: Range = 1.0 – 6.3; mean = 3.4 2010 – 2014: Range = 1.4 – 6.2; mean = 4.3 **Herbicide intensity** 2004 – 2009: Range = 1.0 - 4.6; mean = 2.6 2010 – 2014: Range = 1.2 – 6; mean = 3.3
	Mosaics (treatments vary spatially, but not temporally)	Not assessed	NA	NA
	Combination (treatments vary over time and space; multiple MOAs applied at once)	See temporal cycling	Negative correlation with resistance	See temporal cycling
Cultural	Tillage	• Cultivation intensity score • Plough frequency	Studies show results to be variable depending on combination of frequency and depth of cultivation	**Cultivations** *Mean cultivation intensity scores:* 2004 – 2009: Range = 0.5 - 4; mean = 2.95 2010 – 2014: Range = 0 - 4; mean = 2.82 *Proportion of years field ploughed:* 2004 – 2009: Range = 0 - 1; mean = 0.44 2010 – 2014: Range = 0 - 1; mean = 0.32
	Crop type	PCA axis based on proportion of years field in winter wheat/ autumn sown cereal / cereal crop	Negative correlation with resistance	**Crop Type** *Proportion years in autumn crop:* 2004 – 2009: Range = 0.17 - 1; mean = 0.86 2010 – 2014: Range = 0.4 - 1; mean = 0.89 *Proportion years in cereal crop:* 2004 – 2009: Range = 0.17 - 1; mean = 0.63 2010 – 2014: Range = 0.4 - 1; mean = 0.70 *Proportion years in winter wheat:* 2004 – 2009: Range = 0 - 1; mean = 0.56 2010 – 2014: Range = 0.2 - 1; mean = 0.62

Supplementary Table 2 Additional models of herbicide resistance using data for only one herbicide: Atlantis. Generalized linear mixed effects models (GLMM) were used to determine the effect of farm management histories on two measures of herbicide resistance (survival and dry weight) across two timeframes (old: 2004-2009 and recent: 2010 – 2014). Mean black-grass density state, herbicide, soil type and herbicide parameters (mean number of herbicide application days per harvest year (herbicide intensity), mean number of herbicide MOAs applied within a harvest year (herbicide diversity)) were fitted as fixed effects in the models, and farm name was fitted as a random effect to describe the structure of the data. Observation-level random effects were used to account for over dispersion in the models. Here we present only the final models (black font) with significant predictor terms. A set of secondary analyses (grey font) investigated the additional effect of crop type (derived from the proportion of years the field was in winter wheat/ an autumn sown crop/ a cereal crop), the proportion of years the field was ploughed and a mean cultivation intensity score. R-square values were calculated using MuMIN [39] and parametric bootstrap using Kenward Roger methods [40] (using the 'pbkrtest' package in R) were used for model comparison and calculation of p-values.

OLD

SURVIVAL			Model fit	
Model structure	Effect size (Sum Sq)	P value	R2 GLMM (m)	R2 GLMM (c)
Black-grass Density	30.42	0.001	0.134	0.164
Soil type	9.41	0.01		
Herbicide intensity	16.80	0.001		
+ Crop type (PCA axis 1)	1.92	0.175		
+ Plough frequency	0.10	0.761		
+ Cultivation score	0.01	0.946		

DRY WEIGHT			Model fit	
Model structure	Effect size (Sum Sq)	P value	R2 GLMM (m)	R2 GLMM (c)
Black-grass Density	9.22	0.003	0.126	0.320
Soil type	5.05	0.017		
Herbicide intensity	3.34	0.030		
+ Crop type (PCA axis 1)	0.19	0.624		
+ Plough frequency	0.20	0.593		
+ Cultivation score	1.38	0.163		

RECENT

SURVIVAL			Model fit	
Model structure	Effect size (Sum Sq)	P value	R2 GLMM (m)	R2 GLMM (c)
Black-grass Density	29.60	0.001	0.128	0.161
Soil type	9.16	0.01		
Herbicide intensity	13.30	0.002		
+ Crop type (PCA axis 1)	1.10	0.371		
+ Plough frequency	1.76	0.239		
+ Cultivation score	1.06	0.379		

DRY WEIGHT			Model fit	
Model structure	Effect size (Sum Sq)	P value	R2 GLMM (m)	R2 GLMM (c)
Black-grass Density	9.08	0.003	0.096	0.303
Soil type	4.89	0.017		
+ Crop type (PCA axis 1)	0.46	0.445		
+ Plough frequency	0.24	0.530		
+ Cultivation score	0.001	1.000		

Supplementary Table 3 Model relating weed density to herbicide usage. Weed density was the response variable, and farm entered as a random effect. The number of herbicide applications was used as the predictor, separately for recent and old periods. The significance of these was assessed both using Kenward-Rogers and parametric bootstrap methods: these yielded identical results.

SURVIVAL

Model structure	Effect size (Sum Sq)	P value
Herbicide intensity – recent (2010-2014)	1.92	0.065
Herbicide intensity – old (2004-2009)	0.30	0.475

Supplementary Table 4 Herbicide resistance differs between soil types. Numbers represent mean values ± standard error for populations originating from clay and non-clay soils, for each of the herbicides tested. Note also that there is a significant difference in dry weight between populations from different soil types when zero herbicide has been applied (i.e. control plants from all three experiments).

	Herbicide	Clay	Non-clay	Anova
Survival	ATL	84.97±3.63	72.93±4.61	(F(94,1) = 4.250, p = 0.042)*
	CYC	69.35±3.7	52.81±4.32	(F(94,1) = 8.512, p = 0.004)**
	FEN	93.38±1.41	90.66±1.61	(F(94,1) = 1.608, p = 0.208)
	Herbicide	Clay	Non-clay	
Dry Weight	ATL	146.45±6.01	127.62±7.62	(F(94,1) = 3.801, p = 0.054)
	CYC	137.16±6.18	100.33±6.83	(F(94,1) = 16.04, p < 0.001)***
	FEN	184.44±6.18	158.09±7.14	(F(94,1) = 7.826, p = 0.006)*
	No Herbicide	190.17±3.19	176.63±3.49	(F(283,1) = 8.208, p=0.004)**

Supplementary Table 5 Yield loss resulting from black-grass infestations assuming a wheat price of £115.10/ t (source: Agriculture and Horticulture Development Board, Corn Returns)

	Percentage of field in black-grass density state					Mean ww yield within patches of density state (t/ha)						Economic costs		
Field	absent	low	med	high	very high	absent	low	med	high	very high	Total Yield Loss (%)	Cost of yield loss/ ha (£)	Cost of BG herbicides/ ha (£)	Total cost of BG/ ha (£)
A	69.7	27.9	2.4	0.0	0.0	12.1	12.0	12.0	-	-	0.2	25.62	173.89	199.51
B	64.4	35.6	0.0	0.0	0.0	11.3	11.2	-	-	-	0.5	145.65	173.89	319.55
C	0.0	65.3	22.4	6.6	5.6	-	12.3	11.8	11.9	10.7	1.8	29.26	132.03	161.29
D	0.0	20.4	40.7	30.5	8.4	-	9.7	9.8	9.4	8.3	2.6	148.69	129.88	278.56
E	3.2	57.2	34.8	4.8	0.0	11.6	11.3	11.9	11.1	-	3.5	47.55	105.35	152.90
F	0.0	2.1	18.3	52.1	27.5	-	10.2	10.4	9.8	8.5	7.9	94.46	176.18	270.64
G	0.7	60.7	19.7	6.9	12.1	11.1	11.9	11.9	8.1	6.4	8.0	109.51	106.96	216.47
H	50.0	48.8	1.2	0.0	0.0	10.6	10.6	11.8	-	-	9.8	2.72	160.62	163.33
I	0.0	11.7	32.0	39.8	16.5	-	12.5	11.5	11.2	10.0	10.1	133.78	108.83	242.61
J	0.0	0.0	4.2	13.4	82.4	-	-	10.1	9.1	8.7	12.8	6.89	108.83	115.71

Supplemental Experimental Procedures

Rapid Assessment of *Alopecurus myosuroides* range

In addition to the detailed 20x20m grid field surveys undertaken in 2014, we undertook rapid assessment exercise in 2015 and 2016 to give overall field scale density estimates for a large number of cereal fields across a more widespread geographic area than the detailed density surveys. The location, crop and an estimate of field-scale black-grass density were recorded from the side of each field.

Seed collection

Each field was divided into ten linear sections based around the field tram-lines. A single position along each section was chosen at random, and the stand of black-grass nearest to this point was sampled for seeds. At each point, twenty handfuls of black-grass heads were gently shaken into a polythene bag allowing only mature seeds to be collected. The twenty handfuls of heads were gathered over an approximate 5-10 metre area around the sample point, ensuring that multiple black-grass plants were sampled. This design avoids the potential for preferentially sampling only high abundance patches of black-grass, whilst ensuring that samples were collected from a large number of black-grass individuals across the spatial extent of black-grass within each field.

Seeds were air-dried at room temperature for two weeks, before being cleaned using an air-column seed cleaner to remove unfilled seeds and chaff. The ten cleaned and dried seed samples per field were weighed and combined into a single seed bulk per field. These field scale seed bulks were used to represent each field population of black-grass throughout the subsequent resistance testing.

Resistance testing

Dried seeds were maintained in an incubator in the dark at 30°C for three weeks to break any remaining seed dormancy before experimentation. Seeds were geminated in petri-dishes on Whatman No. 1 filter papers soaked in 20 mmol L^{-1} KNO_3, and incubated for seven days at 17/11°C over a 14/10 hour day/night cycle. Germinated seedlings were transplanted into 3.5 inch pots containing a loam soil pre-mixed with 2 kg m^{-2} osmocote fertiliser. Six pots were sown for each field population of black-grass, with six seedlings sown per pot in an equally spaced ring. Pots were assigned to either control or herbicide treatments (n=3), and arranged over three glasshouse compartments in a randomised block design. Glasshouses were set to 18/12 °C day/night temperatures over 14/10 hours, with supplementary lighting provided by sodium lamps whenever ambient daytime PAR was low.

This experiment was repeated three times over autumn 2014 - spring 2015 to test three herbicides; the ALS inhibitor 'Atlantis' (Mesosulfuron + Iodosulfuron), the 'fop' ACCase inhibitor 'Cheetah' (Fenoxaprop), and the 'dim' ACCase inhibitor 'Laser' (Cycloxydim).

Cultivation intensity scores

Cultivation type was recorded and classified as one of the following: direct drill (i.e. no cultivation), minimum tillage (including drag and scuffle), light cultivation (including discs and tines), deep cultivation (including subsoiling) or plough (inversion tillage and ploughing). These were converted to a numerical scale according to cultivation intensity (where direct drilling = 0, minimum tillage = 1, light cultivation = 2, deep cultivation = 3, ploughing =4) to allow calculation of a mean cultivation intensity scores (on a scale of 0 – 4).