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1 **The factors driving evolved herbicide resistance at a**
2 **national scale**

3

4 Helen L. Hicks¹, David Comont², Shaun R. Coutts¹, Laura Crook², Richard Hull², Ken
5 Norris³, Paul Neve², Dylan Z. Childs¹, Robert P. Freckleton^{1*}

6

7 Affiliations:

8 1: Department of Animal and Plant Sciences, University of Sheffield, Western Bank,
9 Sheffield, S10 2TN, UK.

10 2: Rothamsted Research, West Common, Harpenden, AL5 2JQ, UK.

11 3: Institute of Zoology, Zoological Society of London, Regent's Park, London, NW1 4RY,
12 UK.

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23 *Corresponding Author: r.freckleton@sheffield.ac.uk

24 **Abstract**

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

40 **Introduction**

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

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

54 The primary approach to minimizing the rate of evolution of resistance is through using
55 multiple xenobiotics with contrasting modes of action (MOAs: families of chemicals that target
56 cellular machinery or metabolic processes in different ways). Four principal strategies exist for
57 combining two or more chemical MOAs over space and time, with the objective of delaying the
58 evolution of resistance to pesticides and drugs [9]: *Periodic application* and *Responsive*
59 *alternation* (collectively referred to as ‘temporal cycling’) where treatments vary over time, but
60 not space; *Mosaic* where treatments vary spatially but not temporally; and *Combination* where
61 treatments vary over both space and time (with multiple MOAs administered at once). In
62 medicine, drug *combination* therapies have slowed the evolution of resistance in HIV [10] and are
63 recommended for treating tuberculosis [11] and malaria [12]. In agriculture both the scientific
64 literature and industry advice suggest managing the evolution of resistance with *temporal cycling*

65 and/or *combination* of different MOAs [8, 13-18]. The rate of evolution for herbicide resistance
66 should be slowed more effectively by *combination* (simultaneous use of multiple MOAs) than by
67 *responsive alternation* (annual rotation) of MOAs [13, 14, 16, 17], however this has yet to be
68 tested at large scales and under the usual scenario where resistance has already evolved to some
69 MOAs. Notwithstanding in broad terms current management is founded on the theoretical
70 prediction that increasing the diversity of chemicals used can reduce the rate of evolution of
71 resistance.

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

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

86 In agriculture, resistance management is embedded within Integrated Management (IM),
87 where pests are controlled by varying crops and management practices, including options beyond
88 chemical control [20]. Significantly, mortality from non-chemical control is unaffected by the
89 extent of evolved resistance and should not select for increased xenobiotic resistance. By reducing

90 population sizes independently of chemical control, IM is argued to be effective at both delivering
91 pest control as well as reducing the rate of evolution to xenobiotics [21]. However, it is generally
92 unclear how effective such strategies are, as well as the extent to which managers proactively use
93 these methods.

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

109

110 **Results and Discussion**

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

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

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

124

125 **Is resistance driving high weed densities?** Herbicide resistance was first reported in the 1950's
126 [19] and, as of March 2017, is confirmed in 252 weed species globally, covering a broad range of
127 herbicides [23]. Resistance is widespread in populations of *A. myosuroides* in the UK. The three
128 herbicides tested caused <40% mortality (very high resistance) in 96% (FEN), 82% (ATL) and
129 57% (CYC) of the 138 blackgrass populations, when applied at recommended field rates (see
130 Experimental Procedures for details). Most populations were resistant to multiple herbicides
131 (Figure 2): 79% of populations had high levels of resistance (defined as <80% mortality after
132 exposure) to all three herbicides. This suggests two possibilities: firstly, that target-site resistance
133 combined with extensive gene flow has led to the evolution of resistance to all three MOAs
134 independently, or alternatively, evolution of resistance to one MOA confers cross resistance to the
135 other MOAs (i.e. one that the plant is yet to meet), potentially through metabolic mechanisms.

136 Our data indicate that resistance appears to be a key factor driving the abundance of *A.*
137 *myosuroides*: we find a positive relationship between blackgrass density and herbicide resistance
138 across all three herbicides tested (Figure 3a). The fraction of plants surviving herbicide treatment
139 increased with blackgrass density in the source population, but the relationship differs between

140 herbicides ($\chi^2 (3) = 128.13$, $p < 0.001$. Corrected $R^2 = 0.34$; Figures 3a/b). The dry weight of
141 blackgrass (per plant) after treatment with herbicides also increases with blackgrass density, and
142 the relationship between weed density and biomass differs between herbicides ($\chi^2 (3) = 98.154$,
143 $p < 0.001$. Corrected $R^2 = 0.52$; SI: Figure S1).

144 To further explore the relationship between herbicide resistance and black grass density we
145 analysed the relationship between resistance and densities in successive winter wheat crops. The
146 significant relationship between herbicide resistance and density can be seen in Figure 4a, where
147 fields with higher levels of resistance tended to have a higher mean density state in 2014 ($F_{1,43} =$
148 12.9 , $P = 0.0009$) and 2016 ($F_{1,43} = 11.1$, $P = 0.0017$). As shown in Figure 4b, the relationship
149 between resistance and density drives weed levels in the subsequent crops: there is a close
150 relationship between densities in successive crops, correlated with resistance. Although there is
151 slight evidence for increases in density between 2014 and 2016 (30 out of 45 populations
152 increased in density, sign test $P = 0.036$) the closeness of the relationship between densities in
153 2014 and 2016 ($r = 0.81$, $F_{1,43} = 83.1$, $P < 0.0001$) emphasizes the importance of previous density
154 and, hence historical resistance, in generating long-term infestations.

155

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

165 or reduce rates of population increase through mechanisms unconnected with susceptibility or
166 resistance to xenobiotics.

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

178 Herbicide diversity (mean number of MOA applied within a crop year) is correlated with
179 herbicide intensity (mean number of herbicide application dates within a crop year) (2004 – 2009:
180 $\rho = 0.874$; 2010 – 2014: $\rho = 0.827$). To assess the effect of this correlation we fit models with
181 either herbicide diversity or herbicide intensity. Although there was a relationship between
182 herbicide diversity and resistance, when compared in the same model herbicide diversity was
183 always a *weaker* predictor of resistance than herbicide intensity, and so was not retained in any of
184 the final models. The intensity of herbicide applications (number of applications within a growing
185 season), irrespective of the type of herbicide, is thus the most important management variable
186 correlated with the evolution of resistance.

187 We considered the directionality of the relationship between herbicide usage and
188 resistance. One possibility is that the relationship between volume of herbicide applied and
189 resistance could reflect recent increases in herbicide use in response to high weed densities

190 resulting from resistance. Crucially three findings render this interpretation unlikely. First, as
191 shown in Table 1, the relationships are robust whether we consider management in the past (2004-
192 2009) or recently (2010-2014). Second, these relationships remained when we analysed data on
193 resistance to the most recently introduced product to the market, Atlantis, separately
194 (Supplemental Information: Table S2). Atlantis was only introduced in 2005, however the
195 correlates of resistance remain the same. Thirdly, we found no relationship between weed density
196 and volume of herbicide used either recently (2010-2014) or in the past (2004-2009) indicating
197 that weed density is not a driver of herbicide usage, notwithstanding the correlation of both
198 volume of herbicide and weed density with resistance (See Supplemental Information: Table S3).
199 Taken both individually and together these three results do not support the interpretation that
200 resistance is driving herbicide usage rather than vice versa.

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

207 A recent study in Germany found no relationship between number of MOA used and
208 resistance status of *A. myosuroides* [30]. Alongside our finding that the intensity of herbicide
209 application was a stronger predictor, we found the widespread occurrence of resistance to multiple
210 herbicides in our dataset (Figure 2). This suggests a significant role for multiple herbicide
211 resistance driven by metabolic mechanisms. Multiple herbicide resistance driven by metabolic
212 mechanisms is a significant threat to the sustainability of chemical management because evolution
213 or resistance under selection by one herbicide can lead to resistance to others, including those that

214 populations have not yet been exposed to. Thus, future options for management are constrained if
215 multiple herbicide resistance is widespread.

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

224 Despite widely repeated recommendations that diversity of crop rotation, changes in
225 cultivation and ploughing regimes should be adopted to reduce *A. myosuroides* infestations [32,
226 33], our results fail to detect an effect of cultivation intensity, frequency of ploughing or crop type
227 (PCA axis 1: combining frequency of winter wheat, cereal and autumn sown cropping) on the
228 evolution of herbicide resistance (Table 1). Thus, although such techniques are expected to have
229 demonstrable impacts on population sizes [33], at least in the medium-term, impacts on resistance
230 are undetectable in our dataset.

231

232 **Measuring the impacts of evolved resistance and its management.** Since its widespread
233 emergence, herbicide resistance has become a major threat to global food security [34]. Herbicide
234 resistant weeds are one of the biggest threats to crop yields. Weeds cause average yield losses of
235 35%, worldwide [35], this figure could be much higher without effective herbicides [10]. Yield
236 losses incurred by *A. myosuroides* infestations are thought to make it the most economically
237 important weed in Western Europe [32]; our dataset offers a unique resource to estimate these
238 costs from field to regional scales.

239 At the field scale, our data show total yield losses to range from 0.2% to 12.8% and overall
240 yield decreased significantly with increased weed density ($F_{1,8}=5.643$, $p=0.045$). Within fields, *A.*
241 *myosuroides* only begins to impact wheat yields when it occurs at high densities (Figure 5a).
242 Herbicide treatments targeted at control of *A. myosuroides* cost between £105/ha to £176/ha, but
243 there is no relationship between costs of herbicides applied/ha and weed density
244 ($F_{1,8}=1.061$, $p=0.33$) (Figure 5b). This suggests that farmers do not vary their management
245 approaches with respect to weed density. Combined costs (herbicides + yield loss) ranged from
246 £115/ha to £320/ha, accounting for profit losses of between 4% and 12% (see SI: Table S5). Total
247 cost of *A. myosuroides* (herbicide costs/ha + yield loss) increased significantly with weed density
248 ($F_{1,8}=6.631$, $p=0.033$) (Figure 5c), where an increase in average *A. myosuroides* density, at the
249 field level, to the next density state results in a 2.5% loss in profit. The distribution of *A.*
250 *myosuroides* within a field tends to be clumped, and so average densities were often increased by a
251 larger area of a field developing high density infestations, and yield losses in those areas could be
252 very high (Fig 5). Increasing blackgrass density state explained 34% of the reduction in yield and
253 39% of the increase in total management cost.

254

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

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

273 There is a belief that new compounds will continue to become available in the future [38,
274 39], and so there is no need to change the way we use these valuable chemical tools. The lessons
275 learned from case studies such as this are vital to ensure that the value of any new product is
276 maximized. With resistance evolving over short timescales [4, 5] it is inevitable that any new
277 products will become ineffective if application strategies do not change. A major imminent threat
278 to food production is the growing reliance on glyphosate as a weed management tool (Figure
279 1d/e). Resistance to glyphosate is already present in eight different countries [40]. How long until
280 resistance to glyphosate becomes near universal is uncertain, but in evolutionary terms it is
281 inevitable unless standard management practices change.

282

283 **Author contributions**

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

287

288 **Competing financial interests**

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

291

292 **Data availability statement**

293 Data that support the findings of this study have been deposited in the University of Sheffield
294 Online Research data archive (ORDA) and can be accessed from the following URL:

295 <https://figshare.com/s/eb21f4d1862741d50ceb>.

296

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301

302

303

304 **Figure legends**

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

318
319 **Figure 2** Percentage of fields tested for resistance to three herbicides, where resistance has been
320 confirmed and is highly likely to reduce herbicide effectiveness. 79% of fields were resistant to all
321 three herbicides; 1% of fields were not resistant to any of the herbicides tested. Resistance refers to
322 <80% mortality when herbicide applied at recommended field rate – see Experimental Procedures
323 for details.

324
325 **Figure 3 a**, Relationship between mean blackgrass density state measured on ordinal scale from 0
326 (absent) to 4 (very high) and percentage survival of plants after treatment with each herbicide.
327 Plotted lines represent predicted survival of weeds after treatment with herbicide for differing
328 blackgrass densities; models are mixed effect models with mean blackgrass density state and

329 herbicide as fixed effects and farm name as a random effect. **b**, Heat maps showing percentage
330 survival of plants (as a measure of herbicide resistance) to each of three herbicides. Red colours
331 show high survival rates (i.e. low herbicide effectiveness), green colours show low survival rates
332 (i.e. high herbicide effectiveness).

333

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

339

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

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461 **Table 1** Final models of herbicide resistance. Generalized linear mixed effects models (GLMM)
 462 were used to determine the effect of farm management histories on two measures of herbicide
 463 resistance (survival and dry weight) across two timeframes (old: 2004-2009 and recent: 2010 –
 464 2014). Mean black-grass density state, herbicide, soil type and herbicide parameters (mean number
 465 of herbicide application days per harvest year (herbicide intensity), mean number of herbicide
 466 MOAs applied within a harvest year (herbicide diversity)) were fitted as fixed effects in the models,
 467 and farm name was fitted as a random effect to describe the structure of the data. Observation-level
 468 random effects were used to account for over dispersion in the models. Here we present only the
 469 final models with significant predictor terms. A set of secondary analyses investigated the additional
 470 effect of crop type (derived from the proportion of years the field was in winter wheat/ an autumn
 471 sown crop/ a cereal crop), the proportion of years the field was ploughed and a mean cultivation
 472 intensity score. R-square values were calculated using MuMIN [39] and parametric bootstrap using
 473 Kenward Roger methods [40] (using the ‘pbkrtest’ package in R) were used for model comparison
 474 and calculation of p-values.
 475

OLD					RECENT				
SURVIVAL			Model fit		SURVIVAL			Model fit	
Model structure	Effect size (Sum Sq)	P value	R2 GLMM (m)	R2 GLMM (c)	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	Black-grass Density	23.380	0.001	0.275	0.351
Herbicide	126.364	0.001			Herbicide	124.661	0.001		
Soil type	9.907	0.006			Soil type	9.634	0.006		
Herbicide intensity	17.099	0.002			Herbicide intensity	13.188	0.003		
+ Crop type (PCA axis 1)	2.244	0.168			+ Crop type (PCA axis 1)	0.757	0.447		
+ Plough frequency	0.149	0.718			+ Plough frequency	1.168	0.357		
+ Cultivation score	0.100	0.808			+ Cultivation score	0.736	0.465		

OLD					RECENT				
DRY WEIGHT			Model fit		DRY WEIGHT			Model fit	
Model structure	Effect size (Sum Sq)	P value	R2 GLMM (m)	R2 GLMM (c)	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	Black-grass Density	7.192	0.001	0.258	0.508
Herbicide	49.117	0.001			Herbicide	49.117	0.001		
Soil type	2.992	0.023			Soil type	2.923	0.023		
Herbicide intensity	2.863	0.013							
+ Crop type (PCA axis 1)	0.221	0.513			+ Crop type (PCA axis 1)	0.433	0.394		
+ Plough frequency	0.127	0.622			+ Plough frequency	0.087	0.647		
+ Cultivation score	1.197	0.100			+ Cultivation score	0.003	1.000		

476
477

478 **Methods**

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

484

485 ***Weed population surveys***

486 138 Fields with black-grass present were censused in a six week period from 1st of July 2014.
487 Fields were divided into contiguous 20 x 20m grid squares and weed density was estimated in
488 each grid square. The surveys followed a density-structured approach, recording density state of
489 black-grass rather than numerical abundance. Each grid square was assigned to one of 5 density
490 states that correspond to the number of plants per 20x20m square; 0 (absent), 1 (1-160 plants), 2
491 (160-450 plants), 3 (450-1450 plants) and state 4 (1450+ plants). These density states have been
492 shown to accurately capture the variation within field populations and the 20 x 20m grid size
493 sufficient to be representative of 1m² subplots where blackgrass plants were physically counted
494 [45]. Areas within fields that were sprayed off or cut early were classified as state 4, to reflect
495 management for very high levels of black-grass infestation.

496

497 ***Resistance testing***

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

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

505 Black-grass seeds were collected from ten different locations within each field surveyed in
506 2014, using a semi-random seed collection strategy (See Supplemental Experimental Procedures:
507 *Seed Collection* for further details). *A. myosuroides* seedlings were germinated and allowed to
508 grow for 18-21 days until reaching the three leaf stage before spraying with herbicide. We tested
509 for resistance to three herbicides at the following rates: 'Atlantis' (Mesosulfuron + Iodosulfuron at
510 300 g ha⁻¹), 'Cheetah' (Fenoxaprop at 1.25 L ha⁻¹), and 'Laser' (Cycloxydim at 0.75 L ha⁻¹). These
511 application rates were chosen as previous experimentation has shown them to provide the best
512 approximation of field rate doses under glasshouse conditions and were applied with a track
513 sprayer. Plants remained in the glasshouse for three weeks following herbicide treatment, at which
514 point plant mortality was recorded before harvesting aboveground biomass from each pot. Plant
515 material was dried at 80°C for 48 hours before weighing (See Supplemental Experimental
516 Procedures: *Resistance Testing* for more details).

517

518 ***Field Management Data***

519 Historical field management data was requested for each of the 138 fields that we surveyed for
520 weed density. Data were available for 96 fields and up to 10 years data were collated for each
521 field. For each year we recorded the following: crop, first cultivation type and herbicide
522 applications (product name and date of application). From this we derived herbicide intensity
523 (average number of herbicide application days per year) and herbicide diversity (average number
524 of modes of action applied per year). We also derived cropping patterns (e.g. autumn or spring
525 sown, cereal or non-cereal). Cultivation types were recorded and scored on a scale of intensity
526 from 0-4 (where direct drilling = 0, to ploughing = 4) (See Supplemental Experimental
527 Procedures: *Cultivation Intensity Scores* for more detail). Soil type for each field was extracted

528 from the National Soil Resources Institute, NATMAP1000 database and classified into two groups
529 (clays, non-clays) after [46, 47]. Where available, yield maps were obtained for fields that we
530 surveyed to enable direct comparison of within field black-grass density and crop yield. See
531 Supplemental Information: Table S1 for outlines of chemical/ cultural control techniques and
532 corresponding model input variables.

533

534 **Statistical analyses**

535 ***Does resistance drive weed abundance and the role of diversity of management in the evolution*** 536 ***of resistance?***

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

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

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

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

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

569

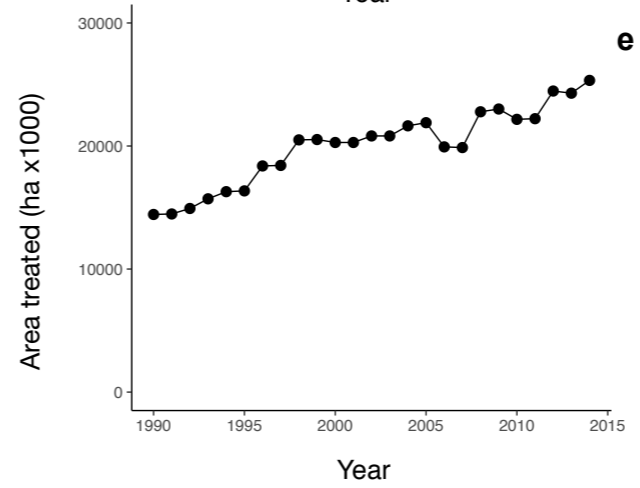
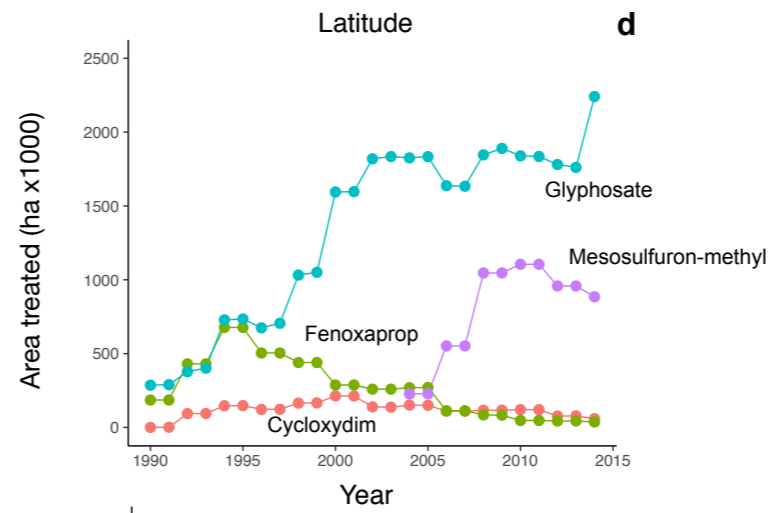
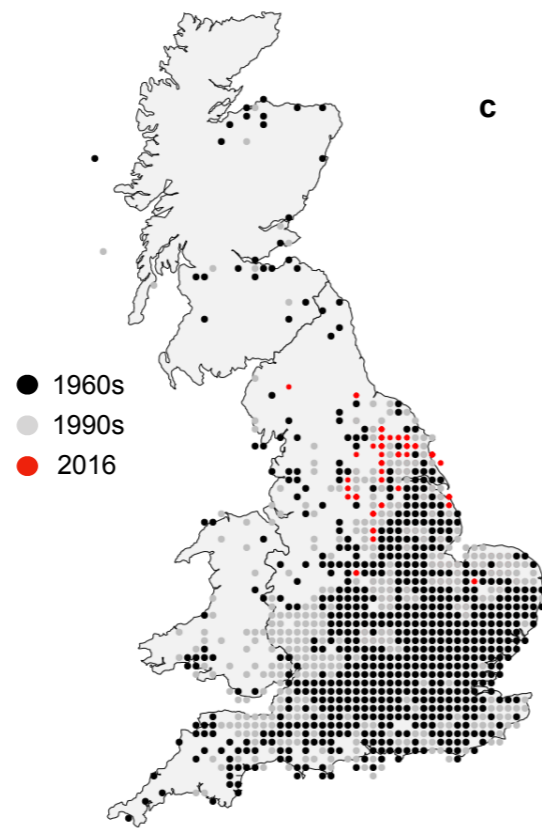
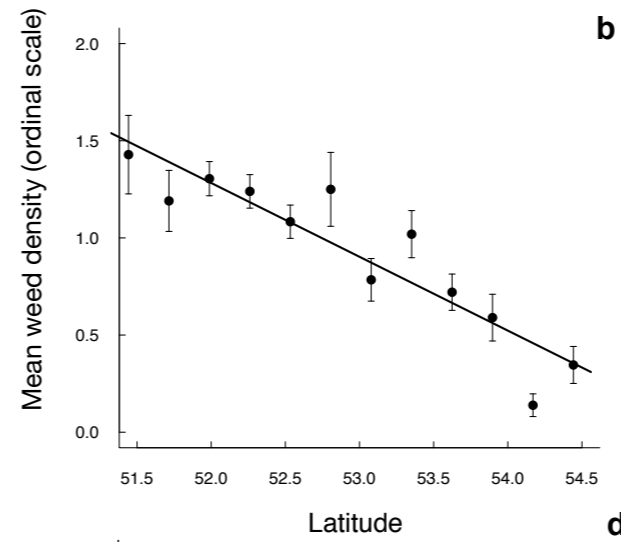
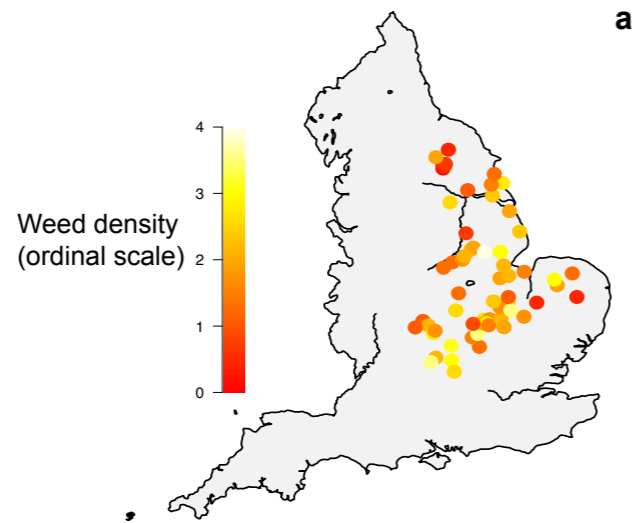
570 ***What impact does a black-grass infestation have on yield?***

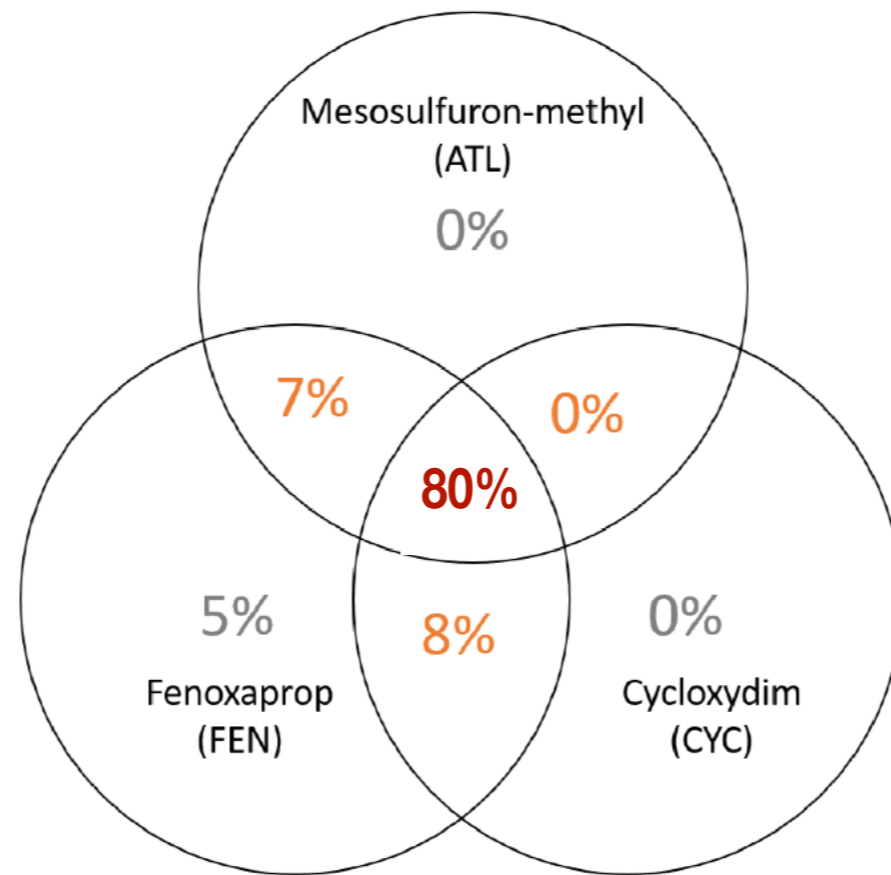
571 For ten fields where high resolution wheat yield data were available black-grass density data were
572 overlaid onto yield maps (in ArcGIS 10.1). Mean yield (t/ha) was extracted for each 20x20m grid
573 square in which black-grass density had been estimated. For each field, details of products applied
574 for control of *A. myosuroides* were obtained within that crop year (product name, date applied, rate
575 applied). Herbicide product prices were obtained from industry sources and prices per hectare
576 were calculated for the application of each herbicide. We assume a wheat price of £115.10/t

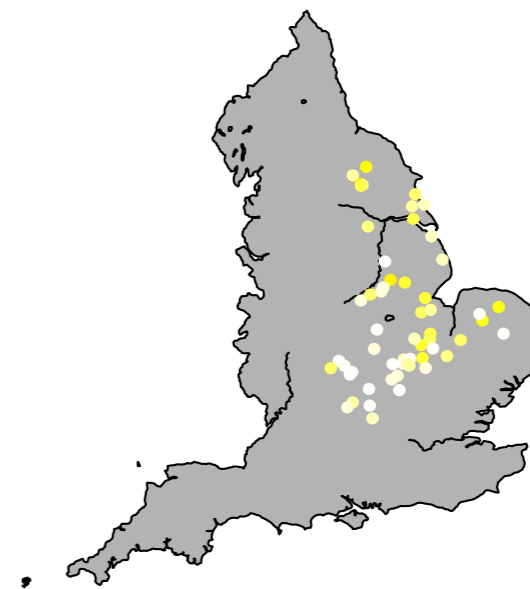
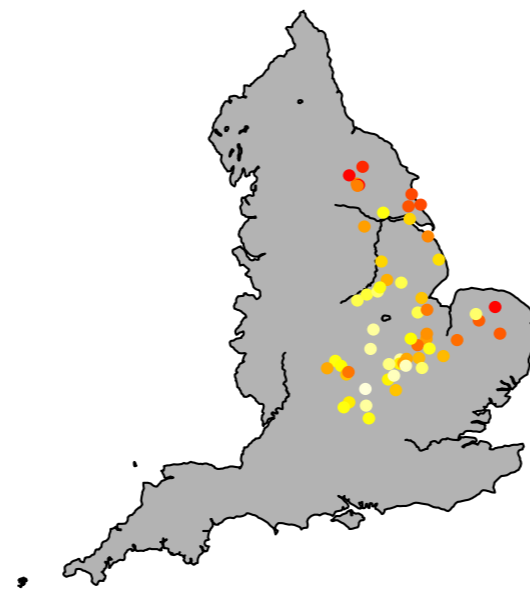
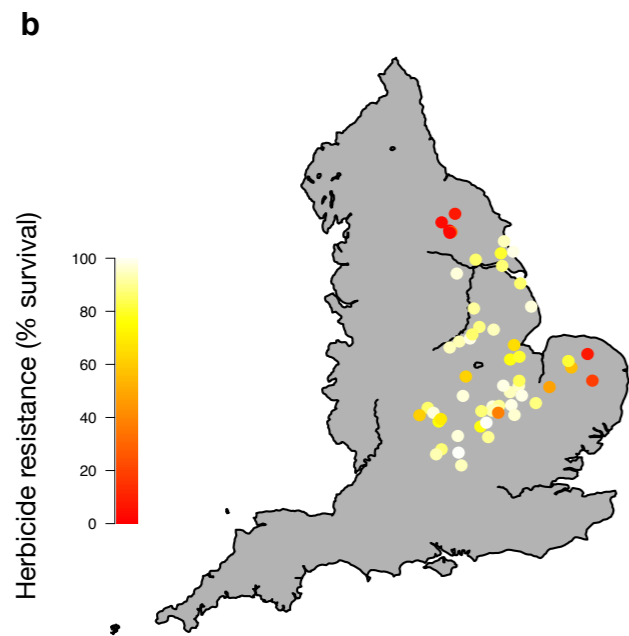
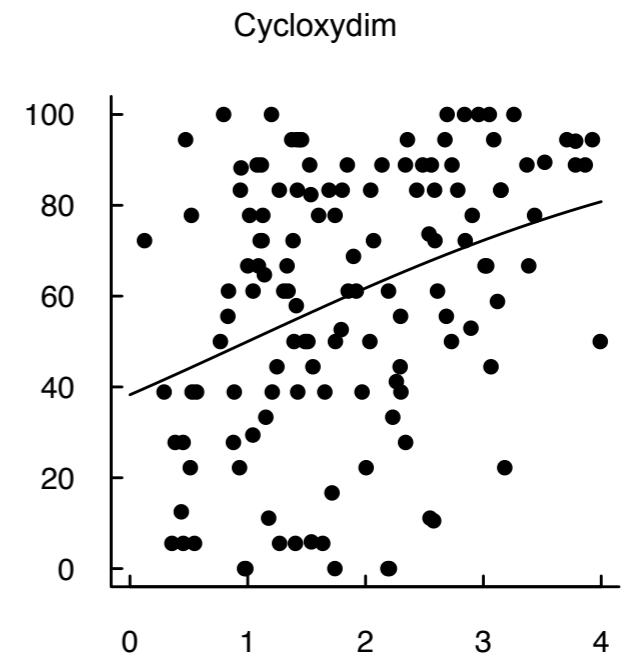
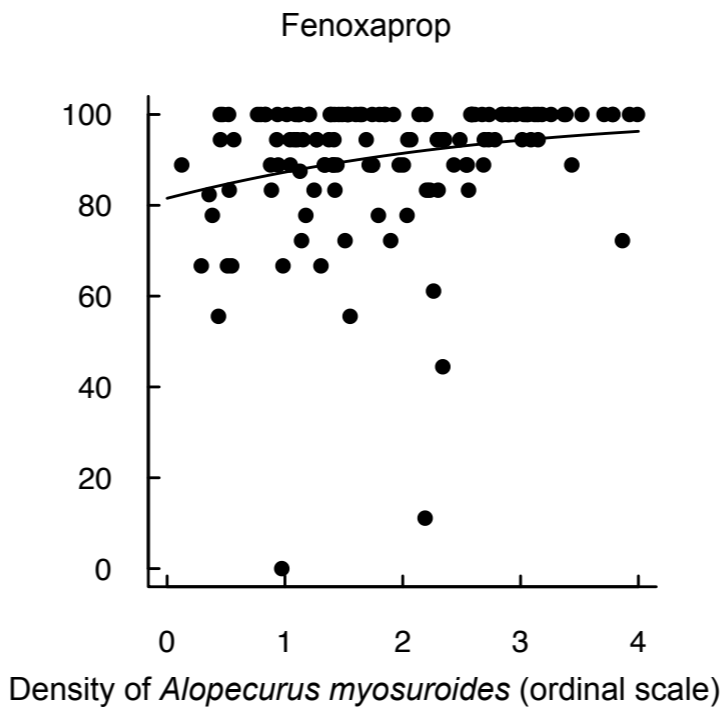
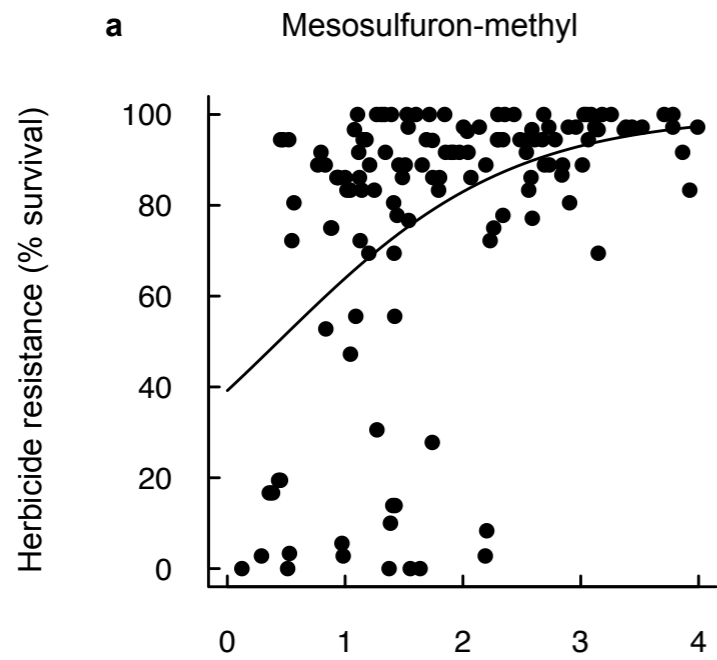
577 (source: Agriculture and Horticulture Development Board, Corn Returns). All costings were
578 calculated at 2014 prices, in line with the time of data collection and weed surveys.

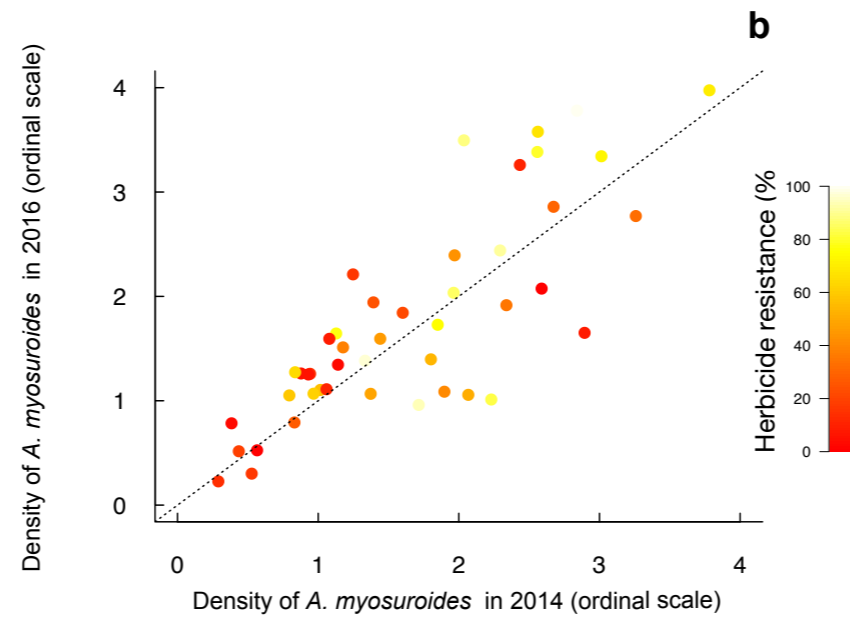
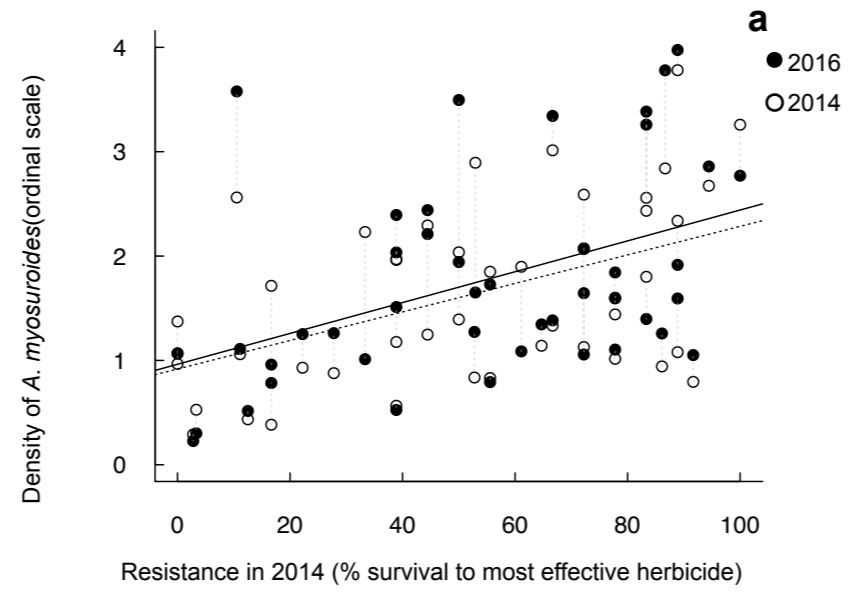
579 We used the linear model $\text{yield} \sim \text{density state} + (\text{density state} \mid \text{field})$ to predict yield at the
580 20m by 20m grid square level (fit using `lmer()` in the 'lme4' package) for the ten fields with high
581 resolution yield data. Density state was treated as categorical to allow a non-linear effect of
582 density on yield, and field was used as a random effect to control for differences between fields.
583 Linear regressions were performed on field scale relationships between weed density and
584 herbicide costs/ha, and weed density and total costs of *A. myosuroides* (herbicide costs + yield
585 loss) for these same ten fields.

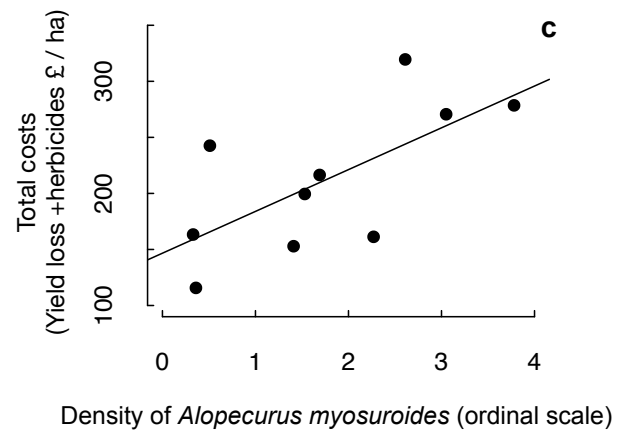
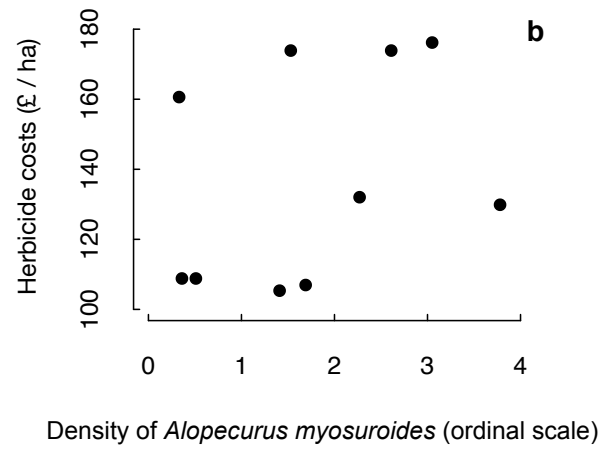
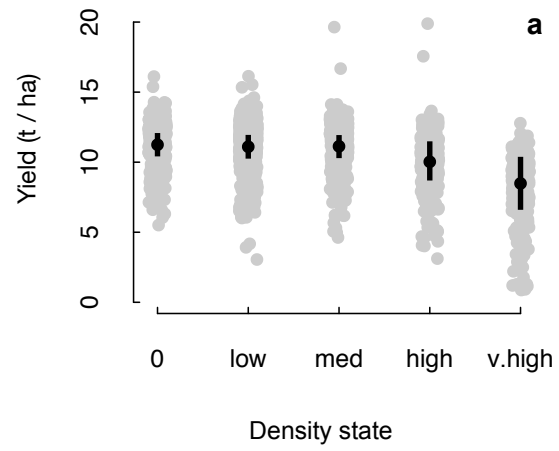
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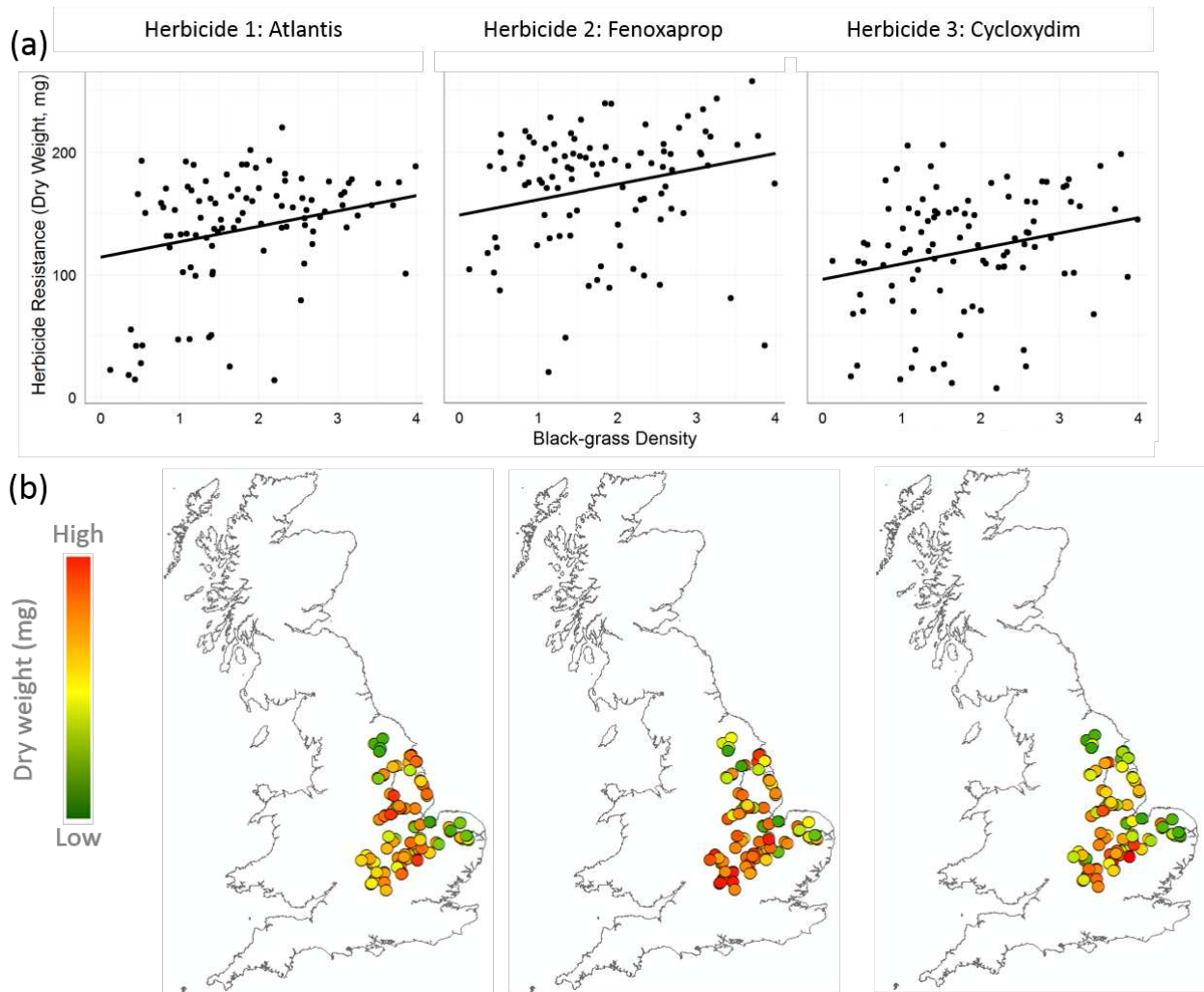


1 **The factors driving evolved herbicide resistance at a**
2 **national scale: Supplementary Information**

3

4 Helen L. Hicks¹, David Comont², Shaun R. Coutts¹, Laura Crook², Richard Hull², Ken
5 Norris³, Paul Neve², Dylan Z. Childs¹, Robert P. Freckleton^{1*}

6



8
 9 **Supplementary Figure 1** a) Relationship between mean black-grass density state and dry weight of plant material
 10 after treatment with each herbicide. Plotted lines represent predicted dry weight of weeds after treatment with
 11 herbicide for differing black-grass densities; models are mixed effect models with mean black-grass density state and
 12 herbicide as fixed effects and farm name as a random effect. (b) Heat maps showing dry weight of plant material (as a
 13 measure of herbicide resistance) after treatment with each of three herbicides. Red colours show larger amounts of
 14 plant material (i.e. low herbicide effectiveness), green colours show lower amounts of plant material (i.e. high
 15 herbicide effectiveness).

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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)	<p>Herbicide diversity: # MOAs applied in single harvest year</p> <p>Herbicide intensity: # herbicide application days in a single harvest year</p>	Negative correlation with resistance	<p>Herbicide diversity 2004 – 2009: Range = 1.0 – 6.3; mean = 3.4 2010 – 2014: Range = 1.4 – 6.2; mean = 4.3</p> <p>Herbicide intensity 2004 – 2009: Range = 1.0 - 4.6; mean = 2.6 2010 – 2014: Range = 1.2 – 6; mean = 3.3</p>
	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	<ul style="list-style-type: none"> • Cultivation intensity score • Plough frequency 	Studies show results to be variable depending on combination of frequency and depth of cultivation	<p>Cultivations <i>Mean cultivation intensity scores:</i> 2004 – 2009: Range = 0.5 - 4; mean = 2.95 2010 – 2014: Range = 0 - 4; mean = 2.82</p> <p><i>Proportion of years field ploughed:</i> 2004 – 2009: Range = 0 - 1; mean = 0.44 2010 – 2014: Range = 0 - 1; mean = 0.32</p>
	Crop type	PCA axis based on proportion of years field in winter wheat/ autumn sown cereal / cereal crop	Negative correlation with resistance	<p>Crop Type <i>Proportion years in autumn crop:</i> 2004 – 2009: Range = 0.17 - 1; mean = 0.86 2010 – 2014: Range = 0.4 - 1; mean = 0.89</p> <p><i>Proportion years in cereal crop:</i> 2004 – 2009: Range = 0.17 - 1; mean = 0.63 2010 – 2014: Range = 0.4 - 1; mean = 0.70</p> <p><i>Proportion years in winter wheat:</i> 2004 – 2009: Range = 0 - 1; mean = 0.56 2010 – 2014: Range = 0.2 - 1; mean = 0.62</p>

25
26

27 **Supplementary Table 2** Additional models of herbicide resistance using data for only one herbicide: Atlantis.
 28 Generalized linear mixed effects models (GLMM) were used to determine the effect of farm management histories on
 29 two measures of herbicide resistance (survival and dry weight) across two timeframes (old: 2004-2009 and recent:
 30 2010 – 2014). Mean black-grass density state, herbicide, soil type and herbicide parameters (mean number of
 31 herbicide application days per harvest year (herbicide intensity), mean number of herbicide MOAs applied within a
 32 harvest year (herbicide diversity)) were fitted as fixed effects in the models, and farm name was fitted as a random
 33 effect to describe the structure of the data. Observation-level random effects were used to account for over dispersion
 34 in the models. Here we present only the final models (black font) with significant predictor terms. A set of secondary
 35 analyses (grey font) investigated the additional effect of crop type (derived from the proportion of years the field was
 36 in winter wheat/ an autumn sown crop/ a cereal crop), the proportion of years the field was ploughed and a mean
 37 cultivation intensity score. R-square values were calculated using MuMIN [39] and parametric bootstrap using
 38 Kenward Roger methods [40] (using the ‘pbkrtest’ package in R) were used for model comparison and calculation of
 39 p-values.
 40

OLD					RECENT				
SURVIVAL					SURVIVAL				
Model structure	Effect size (Sum Sq)	P value	Model fit		Model structure	Effect size (Sum Sq)	P value	Model fit	
			R2 GLMM (m)	R2 GLMM (c)				R2 GLMM (m)	R2 GLMM (c)
Black-grass Density	30.42	0.001	0.134	0.164	Black-grass Density	29.60	0.001	0.128	0.161
Soil type	9.41	0.01			Soil type	9.16	0.01		
Herbicide intensity	16.80	0.001			Herbicide intensity	13.30	0.002		
+ Crop type (PCA axis 1)	1.92	0.175			+ Crop type (PCA axis 1)	1.10	0.371		
+ Plough frequency	0.10	0.761			+ Plough frequency	1.76	0.239		
+ Cultivation score	0.01	0.946			+ Cultivation score	1.06	0.379		
DRY WEIGHT					DRY WEIGHT				
Model structure	Effect size (Sum Sq)	P value	Model fit		Model structure	Effect size (Sum Sq)	P value	Model fit	
			R2 GLMM (m)	R2 GLMM (c)				R2 GLMM (m)	R2 GLMM (c)
Black-grass Density	9.22	0.003	0.126	0.320	Black-grass Density	9.08	0.003	0.096	0.303
Soil type	5.05	0.017			Soil type	4.89	0.017		
Herbicide intensity	3.34	0.030							
+ Crop type (PCA axis 1)	0.19	0.624			+ Crop type (PCA axis 1)	0.46	0.445		
+ Plough frequency	0.20	0.593			+ Plough frequency	0.24	0.530		
+ Cultivation score	1.38	0.163			+ Cultivation score	0.001	1.000		

41

42 **Supplementary Table 3** Model relating weed density to herbicide usage. Weed density was the response variable,
43 and farm entered as a random effect. The number of herbicide applications was used as the predictor, separately for
44 recent and old periods. The significance of these was assessed both using Kenward-Rogers and parametric bootstrap
45 methods: these yielded identical results.
46
47
48

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

49

50 **Supplementary Table 4** Herbicide resistance differs between soil types. Numbers represent mean values \pm standard
 51 error for populations originating from clay and non-clay soils, for each of the herbicides tested. Note also that there is
 52 a significant difference in dry weight between populations from different soil types when zero herbicide has been
 53 applied (i.e. control plants from all three experiments).
 54

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

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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)

Field	Percentage of field in black-grass density state					Mean ww yield within patches of density state (t/ha)					Economic costs			
	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

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60

61 **SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

62
63 **Rapid Assessment of *Alopecurus myosuroides* range**

64 In addition to the detailed 20x20m grid field surveys undertaken in 2014, we undertook rapid assessment exercise in
65 2015 and 2016 to give overall field scale density estimates for a large number of cereal fields across a more
66 widespread geographic area than the detailed density surveys. The location, crop and an estimate of field-scale black-
67 grass density were recorded from the side of each field.

68
69 **Seed collection**

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

77
78 Seeds were air-dried at room temperature for two weeks, before being cleaned using an air-column seed cleaner to
79 remove unfilled seeds and chaff. The ten cleaned and dried seed samples per field were weighed and combined into a
80 single seed bulk per field. These field scale seed bulks were used to represent each field population of black-grass
81 throughout the subsequent resistance testing.

82
83 **Resistance testing**

84 Dried seeds were maintained in an incubator in the dark at 30°C for three weeks to break any remaining seed
85 dormancy before experimentation. Seeds were germinated in petri-dishes on Whatman No. 1 filter papers soaked in 20
86 mmol L⁻¹ KNO₃, and incubated for seven days at 17/11°C over a 14/10 hour day/night cycle. Germinated seedlings
87 were transplanted into 3.5 inch pots containing a loam soil pre-mixed with 2 kg m⁻² osmocote fertiliser. Six pots were
88 sown for each field population of black-grass, with six seedlings sown per pot in an equally spaced ring. Pots were
89 assigned to either control or herbicide treatments (n=3), and arranged over three glasshouse compartments in a
90 randomised block design. Glasshouses were set to 18/12 °C day/night temperatures over 14/10 hours, with
91 supplementary lighting provided by sodium lamps whenever ambient daytime PAR was low.

92
93 This experiment was repeated three times over autumn 2014 - spring 2015 to test three herbicides; the ALS inhibitor
94 'Atlantis' (Mesosulfuron + Iodosulfuron), the 'fop' ACCase inhibitor 'Cheetah' (Fenoxaprop), and the 'dim' ACCase
95 inhibitor 'Laser' (Cycloxydim).

96
97 **Cultivation intensity scores**

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