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Black-grass herbicide resistance initiative (BGRI): Multiple herbicide resistance in grass weeds: from genes to agroecosystems

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1. Abstract

Evolved resistance to herbicides in blackgrass is a major constraint to cereal production in the UK. This large inter-disciplinary project has examined the evolution and management of herbicide resistance in black-grass from the genetic to the agroecosystem level. Multiple herbicide resistance (MHR), whereby black-grass evolves resistance to multiple active agents irrespective of chemistry, or mode of action, has been a particular focus for the project. Arranged into five large work packages, the project has addressed five key questions:

- What is the molecular physiological basis of MHR ?
- What is the extent and impact of MHR ?
- What are the major drivers of resistance evolution ?
- Can applied evolutionary models aid in resistance management ?
- What are the economic and environmental consequences of novel weed and resistance management ?

The project has used a combination of cutting edge foundational research to underpin new understanding of herbicide resistance and its evolution in the field, that has then allowed us to develop new practical tools for black-grass management and to exchange this knowledge with the arable farming industry on a continuous and participatory basis. The key outcomes from the programme are:

1. Identification of key proteins that are causatively linked to MHR and new understanding of how their functions in resistance could be disrupted in future
2. Evidence for sub-types of MHR linked to specific herbicide chemistries
3. The characterisation of latent viruses in blackgrass that could be of value in future biocontrol programmes
4. That epigenetic mechanisms are unlikely to be an evolutionary driver of MHR inheritance
5. The first evidence for active roles for transporter proteins functioning in MHR and their coupled function with detoxifying enzymes
6. Practical diagnostics for MHR in black-grass that can be used to detect resistance in the field in 10 minutes
7. A national audit of resistant blackgrass populations linked to previous management
8. The research resource of a characterized collection of herbicide resistant blackgrass populations
9. Underpinning knowledge that can help reduce the likelihood of resistance evolution
10. Converting predictive models of herbicide resistance to on farm decisions
11. Environmental and economic valuation of cost and mitigation of resistance

2. Introduction

In the advanced agricultural production systems of Northern Europe, weed control in cereal crops has become one of the greatest challenges to sustainable intensification, accounting for higher yield losses and greater input costs than all other biological constraints (pests and diseases). The most problematic weeds in cereals in Northern Europe are the wild grasses, notably blackgrass (*Alopecurus myosuroides*), which has become steadily more difficult to control over the last 30 years due to the evolution of herbicide resistance. This resistance assumes two forms: 1) Target site resistance (TSR), whereby the weeds become highly tolerant of herbicides due to mutations in the proteins targeted by these chemicals rendering them less sensitive to inhibition by that herbicide mode of action. 2) Metabolic or multiple herbicide resistance (MHR), also termed non-target site resistance (NTSR), where weeds become more tolerant of a broad range of herbicides, irrespective of their chemistry or mode of action, due to a general enhancement in the ability to detoxify crop protection agents. While TSR is now quite well understood and can be countered by the rotational use of herbicides with differing modes of action, the molecular basis and evolutionary drivers which promote MHR are poorly understood and the associated grass weeds very difficult to control using conventional methods.

In this 4 year project, we have used a combination of molecular biology and biochemistry, ecology and evolution modeling and integrated pest management to develop better tools to monitor and manage both TSR and MHR in black-grass under field conditions. The project has utilized a novel agri-systems approach, linking our latest understanding in the molecular biology of herbicide resistance to on farm monitoring and modeling based on a quantitative genetics approach to define the effectiveness of different intervention measures. Through a multidisciplinary consortium, we have integrated knowledge about MHR and TSR at the molecular and biochemical levels and related this fundamental understanding to resistance phenotypes observed in the field. Selection and breeding experiments examined the dynamics of selection for resistance, with the intention of determining the genetic architecture of MHR for the first time and its relation to other stresses and life history traits. Data from field monitoring and glasshouse studies was integrated in ecological, evolutionary and management models with the ultimate aim to design novel management to prevent, delay or mitigate the evolution of herbicide resistance. Finally, the environmental and economic impacts of novel management were explored. The project therefore has the primary goal of using state of the art approaches spanning molecular biology, weed science, modeling and agronomy to provide new resistance control measures within the life of the program.

The project was divided into 5 integrated work packages (WPs) which address the following questions;

1. What are the molecular mechanisms that underpin the evolution of metabolic herbicide resistance ?
2. What is the extent of the herbicide resistance problem in UK black-grass populations and what impacts is resistance having on black-grass populations and crop yields ?
3. What are the genetic, ecological and agronomic factors that promote and constrain the emergence of herbicide resistance ?
4. How can applied evolutionary models be used to manage herbicide resistance ?
5. What are the economic and environmental consequences of novel weed and resistance management strategies?

The original proposed major outputs were anticipated to be:

1. A rapid diagnostic toolkit for the on-farm characterization of herbicide resistance.
2. A resistance audit for the extent and distribution of resistance to the major herbicide modes of action in black-grass.
3. A suite of models to address key questions in the emergence and management of resistance.
4. Management recommendations, together with an analysis of their impacts.

3. Materials and methods

Full materials and methods of all published work will be found in the respective papers cited in the report. Materials and methods presented below represent those from currently unpublished studies which are cited in the results sections.

3.1. Characterisation of blackgrass populations

3.1.1. Seed collection and source populations

Blackgrass seeds were collected in 2014 from a series of 132 wheat production fields across England. For full details of the site locations see Hicks et al. (2018). Seeds were sampled using a stratified-random approach from ten locations within each field. At each location, seeds were collected from multiple plants in a circumference of approximately 5-10 m. A single representative seed population for each field was subsequently generated by combining 50% by weight of seed collected at all sampling locations within a field. These field-scale seed populations were used in all phenotypic and genotypic assays reported here. The spatial distribution and abundance of blackgrass in each field was recorded using a density-structured approach (Queenborough et al. 2011), and a mean blackgrass density was calculated per field on a scale of 0-4 (absent – very high).

3.1.2. Field management histories

Field management histories were collected for 89 of the 132 blackgrass populations (Hicks *et al.*, 2018) and provide a mean of seven years data on herbicide usage, soil cultivation, crop rotations, and soil properties. These data were assessed and used to calculate measures representing differences in the intensity and heterogeneity of herbicide selection, alongside measures of cultivation intensity, location, and weed population size.

3.1.3. Resistance audit

We used our national-scale data set of occurrence of blackgrass across the 132 farms in the United Kingdom to uncover the agronomic drivers of herbicide resistance. Weed densities were 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 found that populations resistant to one herbicide are likely to show resistance to multiple herbicide classes.

Finally, we found that the economic costs of evolved resistance are considerable: loss of control through resistance can double the economic costs of weeds.

3.1.4. National-scale survey

We developed a simple, repeatable methodology for estimating field-scale occurrence of blackgrass using rapid roadside surveys. This allowed us to measure the occurrence and density of blackgrass in 4631 locations across England. We also analysed national scale botanical atlas data published over the past 50 years. Populations of blackgrass show a characteristic central distribution in the UK, with densities declining to the north and south, but with significant inter-annual variability. Analyses of atlas data show that the species has been spreading over the past 50 years: the pattern of spread is a localised phalanx, suggesting that spread occurs over short distances, rather than by long-distance dispersal. Significant impacts of soil and rainfall on densities of the weed were determined; with densities increasing with the proportion of heavy soils, and decreasing with increasing rainfall. Comparing our models with independent atlas data we determined that the models provide a good prediction of occupancy at the national scale and have provided the first national maps of current spatio-temporal distributions as well as potential distribution. Our results highlight the localised nature of colonisation, and this emphasises the need for locally focussed management to limit dispersal between fields. We demonstrate that while blackgrass has increased its national distribution in recent decades, there still exist many suitable areas in which increases in both density and occurrence are still possible. This highlights the need for proactive management, particularly in the initial stages of local population establishment.

3.2. Molecular and biochemical studies

3.2.1. Omics studies.

Next generation transcriptome sequencing was carried out on a range of black-grass populations using a range of sequencing platforms including Roche 454, Ion-torrent and Illumina technologies. Differential RNAseq analysis was applied to MHR Vs herbicide susceptible (HS) populations, and after intensive processing, unigene sequences representing open reading frames (ORFs) of interest were compared to those present in the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/) database using the online protein Blast analysis tool (https://blast.ncbi.nlm.nih.gov/) as described (Sabbadin et al., 2017). Proteomics was carried out on total extracts from black-grass plants using large format 2-dimensional gel electrophoresis to resolve individual polypeptides (Tetard-Jones et al., 2018). Changes in the expression of individual proteins in MHR Vs HS plants was achieved using differential dye analysis, with peptides of interest sequenced de novo using mass spectrometry (Tetard-Jones et al., 2018)

3.2.2. **Gene expression studies**

Total RNA was extracted from stem from individual plants using NucleoSpin RNA Plant - MACHEREY-NAGEL and cDNA synthesis was done with iScript™ cDNA Synthesis Kit (Bio-Rad) following manufacturer's instructions. Fold-changes in the expression of individual genes was achieved using PCR with specific primers directed to the sequences of interest and quantified in comparison to the internal standards glyceraldehyde 3-phosphate dehydrogenase and beta-tubulin. Transcriptional connectivity between genes was determined using Pearson correlation coefficient analysis.

3.2.3. **Cloning and expression of novel MHR-related genes.**

Novel blackgrass tau GSTU protein sequences and ABC transporters from the C subfamily were manipulated using SEAVIEW software version4 .6.4 (Gouy et al. 2010). Sequences were aligned with Muscle (Edgar 2004) and subsequent trimmed using trimAI software with defaults settings v.1.3 (Capella-Gutiérrez et al. 2009) accessed through the webserver Phylemon 2 (Sánchez et al. 2011). The trimmed alignment was used to infer maximum likelihood phylogenies with IQ-TREE software using the online server (Nguyen et al. 2015). A previously undescribed full-length tau GST sequence assembled from the next-generation sequencing (NGS) data termed *Am*GSTU2 was checked with respect to its ORF, by amplifying the respective sequence by polymerase chain reaction (PCR) using cDNA prepared from Peldon plants. To identify the potential variants of *Am*GSTU2 present in black-grass, a combination of internal and vector primers were used. The complete ORF of a specific variant termed *Am*GSTU2a, was cloned into the expression vector pET-STRP3, expressed in E. coli and purified using a Strep-Tactin macroprep column (Stratech Scientific Ltd, Soham, UK) and used for enzyme assays.

3.2.4. **Analysis of enzymes linked to MHR.**

GST activities toward 1-chloro-2,4-dinitrobenzene (CDNB) and t-butyl- and cumene hydroperoxides were determined by spectrophotometry. The glutathione conjugation of the herbicides fenoxaprop-ethyl and metolachlor was quantified by High Performance Liquid Chromatography (HPLC) as previously described (Cummins et al., 1999).

3.2.5. Determination of herbicide metabolism in blackgrass plants

Blackgrass plants from eight different populations were grown to the three-five tiller stage. For each population, metabolism assays were carried out on three biological replicates, each consisting of leaf tissue from three plants, cut into 1 cm strips and submerged in ½ MS containing the phenylurea herbicide chlorotoluron (50 µM) for 24 hours. After harvest, samples were frozen in liquid nitrogen and then mixed with 5 x (w/v) methanol at 4 °C overnight. The grass was then

removed and the methanolic extract dried down under nitrogen gas. The sample was re-suspended in 2.5 x (w/v) citrate phosphate buffer, pH 5.0 containing 1 mg ml^{-1} cellulase and incubated at 30 °C overnight. Each sample was extracted twice with equal volume of water saturated ethyl-acetate. The organic fractions were combined and dried down under nitrogen gas. The sample was re-suspended in 1 x (w/v) methanol:water (4:1) and stored at -20 °C prior analysis. Extracts (5 µl) were injected onto an Acquity UPLC® BEH C18 (1.7µm, 2.1 x 50 mm) column at a flow rate of 0.5 ml min^{-1} and separated on a Waters Acquity UPLC I class with a gradient starting at 5 % B rising to 95 % B over 1.5 min. The eluent was analysed using a Waters Xevo G2-XS Q-TOF mass spectrometer after electrospray ionisation (capillary 0.7 kV, source 120 °C, desolvation at 600 °C and desolvation gas flow at 800 L hr^{-1}) in positive polarity and acquired in MS^E mode (high CE 30V, low CE 10 V).

3.2.6. DNA based (qPCR) screening of resistance

Plants from HS (Rothamsted) and MHR (Peldon) blackgrass populations were grown for two weeks as above before harvesting 100 mg of shoot tissue (by flash freezing in liquid nitrogen) for mRNA extraction. mRNA extraction was performed with the RNeasy Plant Mini Kit (Qiagen) and 1 µg was used a template for ss cDNA synthesis using an oligo(dT) primer and the SuperscriptII® Reverse Transcriptase Kit (Invitrogen). An additional on-column DNase digestion with RNase-free DNase set (Qiagen) and implementation of Wizard® SV gel and PCR clean-up system (Promega) was used on the cDNA. Primer pairs were designed with Primer Express® version 3.0 (Applied Biosystems) in order to amplify selected genes identified within the IonTorrent contig library. PCR was performed on 3 separate cDNA biological replicas, with technical triplicates of each and fold-increases were measured in comparison to the internal standard glyceraldehyde 3 phosphate dehydrogenase. QPCR was performed on The StepOnePlus™ Real-Time PCR System, with a reaction volume of 20 µl, containing 0.7 µM each of forward and reverse primers and 10 µl of Fast SYBR® Green Master Mix (Applied Biosystems), together with 2µl of a 1:50 dilution of cDNA template. After an initial denaturation of 20 seconds at 95°C, amplification was achieved following 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Amplification results were analysed with StepOne v 2.2.2 (Applied Biosystems). Primer efficiency test was performed for each primer couple, including dissociation analysis of the products and calculation of squared correlation coefficient (R^2) to ensure efficient and specific amplification.

Populations used in the herbicide resistance phenotyping were assessed for their gene expression against seven genes selected from the whole transcriptome qPCR validation. Methods for the plant growth and qPCR were performed as described above, with eight of the primer sets (see Table 2). QPCR was performed on ten separate cDNA biological replicates, with each analysed in technical

triplicates and fold-changes measured in comparison to the internal standard glyceraldehyde 3 phosphate dehydrogenase (G3PD). QPCR was performed as in the initial confirmation of xenome differential regulation (above) although there was no requirement for primer efficiency tests as they had already been optimised.

3.2.7. Immunological studies with MHR-linked proteins

Antisera to proteins associated with MHR were raised against recombinant AmGSTF1 in rabbits and sheep as described previously (Cummins et al., 2013). Antibodies were then used to test for the presence of AmGSTF1 and its orthologs in other grasses, using a combination of Western blotting (Cummins et al., 1999) and ELISA immunoassay (see section 3.3.4)

3.3. Quantification of resistance in black-grass populations

3.3.1. Post emergent herbicide resistance phenotyping

Phenotypic resistance to three herbicides was established using separate glasshouse dose response experiments conducted over October 2014 – May 2015. Herbicides tested included a commercial formulation of mesosulfuron-methyl + iodosulfuron (site of action; acetolactate synthase, ALS), fenoxaprop-p-ethyl (site of action; acetyl-CoA-carboxylase, ACCase), and cycloxydim (site of action; acetyl-CoA-carboxylase, ACCase). All herbicides are, or have been, used for blackgrass control in wheat crops. In each case, the dose response design consisted of six treatments (five herbicide doses plus a no-herbicide control). For the ALS herbicide mesosulfuron, doses were 75, 150, 300, 600, and 1200 g ha^{-1} (current UK field rate is 400 g ha^{-1}). fenoxaprop doses were 0.3125, 0.625, 1.25, 2.5, and 5 L ha^{-1} (current UK field rate is 1.25 L ha^{-1}), while cycloxydim doses were 0.1875, 0.375, 0.75, 1.5, and 3 L ha^{-1} (current UK field rate is 0.75 L ha^{-1}).

Seeds from each population were pre-germinated in an incubator (Sanyo, MLR-350) with a 17/11°C temperature cycle and a 14/10 hour light/dark cycle, before sowing into 8 cm plastic plant pots filled with a Kettering loam soil, mixed with 2 kg m^{-2} osmocote fertiliser. 18 pots, each containing six seedlings were sown per population, providing three pots (18 plants) at each herbicide dose. Replicate pots were blocked over three glasshouse compartments, with the position of pots within each compartment determined using a randomised alpha design. The glasshouse was set to maintain approximately 16°C during the day and 10°C at night, with a 14/10 hour day/night cycle. Three reference populations (herbicide susceptible, target site resistant and non-target site resistant) were included as positive and negative controls for resistance. Over the three dose-response experiments, 324 plants per population were phenotyped, with over 40,000 plants phenotyped in total. Plants were maintained in the glasshouse for 2-3 weeks until they

reached the 3-4 leaf stage. At this point, pots were removed from the glasshouse and sprayed using a fixed track sprayer with a Teejet 110015VK nozzle placed 50 cm above the plant canopy. The boom speed was set at 0.33 m s^{-1}, and herbicide was applied at a volume of 197 – 213 L ha^{-1}. After spraying, pots were immediately returned to the glasshouse. After three weeks, the number of surviving plants per pot was assessed, and above-ground tissue was harvested on a per pot basis. Leaf tissue was oven-dried at 80°C for 48 hours before weighing to determine plant biomass.

3.3.2. Glyphosate sensitivity screening

Sensitivity to the broad-spectrum herbicide glyphosate was established using another glasshouse based whole-plant assay. Seeds were pre-germinated within an incubator and sown into 8 cm plastic plant pots as previously. In total 2,772 pots (16,632 blackgrass plants) were grown, with 21 pots per population providing three replicate pots (n=3) for each of seven herbicide doses. The three replicate pots at each dose were blocked over three glasshouse compartments, with pot position within each compartment determined using a randomised alpha design. The seven glyphosate doses used were control (no herbicide) plus; 67.5, 135, 270, 405, 540, and 675 g ha^{-1} of glyphosate (current UK glyphosate field rate is 540 g ha^{-1}). Plants were sprayed after three weeks' growth at the three to four leaf stage. For spraying, plants were removed from the glasshouse and herbicide was applied using a fixed track-sprayer. The spray nozzle (Teejet, 110015VK) was mounted 50 cm above the plants, with boom speed set at 0.33 m s^{-1} applying herbicide at a rate of 214 L ha^{-1}. Plants were placed back into the glasshouse immediately following spraying. Three weeks after spraying, plants were visually inspected for survival, and any plant with visible new growth was designated as a survivor.

3.3.3. Quantification of target-site resistance

Known target-site resistance mutations within the ALS and ACCase genes were analysed using pyrosequencing following the approach of Beffa et al., (2012). Briefly, approximately 10mg of air-dried leaf material was homogenised in 400µl of extraction buffer (100 mM Tris(HCl) and 1 M KCl at pH 9.5). A 5µl aliquot of the resulting supernatant was diluted using 250µl PCR grade water (Merck, Darmstadt, Germany) and PCR amplification of target sequences performed using HotStar Taq Master Mix (Qiagen, Hilden, Germany) and biotinylated primers. Two ALS fragments were amplified containing either the 197 or 574 mutation positions, and two further ACCase fragments were amplified containing either the 1781 position or the combined 2027, 2041, 2078 and 2096 positions. The biotinylated ALS and ACCase fragments were used in pyrosequencing reactions to amplify individual target-sites using site-specific primers. For pyrosequencing, 12µl of amplified biotinylated PCR product was combined with a 70µl solution containing binding buffer and streptavidin-coated Sepharose beads and shaken for five minutes, before washing and drying DNA

coated beads at 80°C. Sequencing was carried out using a Pyromark Q96 MD pyrosequencer (Qiagen, Hilden, Germany). In total, genotyping data was generated for 2574 individual plants ($n \geq 16$ plants per population).

3.3.4. Quantification of AmGSTf1 protein concentration

Seeds from each population were pre-germinated as previously above, and seedlings sown into 8 cm plastic plant pots filled with a Kettering loam mixed with 2 kg m^{-2} osmocote fertiliser. Three pots were sown per population, each containing 15 seedlings, and were maintained within a single glasshouse compartment for two weeks until the plants reached the two-leaf stage. The glasshouse was set to maintain approximately 16ºC during the day and 10ºC at night, and the position of pots was fully randomised within the compartment. After two weeks growth, the above-ground material from all 15 plants per pot was harvested in bulk and immediately flash frozen in liquid nitrogen and stored at -80ºC. The frozen samples were homogenised by grinding in liquid nitrogen using a mortar and pestle, with 100mg of frozen ground tissue taken from each sample for protein extraction.

Total proteins were extracted and quantified using the Bradford method (Bradford, 1976). The protein extraction buffer comprised 100mM Tris-HCl, 150mM NaCl and 5mM EDTA, adjusted to pH 7.5 with NaOH, and with the addition by volume of 5% glycerol and 2% PVPP. Samples of 100mg homogenised leaf tissue were vortex mixed with 500μl of the extraction buffer, incubated on ice for 15 minutes, and centrifuged twice at 4ºC for 15 minutes. A 1:10 dilution of supernatant from each sample was prepared using deionised water, and 10μl of diluted samples were plated in triplicate on a 96 well plate. 200μl of Bradford dye reagent (Bio-Rad protein assay kit, Bio-Rad, California) was added per well, incubated at room temperature for 15 minutes, before reading absorbance at 595nm using a plate reader (Spectra max 250, Molecular devices, California, USA). Total protein was calculated from the absorbance readings using a standard curve generated for each plate from a serial dilution of a stock bovine serum albumen (BSA) solution.

A sandwich-type Enzyme-Linked Immunosorbent Assay (ELISA) was carried out to quantify the AmGSTf1 protein concentration in each extracted protein sample. Two antibodies designed to capture and detect the AmGSTf1 protein (S909D and S908D-HRP) were used. A 96-well microtiter plate was incubated overnight at 4°C with 100μl per well of a phosphate-buffered saline solution (PBS) containing the antibody S909D. Plates were washed, then blocked using PBS containing 1% BSA by volume, and incubated for one hour at room temperature. Wells were washed again before adding 100μl of the extracted plant protein samples. A dilution series of purified AmGSTf1 protein was also included on each plate to provide a standard curve for quantification. Plates were incubated for one hour at room temperature whilst shaking at 150 rpm before re-washing using

PBS. 100µl of PBS solution containing 1% BSA and 0.005% of the second antibody, S908D-HRP, was added to each well and incubated for a further hour at room temperature and 150 rpm. After re-washing, 100µl of a tetramethylbenzidine solution (TMB) was added per well and incubated in the dark at room temperature for 30 minutes. Absorbance was measured using a plate reader at 655nm. The reaction was stopped by addition of 50µl 1M HCL before measuring absorbance again at 450nm. Samples and standards were analysed in duplicate, and concentration of the AmGSTf1 protein calculated by comparison with the standard curve for each plate.

3.4. Heritability and fitness studies

3.4.1. Intraspecific competition assay

A target-neighbour design was set up to evaluate establishment and early development of two blackgrass biotypes (MHR: 'R', and susceptible: 'S') with a standardised genetic background, under intraspecific competition. Seeds of the two biotypes were pre-germinated and planted into 25cm pots filled with the same Kettering loam soil as previously. 'Target' plants were established by sowing 120 pots with a single 'R' seedling in the centre, while a further 120 were sown with a single 'S' seedling in the centre. Further 'neighbour' blackgrass plants were then sown at different planting densities in a ring around each of these central plants, to provide varying levels of intraspecific competition. Numbers of neighbour plants were 0, 1, 2, 5, 10, and 25, representing competitor planting densities of 0, 20, 40, 100, 200, and 500 plants m^{-2}. Half of the pots of each target biotype were established with 'R' neighbour plants, while the other half were established with 'S' neighbour plants. In total, 10 replicate pots were established at each planting density for each combination of target and neighbour biotypes (n=10). Pots were maintained outside, exposed to ambient environmental conditions at Harpenden (Hertfordshire, UK) over December - April. On seven occasions, target plants were non-destructively assessed for the number of tillers and the length of the longest tiller. For analysis, the dates of each non-destructive measurement were converted to thermal time, calculated as cumulative growing degree days above a base temperature of 1°C, as per Chauvel et al. (2000).

3.4.2. Interspecific competition assay

A further target-neighbour design was established to evaluate the reproductive productivity of the two blackgrass biotypes when grown under interspecific competition with wheat. 25 cm pots containing Kettering loam soil were sown in another target-neighbour design. In this case, neighbour plants were wheat (variety JB Diego), at planting densities representing 0, 40, 80, 120, 200, and 500 competitor plants m^{-2}. Pots were maintained outdoors under ambient conditions as previously over December to July, with supplementary watering provided by overhead sprinklers as

necessary. At reproductive maturity the number and length of all blackgrass seed-heads per pot was evaluated.

3.4.3. Generation of pedigreed seed families

Nine of the collected blackgrass populations were used to generate a set of 400 pedigree seed families with a paternal ½ sibling crossing design. At the time of seed collection, ten sampling locations were identified within each field using a stratified random approach, with five blackgrass seed heads collected at each location. In all cases, seed heads were collected from different individual plants, with 50 seed heads collected in total from each field. Seeds from each seed head were pre-germinated as previously described, with a single germinated seedling derived from each head transplanted into individual 6-inch plastic plant pots containing compost. Plants were maintained in a glasshouse for three months over September – November, with temperature control set to 16/14°C. Plants were vernalised for one month over December by turning off supplemental heating and lighting in the glasshouse. After vernalisation, plants were maintained at 20/15°C with supplemental lighting provided over a 14/10 hour day to stimulate growth.

Once plants were well established, controlled crossings between paired plants were performed to generate a set of paternal half-sibling seed families for experimentation. In order to do this, one quarter of the plants from each population were randomly chosen and designated as pollen donors (paternal plants). These plants were split apart using a system of vegetative cloning, creating three genetically identical tillers. Each of the three clones was randomly paired with an un-cloned (maternal) individual from the same population, and paired plants were grown together within a glasshouse. At the onset of flowering, paired plants were bagged together using plastic pollination bags, allowing fertilisation of seed heads within each pair, and preventing cross pollination between different pairs. Once seed heads were mature, the pairs were separated, and seeds from each maternal plant were collected by gently shaking heads into a paper bag. As *A. myosuroides* is an obligate outcrossing species, all seeds from the maternal plant were considered to be the result of pollination from the paired paternal plant. In total, 400 pedigreed seed families were produced in this way.

3.4.4. Phenotypic characterisation of pedigreed seed families

Pre-germinated seedlings were sown into 20 cm pots (one seedling per pot) containing Kettering loam soil. Seven replicate pots were sown for each of the 400 blackgrass seed families, providing 2,800 pots in total. Pots were maintained outdoors under ambient conditions as described above over the period of November to July, with supplementary watering provided by overhead sprinklers over late spring - summer. The number of tillers and length of the longest tiller were measured non-destructively on each plant approximately every 2-3 weeks during vegetative development (December – April). As plants matured, the date of first flowering was recorded as the date at

which the first visible flower head was observed, and subsequently the date at which the first seed was seen to have matured and dropped was recorded as the date of first seed shed. At the end of July once all plants had flowered, the aboveground material was harvested and the total number of heads recorded, along with the length of the longest flowering tiller.

4. Results

The following results represent a combination of published work and work in progress/ to be submitted

4.1. Advances in our understanding of the mechanisms of MHR

4.1.1. Proteomic and transcriptomic studies with MHR blackgrass.

A full account of these studies has been published: Tétard-Jones, C., Sabbadin, F., Moss, S, Hull, R., Neve, P. and Edwards, R. (2018) Changes in the proteome of the problem weed blackgrass correlating with multiple herbicide resistance. Plant Journal., DOI:10.1111/tpj.13892

Abstract: Herbicide resistance in grass weeds is now one of the greatest threats to sustainable cereal production in Northern Europe. Multiple herbicide resistance (MHR), a poorly understood multigenic and quantitative trait, is particularly problematic as it provides tolerance to most classes of chemistries currently used for post-emergence weed control. Using a combination of transcriptomics and proteomics, the evolution of MHR in populations of the weed blackgrass (*Alopecurus myosuroides*) has been investigated. While over 4500 genes showed perturbation in their expression in MHR vs. herbicide sensitive (HS) plants, only a small group of proteins showed >2-fold changes in abundance, with a mere 8 proteins consistently associated with this class of resistance. Of the 8, orthologs of three of these proteins are also known to be associated with multiple drug resistance (MDR) in humans, suggesting a cross-phyla conservation in evolved tolerance to chemical agents. Proteomics revealed that MHR could be classified into three sub-types based on the association with resistance to herbicides with differing modes of action (MoA), being either global, specific to diverse chemistries acting on one MoA, or herbicide specific. Furthermore, the proteome of MHR plants were distinct from that of HS plants exposed to a range of biotic (insect feeding, plant-microbe interaction) and abiotic (N-limitation, osmotic, heat, herbicide safening) challenges commonly encountered in the field. It was concluded that MHR in blackgrass is a uniquely evolving trait(s), associated with changes in the proteome that are distinct from responses to conventional plant stresses, but sharing common features with MDR in humans.

4.1.2. Discovery of symptom-less viruses in MHR blackgrass

A full account of these studies have been published: Sabbadin, F., Glover, R., Stafford, R., Rozado-Aguirre, Z., Boonham, N., Adams, I., Mumford, R. and Edwards, R. (2017) Transcriptome sequencing identifies novel persistent viruses in herbicide resistant wild-grasses. Scientific Reports. **7**, 49187. DoI: 10.1038/srep41987

Abstract. Herbicide resistance in wild grasses is widespread in the UK, with non-target site resistance (NTSR) to multiple chemistries being particularly problematic in weed control. As a complex trait, NTSR is driven by complex evolutionary pressures and the growing awareness of the role of the phytobiome in plant abiotic stress tolerance, led us to sequence the transcriptomes of herbicide resistant and susceptible populations of black-grass and annual rye-grass for the presence of endophytes. Black-grass (*Alopecurus myosuroides; Am*) populations, displaying no overt disease symptoms, contained three previously undescribed viruses belonging to the *Partititiviridae* (AMPV1 and AMPV2) and *Rhabdoviridae* (AMVV1) families. These infections were widespread in UK black-grass populations and evidence was obtained for similar viruses being present in annual rye grass (*Lolium rigidum*), perennial rye-grass (*Lolium perenne*) and meadow fescue (*Festuca pratensis*). In black-grass, while no direct causative link was established linking viral infection to herbicide resistance, transcriptome sequencing showed a very high incidence of infection in the NTSR Peldon population. The widespread infection of these weeds by little characterised and persistent viruses and their potential evolutionary role in enhancing plant stress tolerance mechanisms including NTSR warrants further investigation.

4.1.3. Development of DNA based biomarkers of resistance reveals close associations between specific xenoma genes and MHR

Biomarker genes were identified from the transcriptomic studies that were upregulated in the MHR population relative to HS plants and which reflected the different reaction types associated with four phases of xenobiotic detoxification involved in herbicide metabolism (Table 1). For real-time qPCR analysis, primer sets were designed to the three most highly up-regulated transcripts for each of the xenome gene classes. Quality control was performed and the primer sets with the greatest efficiency and fidelity were selected to amplify a representative biomarker for each xenome class. The genes selected were AmOPR1, CYP709, AmGSTF1, AmGSTU, UGT, MATE, ABCI and TMT. As a first step in their validation, qPCR primers were prepared for each gene and used to quantify relative transcript levels in HS and MHR plants relative to that determined using transcriptome sequencing (Table 1). While the relative magnitude of up-regulation was shown in both sets of analysis the absolute fold change between the samples tended to be lower in the qPCR validation as compared with the IonTorrent analysis. For example for Am*OPR1* was 70 fold more abundant in the MHR plants as compared to WTS, as compared with a 101 fold change

based on the IonTorrent sequencing this did not alter the significance of the differential gene regulation. Similar patterns of differential induction (MHR Vs. HS) were shown with the other biomarkers (Table 1).

Table 1. Expression of genes involved in herbicide metabolism in MHR Vs. HS blackgrass

Up-regulated protein function	**Unigene**	**Gene name**	**Species**	**Fold change**
Xenobiotic detoxification Phase 1	O48923	*Cytochrome P450 71D10*	*Glycine max*	8.81
	Q9XHE8	*Cytochrome P450 71D18 ((4S)-Limonene-6-hydroxylase)*	*Mentha spicata*	3.20
	Q9SHG5	*Cytochrome P450 72C1*	*Arabidopsis thaliana*	<6.82
	O48786	*Cytochrome P450 734A1*	*A. thaliana*	<18.41
	B9X287	*Cytochrome P450 734A6*	*Oryza sativa subsp. japonica*	<10.93
	O64636	*Cytochrome P450 76C1*	*A. thaliana*	9.20
	Q42799	*Cytochrome P450 93A2*	*G. max*	12.85
	P48419	*Flavonoid 3',5'-hydroxylase (Cytochrome P450 75A3)*	*Petunia hybrida*	6.67
	D1MI46	*Geraniol 8-hydroxylase (Cytochrome P450 76B10)*	*Swertia mussotii*	5.42
	P93147	*Isoflavone 2'-hydroxylase (Cytochrome P450 81E1)*	*Glycyrrhiza echinata*	<13.16
	P21569	*Peptidyl-prolyl cis-trans isomerase CYP (Cyclosporin A-binding protein)*	*Zea mays*	5.11
	A6YIH8	*Premnaspirodiene oxygenase (Cytochrome P450 71D55)*	*Hyoscyamus muticus*	8.11
	Q05047	*Secologanin synthase (Cytochrome P450 72A1)*	*Catharanthus roseus*	<11.58
Xenobiotic detoxification Phase 2	Q03663	*Glutathione S-transferase*	*Nicotiana tabacum*	13.78
	P32111	*Glutathione S-transferase (Pathogenesis-related protein 1)*	*Solanum tuberosum*	25.97
	O65857	*Glutathione S-transferase GSTF1*	*O. sativa subsp. japonica*	<26.25
	Q06398	*Glutathione S-transferase GSTU6*	*O. sativa subsp. japonica*	<27.20
	Q03664	*Glutathione S-transferase*	*N. tabacum*	41.47
	P46420	*Glutathione S-transferase F4*	*Z. mays*	<8.95
	A2XMN2	*Glutathione S-transferase GSTU1*	*O. sativa subsp. indica*	<27.60
	Q06398	*Glutathione S-transferase GSTU6*	*O. sativa subsp. japonica*	<141.48
	Q9FUS8	*Glutathione S-transferase U17*	*A. thaliana*	<79.29
	Q9FUS9	*Glutathione S-transferase U18*	*A. thaliana*	<73.55
	P46421	*Glutathione S-transferase U5*	*A. thaliana*	10.52
	Q8H8U5	*Glutathione S-transferase GSTZ5*	*O. sativa subsp. japonica*	3.11
	Q9CA75	*Glycosyltransferase 5*	*A. thaliana*	39.91
	D4Q9Z4	*Soyasapogenol B glucuronide galactosyltransferase (UDP-galactose:SBMG-galactosyltransferase)*	*Glycine max*	10.94
	Q9SCP5	*UDP-glycosyltransferase 73C7*	*A. thaliana*	5.54
	O23406	*UDP-glycosyltransferase 75D1*	*A. thaliana*	<116.17
	Q9SGA8	*UDP-glycosyltransferase 83A1*	*A. thaliana*	7.19
	Q9ZWJ3	*UDP-glycosyltransferase 85A2*	*A. thaliana*	3.35
Xenobiotic detoxification Phase 3	Q9LYS2	*ABC transporter C family member 10*	*A. thaliana*	<7.35
	Q9LZJ5	*ABC transporter C family member 14*	*A. thaliana*	3.65
	Q8LGU1	*ABC transporter C family member 8*	*A. thaliana*	<4.26
	Q8LPJ4	*ABC transporter E family member 2*	*A. thaliana*	3.48
	Q9FJH6	*ABC transporter F family member 1*	*A. thaliana*	4.20
	Q9LV93	*ABC transporter F family member 5*	*A. thaliana*	10.00
	Q9LQK7	*ABC transporter I family member 7*	*A. thaliana*	10.13
	Q9LUH2	*MATE efflux family protein ALF5*	*A. thaliana*	7.25

The biomarkers were then tested in biological triplicates for their quantitative expression in the blackgrass populations characterized earlier. The results are represented in ‘heat map’ format showing levels of relative expression in the different lines as referenced to the HS = WTS Rothamsted population (Figure 1). Highly upregulated genes are shown in red, moderately induced in graded orange and down regulation in green.

Of the xenome genes four proved to be useful diagnostic markers of NTSR. The most quantitative of these were the CYP709, GSTF1 and GSTU genes. AmOPR1 was a sensitive maker of NTSR, though its relative expression was not indicative of the level of metabolism based resistance. In contrast, the UGT, MATE, ABCI and TMT genes proved to be poor indicators of NTSR.

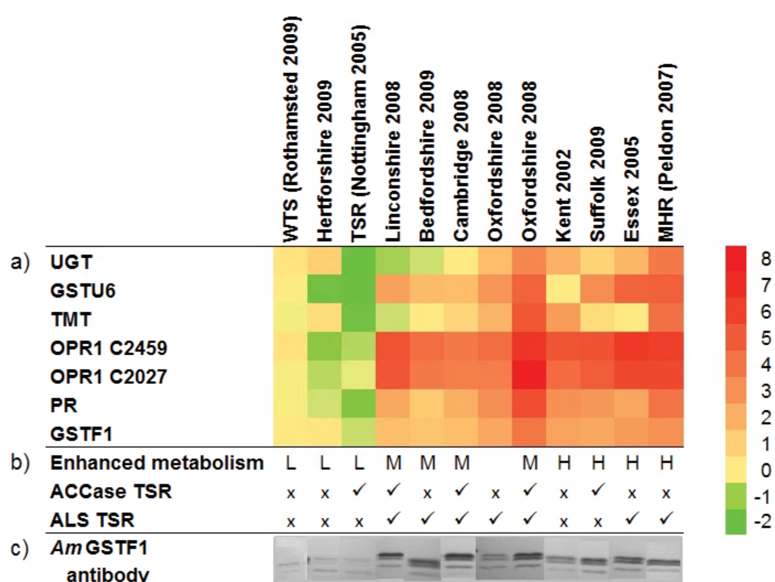

Figure 1: Diagnostic screening of 12 black-grass populations using a) qPCR molecular markers (results displayed as a heat map; red and green indicating up and down regulation); b) interpretation of herbicide application (Low, Medium, or High enhanced metabolism, and absence (x) or presence (✓) of ACCase (Cheetah) or ALS (Atlantis) TSR; c) AmGSTF1 antibody (banding pattern visualised on a western blot).

4.1.4. Identification of novel GSTs and transporters associated with MHR gives new insight into detoxification mechanisms in blackgrass

The transcriptomic data revealed that more than 4000 Unigenes varied more than two-fold between the HS and NTSR populations (Tétard-Jones et al. 2018). To identify the full range of GSTs, along with the ABC and MATE transporter genes associated with MHR in blackgrass, we analysed the transcriptomic data using NCBI species-specific unigene nucleic acid databanks to identify contigs showing differential expression in the transcriptomes of MHR Peldon compared to HS Rothamsted populations (Tétard-Jones et al. 2018). This approach mapped the contigs to 15 distinct GSTs (eight GSTUs, six GSTFs and one GSTL gene), two MATEs, two ABC transporters

from the C subfamily (*Am*ABCC1 and *Am*ABCC2) and two ABC transporters from the B subfamily (*Am*ABCB1 and *Am*ABCB2). Of the eight GSTU putative genes, one sequence corresponded to the previously identified *Am*GSTU1 (Cummins et al. 2009). Hence, the remaining novel unigenes identified were named as *Am*GSTU2-7. Of the GSTF sequences identified in this study, AmGSTF2 and AmGSTF3 sequences were identified in addition to the four AmGSTF1 isoforms.

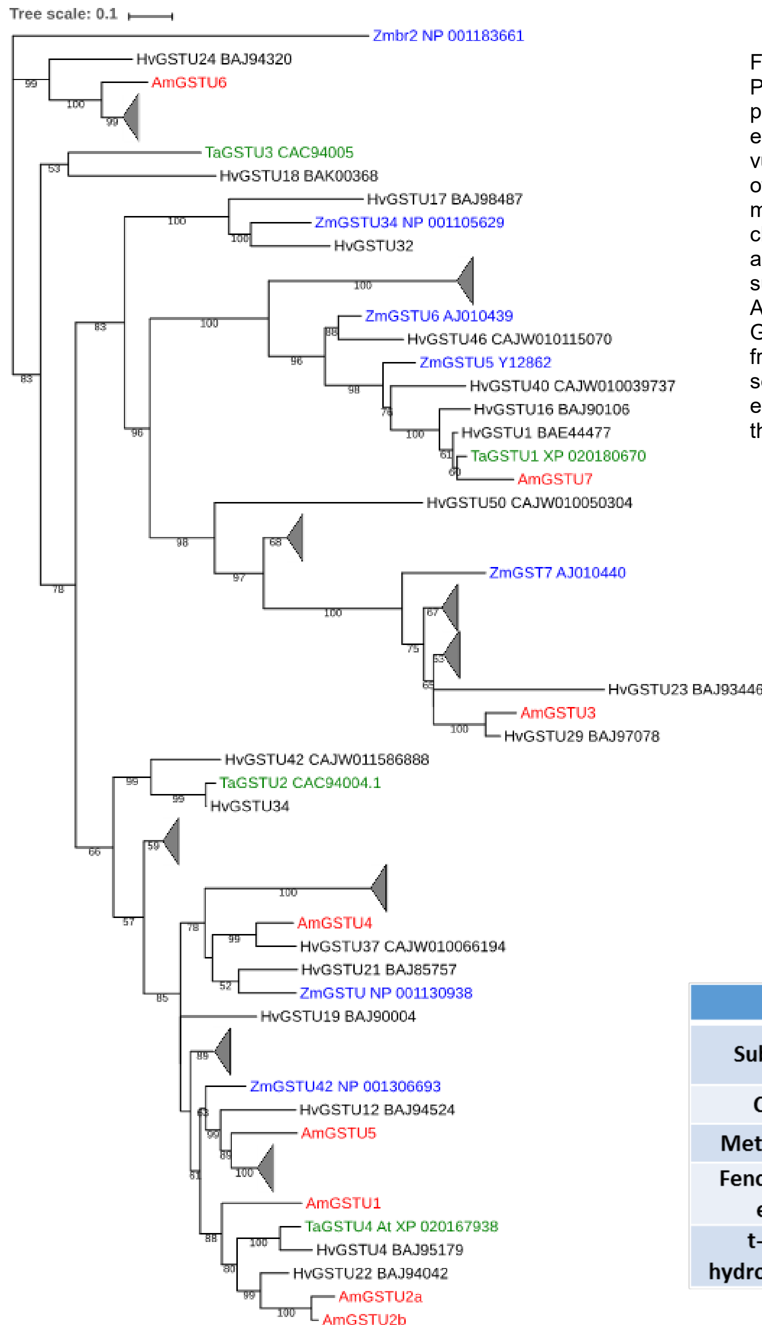

Figure 2 Characterization Black grass GSTU2. **A**. Phylogenetic of GSTU homologues. Maximum likelihood phylogeny inferred from a GSTUs protein alignment with eight black grass (Am, red), all 50 barley (Hv, Hordeum vulgare, black) GSTUs (Rezaei et al, 2013) and a selection of GSTUs from wheat (Ta, Triticum aestivum, green) and maiz (Zm, Zea mays, blue) that includes functionally characterised proteins. Bootstrap support values above 50% are shown and the scale bar indicates the inferred number of substitutions per site. The two AmGSTU2a/b and the AmGSTU1 proteins cluster together whereas the other five GSTU3-7 cluster in other parts of the tree with paralogues from other species. Clans comprising exclusively barley sequences were collapsed into triangles. **B**. AmGSTU2a enzymatic activity against different substrates. The activity of the wt protein was compared with the mutant GSTU2a-S15A.

	Specific activity (nkat mg^{-1} rGST)		
Substrate	**AmGSTF1**	**AmGSTU2a**	**AmGSTU2a S15A**
CDNB	**670 ± 36**	**608 ± 56**	**0.13 ± 0.15**
Metolachlor	**16.9 ± 4.9**	**10.4 ± 1.3**	**0 ± 0**
Fenoxaprop-ethyl	**0.0 ± 0.0**	**0.6 ± 0.1**	**nd**
t-Butyl hydroperoxide	**50.04 ± 13.3**	**0 ± 0**	**0 ± 0**

Using the Peldon cDNA library as a template (Cummins et al. 1999), multiple sequence variants of *Am*GSTU2 were recovered. These were named isoforms *Am*GSTU2a-f with only two entries, AmGSTU2a and AmGSTU2b, being up-regulated in the Peldon transcriptome. The protein sequences of *Am*GSTU that were up-regulated in the transcriptomic data were then subjected to a phylogenetic analysis that included homologues identified in wheat, maize, and barley (Figure 2A). The phylogenetic analysis showed that *Am*GSTU2a and *Am*GSTU2b were most likely derived from a lineage specific duplication as they clustered together (Figure 2A). In contrast, the degree of protein sequence identity between the other six GSTU proteins ranged from 36% to 76%, suggesting they had evolved in isolation from one another. *Am*GSTU2a/b clustered along with *Am*GSTU1, in contrast, the five genes termed *Am*GSTU3-7 were more evolutionarily diverse, clustering to several distinct clades within the GSTU class (Figure 2A). Of the 15 up-regulated GST genes, *Am*GSTU2a showed the highest level of transcript abundance in the NTSR transcriptome therefore it was selected for functional characterization. Enzyme activity assays of the recombinant protein showed that while *Am*GSTU2a conjugated CDNB and the structurally diverse herbicides fenoxaprop-ethyl and metolachlor, the recombinant protein showed no GPOX activity.

Consistent with GSTU2a being an active detoxification enzyme, the catalytic activity of the protein was disrupted when the serine residue in the putative active site domain was mutated to an alanine residue (Figure 2B). The closely related GSTU protein in wheat (*Am*GSTU2 and *Ta*GSTU4 share 69% of sequence identity) has been reported to have detoxification activity against multiple herbicides (Thom et al. 2002). Recently, the capacity of this *Ta*GSTU4 to evolve detoxifying activities toward diverse herbicides has been demonstrated by the directed engineering of the protein (Govindarajan et al. 2015). Together, these suggest that the multiple variants of *Am*GSTU2 are likely to demonstrate a range of conjugating activities toward different herbicides.

Attention was then focussed on the transporter proteins identified in the transcriptomic studies. ABC and MATE transporters are able to confer multi-drug resistance and have been widely study in bacteria, yeast, mammalian cells and arthropod vectors due to their roles in neutralising toxic agents (Chang 2003; Kuroda and Tsuchiya, 2008; Prasad and Goffeau 2012, Epis et al., 2014). In particular, ABC transporters from the C subfamily (MRP) are able to transport a wide range of endogenous substances and xenobiotics conjugated to glutathione (GSH), glucuronate or sulphate (Ishikawa, 1992; Jedlitschky et al., 1996). They were originally identified in cancer cell lines resistance to drugs (Cole et al., 1992). Heterologous expression of Arabidopsis MRPs in yeast showed that AtMRP1 and 2 can transport glucoronate and GSH-conjugated components including the herbicide Metolachlor-GS (Lu et al 1997; Lu et al 1998).

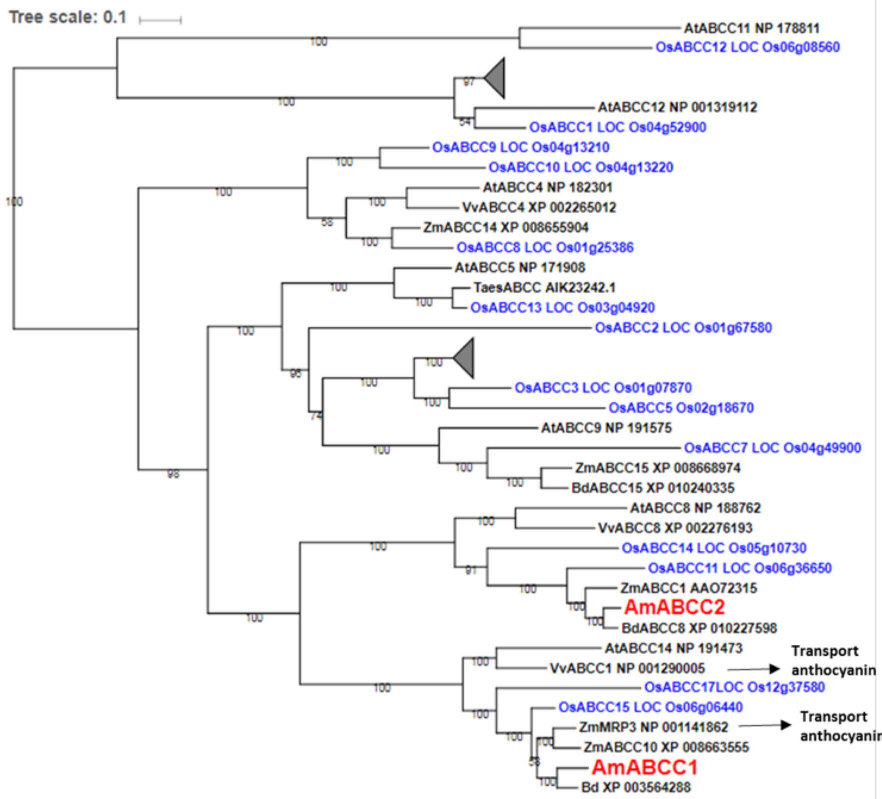

Figure 3. Phylogeny of ABCC homologues. Maximum likelihood phylogeny inferred from an ABCCs protein alignment including (*Am*, red), rice (*Os, Oryza sativa*, blue) and a selection of ABCCs (black) from Arabidopsis (At, Arabidopsis thaliana), maize (Zm, Zea mays), wheat (*Ta*, *Triticum aestivum*), grape vine (Vv , Vitis vinifera) and *Brachypodium distachyon* (*Bd*) that includes functionally characterised proteins. Bootstrap support values above 50% are shown and the scale bar indicates the inferred number of substitutions per site.

Gene clustering analysis was performed based on sequence similarities and the C-family ABC proteins assembled into related clades (Figure 3). *Am*ABCC1 and *Am*ABCC2 have the typical ABC structure with two domains containing a transmembrane (TMD) and nucleotide binding domain (NBD) and an additional TMD at the N-termini, which is present in some ABCC transporters. In yeast, the additional TMD was shown to be important for protein targeting into the vacuole (Mason and Michaelis, 2002). The (*Am*ABCC1 and *Am*ABCC2) proteins share only 41% of identity and in a phylogenetic analysis cluster in different groups (Figure 3).

*Am*ABCC1 cluster in the same group as *Vitis vinifera* *Vv*ABCC1 and maize *Zm*MRP3, both proteins described as being involved in the transport of anthocyanins into the vacuole (Goodman et al., 2004; Francisco et al., 2013)

Having identified these novel AmGSTs and transporter genes, it was then of interest if any putative coupling of functions in detoxification could be linked through associations in their relative expression. Thus, several transcriptomic studies and metabolic network analyses have shown that genes with similar expression patterns are likely to be associated with related metabolic functions (Ihmels et al, 2003, Kim and Buell et al 2015). Most of the ABCC members of Arabidopsis localize to the plant vacuole (Jaquinod et al 2007). Since GSTU2 can conjugate GSH to herbicides, the ABC transporters and GSTs could be part of the same metabolic pathway within the plant xenome (the collections of proteins involve in xenobiotic detoxification), and conferring in that way metabolic resistance to herbicides.

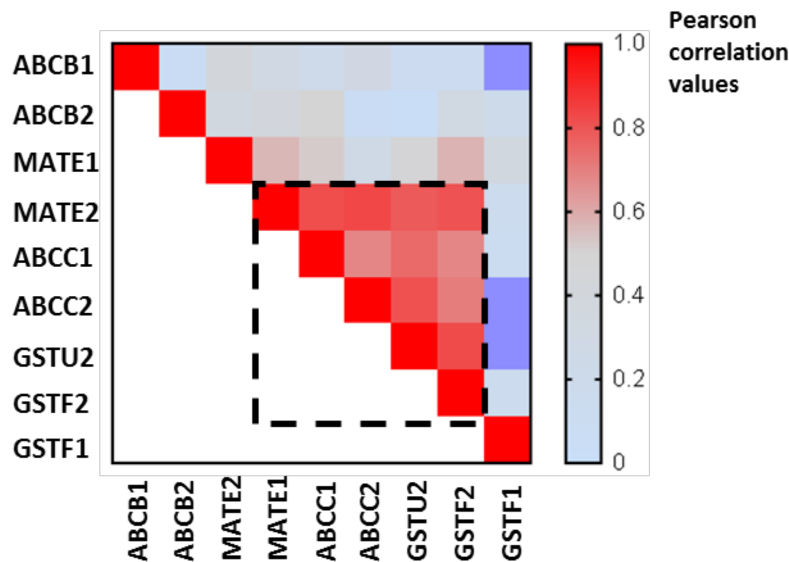

Figure 4 . Heat map showing a Pearson correlation coefficient of the expression of transporters from the ABC and MATE families and 3 genes from the GSTs family, across plants with different susceptibilities to herbicides. Values within the dashed squares are between 0.6 and 0.97. P < 0.05 .

Using eight populations of blackgrass, relative levels of expression of transporter and GST genes were compared to establish any potential coupling in regulation and function in MHR (Figure 4). While the GSTs are up regulated in most populations, ABCC transporters and MATE2 are particularly up regulated in populations with high rate of herbicide detoxification (data no shown). Pearson correlation values indeed suggested that there is a potential functional association between ABC-C1, ABC-C2, MATE2, GSTU2, and GSTF2 respectively (Figure 4).

4.1.5. Investigating the potential role of epigenetics in MHR in blackgrass.

In order to investigate whether Epigenetic mechanisms may be associated with the changes of gene expression observed in MHR blackgrass, efforts were focused on addressing 1) whether the changes in gene expression that occur as a consequence of herbicide treatment maintained in the post-treatment period, 2) is there existing variation in gene expression in susceptible varieties that may form the basis of the resistant individuals that survive treatment and 3) can differences in the DNA methylation status of key genes be detected between susceptible and resistant individuals?

To address question whether enhanced gene expression following herbicide treatment were maintained post-treatment, the upregulation of the highly induced *OPR1* and *GSTF1* genes was used as a proxy. Tillers of individual HS blackgrass (Rothamsted) were split to produce pairs of genetically identical plants. One tiller of each pair was sprayed with a half field rate of the herbicide "Cheetah" (active ingredient Fenoxaprop) and the other left untreated. RNA was extracted from both sprayed and unsprayed individuals 6 weeks post-treatment. Quantitative rtPCR was then used to assess the expression levels of *GSTF1* and *OPR1* transcripts (Figure 5). Elevated expression compared to untreated controls was observed for a proportion of plants suggesting that gene expression changes could be maintained in the absence of continued presence of the herbicide but that this was stochastic in nature, as not all plants showed any elevation in

expression on herbicide treatment. These observations were consistent with the potential of epigenetic change (maintenance of gene expression changes in the absence of the continued presence of the trigger), but did not provide conclusive proof.

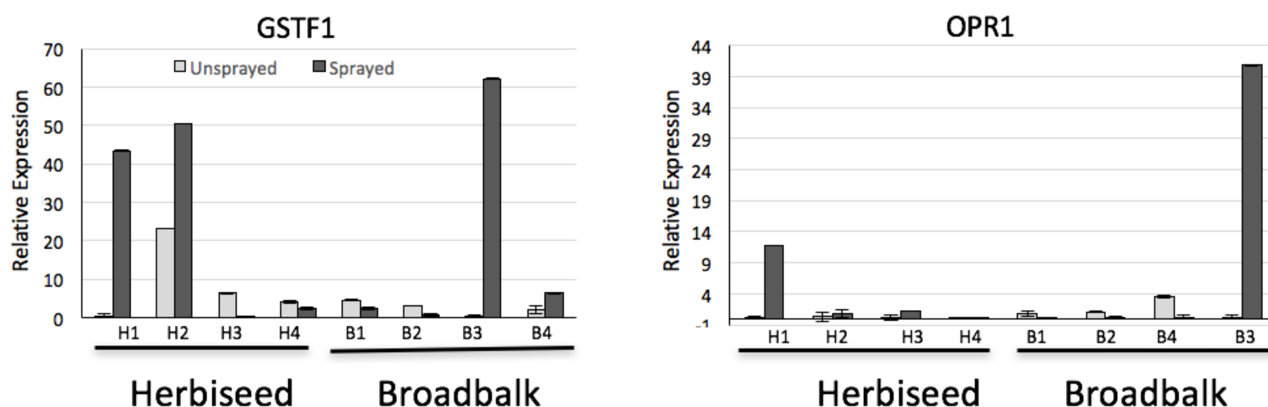

Figure 5. . Expression of GSTF1 and OPR1 in the split-tiller experiment. Data shows expression from 4 sprayed and unsprayed pairs per variety. Elevated expression is seen in some but not all plants.

To address question 2, quantitative PCR was then used to measure expression levels of *OPR1* and *GSTF1* in multiple susceptible and MHR individuals to investigate whether there were pre-existing variations in expression in the HS populations. Variation in expression was observed in both MHR and HS individuals, with transcript levels considerably lower in the susceptible plants (data not shown). There is therefore no strong evidence to suggest that existing variation in gene expression can explain the ease with which herbicide resistance can be selected. However, the data from the split tiller experiment did suggest that there may be differences in the ability of individuals to maintain elevated gene expression post-spray application.

Determination of changes in DNA methylation in *OPR1* and *GSTF1* genes was then used as a test case to determine whether there was evidence of epigenetic changes in the DNA of genes strongly associated with MHR in blackgrass. This was an important experiment, as it is known that stress can induce changes in DNA methylation and that in plants, altered DNA methylation patterns can be heritable. Sodium bisulfite sequencing, which allows analysis of DNA methylation at single-base resolution, was used to investigate the DNA methylation status of the *OPR1* and *GSTF1* genes. Two regions (~300 bp) were analyzed per gene: the promoter region, predicted to overlap with the transcriptional start site and the 5' coding region. No evidence of DNA methylation was found in either region of either gene, with no differences observed between samples from MHR and HS plants. In addition to sodium bisulfite sequencing, two other methods of DNA methylation analysis were used that involved the use of methylation-sensitive restriction enzymes (McrBC, Sau3A), coupled to PCR. These studies also resulted in no evidence of DNA methylation differences in the OPR1 and GSTF1 genes between MHR and HS blackgrass populations. While it cannot be ruled

out that DNA methylation is involved in the changes in gene expression outside of the regions analysed, a genome-wide analysis as opposed to focusing on candidate genes would be required to make definitive conclusions.

4.2. On-farm diagnostic tool for non-target site herbicide resistances in grass weeds

Seed-based testing in petri-dish or spray-testing in glasshouse are the common approaches to assess herbicide resistance status in blackgrass, annual ryegrass and wild oat (Moss, 1999). However, these tests need to be done in a glasshouse or laboratory and take months to complete. To enable growers to do on-farm testing for herbicide resistance in grass weeds, we have developed the world first pocket diagnostic devices to detect MHR in blackgrass in collaboration with partner company, Mologic (Figure 6). The devices are also able to detect MHR in annual and Italian rye-grass and wild oat.

Figure 6. On-farm diagnostic test for MHR in blackgrass

Using the same principle as a pregnancy test, the pocket diagnostic devices are designed to detect a specific biomarker of NTSR in blackgrass, namely the phi (F) class glutathione transferase (AmGSTF1) protein. A high reading of test-line intensity from the diagnostic device corresponds with the high abundance of *Am*GSTF1 protein in blackgrass. To confirm that *Am*GSTF1 is a functional biomarker for NTSR, we analysed its abundance by enzyme-linked immunosorbent assay (ELISA) and using the diagnostic devices in blackgrass populations that have been previously characterised for their herbicide resistance status.

Variation in *Am*GSTF1 protein abundance in black-grass populations were detected by both ELISA and the diagnostic devices (Figure 7A & B). To associate the level of *Am*GSTF1 protein to resistance status the populations were tested for tolerance toward the cell division inhibiting herbicide, pendimethalin using a seed-based petri-dish assay. Resistance to pendimethalin is commonly used as an indicator of enhanced metabolic resistance, one of the important MHR mechanisms. The high abundance of *Am*GSTF1 protein quantified by ELISA and diagnostic devices positively correlated with pendimethalin resistance in the blackgrass populations (Figure 7C). Collectively, our results can demonstrate that the diagnostic devices are reliable and robust tools to detect *Am*GSTF1 abundance and MHR in blackgrass populations.

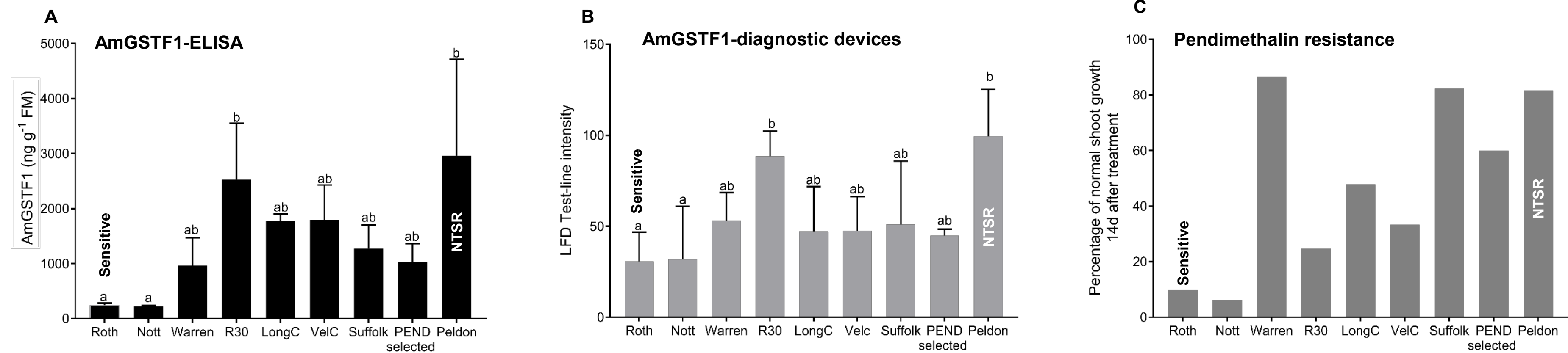

Figure 7 The high abundance of *Am*GSTF1 protein in black-grass populations corresponds with high levels of resistance toward pendimethalin. *Am*GSTF1 protein abundance (Mean± SE, n =3) in leaf tissue of blackgrass populations quantified by ELISA (A) or by diagnostic devices (B). The high test-line intensity corresponds with high *Am*GSTF1 abundance. (C) The resistance to pendimethalin in blackgrass populations. The high percentage of normal shoot growth corresponds with the high resistance to pendimethalin. The levels of *Am*GSTF1 protein were compared among black-grass populations using one-way ANOVA followed by Tukey post-hoc test. Different letters represent statistic differences of *Am*GSTF1 levels among populations.

The on-farm diagnostic devices for herbicide resistance testing in black-grass were launched in June 2018 and made commercially available under the BReD brand (https://www.mologic-bred.co.uk). During the summer of 2018, we demonstrated and evaluated the diagnostic devices through AHDB-sponsored demonstrations around the UK, testing blackgrass samples brought in by growers from different areas. The diagnostic devices were able to detect the variation of *Am*GSTF1 levels in previously uncharacterised blackgrass populations from different fields (Figure 8). These practical demonstrations have further supported the application of the diagnostic devices as on-farm monitoring tool for MHR in blackgrass populations.

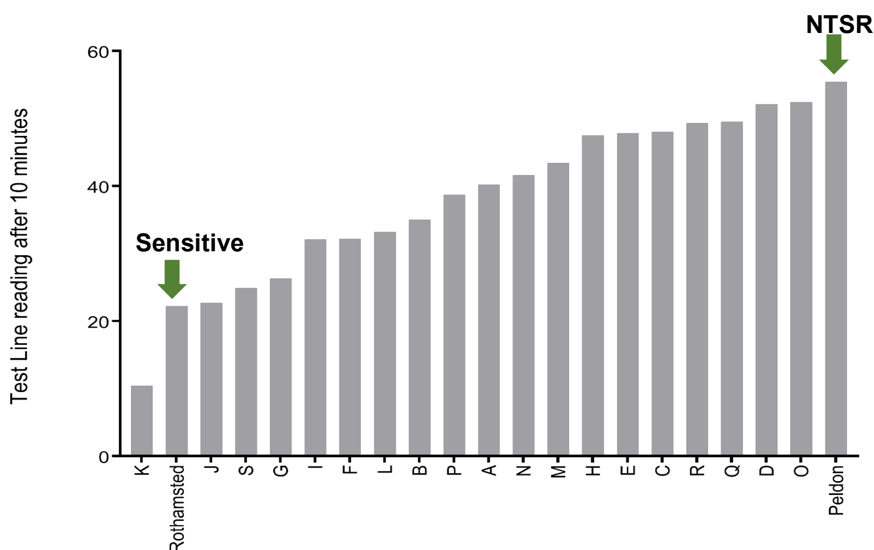

Figure 8. *Am*GSTF1 protein abundance in black-grass populations from different fields in the Suffolk area quantified by diagnostic devices in June 2018. The readings (arrowed) from Rothamsted and Peldon populations were used as an bench-mark indicators of HS and highly MHR populations respectively.

To explore the potential application of the diagnostic devices in other grass weeds, we quantified the abundance of *Am*GSTF1 protein in annual ryegrass and wild oat using ELISA. Interestingly, the protein abundance of *Am*GSTF1 homologues in these grass weeds corresponded with the MHR status of each grass weed population (Figure 9). These results implicate a broader application of diagnostic devices for detecting MHR in other problematic grass weeds.

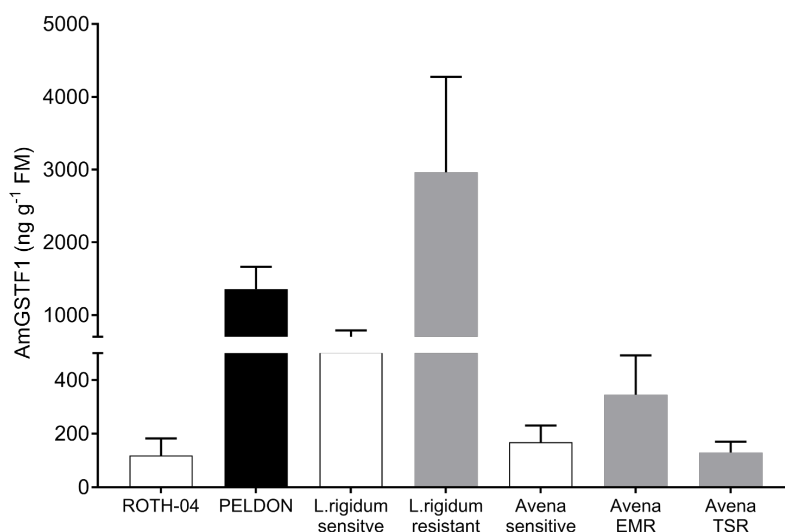

Figure 9 The protein abundance of *Am*GSTF1 homologues in annual ryegrass (*Lolium rigidum*) and wild oat (*Avena fatua*) as quantified by ELISA using *Am*GSTF1 specific antibodies.

4.3. Current extent and evolutionary drivers of resistance

4.3.1. Resistance to post-emergence herbicides

Herbicide resistance screening with the herbicides mesosulfuron, fenoxaprop, and cycloxydim, identified high levels of herbicide resistance amongst UK blackgrass populations, with notable differences at a national scale in the extent of resistance to the three herbicide active ingredients tested (Figure 10). In particular, fenoxaprop resistance was the most widespread, while populations retained the greatest susceptibility to cycloxydim. Some geographical clustering in resistance was observed, with populations in the far Northern and Eastern limits of our sampling range showing lower resistance to the actives mesosulfuron and cycloxydim (Figure 11).

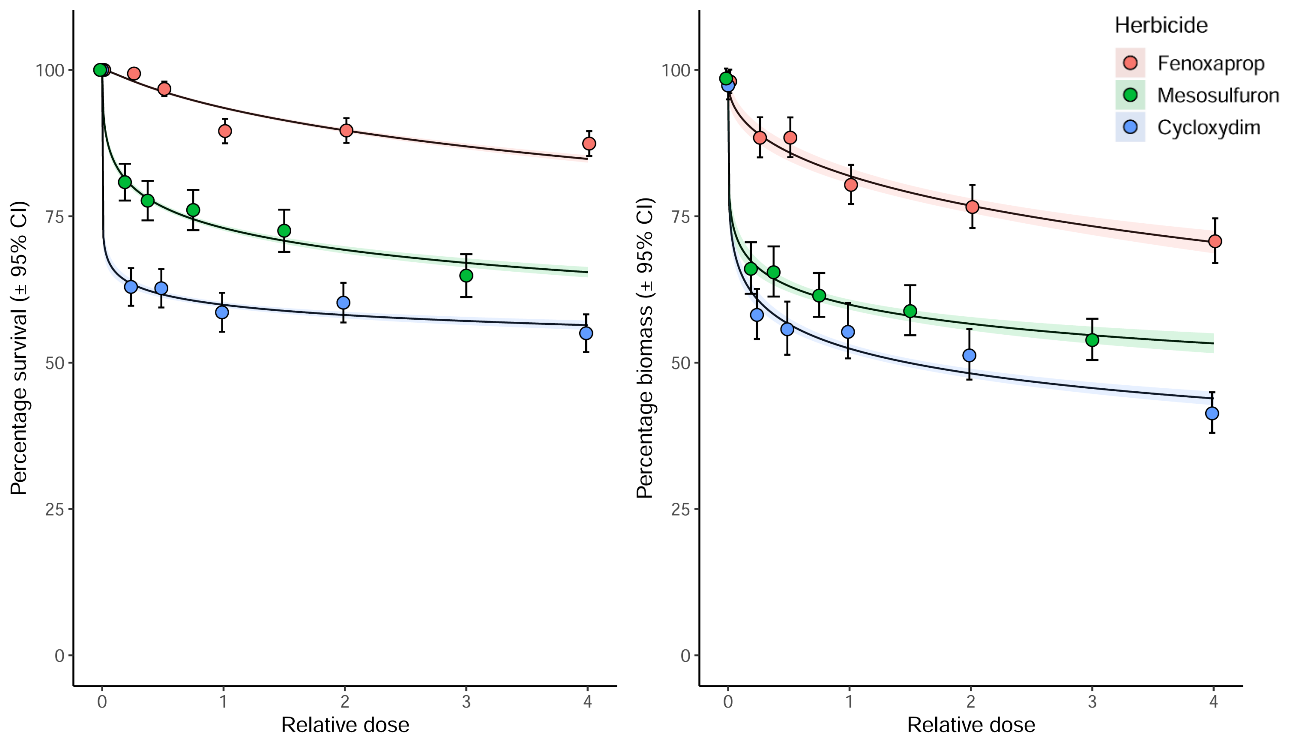

Figure 10: National extent of herbicide resistance in UK *A. myosuroides* populations. Results show fitted dose-response relationships for each of the three tested herbicides using proportional survival and log-transformed relative biomass data from all 132 populations. Bars show the 95% confidence bounds for the fitted relationship at each dose.

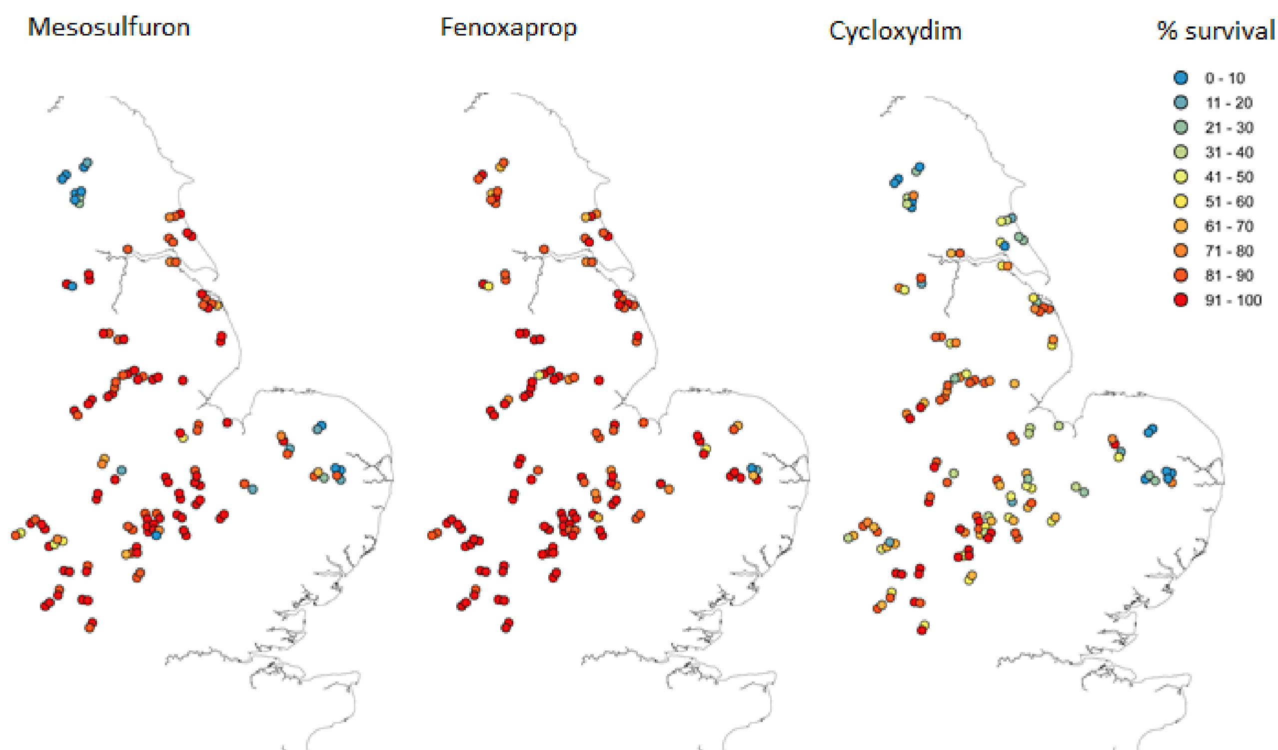

Figure 11: Geographical variation in herbicide resistance in UK A. myosuroides populations. Each point shows the position of a field population of *A. myosuroides,* coloured according to it's percentage survival at a field-rate dose of, from left to right: Mesosulfuron, Fenoxaprop, and Cycloxydim. Mesosulfuron was applied as the commercial formulation 'Atlantis' which also includes the active ingredient Iodosulfuron which has limited herbicidal activity on blackgrass. Fenoxaprop and Cycloxydim were applied as the formulations 'Cheetah' and 'Laser' respectively.

4.3.2. Prevalence of target-site resistance (TSR)

We identified 16 single nucleotide polymorphisms known to result in six ALS and eight ACCase resistance-endowing amino acid substitutions (Table 2). Within the ALS gene, the most prevalent mutation (allele frequency of 0.122) was from cysteine to adenine in the first position of codon 197, resulting in an amino-acid substitution from proline to threonine. Within the ACCase gene, an isoleucine to leucine substitution caused by mutation at the first position of codon 1781 had the highest allele frequency (0.356), and represented the most widespread resistance mutation detected across all tested blackgrass populations (see Table 1 for all detected substitutions and their frequencies). Mutations were widespread across the sampled geographic range, although as with the herbicide survival data, lower levels of TSR were observed in the far Northern and Eastern parts of our surveyed range (Figure 12). Overall, target site mutations within the ACCase gene were more frequent than ALS mutations, with approximately 58% of tested plants carrying one or more (either homozygous or heterozygous) ACCase TSR mutation, and 43% either homozygous or heterozygous for at least one ALS mutation.

Table 2: Frequency of known target-site resistance mutations across all tested UK blackgrass populations. Analysis was performed on 2574 blackgrass plants from populations across the UK using pyrosequencing. Mutations within the ALS and ACCase genes have been characterised, and in each case the nucleotide substitution and resultant amino-acid substitution are shown. 'Frequencies' are the frequency of the mutant allele, calculated from all plants for which sequencing was successful at that position (minimum 1832, maximum 2555 plants).

Gene	Position	WT codon	Mutant Codon	WT protein	Mutant Protein	Frequency
ALS	197	CCC	ACC	Proline	Threonine	0.122
		CCC	TCC	Proline	Serine	0.011
		CCC	GCC	Proline	Alanine	0.001
		CCC	CAC	Proline	Histidine	0.016
		CCC	CTC	Proline	Leucine	0.032
	574	TGG	TTG	Tryptophan	Leucine	0.068
ACCase	1781	ATA	CTA / TTA	Isoleucine	Leucine	0.356
		ATA	GTA	Isoleucine	Valine	0.005
		ATA	ACA	Isoleucine	Threonine	0.003
	2027	TGG	TGC / TGT	Tryptophan	Cysteine	0.010
	2041	ATT	GTT	Isoleucine	Valine	0.000
		ATT	AAT	Isoleucine	Asparagine	0.033
	2078	GAT	GGT	Aspartate	Glycine	0.017
	2096	GGT	GCT	Glycine	Alanine	0.005

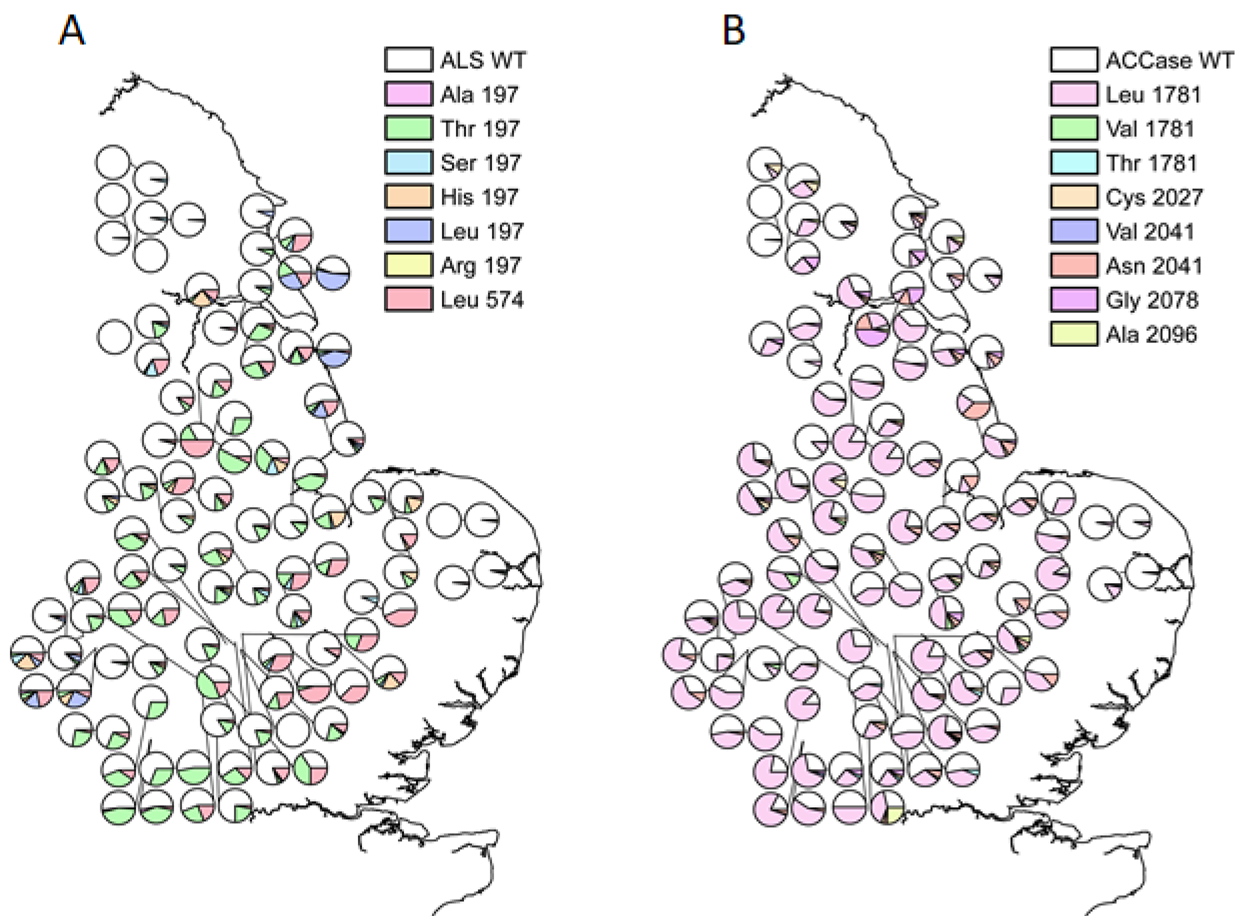

Figure 12: Target-site resistance in UK *A. myosuroides* populations. **A** and **B** show the geographic distribution and relative frequency of amino-acid substitutions in the ALS gene and ACCase gene respectively. White portions of the pie charts show the frequency of wild-type sequence, while coloured portions represent the relative frequencies of each amino-acid substitution (see table 1 for details).

4.3.3. Prevalence of non-target site resistance (NTSR)

The accumulation of AmGSTf1 in blackgrass leaf tissue has previously been shown to correlate with the extent of MHR/NTSR (Cummins *et al.*, 2013). We have assessed foliar concentrations of this protein across our large-scale collection of black-grass populations, to confirm the utility of this biomarker as a diagnostic for this resistance mechanism. Assessment of foliar AmGSTf1 concentrations in populations with a known NTSR phenotype identified significantly greater accumulation of this protein in populations with high NTSR (Figure 13, A). Across all of the collected populations, significant positive relationships were observed between foliar concentrations of this protein and percentage survival when screened with mesosulfuron and fenoxaprop, but not cycloxydim (Figure 13, B). To determine why no relationship was observed with cycloxydim, we also plotted the proportion of TSR individuals against the observed survival for each herbicide (Figure 13, C-E). An approximately 1:1 relationship was found for cycloxydim, demonstrating that TSR alone conveys resistance to this herbicide. Survival of mesosulfuron and

fenoxaprop was considerably greater than expected from the frequency of target-site resistance alone, confirming the importance of NTSR in these actives. These results confirm the utility of the AmGSTf1 protein as a measure of NTSR, providing the first large-scale validation of this protein biomarker in *A. myosuroides*. Additionally, these results provide empirical evidence to support that target-site mechanisms alone convey resistance to the herbicide cycloxydim, but NTSR to mesosulfuron and fenoxaprop is widespread.

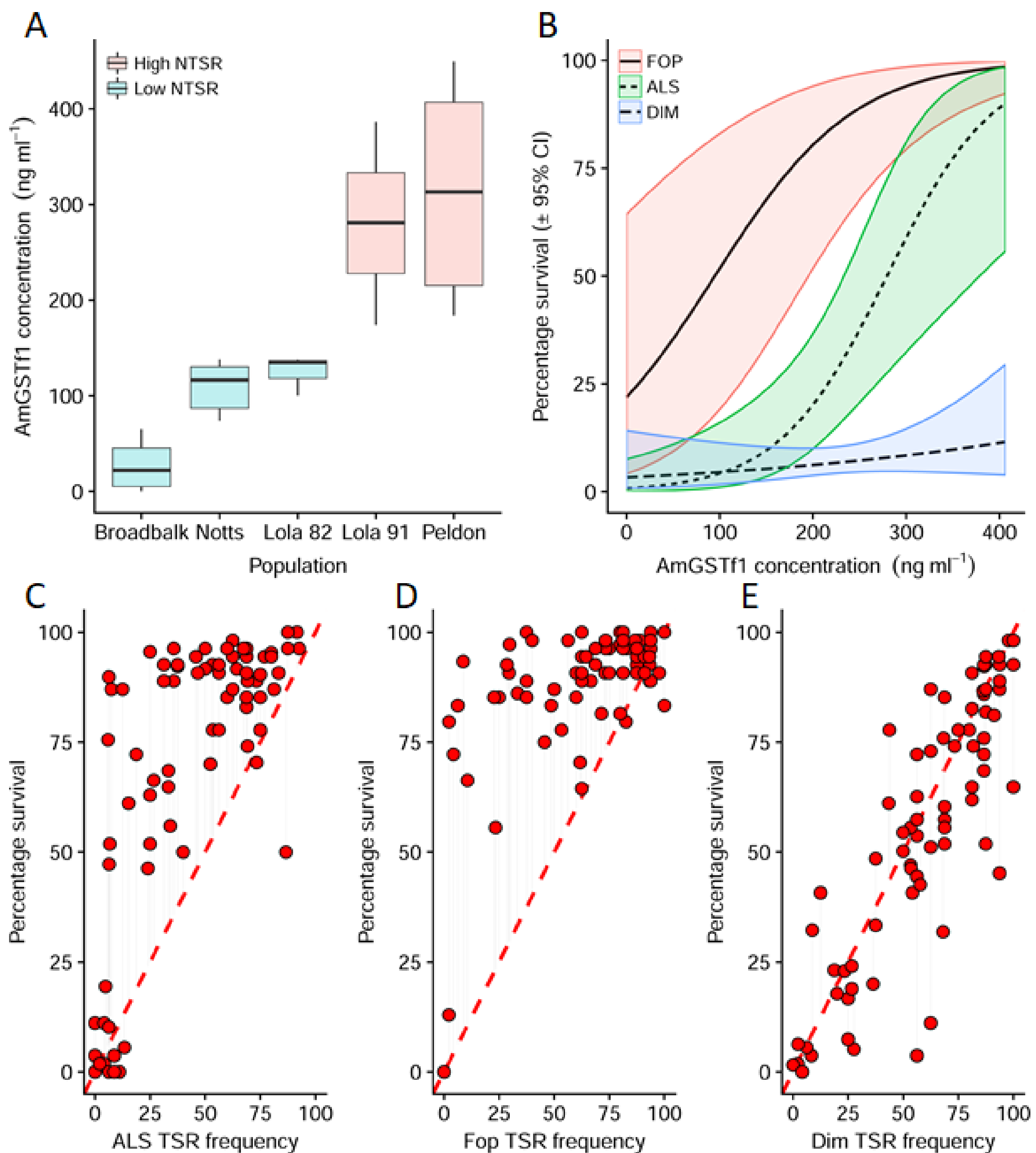

Figure 13: Non-target-site resistance in *A. myosuroides* populations. **A** shows the relative foliar concentration of AmGSTf1 in populations with known contrasting NTSR phenotypes. **B** shows the relationship between AmGSTf1 concentration and herbicide survival for each of the three tested actives. Results are the predicted relationship from a mixed model analysis, with shaded regions representing 95% confidence bounds of the prediction. **C**, **D**, and **E** show the observed proportion of individuals surviving herbicide (Y-axis) versus. the poroportion of individuals carrying a TSR resistance mutation (X-axis) for the ALS, Fop, and Dim herbicides respectivley. An expected 1:1 relationship is shown by the diagonal line.

4.3.4. Variation in glyphosate usage and sensitivity

Glyphosate usage across the UK has risen considerably, with an approximately eight-fold increase in the area of cereal crops receiving glyphosate treatment over 1990 to 2014 (Figure 14, a). To establish if glyphosate usage has changed over time at our survey locations, a set of 67 fields with a full herbicide application history for the period 2006-2014 were assessed for temporal trends in glyphosate usage. A significant increase in glyphosate use over this time was observed (Figure 14, b), confirming that selection pressure for a glyphosate resistance trait is likely increasing within UK agriculture.

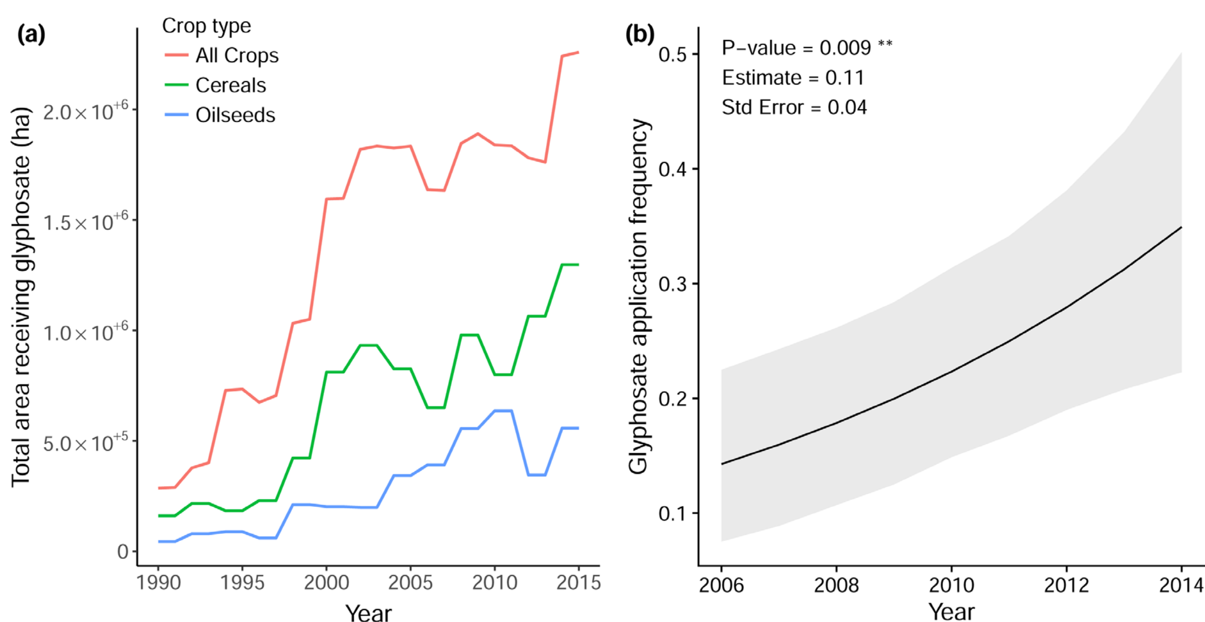

Figure 14: Historical pattern of glyphosate usage within Great Britain. (a) total area (ha) receiving glyphosate treatment. Data collated from the FERA pesticide usage survey (https://www.fera.co.uk). (b) The estimated change in annual frequency of glyphosate applications ± 95% confidence bounds (shaded region) following generalized linear mixed-model analysis of glyphosate usage histories from 67 fields with a complete set of data spanning nine years from 2006-2014. P-value and estimated slope of the relationship are shown (Z-statistic = 2.613 with 598 residual degrees of freedom).

Glyphosate sensitivity screening for all collected *A. myosuroides* populations identified considerable inter-population variability in survival in response to glyphosate treatment. The recommended field application rate for glyphosate in the UK (540 g ha^{-1}) resulted in a mean population level mortality of 95.4% (±0.6 SE). Notably, percentage mortality at a 405g ha^{-1} dose (0.75x field rate) varied from 94% to 15% amongst populations (Figure 15). Estimated LD_{50} values (dose resulting in 50% mortality) ranged from 230 g ha^{-1} to 470 g ha^{-1}. These results demonstrate that, in comparison with the three herbicide actives previously tested, a current field-rate dose of glyphosate remains effective for control of current blackgrass populations. Nevertheless, the levels of variation observed suggest that glyphosate may not provide complete control of treated plants in

field situations, with high levels of survival recorded at doses close to field application rates.

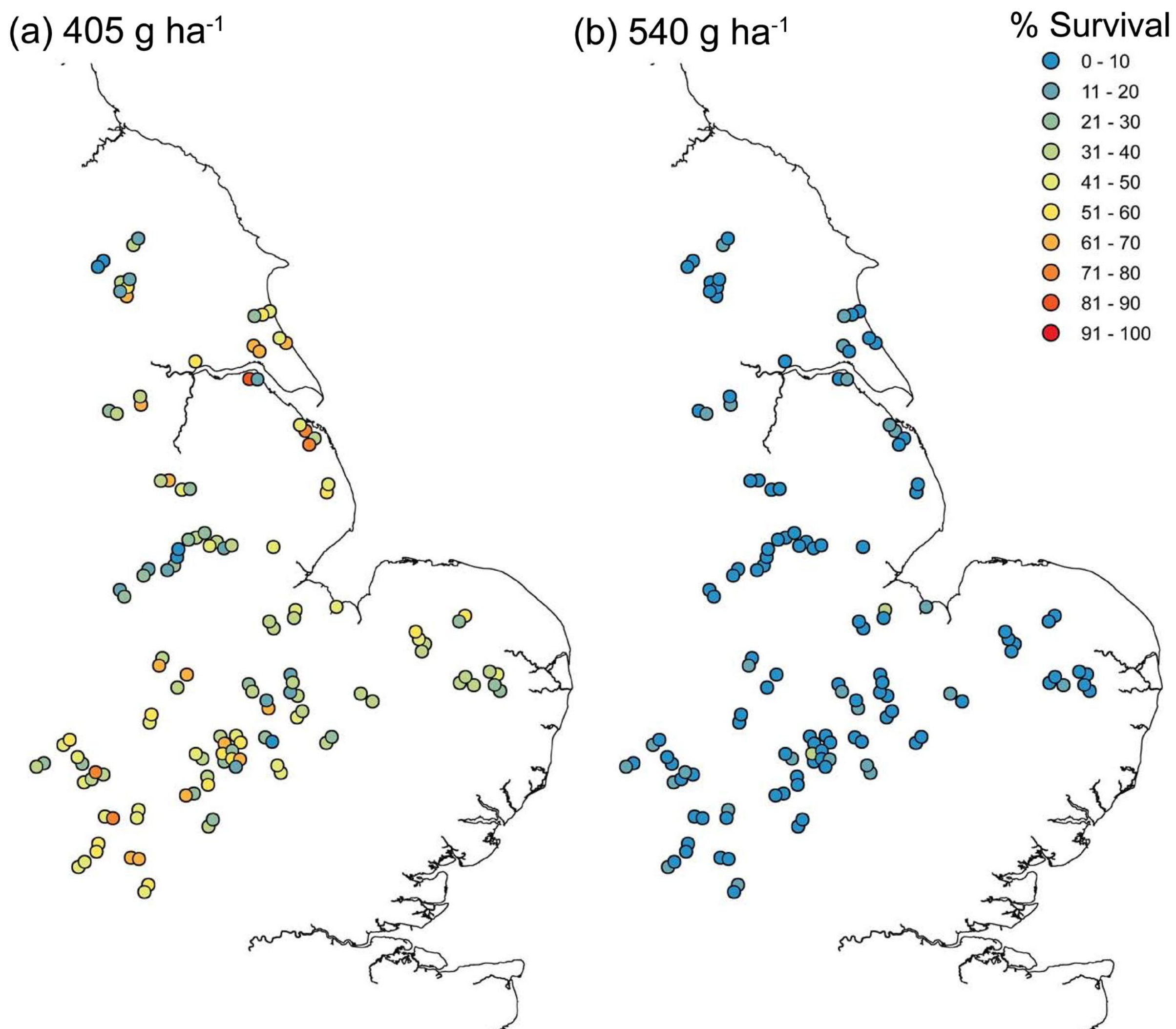

Figure 15: Geographical variation in glyphosate sensitivity amongst UK *A. myosuroides* populations. Colours denote percentage survival of each population following a glasshouse whole-plant assay at (a) 405 g ha^{-1} glyphosate treatment (0.75x field rate), and (b) 540 g ha^{-1} glyphosate treatment (field rate). Survival was assessed for 18 plants per population per dose, three weeks after herbicide application.

4.3.5. Heritability of glyphosate sensitivity and response to glyphosate selection

We found that the fitness of plants following glyphosate application was heritable, demonstrated by a significant response to glyphosate selection amongst a subset of populations from the initial glyphosate sensitivity assay. Dose-response analysis of progeny from survivors of glyphosate application indicates that the LD_{50} values of the once-selected population is significantly greater than for the baseline population (Figure 16), with several survivors at the glyphosate field rate (540 g ha^{-1}). This demonstrates that field-collected populations which showed the least sensitivity to glyphosate have not yet reached a ‘sensitivity threshold’, but instead can still respond to glyphosate selection, further reducing glyphosate sensitivity.

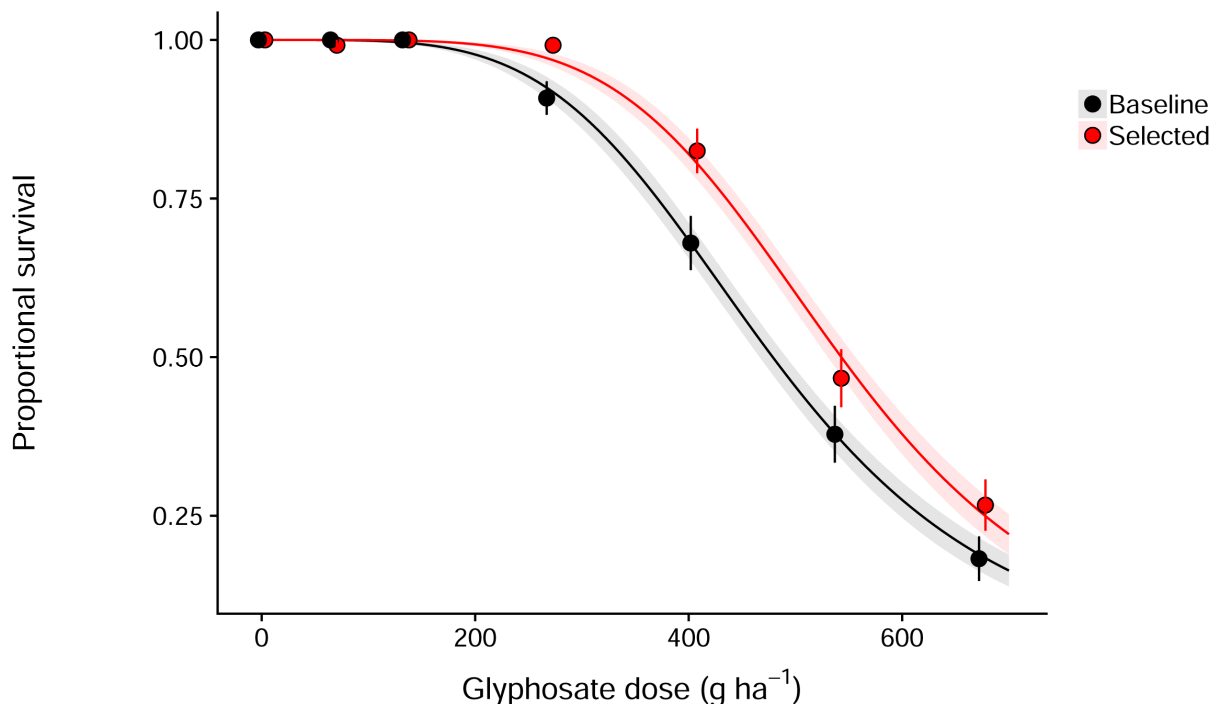

Figure 16: Response of *A. myosuroides* to further experimental glyphosate selection. Dose-response analysis of progeny derived from survivors of glyphosate application. Solid lines show the fitted binomial log-logistic regression models, with shaded regions representing the 95% confidence bounds of the fitted relationship, and points showing the mean proportional survival ±SE at each tested dose.

For populations where historical glyphosate selection pressure could be established from field management histories (*N*=89), selection intensity was found to be a significant predictor of the glyphosate LD_{50} (Table 3). The relationship was positive, indicating that increased selection pressure is driving an evolutionary response for reduced glyphosate sensitivity. The non-herbicidal use of glyphosate as a pre-harvest desiccant was independently assessed as a predictor of the LD_{50}, but no significant relationship was found. The only other significant predictor of glyphosate sensitivity was blackgrass emergence (Table 3). This variable estimates the mean proportion of the blackgrass population that was exposed to glyphosate treatment. The relationship is positive, suggesting that selection pressure is greater when a higher proportion of the population is exposed to glyphosate. Black-grass population density at the field collection site was not significantly related to the glyphosate LD_{50} however, suggesting that population size was not a major driver of higher glyphosate LD_{50} values or vice-versa. Use-histories for other herbicide modes of action, and the previously characterised population-level degree of resistance to those modes of action, were also not predictive of the glyphosate LD_{50}.

Table 3: Results of a mixed model analysis of the effect of field management variables and phenotypic resistance to other herbicide modes of action on glyphosate sensitivity. LD_{50} values from a binomial log-logistic regression of glasshouse dose-response data was used as the response. Variables representing historic field cultivation and herbicide use, and principal components scores representing phenotypic resistance to other herbicides were included as fixed effects, while farm was fitted as a random effect to account for non-independence. Individual terms were added sequentially, and the significance of each term calculated by comparison to the previous model using parametric bootstrapping.

Fixed effects	DF	Estimate	SE	Sum Sq	P value
Population size and cultivation					
Black-grass density	1	-0.007	0.115	0.793	0.217 ns
Proportion Autumn sown	1	0.408	0.134	0.417	0.517 ns
Black-grass emergence	1	0.270	0.143	3.027	0.026 *
Cultivation score	1	0.150	0.118	0.133	0.661 ns
Herbicide usage					
Glyphosate Sep-May	1	0.452	0.156	3.407	0.008 **
HRAC Turnover	1	0.164	0.138	1.129	0.142 ns
HRAC diversity	1	-0.126	0.152	0.379	0.447 ns
HRAC mixing	1	-0.092	0.121	0.060	0.763 ns
Herbicide resistance					
Atl Survival PC	1	0.277	0.149	1.649	0.081 ns
Cyc Survival PC	1	-0.330	0.157	1.612	0.096 ns
Fen Survival PC	1	0.170	0.129	0.865	0.238 ns

R^2 marginal: 0.240 **R^2 conditional**: 0.565

4.4. Heritability of fitness and life-history characteristics

4.4.1. Fitness costs associated with NTSR

The life-history characteristics of two accessions of blackgrass (one NTSR ‘R’ and one HS ‘S’ biotype) with a standardised genetic background were compared under intra- and inter-specific competition. Results showed that the ‘R’ biotype maintained a significantly greater tiller length during early establishment (Figure 17), leading to a slightly increased intraspecific competitive ability. No differences in tiller number were observed however, and the number and total length of seed heads produced under competition with wheat was equal for both biotypes (Figure 18). These results suggest that the presence of NTSR may be associated with specific alteration of life-history characteristics, but shows no evidence for a direct fitness-cost of NTSR on blackgrass fecundity under competition with wheat.

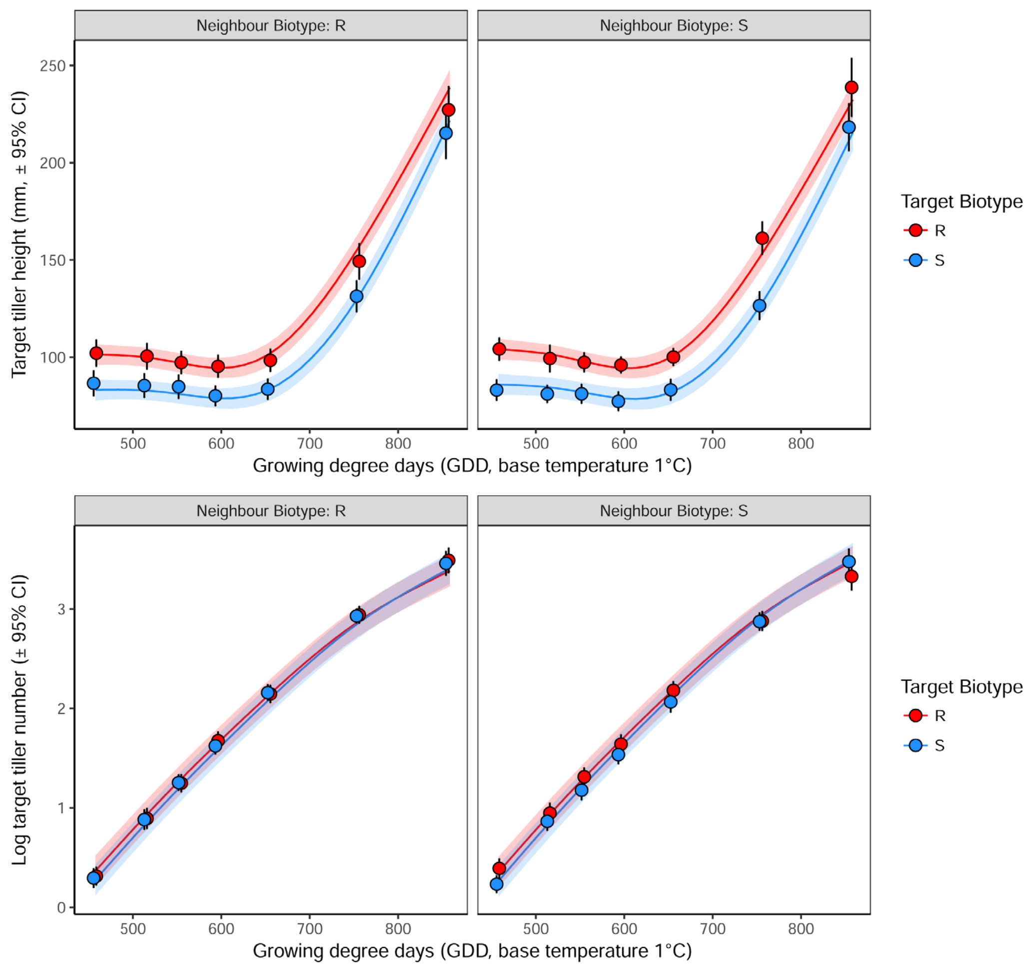

Figure 17: Vegetative development of 'R' and 'S' blackgrass biotypes under intraspecific competition. Results show the maximum tiller length (top) and log transformed number of tillers (bottom) of 'R' and 'S' target plants, under intraspecific competition with either 'R' or 'S' neighbour plants. Measurements were taken non-destructively during vegetative growth. Solid lines show the fitted relationships following analysis using generalised additive mixed models, with shaded regions representing the 95% prediction interval.

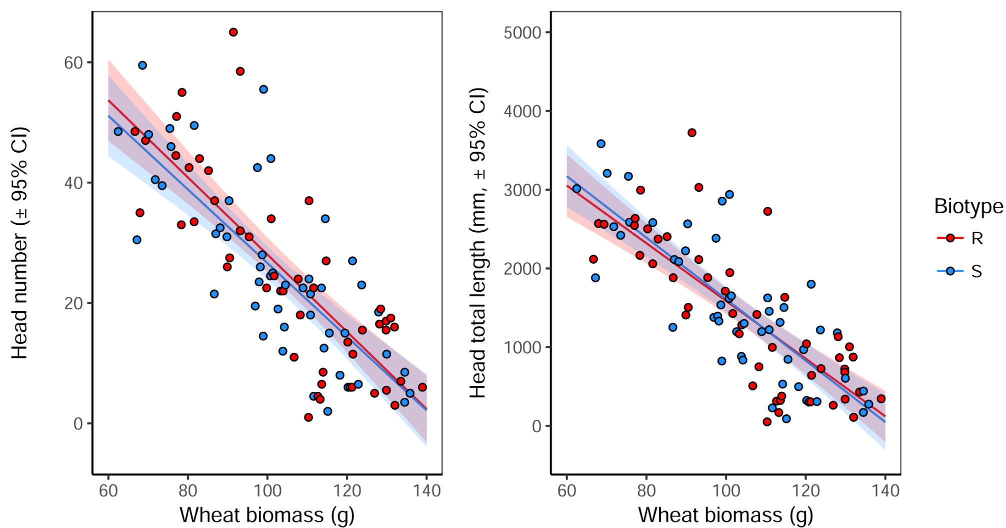

Figure 18: Comparison of 'R' and 'S' blackgrass biotypes at reproductive maturity under interspecific competition with wheat. Solid lines show the linear fitted relationship, with shaded regions representing the 95% prediction interval.

4.4.2. Heritability of life-history traits

Life history characteristics were further assessed in a set of 400 pedigreed blackgrass seed families. Quantitative genetics analysis of this data supports that tiller length has a greater heritability than tiller number, particularly during early growth and establishment (Figure 19). At reproductive maturity, all four measured traits (time to first flower, time to first seed shed, number of flower heads produced, height of the tallest flowering tiller) had a significant heritability (Figure 20, A). In particular the flower head number (representing a measure of fecundity), was found to have the highest heritability. Calculation of the G-matrix revealed both significant positive and negative genetic correlations amongst these traits (Figure 20, B). The time to first flower had a strong positive genetic correlation with time to first seed-shed, confirming that plants which flowered sooner also set seed sooner. Negative genetic correlations were observed between flower height and the time to first seed shed, and between flower height and final flower number. These results demonstrate that plants with shorter flowering tillers generally produce a greater number of heads, and take longer to reach the point of shedding seeds.

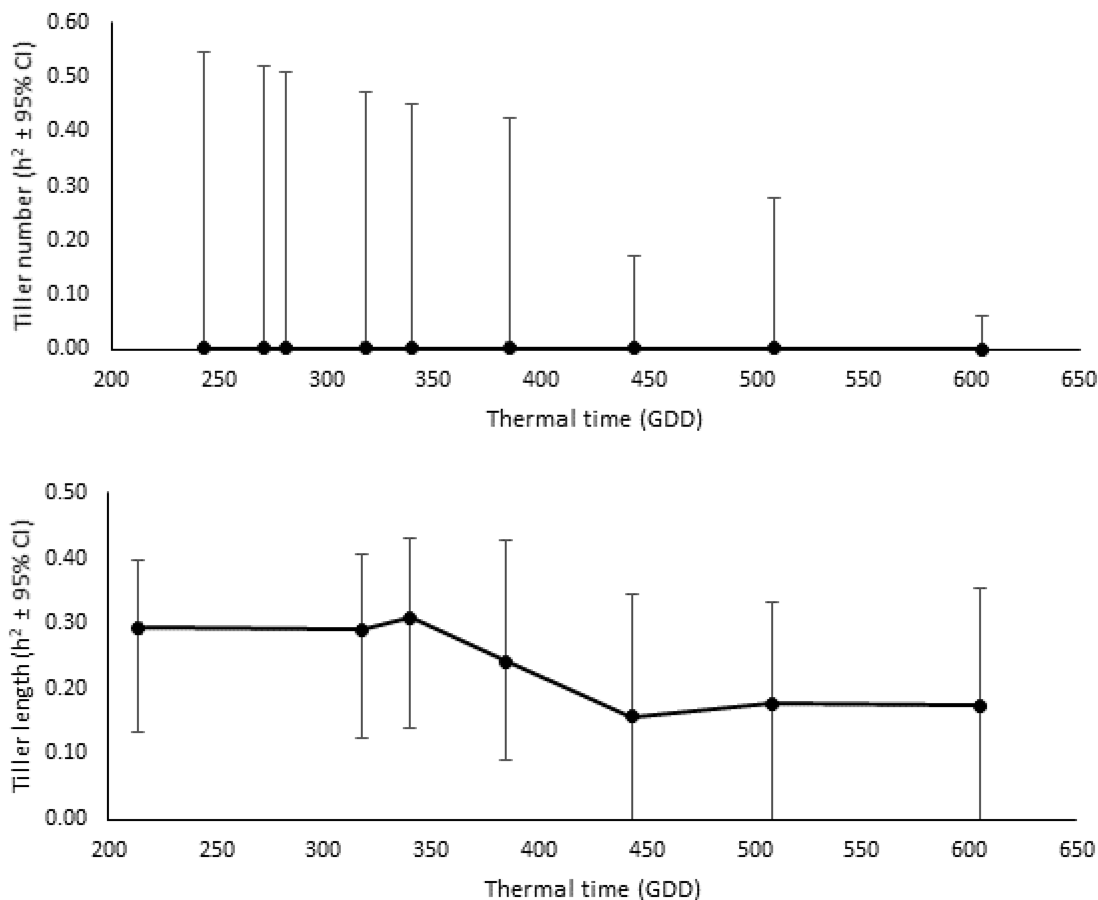

Figure 19: Heritability (h^2) estimates for the number of tillers (top) and length of the longest tiller (bottom), measured non-destructively during establishment and vegetative development for 400 pedigreed *A. myosuroides* families. Points show the heritability estimate, with bars showing the 95% credible interval.

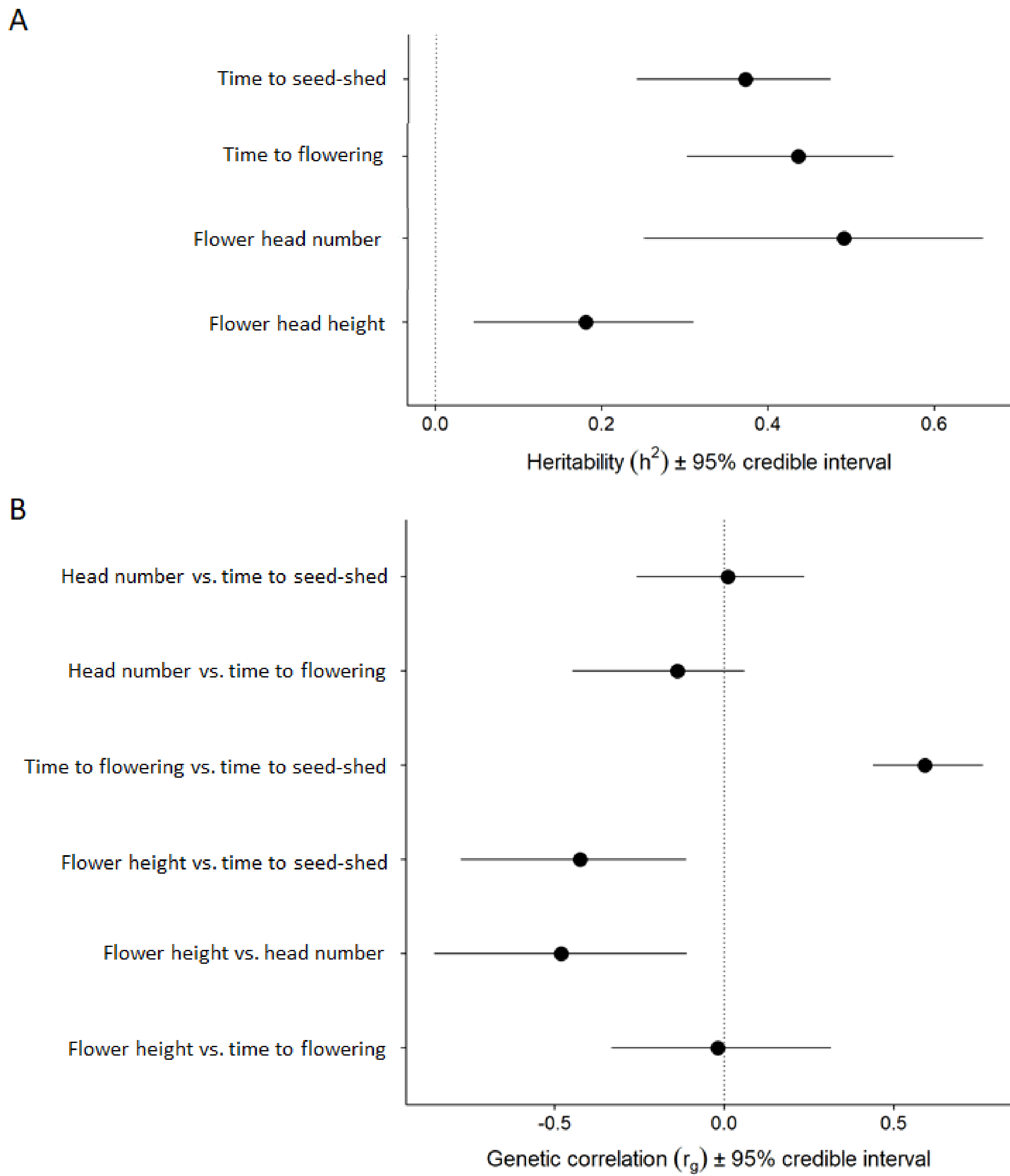

Figure 20: Quantitative genetic analysis of reproductive traits for 400 pedigreed *A. myosuroides* families. **A** shows the heritability estimates (points) with 95% credible intervals (horizontal bars). **B** shows the genetic correlation between each pair of traits. Significant correlations are those whose 95% credible interval does not encompass zero (shown as the vertical dotted line).

4.5. Modelling baseline impacts and potential management strategies

4.5.1. Economic and environmental modelling

A suite of models was developed to estimate (a) the baseline economic impact of black-grass infestation and (b) the environmental and economic implications of changes to farm management strategies aimed at controlling resistant black-grass. The environmental models selected were the Cool Farm Tool (for carbon emissions), DSSAT (for nitrate leaching), and a farmland bird model developed previously by Simon Butler and Ken Norris. Extensive work was needed to convert both the CFT and DSSAT from single-field use to batch use, which is on-going.

An economic model, BGRI-ECOMOD, was constructed that was capable of incorporating a wide range of farm management options and including a user-specified yield penalty for varying levels of weed infestation. It performs gross margin analysis associated with crop enterprises in different years. The model incorporates the effect of variables such as soil type, sowing date, tillage practices, and yield penalties associated with crop sequences. This allows us to estimate the costs associated with a range of management practices aimed at reducing black-grass populations. It is built in R and uses a simple data-entry system. The model can be run for multiple fields and years. This makes it useful not only for estimating economic impacts of current and historical weed infestations, but also for working with very large datasets – thereby enabling more reliable up-scaling to policy-relevant scales – and for aiding within-year decision-making at the field scale or multi-year planning at a farm or landscape scale.

Model tests were carried out on yield and gross margin. For evaluation of yield estimates, we first removed from the dataset any observations (n =13) where a farmer had grown a crop not modelled by BGRI-ECOMOD. The model accurately estimated yield both with (R^2=0.91, slope=1.05) and without (R^2=0.97, slope=1.05) failed crops in the dataset (BGRI-ECOMOD is unable to predict crop failure). We also evaluated yield estimates with the heavy crops (potatoes and sugar beet) removed from the dataset, to remove their influence on the relationship: the model still estimated yield well (R^2=0.74, slope=1.01). Estimated regional gross margin fell within the 95% confidence intervals for the regional values obtained from Farm Business Survey (FBS) data. Furthermore, the model was robust to sensitivity testing on tractor work rates during different tillage operations, which was the management variable for which published data were lacking. We varied the proportions used to calculate tillage work rates in relation to ploughing work rate: the range tested was -30% to +30% (+/-5%, +/-10%, +/-20% and +/-30%) of initial values. There was no effect on the per hectare cost of resistance (results not shown).

The model was, however, sensitive to the yield penalty applied for black-grass infestation. We ran a sensitivity analysis using the lower and upper 95% confidence intervals for the yield estimates at each density state and the consequences of using different yield penalties are given in the results.

We assumed that the estimated costs and yield losses due to black-grass infestation equate to costs and yield losses due to resistance, because resistance drives blackgrass density (Hicks et al, 2018). Three additional factors allow this assumption. First, the yield penalty applied to winter wheat in our model was developed from fields treated with herbicides targeting black-grass: susceptible black-grass would therefore have been controlled, allowing us to ascribe the yield loss and resultant costs to resistant weeds. Second, in the 'no blackgrass' model run, only herbicide applications specifically targeting black-grass were excluded. Other herbicide costs were retained (they were included in the sundry costs). These 'other' herbicide costs included applications targeting a range of weeds: despite not specifically targeting just black-grass, some of these applications would have been effective against susceptible black-grass. Third, baseline yield loss due to incomplete control of susceptible weeds is implicit in the yield estimation function.

To calculate COR across the time span of our dataset (2004 – 2014) we assumed that the density state of a field as recorded in 2014 also applied to all the preceding years for which we had management history data (we had no density data pre-2014). Hicks et al (2018) found slight evidence for a within-field increase in density between 2014 and 2016, and showed that resistance is driving black-grass density. However, this increase in density is not at a magnitude to change the categorical density state of a field unless over a fairly long timescale and could well simply represent normal inter-annual fluctuations. To test the validity of using the entire time span, we re-ran the analysis on just the later part of the time series (2010 – 2014 inclusive). Although this gave slightly higher costs per hectare, the costs estimated using 2010 – 2014 data fell within the 95% CIs estimated using 2004 – 2014 data and *vice versa*. This indicates that the assumption holds for this analysis.

Our aim was to estimate the average cost and yield loss per hectare for different weed densities at a baseline point in time. The baseline chosen was harvest 2014, because this was the first year in which we undertook field surveys of blackgrass density and crop yield. The first step was to derive a yield penalty for each of four weed density states – absent/low, medium, high, very high – using yield and weed density maps (for details, see Hicks et al, 2018). The resulting yield penalties (no/low blackgrass density = 0% reduction in winter wheat yield; medium density = 3% reduction; high density = 12%; very high density 24%) were applied as parameters in BGRI-ECOMOD. We then used BGRI-ECOMOD to estimate costs due to yield loss and herbicide application (chemical + operations costs) for every field in every year for which we had data (maximum date range 2004 – 2014). We did this by running the model both with and without black-grass infestation, and then subtracting the estimated profit (or yield) obtained in the presence of black-grass from the estimated potential profit (or potential yield). Our estimations took into account variability in blackgrass density within each field.

Finally, the costs of resistance in winter wheat were scaled up to regional winter wheat areas (DEFRA, 2014). For each region, we estimated the area of wheat at each blackgrass density state by taking the proportion of BGRI fields at each density state, then multiplying the regional wheat area by these proportions. Next, for each density state and region, these wheat cropping areas were used to scale up the per hectare COR. This methodology ensures that the up-scaling of costs in winter wheat better reflects regional differences in black-grass density. The costs of resistance across rotations were scaled up directly to regional cereal cropping areas (DEFRA, 2014), as we have no data on black-grass density in crops other than wheat.

Because resistance is increasing over time and driving black-grass density (Hicks et al, 2018), we also estimated yield loss and COR in winter wheat under a total loss of herbicide control by assuming that all quadrats in every field were in a very high density state. We do not suggest that such a scenario will occur; however, it is worth estimating these impacts (a) to illustrate the potential consequences of inaction and loss of glyphosate and/or pre-emergence blackgrass herbicides, and (b) to present a frame of reference, allowing the extent of the current situation to be assessed in relation to the worst possible case.

4.5.2. Cost effective integrated pest management in the face of evolving resistance.

While the concept of IPM is well established, for IPM strategies to be adopted by farmers, and used long-term, they must be economically viable. Finding cost effective IPM strategies is extremely challenging. Management tools need to be used in the correct combination and sequence to be effective. This results in a very large number of potential IPM strategies (i.e. different combinations and sequences), even when considering only a handful of management tools and short time horizons. In addition, weed populations are ecologically and evolutionarily dynamic, responding to any management options used.

We framed IPM as a combinatorial optimization problem and used a genetic algorithm to find economically incentivized (measured by gross margin) IPM strategies in the face of evolution. We encode IPM management in a single year as a set of four possible sub-actions, ploughing, crop choice, which (if any) herbicide(s) to apply, and spot control that is cheaper for small weed populations but very expensive for large weed populations. An action is a combination of these four sub-actions taken within one year. An IPM strategy is a sequence of these actions over multiple years. The goal is to find the sequence of actions that maximises time discounted economic reward. While genetic algorithms are not guaranteed to find the optimal IPM strategy they do find a set of actions sequences that perform well on this class of problem, often close to the optimal solution.

We parameterise this problem for blackgrass by modelling a population in which TSR to two herbicides is already present, although possibly at very low frequencies. We assumed resistance to each herbicide is conferred by a single mutation, at independent loci. To allow non-chemical control in the IPM strategies the population model has two seed bank levels so that cultivation can bury seeds, and spring crops and spot control (e.g. by hand weeding) affect survival. We carried out a global sensitivity analysis, testing how those IPM strategies changed across 15,000 plausible parameter combinations incorporating a wide range of economic, biological and psychological factors. This analysis revealed which factors were important in shaping IPM strategies and how those strategies changed. We also tested how IPM strategies changed in response to evolving herbicide resistance. We find that cost effective IPM strategies can change dramatically in response to changes in potential yield losses, and herbicide use is sharply reduced as herbicide resistance evolves.

This problem has four parts: i) a reward function that measures how good a given IPM strategy is based on how much that strategy costs and its effectiveness, we use time discounted gross margin; ii) a population model that translates a given IPM strategy into a population, and thus a reward; iii) an algorithm that finds IPM strategies with higher rewards, the genetic algorithm; iv) finally we need to relate changes in the best IPM strategy found to changes in initial conditions and model parameters. For this we use a meta-modelling global sensitivity analysis based on multi-variate boosted regression trees.

4.5.3. The spatial and temporal scales at which target site and quantitative resistance co-exist.

Pesticide resistance is a complex trait, with a variable number of contributing loci (Warwick 1991, Baucom 2016), complicating efforts to understand and manage its evolution. TSR typically confers very high levels of resistance and incurs few fitness costs (i.e. reductions in survival, growth and/or fecundity) (Baucom 2016, Vila-Aiub MM et al. 2005). At the other extreme, NTSR can be conferred by the small effects of many genes (Delye et al. 2013). NTSR is usually achieved by metabolizing the pesticide, or transporting it away from its binding site (Delye et al. 2013, Baucom 2016), and thus often confers lower levels of resistance and incurs fitness costs (Baucom 2016, Vila-Aiub MM et al. 2005). There is also growing evidence that target site resistance and NTSR exist within the same population (Warwick 1991, Vila-Aiub MM et al. 2005). To test the spatial and temporal scales over which both target site and quantitative resistance can co-exist we developed spatially explicit, density-dependent population models of the evolution of TSR and NTSR in the same population. We then applied this model to blackgrass and tested how initial conditions, with respect to population size and the level of NTSR, influence the evolution of TSR over ecologically and management relevant time scales.

We modelled selection on a single TSR locus where a dominant resistance allele confers resistance with no fitness costs (perfect TSR), against a background of additive genetic variance for the costly quantitative, NTSR trait. NTSR is modelled assuming the infinitesimal model of inheritance and begins with a spatially implicit model that then develops a spatial model where the population exists on a 1D landscape. We then use a discrete time, continuous space, modelling framework that can track the evolution of both continuous (quantitative resistance) and discrete (target site resistance) state variables.

4.5.2 Cost effective integrated pest management in the face of evolving resistance.

Across a wide range of parameter combinations, five parameters were important in shaping IPM strategies (Figure 21a), two related to the yield loss function (Figure 21b) and three biological parameters that control how large the blackgrass population can become if left unmanaged (f_m, f_d and φ_b). Together these top five parameters control the immediate, or potential maximum, economic impact of the weed. The yield loss function describes the relationship between weed density and crop yield (Figure 21b). We could then use a linear yield loss function fit to data from 10 fields, with two parameters, yield when the weed was absent (Y_0) and the effect of weed density on yield (Y_D).

a)

	parameter	rel. inf.
reduction in wheat yield (£.plant^{-1}.ha^{-1})	Y_D	16.5
effect of density on fecundity	f_d	13
yield of wheat when weed absent (£.ha^{-1})	Y_0	10.2
fecundity when density 0 (seeds.plant^{-1})	f_m	9.2
seed survival	ϕ_b	8.1
initial frequency of rarest TSR allele	G_{min}	5.4
discount rate on future returns	γ	4.7
fixed cost of spot control (£.ha^{-1})	η_s^0	4.4
survival under spot control	β	4.4
yield of spring crop (£.ha^{-1})	ϑ	2.9
yield penalty for repeat crop	ϖ	2.9
initial frequency of most common TSR allele	G_{max}	2.8
initial seed bank size	N_{int}	2.6
proportion of population exposed to herbicide	θ	2.4
cost of herbicide application	η_h	2.1
proportion of seed bank moved by ploughing	I	2.1
cost of ploughing	η_b	1.8
variable cost of spot control (£.plant^{-1}.ha^{-1})	η_s	1.6
survival under spring crop	α	1.6
germination probability	ϕ_e	1.5

b)

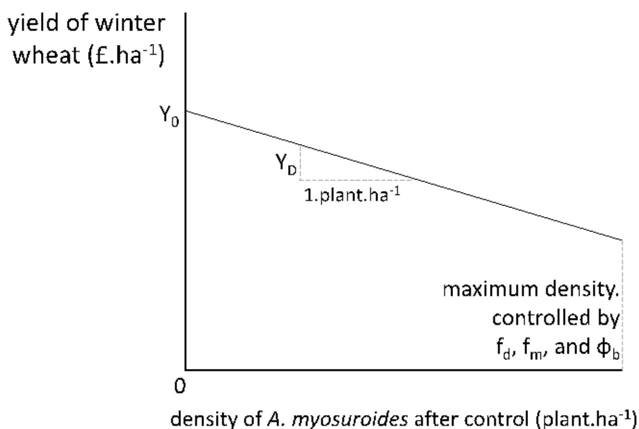

Figure 21. a) Relative influence of each parameter on IPM strategy. Higher values indicate parameters with more influence on the structure of incentivized IPM strategies. Because only relative values are meaningful the index is re-scaled to sum to 100 across all parameters. To find which parameters were crucial in shaping IPM with high gross margin we used multi-variate boosted regression trees to perform a global sensitivity analysis. **b**) How the most important parameters relate to the yield loss function, which maps post-control weed density to the yield of winter wheat.

The shape of the yield loss function is important because it determines how much control is justified. However, knowing which type of IPM strategy to employ may only require an estimate of the pest free yield (Y_0), and one or two threshold values of weed density where a new IPM strategy becomes advantageous (Figure 22):

1. When the yield of winter wheat in the absence of blackgrass (Y_0) is low little management is justified because the value of the protected crop is low. What management interventions are used relies on crop rotation and infrequent, tactical use of herbicide (Figure 22, 'Y_0 low').
2. The strategy changes little when the effect of weed density on yield (Y_D) increases. However, more herbicide is used when the value of Y_D increases from a very low value to a slightly higher value (1% to 12% losses at high densities of blackgrass; Figure 22 g,e).

When Y_0 is high, Y_D shows two thresholds where IPM strategy changes.

1. When Y_D is very low, even the maximum blackgrass population does not cause yield losses high enough to justify expenditure on control and the best strategy is to do nothing (Figure 22h).
2. When Y_D increases slightly the IPM strategy shifts to intermittent use of herbicide and ploughing (Figure 22f).
3. Once Y_D increase enough to justify intensive control the IPM strategy includes continuous herbicide use until resistance develops (Figure 22b,d), at which point herbicide use is reduced (Figure 24). Spot control is used continuously, and the seedbank is rotated every other year. Further increases in Y_D did not change the IPM strategy (Figure 22b,d).

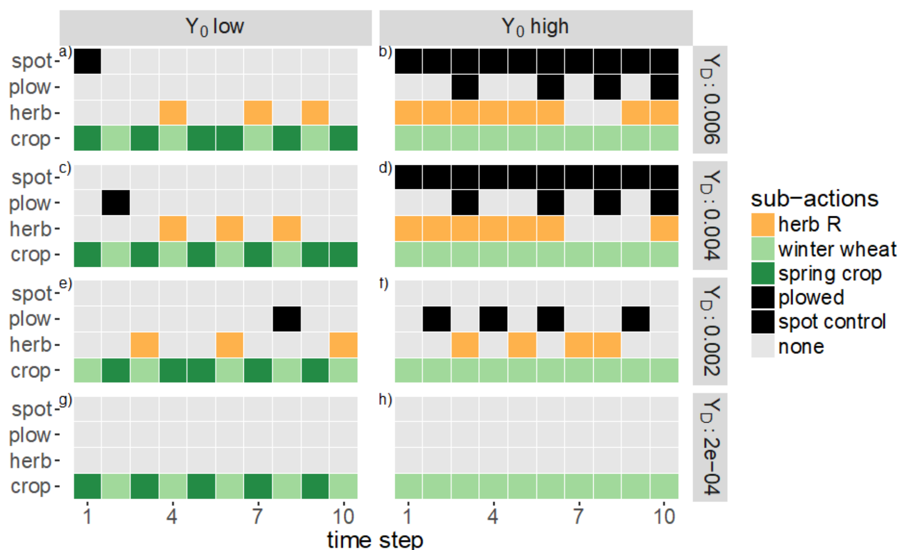

Figure 22. IPM strategies under high (£1668·ha^{-1}) and low (£986·ha^{-1}) values of Y0 (yield of winter wheat with no blackgrass), under increasing values (rows) of YD (£·plant^{-1}·ha^{-1}). At the lower limit of YD very high blackgrass densities result in a 1% yield loss under the high Y0 scenario, and the upper limit implies a yield loss of 35%. There is initially one effective herbicide (R_{int} = 0.0001, Q_{int} = 0.9). Sub-actions are: crop (winter wheat or spring barley), herbicide (herb R, resistance conferred by allele R), plow (to plough or not), spot (to carry out spot control or not).

Intensive management is costly, and so requires returns over several years to justify. As a result, continuous herbicide and spot control to reduce the seed bank is only selected through the genetic algorithm when future returns are given more value (higher values of the discount rate, γ, Figure 23). When crop yields are low the time to pay back investments in weed control increases, and future returns have to be valued even more highly to justify intensive control to drive down the seed bank (Figure 23b).

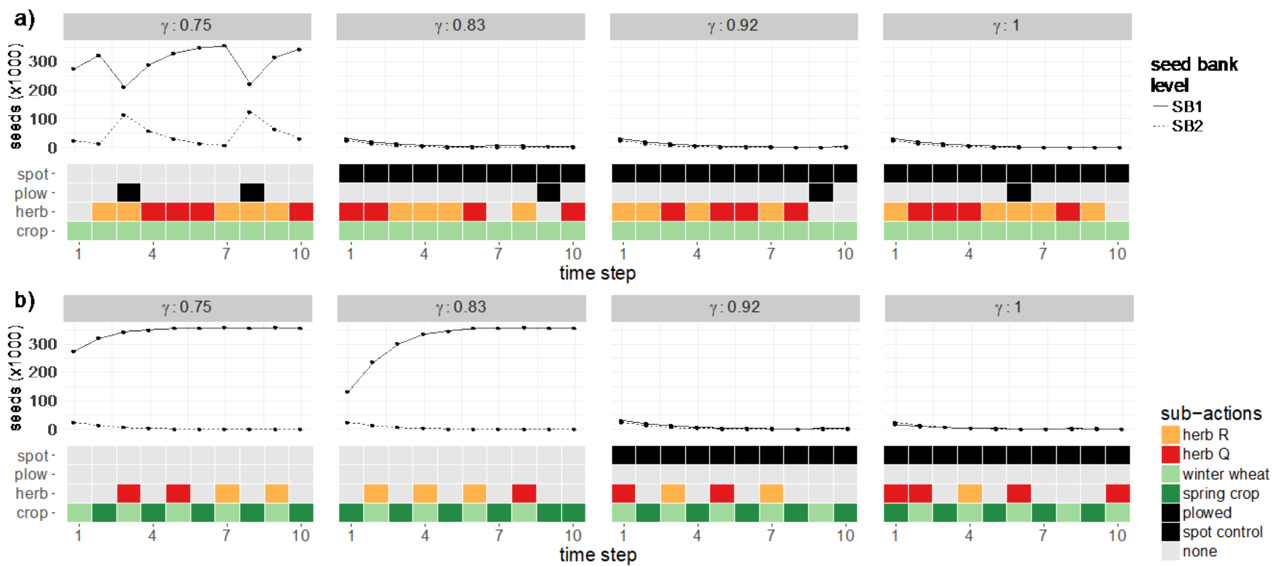

Figure 23. The effect of discount rate (γ) on the seed bank and IPM strategy (tile plots) when yields from winter wheat are high (a; £1668·ha^{-1}) and low (b; £986·ha^{-1}). When γ = 0.75 rewards 5 years in the future are valued at 23% of current returns and when γ = 1 present and future rewards are valued equally. In both cases the slope of the yield function (Y_D) is high (£0.006·plant^{-1}·ha^{-1}). Sub-actions are: crop (winter wheat or spring barley), herbicide (herb R/Q, resistance conferred by allele R or Q), plow (to plough or not), spot (to carry out spot control or not). Although only the first 10 years of IPM strategy are shown, the discounted returns over 25 years are considered by the genetic algorithm.

In our system herbicide resistance incurs a high cost, reducing gross margin (reward) by up to a quarter once high levels of resistance evolve (Figure 24c,d). As a result, IPM strategies are responsive to increasing resistance, drastically reducing herbicide use as higher levels of resistance evolve (Figure 24b–d). Over a wide range of parameters, when both herbicides are effective the preference is to cycle herbicides (using different compounds sequentially; e.g. Figure 23 and 24a) rather than stacking them (using different compounds at the same time). Cycling is favoured over stacking because the application of each herbicide is reduced, prolonging their useful life, without reducing the number of times herbicide was applied. An important assumption behind this result is that we modelled reactive management, where resistant genotypes are already established in the population.

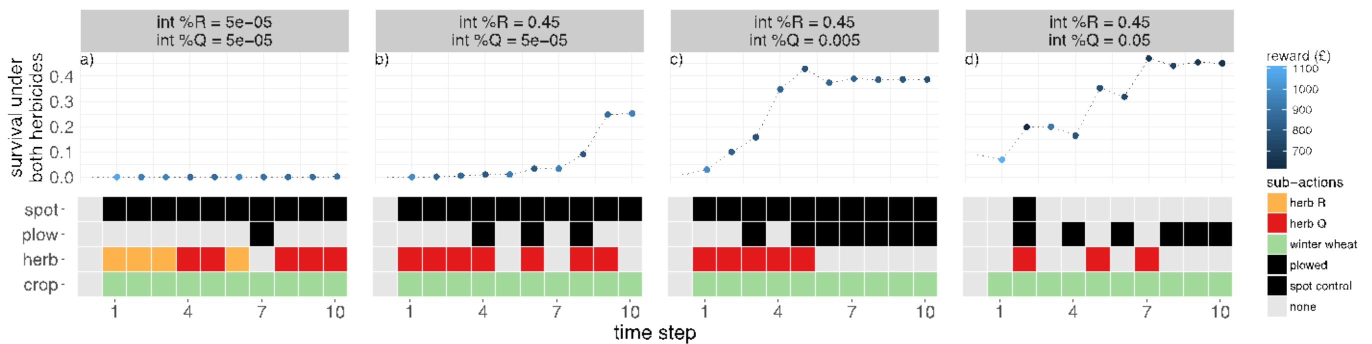

Figure 24. The effect of initial resistance to herbicide R (resistance conferred by allele R) and herbicide Q on the selected IPM strategy (tile plots) and the evolution of herbicide resistance (% survival to under both herbicides). Lighter coloured points indicate higher reward (gross margin) obtained in that time step. The slope of the yield function is high (YD =£0.006·plant^{-1}·ha^{-1}), as is yield of winter wheat (Y0 = £1668·ha^{-1}). Sub-actions are: crop (winter wheat or spring barley), herbicide (herb R/Q, resistance conferred by allele R or Q), plow (to plough or not), spot (to carry out spot control or not).

4.5.3 The spatial and temporal scales at which target site and quantitative resistance co-exist.

As expected, in the absence of TSR, quantitative NTSR can evolve very quickly (within 20 generations). TSR evolves slowly when introduced to the population after quantitative resistance had already evolved (solid pink line Figure 25a). In contrast, TSR evolves very quickly when quantitative resistance is remains low (dotted pink line Figure 25a). Quantitative resistance slows the evolution of TSR through two processes. First, quantitative resistance continued to evolve after the introduction of target site resistance (Figure 25a), increasing target site susceptible survival and thus their average reproduction (since survival is a prerequisite for reproduction). Secondly, fitness costs depend only on the background level of NTSR. As a result, TSR individuals suffer the fitness costs incurred by high quantitative resistance through crossing with target site susceptible individuals, which initially dominate the population. These redundant (for target site resistant mutants) fitness costs maintain a fitness difference between target site susceptible and resistant mutants low for a prolonged period (50 generations, Figure 25b).

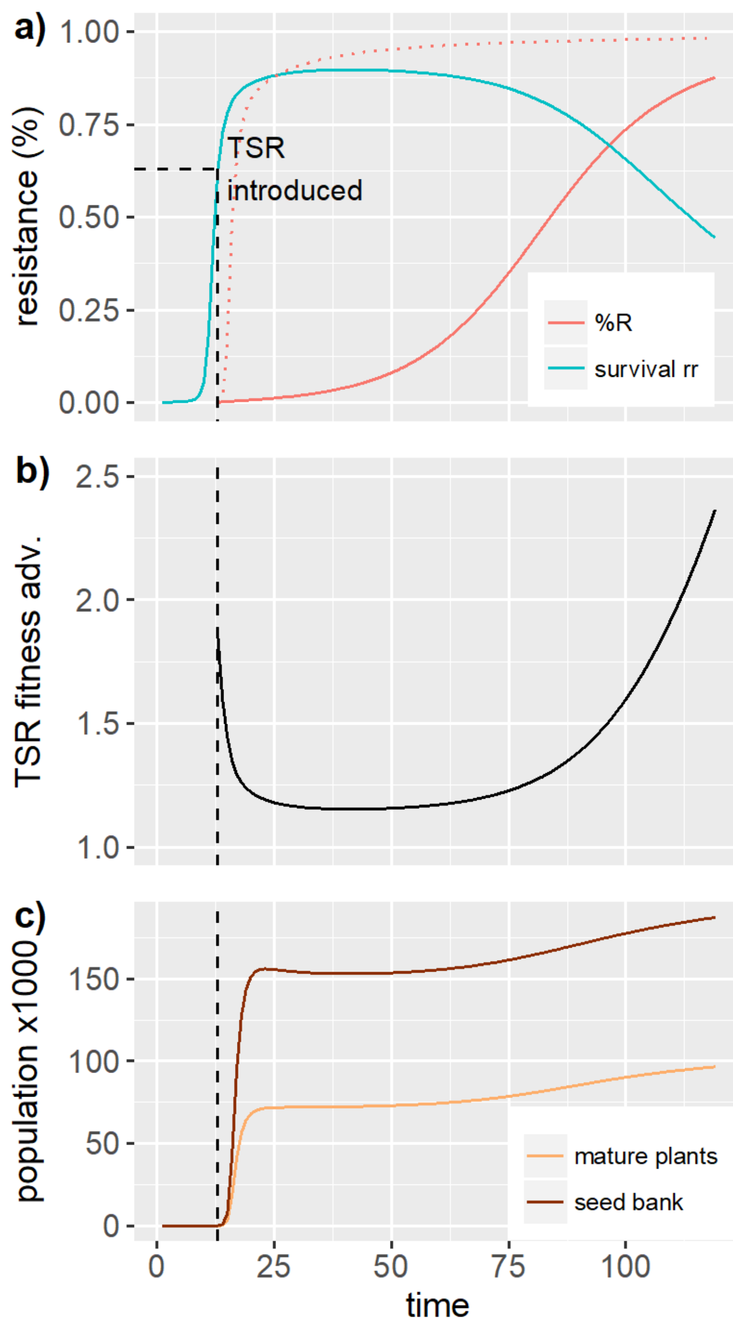

Figure 25. The evolutionary dynamics of target site (TSR) and quantitative resistance over time under herbicide application (a and b) and the population response (c). The initial population of 10 seeds had no target site resistance and low quantitative resistance. This population was exposed to herbicide continuously, and target site resistant mutants where introduced (at a frequency of %R = 0.001) in the first time-step after quantitative resistance conferred more than 50% survival under herbicide. We use three metrics to summarise the evolutionary dynamics, %R, survival of target site susceptible individuals (a) and the fitness advantage of target site resistant individuals (b). The dotted pink line in (a) shows the very rapid evolution of target site resistance under a control run where there was no quantitative resistance.

The rate that TSR could evolve depended on the genetic background into which the target site resistance was introduced. When quantitative resistance was low target site resistance evolved rapidly, reaching %R > 0.7 within 10 generations, unless target site resistant alleles (R) were introduced at a low frequency, and even then 177 %R > 0.7 was reached within 20 generations (Figure. 26c). When high quantitative resistance had already developed two different dynamics occur depending on the initial frequency of target site resistance (Figure 26a). When initial %R = 0.1, TSR could develop rapidly, making 50% of all target site alleles within 10 generations (dark green, Figure. 26a). At the same time quantitative resistance initially increases slightly, but then the dynamic seen in Figure 25b starts to drive down the level of quantitative resistance within four generations.

In contrast, when target site resistance was introduced at a lower frequency (%R ≤ 0.01) quantitative resistance continued to increase rapidly for the next 10 generations and did not begin to decline until after 20 generations. In these cases, the decline in NTSR can confer significant resistance before substantial levels of target site resistance evolve (%R < 0.1; Figure 26a). Again, these marked evolutionary dynamics had little effect on the population dynamics (Figure 26a).

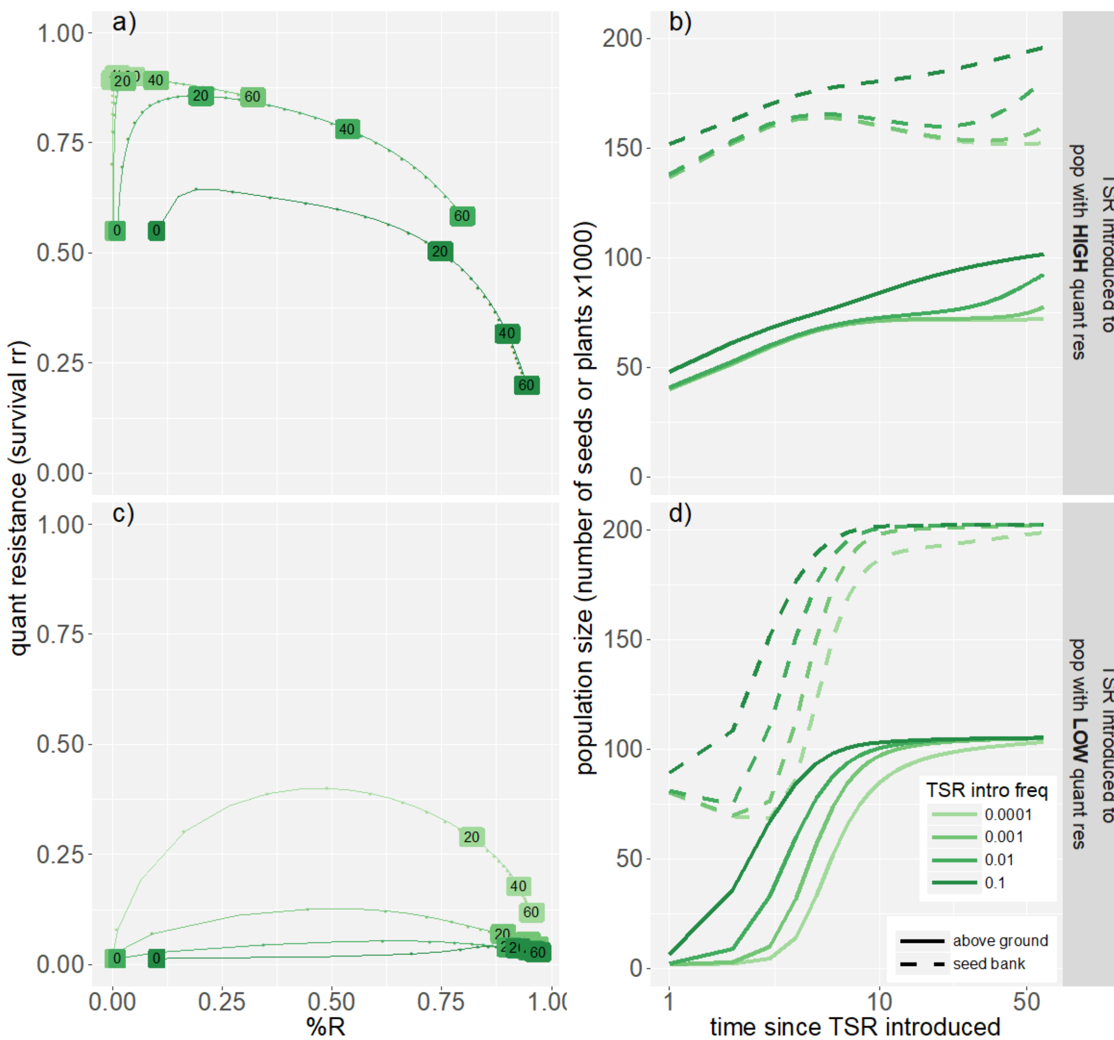

Figure 26. The evolutionary dynamics of target site (TSR) and quantitative resistance over time under herbicide application (a and c) and the population response (b and d) under different initial levels of quantitative resistance and frequency of target site resistance. The initial population of 10 seeds had no target site resistance and low quantitative resistance. This population was exposed to herbicide continuously, and target site resistant mutants where introduced in the first time-step after quantitative resistance conferred more than 50% (HIGH scenario; a, b) or 1% (LOW scenario; c, d) survival under herbicide. In plots of resistance trait space (a, c) points mark two generation intervals and numbered markers show 20 generation intervals, thus the closer markers are together the slower the evolutionary dynamics.

As in the non-spatial model, in the spatial model TSR evolved rapidly at the focal introduction location when the resident population had low levels of NTSR, and evolved much more slowly when the resident population had high levels of quantitative resistance (Figure 27). However, in both cases the spread of target site resistance across the landscape was much slower than its initial evolution at the introduction location. This leads to very rapid changes in target site resistance over space.

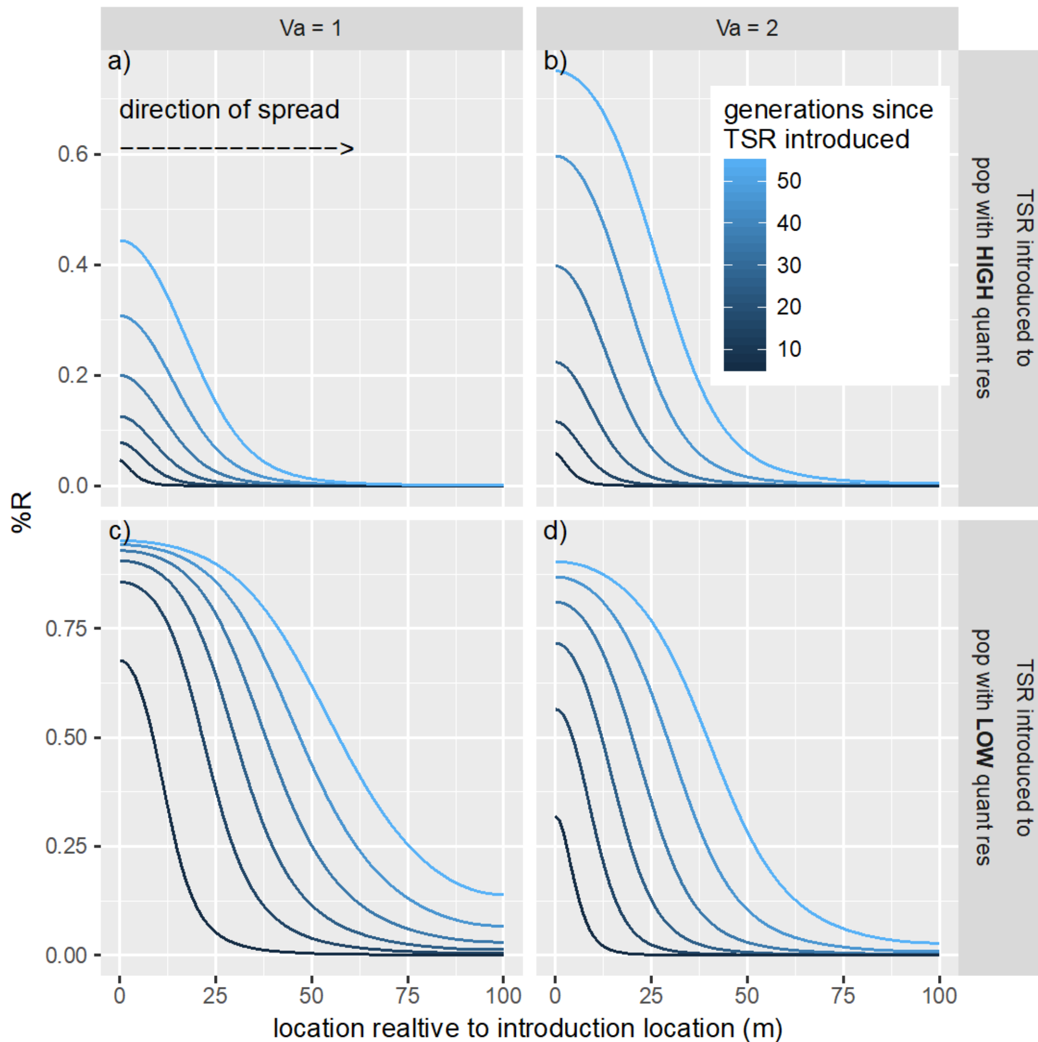

Figure 27. The spread of TSR over a 1D landscape that already contains a target site susceptible population. The resident population was exposed to herbicide continuously, and target site resistant mutants where introduced (at a frequency of %R = 0.1) only to the centre location in the landscape, in the first time step after quantitative resistance conferred more than 50% (HIGH scenario; a, b) or 1% (LOW scenario; c, d) survival under herbicide. Each line shows %R at 10 generation intervals for each location.

4.6. Advances in understanding of baseline impacts and potential management strategies

4.6.1. Economic cost of herbicide resistant black-grass

We calculate that the cost of herbicide-resistant blackgrass in England is £0.5bn p.a., and is associated with an annual wheat yield loss is 1 million tonnes. We then project that a total loss of

herbicide control against blackgrass would cost £1bn and result in 6.8 million tonnes of lost wheat yield annually.

Mean yield loss in winter wheat was 0.5t ha^{-1}, or 6% of the average estimated potential wheat yield (8.3 t ha^{-1}) in the absence of herbicide resistant blackgrass. At the highest blackgrass densities, mean yield loss was calculated at 1.9t ha^{-1}, becoming negligible at low densities (Table 4 & Figure 28a). The economic cost of resistance (defined as the additional costs of herbicide applications and production losses due to resistant black-grass) in winter wheat was, on average, £114 ha^{-1} at low black-grass densities and £473 ha^{-1} at very high densities (Table 4 & Figure 28c). These costs represent 10% and 39%, respectively, of the potential gross profit from winter wheat in the absence of herbicide-resistant black-grass, and compare to average total agricultural costs (English cereal farms, 2014) of £1,076 ha^{-1}. Across all density states, the mean cost of resistance (COR) in winter wheat was £197 ha^{-1}, representing 18% of potential gross profit. COR within density states varied widely, ranging from £0.5-498 ha^{-1} in fields with low densities of black-grass, to £379-802 ha^{-1} in fields with very high densities (raw data not shown). Across a rotation (Table 4 & Figure 28d), the mean COR in low density fields was £89 ha^{-1}, and £292 ha^{-1} in very high density fields. The COR in winter wheat was higher due to the negative impact of the weed on wheat yield (no yield penalties were applied to other crops in the rotation). Overall, field-scale resistance impacts are greater in regions with higher weed densities, especially in winter wheat crops (Figure 28).

Table 4 The field-scale yield loss and economic costs of resistance at different densities of Black-grass in England. Figures in brackets show 95% confidence intervals generated by bootstrapping.

Average Black-grass density state of field	**Average yield loss in winter wheat (t /ha)**	**Average cost (£ /ha)**	
		in winter wheat	**across rotations**
	Mean (95%CI)	Mean (95%CI)	Mean (95%CI)
absent/low	0.1 (0.0, 0.2)	114 (97, 131)	89 (77, 102)
medium	0.5 (0.4, 0.6)	182 (172, 193)	137 (127, 148)
high	1.0 (0.9, 1.1)	308 (292, 323)	211 (199, 222)
very high	1.9 (1.8, 2.0)	473 (457, 489)	292 (275, 310)

Farmer decision-making regarding black-grass herbicide use is not driven by black-grass density (mean herbicide use for black-grass control was not related to weed density, χ^2_1=0.0982, p=0.754, Figure 29). As a result, at low densities of black-grass where total COR was lowest and the costs due to lost yield were small, the cost of herbicides targeting black-grass was the major contributor (76%) to overall COR (Figure 29).

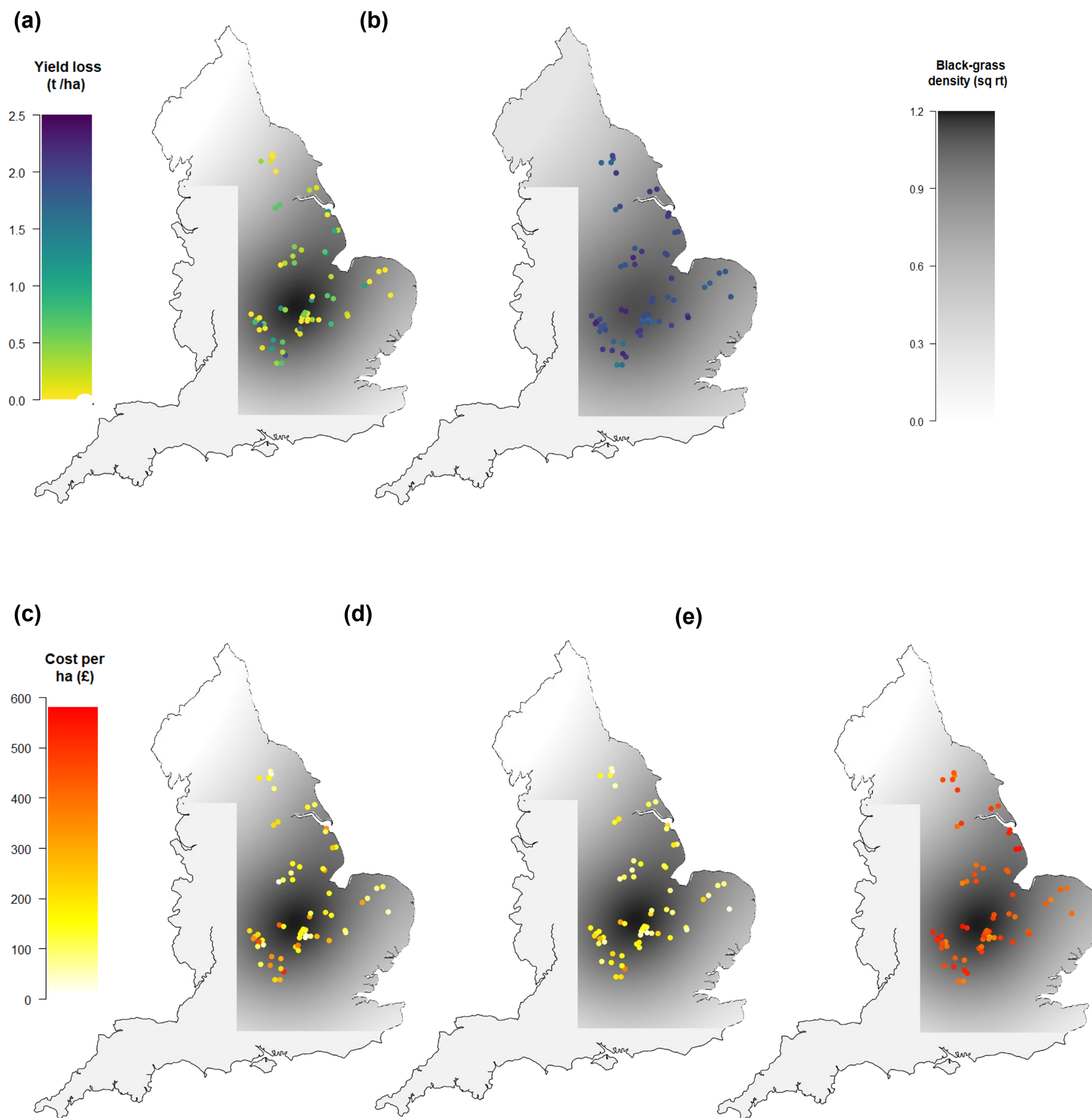

Figure 28 (a) and **(b)** show yield loss (t ha^{-1}) due to resistant Black-grass: **(a)** average field-scale yield losses in winter wheat **(b)** maximum field-scale yield loss in winter wheat in the event of total loss of herbicide control. **(c)** – **(e)** show cost of resistance (£ ha^{-1}): average field-scale cost of resistance for **(c)** years in winter wheat crops and **(d)** all years' data, i.e. across a rotation; **(e)** maximum field-scale costs in the event of total loss of herbicide control. Fields are overlaid on a map of modelled density (square root) of Black-grass averaged over 2015-2017. This density map was generated by fitting a generalized additive model to the data reported in Hicks et al. 2018, with spatial covariates representing latitude and longitude.

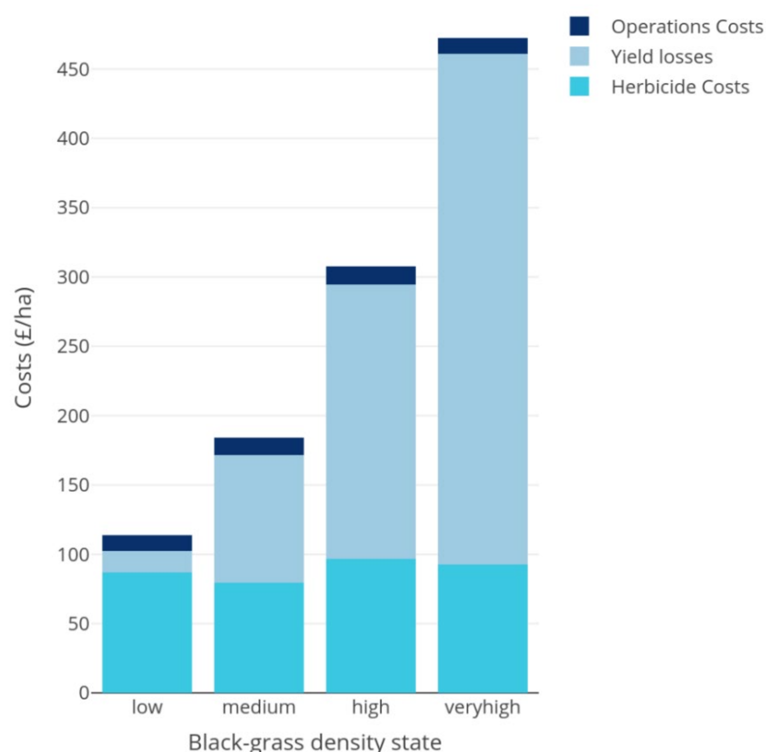

Figure 29 For winter wheat crops, the relative contribution of herbicide costs, lost yield and operations costs to the overall cost of resistance caused by herbicide-resistant black-grass. Herbicide costs consider only those herbicide applications targeting black-grass. (Error bars intentionally omitted as the purpose is to illustrate the contribution of component parts and, when data are presented in this way, error bars of individual components influence each other and are misleading).

Table 5 Cost of resistance and yield loss scaled to regional level and to England

Region [1]	Total yield loss, winter wheat (tonnes) (+/- 95% CI)[2]	Total cost in winter wheat crops (£) (+/- 95% CI)[2]	Total cost across a rotation (all crops)[3] (£) (+/- 95% CI)[2]
YH	46,489 (19,262 – 59,682)	24,918,186 (23,026,838 - 26,808,925)	66,609,634 (57,992,748 - 75,231,303)
EM	122,102 (92,392 – 153,850)	48,833,040 (45,408,646 - 52,258,037)	93,786,918 (81,654,271 - 105,926,300)
WM	70,756 (49,184 – 82,561)	45,135,928 (41,990,471 - 48,284,849)	41,829,899 (36,418,617 - 47,244,184)
EE	271,112 (217,712 – 328,965)	93,548,087 (85,029,554 - 102,073,603)	132,057,394 (114,973,927 - 149,150,344)
SE	250,480 (216,166 – 273,770)	83,283,795 (79,003,160 - 87,561,392)	61,792,924 (53,799,147 - 69,791,139)
Total, BGRI regions	760,938 (594,716 – 898,827)	295,719,037 (274,458,669 - 316,986,806)	396,076,769 (344,838,710 - 447,343,270)
ENGLAND	**1,014,435** (854,291 – 1,213,637)	**375,917,307** (347,879,514 - 403,950,393)	**481,684,921** (419,372,252 - 544,032,179)

[1] These are UK Government Office Regions. Only those where the Black-Grass Resistance Initiative project (BGRI) had fields were used. EE East of England; SE South East; YH Yorkshire and the Humber; EM East Midlands; WM West Midlands; Total, regions = all previous regions summed; England = this includes all UK Government Office Regions in England, so we are extrapolating beyond the regions where the BGRI has field sites.

[2] 95% CIs were calculated using bootstrapping

[3]For rotation costs, scaling up was done using the total area of the following crops: wheat, total barley, OSR, field beans & dried peas, and, for regions EM, EE and for England, also sugar beet.

At a national scale, the total annual wheat yield loss for England was 1 million tonnes (mt; Figure 30a). Economic costs were £0.5bn p.a. across all crops (termed *rotation cost* from now on) and £0.4bn p.a. in winter wheat (Figures 30b & c; for 95% CIs see Table 5). Across the five regions in which field sites were located, the total rotation cost was £0.4bn p.a. and in winter wheat £0.3bn p.a..

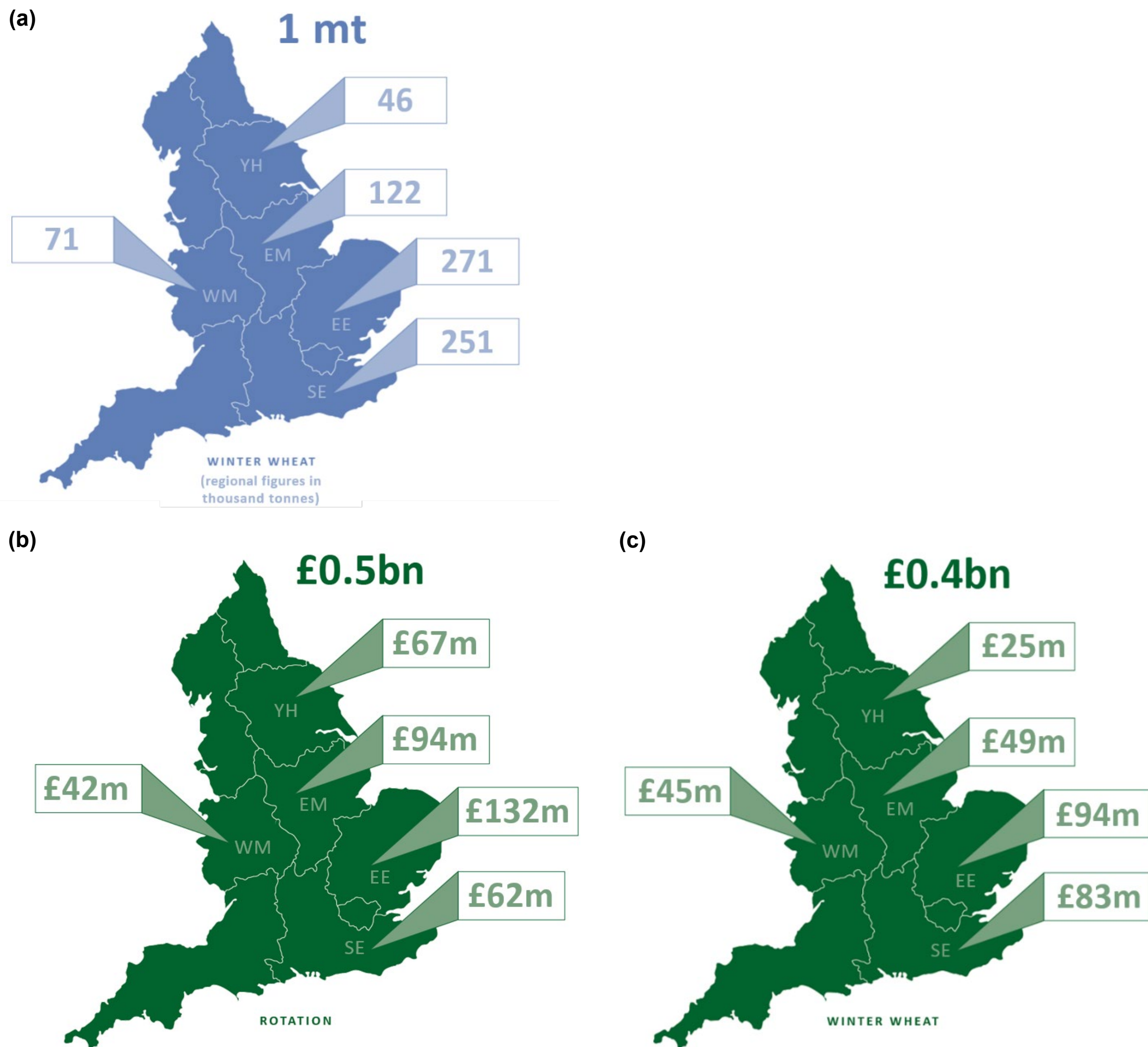

Figure 30 Annual impacts of herbicide resistant black-grass at regional and national scales. Annual yield losses in winter wheat **(a)**. National yield loss given in million tonnes; regional figures in thousand tonnes. Annual economic costs (£) across all crops **(b)** and in winter wheat crops **(c).** 95% confidence intervals given in Table 5. Regions are UK Government Office regions: EE East of England; SE South East; YH Yorkshire and the Humber; EM East Midlands; WM West Midlands.

At a regional-scale, some rotation costs are higher than those in winter wheat. This is because, although field-scale rotation costs are lower than those in winter wheat, the total cereal crop area is much larger than the winter wheat area and so, once costs have been scaled up, rotation costs are relatively higher. In the West Midlands (WM) and South East (SE) the average cost per ha in winter wheat crops was particularly high compared to other regions (WM £328/ha; SE 304/ha; EM

£191/ha; EE £242/ha; YH £163/ha; abbreviations as in Figure 30); as a result, the scaled-up costs in these two regions remained higher in winter wheat than across rotations.

Under the ‘worst-case’ scenario of ubiquitous very high black-grass density, wheat yield loss ranged from 1.6 – 2.4 t ha^{-1} and on average was 2 t ha^{-1}, representing almost a quarter (24%) of average potential estimated wheat yield (8.3 t ha^{-1}) in the absence of resistant black-grass. Scaling up these ‘worst-case’ estimates we find that potential yield loss in winter wheat under a scenario of total loss of herbicide control is 6.8mt yr^{-1} (95% CI 6.4 – 7.1mt), representing almost half of UK domestic wheat production. Potential yield loss is almost seven times greater than baseline yield loss, reflecting the very large jump in yield loss from lower to higher black-grass density states. Potential annual COR across all crops is £1bn (95% CI £0.9bn – £1.1bn). To present a more conservative worst-case estimate, we also estimated costs using just those fields in the top 25% and top 10% of the black-grass density range: these gave potential annual yield losses in winter wheat of 2.3mt and 2.8mt respectively, and rotation COR of £0.8bn and £0.9bn respectively.

5. Discussion

5.1. MHR mechanisms

Identification of key proteins that are causatively linked to MHR and new understanding of how their functions in resistance could be disrupted in future.
The project has identified a central role for AmGSTF1 as a regulator and coordinator of MHR in blackgrass, with the inference that the protein plays similar role in wild oat and rye grass. Based on our earlier chemical biology work (Cummins et al., 2013), we have demonstrated that this protein can be targeted for chemical disruption, with the work from the current study illustrating the potential value of following this approach in targeting AmGSTF with herbicide synergists that counteract MHR. Similarly, the proteomic studies identified a series of other proteins that are functionally important in MHR and could have future value as druggable targets for resistance busting.

Evidence for sub-types of MHR linked to specific herbicide chemistries. The work with the DNA diagnostic biomarkers and the quantitative proteomics (Tetard-Jones et al., 2018), demonstrate at least three sub-types of MHR; a form that gives broad ranging resistance to multiple chemistries, a type that gives metabolic based resistance to single classes of chemistry and a type that is herbicide specific. By using these multiple biomarkers allied to multiplex diagnostics it would be possible to tailor herbicide control strategies to deal with specific field populations. Based on the pocket diagnostics we have already developed, this offers real time decision making tools for agronomists and farmers when attempting to reassert chemical control over problem grass weed populations and is a powerful new concept in linking diagnostics to sustainable chemical weed control.

The characterisation of latent viruses in blackgrass that could be of value in future biocontrol programmes. Two different classes of latent viruses have been found to form specific associations with grass weeds but are not present in crops. Our original interest in such viruses was their capacity to prime herbicide resistance in blackgrass. We propose that while such viruses cannot directly cause MHR, they may be a contributing factor in allowing weeds to evolve such resistance more readily as a consequence of altering transcriptional gene control in infected populations. From a practical standpoint, such viruses may present an opportunity as future biocontrol agents, either through finding weed specific virulent pathovars, or through using the viruses to deliver resistance suppressing genes (eg: gene silencing of AmGSTF1).

That epigenetic mechanisms are unlikely to be an evolutionary driver of MHR inheritance.
The evidence for epigenetic inheritance of MHR is not supported by our current findings (though it

cannot be entirely ruled out). This is an important finding based on the assertions of some in science community who have suggested that herbicide-imposed stress and safener treatment have been direct genetic drivers of resistance acquisition through genetic reprograming of the affected plants. The work presented here does not support that hypothesis, and taken together with the work in the proteomics studies showing safening and resistance present distinct biochemical phenotypes argues strongly against the proposal that agrochemicals are epigenetic drivers of herbicide resistance evolution. We conclude that MHR is driven primarily by selection for resistance traits that evolve 'naturally' in genetically diverse blackgrass populations.

The first evidence for active roles for transporter proteins functioning in MHR and their coupled function with detoxifying enzymes. The finding that MHR is associated with the upregulation of specific transporter proteins offers completely new insight into resistance mechanisms founded on enhanced detoxification. Further, we have found evidence that such transporters function with specific detoxifying enzymes giving us a rationale as to how it is possible to derive multiple subtypes of MHR.

Practical diagnostics for MHR in black-grass that can be used to detect resistance in the field in 10 minutes. This is a major output form the project and a world first in this technology as applied to herbicide resistance and weed management. The practical utility of using the technology has been demonstrated by working with agronomists at Frontier and ADAS, the units have been used to map MHR blackgrass populations on a whole farm and the diagnostics have attracted national interest in the farming community and press. The further development of this type of approach would be well suited to a continued activity with AHDB now that we have secured a long term manufacturing capability with MoLogic.

5.2. Extent and drivers of resistance

Resistance to ALS and ACCase post-emergence herbicides is widespread. These findings provide a detailed characterisation of the extent and distribution of herbicide resistance within UK populations of *A. myosuroides*. Although variation in efficacy was observed between the actives, and between geographical regions, the results demonstrate that resistance to these herbicides is now extremely widespread. The discovery that 79% of the tested populations were resistant to all three tested actives highlights the degree to which cross-resistance has become prevalent in this species.

Both TSR and NTSR mechanisms are common, and co-occur within populations. Evidence for both ALS and ACCase target-site mutations suggests that TSR is common amongst these populations, in accordance with studies in other countries. Strong correlation between the

frequency of target-site resistance and survival of cycloxydim provides tangible evidence that resistance to this herbicide is conveyed by TSR alone. Nevertheless, the observations from the mesosulfuron and fenoxaprop assays demonstrate considerably greater survival than is explained by TSR alone, confirming the importance of NTSR for these actives. The widespread co-occurrence of these mechanisms demonstrates that both mechanisms can be concurrently selected within the same field population of blackgrass.

Foliar AmGSTf1 concentrations are predictive of NTSR in field populations of *A. myosuroides*. The observed correlations between AmGSTf1 concentration and herbicide resistance to mesosulfuron and fenoxaprop demonstrates the utility of this marker as a proxy for NTSR. This is important as it provides robust scientific validation of the use of this protein within the rapid in-field diagnostic kit for NTSR. The experimental protocol for quantification of AmGSTf1 is simple and high-throughput, facilitating further work to understand the extent and evolution of NTSR in this species.

Sensitivity to glyphosate is heritable, and responding to current glyphosate selection in the field. The finding that populations vary in their sensitivity to glyphosate is of considerable importance. The confirmation by two separate approaches that this variation has a heritable genetic basis, and responds to selection, demonstrates the potential for evolution of a resistance mechanism. Association between historic glyphosate usage and current glyphosate sensitivity compound this, demonstrating that such selection is occurring in field populations of *A. myosuroides*. The current data can't confirm the extent to which glyphosate sensitivity can be reduced by this means, but highlights that further study of this trait and any potential mechanism should be a priority.

5.3. Heritability of fitness and life-history characteristics

Fitness of NTSR in blackgrass. No consistent evidence was found to support the hypothesis that NTSR is associated with a fitness cost in *A. myosuroides*. There were clearly age- and growth-stage- specific effects of plant biotype on aspects of life-history. In particular, NTSR plants were found to have greater tiller elongation during early establishment and vegetative growth. Nonetheless, effects on plant productivity were either negligible, or of an insufficient size to have an important impact on an agronomic time-scale.

Heritable components of blackgrass life-history. The 400 pedigreed blackgrass lines represent the first quantitative genetic study of this scale to be applied within weed research, and results demonstrate that a quantitative genetic approach can be successfully applied to study such species. In particular, characterisation of the heritability of glyphosate sensitivity provides an

additional means to characterise this trait. Application of quantitative genetics to measures of life-history reveals that several components of reproductive development and timing have a heritable genetic basis, with some genetic constraints revealed by evaluation of the G-matrix. These results have utility when considering potential responses to non-chemical selection in this system, for example the potential for phenological shifts in flowering time and seed shed.

5.4. Baseline impacts and potential management strategies

5.4.1. Baseline estimates of the impact of herbicide resistant black-grass

Our findings illustrate that pesticide resistance has implications for national food security and economics. Annual potential losses of the order of 2mt and £0.7bn are large enough that national-scale policy measures are needed to reduce the impact and spread of resistance.

Field-scale economics of herbicide resistant black-grass. At the highest black-grass densities, the average cost of resistance represents nearly 40% of the potential gross profit from winter wheat in the absence of resistant black-grass and highlights the severity of the problem English farmers are facing.

Farmer decision-making regarding black-grass herbicide use is not driven by black-grass density. Our results suggest that there may be scope for a more strategic approach to herbicide applications for blackgrass management: for example, at low weed densities, greater profits might be achieved by dramatically reducing blackgrass herbicide use and tolerating small yield losses. Co-benefits of this strategy would be preservation of pesticide efficacy and less damage to the environment. A proviso would be that cultural control methods be employed to prevent weed populations increasing, because at high and very high blackgrass densities, yield loss became the single main source of lost income (64% and 78% respectively). This provides a strong incentive to prevent population increases in fields which currently have low to medium blackgrass densities. Furthermore, our results imply that leaving resistance management to individual practitioners is an inadequate approach and that a national, targeted response is required.

National-scale yield impact of herbicide resistant black-grass. This is the first time that national-scale yield loss due to herbicide resistance has been estimated. Total annual wheat yield loss for England was 1 million tonnes, although sensitivity analyses suggest that annual wheat yield losses in England may be as low as 0.4 mt or as high as 2 mt given uncertainties in our yield penalty estimates. Whichever figure we accept, our estimates run counter to global goals of increased yields and are particularly concerning in view of the current wheat yield stagnation in NW Europe.

National-scale economic impact of herbicide resistant black-grass. This is the first time that national-scale costs due to herbicide resistance have been estimated. Across all crops, the cost

was £0.5bn p.a. and in winter wheat it was £0.4bn p.a.. Sensitivity analyses showed that annual rotation cost might be as low as £0.3bn p.a. or as high as £0.7bn p.a.. Nevertheless, even at the lower end, the costs are very large. To put these figures into perspective, UK annual domestic wheat production is in the region of 15 million tonnes and total income from all types of farming in England was £3.9bn in 2014 (DEFRA). Herbicide resistance is therefore having a severe impact on English arable farming. Our estimates indicate that low black-grass densities currently account for just over half of England's wheat producing area, so there is a strong incentive to prevent densities increasing.

Our results underscore the need to manage resistance through coordinated action at a national level. A comparison of the current costs with potential costs shows that we are about half way to the worst case scenario, except towards the northern edge of the black-grass range: here, potential COR is around 4.7 times current COR, reflecting the fact that herbicide resistant black-grass is not yet such a pressing problem in these areas. Our findings should be a catalyst for the UK to develop a national, government-led, pesticide resistance strategy.

5.4.2 Cost effective integrated pest management in the face of evolving resistance.

The immediate or potential impact of a weed shapes IPM strategy

Across a wide range of parameter combinations, five parameters control the immediate, or potential maximum, economic impact of the weed, and in particular the yield loss function. Although yield loss functions have been estimated for major weeds, there is evidence that yield loss functions vary substantially between fields, and little attention has been paid to this variation and understanding its causes.

This is a major gap in our knowledge as the cost effective IPM strategies changed substantially in response to changes in the yield loss function. Historically, farmers do not change their management in response to yield losses and apply the same amount of herbicide (the primary management tool in this system) regardless of the impact weeds have on crop yields (Hicks et al. 2018). This is a potentially expensive choice, as farmers may incur management costs with little reward.

A greater value on future returns increases investment in weed control

Intensive management is costly, and so requires returns over several years to justify. As a result, continuous herbicide and spot control to reduce the seed bank were only part of the most cost-effective strategy when future returns are given more value. In agricultural systems land tenure has a crucial effect on how investments in weed control are valued. Those who own fields can benefit

from long-term investments like weed control campaigns and soil conservation, whereas those who rent fields do not.

Selected IPM strategies are responsive to herbicide resistance

Cost effective IPM strategies are responsive to increasing resistance, drastically reducing herbicide use as higher levels of resistance evolve. In contrast, multiple herbicide applications per year are the norm in this cropping system, despite high levels of resistance. This disparity could arise from a number of contributing factors. Some managers may believe that even a little control (mortality of a few susceptible individuals) is better than no control and inaction is seen as the worst approach to weed management. In addition, IPM strategies are often seen as complex and more difficult to implement than the routine application of chemicals. There may be a belief that new herbicides will become available, despite no new modes of action being marketed for over 20 years. Finally, uncertainty in the efficacy of non-chemical control is a major impediment to adopting IPM, and farmers may prefer to stick with known chemical control, even if it is only partially effective.

The eventual solution to pesticide resistance will require reducing their use. Any strategy to drive this behaviour change at scale will need to mitigate farmers economic incentives where they encourage short-term intensive use of pesticides. Where such intensive use is sub-optimal, for example when high levels of resistance have already evolved, other factors encouraging continued over-use, such as land tenure and social norms that favour action over inaction, will need to be recognized and addressed. The alternative is to maintain the current management paradigm in spite of herbicide resistant weeds, which could cost hundreds of millions annually in extra control costs and yield losses.

5.4.3 The spatial and temporal scales at which target site and quantitative resistance co-exist.

The evolution of target site resistance was greatly slowed by the quantitative resistance in the non-spatial case, and its invasion was very slow, causing rapidly changes in target site resistance over small spatial scales. This occurred even though target site resistant individuals had a substantial fitness advantage. Where fitness costs have been measured, target site resistance can incur fitness costs and the fitness costs of quantitative resistance in *A. myosuroides* are modest. Thus, at least in some cases our results may overestimate the fitness advantage of target site resistance and how quickly it can evolve in the presence of quantitative resistance.

It has been suggested that the slow spread of resistance is due to limited dispersal of seeds and pollen. However, in our case the landscape is small relative to the pollen dispersal kernel in particular. Instead, our slow spread for target site resistance was due to the local genetic background. We saw this pattern in both the spatially implicit model and the one on a 1D

landscape. We expect this effect of the genetic background to be even stronger in 2D. On the 1D landscape the genetic background of the neighbours could only effect a location from two directions. On a 2D landscape every location has many more neighbours, and thus is more affected by their genetic background. These spatial dynamics suggests that applying herbicide in a spatial mosaic (a strategy recommended to slow the evolution of resistance), may be effective in managing target site resistance, but be less effective in managing quantitative resistance. Especially where stress tolerance pathways provide some pre-adaptation to herbicides across the whole population (Baucom 2016).

6. References

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