GENETIC AND PHYSIOLOGICAL CHARACTERIZATION AND ECOLOGICAL MANAGEMENT OF NON-TARGET SITE RESISTANCE IN MULTIPLE HERBICIDE RESISTANT *AVENA FATUA* L.

by

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ABSTRACT

Extensive herbicide usage has led to the evolution of resistant weed populations that cause substantial crop yield losses and increase production costs. The multiple herbicide resistant (MHR) *Avena fatua* populations utilized in this study are resistant to members of all selective herbicide families, across five modes of action, available for *A. fatua* control in small grain production, and thus pose significant agronomic and economic threats. Resistance to ALS and ACCase inhibitors is not conferred by target site mutations, indicating that non-target site resistance (NTSR) mechanisms are involved. The objectives of this dissertation were to investigate the following features of NTS MHR in comparison to herbicide susceptible (HS) *A. fatua*: 1) inheritance and genetic control, 2) the involvement of glutathione-related enzymes, 3) differentially expressed stress-related proteins in HS and MHR redox and phosphoproteomes, and 4) the influence of abiotic and biotic stress on *A. fatua* demography. First, NTSR in MHR *A. fatua* is controlled by three separate, closely-linked nuclear genes for flucarbazone-sodium, imazamethabenz-methyl, and pinoxaden. Second, a combination of proteomic, biochemical, and immunological approaches showed that constitutive glutathione-*S*-transferase (GST) activities and other glutathione-related enzymes are not directly involved in enhanced rates of fenoxaprop-P-ethyl and imazamethabenz-methyl metabolism in MHR *A. fatua*. Instead, we hypothesize that constitutively elevated GST enzyme rates and amounts are representative of a global suite of abiotic stress-related changes in MHR plants. Third, post-translation modifications including protein phosphorylation and oxidation were compared in two-dimensional gels, showing that MHR plants contain constitutive changes very similar to systemic acquired resistance and systemic acquired acclimation to biotic and abiotic stressors, respectively. And fourth, we investigated the influence of nitrogen fertilizer rate and spring *Triticum aestivum* seeding density on the demography of MHR and HS *A. fatua* under two cropping systems (continuous cropping and crop-fallow rotation). In both systems, MHR seedbank densities were negatively impacted by increasing nitrogen fertilization rate and *T. aestivum* density, and density-dependent seed production was the most influential parameter impacting population growth rate. Overall, this work provides significant insights into the genetic and physiological mechanisms that confer NTS MHR in *A. fatua*, and presents realistic ecologically-based approaches for management.

CHAPTER ONE

INTRODUCTION

Herbicide Resistance

Weedy plants, as a main biotic cause of crop yield loss, have played a central role in agriculture since its beginning about ten thousand years ago (Délye, Jasieniuk & Le Corre 2013). In the early 1950s synthetic herbicides revolutionized agriculture and have been at the foundation of both weed science research and the intensification and expansion of industrialized agriculture (Liebman, Mohler & Staver 2001). However, their extensive usage worldwide imposes strong selection pressure for resistant weed populations, threatening our ability to successfully manage weed populations (Menalled *et al.* 2016). Herbicide resistance is a rapidly growing worldwide problem that causes significant crop yield losses and increases production costs (Shaner, Lindenmeyer & Ostlie 2012; Heap 2017). To date, approximately 252 species have evolved resistance to 23 of the 26 known herbicide modes of action, representing over 161 different herbicides (Heap 2017). Furthermore, 37% of these species are resistant to two or more modes of action, a phenomenon known as multiple herbicide resistance (MHR). MHR, is a particularly ominous development in many cropping systems worldwide that threatens the very basis of herbicidal weed management.

Herbicide Resistance Mechanisms

Herbicide resistance can be conferred by target site (TSR) or non-target site resistance (NTSR) mechanisms. TSR is conferred by mutations that alter herbicide binding or target site gene overexpression. TSR to acetolactate synthase (ALS) inhibitors and acetyl-CoA carboxylase (ACCase) inhibitors, two large classes of herbicides used to control grass weeds, is the most widely documented mechanism of resistance (Heap 2017). In contrast, NTSR mechanisms include enhanced rates of herbicide metabolism, reduced absorption/translocation, sequestration, or changes in generalized abiotic stress defense networks (Délye *et al.* 2011).

Enhanced rates of herbicide metabolism implicated in NTSR is, in general, a three phase process. Phase I reactions increase the polarity of the herbicide and involve oxidation, reduction, or hydrolysis which form free amino, hydroxyl, or carboxylic acid groups (Yuan, Tranel & Stewart Jr 2007). The most common phase I reactions are oxidation reactions carried out by cytochrome P450 monooxygenases (P450s) (Werck-Reichhart, Hehn & Didierjean 2000; Siminszky 2006). P450s are a large superfamily of enzymes and catalyze oxygen- and NADPH-dependent monooxygenase reactions. P450s have been cited in playing a role in NTSR (Hu *et al.* 2009) with over 11 different species implicated having cytochrome P450 mediated metabolism conferring resistance to several herbicide families (Powles & Yu 2010).

Phase II reactions are commonly catalyzed by the glutathione *S*-transferase (GST) superfamily which is large and diverse (Chronopoulou *et al.* 2014; Labrou *et al.* 2015). Soluble GSTs are typically dimers of 24-27 kDa subunits, with isoelectric points near pH

5 (Dixon, Davis & Edwards 2002). Higher plants have at least ten different GST classes, of which the predominant phi and tau classes have broad substrate specificities and are primarily responsible for herbicide detoxification (Edwards & Dixon 2000). GSTs conjugate glutathione to oxidized xenobiotics, and individual GSTs of several classes are key players in NTSR to herbicides (Reade, Milner & Cobb 2004). The best-characterized role of GSTs in NTSR is for the 'Peldon' MHR *Alopecurus myosuroides* populations that are resistant to the photosystem II inhibitor chlorotoluron and several ACCase inhibiting herbicides (Cummins *et al.* 2013). In addition to GSTs, the glycosyltransferase enzyme family is also involved in phase II of herbicide metabolism. Specifically, glycosyltransferases conjugate herbicides directly or conjugate a sugar molecule to a variety of lipophilic molecules including xenobiotics (Yuan, Tranel & Stewart Jr 2007). Glycosyltransferases have been shown to metabolize many herbicides (Brazier-Hicks *et al.* 2007), and have important roles in conferring tolerance to other abiotic stresses such as salt, cold, and drought, by modifying anthocyanin accumulation (Li *et al.* 2016).

The third phase of herbicide metabolism involves compartmentalization and transportation of the conjugated herbicide into the vacuole or extracellular space (Hu *et al.* 2009). The most common transporters in phase III are ABC transporters. ABC transporters have been shown to transport herbicide metabolites of primisulfuron (Gaillard *et al.* 1994), glutathione conjugated herbicide metachlor (Lu, Li & Rea 1997), and have potential roles in conferring NTSR to glyphosate in *Conyza canadensis* (Yuan *et al.* 2010).

Another NTSR mechanism includes changes in generalized stress defense networks (Délye *et al.* 2011). Plants are sessile organisms and as a consequence cannot move away from attack. Due to this sessile nature plants have evolved elaborate strategies for responding to changing abiotic and biotic stressors. Exposure to one environmental stress has been found to affect subsequent tolerance to other environmental stressors (Rejeb, Pastor & Mauch-Mani 2014) and there are significant overlaps between plant responses to both abiotic and biotic stresses [reviewed in Atkinson and Urwin (2012) and Fujita *et al.* (2006)]. An evolutionarily recent abiotic stress is the application of herbicides to control weedy plants. Herbicide application is an abiotic stress and thus should trigger preexisting stress pathways (Délye 2013). This idea is supported by previous studies that have shown changes in the plant transcriptome resulting from herbicide stress parallel transcriptome changes resulting from abiotic and biotic stress (Das *et al.* 2010; Unver *et al.* 2010).

Overall, due to the mechanisms outlined above the selection of NTSR is particularly threatening as it is associated with unpredictable patterns of resistance, e.g. populations with resistance to unrelated modes of action and to herbicides not yet commercially available (Petit *et al.* 2010a).

Non-Target Site Resistance Evolution

It has long been recognized that the underlying genetics and inheritance of herbicide resistance have profound implications for the occurrence, evolution, spread, and management of resistant populations (Jasieniuk, Brûlé-Babel & Morrison 1996). It is

particularly important to understand the genetic determinants of NTSR, which is now considered the prominent type of resistance to the three most important herbicide mechanisms of action worldwide: ACCase inhibitors, ALS inhibitors (for grasses), and 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase inhibitors (Délye 2013).

NTSR is thought to evolve via a gradual selective process over multiple generations by 'gene stacking' in which herbicide application initially selects for genotypes with slightly reduced sensitivity, which then cross-pollinate to create recombinant genotypes with combinations of parental alleles that confer increasing levels of herbicide resistance over multiple generations (Délye *et al.* 2011). Significantly, this hypothesis pertains only to allogamous weed species, which represent the vast majority of NTSR and MHR species studied to date (Heap 2017). For example in populations of *A. myosuroides*, *Lolium rigidum*, and *Amaranthus tuberculatus* NTSR is conferred by multiple mechanisms and alleles (Busi, Vila-Aiub & Powles 2010; Petit *et al.* 2010b; Huffman *et al.* 2015). In an extreme case, NTSR in an allogamous *A. myosuroides* population resistant to ACCase and ALS inhibitors is controlled by three loci for each herbicide (Petit *et al.* 2010b). In contrast to allogamous species in which NTSR has been well studied, the few studies investigating NTSR inheritance in autogamous species showed that it was controlled by a single gene (Zelaya, Owen & VanGessel 2004; Iwakami *et al.* 2014).

Ecologically-Based Tactics for Weed Management

MHR populations pose significant agronomic and economic threats to agriculture and demand the development and implementation of alternative, ecologically-based tactics for sustainable management. One such widely successful tactic involves maximizing the competitive ability of the crop through increasing planting densities, planting competitive crop cultivars, fertilizer management, and decreasing crop row spacing (Liebman, Mohler & Staver 2001). For example, planting competitive *Hordeum vulgare* L. varieties at high seeding rates can reduce *A. fatua* seed production by 60% (O'Donovan *et al.* 2007). Nitrogen (N) is the main form of fertilizer added to increase crop yield, but the effects of added N on crop-weed interactions are often species-specific, site-specific, year-specific, and field history-specific (Blackshaw & Brandt 2008).

The short-term impact of ecologically-based weed management tactics can be investigated by field experiments, but evaluations of their long-term consequences requires the use of population dynamics models that allow for the range of outcomes based on variability in demographic data (Holst, Rasmussen & Bastiaans 2007). Population dynamics models are vital tools in weed management and previous studies have used population dynamics models to understand the impact of various management strategies on weed population growth (Davis, Dixon & Liebman 2004) and the evolution and spread of herbicide resistance (Maxwell, Roush & Radosevich 1990). Furthermore, elasticity analysis of the models can be used to identify demographic processes with the largest negative impact on population growth and thus can be the focus of management

efforts (Caswell 2001; Davis, Dixon & Liebman 2004). Ultimately, knowledge gained from these models can lead to the design of ecologically-based weed management programs that reduce and prevent MHR weed population growth.

Avena fatua

The NTS MHR weed of interest in this research is *Avena fatua* L. (Poaceae, Pooideae, Aveneae; wild oat) which is an annual, allohexaploid, predominantly self-pollinating monocot that is ranked as one of the world's worst weed species (Holm *et al.* 1991). It is particularly well-adapted to temperate regions of Europe and North America, and infests over 11 million ha of cropland in the Northern Great Plains alone causing crop losses of over $1 billion annually (Beckie, Francis & Hall 2012). Due to extensive use of herbicides that inhibit ACCase, ALS, and other targets, populations of resistant *A. fatua* have evolved in the United States (Kern *et al.* 1996; Lehnhoff *et al.* 2013b; Keith *et al.* 2015) and worldwide (Heap 2017).

In Montana, producer complaints from a malt barley production area led us to investigate *A. fatua* populations that were not controlled by pinoxaden. This complaint was unusual because it was the first year pinoxaden was commercially available, and due to its novel chemical characteristics, successful control was expected. From these populations, we derived the *A. fatua* MHR3 and MHR4 populations that are resistant to the ACCase inhibitors fenoxaprop-P-ethyl, tralkoxydim, and pinoxaden, the ALS inhibitors imazamethabenz-methyl and flucarbazone-sodium, the growth inhibitor difenzoquat, the photosystem I inhibitor paraquat (MHR3 only), and the very long chain

fatty acid (VLCFA) biosynthesis inhibitor triallate, with resistance/susceptible ratios ranging from 1.4 to 57 (Lehnhoff *et al.* 2013b; Keith *et al.* 2015). The MHR *A. fatua* populations are thus resistant to members of all selective herbicide families available for *A. fatua* control in small grain crops. Greenhouse experiments have demonstrated that fitness costs are limited in these MHR populations, with a trend of fewer tillers and seeds produced by MHR plants compared to HS plants (Lehnhoff *et al.* 2013b). Resistance to ALS- and ACCase-inhibiting herbicides is not due to known target site mutations, and the P450 inhibitor malathion partially reversed the resistance phenotype for several herbicides (Keith *et al.* 2015), indicating that NTSR mechanisms are involved.

Project Significance

Overall, the occurrence of NTSR and MHR is on the rise and can cause significant crop yield loss, increase production cost, and threaten current weed management strategies (Shaner, Lindenmeyer & Ostlie 2012; Heap 2017). Furthermore, no new herbicide mode of action has been introduced to the market for approximately 25 years, suggesting that herbicides appear to be non-renewable resources (Duke 2012). Current herbicide resistance management strategies are centered on using different herbicide mechanisms of action in annual rotations, tank mixtures, and/or sequential applications (Norsworthy *et al.* 2012). However, these strategies will be ineffective at controlling or slowing the evolution of NTSR, since it is associated with unpredictable patterns of resistance and to herbicides not yet commercially available (Petit *et al.* 2010a).

Therefore, the below objectives evaluated the genetic and physiological mechanisms that confer NTS MHR and ecological management of NTS MHR *A. fatua*. The ultimate goals and outcome of this research were to 1) provide information about a weed population's response to intense herbicide selection from multiple herbicides and 2) use this knowledge to construct alternative ecologically-based approaches for sustainable weed management which will extend the utility of herbicides and lead to successful management of current and future NTS MHR weed populations.

Research Objectives

1) Determine the inheritance patterns and genetic control of NTSR in MHR *A. fatua* populations.

2) Investigate the potential involvement of glutathione-related enzymes in conferring the MHR *A. fatua* phenotype using a combination of proteomic, biochemical, and immunological approaches.

3) Investigate post-translational modifications associated with NTSR in MHR *Avena fatua*, and its commonalities with systemic acquired resistance and systemic acquired acclimation.

4) Construct a population dynamics model using demographic data obtained in field conditions from MHR and HS *A. fatua* populations, under factorial combinations nitrogen fertilizer rate and spring *Triticum aestivum* seeding density in two different cropping systems to identify demographic processes that could be exploited for non-herbicide management of the resistant phenotype populations.

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CHAPTER TWO

NON-TARGET SITE HERBICIDE RESISTANCE IS CONTROLLED BY A SINGLE GENE IN *AVENA FATUA* L.

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Summary

Extensive herbicide usage has led to the evolution of resistant weed populations that cause substantial crop yield losses and increase production costs. The multiple herbicide resistant (MHR) *Avena fatua* L. populations utilized in this study are resistant to members of all selective herbicide families, across five modes of action, available for *A. fatua* control in U.S. small grain production, and thus pose significant agronomic and economic threats. Resistance to ALS and ACCase inhibitors is not conferred by target site mutations, indicating that non-target site resistance (NTSR) mechanisms are involved. Understanding the inheritance of NTS MHR is of upmost importance for continued agricultural productivity in the face of the rapid increase in resistant weed populations worldwide. Since few studies have examined the inheritance of NTSR in autogamous weeds, we investigated the inheritance and genetic control of NTSR in the highly autogamous, allohexaploid species *A. fatua*. We found that NTSR in MHR *A. fatua* is controlled by three separate, closely-linked nuclear genes for flucarbazone-sodium, imazamethabenz-methyl, and pinoxaden. The single-gene NTSR inheritance patterns reported here contrast with other examples in allogamous species, and illustrate the diversity of evolutionary responses to strong selection.

Introduction

Agricultural weeds are the primary cause of crop yield loss (Oerke 2006), and herbicides have been the most prominent and effective weed control practice for over 50 years (Liebman, Mohler & Staver 2001). However, their extensive usage worldwide imposes strong selection for resistant weed populations, threatening their continued efficacy. Populations of approximately 250 species have evolved resistance to 23 of the 26 known herbicide mechanisms of action, representing over 160 different herbicides (Heap 2017). Furthermore, 34% of these populations are resistant to two or more mechanisms of action, a phenomenon known as multiple herbicide resistance (MHR).

Resistance can be conferred by target site overexpression or mutations that alter herbicide binding, or non-target site based resistance (NTSR) alterations such as enhanced rates of herbicide metabolism, reduced absorption and/or translocation, sequestration, or more generalized stress defense networks (Délye 2013). The selection of NTSR is particularly threatening, as it is associated with unpredictable patterns of resistance to unrelated mechanisms of action (Délye, Jasieniuk & Le Corre 2013) and to herbicides not yet commercially available (Petit *et al.* 2010a).

It has long been recognized that the underlying genetics and inheritance of herbicide resistance have profound implications for the occurrence, evolution, spread, and management of resistant populations (Jasieniuk, Brûlé-Babel & Morrison 1996). It is particularly important to understand the genetic determinants of NTSR, which is considered the prominent type of resistance to the three most widely used herbicide mechanisms of action worldwide: acetyl-CoA carboxylase (ACCase) inhibitors,

acetolactate synthase (ALS) inhibitors (for grasses), and 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase inhibitors (Délye 2013).

NTSR results from a gradual selective process over multiple generations, and previous research in *Alopecurus myosuroides*, *Lolium rigidum*, and *Amaranthus tuberculatus* demonstrates that NSTR is conferred by multiple mechanisms and alleles (Busi, Vila-Aiub & Powles 2010; Petit *et al.* 2010b; Huffman *et al.* 2015). In an extreme case, resistance to ACCase and ALS inhibitors in an NTSR population of the allogamous species *A. myosuroides* is controlled by three loci for each herbicide (Petit *et al.* 2010b). In contrast to the numerous studies of NTSR in allogamous species (Preston 2003; Busi, Vila-Aiub & Powles 2010; Petit *et al.* 2010b; Busi, Neve & Powles 2012; Han *et al.* 2014; Huffman *et al.* 2015; Rosenhauer *et al.* 2015), only a few studies have investigated NTSR inheritance in autogamous species, showing that it was controlled by a single gene in *Echinochloa phyllopogon* (Iwakami *et al.* 2014) and *Conyza canadensis* (L.) Cronq. (Zelaya, Owen & VanGessel 2004).

Avena fatua L., an annual, predominantly autogamous, allohexaploid monocot, is a successful, persistent, and economically important weed around the world, especially in the Northern Great Plains and Pacific Northwest of North America, where it infests over 11 million ha and causes crop losses of over $1 billion annually (Beckie, Francis & Hall 2012). Due to extensive use of herbicides that inhibit ACCase, ALS, and other targets, populations of resistant *A. fatua* have evolved in the United States (Kern *et al.* 1996; Lehnhoff *et al.* 2013b; Keith *et al.* 2015) and worldwide (Heap 2017). In Montana, producer complaints from a malt barley production area led us to investigate *A. fatua*

populations that were not controlled by pinoxaden. This complaint was unusual because it was the first year pinoxaden was commercially available, and due to its novel chemical characteristics, successful control was expected. From these populations, we derived the *A. fatua* MHR3 and MHR4 populations that are resistant to the ACCase inhibitors fenoxaprop-P-ethyl, tralkoxydim, and pinoxaden, the ALS inhibitors imazamethabenz-methyl and flucarbazone-sodium, the growth inhibitor difenzoquat, the photosystem I inhibitor paraquat (MHR3 only), and the very long chain fatty acid (VLCFA) biosynthesis inhibitor triallate, with R/S ratios ranging from 1.4 to 57 (Lehnhoff *et al.* 2013b; Keith *et al.* 2015). These MHR populations are thus resistant to members of all selective herbicide families available for *A. fatua* control in small grain crops. The cytochrome P450 monooxygenase inhibitor malathion partially reversed the resistance phenotype for several herbicides (Keith *et al.* 2015), indicating that NTSR mechanisms are involved.

Since relatively few studies have examined the inheritance of NTSR in autogamous weed species, we investigated the inheritance and genetic control of NTSR in MHR populations of the mostly autogamous species *A. fatua*.

Materials and Methods

Parental populations

The MHR3 and MHR4 parental populations were derived from seeds collected in 2006 from two *A. fatua* populations not controlled by 60 g a.i. ha^{-1} pinoxaden in two production fields separated by approximately 8 km in Teton County, Montana, USA. Field-collected seeds (about 90% of which were resistant to 60 g a.i. ha^{-1} pinoxaden, data

not shown) were subjected to two generations of recurrent group selection (50 plants each generation) by spraying with the same dose of pinoxaden (for MHR3 and MHR4) or surfactant only (susceptible population), followed by five additional generations with no herbicide selection in the greenhouse. From each generation of 50 plants, all seeds were harvested and a random selection of 50 seeds was used to initiate the next generation. Recurrent group selection was used as opposed to single seed descent in order to maintain representative genetic backgrounds in the MHR3 and MHR4 populations. Further characterization of these populations is reported in Lehnhoff *et al.* (2013b) and Keith *et al.* (2015). The herbicide susceptible parental population HS2, is the highly inbred nondormant SH430 line used in seed dormancy research (Naylor & Jana 1976; Johnson *et al.* 1995). All plants used in this research were grown under a 16-hr photoperiod of natural sunlight supplemented with mercury vapor lamps (165 μmol m^{-2} sec^{-1}) at 25 ± 4 C in standard greenhouse soil mix [1:1:1 (by vol) Bozeman silt loam:Sunshine mix #12 (Sun Gro Horticulture, Inc., Bellvue, WA):perlite] and fertilized weekly with 100 ppm of Jack's Classic 20-20-20 All Purpose fertilizer (JR Peters Inc., Allentown, PA).

Establishing F_1-F_3 populations

Homozygous MHR3, MHR4, and HS2 plants were used as parents in reciprocal crosses to generate F_1 seed. Homozygosity of parental resistance was confirmed for MHR3 and MHR4 by zero injury from treatment with field use rates of flucarbazone-sodium (flucarbazone hereafter; 30 g a.i. ha^{-1}), imazamethabenz-methyl (imazamethabenz hereafter; 94 g a.i. ha^{-1}), or pinoxaden (58 g a.i. ha^{-1}) as previously reported (Lehnhoff *et al.* 2013b; Keith *et al.* 2015), and susceptibility of HS2 plants by 100% injury from the

same treatments (data not shown). Reciprocal crosses were performed using the methods of Brown (1980) for *Avena sativa* cross-pollination. The resulting 151 F_1 seeds were grown in the greenhouse, self-pollinated by wrapping individual panicles in clear polypropylene pollen-proof bags, and F_2 seeds collected. Fifty-five successful F_1 crosses were confirmed by Touchdown PCR assays (Don *et al.* 1991) using primer pairs AJ010728 (Holland *et al.* 2001) and AM83 (Pal *et al.* 2002). Of these, four F_1 plants representing each pairwise reciprocal cross [(MHR3 x HS2), (HS2 x MHR3), (MHR4 x HS2), (HS2 x MHR4)] were chosen for further testing. Ten F_2 families from each of the four F_1 plants were advanced to the F_3 generation. In total 100 F_3 plants from each pairwise cross (10 from each of the 10 F_2 families) were screened for herbicide response. Family sizes were calculated as described in Jasieniuk, Brûlé-Babel and Morrison (1996).

Herbicide screening

F_3 and parental HS2, MHR3, and MHR4 plants were screened for herbicide response using previously determined doses that were lethal to HS2 plants while not severely injuring MHR plants (Lehnhoff *et al.* 2013b). One ACCase- and two ALS-inhibiting herbicides (127 g a.i. ha^{-1} imazamethabenz, 30 g a.i. ha^{-1} flucarbazone plus 0.125% (v/v) nonionic surfactant, and 29 g a.i. ha^{-1} pinoxaden) were applied separately to 3-leaf stage plants using a moving nozzle sprayer in 94 L water ha^{-1}. When the spray solution had dried, plants were returned to the greenhouse conditions described above for 3 wk, after which plants were scored for visual injury (0 = no injury to 4 = plant death; Fig. 1). All herbicide applications were made in mid-morning to minimize potential

environmental- and circadian-induced changes in plant response. In total, 100 F_3 plants from each pairwise cross and 20 parental plants were screened for each herbicide.

Data analysis

F_3 plants were classified as homozygous resistant or homozygous susceptible when injury ratings were within two standard errors of the mean injury rating for MHR or HS parents, respectively, or segregating for resistance if the above criteria were not met (Table 2.1). Because hexaploid *A. fatua* exhibits bivalent chromosome pairing with disomic inheritance (Thomas, Marshall & Sorrells 1992), results were compared to Mendelian disomic inheritance segregation ratios. To confirm homozygosity, a second set of herbicide treatments was applied as described above to 20 additional plants from each $F_{2:3}$ family that were scored as homozygous resistant or susceptible.

To compare injury ratings among $F_{2:3}$ families and determine if $F_{2:3}$ families from each F_1 cross could be combined for data analysis, a linear model was constructed for each herbicide screened, with fixed effects for F_1, F_2, and F_3. Models were fit using the lm function in R and means were separated using Tukey's HSD post hoc tests (R 2016). When appropriate, data were pooled and chi-square tests were performed to determine the goodness-of-fit of $F_{2:3}$ segregation ratios to known genetic models (Strickberger 1985). Tests for linkage among resistance genes to the three herbicides were conducted using both logarithm of odds (LOD) score analysis and recombination frequency analysis following the methods of Lathrop *et al.* (1984). Correlation analyses using the mean F_2 injury ratings were conducted in R using the rcorr function in the Hmisc package (R

2016) to examine the pairwise correlation of injury ratings to the three herbicides tested in this study.

Results

Genetic analysis

Data were pooled for analysis based on non-significant injury rating differences among reciprocal crosses (data not shown) for plants screened with flucarbazone, imazamethabenz, and pinoxaden. $F_{2:3}$ families for each herbicide segregated in a 1 homozygous resistant: 2 segregating: 1 homozygous susceptible ratio (Table 2.2), indicating that resistance is conferred by one gene for each herbicide. A lack of reciprocal cross differences indicated that resistance genes are nuclear and not cytoplasmic.

Linkage analysis

Data obtained from F_3 plants were used to derive the genotypes of $F_{2:3}$ families. For linkage analysis, non-parental recombinants were classified as $F_{2:3}$ families that were resistant to only one of the herbicides screened. All other phenotypes, resistant to all or susceptible to all herbicides, were classified as parental types. The presence of non-parental recombinants indicates that the same gene is not responsible for resistance to all three herbicides screened in this study. Furthermore, if resistance genes segregate independently, then the $F_{2:3}$ families should segregate in a 9:3:3:1 ratio. For example, for resistance to flucarbazone and pinoxaden, the $F_{2:3}$ families should segregate in the ratio of 9 resistant to both flucarbazone and pinoxaden: 3 resistant to flucarbazone and susceptible to pinoxaden: 3 susceptible to flucarbazone and resistant to pinoxaden: 1 susceptible to both flucarbazone and pinoxaden. Data for the resistance genes for

flucarbazone/imazamethabenz and flucarbazone/pinoxaden did not fit this ratio, suggesting that the resistance genes for these herbicides are linked (Table 2.3). In contrast, data for imazamethabenz/pinoxaden fit a 9:3:3:1 ratio, suggesting independence of resistance genes.

To further explore if resistance genes are linked, recombination frequency analysis and the LOD score method were independently employed. Recombination frequencies were 32-33% for flucarbazone/imazamethabenz, 13% for flucarbazone/pinoxaden, and 32-33% for imazamethabenz/pinoxaden, respectively (Table 2.4). LOD scores for flucarbazone/imazamethabenz, imazamethabenz/pinoxaden, and flucarbazone/pinoxaden linkage were 1.14 ($p = 0.011$), 1.14 ($p = 0.011$), and 5.01 ($p \leq 0.0001$), respectively, indicating that the likelihood of linkage is 14, 14, and 102,329 times greater than no linkage, respectively.

Correlation analysis of the mean F_2 injury ratings between each pairwise comparison of herbicides resulted in correlation coefficients of 0.61 ($p = < 0.0001$) for flucarbazone/imazamethabenz injury ratings, 0.70 ($p = < 0.0001$) for flucarbazone/pinoxaden injury ratings, and 0.72 ($p = < 0.0001$) for imazamethabenz/pinoxaden injury ratings (Fig. 2.2), where an absolute value of 1 indicates a perfect linear relationship and 0 indicates no linear relationship (Lawrence & Lin 1989).

Discussion

This study shows that NTSR in MHR3 and MHR4 *A. fatua* is controlled by a separate nuclear gene each for flucarbazone, imazamethabenz, and pinoxaden. The most

parsimonious explanation for these results would be a mutation in the genes encoding these herbicides' respective target enzymes. Target site resistance (TSR) for ALS and ACCase inhibitors is the most widely documented mechanism of resistance worldwide (Heap 2017), and it has been documented for *A. fatua* populations resistant to diclofop-methyl and flucarbazone herbicides as reviewed in Beckie, Francis and Hall (2012). However, we previously showed that MHR3 and MHR4 do not contain known target site mutations for ALS or ACCase inhibitors via transcriptome sequencing, cDNA and genomic DNA sequencing, and allele-specific PCR assays (Keith *et al.* 2015). While it is possible that MHR3 and MHR4 contain novel target site mutations, our recent RNA-Seq results strongly support the involvement of NTSR mechanisms (Keith et al. submitted).

Although our results show that NTSR in MHR3 and MHR4 *A. fatua* is controlled by a separate nuclear gene for each herbicide screened, it is possible that numbers of non-parental types and subsequent estimates of recombination could have been inflated by 1) insufficient F_3 plants in which heterozygous $F_{2:3}$ families might be mistaken for homozygous due to chance, 2) plants scored as susceptible may have been injured by another factor that caused a homozygous resistant family to appear to be segregating, or 3) plants scored as resistant were escapes from herbicide treatment. We believe that 2) can be eliminated because no untreated F_3 plants exhibited significant injury symptoms, and our extensive experience with herbicide applications rules out 3). Factor 1) is nonetheless possible but highly unlikely, since F_2 families like 40j were scored as homozygous resistant to flucarbazone and segregating for pinoxaden resistance,

demonstrating the presence of recombination and that resistance phenotypes are not controlled by the same gene (Table A1).

Linkage analysis indicates that the resistance genes for these herbicides are located in close proximity on the same chromosome. Similarly, positive correlations between pairwise comparisons of mean F_2 injury ratings in response to each herbicide (Fig. 2.2) further demonstrate that independent segregation of individual resistance genes is not occurring. Although data for imazamethabenz/pinoxaden linkage fit the 9:3:3:1 ratio (suggesting independence of resistance genes), these data may be have been influenced by the large range of injury scores from the parental MHR3 and MHR4 populations treated with imazamethabenz (Table 2.1), indicating that the herbicide screening dose was above that required for clearly distinguishing between resistant and susceptible phenotypes. While the 9:3:3:1 ratio was not rejected in this case, correlation data nonetheless support some degree of linkage between genes for imazamethabenz and pinoxaden resistance.

The linkage patterns documented here may help clarify why these populations were resistant to pinoxaden prior to its first field use. Adoption of the ALS inhibitors imazamethabenz and flucarbazone occurred in 1988 and 1998, respectively, and the ACCase inhibitor pinoxaden was introduced in 2005. Cross-resistance between these two families is well documented (Bagavathiannan *et al.* 2014), and it is possible that previous applications of these two ALS inhibitors simultaneously selected for pinoxaden resistance due to linkage of resistance genes. This idea is supported by the high LOD score

associated with flucarbazone and pinoxaden resistance, reflecting that the likelihood of linkage is 102,329 times greater than no linkage.

Linkage of NTSR genes has also been reported in *L. rigidum*, where resistance genes for diclofop-methyl/chlorsulfuron (Busi, Vila-Aiub & Powles 2010) and simazine/chlorotoluron (Preston 2003) are linked, as well as in *A. myosuroides* where resistance genes for fenoxaprop/clodinafop (Petit *et al.* 2010b), fenoxaprop/pinoxaden (Petit *et al.* 2010b; Rosenhauer *et al.* 2015), and fenoxaprop/flufenacet (Rosenhauer *et al.* 2015) are linked. These results support the hypothesis that accumulations of adaptive mutations are generally located in regions of strongly reduced recombination (Aeschbacher & Bürger 2014).

Our findings are similar to those of Karlowsky *et al.* (2006) who investigated the inheritance of MHR in the UMWO12-01 and UMWO12-03 Canadian *A. fatua* populations. They reported that separate single dominant or semi-dominant nuclear genes conferred resistance to imazamethabenz, flamprop-M-methyl, and fenoxaprop-P-ethyl in both populations. Furthermore, genes conferring resistance to imazamethabenz and flamprop-M-methyl were linked. However, the physiological mechanisms conferring MHR in UMWO12-01 and UMWO12-03 are unknown (Friesen *et al.* 2000; Karlowsky 2004), in contrast to the MHR3 and MHR4 *A. fatua* populations discussed here. However, since TSR is generally much more prevalent than NTSR (Heap, 2016), it seems likely that resistance in UMWO12-01 and UMWO12-03 is due to TSR.

Monogenic control of TSR is widespread, since a single point mutation in the gene encoding a herbicide's target enzyme is frequently sufficient to confer resistance

(Jasieniuk, Brûlé-Babel & Morrison 1996; Yuan, Tranel & Stewart Jr 2007). In contrast, NTSR is generally thought to evolve by 'gene stacking' in which herbicide application initially selects for slightly less sensitive genotypes, which then cross-pollinate over multiple generations to create recombinant genotypes with combinations of parental alleles conferring enhanced herbicide resistance (Délye *et al.* 2011). Significantly, this hypothesis pertains only to allogamous weed species, which are represented by the vast majority of NTSR and MHR populations studied to date [(Heap 2017); see examples below]. However, our results from the highly autogamous species *A. fatua* and those of Iwakami *et al*. (2014) and Zelaya, Owen & VanGessel (2004) support an alternative hypothesis more relevant for autogamous species: NTSR evolves through a 'step-by-step' process of consecutive selection events in the same individuals over time (Neve, Vila-Aiub & Roux 2009).

Our results also contrast with the majority of NTSR inheritance studies reporting that NTSR is multigenic in populations of the allogamous species *L. rigidum* (Preston 2003; Busi, Vila-Aiub & Powles 2010; Busi, Neve & Powles 2012; Han *et al.* 2014), *A. myosuroides* (Petit *et al.* 2010b; Rosenhauer *et al.* 2015), *A. tuberculatus* (Huffman *et al.* 2015), and *Papaver rhoeas* (Scarabel, Pernin & Délye 2015). In contrast, monogenic NTSR has only been reported in the autogamous species *Conyza canadensis* (Zelaya, Owen & VanGessel 2004) and *Echinochloa phyllopogon* (Iwakami *et al.* 2014), with one report in the allogamous species *L. rigidum* (Yu *et al.* 2009). Unlike multigenic NTSR in which resistance is conferred by enhanced herbicide metabolism, monogenic NTSR resistance is conferred by reduced translocation in the two known cases in *C. canadensis*

resistant to glyphosate (Zelaya, Owen & VanGessel 2004) and in paraquat-resistant *L. rigidum* (Yu *et al.* 2009). We have no evidence that reduced herbicide translocation is present in the MHR *A. fatua* populations used here.

In addition to contrasting mating systems, differences in genome structure and ploidy level may influence the evolution of NTSR. The majority of research investigating NTSR inheritance has been conducted with allogamous diploid weedy species as discussed above, which maintain high levels of heterozygosity and allow resistance alleles to spread quickly through cross-pollination. In contrast, autogamy promotes homozygosity and resistance alleles are not spread via pollen flow. In addition to being a highly autogamous species, *A. fatua* is also an allohexaploid (2n = 6 x = 42), with six chromosome sets from three progenitor species (Yang *et al.* 1999a). Polyploidy results in genome-wide genetic redundancy, providing novel opportunities for adaptive evolution (Wendel, 2000). For example, gene duplication after polyploidization leads to the retention of functional gene copies, or homoeologs, at many loci. The result is that *A. fatua* and other polyploid weedy species can accumulate and fix normally deleterious herbicide resistance gene mutations, facilitating the evolution of NTSR.

To date the only study investigating the impacts of polyploidy on herbicide resistance evolution was conducted on *A. fatua* populations with known target site mutations in the three homoeologous ACCase genes ACC1;1, ACC1;2, and ACC1;3 (Christoffers, Berg & Messersmith 2002). Yu *et al.* (2013) subsequently reported that the low levels of resistance observed in these populations may be due to dilution effects of resistance mutations by the maintenance of sensitive homoeologs. Thus polyploidy may

play contrasting roles in herbicide resistance evolution by 1) minimizing the deleterious effects of herbicide resistance gene mutations, while at the same time 2) buffering the effect of herbicide resistance genes with homoeologous sensitive alleles. This gene dosage effect has not yet been investigated in weedy species with NTSR, so the implications drawn from Yu *et al.* (2013) may not be directly applicable to the current findings. Furthermore, the details of homoeologous gene expression, gene silencing, and possible epigenetic regulation of homoeologs are essentially unknown in weedy species including *A. fatua*, although they provide valuable opportunities for developing a deeper understanding of NTS herbicide resistance evolution.

Understanding the inheritance of NTS MHR is of upmost importance for continued agricultural productivity in the face of rapid increases in resistant weed populations worldwide. The single-gene NTSR inheritance patterns reported here add to our understanding, and illustrate the diversity of evolutionary responses to strong selection. Overall, these results from *A. fatua* demonstrate the importance of investigating the genetic basis of NTSR on a species by species, population by population level in order to develop a complete understanding of this phenomenon. Current herbicide resistance management strategies include using different herbicide mechanisms of action in annual rotations, tank mixtures, and/or sequential applications (Norsworthy *et al.* 2012). However, these strategies will be ineffective at controlling or slowing the evolution of NTSR as described here, since it is associated with unpredictable patterns of resistance and to herbicides not yet commercially available (Petit *et al.* 2010a). It is clear that, if herbicides are indeed non-renewable resources (Duke 2012), alternative

ecologically-based approaches for sustainable weed management will be required in order to extend the utility of herbicides and lead to successful management of current and future NTS MHR weed populations.

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Table 2.1. Mean injury ratings (standard errors) used for $F_{2:3}$ family classifications of parental populations HS2, MHR3, and MHR4 *Avena fatua* treated with flucarbazone, imazamethabenz-methyl, or pinoxaden.

Parental population	Herbicide	Mean injury[a]
HS2	Flucarbazone	3.10 (0.067)
MHR3	Flucarbazone	0.44 (0.056)
MHR4	Flucarbazone	0.20 (0.082)
HS2	Imazamethabenz	3.00 (0.000)
MHR3	Imazamethabenz	0.95 (0.189)
MHR4	Imazamethabenz	1.53 (0.230)
HS2	Pinoxaden	3.06 (0.056)
MHR3	Pinoxaden	0.50 (0.065)
MHR4	Pinoxaden	0.69 (0.091)

[a]Injury rating using scale of 0 = no injury to 4 = plant death

Table 2.2. Segregation of resistance to flucarbazone, imazamethabenz-methyl, and pinoxaden in the $F_{2:3}$ generation of pooled MHR3-4/HS2 *Avena fatua* crosses.

Herbicide	Resistant	Segregating	Susceptible	Ratio	χ^2	Probability[a]
		number of families				
Flucarbazone	10	20	8	1:2:1	0.316	0.85
Imazamethabenz	10	18	10	1:2:1	0.105	0.95
Pinoxaden	8	24	6	1:2:1	2.842	0.24

[a]Probability values ≥ 0.05 indicate that data do not differ significantly from the test ratio

Table 2.3. Chi-squared tests to determine the presence of linkage between flucarbazone, imazamethabenz-methyl, and pinoxaden resistance based on derived F_2 genotypes from $F_{2:3}$ family screening.

Herbicide	Test ratio (observed)	χ^2	Probability[d]
Flucarbazone/Imazamethabenz	9:3:3:1 (20:10:2:6)[a]	10.47	0.015
Flucarbazone/Pinoxaden	9:3:3:1 (28:2:2:6)[b]	14.96	0.002
Imazamethabenz/Pinoxaden	9:3:3:1 (21:7:5:5)[c]	3.54	0.315

[a]Test ratio 9 resistant to both flucarbazone and imazamethabenz-methyl: 3 resistant to flucarbazone and susceptible to imazamethabenz: 3 susceptible to flucarbazone and resistant to imazamethabenz: 1 susceptible to both flucarbazone and imazamethabenz
[b]Test ratio 9 resistant to both flucarbazone and pinoxaden: 3 resistant to flucarbazone and susceptible to pinoxaden: 3 susceptible to flucarbazone and resistant to pinoxaden: 1 susceptible to both flucarbazone and pinoxaden
[c]Test ration 9 resistant to both imazamethabenz and pinoxaden: 3 resistant to imazamethabenz and susceptible to pinoxaden: 3 susceptible to imazamethabenz and resistant to pinoxaden: 1 susceptible to both imazamethabenz and pinoxaden
[d]Probability values ≥ 0.05 indicate that data do not differ significantly from the test ratio

Table 2.4. Linkage analysis of resistance genes in the $F_{2:3}$ generation of pooled MHR3-4/HS2 *Avena fatua* crosses.

Herbicide comparison	LOD recombination frequency	LOD score	p-value	Recombination frequency
Flucarbazone vs imazamethabenz	0.33	1.14	0.011	0.32
Flucarbazone vs pinoxaden	0.13	5.01	<0.001	0.13
Imazamethabenz vs pinoxaden	0.33	1.14	0.011	0.32

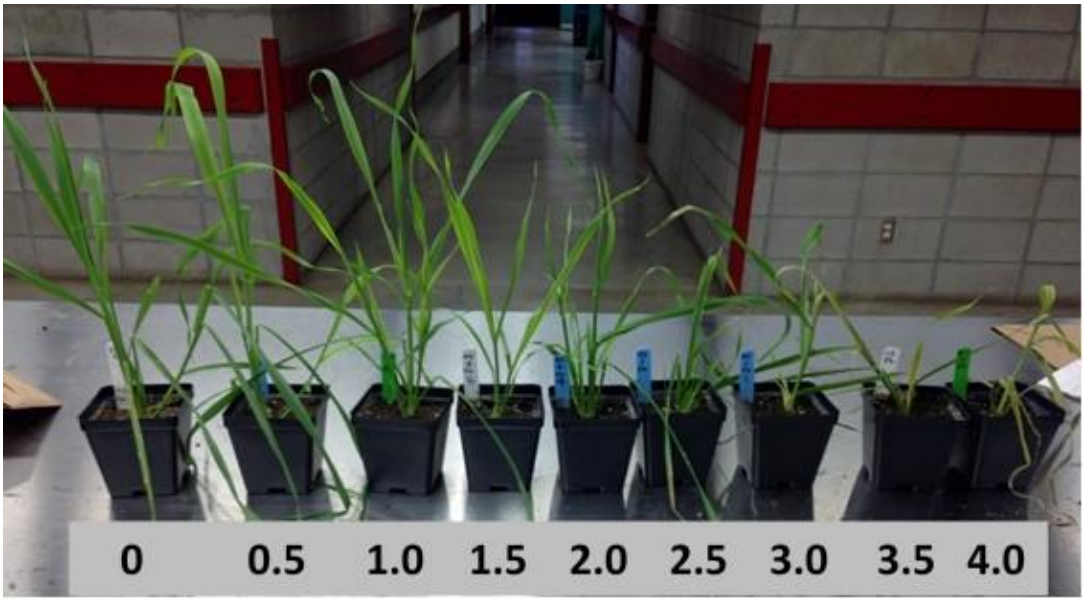

Figure 2.1. Visual injury rating scale of *Avena fatua* F_3 plants used for genetic analysis of non-target site resistance.

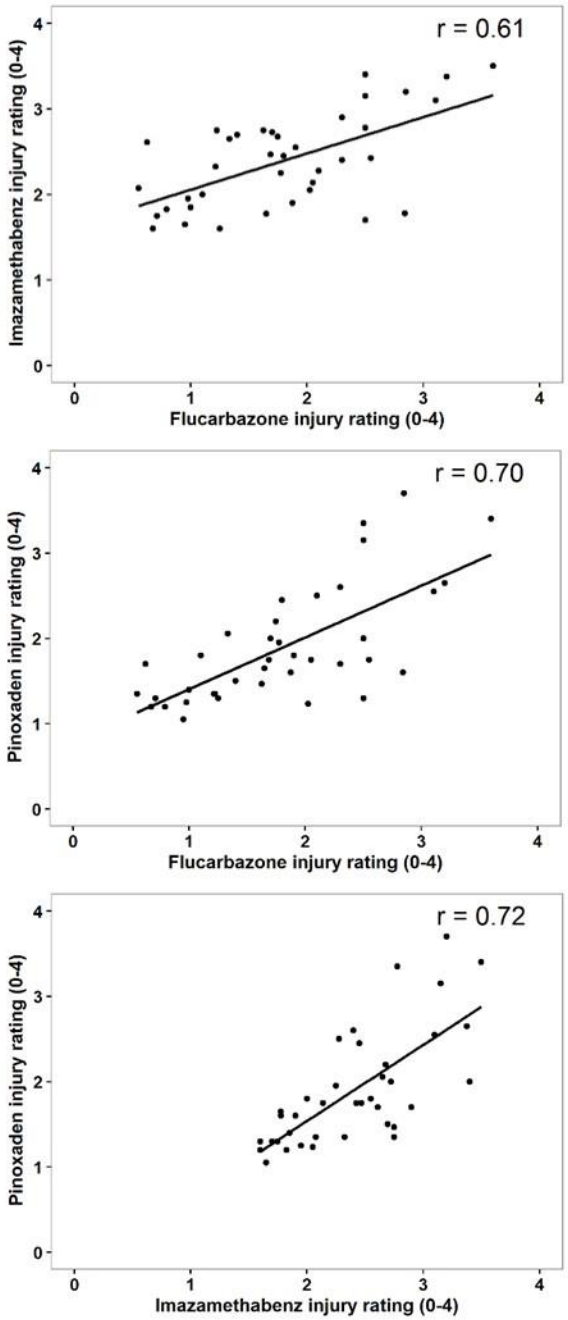

Figure 2.2. Correlation plots of mean pairwise *Avena fatua* F_2 injury ratings 3 wk after flucarbazone, imazamethabenz-methyl, or pinoxaden herbicide application.

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CHAPTER THREE

PROTEOMIC AND BIOCHEMICAL ASSAYS OF GLUTATHIONE-RELATED PROTEINS IN SUSEPTIBLE AND MULTIPLE HERBICIDE RESISTANT *AVENA FATUA* L.

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Abstract

Extensive herbicide usage has led to the evolution of resistant weed populations that cause substantial crop yield losses and increase production costs. The multiple herbicide resistant (MHR) *Avena fatua* L. populations utilized in this study are resistant to members of all selective herbicide families, across five modes of action, available for *A. fatua* control in U.S. small grain production, and thus pose significant agronomic and economic threats. Resistance to ALS and ACCase inhibitors is not conferred by target site mutations, indicating that non-target site resistance mechanisms are involved. To investigate the potential involvement of glutathione-related enzymes in the MHR phenotype, we used a combination of proteomic, biochemical, and immunological approaches to compare their constitutive activities in herbicide susceptible (HS1 and HS2) and MHR (MHR3 and MHR4) *A. fatua* plants. Proteomic analysis identified three tau and one phi glutathione *S*-transferases (GSTs) present at higher levels in MHR compared to HS plants, while Western analyses revealed elevated levels of lambda, phi, and tau GSTs. GST specific activity towards 1-chloro-2,4-dinitrobenzene was 1.2-fold higher in MHR4 than in HS1 plants and 1.3- and 1.2-fold higher in MHR3 than in HS1 and HS2 plants, respectively. However, GST specific activities towards fenoxaprop-P-ethyl and imazamethabenz-methyl were not different between untreated MHR and HS plants. Dehydroascorbate reductase specific activity was 1.4-fold higher in MHR than HS plants. Pretreatment with the GST inhibitor NBD-Cl did not affect MHR sensitivity to fenoxaprop-P-ethyl application, while the herbicide safener and GST inducer mefenpyr reduced the efficacy of low doses of fenoxaprop-P-ethyl on MHR4 but not MHR3 plants.

Mefenpyr treatment also partially reduced the efficacy of thiencarbazone-methyl or mesosulfuron-methyl on MHR3 or MHR4 plants, respectively. Overall, the GSTs described here are not directly involved in enhanced rates of fenoxaprop-P-ethyl or imazamethabenz-methyl metabolism in MHR *A. fatua*. Instead, we propose that the constitutively elevated GST proteins and related enzymes in MHR plants are representative of a larger, more global suite of abiotic stress-related changes.

1. Introduction

Herbicide resistance is a rapidly growing worldwide problem that causes significant crop yield losses, increases production costs, and threatens our ability to successfully manage weed populations (Shaner, Lindenmeyer & Ostlie 2012; Heap 2017). Resistance can be conferred by target site overexpression or mutations that alter herbicide binding, or non-target site resistance (NTSR) mechanisms like enhanced rates of herbicide metabolism, reduced absorption/translocation, sequestration, or changes in generalized abiotic stress defense networks (Délye *et al.* 2011). Multiple herbicide resistance (MHR), in which weeds are resistant to two or more unrelated herbicides, is a particularly ominous development in many cropping systems worldwide, and threatens the very basis of herbicidal weed management.

Avena fatua (Poaceae, Pooideae, Aveneae; wild oat) is an annual, allohexaploid, predominantly self-pollinating monocot that is ranked as one of the world's worst weed species (Holm *et al.* 1991). It is particularly well-adapted to temperate regions of Europe and North America, and infests over 11 million ha of cropland in the Northern Great Plains alone (Beckie, Francis & Hall 2012). *A. fatua* populations that are resistant to one or more herbicides have been reported in the USA (Kern *et al.* 1996; Nandula & Messersmith 2002; Keith *et al.* 2015) and elsewhere (Heap 2017). We recently described the *A. fatua* MHR3 and MHR4 populations that are resistant to the acetyl-CoA carboxylase (ACCase) inhibitors fenoxaprop-P-ethyl, tralkoxydim, and pinoxaden, the acetolactate synthase (ALS) inhibitors imazamethabenz-methyl and flucarbazone, the growth inhibitor difenzoquat, the photosystem I inhibitor paraquat (MHR3 only), and the

very long chain fatty acid (VLCFA) biosynthesis inhibitor triallate, with resistant/susceptible ED_{50} ratios ranging from 1.4 to 57 (Lehnhoff *et al.* 2013a; Keith *et al.* 2015). The MHR *A. fatua* populations are thus resistant to members of all selective herbicide families available for *A. fatua* control in U.S. small grain production. Resistance to ALS- and ACCase-inhibiting herbicides is not due to known target site mutations, and the cytochrome P450 monooxygenase inhibitor malathion partially reversed the resistance phenotype for several herbicides (Keith *et al.* 2015), indicating that NTSR mechanisms are involved.

The central component of plant response to abiotic stresses like herbicides is activation of the xenome, or the xenobiotic detection, transport, and detoxification network (Edwards *et al.* 2011). Glutathione (GSH)-related enzymes such as glutathione *S*-transferases (GSTs) and others are critical xenome players that are involved in enhanced degradation rates of several herbicides (reviewed in (Hatton *et al.* 1996; Cummins *et al.* 2011)). Compounds like the herbicide safener and GST inducer mefenpyr (Scalla & Roulet 2002; Cummins, Bryant & Edwards 2009) and the GST inhibitor 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) (Ricci *et al.* 2005; Cummins *et al.* 2013) have proven invaluable in dissecting the contributions of GSTs in herbicide metabolism and MHR.

Our previous work showing that enhanced metabolism was likely involved in *A. fatua* resistance to several herbicides (Keith *et al.* 2015) led us to investigate the potential involvement of GSH-related enzymes in the MHR phenotype. We used a combination of

proteomic, biochemical, and immunological approaches to compare the enzymes of GSH conjugation and related activities in HS and MHR *A. fatua* plants.

2. Materials and methods

2.1. Plant material

The MHR3 and MHR4 populations were derived from seeds collected in 2006 from two *A. fatua* populations not controlled by 60 g a.i. ha^{-1} pinoxaden (Axial, Syngenta Crop Protection; ACCase inhibitor) in two production fields separated by approximately 8 km in Teton County, Montana, USA. Field-collected seeds (about 90% of which were resistant to 60 g a.i. ha^{-1} pinoxaden, data not shown) were subjected to two generations of recurrent group selection (50 plants each generation) by spraying with the same dose of pinoxaden (for MHR3 and MHR4) or surfactant only (susceptible populations), followed by five additional generations with no herbicide selection in the greenhouse. From each generation of 50 plants, all seeds were harvested and a random selection of 50 seeds was used to initiate the next generation. The herbicide susceptible population HS1 was derived from seeds collected from untreated border plants in an adjacent field, and was subsequently confirmed to be 100% susceptible to the herbicides used in these studies (Lehnhoff *et al.* 2013b). A second susceptible population, HS2, is the inbred nondormant SH430 line used in seed dormancy research (Naylor & Jana 1976; Johnson *et al.* 1995). Plants were grown under a 16-hr photoperiod of natural sunlight supplemented with mercury vapor lamps (165 μmol m^{-2} sec^{-1}) at 25 ± 4 ˚C in standard greenhouse soil mix [1:1:1 (by vol) Bozeman silt loam:Sunshine mix #12 (Sun Gro Horticulture, Inc., Bellvue, WA):perlite] and fertilized weekly with Jack's water soluble 20 N-20 P-20 K

(JR Peters Inc., Allentown, PA). All plants for each experiment were grown on the same greenhouse bench and were harvested in mid-morning to minimize environmental- and circadian-induced changes in protein levels.

2.2. GSH affinity chromatography

Shoot tissue (250 g) from fully tillered (BBCH stage 29) HS1 and MHR4 plants was ground under liquid nitrogen and suspended in ice cold extraction buffer (EB) containing 0.1 M Tris HCl (pH 7.5), 2 mM EDTA , 1 mM DTT, 1 mM PMSF, and 5% (w/v) PVPP. The slurries were homogenized (Polytron Homogenizer, Brinkman Instruments, Westbury NY) for 4 min on ice and filtered through Miracloth (EMD Millipore, Merck KGaA, Darmstadt, Germany). The filtrates were centrifuged at 19,600 x g for 20 min at 4 ˚C and the supernatants subjected to 0-80% ammonium sulfate precipitation at 0 ˚C. After centrifugation at 14,300 x g for 15 min at 4 ˚C, the resulting protein pellets were redissolved in EB and dialyzed against the same buffer for 16 hr at 4 ˚C. Following addition of Triton X-100 to 1% (v/v), protein samples (10 mg ml^{-1}) were subjected to GSH-agarose (Sigma-Aldrich G4510) affinity chromatography according to manufacturer's instructions. GSH-binding proteins were eluted with 10 mM reduced GSH, precipitated twice with acetone at -20 ˚C overnight and resuspended in DIGE buffer (7 M urea, 2 M thiourea, 30 mM Tris pH 8.5, 4% [w/v] CHAPS, 1X protease inhibitor and nuclease mix [GE Healthcare Life Sciences, Pittsburgh, PA], and 0.1% [w/v] bromophenol blue) at 2 mg ml^{-1}. Protein concentrations were determined (Bradford 1976) using bovine serum albumin (BSA) fraction V as standard.

2.3. 2D-DIGE

Two-dimensional difference gel electrophoresis (2D-DIGE) was initiated by minimal fluorescent labeling of lysine side-chains (1 per 100) with N-hydroxysuccinimide ester cyanine dyes (Z-CyDyes; Z-Cy3, Z-Cy5, Z-Cy2; (Epstein *et al.* 2013)) according to the manufacturer's protocol (GE Healthcare Bio-Sciences Corp. Piscataway, NY). Briefly, GSH affinity chromatography extracts containing 50 ug protein, were labeled separately on ice with 400 pmol of either Z-Cy3 or Z-Cy5 Cydye DIGE fluors dissolved in DMF. The internal standard, an equimolar mixture of all protein extracts, was labeled with Z-Cy2. Labeling reactions were quenched with 1 µl of 10 mM lysine, held on ice for 10 min, combined appropriately (ex. HS1-Z-Cy3, MHR4-Z-Cy5, and internal standard-Z-Cy2), diluted to a final volume of 450 µl with isoelectric focusing (IEF) buffer (DIGE buffer containing 50 mM DTT and 0.5% (v/v) IPG buffer 3-11 NL (GE Healthcare Bio-Sciences Corp. Piscataway, NY), and incubated for 1 hr at room temperature. IEF and SDS-PAGE followed the methods of Maaty *et al.* (2012).

2.3.1. Image acquisition and analysis

Gels were scanned using a Typhoon Trio Imager according to the manufacturer's protocol (GE Healthcare Bio-Sciences Corp. Piscataway, NY 08855) at 100 µm resolution and 640 V for PMT. Images were subjected to an automated difference in-gel analysis using Progenesis SameSpots v 3.0.2 software (Nonlinear Dynamics Ltd. Newcastle, UK). Individual gel spots were co-detected as DIGE image pairs and linked to the corresponding in-gel Z-Cy2 standard to allow between-gel comparisons and statistical analyses. Gels used for protein identification contained 400 µg of protein each and were

stained with colloidal coomassie stain (Dyballa & Metzger 2009), destained in 10% acetic acid, and stored at 4 ˚C in 1% acetic acid until spot excision.

2.3.2 Protein mass determination and analysis

Protein spots of interest were excised, digested with porcine trypsin (Promega Corp. Madison, WI 53711), and eluted as described in Shevchenko *et al.* (2006). The resulting peptides were subjected to mass analysis performed on Bruker maXis Impact with Dionex 3000 nano-uHPLC controlled with Chromeleon Xpress (2.13 for Hystar). Briefly, samples of 20 µl each were processed via Dionex Ultimate 3000 nano UHPLC, with an Acclaim PepMap100 C18 column (300 µm x 5 mm) used for both trapping and final peptide separation. Chromatography was as follows: solvent consisted of H_2O with 0.1% (v/v) formic acid for channel "A" and acetonitrile for channel "B". Following sample trapping for 2 min at a flow rate of 20 µl min^{-1}, the HPLC valve was switched to elution position. From 0.0 min to 2.5 min, the elution solvent pump composition was held at 5% B. From 2.5 to 20 min, the elution solvent gradient was linearly changed from 5% to 30% B. From 20 to 23 minutes, the gradient was ramped from 30% to 95% B. From 23 to 28 min, the solvent was held at 95% B, and from 28 min to 30 min the solvent was linearly ramped from 95% B to 7% B. During the entire run, the loading pump solvent was held at 20 µl min^{-1} of 97% water, 3% acetonitrile, and 0.1% formic acid. The mass spectrometer used was a Bruker maXis Impact with CaptiveSpray ESI source with a resolution and accuracy of approximately 40,000 and <5 ppm, respectively. Spectra were collected in positive mode from 200 to 2500 m/z at a maximum rate of 2 Hz for both precursor and fragment spectra and with adaptive acquisition time for highly-abundant

ions. The resulting data files were processed to select fragmentation spectra with Bruker DataAnalysis and exported as MGF files.

Protein identification followed the methods outlined in Mason *et al.* (2016) using SearchGUI (V3.0.3) and Peptideshaker (V1.12.3), initiated by generating a custom protein sequence file (.FASTA) from the *Oryza sativa* subsp. japonica (retrieved 2016; http://www.UniProt.org; containing 121,989 entries) and *Brachypodium distachyo*n (retrieved 2016; http://www.UniProt.org; containing 50,507 entries) databases. Sequences for potential contaminants human keratin (retrieved 2016; http://www.UniProt.org; containing 49 entries) and porcine trypsin (retrieved 2016; http://www.UniProt.org; containing 1 entry) were added to the sequence file to create a final target-decoy library. The library was queried against data files using the following search parameters: up to two missed cleavages allowed, precursor charges +2; +3; +4, precursor ion mass tolerance 30 ppm, and fragmentation mass tolerance of 0.5 Da. Post translational modifications were defined as oxidation of M, acetylation of N-terminus, and carboxylation of C-terminus. Two or more significant peptides with an FDR $\leq$ 1.0% (PeptideShaker software (Vaudel *et al.* 2015)) were required for annotation of each protein from the PaxDb database (Wang *et al.* 2012) as accessed through UniProtKB and the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).

2.4. Enzyme assays

Proteins were extracted from four replicate three-leaf stage (BBCH stage 13) HS and MHR plants as described above except using a 0-80% ammonium sulfate precipitation step and desalting with Zeba™ spin desalting columns (ThermoFischer

Scientific, Waltham, MA). Samples were held on ice for immediate use or stored at -80 ˚C. Total protein concentrations were determined as described above and results are expressed as enzyme specific activities.

2.4.1. Glutathione *S*-transferase

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined as described in Habig, Pabst and Jakoby (1974) in 0.1 M KPO_4 buffer (pH 7.6) containing 1 mM reduced glutathione (GSH), 1 mM CDNB, and 37.5 µg of plant protein. Enzyme activity was determined by measuring the increase in absorbance at 340 nm over 5 min at 21 ˚C.

2.4.2. Glutathione peroxidase

Glutathione peroxidase (GPx) activity was determined as described in Weydert and Cullen (2010) in 50 mM Tris-HCl (pH 7.6) containing 5 mM EDTA, 1 mg ml^{-1} BSA fraction V, 0.2 mM NADPH, 1 mM GSH, 1 U ml^{-1} glutathione reductase, 315.8 µg protein extract, and either 1.5 mM cumene hydroperoxide or linoleic acid hydroperoxide. Linoleic acid hydroperoxide was synthesized according to Edwards and Dixon (2005). Enzyme activity was determined by measuring the decrease in absorbance at 340 nm over 5 min at 21 ˚C.

2.4.3. Dehydroascorbate reductase

Dehydroascorbate reductase (DHAR) activity was determined as described in Dixon, Davis and Edwards (2002) in 90 mM KPO_4 buffer (pH 6.5) containing 5 mM GSH, 0.5 mM dehydroascorbate, and 356 µg protein extract. Enzyme activity was determined by measuring the increase in absorbance at 265 nm over 120 s at 21 ˚C.

2.4.4. 2-Hydroxyethyl disulfide

2-Hydroxyethyl disulfide (HED) thioltransferase activity was determined as described in Dixon, Davis and Edwards (2002) in 0.1 M Tris-HCl (pH 7.8) containing 2 mM EDTA, 1 mM GSH, 0.6 U ml^{-1} glutathione reductase, 0.7 mM HED, 0.25 mM NADPH, and 480 µg protein extract. Following HED addition, the assay mixture was incubated at 30 ˚C for 3 min, after which enzyme activity was determined by measuring the decrease in absorbance at 340 nm over 120 s at 21 ˚C.

2.5. HPLC assays

Proteins were extracted from four replicate three-leaf stage (BBCH stage 13) HS1 and MHR4 plants as described above except the extraction buffer contained 100 mM KPO_4 (pH 7.0), 1 mM DTT, 2 mM EDTA, and 500 mM NaCl. Total protein concentrations were determined as described above. GST activity assays (250 µl) contained 50 mM KPO_4 buffer (pH 7.0), 25 µg fenoxaprop-P-ethyl (96.6% purity; Bayer CropScience) or fenoxaprop-acid (98% purity; Sigma-Aldrich) or 25 µg imazamethabenz-methyl (90% purity; American Cyanamid Company), 1 mM reduced glutathione, and 100 µg plant protein. Blank assays contained 50 mM KPO_4 buffer (pH 7.0), extraction buffer, fenoxaprop-P-ethyl and fenoxaprop acid or imazamethabenz-methyl and imazamethabenz acid (synthesized from imazamethabenz-methyl). Assays were incubated at 30 C for 30, 60, 180, 300, or 1440 min, reactions were stopped by addition of an equal volume of methanol, held at -20 ˚C for 4 hr, and centrifuged for 20 min at 14,000 x g at 4 ˚C. Supernatants were subjected to HPLC separation using a Phenomenx PLRP-S 150 mm column at 60 ˚C with a flow rate of 0.5 ml min^{-1} on an

Agilent 1100 binary pump with an Agilent UV detector at 180 nm. For fenoxaprop-P-ethyl assays, Solvent A was water containing 0.1% (v/v) formic acid and solvent B was acetonitrile. Following column equilibration for one min at 20% B, the sample was injected and a gradient was linearly ramped from 60% to 90% B over the 10 min run. All other HPLC methods followed those of Marles, Devine and Hall (1993).

Mass analysis of herbicide-GSH conjugates was conducted using an Agilent 6538 Q-TOF with dual-ESI source. Resolution was approximately 20,000, accuracy was < 5 ppm, and source parameters were: drying gas 12 L min^{-1}, nebulizer 55 psi, capillary voltage 3500 V, and capillary exit 120 V. Spectra were collected in positive mode from 50 to 1000 m/z at a rate of 2 Hz. Resulting spectral data were processed using the Bruker Daltonics Compass data analysis 4.2 SR2 open lab CDS, Chemstation edition for LC and LCMS systems (Agilent Technologies rev C.01.05).

For enzyme and HPLC assays, linear mixed effects models were constructed to compare the rates of enzyme specific activity among populations. Models were fit using the lme function from the nlme package in R (R 2016). Differences in mean enzyme specific activities were separated using Tukey's HSD post hoc tests in the R lsmeans package.

2.6. GST inducer and reversal experiments

For GST inducer experiments, three-leaf stage (BBCH stage 13) HS and MHR seedlings were treated with five doses of the following technical grade (active ingredient only) herbicides with and without their safener (mefenpyr-diethyl) components: fenoxaprop-P-ethyl (Puma, Bayer CropScience), thiencarbazone-methyl (Varro, Bayer

CropScience) plus 0.125% (v/v) nonionic surfactant, or mesosulfuron-methyl (Osprey, Bayer CropScience) plus 0.125% (v/v) nonionic surfactant. Technical grade herbicides and the safener were applied at 0, 0.1x, 0.3x, 1x, 3x, and 6x doses, with 1x being the manufacturer's recommended application dose for small grains, which are 86 g a.i. ha^{-1} for fenoxaprop-P-ethyl, 5 g a.i. ha^{-1} for thiencarbazone-methyl, 15 g a.i. ha^{-1} for mesosulfuron-methyl. The 1x field rate of mefenpyr-diethyl used in this study was 30 g a.i. ha^{-1} for mesosulfuron-methyl and thiencarbazone-methyl treatments and 25.2 g a.i. ha^{-1} for fenoxaprop-P-ethyl treatments (safener rate information provided by Bayer CropScience). For GST inhibitor experiments, three-leaf stage (BBCH stage 13) HS and MHR seedlings were treated with 80 g ha^{-1} 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) plus 0.125% (v/v) nonionic surfactant 48 hr before application of 86 g a.i. ha^{-1} fenoxaprop-P-ethyl. All treatments were applied using a moving nozzle sprayer in 94 L water ha^{-1}. When the spray solution had dried, plants were returned to the greenhouse conditions described above for 3 wk, after which aboveground biomass was harvested, dried for 1 wk at 40 ˚C, and weighed. There were four biological replications of each herbicide dose, and the experiments were conducted twice.

To compare the effects of safener treatment within a herbicide application rate, linear mixed effects models were constructed with fixed effects for treatment and population and a random effect for experimental run. Models were fit using the lme function in R (R 2016), and means were separated using Tukey's HSD post hoc tests. To compare GST inhibitor effects, linear mixed effects models were constructed with fixed effects for treatment and population and a random effect for experimental run. Models

were fit using the lme function in R (R 2016), and means were separated using Tukey's HSD post hoc tests.

2.7. GST Immunoassays

2.7.1. 1D PAGE

Protein extracts (20 µg per lane) obtained as described above were heated at 95 ˚C for 5 min in 1x Laemmli SDS sample buffer (Cleveland *et al.* 1977) before being subjected to SDS-PAGE in 12.5% gels at 2.5 mA cm^{-1} for 3 hr with water cooling.

2.7.2. 2D PAGE

Protein extracts obtained as described above and were subjected to the isoelectric focusing and electrophoresis methods of Hochstrasser *et al.* (1988) with the following modifications. Proteins were precipitated with 10% (v/v) TCA at 0 ˚C, centrifuged at 14,000 x g for 2 min at 4 ˚C, and the pellets washed twice with ether. After brief drying, the pellets were resuspended in IEF sample buffer (5 mg protein ml^{-1}) at 37 ˚C, centrifuged at 14,000 x g for 1 min at 25 ˚C, and the supernatant loaded onto 0.7 mm IEF capillary tube gels (30:0.8 acrylamide:piperazine diacrylamide) containing 4% (v/v) pH 3-10 and 1% (v/v) pH 4-6 ampholytes (Bio-Rad 163-1112 and 163-1142). Isoelectric focusing was done for 16 hr at 850 V with water cooling, and SDS-PAGE performed as described above.

2.8. Western blots

Proteins were electroblotted from SDS gels onto PVDF membrane (Pall Corporation, Radnor, PA) and probed with rabbit polyclonal antisera against either lambda (Theodoulou *et al.* 1999), tau (Cummins, Cole & Edwards 1997), or phi (Dixon,

Cole & Edwards 1998) GSTs diluted 1:5,000 (v/v) in blotto (10% [w/v] nonfat dry powdered milk/phosphate-buffered saline/0.001% [w/v] sodium azide) followed by detection with goat anti-rabbit IgG/alkaline phosphatase (Sigma-Aldrich AP132A) diluted 1:10,000 (v/v) in blotto. Densitometric analyses were performed using ImageJ (Schneider, Rasband & Eliceiri 2012) to compare amounts of immunoreactive proteins among populations.

3. Results

3.1. Proteomic analysis

2D-DIGE was used to compare constitutive profiles of abundant, soluble GSH-binding proteins from untreated MHR4 and HS1 plants (Figure 3.1, Table 3.1). Statistical analysis identified 33 differential spots corresponding to 24 unique proteins, identified here by the best annotation from UniProt and the Rice Genome Annotation Project. Of these, five proteins were detected at higher levels in MHR4 plants, including three tau class GSTs (OsGSTU1, OsGSTU5, OsGSTU27), one phi class GST (OsGSTF3), and an unclassified GST (Table 3.1). In addition to GSTs, eight other protein spots (13, 17, 19-23, 30) corresponding to five unique proteins were identified as GSH-binding proteins from non-plant systems (Table 3.1). DIGE gels also revealed 17 differential spots corresponding to 14 unique proteins with no known GSH-binding activity in plants (Table B1).

3.2. Biochemical analysis

3.2.1 Enzyme assays

GST specific activity towards CDNB (GST(CDNB)) was 1.2-fold ($p = 0.018$) higher in MHR4 than in HS1 plants and 1.3- ($p \leq 0.0001$) and 1.2-fold ($p = 0.007$) higher in MHR3 than in HS1 and HS2 plants, respectively (Table 3.2). There were no differences in GST(CDNB) activity between MHR4 and HS2 plants, between HS1 and HS2 plants, or between MHR3 and MHR4 plants.

GST specific activity assays on fenoxaprop-P-ethyl were conducted using both fenoxaprop-P-ethyl and fenoxaprop acid to account for non-enzymatic GSH conjugation (Cummins *et al.* 1997). Metabolism of imazamethabenz-methyl in *A. fatua* (Nandula & Messersmith 2002) and other monocots proceeds mainly via glucose conjugation, although GSH conjugation of related herbicides is observed in other species (Jablonkai 2015). For both herbicides, GST activities were very low and were not significantly different between untreated MHR4 and HS1 plants (Table 3.2). Mass analyses of fenoxaprop acid-glutathione and imazamethabenz acid-glutathione conjugates confirmed their molecular masses as 458.87 and 581.64, respectively.

DHAR specific activity was 1.4-fold higher ($p \leq 0.0001$) in MHR versus HS plants, and there were no significant differences between HS1 and HS2 plants, or between MHR3 and MHR4 plants (Table 3.2). HED and GPx specific activities were not significantly different among any populations (Table 3.2). However, MHR plants had slightly but not significantly higher GPx activity on linoleic acid than HS plants.

3.2.2. GST inducer

There were no differences in mean dry weights (DWs) of HS1, HS2, and MHR3 plants treated with mesosulfuron-methyl alone as compared to plants treated with mesosulfuron-methyl plus the GST inducer mefenpyr at any herbicide dose (Table B2). For MHR4, plant DW was significantly larger for plants treated with mesosulfuron-methyl plus mefenpyr, as compared to plants treated with mesosulfuron-methyl alone, at the 15 g a.i. ha^{-1} ($p = 0.0947$) and 45 g a.i. ha^{-1} ($p = 0.0499$) doses. For thiencarbazone-methyl, there were no differences in DWs of HS1, HS2, and MHR4 plants treated with herbicide alone as compared to plants treated with herbicide plus mefenpyr at any herbicide dose (Table B2). However, MHR3 plants treated with thiencarbazone-methyl plus mefenpyr at 0.5 g a.i. ha^{-1} had significantly more DW ($p = 0.069$) than plants treated with thiencarbazone-methyl alone. For fenoxaprop-P-ethyl, there were no differences in DW of MRH4 plants treated with the herbicide alone or in combination with mefenpyr at any dose (Table B2). In contrast, HS1, MHR3, and MHR4 plants treated with 8.6 g a.i. ha^{-1} fenoxaprop-P-ethyl plus mefenpyr had significantly more DW ($p \leq 0.001$, $p = 0.024$, and $p = 0.061$, respectively) than their counterparts treated with the same dose of herbicide alone.

3.2.3. GST inhibitor

HS1 and HS2 plants treated with fenoxaprop-P-ethyl alone were significantly injured ($p \leq 0.0001$), and pre-treatment with the GST inhibitor NBD-Cl did not alter their sensitivity (Fig. 3.2). MHR3 plants were slightly but not significantly injured by treatment with fenoxaprop-P-ethyl, while DWs of MHR4 plants were not reduced by this

treatment. Pre-treatment of MHR plants with NBD-Cl did not affect their resistance to fenoxaprop-P-ethyl. As compared to all other treatments, DWs of both HS and MHR plants were significantly increased ($p \leq 0.0001$) by treatment with NBD-Cl alone.

3.3. GST immunoassays

3.3.1. 1D PAGE

We used polyclonal antibodies raised against lambda, phi, and tau GSTs in Western immunoassays to detect immunoreactive proteins in HS and MHR *A. fatua* plants (Fig. 3.3a). These antibodies have been successfully used to identify GST orthologs in *Triticum aestivum* (Theodoulou *et al.* 2003; Taylor *et al.* 2013), *A. myosuroides* (Cummins *et al.* 1997; Brazier, Cole & Edwards 2002), and other species.

Antibodies to lambda GSTs recognized 29, 27, and 25 kDa proteins in *A. fatua* plants (Fig. 3.3a). Levels of the 29 kDa protein were 2.2- and 6.1-fold higher in MHR3 than in HS1 and HS2 plants, respectively. Similarly, levels of the 29 kDa protein were 1.7- and 4.8-fold higher in MHR4 than in HS1 and HS2 plants, respectively. The 27 and 25 kDa proteins were not detectable in either HS population under these conditions.

Four *A. fatu*a proteins of 28, 28.5, 26, and 25 kDa were recognized by anti-tau GST antibodies (Fig. 3.3a). Levels of the 28 kDa protein were 2.1-, 2.3-, and 4.6-fold higher in HS2, MHR3, and MHR4 plants compared to HS1 plants, respectively. Levels of the other tau immunoreactive proteins were similar between HS and MHR plants.

Anti-phi GST antibodies recognized four proteins of 29, 28.5, 27, and 26 kDa in *A. fatua* plants (Fig. 3.3a). Levels of the 29 kDa protein were 4.7- and 5.9-fold higher in MHR3 than in HS1 and HS2 plants, respectively. This protein was also 6.9- and 8.6-fold

higher in MHR4 than in HS1 and HS2 plants, respectively. Levels of the 28.5 kDa protein were 1.8- and 3.0-fold higher in MHR3 than in HS1 and HS2 plants, respectively. The 28.5 kDa protein was also present at 2.7- and 4.4-fold higher levels in MHR4 plants as compared to HS1 and HS2 plants, respectively. Levels of the 27 and 26 kDa phi immunoreactive proteins were similar among plants of all populations.

3.3.2. 2D PAGE

Differential levels of the 29 kDa phi GST protein were further examined using 2D electrophoresis and Western immunoassay (Fig. 3.3b). The 29 kDa protein was resolved into two proteins (arrows) with pIs of approximately 4 and 6 that were detected in MHR3 but not HS2 plants. Significantly, amounts of the proteins recognized by anti-lambda, -tau, and -phi GST antibodies did not change in HS or MHR plants 24 hr after treatment with recommended field use rates of 94 g a.i. ha^{-1} imazamethabenz-methyl, 106 g a.i. ha^{-1} difenzoquat, or 86 g a.i. ha^{-1} fenoxaprop-P-ethyl (data not shown).

4. Discussion

Proteomic comparisons have been used by a number of laboratories to investigate changes in GSH-related proteins associated with xenome activation. In most cases, GSH-related enzymes are induced or otherwise modified rapidly after the imposition of stress, illustrating the central role of redox maintenance. For example, 2D-DIGE analyses of *O. sativa* response to bentazon treatment (Fang *et al.* 2015) and *Anabena* spp. tolerance to butachlor (Agrawal *et al.* 2014) demonstrated the upregulation of several xenome enzymes including GSTs. Smith *et al.* (2004) used proteome comparisons to identify specific phi and tau GSTs that were induced by the herbicide safener benoxacor or

copper. We used a similar approach, but instead compared constitutive levels of GSH-binding proteins in MHR and HS plants in the absence of herbicide treatment. Our results confirm that *A. fatua* MHR plants display constitutive changes in the levels of several GSH-related proteins.

Of all plant GSH-related enzymes, the GST superfamily is especially large and diverse (Chronopoulou *et al.* 2014; Labrou *et al.* 2015). Soluble GSTs are typically dimers of 24-27 kDa subunits, with isoelectric points near pH 5 (Dixon, Davis & Edwards 2002). Higher plants have at least ten different GST classes, of which the predominant phi and tau classes have broad substrate specificities and are primarily responsible for herbicide detoxification (Edwards & Dixon 2000). GSTs conjugate GSH to oxidized xenobiotics as part of Phase II metabolism, and individual GSTs of several classes are key players in NTSR to herbicides (Reade, Milner & Cobb 2004). For example, resistance to quinclorac in *Echinochloa crus-galli* was associated with increased expression of a GST (Li *et al.* 2013), and transgenic overexpression of a lambda GST in *O. sativa* (Hu 2014) or a tau GST in *Nicotiana tabacum* (Cicero *et al.* 2015) conferred resistance to several herbicides. The best-characterized role of GSTs in NTSR is for the ‘Peldon’ MHR *A. myosuroides* population that is resistant to the PSII inhibitor chlorotoluron and several ACCase inhibitor graminicides. Early work showed that MHR plants had higher GST specific activity towards fenoxaprop-P-ethyl, elevated levels of immuno-recognized GST proteins, and higher concentrations of glutathione and hydroxymethylglutathione than susceptible plants (Cummins *et al.* 1997). Subsequently, Cummins, Bryant and Edwards (2009) reported that antioxidant changes induced by

herbicide safeners closely mirrored, but did not fully account for, the MHR phenotype. Finally and most recently, the same authors elegantly showed that MHR in *A. myosuroides* was conferred by overexpression of the phi class AmGSTF1, which acted not through traditional degradation, but through a novel and direct regulatory control on flavonoid metabolism (Cummins *et al.* 2013).

In *A. fatua*, GSH affinity chromatography and 2D-DIGE revealed constitutively higher levels of three tau class (OsGSTU1, OsGSTU5, and OsGSTU27) GSTs and one phi class (OsGSTF3) GST in MHR4 as compared to HS1 plants. Tau and phi GSTs are the largest plant-specific class of GSTs with known roles in herbicide metabolism (Cummins *et al.* 2011). In this regard, Cho and Kong (2007) showed that OsGSTF3 and OsGSTU5 had high specific activities towards several chloroacetanilide herbicides and the diphenylether herbicide fluorodifen, respectively. In addition to herbicide detoxification, the GST orthologs identified in this study also have roles in abiotic and biotic stress response. For example, Jain, Ghanashyam and Bhattacharjee (2010) reported that OsGSTU5 was up-regulated in *O. sativa* plants in response to desiccation, salt, and cold abiotic stresses, while both OsGSTU1 and OsGSTU5 were up-regulated in *O. sativa* plants grown in arsenate-contaminated soil and OsGSTU1 was induced after infection with the fungal pathogen *Magnaporthe grisea*. In addition to the *A. fatua* proteins identified via 2D-DIGE having well-known GSH-binding roles in plants, we found constitutively lower levels of polyubiquitin, fructose-bisphosphate aldolase, phosphoribulokinase, and malate dehydrogenase in MHR4 as compared to HS1 plants. These proteins are known to bind or are regulated by GSH in non-plant systems, such as

human polyubiquitin and fructose-bisphosphate aldolase which are reduced by GSH during recovery from oxidative stress (Bond & Offermann 1980; Jahngen-Hodge *et al.* 1997). Lower levels of these proteins may reflect a constitutively altered redox state in MHR4 plants.

OsGSTU5, OsGSTU1, and OsGSTF3 were each identified from two unique spots, as illustrated by the spot pairs 6/7, 8/9, and 10/11, respectively, shown in Figure 3.1. Similar 2D gel patterns for GSTs have been reported in maize (Edwards *et al.* 2005) and other plant species (Dixon & Edwards 2009), and are proposed to arise from differential oxidation of methionine and cysteine residues, or as a result of partial protein degradation (Dixon & Edwards 2010). The *A. fatua* protein spot 9 was predicted to contain a dehydrated glutamic acid residue, although the biological significance of this modification is unknown. Comparisons of the predicted GST peptide sequences reported here against *A. fatua* transcript sequences from our RNA-Seq transcriptome analysis (Keith et al. submitted) revealed that annotations within each GST class agreed between the two independent analyses, but peptide sequences did not directly correlate to individual *A. fatua* transcripts (data not shown).

In untreated *A. fatua*, GST specific activity towards the model substrate CDNB was slightly but significantly higher in MHR than in HS plants, likely reflecting the constitutive changes in individual GSTs seen in our proteomic and Western assays. However, the very low GST specific activities towards imazamethabenz-methyl and fenoxaprop-P-ethyl were not different between HS1 and MHR4 *A. fatua* plants,

indicating that enhanced rates of metabolism are not directly responsible for the resistance phenotype. We did not test GST activities using hydroxymethylglutathione.

DHAR specific activity, a key step in the glutathione-ascorbate reactive oxygen species protective pathway (Asada 2006), was also slightly but significantly higher in untreated MHR than HS plants. Elevated DHAR and GST(CDNB) activities were also reported for untreated MHR *A. myosuroides* plants (Cummins *et al.* 1997). GSH-dependent peroxidase (GPx) activity, which reduces oxidative stress-generated hydroperoxides to monohydroxyalcohols, and HED thioltransferase activity were not significantly different between untreated MHR and HS *A. fatua* plants.

Safeners like mefenpyr are chemicals applied with herbicides that protect cereal crops from injury while not reducing weed control efficacy (Kraehmer *et al.* 2014). They function by inducing herbicide catabolic enzymes in crop plants, such as GSTs, P450s, and glucosyltransferases (Taylor *et al.* 2013). In particular, mefenpyr is known to safen ACCase-inhibitor herbicides like fenoxaprop-P-ethyl (Taylor *et al.* 2013) and the ALS inhibitor mesosulfuron-methyl (Hacker *et al.* 2001) in cereals. An ongoing question concerning safener use is whether they also induce similar systems in weedy plants, especially in MHR populations, and thus enhance resistance levels. We treated MHR *A. fatua* plants with a range of herbicide doses with and without mefenpyr to examine this question in detail. Our results show that mefenpyr reduced the efficacy of low doses of fenoxaprop-P-ethyl on MHR4 but not MHR3 plants. Mefenpyr treatment of MHR3 or MHR4 *A. fatua* plants also partially reduced the efficacy of thiencarbazone-methyl or mesosulfuron-methyl, respectively. These mixed results indicate that mefenpyr may

slightly reduce herbicide efficacy on MHR *A. fatua* populations, but that the effect is likely herbicide- and population-specific. In this regard, our results also support current resistance management recommendations of applying full labelled doses, to avoid the potential rapid evolution of MHR under reduced doses (Neve & Powles 2005a). The subtle differences between MHR3 and MHR4 responses to mefenpyr-containing treatments likely reflect alterations in their resistance mechanisms to individual ALS (thiencarbazone-methyl and mesosulfuron-methyl) and ACCase (fenoxaprop-P-ethyl) inhibitors. In support of this idea, we previously documented significant ED_{50} (dose causing 50% injury) differences between these two populations for paraquat (Keith *et al.* 2015), difenzoquat, flucarbazone, and imazamethabenz-methyl (Lehnhoff *et al.* 2013b). In contrast to MHR4 *A. fatua*, MHR *A. myosuroides* plants were not rendered more resistant to fenoxaprop-P-ethyl by the addition of mefenpyr (Cummins, Bryant & Edwards 2009).

For HS *A. fatua* plants, mefenpyr did not reduce the efficacy of thiencarbazone-methyl or mesosulfuron-methyl, and slightly reduced the efficacy of a low rate of fenoxaprop-P-ethyl, in contrast to its effects in cereal crops and HS *A. myosuroides* (Cummins, Bryant & Edwards 2009). Additionally, the lack of effect of the GST inhibitor NBD-Cl on fenoxaprop-P-ethyl activity against HS or MHR *A. fatua* is in stark contrast to its effects on MHR *A. myosuroides*, where it significantly enhanced the phytotoxicity of chlorotoluron, fenoxaprop-P-ethyl, and clodinafop-propargyl (Cummins *et al.* 2013). Such comparisons show that HS and MHR populations of these two weedy monocot species respond differently to a GST inducer (mefenpyr) and inhibitor (NBD-Cl),

supporting the idea that subtle differences in their xenome activation regulatory networks can give rise to quantitatively different MHR phenotypes. The growth stimulation of *A. fatua* plants caused by mefenpyr treatment was similar to that reported for wheat (Taylor *et al.* 2013), while a similar effect by NBD-Cl was unexpected, and deserves further scrutiny.

Western analyses revealed detectable increases in proteins recognized by anti-lambda, -tau, and -phi GST antibodies in untreated MHR versus HS *A. fatua* plants. These changes are similar to those reported for untreated *A. myosuroides* plants, including the detection of specific phi GSTs in MHR but not HS plants (Cummins, Bryant & Edwards 2009). However, none of the immunoreactive GST proteins in HS or MHR *A. fatua* were induced to higher levels after treatment with three different herbicide mechanisms of action, in direct contrast to results from *A. myosuroides* (Cummins, Cole & Edwards 1999), maize (Pang *et al.* 2012), and other species (Flury, Adam & Kreuz 1995). The *A. fatua* results are supported by a lack of transcriptional induction of four GSTs in response to flucarbazone-sodium treatment of HS plants (Keith et al. submitted). Together, these findings indicate that constitutively increased GST levels in *A. fatua* plants may be sufficient for their possible role(s) in the MHR phenotype without the need for additional induction after herbicide treatment, or that they are unrelated.

Anti-lambda, -tau, and -phi GST antibodies recognized three, four, and four proteins, respectively, in allohexaploid *A. fatua* plants, as compared to one, one, and two proteins in the closely related diploid *A. myosuroides*, respectively (Cummins, Bryant & Edwards 2009). These differences likely reflect gene duplication events (i.e., creation of

homoeologs) following polyploidization of *A. fatua* from its diploid progenitors (Yang *et al.* 1999b). The resulting greater heterozygosity in *A. fatua* and other polyploids likely contributes to their over-representation among successful weedy species (te Beest *et al.* 2011) and has provided a diverse genetic background for the evolution of MHR. For *A. fatua*, enhanced heterozygosity conferred by hexaploidy is likely offset in part by its predominantly autogamous pollination behavior. The prevailing view that NTSR evolves through selection and introgression of individual alleles through outcrossing (Délye 2013) may thus be less applicable to this species. MHR and HR populations from the Poaceae are over-represented (Holm *et al.* 1991) among 'the world's worst weeds' families (Holt *et al.* 2013), and HR *A. fatua* populations probably have a greater worldwide economic impact than those of any other species (Heap 2017).

In conclusion, the GSTs and other GSH-related proteins described here do not appear to be directly involved in enhanced rates of fenoxaprop-P-ethyl and imazamethabenz-methyl metabolism in MHR *A. fatua*. Instead, we propose that constitutively elevated GST(CDNB) and DHAR enzyme activities, as well as the increased levels of lambda, phi, and tau GSTs documented here are representative of a larger, more global suite of abiotic stress-related changes in MHR plants. We recently showed that a significant number of such transcripts and proteins are constitutively elevated in MHR *A. fatua* plants (Keith et al. submitted), similar to other stress-tolerant plant species and varieties. Of 25 transcripts examined in detail, differential expression of 21 co-segregated with the flucarbazone resistance phenotype in F_3 families. Further, 15 transcripts and proteins constitutively expressed at higher levels in MHR plants have

functions in redox maintenance and stress response (Keith et al. submitted). It thus appears that intensive herbicide usage over several decades has selected for MHR plants with a constitutively induced abiotic stress-response pathway, and that elements of this pathway overlap with xenome activation.

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Table 3.1. Differentially expressed proteins from untreated herbicide susceptible (HS1) and multiple herbicide resistant (MHR4) *Avena fatua* plants identified using two-dimensional fluorescence difference gel electrophoresis.

Spot number	Uniprot	*Oryza sativa* number	Annotation	Fold change[a]	Number of peptides	Peptide sequences[b]
6	Q9FUE5	Os09g0367700	Glutathione *S*-transferase OsGSTU5	1.6	2	FWADYVDK FWADYVDKKLFDCQTR
7	Q9FUE5	Os09g0367700	Glutathione *S*-transferase OsGSTU5	3.1	2	DAAHEQAKRDMAEALGTLEA FWADYVDKK
8	Q10CE7	Os03g0785900	Glutathione *S*-transferase OsGSTU1	2.5	3	HLPSPEKVYDFVGVLK HLPSPEKVYDFVGVLKK EEKELVLLDFWVSPFGQR
9	Q10CE7	Os03g0785900	Glutathione *S*-transferase OsGSTU1	1.8	2	HLPSPEKVYDFVGVLKK *E*LVLLDFWVSPFGQR
10	Q9FUD8	Os03g0135300	Glutathione *S*-transferase OsGSTF3	2.3	3	ATDMAVVEQNEAK LQPFGQVPAFK AAPVTVYGPMISPAVAR
11	Q9FUD8	Os03g0135300	Glutathione *S*-transferase OsGSTF3	2.4	2	ATDMAVVEQNEAK LQPFGQVPAFK
12	Q60DP1	Os03g0643700	Glutathione *S*-transferase	3.5	2	TLEAELGGRRYFGGGGEALGYVDVALAPFTAWF FIDEEFAGVGEPLLPA
13	B7ERQ1	Os07g0638300	Peroxiredoxin	1.6	2	AIKQLNMVDPDEKD IPPGVSDDEAKEKFPQGFDTAD
17	I1GW31	Os04g0628100	Polyubiquitin	-7.5	2	ESTLHLVLR DKEGIPPDQQRLIFAGKQLEDGRT
19	I1IN47	Os11g0171300	Fructose-bisphosphate aldolase, chloroplast precursor	-5.5	10	IVDILVEQGIVPGIK IVDILVEQGIVPGIKVDK KIVDILVEQGIVPGIK LDSIGLENTEANR RLDSIGLENTEANR

						TLLVTPPGLGNYISGAILFEETLYQSTVD TLLVTPPGLGNYISGAILFEETLYQSTVDG TVVSIPNGPSELAVK TWGGRPENVAAAQEALLLR YAAISQDNGLVPIVEPEILLDGEHGIDR
20	I1IN47	Os11g0171300	Fructose-bisphosphate aldolase, chloroplast precursor	7.7	11	ASAYADELVK *E*AAWGLAR IVDILVEQGIVPGIK IVDILVEQGIVPGIKVDK KIVDILVEQGIVPGIK LDSIGLENTEANR RLDSIGLENTEANR TLLVTPPGLGNYISGAILFEETLYQSTVD TVVSIPNGPSELAVK TWGGRPENVAAAQEALLLR YAAISQDNGLVPIVEPEILLDGEHGIDR
21	I1IN47	Os11g0171300	Fructose-bisphosphate aldolase, chloroplast precursor	-6.9	15	AKANSLAQLGK ANSLAQLGK ASAYADELVK IVDILVEQGIVPGIK IVDILVEQGIVPGIKVDK KIVDILVEQGIVPGIK KIVDILVEQGIVPGIKVDK LDSIGLENTEANR QAYRTLLVTPPGLGNYISGAILFEETLYQSTV RLDSIGLENTEANR TLLVTPPGLGNYISGAILFEETLYQSTVDGK TLLVTPPGLGNYISGAILFEETLYQSTVDGKK

						TVVSIPNGPSELAVK TWGGRPENVAAAQEALLLR YAAISQDNGLVPIVEPEILLDGEHGIDR
22	Q6Z8F4	Os02g0698000	Phosphoribulokinase	-8.2	11	ANDFDLMYEQVK DLYEQIIAER FYGEVTQQMLK HADFPGSNNGTGLFQTIVGLK IRDLYEQIIAER KPDFDAFIDPQK KPDFDAFIDPQKQYADAVIEVLPTQLIPDDNE KPDFDAFIDPQKQYADAVIEVLPTQLIPDDNEG LDELIYVESHLSNLSTK QYADAVIEVLPTQLIPDDNEGK QYADAVIEVLPTQLIPDDNEGKVLR
23	Q6Z8F4	Os02g0698000	Phosphoribulokinase	-7.3	10	ANDFDLMYEQVK DLYEQIIAER FYGEVTQQMLK HADFPGSNNGTGLFQTIVGLK IRDLYEQIIAER KPDFDAFIDPQK KPDFDAFIDPQKQYADAVIEVLPTQLIPDDNE LDELIYVESHLSNLSTK QYADAVIEVLPTQLIPDDNEGK QYADAVIEVLPTQLIPDDNEGKVLR
30	Q7XDC8	Os10g0478200	Malate dehydrogenase, cytoplasmic	-7.2	4	EFAPSIPEK MDATAQELSEEK
31	Q69LE6	Os07g0168300	glutathione *S*-transferase OsGST27	2.6	2	TPLLVAWAER EEEIGGGKSELLLA

[a]Positive and negative fold change values indicate higher and lower levels in MHR4 compared to HS1 plants, respectively.

[b]Italic letter indicates dehydrated, underlined letter indicates ammonia loss post translational modifications, respectively.

Table 3.2. Glutathione-related enzyme activities from untreated multiple herbicide resistant (MHR3 and MHR4) and herbicide susceptible (HS1 and HS2) *Avena fatua* plants.

Enzyme[a]	Population	Enzyme Rate(SE)[b]
GST(CDNB)	HS1	93.8(3.9)a[c]
	HS2	98.9(3.8)ab
	MHR3	119.8(2.4)c
	MHR4	112.1(7.9)bc
GST(fenoxaprop-P-ethyl)	HS1	-0.06(4.3)a
	MHR4	15.2(10.0)a
GST(imazamethabenz-methyl)	HS1	3.0(26.7)a
	MHR4	5.8(0.9)a
GPx(cumene)	HS1	27.1(1.5)a
	HS2	29.4(1.5)a
	MHR3	29.5(2.2)a
	MHR4	28.4(0.8)a
GPx(linoleic)	HS1	10.7(1.2)a
	HS2	11.0(1.2)a
	MHR3	12.6(1.2)a
	MHR4	14.1(1.5)a
DHAR	HS1	230.3(5.3)a
	HS2	224.0(3.0)a
	MHR3	324.2(10.4)b
	MHR4	323.3(12.0)b
HED	HS1	82.4(6.0)a
	HS2	60.2(3.0)a
	MHR3	60.9(3.0)a
	MHR4	79.0(12.0)a

[a]GST(CDNB) = glutathione-*S*-transferase activity towards 1-chloro-2,4-dinitrobenzene, GST(fenoxaprop-P-ethyl) = glutathione-*S*-transferase activity towards fenoxaprop-P-ethyl, GST(imazamethabenz-methyl) = glutathione-*S*-transferase activity towards imazamethabenz-methyl, GPx(cumene) = glutathione peroxidase activity towards cumene hydroperoxide, GPx(linoleic) = glutathione peroxidase activity towards linoleic acid hydroperoxide, DHAR = dehydroascorbate reductase, HED = 2-Hydroxyethyl disulfide

[b]Enzyme specific activities for GST(CDNB), GPx(cumene), GPx(linoleic), DHAR, and HED assays are expressed in nkat mg fresh weight^{-1} min^{-1}, enzyme rates for GST(fenoxaprop-P-ethyl) and GST(imazamethabenz-methyl) are expressed in ng mg fresh weight^{-1} min^{-1}

[b]Means (standard error) within an enzyme activity followed by the same letter are not significantly different ($p \leq 0.05$)

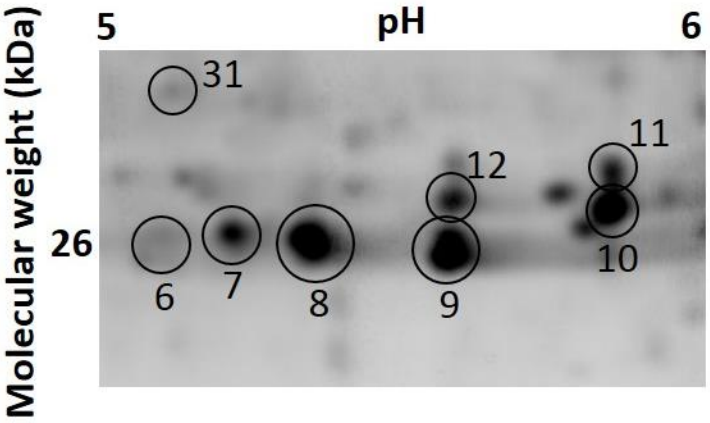

Figure 3.1. Selected area of a two dimensional fluorescence difference electrophoresis gel of the *Avena fatua* GSH-affinity proteome. Numbered and additional spots were excised from the gel and proteins identified using in-gel proteolysis followed LC-MS/MS.

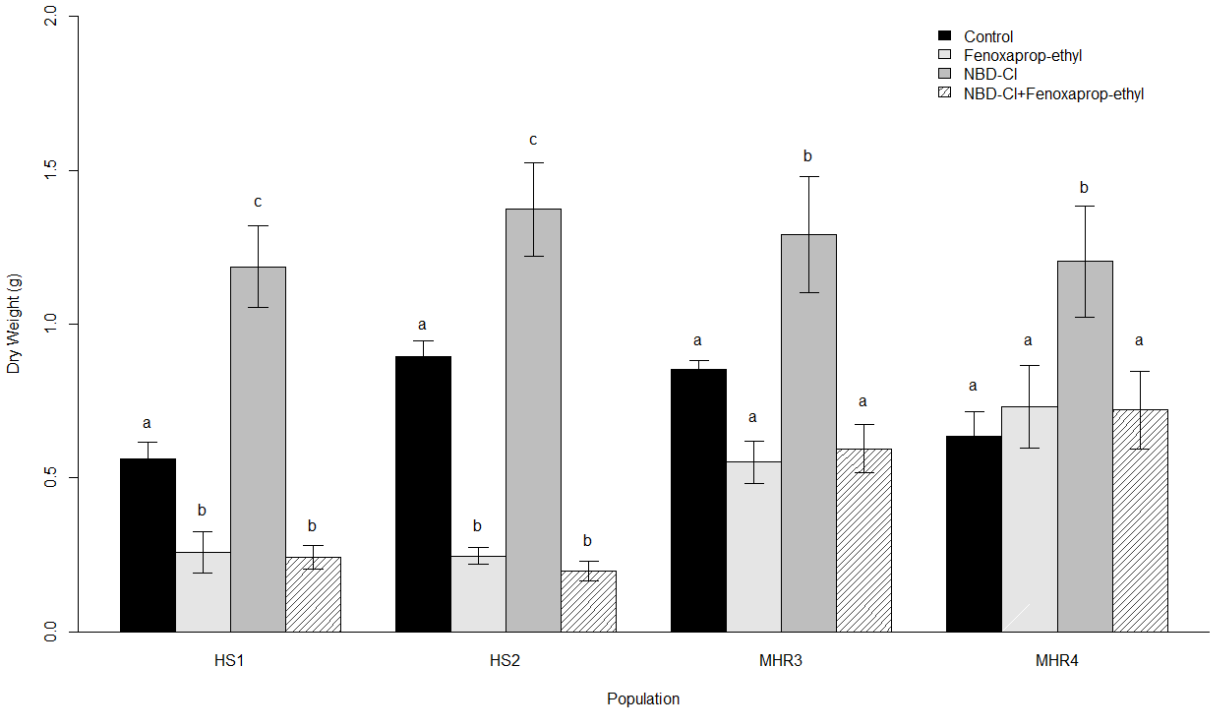

Figure 3.2. Mean dry weight (g) of herbicide susceptible (HS1 and HS2) and multiple herbicide resistant (MHR3 and MHR4) *Avena fatua* plants treated with fenoxaprop-P-ethyl, the GST inhibitor NBC-Cl, or fenoxaprop-P-ethyl plus NBC-Cl. Means within a population followed by the same letter are not significantly different ($p \leq 0.05$).

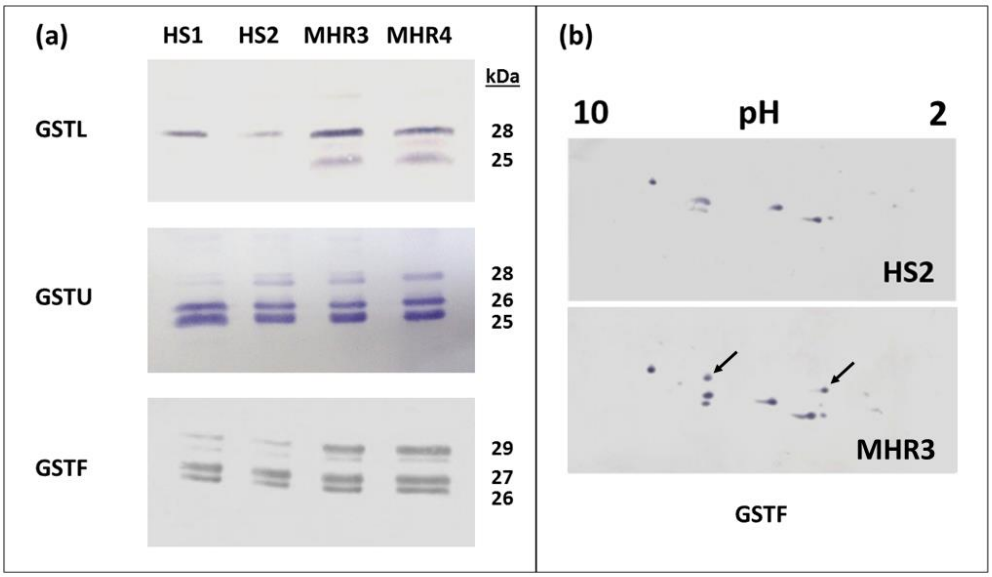

Figure 3.3. (a) 1D immunoblot of proteins recognized by anti-lambda, -tau, and -phi GST antibodies from untreated herbicide susceptible (HS1 and HS2) and multiple herbicide resistant (MHR3 and MHR4) *Avena fatua* plants. (b) 2D immunoblot of proteins recognized by anti-lambda GST antibodies from untreated herbicide susceptible (HS2) and multiple herbicide resistant (MHR3) plants. Arrows indicate protein spots not detected in HS2.

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CHAPTER FOUR

CONSTITUTIVE REDOX AND PHOSPHOPROTEOME CHANGES IN MULTIPLE HERBICIDE RESISTANT *AVENA FATUA* L. ARE SIMILAR TO THOSE OF SYSTEMIC ACQUIRED RESISTANCE AND SYSTEMIC ACQUIRED ACCLIMATION

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Abstract

Plants are routinely confronted with numerous biotic and abiotic stressors and in response have evolved highly effective strategies of systemic acquired resistance (SAR) and systemic acquired acclimation (SAA), respectively. An evolutionarily recent abiotic stress is the application of herbicides to control weedy plants, and intensive herbicide use has selected for resistant weed populations that cause substantial crop yield losses and increase production costs. Non-target site resistance (NTSR) to herbicides is rapidly increasing worldwide and is associated with alterations in generalized stress defense networks. This work sought to investigate the post-translational modifications associated with NTSR in multiple herbicide resistant (MHR) *Avena fatua*, and its commonalities with SAR and SAA. We used proteomic, biochemical, and immunological approaches to survey and compare constitutive protein profiles in MHR and herbicide susceptible (HS) *A. fatua* populations. Analyses of superoxide dismutase activity and enzyme levels did not detect differences between MHR and HS plants. However, phosphoproteome and redox proteome surveys showed that post-translational modification patterns of a number of proteins with functions in core cellular processes, xenobiotic and stress response, reactive oxygen species detoxification and redox maintenance, heat shock response, and intracellular signaling were altered between MHR and HS plants. MHR plants contained constitutively elevated levels of three protein kinases including the lectin S-receptor-like serine/threonine-protein kinase LecRK2, a well-characterized receptor and signal initiator in SAR. Results support the idea that herbicide stress is perceived much the same as other abiotic stresses, and that NTSR shares similar features with SAR and SAA. We speculate

that MHR *A. fatua*'s previous exposure to sublethal herbicide doses, as well as pre-agricultural evolution under a diversity of abiotic and biotic stressors, has led to a heightened state of stress preparedness that includes NTSR to a number of unrelated herbicides.

1. Introduction

Plants are regularly confronted with numerous external stresses from both biotic and abiotic sources during their evolution. To combat biotic invasions by pathogenic bacteria and fungi, they have evolved highly effective innate and induced immune responses (Dodds & Rathjen 2010). The components and pathways of pattern-triggered immunity (PTI) (Boller & Felix 2009) and systemic acquired resistance (SAR) (Fu & Dong 2013) are well characterized, involving recognition, signal transduction, and transcriptional reprogramming.

Similarly, plants have evolved related strategies to respond and acclimate to numerous abiotic stresses such as heat, cold, and salt. Systemic acquired acclimation (SAA) describes a systemic accumulation of transcripts and proteins that confer enhanced resistance to subsequent abiotic stresses (Shah *et al.* 2014). Although less detail is known about SAA than SAR, there are a number of commonalities between the two (Fujita *et al.* 2006; Atkinson & Urwin 2012). For example, both systems include stress recognition proteins such as receptor-like kinases (RLKs) and lectin receptor kinases (LRKs), the key roles of reactive oxygen species (ROS) and redox state, and mitogen-activated protein (MAP) kinase-mediated signal transduction cascades (Mittler & Blumwald 2015).

An evolutionarily recent abiotic stress imposed on plants is the application of herbicides. Herbicides are obviously designed to control plants, but they can also merely cause injury, due to sublethal dosages (improper applications or equipment problems), adverse environmental conditions that reduce efficacy, or delayed applications on older, more tolerant plants (Caseley & Walker 1990). Under these conditions, susceptible plants

respond much the same as they do to other abiotic stresses (reviewed in (Alberto *et al.* 2016)). Both abiotic (Dietz, Mittler & Noctor 2016) and herbicide stresses (Dayan & Watson 2011; Sewelam, Kazan & Schenk 2016) induce rapid ROS generation, and even though herbicides are designed to inhibit a specific biochemical target, both have effects on multiple pathways and cellular components (Délye 2013; Suzuki *et al.* 2014). Plant transcriptome changes caused by herbicide stress are very similar to those resulting from abiotic and biotic stresses (Das *et al.* 2010; Unver *et al.* 2010).

Worldwide intensive herbicide use has led to the evolution of resistant weed populations that cause substantial crop yield losses and increase production costs (Heap 2014). Resistance can be conferred by target site overexpression or mutations that alter herbicide binding, or non-target site based resistance (NTSR) mechanisms like enhanced rates of herbicide metabolism, reduced absorption and/or translocation, sequestration, or more generalized stress defense networks (Délye 2013). Selection of herbicide-resistant (HR) plants is thought to operate on standing genetic variation as affected by biological (species characteristics) and operational (herbicide dose and use patterns) factors (Georghiou & Taylor 1986; Jasieniuk, Brûlé-Babel & Morrison 1996). NTSR evolves gradually, appears to be controlled by multiple alleles, and is conferred by one or more constitutive and/or induced physiological mechanisms (reviewed in (Délye 2013)). Recent transcriptome analyses of constitutive changes in HR or multiple herbicide resistant (MHR) populations show that several genes representing functions in xenobiotic metabolism are more abundant prior to herbicide treatment (Peng *et al.* 2010; Gaines *et al.* 2014; Hofer, Felsenstein & Petersen 2014).

We recently described populations of MHR *Avena fatua* L. that are resistant to members of all selective herbicide families available for *A. fatua* control in small grain crops (Lehnhoff *et al.* 2013b; Keith *et al.* 2015). MHR3 and MHR4 plants do not contain known target site mutations for ALS or ACCase inhibitors, and the cytochrome P450 monooxygenase (P450) inhibitor malathion partially reversed the resistance phenotype for several herbicides (Keith *et al.* 2015), indicating that NTSR mechanisms are involved. Transcriptome and proteome analyses show that MHR *A. fatua* plants have constitutively altered levels of a number of stress-related differentially expressed genes (DEGs) and proteins (Keith et al. submitted). Specifically, DEGs with functions in xenobiotic catabolism, stress response, redox maintenance, and transcriptional regulation, as well as functionally similar proteins are constitutively elevated in untreated MHR plants. We now extend these transcriptional and translational investigations to include surveys of post-translational modifications (PTMs) including protein phosphorylation and redox state of individual proteins.

2. Materials and Methods

2.1. Plant material

The MHR3 and MHR4 populations were derived from seeds collected in 2006 from two *A. fatua* populations not controlled by 60 g a.i. ha^{-1} pinoxaden (Axial, Syngenta Crop Protection; ACCase inhibitor) in two production fields separated by approximately 8 km in Teton County, Montana, USA. Field-collected seeds (about 90% of which were resistant to 60 g a.i. ha^{-1} pinoxaden, data not shown) were subjected to two generations of recurrent group selection (50 plants each generation) by spraying with the same dose of

pinoxaden (for MHR3 and MHR4) or surfactant only (susceptible populations), followed by five additional generations with no herbicide selection in the greenhouse. From each generation of 50 plants, all seeds were harvested and a random selection of 50 seeds was used to initiate the next generation. The herbicide susceptible population HS1 was derived from seeds collected from untreated border plants in an adjacent field, and was subsequently confirmed to be 100% susceptible to the herbicides used in these studies (Lehnhoff *et al.* 2013b; Keith *et al.* 2015). A second susceptible population, HS2, is the inbred nondormant SH430 line used in seed dormancy research (Naylor & Jana 1976; Johnson *et al.* 1995). Plants were grown under a 16-hr photoperiod of natural sunlight supplemented with mercury vapor lamps (165 μmol m^{-2} sec^{-1}) at 25 ± 4 C in standard greenhouse soil mix [1:1:1 (by vol) Bozeman silt loam:Sunshine mix #12 (Sun Gro Horticulture, Inc., Bellvue, WA):perlite] and fertilized weekly with Jack's water soluble 20 N-20 P-20 K (JR Peters Inc., Allentown, PA). All plants for each experiment were grown on the same greenhouse bench and were harvested in mid-morning to minimize potential environmental- and circadian-induced changes in protein levels.

2.2. Protein extraction

For phosphoproteome analysis, leaf tissue (200 mg) from each of three replicate three-leaf HS1 and MHR4 plants was ground under liquid nitrogen and suspended in ice cold extraction buffer containing 0.1 M Tris HCl (pH 7.5), 2 mM EDTA , 1 mM DTT, 1 mM PMSF, and 5% (w/v) PVPP. The slurries were filtered through Miracloth (EMD Millipore, Merck KGaA, Darmstadt, Germany) and filtrates were centrifuged at 21,380 x g for 10 min at 4 C. Proteins were concentrated by precipitation with four volumes of ice-

cold acetone containing 10% TCA at -80 C overnight. Two additional 100% acetone precipitations were performed for 3 hr each at -80 C, and proteins were resuspended in DIGE buffer (30 mM Tris pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS, 1x protease inhibitor/nuclease mix [GE Healthcare Life Sciences, Pittsburgh, PA], and 0.1% [w/v] bromophenol blue) to a final concentration of 2 mg ml^{-1}. Protein concentrations were determined (Bradford 1976) using bovine serum albumin fraction V as standard.

For redox proteome analysis, leaf tissue (200 mg) from each of three replicate three-leaf HS1 and MHR4 plants was ground under liquid nitrogen and extracted using the blocking 5-(iodoacetamido)fluorescein (IAF) labeling method described in Wang *et al.* (2011). Thiol groups of oxidized proteins were labeled with Rhodamine Red® C_2 maleimide (Thermo Fisher Scientific, Waltham MA) in a final concentration of 40 µM, incubated at room temperature in the dark for 30 min, and then resuspended in DIGE buffer to a final concentration of 2 mg ml^{-1} (Waszczak *et al.* 2015). Protein concentrations were determined (Bradford 1976) using bovine serum albumin fraction V as standard.

2.3. 2D electrophoresis

Extracts containing 150 or 50 µg of protein for phosphoproteome or redox proteome analysis, respectively, were diluted to a final volume of 450 µl with isoelectric focusing (IEF) buffer (DIGE buffer containing 50 mM DTT and 0.5% (v/v) IPG buffer 3-11 NL (GE Healthcare), and incubated for 1 hr at room temperature. Samples were loaded on separate IPG strips (pH 3-11 NL, non-linear, 24 cm length; GE Healthcare) and IEF and SDS-PAGE were carried out as described by Maaty *et al.* (2012).

2.3.1. Image acquisition and analysis

After electrophoresis, phosphoproteome gels were stained with the fluorescent phosphoprotein specific stain Pro-Q Diamond (Invitrogen Corp. Carlsbad, CA) following the methods of Agrawal and Thelen (2005). Gels were then scanned with a Typhoon Trio Imager following the Invitrogen protocol. Redox proteome gels were scanned with the same imager using the Thermo Fisher Scientific protocol. All gels were scanned at 100 µm resolution and 640 V for PMT. Images were subjected to an automated in-gel analysis using Progenesis SameSpots v 3.0.2 software (Nonlinear Dynamics Ltd. Newcastle, UK). Individual gel spots were detected and spot volumes were provided for statistical analysis. Gels used for protein identification contained 400 µg of protein each and were stained with colloidal coomassie stain (Dyballa & Metzger 2009), destained in 10% acetic acid, and stored at 4 C in 1% acetic acid until spot excision.

2.3.2. Protein mass determination and analysis

Protein spots of interest were excised, digested with porcine trypsin (Promega Corp. Madison, WI 53711), and eluted as described in Shevchenko *et al.* (2006). The resulting peptides were subjected to mass analysis performed on Bruker maXis Impact with Dionex 3000 nano-uHPLC controlled with Chromeleon Xpress (2.13 for Hystar) following the methods of Maaty *et al.* (2012).

Protein identification followed the methods outlined in Mason *et al.* (2016), initiated by generating a custom protein sequence file (.FASTA) from the *Oryza sativa* subsp. japonica (retrieved 2016; http://www.UniProt.org; containing 121,989 entries) and *Brachypodium distachyo*n (retrieved 2016; http://www.UniProt.org; containing 50,507

entries) databases. Sequences for potential contaminants human keratin (retrieved 2016; http://www.UniProt.org; containing 49 entries) and porcine trypsin (retrieved 2016; http://www.UniProt.org; containing 1 entry) were added to the sequence file to create a final target-decoy library. The library was queried against data files using the following search parameters: up to two missed cleavages allowed, precursor charges +2; +3; +4, precursor ion mass tolerance 30 ppm, and fragmentation mass tolerance of 0.5 Da. Post translational modifications were defined as: oxidation of M, acetylation of N-terminus, and carboxylation of C-terminus. Two or more significant peptides with an FDR ≤ 1.0% (PeptideShaker software (Vaudel *et al.* 2015)) were required for annotation of each protein from the PaxDb database (Wang *et al.* 2012) as accessed through UniProtKB and the Rice Genome Annotation Project (Kawahara *et al.* 2013).

2.4. 1D PAGE

Protein extracts from HS1, HS2, MHR3, and MHR4 plants (20 µg per lane) obtained as described above were heated at 95 C for 5 min in 1x Laemmli SDS sample buffer (Cleveland *et al.* 1977) before being subjected to SDS-PAGE in 12.5% gels at 2.5 mA cm^{-1} for 3 hr with water cooling.

2.5. Western blots

Proteins were electroblotted from SDS gels onto PVDF membrane (Pall Corporation, Radnor, PA) and probed with rabbit polyclonal antisera against either cytosolic or chloroplastic CuZn SODs (Kanematsu & Asada 1990) diluted 1:5,000 (v/v) in blotto (10% [w/v] nonfat dry powdered milk/phosphate-buffered saline/0.001% [w/v] sodium azide) followed by detection with goat anti-rabbit IgG/alkaline phosphatase

(Sigma-Aldrich AP132A) diluted 1:10,000 (v/v) in blotto. Densitometric analyses were performed using ImageJ (Schneider, Rasband & Eliceiri 2012) to compare amounts of immunoreactive proteins among populations.

2.6. Superoxide dismutase activity gels

Proteins extracts from HS1, HS2, MHR3, and MHR4 plants (20 µg per lane) obtained as described above were subjected to native electrophoresis in 12.5% polyacrylamide gels at 110V for 1 h with water cooling. Superoxide dismutase (SOD) activity was measured photochemically according to the methods of Chen and Pan (1996), gels were illuminated with 30 µE s^{-1} m^{-2} intensity light for 5 min and then photographed. Densitometric analyses were performed using ImageJ (Schneider, Rasband & Eliceiri 2012) to compare SOD activities among populations.

3. Results and Discussion

2D electrophoresis was used to compare constitutive profiles of abundant, soluble phosphorylated (Fig. 4.1, Table 4.1) and redox-sensitive proteins (Fig. 4.2, Table 4.2) from untreated MHR4 and HS1 plants. Statistical analysis of phosphoproteome gels identified 24 differential protein spots, of which 14 proteins were detected at higher levels in MHR4 compared to HS1 plants. Similar analysis of redox proteome gels identified 23 differential spots, of which 15 proteins were detected at higher levels in MHR4 compared to HS1 plants. All proteins were identified by the best annotation from UniProt and the Rice Genome Annotation Project (Kawahara *et al.* 2013). Differentially expressed phosphorylated and redox-sensitive proteins are discussed below in five functional categories: core cellular processes, xenobiotic and stress response, ROS

detoxification and redox maintenance, chaperones and heat shock proteins, and signaling proteins. Overall, proteins with functions in core cellular processes, photosynthesis, and translation were reduced in MHR plants, while those involved in xenobiotic response, ROS metabolism, redox maintenance, heat shock response, and signaling were elevated in MHR compared to HS plants.

3.1. Core cellular processes

Six of the nine differentially expressed phosphorylated proteins involved in basic metabolism were reduced in MHR plants (Table 4.1), including hexokinase, fructose-bisphosphate aldolase, sedoheptulose-1,7-bisphosphatase, granule-bound starch synthase I, phosphoribulokinase, and 2-dehydro-3-deoxyphosphooctonate aldolase (spots 16-21, Fig. 4.1). These enzymes are involved in the biosynthesis or sensing of primary metabolites, specifically glycolysis, starch synthesis, lipopolysaccharide biosynthesis, the Calvin cycle, and glucose sensing. In contrast, glutamine synthetase, ATP synthase CF1 beta subunit, and transketolase (spots 6, 7, and 14, Fig. 4.1) were elevated in MHR4 plants. Phosphorylated glutamine synthetase, transketolase, and fructose-bisphosphate aldolase were identified in *Arabidopsis thaliana* and *O. sativa* in response to pathogen attack (Jones *et al.* 2006) and abscisic acid application (He & Li 2008), respectively.

Differential expression levels of redox-sensitive proteins involved in photosynthesis were mixed. A photosystem I reaction center subunit II (spot 41) and a 23 kDa polypeptide of photosystem II (spot 39) were reduced and elevated in MHR4 plants, respectively (Fig. 4.2, Table 4.2). Photosystem I and II reaction centers are a primary site of ROS generation (Asada 2006), and a redox sensitive photosystem I light harvesting

complex protein was identified in response to methyl jasmonate treatment in *A. thaliana* (Alvarez, Zhu & Chen 2009).

Three of four redox-sensitive proteins involved in protein translation were differentially reduced in MHR4 plants, including a chloroplast translational elongation factor Tu, plastid-specific 30S ribosomal protein 2, and 40S ribosomal protein S14 (spots 40, 45, and 47, respectively) (Fig. 4.2, Table 4.2). In contrast, an elongation factor 1-beta 1 (spot 31, Fig. 4.2) was elevated in MHR4 as compared to HS1 plants. Ribosomal and elongation factor proteins were determined to be redox-sensitive in *A. thaliana* (Wang *et al.* 2011), while the Tu elongation factor was induced by hypochlorite and nitric oxide treatments in *E. coli* (Leichert *et al.* 2008).

Other phosphorylated proteins identified at reduced constitutive levels in MHR plants but with weak annotation include a putative polyprotein, poly(ADP-ribose) polymerase, a NAD(P)-binding domain containing protein, and aminomethyltransferase (spots 15, 22-24) (Fig. 4.1, Table 4.1). Of these, the aminomethyltransferase is of interest since it was also identified at lower levels in salt-stressed *Beta vulgaris* (Wakeel *et al.* 2011) and was phosphorylated in response to pathogen attack in *A. thaliana* (Jones *et al.* 2006).

These overall reductions in proteins involved in core cellular processes in MHR plants may represent an energetic tradeoff as predicted by the resource-based allocation theory (Coley, Bryant & Chapin 1985). Briefly, heritable resistance to an environmental stress (SAA) may require the reallocation of carbon away from core processes to stress-related pathways, resulting in a new homeostasis (Kosová *et al.* 2011). More specifically,

translational and post-translational regulation is often employed under stress conditions because it allows for both immediate and selective changes in protein levels (Holcik & Sonenberg 2005). The physiological result of these changes is often manifested in fitness costs and a resulting ecological disadvantage (Bazzaz *et al.* 1987). Fitness costs associated with SAR (Bergelson & Purrington 1996), SAA (Zhen, Dhakal & Ungerer 2011), and herbicide resistance (Vila-Aiub, Neve & Powles 2009) are well documented. For MHR *A. fatua*, greenhouse experiments have demonstrated that fitness costs are limited in these MHR populations, with a trend of fewer tillers and seeds produced by MHR plants compared to HS populations (Lehnhoff *et al.* 2013b), indicating that additional work may be required to further elucidate potential fitness costs.

3.2. Xenobiotic and stress response

Five redox-sensitive proteins, anthocyanin 5-O-glucosyltransferase, cytochrome P450 monooxygenase, a putative carboxylesterase, Ulp1 protease, and a glycoside hydrolase (spots 28, 37, 32, 34, and 29, respectively, Fig. 4.2), were constitutively elevated in MHR4 as compared to HS1 plants (Table 4.2). The large family of plant glycosyltransferases have roles in Phase II herbicide metabolism (Yuan, Tranel & Stewart Jr 2007) as well as conferring tolerance to abiotic stresses like salt, cold, and drought by modifying anthocyanin accumulation (Li *et al.* 2016). We recently reported on a closely related anthocyanidin 3-O-glucosyltransferase DEG as one of four glucosyltransferase DEGs constitutively elevated in MHR4 plants, three of which co-segregated with flucarbazone-sodium herbicide resistance in F_3 families (Keith et al. submitted). Similarly, P450s are well-known participants in xenobiotic catabolism,

especially in Phase I herbicide metabolism (Yuan, Tranel & Stewart Jr 2007). Our transcriptome study (Keith et al. submitted) also identified two P450s elevated in MHR4 plants, one of which co-segregated with flucarbazone resistance in F_3 families. Higher constitutive levels of an oxidized carboxylesterase in MHR4 plants may be related to differential de-esterification of pro-herbicides like fenoxaprop-P-ethyl and imazamethabenz-methyl to their respective toxic carboxylic acids (Cummins, Burnet & Edwards 2001). Elevated levels of Ulp1 proteases, regulators of protein sumoylation (Novatchkova *et al.* 2004) are linked to pathogen attack (Hanania *et al.* 1999) and abiotic stress response (Kurepa *et al.* 2003) in plants. And finally, glycoside hydrolases, enzymes with functions in polysaccharide metabolism, are also involved in plant defense against pathogens (Minic 2008).

3.3. ROS detoxification and redox maintenance

Abiotic stresses including herbicides cause rapid ROS generation and thus disrupt redox homeostasis (Dayan & Watson 2011; Dietz 2014; Demidchik 2015). We expect MHR4 plants to exhibit enhanced capacity for ROS degradation and redox recovery, given that they are resistant to known ROS producing herbicides and families like paraquat (Dodge 1971), difenzoquat (Kovacic & Somanathan 2014), ALS inhibitors (Zulet *et al.* 2015), and ACCase inhibitors (Luo, Sunohara & Matsumoto 2004). In this regard, two phosphorylated peroxiredoxins (spots 2 and 11, Fig. 4.1), thioredoxin-dependent peroxidases that reduce H_20_2 and organic peroxides, were constitutively elevated in MHR4 plants (Table 4.1). Increased levels of these enzymes should improve the capacity for ROS degradation (Muthuramalingam *et al.* 2009) in MHR plants.

However, phosphorylation of a human 1-Cys peroxiredoxin reduced its peroxidase activity by 80%, and the resulting hydrogen peroxide accumulation was shown to improve cell cycle progression (Chang *et al.* 2002). Similarly, redox-sensitive spots 33 and 36 (Fig. 4.2), constitutively elevated in MHR4 plants (Table 4.2), were annotated as a peroxidase and 1-Cys peroxiredoxin, respectively. Both enzymes were elevated in *A. thaliana* following H_20_2 treatment (Wang *et al.* 2011). We also identified a peroxiredoxin DEG that was constitutively elevated in MHR plants in our transcriptome study (Keith et al. submitted).

Nearly half of the redox-sensitive proteins identified in this study have roles in redox maintenance, with the majority of them elevated in MHR4 plants (Table 4.2). Two proteins, L-ascorbate oxidase and iron/ascorbate-dependent oxidoreductase (spots 30 and 38, Fig. 4.2) are involved in ascorbate metabolism, and thus play a significant role in defense against oxidative stress (Smirnoff 2000). We recently demonstrated that the constitutive specific activity of dehydroascorbate reductase, a key step in the glutathione-ascorbate ROS protective pathway (Asada 2006), was 1.4-fold higher in MHR4 versus HS1 plants (Burns et al. submitted). These results provide evidence that ascorbic acid-mediated ROS protection is elevated in MHR plants, potentially ameliorating the oxidative damage imposed by herbicides.

Two redox-sensitive proteins with roles in singlet oxygen metabolism were constitutively elevated in MHR4 plants (Table 4.2). Spot 26 (Fig. 4.2), annotated as a DUF3506 domain-containing protein, shares strong similarity with the EX1 and EX2 proteins from *A. thaliana* that detoxify light-stress generated singlet oxygen (Wagner *et*

al. 2004). Spot 35 (Fig. 4.2) was annotated as pyridoxal 5'-phosphate synthase subunit PDX1.2, which participates in vitamin B6 biosynthesis and thus indirectly contributes to ROS tolerance (Titiz *et al.* 2006).

Superoxide dismutases (SODs) play a key role in the early defense against ROS in chloroplasts, mitochondria, and peroxisomes (Gill *et al.* 2015). We compared SOD activity and enzyme levels in MHR and HS plants to further investigate ROS metabolism. Constitutive SOD activities as determined in native gels showed one major band of activity with no detectable differences between HS and MHR plants (Fig. 4.3a). Polyclonal antibodies against chloroplastic and cytosolic CuZn SODs (Kanematsu & Asada 1990) were subsequently used in western immunoassays to compare levels of immunoreactive proteins in MHR and HS plants (Fig. 4.3b-c). These antibodies have been successfully used to identify cytosolic or chloroplastic CuZn SODs in *Spinacia oleracea*, *O. sativa*, and *Equisetum arvense* (Kanematsu & Asada 1990). Both antibodies recognized two proteins of approximately 17 and 19 kDa in extracts from *A. fatua* plants (Fig. 4.3b-c), and densitometric scans confirmed that constitutive protein amounts were not different between HS and MHR plants (data not shown). Lack of SOD differences between MHR and HS plants may indicate that other ROS detoxification enzymes and redox management pathways are sufficient to ameliorate herbicide-induced ROS damage.

3.4. Chaperones and heat shock proteins

Six protein spots were annotated with chaperone activity: five phosphorylated heat shock proteins (HSPs) Hsp60 (spots 5, 8, 10, and 13; Fig. 4.1) and Hsp70 (spot 3; Fig. 4.1), and a single redox-sensitive protein annotated as a CCt8 protein (spot 25; Fig.

4.2). All were constitutively elevated in MHR4 plants (Tables 4.1 and 4.2). HSPs have well-known roles as molecular chaperones that prevent protein misfolding, some regulate transcription factors (TFs) for a diverse set of genes (Morimoto 2002), and are themselves regulated by phosphorylation (Muller *et al.* 2013). Higher constitutive levels of phosphorylated HSPs in MHR plants may allow them to better protect key proteins during herbicide-mediated stress. Although less is known about phosphorylated HSPs in plants, phosphorylation was shown to protect against oxidative stress in humans (Kalmar & Greensmith 2009) and *Trichinella spiralis* (Martinez *et al.* 2002).

In contrast to the above proteins, two redox-sensitive Hsp60 proteins (spots 42 and 43; Fig. 4.2) and a FACT complex subunit SSRP1-A (spot 44; Fig. 4.2) were constitutively reduced in MHR4 plants (Table 4.2). FACT complexes act as histone chaperones during transcription elongation and were reduced by salt stress in rice (Pandit *et al.* 2011).

3.5. Signaling

Five proteins involved in cell signaling were constitutively elevated in MHR4 plants (Tables 4.1 and 4.2). Phosphorylated proteins include serine carboxypeptidase II (spot 12; Fig. 4.1), three protein kinases (spots 1, 4, and 9; Fig. 4.1), and a redox-sensitive protein annotated as a nucleotide-binding site leucine-rich repeat (NBS-LRR) protein (spot 27; Fig 4.2). Serine carboxypeptidases have well-known roles in storage protein turnover, and some are involved in brassinosteroid and receptor-like kinase signaling (Zhou & Li 2005). NBS-LRR proteins constitute the majority of disease resistance genes in plants, confer resistance to a diverse array of pathogens (Marone *et al.* 2013), provide

enhanced drought and salt tolerance (Xinlong *et al.* 2016), and induce plant defense responses such as an oxidative burst (Belkhadir, Subramaniam & Dangl 2004). Our transcriptome study similarly identified a NBS-LRR DEG that was constitutively elevated in MHR4 plants and co-segregated with flucarbazone resistance in F_3 families (Keith et al. submitted). A redox-sensitive CBL-interacting serine/threonine-protein kinase (spot 46; Fig 4.2) was constitutively reduced in MHR4 plants (Table 4.2). The *O. sativa* ortholog was shown to phosphorylate and thus activate the plasma membrane Na+/H+ antiporter involved in salt tolerance (Martínez-Atienza *et al.* 2007).

Phosphorylated spots 1, 4, and 9 (Fig. 4.1) were annotated as protein kinases and were constitutively elevated in MHR4 plants (Table 4.1). Specifically spot 4 was annotated as the lectin S-receptor-like serine/threonine-protein kinase LecRK2, a well-characterized receptor in SAR and PTI, elements of the plant innate immune response (Boller & Felix 2009). PTI is initiated by perception of microbial, pathogen, or damage-associated patterns by surface-localized receptor-like proteins or RLKs, some of which possess an extracellular lectin motif and an intracellular kinase domain (LecRKs) (Singh & Zimmerli 2013). In general, the linkage between RLK ligand perception and signaling initiation/specificity is tightly regulated by the state of RLK phosphorylation (Macho & Zipfel 2014), and the activities of some LecRKs are enhanced by phosphorylation (Nishiguchi *et al.* 2002; Vaid, Pandey & Tuteja 2016).

PTI and SAA can be ‘primed’ by initial plant exposure to pathogens, abiotic stress, or disparate chemicals (Pastor *et al.* 2013), and primed plants exhibit enhanced and durable resistance to pathogens and subsequent abiotic stresses (Zimmerli *et al.* 2008;

Savvides *et al.* 2016). Priming is thought to result from the accumulation of inactive protein kinases, TFs, or other defense signaling components, that are rapidly activated upon stress leading to more effective defense signaling (Pastor *et al.* 2014). For example, MAPK3 and MAPK6 in *A. thaliana* were strongly activated in plants primed by exposure to biotic or abiotic stresses (Beckers *et al.* 2009). LecRK2 and two additional LecRK genes were shown to confer broad-spectrum and durable resistance against insects pests in rice (Liu *et al.* 2015). Thus, the elevation and/or differential phosphorylation of LecRK2 and two other protein kinases demonstrates that MHR4 plants possess a constitutively-regulated system of stress-related proteins. If in fact these changes are associated with 'priming' the MHR4 phenotype, it may indicate that LecRK2 is responding to features related to herbicide entry or early stress events. Related L-type LecRKs have been shown to mediate plant response to such diverse stresses as salt (Deng *et al.* 2009), allyl-isothiocyanate (Kissen *et al.* 2016), and perhaps ATP as an extracellular signaling molecule (Cao *et al.* 2014).

4. Conclusion

This work seeks to better understand the molecular mechanisms of NTSR in MHR *A. fatua* by surveying differentially-expressed phosphorylated and redox-sensitive proteins. Our results demonstrate that constitutive PTMs of a number of proteins with functions in core cellular processes, xenobiotic and stress response, ROS detoxification and redox maintenance, chaperones and heat shock response, and intracellular signaling are altered in MHR4 as compared to HS1 plants. Clearly additional work will be required before any causal relationship can be assigned between these proteins and MHR. Using a

candidate gene approach, individual proteins can be pursued biochemically, and the rudimentary mapping populations we have developed (Burns et al. submitted) could form the basis for additional genetic or QTL-based strategies with the aid of the recently published *Avena sativa* linkage map (Chaffin *et al.* 2016).

Regardless of their potential roles in MHR, functions of the proteins described above are shared with biotic (SAR) and abiotic (SAA) stress response pathways. For example, elevated stress responses, enhanced ROS detoxification, alterations in protein kinases and other signaling molecules, and fitness penalties (with implied reduced biosynthetic activities) have been documented in SAR and SAA. The MHR *A. fatua* PTMs related to ROS detoxification and protein kinases including LecRK2 are especially relevant, since both features play fundamental roles in cross-talk between biotic and abiotic stresses (Atkinson & Urwin 2012; Rejeb, Pastor & Mauch-Mani 2014). More specifically, MAP kinase cascades are known to transform ROS signals into PTMs like protein phosphorylation and oxidation, resulting in signal transduction modulation that are needed for stress perception, SAR, and SAA (Kosová *et al.* 2011; Waszczak *et al.* 2015; Dietz, Mittler & Noctor 2016).

Priming of PTI can require multiple exposures to modest stresses (Boller & Felix 2009), as has been noted for the evolution of NTSR (Neve & Powles 2005b; Délye, Jasieniuk & Le Corre 2013). In this regard, the NTSR *A. fatua* phenotype was only detected after > 30 years of annual herbicide applications (Keith *et al.* 2015). The idea that herbicide resistance is similar to acclimation was first introduced by Vila-Aiub and Ghersa (2005), who showed that recurrent treatment of the allogamous species *Lolium*

rigidum with a sublethal diclofop-methyl dose selected for non-heritable resistance. Although empirical evidence supporting the emerging hypothesis that NTSR is similar to SAR and SAA is scant (Molina *et al.* 1999; Délye, Jasieniuk & Le Corre 2013; Perez & Brown 2014; Dubey, Mishra & Prasad 2016), we suggest that the constitutive changes documented here support the idea that herbicide stress is perceived much the same as other abiotic stresses, and that NTSR shares a number of similar features with SAR and SAA. We speculate that MHR *A. fatua*'s previous exposure to sublethal herbicide doses, as well as pre-agricultural evolution under a diversity of abiotic and biotic stressors, has led to a heightened state of stress preparedness that includes NTSR to a number of unrelated herbicides. Further investigations into the evolution of these related responses through the lens of phenotypic convergence (Losos 2011; Baucom 2016) may provide valuable insights into plant responses to strong selection by biotic, abiotic, and herbicide stressors.

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Table 4.1. Differentially expressed phosphorylated proteins from untreated herbicide susceptible (HS1) and multiple herbicide resistant (MHR4) *Avena fatua* plants identified using two-dimensional fluorescence difference gel electrophoresis.

Functional Group	Spot number	Uniprot number	Annotation	Fold change[a]	P-value	Peptides[b]	Peptide sequences
Core cellular processes	6	P14655	Glutamine synthetase	7.4	0.04	3	TISKPVEDPSELPK
							WNYDGSSTGQAPGEDSEVILYPQAIFK
							WNYDGSSTGQAPGEDSEVILYPQAIFK
	7	I1HI10	ATP synthase CF1 beta subunit	2.1	0.06	4	FVQAGSEVSALLGR
							IFNVLGEPVDNLGPVDSSATFPIHR
							TVLIMELINNIAK
							YKELQDIIAILGLDELSEEDRLTVAR
	14	Q5VNW1	Transketolase	3.0	0.05	2	IKVTTTIGFGSPNKANSYSVH
							QKDTPEERNVRFGVREHGMGAICNGIAL
	15	Q6ATP4	Putative polyprotein	-5.2	0.02	2	DVAPSEEDRPHRKVQRPVYFVSEAL
							EWTPAPEPVSVPEASSGPSQLPHTAH
	16	Q8LH82	Hexokinase	-2.4	0.07	2	EGCACAAPPAAAAPPMPK
	17	Q40677	Fructose-bisphosphate aldolase, chloroplastic	-2.5	0.05	2	RMVVEVCDIVATRAARLAAAGIV
							LASIGLENTEANR
							TVVSIPNGPSELAVK
	18	B9F813	Sedoheptulose-1,7-bisphosphatase	-5.9	0.08	2	LLFEVAPLGFLIEK
	19	B8XEK6	Granule-bound starch synthase I	-7.3	0.00	2	TNFTVGTIFGVWPGDKLTGVTGGDQ
							RFPSVVVYATGAGMNVVFVGAEMAP
							APRILNLNNNPYFKGTSGEDVVFVCN
	20	Q6Z8F4	Phosphoribulokinase	-8.8	0.04	6	ANDFDLMYEQVK
							FYGEVTQQMLK
							HADFPGSNNGTGLFQTIVGLK
							IRDLYEQIIAER
							KPDFDAFIDPQK
							QYADAVIEVLPTQLIPDDNEGK

	21	Q0IPE5	2-dehydro-3-deoxyphosphooctonate aldolase	-2.4	0.06	3	LYGQLKAAQPFFLLAGPN PVVTDVHESHQCEAVGRV
	22	I1J387	Poly(ADP-ribose) polymerase	-2.5	0.02	2	EWLREANCPVVADVTHALQQ SKDFLFVRDLFLSGMGSFAT
	23	Q2QSR7	NAD(P)-binding domain containing protein	-2.6	0.05	2	LLETLKKLHYCPSLWNKSSI APITQQLPGESDAEYAEFSSK VKDLATAFVLALGNPKASKQ
	24	Q7XPR2	Aminomethyltransferase	-7.8	0.01	2	LAGAAEAAEAELKKTALYDF
ROS detoxification and redox maintenance	2	P0C5D1	1-Cys peroxiredoxin	2.4	0.04	2	LEKSEGKVRLTGLGARDSL GLTLGDVVPDLELDTTHGK
	11	B7ERQ1	Perixiredoxin	3.6	0.06	2	ALHIVGPDKKVKLSFLFPACTG STHGKIRIHDFVGDTY
Chaperones and heat shock	3	Q10NA9	70 kDa heat shock protein	3.2	0.04	7	VRAVDALQTAAKHAVAT ATAGDTHLGGEDFDNR DAGVISGLNVMR NQVAMNPINTVFDAK STVHDVVLVGGSTR TTPSYVAFTDSER VQDLLLLDVTPLSQGLETAGGV VQQLLQDFFNGK
	5	Q2QU06	60 kDa chaperonin alpha subunit	6.6	0.03	9	AVLQDIAIVTGAEFLAK GILNVAAIKAPSFGER GYISPQFVTNLEK LANAVGVTLGPR NVVLDEYGSPK NVVLDEYGSPKVVNDGVT VGAATETELEDR VTIHQTTTTLIADAASKDEI VVNDGVTIAR

	8	Q7G2N7	Chaperonin CPN60-1, mitochondrial	2.1	0.03	6	EGVITIADGNTLYNELEVVE EGVITIADGNTLYNELEVVEG LLEQDNTDLGYDAAK SVAAGMNAMDLR TALVDAASVSSLMTTTESIIVEIP
	10	Q6ZFJ9	60 kDa chaperonin beta subunit	2.0	0.04	2	VTVSKDDTVILDGAGDKK EVELEDPVENIGAK LADLVGVTLGPK
	13	Q8H903	60 kDa chaperonin, mitochondrial	2.3	0.01	2	DGNTLYNELEVVEGMKLDRGYISP AGDKKSIEERAEQIRSAIELSTSDY
Signaling	1	Q7EYF8	Protein kinase	2.1	0.03	2	GGLVVSADELGAPR RGPPLTWAQRLKIAVDVARGLNY
	4	Q7FAZ2	Lectin S-receptor-like serine/threonine protein kinase LecRK2	2.2	0.05	2	DPSGNEVWNPRVTDVGYARMLDTGNFR SPSMISSGSSKWKKDKKYWILGSSLFF
	9	Q6ZF83	Serine/threonine protein kinase	2.8	0.07	2	TTGVSQRLVPWRNNANPSPGLFSL ALIILAIVLFIVFQKCRRDRTLR
	12	Q6H7I7	Serine carboxypeptidase II	2.7	0.05	2	NWTHCSDVIGKWRDAPFSTLPIIRKLV FPQYKSHDFYIAGESYAGHYVPQL

[a]Positive and negative fold change values indicate higher and lower levels in multiple herbicide resistant compared to herbicide susceptible *Avena fatua* plants, respectively.
[b]Numbers of uniquely matched peptides

Table 4.2. Differentially expressed redox sensitive proteins from untreated herbicide susceptible (HS1) and multiple herbicide resistant (MHR4) *Avena fatua* plants identified using two-dimensional fluorescence difference gel electrophoresis.

Functional Group	Spot number	Uniprot number	Annotation	Fold change[a]	P-value	Peptides[b]	Peptide sequence
Core cellular processes	31	Q40680	Elongation factor 1-delta 1	1.4	0.10	2	NVKMEGLLWGASK
							AAEERAAAVKASGK
	39	B0FFP0	23 kDa polypeptide of photosystem II	1.5	0.08	2	AANVFGKPKTNTEF
							HQLITATVNDGKLYICKAQAGD
	40	I1IB68	Chloroplast translational elongation factor Tu	-1.4	0.00	6	GITINTATVEYETETR
							NATVTGVEMFQK
							TMDDAIAGDNVGLLLR
							TTDVTGNVTNIMNDKDEEAK
							VGDPVDLVGIR
							VGDPVDLVGIRETR
	41	Q84PB4	Photosystem I reaction center subunit II	-2.3	0.06	3	EQVFEMPTGGAAIMR
							FTGKNTFDV
	45	Q6H443	Plastid-specific 30S ribosomal protein 2	-2.1	0.10	2	ARKEQCLALGTRLRSKYKINYQ
							SRLTVGAARWWARRRQPAVVVR
							AARKLYVGNIPRTVT
	47	B1NEV4	40S ribosomal protein S14	-3.0	0.10	4	KRGKVQKEEVQ
							HIFASFNDTFVHVTDLS
							MLAAQDVAEKCKSLGI
Xenobiotic and stress response	28	I1IUI3	Anthocyanin 5-O-glucosyltransferase	5.2	0.07	2	RATGGNKTKTPGPGAQSALRA
							DFPTFLVDTTGSDIASSVNEALR
							VLAAYYHFFHDDGGHYK
	29	Q10A56	Glycoside hydrolase	1.3	0.10	4	GVGEPLNEVVCVDQKCDGLVAR
							AEQAFFQRWWAEKSPKIQAIV
							RNMDRLINYVNKDGRVHALYSTP
							EVEYTIGPIPVDDDDDIGK
	32	Q0JG98	Esterase PIR7A, putative carboxylesterase	1.5	0.05	3	FPDKVAAAVFLAACMPAAGK
							AAAGAHPARADEVGSLEE
							EGNYGSVKRVFLVAMDDASSDE

	34	Q8LMC7	Ulp1 protease	1.6	0.08	3	RFHFPCAKQDQR
							YNTEFHWVLLFFD
	37	Q2QS17	Cytochrome P450 family protein	1.4	0.05	3	YSTLSKTPCLYGSTPRSTKA AFTDVLGDLLGGGIFNADGERWFAQRK QLLAAARGRDDLVSRM RMEAIWGADAGEFRPGRWLAAAA
ROS detoxification and redox maintenance	26	I1HB66	DUF3506 domain containing protein	2.7	0.10	2	GNEDTEEKTQDVGNTK
	30	Q942J6	Putative L-ascorbate oxidase	1.3	0.04	2	VKLFISGVVHNKEDMAGAKS AEDPYHFFDWK TRTIMDVAQKVMLINDMFPGPTI
	33	Q5U1J1	Peroxidase 7	1.5	0.05	2	LFFHDFAVQGIDASVLVDSPGSERYAK
	35	Q8W3D0	Probable pyridoxal 5'-phosphate synthase subunit PDX1.2	2.4	0.08	2	YWPLMYGRKDGRRSSMVDA AIVQAVTHYSDPK EVSSGLGEAMVGINLSD
	36	B7ERQ1	1-Cys peroxiredoxin	1.5	0.07	2	QLNMVDPDEK
	38	Q6EQV9	Iron/ascorbate-dependent oxidoreductase	2.2	0.10	2	KLLGISCDDVQSHKDWIKDIEAYKP DVLRAMARIAGLDDDDQHFVDQLG RFNYYPPCPRPDLVMGIKPHSDG
Chaperones and heat shock	25	C6F1N7	CCt8 protein like	1.9	0.10	2	TSLGPNGMNKMVINHLDK YAIAKFAESFEMVPRTLAENAGLSAMEV
	42	Q9LWT6	60 kDa chaperonin	-2.3	0.04	3	EVELEDPVENIGAK IVNDGVTVAREVELEDPVENIGAK LAGGVAVIQVGAQTETELKEK
	43	Q6ZFJ9	60 kDa chaperonin beta	-1.2	0.10	2	EVELEDPVENIGAK LAGGVAVIQVGAQTETELKEK
	44	Q9LGR0	FACT complex subunit SSRP1-A	-1.6	0.01	2	TDGHLFNNILLGGRAGSNPGQFK SPTDDSGGEDSDASESGGEKEKLSKKEA
Signaling	27	Q67WN0	L-zip+NBS+LRR-like protein	1.3	0.10	3	SNFIEDSSMAEDK IGSSNLALIALKINDSGSSSDIV

46	Q69Q47	CBL-interacting serine/threonine-protein kinase 24	-1.4	0.10	2	WKSIPHLELLNITELTIDKCVDSCPVPK YFQQLIDAINYCHSKGVYHR SPFAVVLQVFEVAPSLFMVDVR

[a]Positive and negative fold change values indicate higher and lower levels in multiple herbicide resistant compared to herbicide susceptible *Avena fatua* plants, respectively.
[b]Numbers of uniquely matched peptides

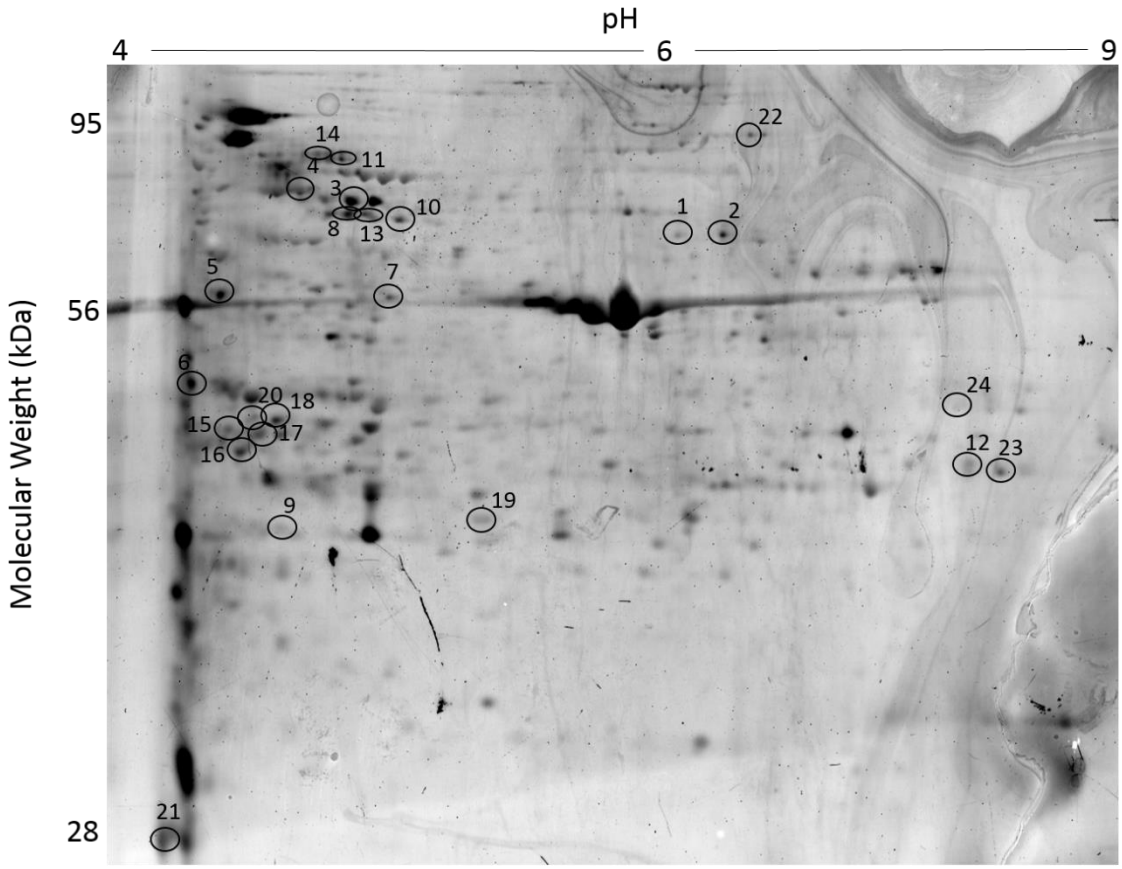

Figure 4.1. Two dimensional electrophoresis gel of the *Avena fatua* phosphoproteome. Numbered spots were excised from the gel and proteins identified using in-gel proteolysis followed LC-MS/MS.

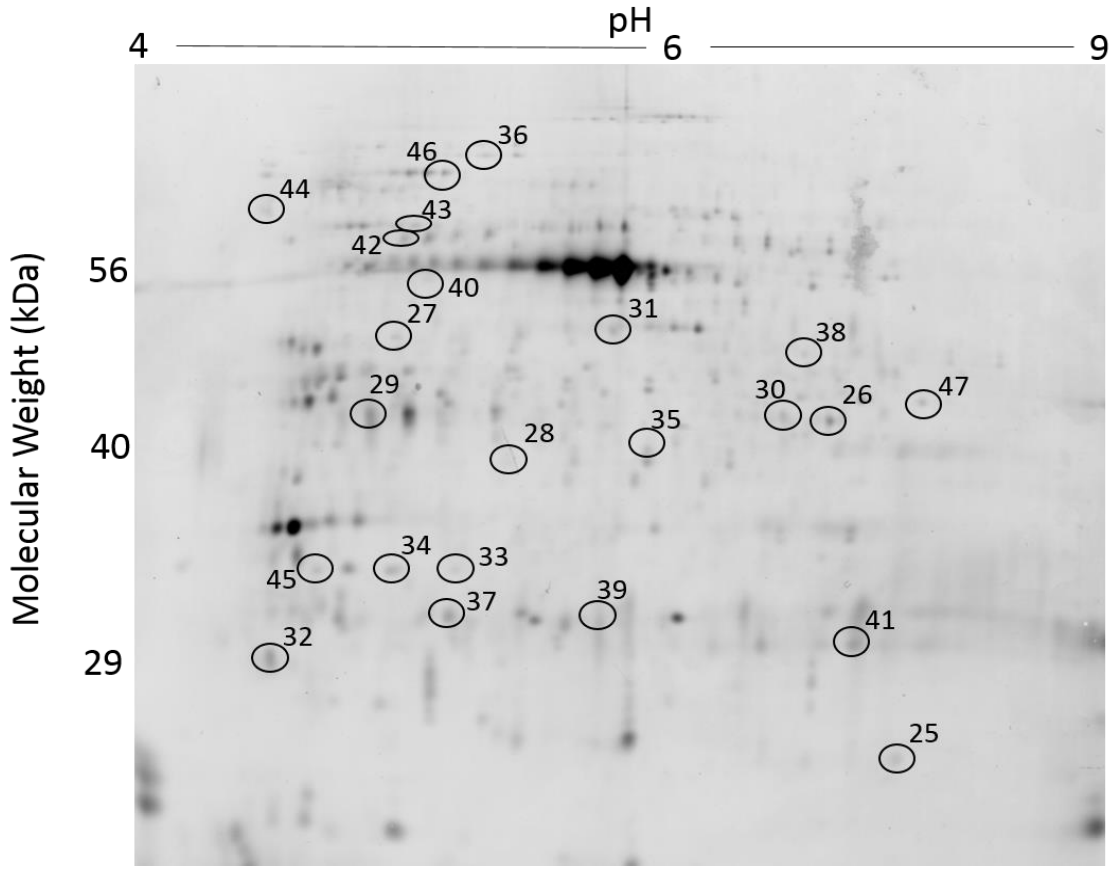

Figure 4.2. Two dimensional electrophoresis gel of the *Avena fatua* redox sensitive proteome. Numbered spots were excised from the gel and proteins identified using in-gel proteolysis followed LC-MS/MS.

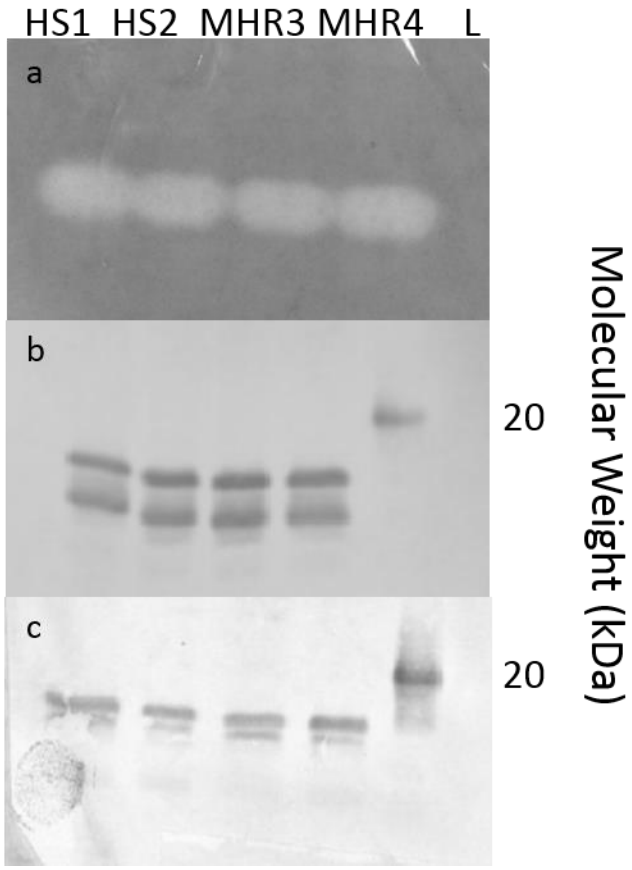

Figure 4.3. (a) Superoxide dismutase activity gel from untreated herbicide susceptible (HS1 and HS2) and multiple herbicide resistant (MHR3 and MHR4) *Avena fatua* plants. (b) 1D immunoblot of proteins recognized by anti-chloroplastic CuZn superoxide dismutase antibodies from untreated herbicide susceptible (HS1 and HS2) and multiple herbicide resistant (MHR3 and MHR4) *Avena fatua* plants, L = Western protein standard ladder. (c) 1D immunoblot of proteins recognized by anti-cytocolic CuZn superoxide dismutase antibodies from untreated herbicide susceptible (HS1 and HS2) and multiple herbicide resistant (MHR3 and MHR4) *Avena fatua* plants, L = Western protein standard ladder.

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CHAPTER FIVE

YOU CAN'T FIGHT FIRE WITH FIRE: MODEL SUGGESTS ALTERNATE APPROACHES TO MANAGE MULTIPLE HERBICIDE RESISTANT *AVENA FATUA* L.

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Summary

1. Multiple herbicide resistant weed populations pose significant agronomic and economic threats to agriculture and demand the development and implementation of ecologically-based tactics for sustainable management.
2. We investigated the influence of nitrogen fertilizer rate and spring *Triticum aestivum* L. seeding density on the demography of two multiple herbicide resistant and one herbicide susceptible *Avena fatua* L. populations under two cropping systems (continuous cropping and crop-fallow rotation). To represent a wide range of environmental conditions, data were obtained in field conditions over three trials (2013-2015). A stochastic density-dependent population dynamics model was constructed using the demographic data to project *A. fatua* populations 20 years forward. To identify demographic processes with negative impacts on population growth that can be exploited in management tactics, elasticity analysis was used to quantify the proportional change in the *A. fatua* seedbank resulting from a proportional change in model vital rates.
3. In the continuous *T. aestivum* and *T. aestivum*-fallow cropping systems, multiple herbicide resistant seedbank densities were negatively impacted by increasing nitrogen fertilization rate and *T. aestivum* density in which mean seedbank densities stabilized near zero by the fifth year of the projection. Overall, multiple herbicide resistant seedbank densities were larger in the *T. aestivum*-fallow cropping system compared to the continuous *T. aestivum* cropping system. In both

cropping systems, density dependent seed production was the most influential parameter impacting population growth rate.

4. The short-term impact of ecologically-based weed management tactics can be investigated by field experiments, but evaluations of their long-term consequences requires the use of population dynamics models that allow for the range of outcomes based on variability in demographic data. Our results suggest that multiple herbicide resistance is not necessarily an unsolvable problem as long as solutions are expanded beyond herbicide based approaches to weed management. Demographic models as the one constructed in this study will aid in selecting ecologically-based weed management tactics such as appropriate resource availability and modification to crop competitive ability to reduce the spread and impact of multiple herbicide resistance.

Introduction

Weedy plants, as the main biotic cause of crop yield loss, have played a central role in agriculture since its beginning about ten thousand years ago (Délye, Jasieniuk & Le Corre 2013). In the early 1950s synthetic herbicides revolutionized agriculture, and have been at the foundation of both weed science research and the intensification and expansion of industrialized agriculture (Liebman, Mohler & Staver 2001). However, their extensive usage worldwide imposes strong selection for resistant weed populations, threatening their continued efficacy (Menalled *et al.* 2016). To date, approximately 250 species have evolved resistance to 23 of the 26 known herbicide modes of action, representing over 160 different herbicides (Heap 2017). Furthermore, 37% of these species are resistant to two or more modes of action, a phenomenon known as multiple herbicide resistance (MHR) and considered a "wicked problem" because there are fewer and fewer herbicides to kill important weeds (Shaw 2016).

Resistance can be conferred by target site mutations that alter herbicide binding, or by non-target site based resistance (NTSR) alterations such as enhanced rates of herbicide metabolism, reduced absorption and/or translocation, sequestration, or more generalized stress defense networks (Délye, Jasieniuk & Le Corre 2013). The selection of NTSR is particularly threatening as it is associated with unpredictable patterns of resistance, e.g. populations with resistance to unrelated modes of action and to herbicides not yet commercially available (Petit *et al.* 2010a).

Avena fatua L. (wild oat), an annual, autogamous, allohexaploid monocot is an important weed worldwide. Across the Northern Great Plains and Pacific Northwest of

North America *A. fatua* infests over 11 million ha, causing annual crop losses of over $1 billion (Beckie, Francis & Hall 2012). Due to extensive use of herbicides that inhibit ACCase, ALS, and shoot growth, several populations of resistant *A. fatua* have evolved worldwide (Heap 2017). We recently characterized two MHR *A. fatua* populations in Montana, USA, that are resistant to members of all selective herbicide families available for *A. fatua* control in cereal crops, representing five modes of action. Greenhouse experiments have demonstrated that fitness costs are limited in these MHR populations, with a trend of fewer tillers and seeds produced by MHR plants compared to herbicide sensitive (HS) populations (Lehnhoff *et al.* 2013b). We further showed that MHR plants did not possess known target site mutations, suggesting the involvement of NTSR mechanisms (Keith *et al.* 2015). One must speculate that most remaining herbicides used to kill *A. fatua* in crop or in fallow are likely to be added to the list of non-effective herbicides.

MHR populations pose significant agronomic and economic threats to agriculture and demand the development and implementation of alternative, ecologically-based tactics for sustainable management. One such widely successful tactic involves maximizing the competitive ability of the crop through increasing planting densities, planting competitive crop cultivars, fertilizer management, and decreasing crop row spacing (Liebman, Mohler & Staver 2001). For example, planting competitive *Hordeum vulgare* L. varieties at high seeding rates can reduce *A. fatua* seed production by 60% (O'Donovan *et al.* 2007). Nitrogen (N) is the main form of fertilizer added to increase crop yield, but the effects of added N on crop-weed interactions are often species-

specific, site-specific, year-specific, and field history-specific (Blackshaw & Brandt 2008).

The short-term impact of ecologically-based weed management tactics can be investigated by field experiments, but evaluations of their long-term consequences requires the use of population dynamics models that allow for the range of outcomes based on variability in demographic data (Holst, Rasmussen & Bastiaans 2007). Previous studies have used population dynamics models to understand the impact of various management strategies on weed population growth (Davis, Dixon & Liebman 2004) and the evolution and spread of herbicide resistance (Maxwell, Roush & Radosevich 1990). Furthermore, elasticity analysis of the models can be used to identify demographic processes with the largest negative impact on population growth and thus can be the focus of management efforts (Caswell 2001; Davis, Dixon & Liebman 2004). Ultimately, knowledge gained from these models can lead to the design of ecologically-based weed management programs that reduce and prevent MHR weed population growth. The objective of this study was to construct a population dynamics model using demographic information obtained in field cropped conditions from two MHR *A. fatua* populations resistant to five herbicide modes of action (Keith *et al.* 2015) and a HS population, under factorial combinations of abiotic (N fertilizer rate) and biotic (spring *Triticum aestivum* seeding density) stressors as well as two different cropping systems (continuous cropping and crop-fallow rotation). The model was then used to identify demographic processes that could be exploited for non-herbicide management of the resistant phenotype populations.

Materials and Methods

Experimental site

The experiment consisted of three trials conducted during the 2013-2015 growing seasons at the Montana State University Arthur H. Post Research Farm near Bozeman, Montana, USA (45° 40’ 29” N; 111° 09’ 14” W, 1432 m elevation). The soil at the site is an Amsterdam-Quagle silt loam, pH 7.8 (NRCS-USDA 2008). Long-term average annual precipitation (1966-2015) at the site was 410 mm and total precipitation in 2013, 2014, and 2015 was 341, 408, and 361 mm, respectively (Western Regional Climate Center 2016). Mean monthly temperatures and total monthly precipitation at the site varied during the course of the study (Fig. 5.1).

Population selection

The MHR populations used in this study, MHR3 and MHR4, were derived from seeds collected in 2006 from two *A. fatua* populations not controlled by 60 g a.i. ha^{-1} pinoxaden in two *H. vulgare* production fields separated by 8 km in Teton County, Montana, USA. Field-collected seeds (90% were resistant to 60 g a.i. ha^{-1} pinoxaden, data not shown) were separately subjected to two generations of recurrent group selection (50 plants in each generation) by spraying pinoxaden at 60 g a.i. ha^{-1} to remove susceptible individuals and obtain the MHR3 and MHR4 populations. These two populations were used to successfully characterize the biochemical mechanisms responsible for MHR (Keith *et al.* 2015) as well as the physiological and ecological consequences of MHR (Lehnhoff *et al.* 2013a; Lehnhoff *et al.* 2013b). The susceptible HS1 population used in this study was derived from seeds of untreated plants bordering an adjacent *H. vulgare*

production field and was confirmed to be 100% susceptible to field rates of pinoxaden (Lehnhoff *et al.* 2013b).

To increase seed stocks, populations were grown at the Arthur H. Post Research Farm in 2012. Seeds were planted into bare ground covered with black landscape fabric in the spring. *Avena fatua* is mostly self-pollinating with outcrossing estimated at 0.05% when grown in crop (Murray, Morrison & Friesen 2002). To minimize cross-pollination, populations were separated by 5 m buffers planted with *Zea mays* L. *Avena fatua* plants were watered regularly and seeds harvested in the fall and stored at 10 C until use.

Experimental design and data collection

Each year, the study was conducted at different locations within the Arthur H. Post Research Farm following a split-plot randomized block design with four replications. Whole plots (23 x 23 m) were assigned hard red spring *T. aestivum* var. Duclair seeded at a density of 67.3 kg ha^{-1} or 101 kg ha^{-1}. Sub-plots (3 x 3 m) were combinations of one of four targeted nitrogen (N) levels (56, 112, 168, or 224 kg N ha^{-1}) and one of three *A. fatua* treatments (HS1, MHR3, or MHR4). Additionally, fallow plots planted with *A. fatua* only were assigned to two sub-plots per replication.

Prior to the initiation of each year's study, the *A. fatua* germinable seedbank abundance was assessed by sampling soil at three random locations per whole plot using handheld bulb planters (10 cm deep, 6.5 cm in diameter). Exhumed soil was placed in flats (30.5 x 21.5 cm), watered regularly, and *A. fatua* emergence monitored for 30 d in the greenhouse. Each year, *A. fatua* germinable seedbank populations were found to be negligible (data not shown). To estimate N availability prior to planting, soil cores (three

per whole plot) were taken each fall to a depth of 61 cm, and N was analyzed by AgVise laboratories (Northwood, North Dakota). Soil nitrate N ranged between 2-19.5 ppm (data not shown), and these values were taken into account when calculating target N levels. In October of each year, *A. fatua* seeds were planted 3 cm deep in the center (1.5 m x 1.5 m) of each sub-plot at a targeted density of 1000 seeds m^{-2} to represent a realistic *A. fatua* seedbank density (Beckie, Francis & Hall 2012). The following May of each year, *T. aestivum* was planted 2.5 cm deep in whole plots utilizing a no-till drill seeder with 25.4 cm row spacing. Starter fertilizer (N-P-K at 6-19-28 kg ha^{-1}) was applied in the furrow at seeding. Nitrogen treatments in the form of urea were applied by hand at the sub-plot level following *T. aestivum* planting. After *T. aestivum* and *A. fatua* emergence in the spring, the entire study, excluding the fallow plots, was sprayed with flucarbazone (30 g a.i. ha^{-1}) at the *A. fatua* 3-leaf stage to represent common producer practices. Bromoxynil plus MCPA (689 g a.i. ha^{-1}) was applied at the same time to control broadleaf weeds.

To assess overwinter seed survival, three nylon mesh bags per block containing either 100 HS1, MHR3, or MHR4 seeds with percent viability known and 98 cm^3 of field soil were buried each fall 3 cm deep in random locations within blocks. Each spring, bags were exhumed and *A. fatua* seeds were placed on moistened filter paper lined Petri dishes (100 x 15 mm) and held at 21 C for 30 d. Germinated seeds were removed and counted daily.

Every year and prior to herbicide application, *A. fatua* emergence was estimated in each sub-plot via nine randomly-placed 100 cm^2 metal rings. After herbicide application, *A. fatua* seedling survivors were counted within rings. In the fall of each

study year, *A. fatua* reproductive tiller and seed counts were recorded. In the spring of each year, *T. aestivum* emergence estimates were made by counting the number of plants per 40 cm in 5 random locations within each split-plot. In the fall of each year, *T. aestivum* yield was measured by harvesting all individuals within the center (1.5 m x 1.5 m) of each sub-plot. Plants were dried for 1 wk at 40 C, threshed using a Vogel thresher, and resulting seeds weighed.

Parameterization of the population dynamics model

A stochastic density-dependent population dynamics model was constructed using a series of equations to project *A. fatua* populations forward 20 years under the previously described treatment combinations. The model parameters included: seedling emergence, seedling establishment, seedling survival, asymptotic density dependent reproductive tiller density, asymptotic density dependent seeds produced per plant, seed predation, yearlong seedbank survival, overwinter seedbank survival, and over summer seedbank survival (Table 1). Yearlong seedling survival was estimated using data from an 11 year (1993-2004) *A. fatua* seedbank dynamics experiment conducted at the same location (Maxwell, Smith & Brelsford 2007). Seed predation was quantified using data from an experiment conducted at the same location from 2004-2006 (Harbuck 2007). All other parameters were fit using data obtained from this experiment. Nonlinear least-squares estimates of the asymptotic density dependent parameters for reproductive tillers (z, q) and seeds produced per plant (i, a) were fit using the nls function in R (R 2016).

The population dynamics model was constructed under two scenarios: continuous *T. aestivum* cropping and a *T. aestivum*-chemical fallow rotation. Within each scenario,

populations were projected forward for each one of the eight seeding density and N rate combinations with a starting *A. fatua* seedbank population of 1000 seeds m^2. From this initial population, the model randomly chose vital rates from a sub-plot and year within that seeding density and N rate combination and then projected the population forward one year. The second year started with year one projected seedbank population and the model once again randomly chose vital rates from a sub-plot and year and then projected the population forward one year. The utilization of independent plots for each year of the simulation served to maximize potential variation and relieved the time series analysis constraint associated with sequential population dynamics. This process was repeated over 20 years which constitutes one simulation. The population projection was modeled for 500 simulations for each population and treatment combination. Under the continuous *T. aestivum* cropping scenario, randomly selected sub-plots planted with *A. fatua* and *T. aestivum* were used in each year of the model. Under the *T. aestivum*-chemical fallow cropping rotation, the model randomly selected a sub-plot planted with *T. aestivum* and *A. fatua* for the first year of the projection followed by a randomly selected *A. fatua* only sub-plot (i.e., fallow plot). This pattern was repeated over 20 years. In the fallow years, a simulated glyphosate application was performed and assumed to have an efficacy rate of 90 ± 5% (Adkins *et al.* 1998).

To assess the overall impacts of *T. aestivum* seeding density and N fertilization on the dN/dt (the change in the number of individuals [dN] per change in time [dt]) for the MHR *A. fatua* populations, a linear mixed effect model was constructed with fixed effects for the 3-way interaction between *A. fatua* population, N rate, and *T. aestivum*

seeding density and random intercept effect for cropping system. The model was fit using the lmer function from the lme4 package in R (R 2016). Differences in means were further investigated using Tukey's HSD post hoc tests in the lsmeans package in R (R 2016).

Elasticity of population growth rate to model parameters

Elasticity analysis was used to quantify the proportional change in the *A. fatua* seedbank resulting from a proportional change in vital rates (Caswell 2001). Elasticities were calculated as:

$$E_{ij} = \frac{a_{ij}}{SB}\frac{\delta SB}{\delta a_{ij}}$$

where E*ij* is the elasticity of the seedbank (SB) to a proportional change in the parameter a*ij* (Caswell 2001). Elasticity analysis was performed on the deterministic form of the population dynamics model described above for the continuous *T. aestivum* and *T. aestivum*-fallow rotations.

Results

Demographic parameters and analysis are included in Appendix C.

Continuous *Triticum aestivum* cropping system

The density dependent population projection simulations indicated that N fertilization and *T. aestivum* seeding density impacted *A. fatua* population responses in the continuous *T. aestivum* cropping system scenario (Fig. 5.2). Overall, HS1 seedbanks declined within three to five years regardless of N fertilization rate and *T. aestivum* seeding density treatments (Fig. 5.2). The MHR3 population indicated a consistent

advantage over MHR4 and HS1 based on simulated seedbank densities and the advantage was amplified with increased N fertilization rate and crop seeding density.

At the end of the simulations and under the low *T. aestivum* seeding density and 56 kg ha^{-1} N rate scenario, MHR seedbank densities were larger than the initial 1000 seeds m^{-2}, with 1437 ± 40 m^{-2} (mean ± one standard error) for MHR3 and 1012 ± 32 seeds m^{-2} for MHR4 (Fig. 5.2a) populations. Under the same N fertilization treatment, 56 kg ha^{-1} N, but high *T. aestivum* seeding density, MHR3 populations increased overtime with a mean seedbank size of 1459 ± 43 seeds m^{-2} in year 20 (Fig. 5.2b). In contrast MHR4 population declined steadily to 77 ± 3 seeds m^{-2} by year 20.

Under the second lowest N fertilization treatment, 112 kg ha^{-1} N, and low *T. aestivum* seeding density, MHR3 simulated seedbank densities were lower than the 56 kg ha^{-1} N rate, but slightly increasing with a final value of 1048 ± 36 seeds m^{-2} (Fig. 5.2c). In contrast, the MHR4 population declined steadily to 47 ± 1 seeds m^{-2}, but did not go extinct over the 20 year period. Increasing the simulated *T. aestivum* seeding density combined with the 112 kg ha^{-1} N led to a MHR3 seedbank of 868 ± 14 seeds m^{-2}, at the end of the 20 years simulation period (Fig. 5.2d). Increasing the *T. aestivum* seeding density at 112 kg ha^{-1} N had little additional impact on MHR4 and while the population declined to 121 ± 4 seeds m^{-2}, it was not extinct at the end of the simulation period.

Increasing the N fertilization rate to 168 kg ha^{-1} N under the low *T. aestivum* seeding density resulted in a steady decline of the MHR3 population stabilizing at 92 ± seeds m^{-2} (Fig. 5.2e). The MHR4 seedbank declined slightly and stabilized at 671 ± 26 seeds m^{-2}. Under the same fertilization rate, but high *T. aestivum* seeding density, MHR3

and MHR4 seedbanks declined rapidly within 5 years and had 88 and 103 seeds m^{-2}, respectively at the end of the simulation (Fig. 5.2f).

In the 224 kg ha^{-1} N and low *T. aestivum* seeding density treatment, MHR seedbanks declined rapidly (Fig. 5.2g). The MHR3 seedbank stabilized at 115 ± 3 seeds m^{-2} while the MHR4 seedbank declined fast and stabilized at 23 ± 0.5 seeds m^{-2}. Increasing the *T. aestivum* seeding density at the highest N treatment had the most severe impact and both MHR seedbank abundances and were nearly extinct by year 5 with 41 ± 1 and 13 ± 0.4 seeds m^{-2} for MHR3 and MHR4 populations, respectively (Fig. 5.2h).

Elasticity analysis in continuous *Triticum aestivum* cropping system

Relative rankings of the seedbank density elasticity to a 10% change in model parameters were similar in all HS1 population models, regardless of cropping system, N fertilizer and *T. aestivum* seeding rate treatments (Table C1). Overwinter seed survival had the greatest impact on *A. fatua* seedbank density followed by over summer seed survival and maximum seed produced per plant.

Regardless of treatment, the most elastic parameter in both MHR3 and MHR4 populations was *a*, the shape parameter in the asymptotic density dependent seed production function, which dictates the maximum proportional seed production reduction at high densities (Table C1). Overwinter seed survival was the second most elastic parameter across treatments followed closely by maximum seed produced per plant.

***Triticum aestivum*-fallow cropping system**

Under the *T. aestivum*-fallow rotation, *A. fatua* mean seedbank density over the 20 year simulation period showed differences among populations, N fertilization rates,

and *T. aestivum* seeding densities. Overall, HS1 populations declined to near zero within three to five years, regardless of N fertilization rate and *T. aestivum* seeding density (Fig. 5.3). MHR seedbank populations differed from each other in response to treatments. The fallow years of the rotation were easily distinguishable due to the large increase in MHR seedbanks by individuals that survived glyphosate treatment and lack of *T. aestivum* competition (Fig. 5.3). HS1 survived the glyphosate treatment at the same rate as the MHR populations, but the increase is not evident because HS1 seedbanks were very low after herbicide treatment in the *T. aestivum* year.

As in the continuous *T. aestivum* cropping system, MHR populations had the largest seedbank densities in the lowest N and low *T. aestivum* seeding density treatment (Fig. 5.3a). At the end of the 20 year projection, MHR3 and MHR4 produced 1580 ± 77 and 2158 ± 118 seeds m^{-2}, respectively. Increased *T. aestivum* seeding density at the lowest N treatment did not negatively impact the MHR3 seedbank (Fig. 5.3b). Unlike MHR3, the increase in *T. aestivum* seeding density at the lowest N rate resulted in a decline of the MHR4 seedbank to 347 ± 31 seeds m^{-2}.

Increasing the N fertilization rate to 112 kg ha^{-1} N reduced the seedbank density of both MHR populations (Fig. 5.3c-d). MHR3 mean seedbank density at the end of the simulation was similar under low and high *T. aestivum* seeding densities with 964 ± 54 and 1045 ± 35 seeds m^{-2}, respectively. MHR4 mean seedbank density was lower than MHR3 in both *T. aestivum* seeding densities although there were slight differences in the rate of decline. In the low *T. aestivum* seeding density, MHR4 declined more rapidly than

in the high *T. aestivum* seeding density and stabilized at 111 ± 10 seeds m^{-2} compared to 436 ± 32 seeds m^{-2} in the high density (Fig. 5.3c-d).

Similar to the continuous *T. aestivum* cropping system, MHR4 had a larger seedbank density than MHR3 in the 168 kg ha^{-1} N-low *T. aestivum* seeding density treatment (Fig. 5.3e). MHR3 population declined gradually for the first 5 years to 234 ± 13 seeds m^{-2}, while MHR4 populations slightly increased to 1023 ± 62 seeds m^{-2} (Fig. 5.3e). Increasing the *T. aestivum* seeding density had a slight negative impact on MHR3 populations, resulting in a seedbank density of 136 ± 8 seeds m^{-2} (Fig. 5.3f). Increasing *T. aestivum* seeding density led to a large decline in MHR4 seedbank density with 248 ± 17 seeds m^{-2}.

Under the highest N fertilizer rate, MHR3 seedbank abundance stabilized at a slightly higher value than MHR4 in the low *T. aestivum* seeding density with 268 ± 20 seeds m^{-2} (Fig. 5.3g). With increased *T. aestivum* seeding density, MHR3 population decreased to 96 ± 6 seeds m^{-2} (Fig. 5.3h). Under the highest N fertilizer rate, MHR4 seedbanks declined rapidly in the low and high *T. aestivum* seeding density to 39 ± 2 and 30 ± 3 seeds m^{-2}, respectively (Fig. 5.3g-h).

Elasticity analysis in *Triticum aestivum*-fallow cropping system

Relative rankings of the seedbank elasticity to a 10% change in model parameters were similar in HS1 populations, irrespective of the N fertilization or *T. aestivum* seeding density treatments (Table C2). The most elastic parameter in the HS1 population was the glyphosate kill rate. Overwinter seed survival was the second most elastic although

values were fairly low for all treatment combinations except the 56 and 112 kg ha^{-1} N-low *T. aestivum* seeding density treatment. All other elasticities were generally very low.

Similar to the continuous *T. aestivum* cropping system and regardless of the N fertilization or *T. aestivum* seeding density treatment, for MHR populations the most elastic parameter was a (Table C2). Glyphosate kill rate was the second most elastic parameter across treatments for all MHR populations except the MHR4 population in the 56 kg ha^{-1} N-low *T. aestivum* seeding density treatment in which it was second. Overwinter seed survival was the third most elastic parameter for both MHR populations. All other elasticities were generally very low.

Nitrogen and *Triticum aestivum* seeding density impacts on dN/dt across rotations

There was a significant 3-way interaction between *A. fatua* population, N rate, and *T. aestivum* seeding density on the simulated *A. fatua* dN/dt ($p \leq 0.0001$). This interaction was further supported by the visual model projections in Figures 5.2 and 5.3. Within an N rate the simulated MHR3 dN/dt was larger when the *T. aestivum* seeding density increased under 56 kg ha^{-1} N and smaller under 112 and 224 kg ha^{-1} N (Table 5.2). There was no difference in dN/dt when the *T. aestivum* seeding density increased under 168 kg ha^{-1} N. Within an N rate the simulated MHR4 dN/dt was smaller when the *T. aestivum* seeding density increased in 56 and 168 kg ha^{-1} N and larger in 112 kg ha^{-1} N (Table 5.2). There was no difference in dN/dt when the *T. aestivum* seeding density increased in 224 kg ha^{-1} N.

Within the low *T. aestivum* seeding density the simulated MHR3 dN/dt, an increase in N rate decreased the dN/dt for all N rate treatment combinations except 168-

224 kg ha^{-1} in which there was no difference (Table 5.2). A similar pattern was observed within the high *T. aestivum* seeding density and N rate treatment combinations. Within the low *T. aestivum* seeding density, the simulated MHR4 dN/dt an increase in N rate resulted in a decrease dN/dt for all N rate treatment combinations, except for the 112 and 168 kg ha^{-1} N treatments in which it was larger and 112-224 kg ha^{-1} in which there was no difference (Table 5.2). Within the high *T. aestivum* seeding density the simulated MHR4 dN/dt an increase in N rate decreased dN/dt for all N rate treatment combinations except 56-112, 56-168, and 112-168 in which there was no difference.

Within an *T. aestivum* seeding density and N rate the simulated dN/dt was larger for MHR3 compared to MHR4 for all treatment combinations except the low *T. aestivum* seeding density 168 kg ha^{-1} N in which MHR4's dN/dt was larger and the high *T. aestivum* seeding density-168 or 224 kg ha^{-1} N treatments in which there was no difference (Table 5.2).

Discussion

Population dynamics models are vital tools in weed management but most are built following a static demographic approach (Davis, Dixon & Liebman 2004; Tidemann *et al.* 2016). Such models are usually constructed using data from a single population and parameter values are mean responses of the population, ignoring intra-population variability (Neve, Vila-Aiub & Roux 2009). As such, these models have inherent limitations that diminish their capability to accurately predict long-term population dynamics. To avoid these limitations, and to incorporate inter- and intra-population variability, we constructed *A. fatua* population models for one susceptible and two MHR

populations with vital rates randomly chosen from field study sub-plots and years to provide realistic basis for simulating response to agroecosystem management factors that could differentially influence population dynamics.

In the continuous *T. aestivum* and *T. aestivum*-fallow cropping systems, HS1 seedbank densities rapidly declined regardless of N fertilizer rate and *T. aestivum* seeding density. The poor performance of HS1 was a result of being highly susceptible to the herbicide applied in this study (Lehnhoff *et al.* 2013b). In the continuous *T. aestivum* and *T. aestivum*-fallow cropping systems, MHR mean seedbank densities of both populations were negatively impacted by increasing N fertilization rate. Previous greenhouse research (Blackshaw & Brandt 2008; Lehnhoff *et al.* 2013a) determined that N fertilization may have a neutral or positive effect on *A. fatua* growth and competitive ability with *T. aestivum*. It is possible that the lower N rates used in those studies, together with the moisture, light intensity, and temperature controlled greenhouse conditions where they were conducted masked the mechanisms driving crop-weed competition occurring in field settings (Freckleton & Watkinson 2000).

In both the continuous *T. aestivum* and *T. aestivum*-fallow cropping systems, MHR mean seedbank density was generally negatively impacted by increasing *T. aestivum* seeding rate. Improving crop competitiveness via increasing crop seeding densities is a well-known strategy to reduce weed interference (Maxwell *et al.* 2007). Specifically, increasing *T. aestivum* seeding density and seed size has been shown to reduce *A. fatua* seed production and increase crop yield and biomass (Stougaard & Xue 2004).

The relative impact of N fertilizer rate and *T. aestivum* seeding density on MHR *A. fatua* seed production differed among populations. This differential impact implied that while the MHR3 seedbank density was not reduced by the increase in *T. aestivum* seeding density at the two lowest N fertilizer rates, MHR4 populations experienced a large decline in mean seedbank size within the first 5 years of the simulation at high *T. aestivum* seeding density. Previous population dynamics models used data collected from a single population and thus inter-population variability was not incorporated (Neve, Vila-Aiub & Roux 2009). Our findings highlight the importance of using multiple locally adapted MHR populations thus enhancing the capability of simulation models to realistically predict long-term dynamics under field conditions.

In general, MHR seedbank densities at year 20 of the projection were larger in the *T. aestivum*-fallow cropping system compared to the continuous *T. aestivum* cropping system. We utilized a glyphosate efficacy 90 ± 5% (Adkins *et al.* 1998) and the large numbers of seeds produced by MHR plants escaping glyphosate treatment in the fallow year of the cropping system resulted in the large peaks in seedbank density. These peaks were not as defined in HS1 due to the extremely low survival rate resulting from flucarbazone application in the first year of the simulation. Although MHR *A. fatua* populations did not experience these peaks of seed production and stabilized at lower seedbank densities in the continuous *T. aestivum* cropping system, there are many negative effects of such continuous cropping that were not incorporated into the model including insect pest and disease outbreaks, depleted soil quality, reduced soil organic

carbon, soil moisture depletions, and general lack of resilience in a changing climate (Lin 2011; Miller, Menalled & Burrows 2013).

In both the continuous and *T. aestivum*-fallow cropping systems, the maximum proportional impact of crop and weed density on seed production (*a*) was the most influential parameter impacting population growth rate. Since these populations were not affected by herbicide applications, the numbers of seeds MHR populations could produce became an important life stage parameter. Thus, our results suggest that management practices should target *A. fatua* seed production, a goal that can be reached by maximizing the competitive effect of the crop. Common practices to increase crop competitiveness include increasing planting densities, planting competitive crop cultivars, fertilizer management, and decreasing crop row spacing (Liebman, Mohler & Staver 2001).

Overwinter seed survival was the second and third most impactful parameter on MHR population growth rates across agronomic treatment combinations in the continuous *T. aestivum and T. aestivum*-fallow cropping systems, respectively. This is not surprising given *A. fatua* lifecycle of a summer annual in which all individuals must survive overwinter in the seedbank to be part of the following year's population. Previous studies have also identified this life stage as highly sensitive to changes in management of annual weeds (Davis, Dixon & Liebman 2004; Tidemann *et al.* 2016), representing a 'choke-point' in the MHR populations in which management would be effective. Studies evaluating burial depth and *A. fatua* seed survival indicated that 80% of seeds placed on the soil surface were characterized as non-viable after six months (Zorner, Zimdahl &

Schweizer 1984). No-till and conservation tillage practices would greatly increase the number of seeds remaining on the soil surface, improve soil quality, and enhance the soil microbial community therefore exposing more seeds to microbial decay (Gallandt, Liebman & Huggins 1999).

Fallow year weed management efficacy was the second most elastic parameter for all MHR treatment combinations in the *T. aestivum*-fallow cropping system, even though MHR populations were not resistant to imposed management (here assumed to be chemical-fallow with glyphosate). *Avena fatua* seedlings escaping management in the fallow year of the rotation produced large amounts of seeds due to lack of competition from *T. aestivum* a crop. Thus, weed management in the fallow year must be strategically applied to minimize seed production without increasing selection for multiple herbicide resistance.

Management Implications

Herbicide resistance in plants is a growing problem and threatening the utility of conventional agricultural practices (Powles & Yu 2010). Specifically troublesome is the characterization of two MHR *A. fatua* populations utilized in this study which are resistant to members of all selective herbicide families available for *A. fatua* control in cereal crops (Lehnhoff *et al.* 2013b; Keith *et al.* 2015). Therefore, alternative ecologically based approaches for sustainable weed management are required. Our results suggest that increasing N fertilization rate and *T. aestivum* seeding density are efficient tools to manage MHR *A. fatua* populations. In addition, decreasing seed production and increasing mortality of *A. fatua* in the fallow years using cover or pulse crops may

represent alternatives to using a herbicide that runs the risk of being included in the multiple herbicide resistance list associated with the MHR phenotype.

During the three years of this study, the average spring *T. aestivum* yield was 2358 and 3705 kg ha^{-1} for the state of Montana and the county in which this study was conducted, respectively (USDA 2015). The recommended N fertilizer rate to achieve these yields is 130 and 204 kg ha^{-1} (Jacobsen, Jackson & Jones 2005). Based off these averages and findings in this study, the most effective of the management practices to adequately control MHR *A. fatua* were N application of 168 kg ha^{-1} in combination with a high *T. aestivum* planting density, or N application of 224 kg ha^{-1}, regardless of *T. aestivum* density. In these treatment combinations, *T. aestivum* yield ranged from 3379-4332 kg ha^{-1} (full data set not shown) which was similar to state and county averages. Choice of which treatment combinations to implement involve environmental and economic tradeoffs. Excess N fertilizer application can have negative environmental effects which include surface runoff, volatilization, and nitrate leaching (Raun & Johnson 1999). Additionally N fertilizers pose a significant cost and this must be factored in when trying to offset crop yield loss from MHR competition. *Triticum aestivum* variety selection should also be taken into account. For example, viability of *A. fatua* seeds collected below a *H. vulgare* canopy was 10-30% less than seeds collected from above the crop canopy in a study conducted in Montana (Lehnhoff *et al.* 2013c). Furthermore, seeds collected below the canopy were more susceptible to pathogen attack which contributed to the observed reduction in viability. Thus, planting taller *T. aestivum*

varieties may force the majority of *A. fatua* to produce seeds under the crop canopy and result in a seedbank composed of less viable seeds thereby reducing future populations.

To our knowledge this study was the first to utilize HS and MHR *A. fatua* field data obtained in a range of environmental conditions to construct a stochastic population dynamics model aimed at providing management recommendations. While other studies have evaluated the impacts of resistance on population dynamics, they did not directly use seed of resistant plants, but rather they simulated herbicide resistance using reduced herbicide rates (Tidemann *et al.* 2016). The use of field evolved MHR population dynamics modelling represents a novel practice that can inform proactive ecological weed management strategies. Duke (2012) stated that herbicides appear to be non-renewable resources, and alternative weed control methods must be investigated before the utility of herbicides are lost. Our results suggest that herbicide resistance is not necessarily a "wicked problem" (Shaw 2016) as long as the solutions are expanded beyond herbicide based approaches to weed management (Menalled *et al.* 2016). Demographic models such as the one constructed in this study will aid in selecting ecologically-based weed management tactics to both control MHR weed populations and extend the utility of weed sciences' non-renewable resources.

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Table 5.1. Abbreviations and estimators for *Avena fatua* parameters used in population dynamics model.

Parameter	Abbreviation	Equation
Emergence	estab	number of seedlings/1000
Seedling density	sdl	seedbank density * estab
Reproductive tiller density	rprt	$(z*sdl)/(1+z*sdl/q)^{*}$
Seeds produced per plant	spp	$sppmax*(1-(i*rprt)/(1 + i*rprt/a))^{\dagger}$
Seeds produced m^2	sp	rprt * spp
Seeds in seedbank in previous fall	seeds_fall1	$(seedbank/owss)^{\ddagger}$
Seeds remaining in seedbank current fall	seeds_fall2	$(seeds_fall1*yss)-sdl^{\P}$
Over summer seed survival rate	osss	seeds_fall2/seeds_fall1
Seeds in next spring's seedbank	seed	$(((seedbank-sdl)*osss)+(sp*(1-pred)))^{\S}$

* z, q asymptotic density dependent shape parameters

† sppmax maximum carrying capacity; i, a asymptotic density dependent shape parameters

‡ owss overwinter seedbank survival rate

¶ yss yearlong seedbank survival rate

§ pred seed predation rate

Table 5.2. Multiple herbicide resistant (MHR3-4) *Avena fatua* mean (standard error) dN/dt under two *Triticum aestivum* seeding densities and four nitrogen fertilizer rates.

Population	Seeding density	Nitrogen rate	dN/dt
	kg ha^{-1}		
MHR3	67	56	87 (5.9)a[1]
MHR4	67	56	-38 (5.2)b
MHR3	101	56	114 (6.8)c
MHR4	101	56	-161 (1.4)de
MHR3	67	112	10 (5.3)f
MHR4	67	112	-184 (0.5)ghi
MHR3	101	112	-23 (2.3)j
MHR4	101	112	-159 (1.3)de
MHR3	67	168	-176 (0.5)egh
MHR4	67	168	-43 (4.4)k
MHR3	101	168	-180 (0.4)ghi
MHR4	101	168	-170 (0.8)deg
MHR3	67	224	-163 (1.1)de
MHR4	67	224	-194 (0.1)i
MHR3	101	224	-186 (0.7)hi
MHR4	101	224	-194 (0.6)i

[1]Means followed by the same letter are not significantly different ($p \leq 0.05$).

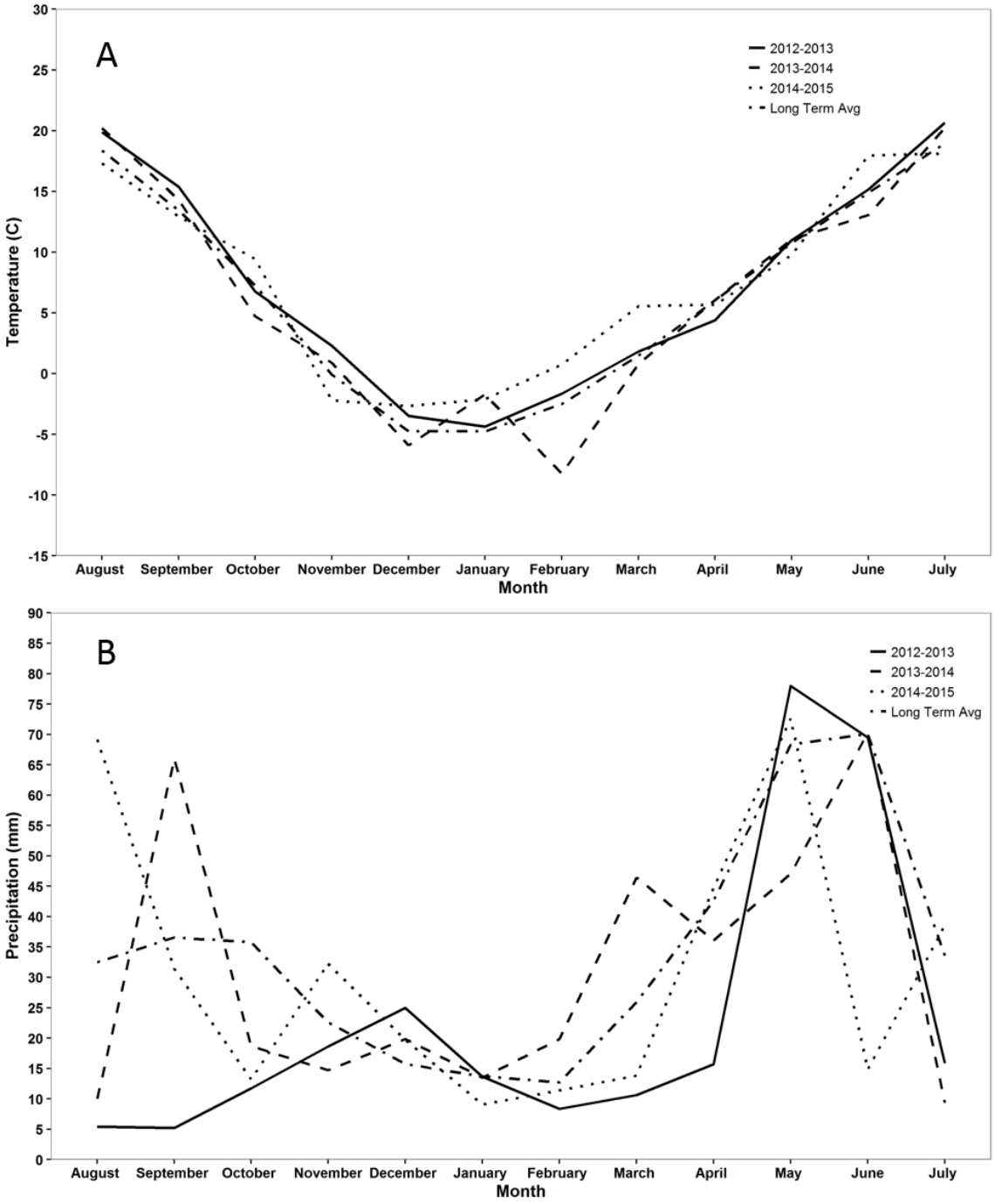

Figure 5.1. Mean monthly air temperature (A) and total monthly precipitation (B) in 2012-2015 and long term averages (1966-2016) at research site near Bozeman, Montana, USA.

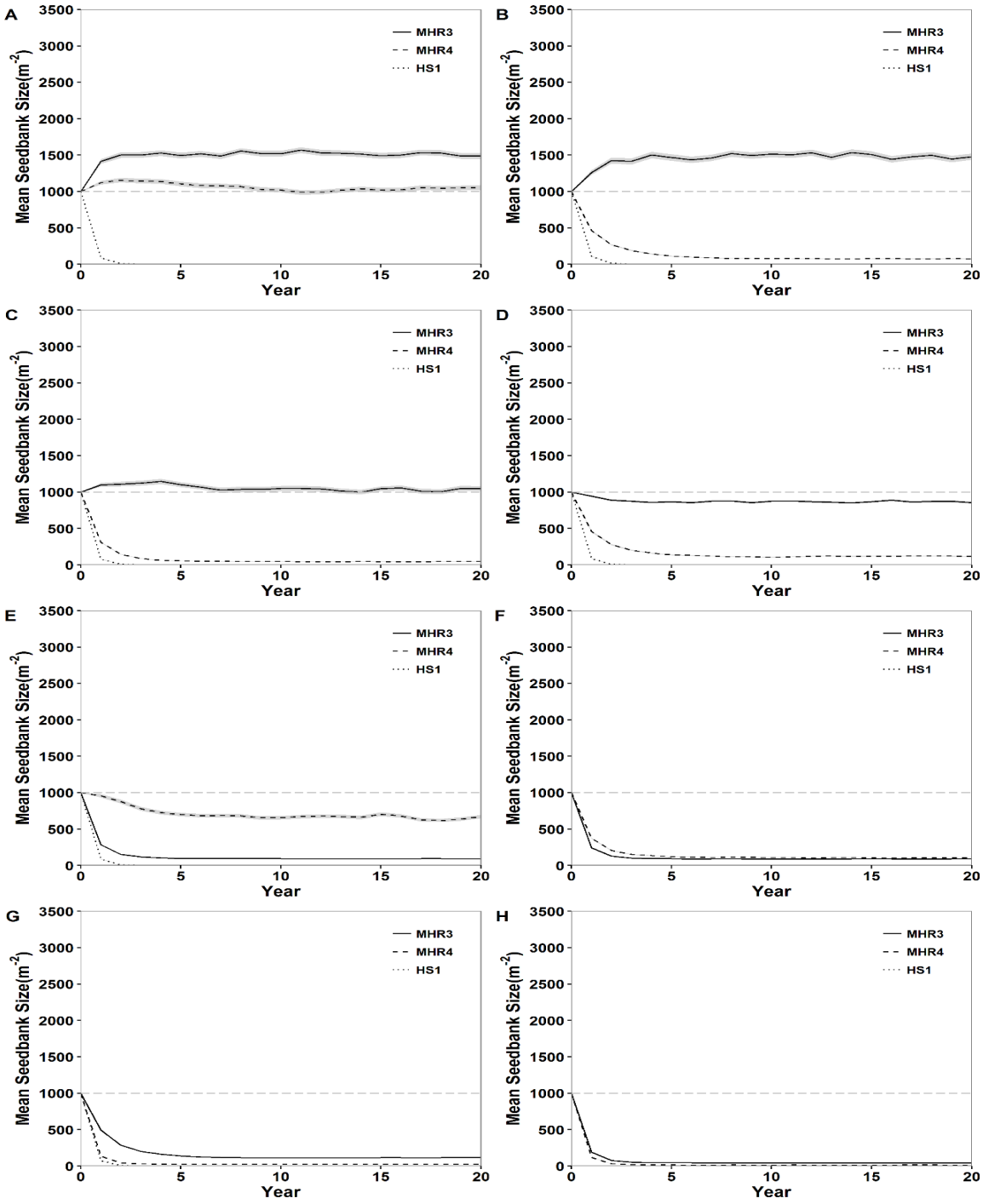

Figure 5.2. Mean density dependent population projections for multiple herbicide resistant (MHR3-MHR4) and herbicide susceptible (HS1) *Avena* fatua populations under two *Triticum aestivum* seeding densities (kg ha^{-1}) and four nitrogen (N) fertilizer rates (kg ha^{-1}) in a continuous *T. aestivum* cropping system. Lines represent the mean and shading ± 1 SEM. A-B 56 N. C-D 112 N. E-F 168 N. G-H 224 N. A, C, E, G 67 seeding density. B, D, F, H 101 seeding density.

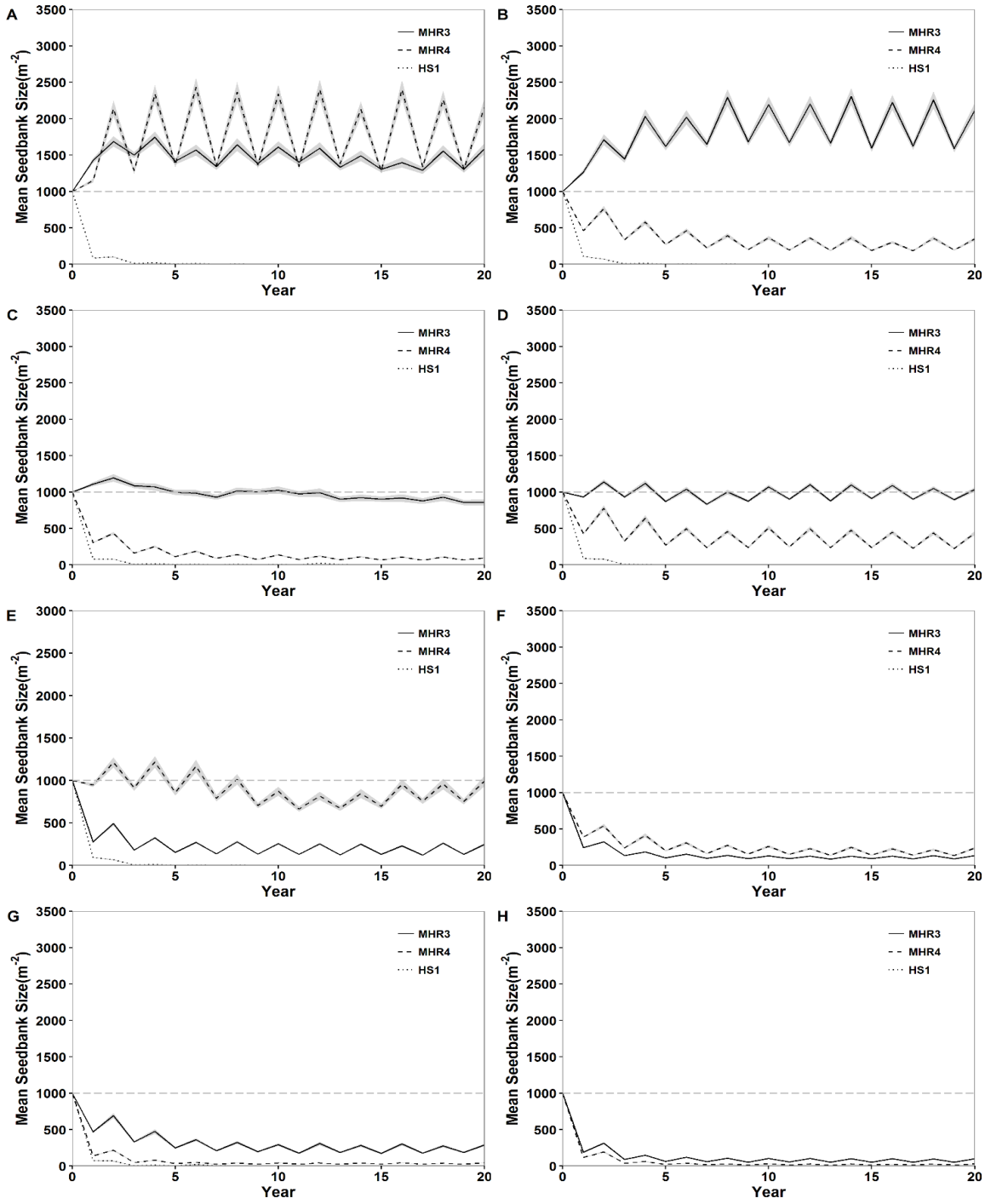

Figure 5.3. Mean density dependent population projections for multiple herbicide resistant (MHR3-MHR4) and herbicide susceptible (HS1) *Avena* fatua populations under two *Triticum aestivum* seeding densities (kg ha^{-1}) and four nitrogen (N) fertilizer rates (kg ha^{-1}) in a *T. aestivum*-fallow cropping system. Lines represent the mean and shading ± 1 SEM. A-B 56 N. C-D 112 N. E-F 168 N. G-H 224 N. A, C, E, G 67 seeding density. B, D, F, H 101 seeding density.

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CHAPTER SIX

CONCLUSIONS

In this conclusions section, I will first summarize the contribution that this work has made towards the study and understanding of the genetic, physiological, and ecological aspects of non-target site (NTS) multiple herbicide resistance (MHR) in *Avena fatua*. I will then highlight future questions for continued work and study of NTS MHR in *A. fatua*. Finally, I will discuss the management implications derived from the results of this dissertation.

Genetic Characterization

This study showed that NTSR in the MHR *A. fatua* populations utilized in this study is controlled by a separate nuclear gene for each flucarbazone-sodium (acetolactate synthase (ALS) inhibitor), imazamethabenz-methyl (ALS inhibitor), and pinoxaden (acetyl-CoA carboxylase (ACCase) inhibitor) herbicides. This finding is in contrast to how NTSR is believed to evolve by 'gene stacking' in which herbicide application initially selects for genotypes with slightly reduced sensitivity, which then cross-pollinate to create recombinant genotypes with combinations of parental alleles that confer increasing levels of herbicide resistance over multiple generations (Délye *et al.* 2011). This hypothesis pertains only to allogamous weed species, which represent the vast majority of NTSR and MHR species studied to date [(Heap 2017); see examples below]. However, our results from the highly autogamous *A. fatua* do not support this scenario,

since target-site resistance has been ruled out (Keith *et al.* 2015) and yet NTSR to individual herbicides is conferred by single genes. An alternative hypothesis that may apply here is that NTSR evolves through a 'step-by-step' process of consecutive selection events in the same individuals over time (Neve, Vila-Aiub & Roux 2009).

Our results are also in contrast to the majority of NTSR inheritance studies which report that NTSR is multigenic in populations of the allogamous species *Lolium rigidum* (Preston 2003; Busi, Vila-Aiub & Powles 2010; Busi, Neve & Powles 2012; Han *et al.* 2014), *Alocpecurs myosuroides* (Petit *et al.* 2010b; Rosenhauer *et al.* 2015), *Amaranthus tuberculatus* (Huffman *et al.* 2015), and *Papaver rhoeas* (Scarabel, Pernin & Délye 2015). In contrast, monogenic NTSR has only been reported in the autogamous species *Conyza canadensis* (Zelaya, Owen & VanGessel 2004) and *Echinochloa phyllopogon* (Iwakami *et al.* 2014), with one report in the allogamous species *L. rigidum* (Yu *et al.* 2009). Overall, the results from *A. fatua* demonstrate the importance of investigating the genetic basis of NTSR on a species by species, population by population level in order to develop a complete understanding of this phenomenon.

In addition to the monogenic resistance reported here, another interesting conclusion is that linkage analysis indicates that the resistance genes for these herbicides are linked. The linkage patterns documented here may help clarify why these populations were resistant to pinoxaden prior to its first field use. Adoption of the ALS inhibitors imazamethabenz and flucarbazone occurred in 1988 and 1998, respectively, and the ACCase inhibitor pinoxaden was introduced in 2005. It is possible that repeated

applications of these two ALS inhibitors simultaneously selected for pinoxaden resistance due to linkage of resistance genes.

Physiological Characterization

The primary conclusions from chapter three focusing on the physiological characterization of NTS MHR in *A. fatua* is that the known herbicide metabolizing glutathione-*S*-transferases (GSTs) enzymes and glutathione-related proteins do not appear to be directly involved in enhanced rates of fenoxaprop-P-ethyl and imazamethabenz-methyl metabolism in MHR *A. fatua*. Our findings are in contrast to the thoroughly characterized roles of GSTs in NTS 'Peldon' MHR *A. myosuroides* populations that are resistant to the photosystem II inhibitor chlorotoluron and several ACCase inhibiting herbicides. Early work showed that MHR plants had higher GST specific activity towards fenoxaprop-P-ethyl, elevated levels of immuno-recognized GST proteins, and higher concentrations of glutathione than susceptible plants (Cummins *et al.* 1997). Subsequently, Cummins *et al.* (2013) showed that MHR in *A. myosuroides* was conferred by overexpression of the phi class AmGSTF1, which acted not through traditional degradation, but through a novel and direct regulatory control on flavonoid metabolism. Overall, the conflicting results from *A. fatua* once again demonstrate the importance of investigating the physiological basis of NTSR on a species by species and population by population level.

We propose that the slightly constitutively elevated glutathione related enzyme activities, as well as the increased levels of lambda, phi, and tau GSTs documented in

chapter three are representative of a larger, more global suite of abiotic stress-related changes in MHR *A. fatua* plants. This conclusion is supported by the findings in chapter four in which a number of proteins with roles in abiotic and biotic stress pathways were found to be elevated in MHR *A. fatua* plants.

Plants are routinely confronted with numerous biotic and abiotic stresses and have evolved highly effective strategies for responding to changing environmental stressors (Vert & Chory 2011). Specifically, systemic acquired resistance (SAR) to combat biotic invasions (Fu & Dong 2013) and systemic acquired acclimation (SAA) that confers resistance to abiotic stresses (Shah *et al.* 2014). Although less detail is known about SAA than SAR, there are a number of commonalities between the two (Fujita *et al.* 2006; Atkinson & Urwin 2012). For example, both systems include stress recognition proteins such as receptor-like kinases (RLKs) and lectin receptor kinases (LRKs), the key roles of reactive oxygen species (ROS) and redox state, and mitogen-activated protein (MAP) kinase-mediated signal transduction cascades (Mittler & Blumwald 2015).

An evolutionarily recent abiotic stress imposed on plants is the application of herbicides. Herbicides are obviously designed to control weedy plants, but they can also merely cause injury, due to sublethal dosages (improper applications or equipment problems), adverse environmental conditions that reduce efficacy, or delayed applications on older, more tolerant plants (Caseley & Walker 1990). Under these conditions, susceptible plants respond much the same as they do to other abiotic stresses (reviewed in (Alberto *et al.* 2016)). Both abiotic (Dietz, Mittler & Noctor 2016) and herbicide stresses (Dayan & Watson 2011; Sewelam, Kazan & Schenk 2016) induce rapid ROS generation,

and even though herbicides are designed to inhibit a specific biochemical target, both have effects on multiple pathways and cellular components (Délye 2013; Suzuki *et al.* 2014). Plant transcriptome changes caused by herbicide stress are very similar to those resulting from abiotic and biotic stresses (Das *et al.* 2010; Unver *et al.* 2010).

Chapter four sought to better understand the molecular mechanisms of NTSR in MHR *A. fatua* by surveying differentially-expressed phosphorylated and redox-sensitive proteins. Our results demonstrate that constitutive post-translational modifications (PTMs) of a number of proteins with functions in core cellular processes, xenobiotic and stress response, ROS detoxification and redox maintenance, chaperones and heat shock response, and intracellular signaling are altered in MHR as compared to HS plants. Clearly additional work will be required before any causal relationship can be assigned between the proteins described here and MHR.

Regardless of their potential roles in MHR, many protein functions described in chapter four are shared with biotic (SAR) and abiotic (SAA) stress response pathways. For example, elevated stress responses, enhanced ROS detoxification, alterations in protein kinases and other signaling molecules, and fitness penalties (with implied reduced biosynthetic activities) have been documented in SAR and SAA. The MHR *A. fatua* PTMs related to ROS detoxification and protein kinases including LecRK2 are especially relevant, since both features play fundamental roles in cross-talk between biotic and abiotic stresses (Atkinson & Urwin 2012; Rejeb, Pastor & Mauch-Mani 2014). More specifically, MAP kinase cascades are known to transform ROS signals into PTMs like protein phosphorylation and oxidation, resulting in signal transduction modulation that

are needed for stress perception, SAR, and SAA (Kosová *et al.* 2011; Waszczak *et al.* 2015; Dietz, Mittler & Noctor 2016).

Priming of SAR can require multiple exposures to modest stresses (Boller & Felix 2009), as has been noted for the evolution of NTSR (Neve & Powles 2005b; Délye, Jasieniuk & Le Corre 2013). In this regard, the NTSR *A. fatua* phenotype was only detected after > 30 years of annual herbicide applications (Keith *et al.* 2015). However, empirical evidence supporting the emerging hypothesis that NTSR is similar to SAR and SAA is scant (Molina *et al.* 1999; Délye, Jasieniuk & Le Corre 2013; Perez & Brown 2014; Dubey, Mishra & Prasad 2016). Nonetheless, we believe that the constitutive changes documented in chapter four support the idea that herbicide stress is perceived much the same as other abiotic stresses, and that NTSR shares a number of similar features with SAR and SAA. We speculate that MHR *A. fatua*'s previous exposure to sublethal herbicide doses, as well as pre-agricultural evolution under a diversity of abiotic and biotic stressors, has led to a heightened state of stress preparedness that includes NTSR to a number of unrelated herbicides.

Ecological Management

In addition to understanding the genetic and physiological aspects conferring NTS MHR in *A. fatua*, we also sought to understand the ecological management of such populations. MHR populations pose significant agronomic and economic threats to agriculture and demand the development and implementation of alternative, ecologically-based tactics for sustainable management. The short-term impact of ecologically-based

weed management tactics can be investigated by field experiments, but evaluations of their long-term consequences requires the use of population dynamics models that allow for the range of outcomes based on variability in demographic data (Holst, Rasmussen & Bastiaans 2007). Therefore the objective of chapter five was to construct a population dynamics model using demographic information obtained in field cropped conditions from two MHR *A. fatua* populations and a HS population, under factorial combinations of abiotic (nitrogen (N) fertilizer rate) and biotic (spring *Triticum aestivum* seeding density) stressors as well as two different cropping systems (continuous cropping and crop-fallow rotation). The model was then used to identify demographic processes that could be exploited for non-herbicide management of the resistant phenotype populations.

We found that in both cropping systems, MHR and HS mean seedbank densities were negatively impacted by increasing N fertilization rate. Previous greenhouse research (Blackshaw & Brandt 2008; Lehnhoff *et al.* 2013a) determined that N fertilization may have a neutral or positive effect on *A. fatua* growth and competitive ability with *T. aestivum*. It is possible that the lower N rates used in those studies, together with the moisture, light intensity, and temperature controlled greenhouse conditions where they were conducted masked the mechanisms driving crop-weed competition occurring in field settings (Freckleton & Watkinson 2000). In both cropping systems, MHR mean seedbank density was negatively impacted by increasing *T. aestivum* seeding rate. Improving crop competitiveness via increasing crop seeding densities is a well-known strategy to reduce weed interference (Maxwell *et al.* 2007).

In general, MHR seedbank densities at the end of the model projection were larger in the *T. aestivum*-fallow cropping system compared to the continuous *T. aestivum* cropping system. We utilized a glyphosate efficacy 90 ± 5% (Adkins *et al.* 1998) and the large numbers of seeds produced by MHR plants escaping glyphosate treatment in the fallow year of the cropping system resulted in the large peaks in seedbank density. Although MHR *A. fatua* populations did not experience these peaks of seed production and stabilized at lower seedbank densities in the continuous *T. aestivum* cropping system, there are many negative effects of continuous cropping that were not incorporated into the model including insect pest and disease outbreaks, depleted soil quality, reduced soil organic carbon, soil moisture depletions, and general lack of resilience in a changing climate (Lin 2011; Miller, Menalled & Burrows 2013).

In both cropping systems, the maximum proportional impact of crop and weed density on seed production was the most influential parameter impacting population growth rate. Since these populations were not affected by herbicide applications, the numbers of seeds MHR populations could produce became an important life stage parameter. Thus, our results suggest that management practices should target *A. fatua* seed production, a goal that can be reached by maximizing the competitive effect of the crop.

Overwinter seed survival was the second and third most impactful parameter on MHR population growth rates across agronomic treatment combinations in the continuous *T. aestivum and T. aestivum*-fallow cropping systems, respectively. This is not surprising given *A. fatua* lifecycle of a summer annual in which all individuals must

overwinter in the seedbank to be part of the following year's population, representing a 'choke-point' in the MHR populations in which management could be effective.

Fallow year weed management efficacy was the second most elastic parameter for all MHR treatment combinations in the *T. aestivum*-fallow cropping system. *Avena fatua* seedlings escaping management in the fallow year of the rotation produced large amounts of seeds due to lack of competition from *T. aestivum*. Thus, weed management in the fallow year must be strategically applied to minimize seed production without increasing selection for MHR.

Overall, our results suggest that increasing N fertilization rate and *T. aestivum* seeding density are efficient tools to manage MHR *A. fatua* populations. In addition, decreasing seed production and increasing mortality of *A. fatua* in the fallow years using cover or pulse crops may represent alternatives to using a herbicide that runs the risk of being included in the list associated with the MHR *A. fatua* phenotype. Demographic models such as the one constructed in this study will aid in selecting ecologically-based weed management tactics to both control and prevent MHR weed population growth.

Future Questions

This work raises further questions on the genetic and physiological mechanisms that confer NTS MHR and ecological management of NTS MHR weed populations. We found significant differences between the potential mechanisms/genetics controlling NTS MHR in *A. fatua* an autogamous polyploid weed compared to those in the allogamous diploid weeds namely, *A. myosuroides* and *L. rigidum*. Implications different mating

systems and genome structure have on the evolution of NTS MHR have simply been speculated on and detailed studies are called for to fully understand this interesting fundamental difference in NTS MHR weed species. For example, NTSR is thought to evolve by 'gene stacking' in which herbicide application initially selects for less sensitive genotypes, which then cross-pollinate to create recombinant genotypes with combinations of parental alleles conferring enhanced herbicide resistance over multiple generations (Délye *et al.* 2011). Significantly, this hypothesis pertains only to allogamous weed species our results from the highly autogamous species *A. fatua* do not support this idea. An alternative, more relevant hypothesis is that NTSR evolves through a 'step-by-step' process of consecutive selection events in the same individuals over time (Neve, Vila-Aiub & Roux 2009). Directly testing these hypotheses on a variety of autogamous and allogamous NTSR weed species would fill in this fundamental knowledge gap and reveal patterns of how NTSR evolves.

In addition to contrasting mating systems, differences in genome structure and ploidy level may influence the evolution of NTSR which raises additional questions for future research. Polyploidy results in genome-wide genetic redundancy, providing novel opportunities for adaptive evolution (Wendel 2000). Polyploidy may play contrasting roles in NTSR evolution by 1) minimizing the deleterious effects of herbicide resistance gene mutations, while at the same time 2) buffering the effect of herbicide resistance genes with homoeologous sensitive alleles. This gene dosage effect has not yet been investigated in weedy species with NTSR. Furthermore, the details of homoeologous gene expression, gene silencing, and possible epigenetic regulation of homoeologs are

essentially unknown in weedy species, although they provide valuable opportunities for developing a deeper understanding of NTS herbicide resistance evolution.

Further testing the hypothesis that MHR *A. fatua* plants are in a heightened state of stress preparedness would also be of interest in understanding how prior stress exposure may mediate a plants response to subsequent exposures. Multiple studies have explored plant responses to different abiotic and biotic stresses occurring simultaneously or sequentially (Suzuki *et al.* 2014; Ramegowda & Senthil-Kumar 2015; Coolen *et al.* 2016), but this has not been conducted in NTSR or MHR weed populations.

The constitutive differentially expressed proteins reported in chapter four comprise a list of candidate NTSR proteins. Further testing which includes determining if their differential regulation co-segregates with herbicide resistance in F_3 families would help to reduce this list to the most likely candidates conferring NTS MHR in *A. fatua*. Ultimately transforming a sensitive plant with a candidate NTSR protein in which the transgenic plant subsequently acquires resistance to multiple herbicides would provide definitive proof of the role that protein plays in NTS MHR.

The population dynamics study in chapter five revealed that we can control MHR *A. fatua* without herbicides through the manipulation of N fertilizer and *T. aestivum* seeding rates. Investigation into how MHR populations would be impacted by additional ecologically-based weed management tactics such as planting competitive crop cultivars, decreasing crop row spacing, row orientation, and alternative non-chemical fallow year replacements e.g. planting cover or pulse crops, grazing, would greatly enhance the tools available to design ecologically-based weed management programs that reduce and

prevent MHR weed population growth. In addition testing the utility of this model on other weed species and conducting a detailed economic analysis are interesting future research avenues.

Management Implications

Herbicide resistance in plants is a growing problem threatening the utility of conventional agricultural practices (Powles & Yu 2010). Specifically troublesome is the characterization of two MHR *A. fatua* populations utilized in this dissertation which are resistant to members of all selective herbicide families available for *A. fatua* control in cereal crops (Lehnhoff *et al.* 2013b; Keith *et al.* 2015). While direct management implications can easily be drawn from the population dynamics study in chapter five, understanding the genetic and physiological mechanisms involved in NTS MHR in *A. fatua* can also inform herbicide resistance management.

Understanding the inheritance of NTS MHR is of upmost importance for continued agricultural productivity in the face of rapid increases in resistant weed populations worldwide. The single-gene NTSR inheritance patterns reported in chapter two add to our understanding and illustrate the diversity of evolutionary responses to strong selection. Overall, these results from *A. fatua* demonstrate the importance of investigating the genetic basis of NTSR on a species by species, population by population level in order to develop a complete understanding of this phenomenon.

Current herbicide resistance management strategies include using different herbicide mechanisms of action in annual rotations, tank mixtures, and/or sequential

applications (Norsworthy *et al.* 2012). However, these strategies will be ineffective at controlling or slowing the evolution of NTSR as described here due to the linkage of resistance genes. Inheritance research can also inform management practices associated with the adoption of genetically modified crops that have stacked two or more resistance genes by demonstrating that NTS resistance genes can be linked and thus solely relying on alternating the two herbicides in the stack will not be an effective management strategy.

Overall, understanding the genetic and physiological mechanisms that confer NTS MHR and ecological management of NTS MHR *A. fatua* have implications on understanding how a population responds to intense herbicide selection from multiple herbicides, but also can be extended to understanding mechanisms conferring resistance to other abiotic stressors such as drought and heat, important factors in our changing climate and biotic stressors such as bacteria, viruses, fungi, and parasites.

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APPENDICES

APPENDIX A

SUPPLEMENTAL INFORMATION FOR CHAPTER TWO

Table A1. *Avena fatua* mean (standard errors) F_2 injury ratings 3 weeks after herbicide treatment.

Cross[a]	F_2	Mean F_2 Injury[b]		
		Flucarbazone	Imazamethabenz	Pinoxaden
MHR3xHS2	316a	1.78(0.27)	2.25(0.28)	1.95(0.27)
	316b	2.05(0.20)	2.14(0.13)	1.75(0.29)
	316c	0.55(0.05)	2.08(0.27)	1.35(0.15)
	316d	1.88(0.32)	1.90(0.15)	1.60(0.24)
	316e	1.90(0.40)	2.55(0.23)	1.80(0.28)
	316f	1.40(0.25)	2.69(0.13)	1.50(0.22)
	316g	2.84(0.09)	1.78(0.24)	1.60(0.31)
	316h	0.95(0.16)	1.65(0.11)	1.05(0.09)
	316i	2.30(0.25)	2.90(0.18)	1.70(0.17)
	316j	2.03(0.34)	2.05(0.25)	1.23(0.12)
HS2xMHR3	38a	1.63(0.31)	2.75(0.25)	1.47(0.11)
	38b	1.70(0.25)	2.73(0.23)	2.00(0.29)
	38c	1.65(0.33)	1.78(0.31)	1.65(0.15)
	38d	0.79(0.09)	1.83(0.21)	1.20(0.27)
	38e	3.20(0.13)	3.38(0.15)	2.65(0.15)
	38f	1.69(0.12)	2.47(0.26)	1.75(0.25)
	38g	2.55(0.20)	2.43(0.23)	1.75(0.37)
	38h	2.10(0.26)	2.28(0.27)	2.50(0.31)
	38i	1.21(0.14)	2.33(0.20)	1.35(0.11)
	38j	1.75(0.26)	2.68(0.29)	2.20(0.37)
MHR4xHS2	40a	2.50(0.14)	3.15(0.18)	3.15(0.28)
	40b	2.50(0.31)	3.40(0.19)	2.00(0.27)
	40c	3.11(0.09)	3.10(0.15)	2.55(0.31)
	40d	0.98(0.24)	1.95(0.11)	1.25(0.17)
	40e	1.80(0.29)	2.45(0.17)	2.45(0.26)
	40f	2.50(0.08)	2.78(0.17)	3.35(0.27)
	40g	0.68(0.07)	1.60(0.16)	1.20(0.23)
	40h	1.33(0.21)	2.65(0.22)	2.06(0.24)
	40i	1.23(0.33)	2.75(0.19)	1.35(0.21)
	40j	0.63(0.05)	2.61(0.39)	1.70(0.40)
HS2xMHR4	45a	1.25(0.11)	1.60(0.10)	1.30(0.12)
	45b	2.50(0.22)	1.70(0.12)	1.30(0.34)
	45c	3.60(0.19)	3.50(0.27)	3.40(0.24)
	45d	2.85(0.10)	3.20(0.20)	3.70(0.20)
	45e	0.71(0.06)	1.75(0.22)	1.30(0.44)
	45f	1.00(0.00)	1.85(0.15)	1.40(0.19)
	45i	2.30(0.37)	2.40(0.40)	2.60(0.51)

45j	1.10(0.10)	2.00(0.00)	1.80(0.20)

[a]MHR3-MHR4 multiple herbicide resistant, HS2 herbicide susceptible populations
[b] Injury rating using scale of 0 = no injury to 4 = plant death

APPENDIX B

SUPPLEMENTAL INFORMATION FOR CHAPTER THREE

Table B1. Differentially expressed proteins from untreated herbicide susceptible (HS1) and multiple herbicide resistant (MHR4) *Avena fatua* plants identified using two-dimensional fluorescence difference gel electrophoresis.

Spot number	Uniprot	Annotation	Fold change[a]	Peptides	Peptide sequences[b]
1	I1IWH6	Ribulose 1,5-bisphosphate carboxylase small subunit	-9	3	KFETLSYLPPLSVEALLKQIEYLIR FETLSYLPPLSVEALLK FETLSYLPPLSVEALLKQIEYLIR
2	I1IWH6	Ribulose 1,5-bisphosphate carboxylase small subunit	-7.4	2	CMQVWPIEGIK KFETLSYLPPLSVEALLKQIEYLIR
3	I1IWH6	Ribulose 1,5-bisphosphate carboxylase small subunit	-16.3	2	KFETLSYLPPLSVEALLKQIEYLIR FETLSYLPPLSVEALLKQIEYLIR
4	Q850K7	Expansin-like B1	-6.4	2	MAQSTDAGTALLTLGVVGIEYR TLNNGDVSASA
5	Q6YPG5	Nitric oxide synthase	-5.3	2	**A**APPLLSLSQR GPKKLKINMV
6	Q9FUE5	Glutathione *S*-transferase OsGSTU5	1.6	2	FWADYVDK FWADYVDKKLFDCQTR
7	Q9FUE5	Glutathione *S*-transferase OsGSTU5	3.1	2	DAAHEQAKRDMAEALGTLEA FWADYVDKK
8	Q10CE7	Glutathione *S*-transferase OsGSTU1	2.5	3	HLPSPEKVYDFVGVLK HLPSPEKVYDFVGVLKK EEKELVLLDFWVSPFGQR
9	Q10CE7	Glutathione *S*-transferase OsGSTU1	1.8	2	HLPSPEKVYDFVGVLKK *E*LVLLDFWVSPFGQR[c]
10	Q9FUD8	Glutathione *S*-transferase OsGSTF3	2.3	3	ATDMAVVEQNEAK LQPFGQVPAFK AAPVTVYGPMISPAVAR
11	Q9FUD8	Glutathione *S*-transferase OsGSTF3	2.4	2	ATDMAVVEQNEAK LQPFGQVPAFK
12	Q60DP1	Glutathione *S*-transferase	3.5	2	RRYFGGGGEALGYVDVALAPFTAWFATYER FIDEEFAGVGEPLLPA

13	B7ERQ1	Peroxiredoxin	1.6	2	AIKQLNMVDPDEKD IPPGVSDDEAKEKFPQGFDTAD
14	Q7XTG5	Glycosyltransferase	1.8	2	LDDEAAAAAAADGAAR AWGLAGSGYAFLWNLRPDLVKGDGG
15	F4MG97	Methylthioribose-1-phosphate isomerase	-9.8	2	IASGGASSTRSSTPPDSPGFLSISAEFR DKIASGGASSTRSSTPPDSPG
16	B9F2M9	Terpene cyclase/mutase family member	-8.5	2	*E*EIYPVHYLTIDWAQARSACAK HRHRSKGSWTLSS
17	I1GW31	Polyubiquitin	-7.5	2	ESTLHLVLR DKEGIPPDQQRLIFAGKQLEDGRT
18	P41344	Ferredoxin-NADP reductase, leaf isozyme, chloroplastic	10.2	4	ITADDAPGETWHMVFSTEGEIPYR LYSIASSALGDFGDSK MAEYKEELWELLK MAEYKEELWELLKK
19	I1IN47	Fructose-bisphosphate aldolase, chloroplast precursor	-5.5	10	IVDILVEQGIVPGIK IVDILVEQGIVPGIKVDK KIVDILVEQGIVPGIK LDSIGLENTEANR RLDSIGLENTEANR TLLVTPPGLGNYISGAILFEETLYQSTVDGK TLLVTPPGLGNYISGAILFEETLYQSTVDGKK TVVSIPNGPSELAVK TWGGRPENVAAAQEALLLR YAAISQDNGLVPIVEPEILLDGEHGIDR
20	I1IN47	Fructose-bisphosphate aldolase, chloroplast precursor	7.7	11	ASAYADELVK *E*AAWGLAR IVDILVEQGIVPGIK IVDILVEQGIVPGIKVDK

					KIVDILVEQGIVPGIK LDSIGLENTEANR RLDSIGLENTEANR TLLVTPPGLGNYISGAILFEETLYQSTVDGK TVVSIPNGPSELAVK TWGGRPENVAAAQEALLLR YAAISQDNGLVPIVEPEILLDGEHGIDR
21	I1IN47	Fructose-bisphosphate aldolase, chloroplast precursor	-6.9	15	AKANSLAQLGK ANSLAQLGK ASAYADELVK IVDILVEQGIVPGIK IVDILVEQGIVPGIKVDK KIVDILVEQGIVPGIK KIVDILVEQGIVPGIKVDK LDSIGLENTEANR QAYRTLLVTPPGLGNYISGAILFEETLYQSTVDGK RLDSIGLENTEANR TLLVTPPGLGNYISGAILFEETLYQSTVDGK TLLVTPPGLGNYISGAILFEETLYQSTVDGKK TVVSIPNGPSELAVK TWGGRPENVAAAQEALLLR YAAISQDNGLVPIVEPEILLDGEHGIDR
22	Q6Z8F4	Phosphoribulokinase	-8.2	11	ANDFDLMYEQVK DLYEQIIAER FYGEVTQQMLK HADFPGSNNGTGLFQTIVGLK IRDLYEQIIAER KPDFDAFIDPQK

					KPDFDAFIDPQKQYADAVIEVLPTQLIPDDNEGK KPDFDAFIDPQKQYADAVIEVLPTQLIPDDNEGKVLR LDELIYVESHLSNLSTK QYADAVIEVLPTQLIPDDNEGK[d] QYADAVIEVLPTQLIPDDNEGKVLR
23	Q6Z8F4	Phosphoribulokinase	-7.3	10	ANDFDLMYEQVK DLYEQIIAER FYGEVTQQMLK HADFPGSNNGTGLFQTIVGLK IRDLYEQIIAER KPDFDAFIDPQK KPDFDAFIDPQKQYADAVIEVLPTQLIPDDNEGK LDELIYVESHLSNLSTK QYADAVIEVLPTQLIPDDNEGK QYADAVIEVLPTQLIPDDNEGKVLR
24	C7IXG8	Phosphoglycerate kinase	-7.5	6	ADDVIGPEVEK FSLAPLVPR IGVIESLLEK KSVGDLAAADLEGKR SVGDLAAADLEGK SVGDLAAADLEGKR
25	Q6ZFJ9	60 kDa chaperonin beta subunit	-2.6	7	*E*VELEDPVENIGAK GYISPYFVTDSEK LADLVGVTLGPK LRVEDALNATK NAGVNGSVVTEK VDAIIETLENDEQKVGAEIVR YGYNAATGKYEDLMAAGIIDPTK

26	Q6ZFJ9	60 kDa chaperonin beta subunit	-3.5	6	EVELEDPVENIGAK GYISPYFVTDSEK LADLVGVTLGPK LRVEDALNATK VDAIIETLENDEQKVGAEIVR YGYNAATGKYEDLMAAGIIDPTK
27	Q5VNW1	Transketolase	-7.3	5	FAEYEKKYPEDAATLK FEALGWHTIWVKNGNDGYDEIR GGYIVSDNSTGNKPDFIVMSTGSELEIVAK NPYWFNR VTTTIGFGSPNK
28	A3AXK2	Aminomethyltransferase	6.9	4	KAEGGFLGADVILK AEGGFLGADVILK TGYTGEDGFEISVPSENAVDLAK MYFSDFK
29	Q2QLY4	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	-8.4	2	**A**SHIVGYPR YLFAGVVDGR
30	Q7XDC8	Malate dehydrogenase, cytoplasmic	-7.2	4	EFAPSIPEK MDATAQELSEEK MELVDAAFPLLK VLVVANPANTNALILK
31	Q69LE6	glutathione *S*-transferase OsGSTU27	2.6	2	TPLLVAWAER EEEIGGGKSELLLA
32	Q2QSR7	NAD(P)-binding	-3.8	11	AGGFPEPEIVHYNPK AGGFPEPEIVHYNPKDFDFGK AGGFPEPEIVHYNPKDFDFGKK AGRPIPVPGAGNQITQLGHVK EGHQVTLFTR GDRQDFDFVK

					GFDVVYDINGR NILVMGGTR SDLLPHFETDAVDPK VLHLKGDRQDFDFVK YVTFDGLAR
33	I1H055	Glycosyl transferase, family 31 protein	3	2	**P**EHGAFVALEARAHGDVLLLDCAENAK MYDYKSAEAKDFLENTIGVHQLKEDLQW

[a]Positive and negative fold change values indicate higher and lower levels in MHR4 compared to HS1 plants, respectively.
[b]Bold letter indicates actyl, italic letter indicates dehydrated, underlined letter indicates ammonia loss post translational modifications, respectively.

Table B2. Mean dry weights (g) of multiple herbicide resistant (MHR3 and MHR4) and herbicide susceptible (HS1) *Avena fatua* plants 3 weeks after treatment with mesosulfuron, thiencarbazone, or fenoxaprop-P-ethyl herbicides with and without the safener mefenpyr.

Population	Dose (x)	ME[a]	Mean (SE)	TH[b]	Mean (SE)	FE[c]	Mean (SE)
HS1	0	-[d]	1.45 (0.16)a[f]	-	1.55 (0.35)a	-	1.68 (0.12)a
HS1	0	+[e]	1.48 (0.16)a	+	1.58 (0.35)a	+	1.70 (0.12)a
HS1	0.1	-	1.84 (0.35)a	-	0.76 (0.22)a	-	1.14 (0.13)a
HS1	0.1	+	1.32 (0.14)a	+	1.10 (0.34)a	+	2.00 (0.27)b
HS1	0.3	-	0.32 (0.02)a	-	0.75 (0.20)a	-	1.44 (0.12)a
HS1	0.3	+	0.57 (0.07)a	+	0.44 (0.11)a	+	1.60 (0.12)a
HS1	1	-	0.30 (0.05)a	-	0.35 (0.09)a	-	0.27 (0.05)a
HS1	1	+	0.50 (0.06)a	+	0.59 (0.21)a	+	0.30 (0.04)a
HS1	3	-	0.30 (0.06)a	-	0.26 (0.05)a	-	0.21 (0.05)a
HS1	3	+	0.27 (0.02)a	+	0.30 (0.06)a	+	0.31 (0.04)a
HS1	5	-	0.27 (0.02)a	-	0.23 (0.05)a	-	0.24 (0.04)a
HS1	5	+	0.30 (0.01)a	+	0.20 (0.03)a	+	0.27 (0.04)a
HS2	0	-	1.78 (0.29)a	-	1.78 (0.35)a	-	1.80 (0.14)a
HS2	0	+	1.82 (0.29)a	+	1.81 (0.35)a	+	1.83 (0.14)a
HS2	0.1	-	1.73 (0.33)a	-	1.04 (0.27)a	-	1.50 (0.09)a
HS2	0.1	+	1.41 (0.21)a	+	1.28 (0.34)a	+	2.06 (0.25)b
HS2	0.3	-	0.32 (0.02)a	-	0.71 (0.15)a	-	1.64 (0.22)a
HS2	0.3	+	0.58 (0.10)a	+	0.80 (0.21)a	+	1.64 (0.20)a
HS2	1	-	0.31 (0.03)a	-	0.41 (0.09)a	-	0.42 (0.09)a
HS2	1	+	0.71 (0.16)a	+	0.45 (0.10)a	+	0.42 (0.09)a
HS2	3	-	0.38 (0.04)a	-	0.33 (0.06)a	-	0.29 (0.05)a
HS2	3	+	0.47 (0.08)a	+	0.33 (0.07)a	+	0.38 (0.04)a
HS2	5	-	0.30 (0.05)a	-	0.33 (0.07)a	-	0.33 (0.07)a
HS2	5	+	0.34 (0.02)a	+	0.33 (0.07)a	+	0.31 (0.04)a
MHR3	0	-	1.30 (0.18)a	-	1.37 (0.17)a	-	1.35 (0.10)a
MHR3	0	+	1.32 (0.18)a	+	1.35 (0.17)a	+	1.38 (0.10)a
MHR3	0.1	-	1.52 (0.20)a	-	1.15 (0.16)a	-	1.35 (0.16)a
MHR3	0.1	+	1.16 (0.17)a	+	1.56 (0.16)b	+	1.81 (0.26)b
MHR3	0.3	-	1.03 (0.20)a	-	1.07 (0.11)a	-	1.49 (0.16)a
MHR3	0.3	+	0.86 (0.13)a	+	1.33 (0.09)a	+	1.35 (0.08)a
MHR3	1	-	0.70 (0.12)a	-	0.83 (0.15)a	-	0.81 (0.19)a
MHR3	1	+	0.92 (0.19)a	+	1.06 (0.19)a	+	1.14 (0.19)a
MHR3	3	-	0.39 (0.04)a	-	0.73 (0.15)a	-	0.48 (0.12)a
MHR3	3	+	0.86 (0.10)a	+	0.94 (0.19)a	+	0.36 (0.09)a
MHR3	5	-	0.34 (0.04)a	-	0.53 (0.12)a	-	0.33 (0.08)a
MHR3	5	+	0.60 (0.10)a	+	0.60 (0.14)a	+	0.34 (0.07)a

MHR4	0	-	1.12 (0.08)a	-	0.90 (0.03)a	-	0.98 (0.06)a
MHR4	0	+	1.08 (0.08)a	+	0.91 (0.03)a	+	0.97 (0.06)a
MHR4	0.1	-	1.25 (0.16)a	-	0.87 (0.05)a	-	1.08 (0.05)a
MHR4	0.1	+	1.20 (0.17)a	+	0.84 (0.04)a	+	1.03 (0.06)a
MHR4	0.3	-	1.07 (0.15)a	-	1.22 (0.12)a	-	1.07 (0.06)a
MHR4	0.3	+	0.95 (0.08)a	+	0.92 (0.03)a	+	0.87 (0.06)a
MHR4	1	-	0.57 (0.10)a	-	0.80 (0.05)a	-	1.06 (0.07)a
MHR4	1	+	0.95 (0.12)b	+	0.93 (0.07)a	+	0.99 (0.08)a
MHR4	3	-	0.37 (0.02)a	-	0.74 (0.04)a	-	0.78 (0.05)a
MHR4	3	+	0.78 (0.09)b	+	0.78 (0.01)a	+	0.65 (0.04)a
MHR4	5	-	0.45 (0.05)a	-	0.72 (0.04)a	-	0.92 (0.20)a
MHR4	5	+	0.61 (0.08)a	+	0.62 (0.03)a	+	0.64 (0.05)a

[a]ME = 1x Mesosulfuron = 15 g a.i. ha^{-1}

[b]TH =1x Thiencarbazone = 5 g a.i. ha^{-1}

[c]FE = 1x Fenoxaprop-P-ethyl = 86 g a.i. ha^{-1}

[d]- = no mefenpyr application

[e]+ = mefenpyr application, 1x = 30 g a.i. ha^{-1} for mesosulfuron-methyl and thiencarbazone-methyl treatments and 25.2 g a.i. ha^{-1} for fenoxaprop-P-ethyl treatments

[f]Means within a dose followed by the same letter are not significantly different ($p \leq 0.05$)

APPENDIX C

SUPPLEMENTAL INFORMATION FOR CHAPTER FIVE

Appendix C1. *Avena fatua* demographic data

Statistical analysis

Linear mixed models (LMM) were constructed to assess the overall effects of *T. aestivum* seeding density and nitrogen (N) fertilization on MHR and HS *A. fatua* demographic parameters: overwinter seed survival (%), emergence (per m^2), reproductive tillers (per m^2), and seeds produced (per m^2). In all models, the response variables (demographic parameters) were log transformed due to near-zero tiller and seed counts for HS1 plants as a result of herbicide application. The transformed response variables were analyzed using a LMM with fixed effects for the 3-way interaction between *A. fatua* population, N rate, and *T. aestivum* seeding density and random intercept effects for year and block (nested within year). To account for the non-constant variance of the *A. fatua* population, each population level was allowed to have its own unique variance in the model. Models were fit using the lme function from the nlme package in R which allows non-constant variance and random effects to be fit simultaneously (R 2016). Differences in means were further investigated using Tukey's HSD post hoc tests in the lsmeans package in R.

Results Overall demography

Avena fatua overwinter seed survival differed among MHR and HS populations ($p < 0.0001$). HS1 had the lowest survival rate (40 ± 3%, mean ± one standard error) compared to the two MHR populations. There was no difference in overwinter seed survival between the two MHR populations, averaging 57 ± 2% for both MHR populations.

Avena fatua seedling emergence differed among N rates ($p = 0.0206$) and MHR and HS populations ($p < 0.0001$, Table C1). Increasing N rate had a negative impact on *A. fatua* seedling emergence, although only the 56 kg ha^{-1} N treatment is different from the other three N treatments. Averaged across *A. fatua* populations and *T. aestivum* seeding densities *A. fatua* emergence in the lowest N rate was 132 ± 2 plants while in the highest N treatments *A. fatua* emergence was 88 ± 2 plants. Averaged across *T. aestivum* seeding densities and N rates, HS1 seedlings emerged at a rate of 70% less than MHR3 and MHR4 populations. There was no difference in MHR3 and MHR4 seedling emergence (Table C1).

Density of *A. fatua* reproductive tillers was negatively affected by *T. aestivum* seeding density ($p = 0.0322$), N rate ($p < 0.0001$), and *A. fatua* population ($p < 0.0001$, Table C1). Averaged across *A. fauta* MHR and HS populations and N rates, reproductive tiller production was 30% less in the high *T. aestivum* seeding density treatment compared to the low seeding density. Higher N application rates negatively affected the number of reproductive tillers, although only the 56 kg ha^{-1} N treatment was different from the other three N treatments. Averaged across *A. fatua* populations and *T. aestivum* seeding densities, *A. fatua* reproductive tiller density in the lowest N rate was 35 ± 1.7 tillers while in the highest N treatments *A. fatua* reproductive tillers was 12.3 ± 1.7 tillers. *Avena fatua* reproductive tillers differed among MHR and HS populations. Averaged across *T. aestivum* seeding density and N rates, HS1 seedlings produced only 2 ± 2 tillers while MHR3 and MHR4 populations produced 61 and 54 ± 2 tillers, respectively. There was no difference in MHR3 and MHR4 tiller production (Table C1).

Avena fatua seed production (m^{-2}) was marginally affected by *T. aestivum* seeding density ($p = 0.1023$), N rate ($p < 0.0001$), and *A. fatua* population ($p < 0.0001$, Table C1). Averaged across *A. fauta* MHR and HS populations and N rates, seed production was 30% less in the high *T. aestivum* seeding density treatment compared to the low seeding density. Higher N application rates negatively affected seed production, although only the 56 kg ha^{-1} N treatment is different from the other three N treatments. Averaged across *A. fatua* MHR and HS populations and *T. aestivum* seeding densities *A. fatua* seed production in the lowest N rate was 365 ± 2.4 seeds while in the highest N treatments *A. fatua* seed production was 89 ± 2.4 seeds. *Avena fatua* seed production differed among populations. Averaged across *T. aestivum* seeding densities and N rates, HS1 produced only 2.2 ± 2.4 seeds while MHR3 and MHR4 populations produced 1236.5 and 1118.8 ± 2.4 seeds, respectively. There was no difference in MHR3 and MHR4 seed production (Table C1).

Table C1. Multiple herbicide resistant (MHR3 and MHR4) and herbicide susceptible (HS1) *Avena fatua* mean emergence, reproductive tillers, and seed production under two *Triticum aestivum* seeding densities and four nitrogen fertilization rates in a three year study (2012-2015).

		Spring wheat 67 kg ha^{-1}				Spring Wheat 101 kg ha^{-1}			
Variable	Population	56	112	168	224	56	112	168	224
		kg ha^{-1}							
Emergence (m^2)$^{\theta}$	HS1	288 (82)a[1]	179 (65)b	140 (44)b	132 (42)b	172 (64)a	191 (70)b	166 (69)b	149 (45)b
	MHR3	285 (74)a*	207 (48)b*	282 (66)b*	257 (88)b*	372 (101)a*	238 (44)b*	198 (73)b*	216 (55)b*
	MHR4	403 (93)a*	200 (49)b*	264 (70)b*	190 (41)b*	293 (80)a*	316 (74)b*	258 (85)b*	195 (43)b*
Reproductive tillers (m^2)$^{\Delta}$	HS1	33 (20)a	6 (4)b	8 (7)b	2 (2)b	17 (10)a	1 (1)b	0 (0)b	0 (0)b
	MHR3	202 (49)a*	192 (64)b*	105 (32)b*	103 (47)b*	212 (64)a*	122 (18)b*	72 (23)b*	82 (36)b*
	MHR4	272 (73)a*	93 (29)b*	149 (44)b*	94 (22)b*	128 (34)a*	107 (27)b*	78 (30)b*	65 (15)b*
Seeds (m^2)$^{+}$	HS1	396 (236)a	65 (65)b	120 (105)b	22 (22)b	198 (115)a	22 (22)b	0 (0)b	0 (0)b
	MHR3	7328 (2065)a*	6040 (2181)b*	3155 (1261)b*	2692 (1047)b*	9244 (3265)a*	4795 (1441)b*	2520 (1023)b*	3215 (1448)b*
	MHR4	7064 (1670)a*	3098 (863)b*	6072 (1919)b*	2953 (888)b*	4304 (1879)a*	3438 (888)b*	3368 (1472)b*	1906 (525)b*

[1]Means within a variable followed by the same letter are not significantly different ($p \leq 0.05$) for the main effect of nitrogen.
*Means within a variable followed by an asterisk are not significantly different ($p \leq 0.05$) for the main effect of population.
$^{\theta}$Non-significant main effect of spring wheat seeding rate ($p \geq 0.1$).
$^{\Delta}$Significant ($p \leq 0.05$) main effect of spring wheat seeding rate.
$^{+}$Marginally significant ($p \leq 0.1$) main effect of spring wheat seeding rate

Table C2. Seedbank elasticity under continuous *Triticum aestivum* cropping system.

Treatment	estab[a]	q	z	sppmax	a	i	osss	owss	pred
HS1[b]									
56-L[c]	0.923	0.004	0.944	**0.960**	0.251	0.096	0.214	***1.000***	0.034
112-L	0.840	0.009	0.876	**0.889**	0.099	0.058	0.481	***1.000***	0.029
168-L	0.519	0.010	0.634	0.644	0.010	0.016	**0.814**	***1.000***	0.016
224-L	0.062	0.000	0.232	0.235	0.006	0.005	**0.964**	***1.000***	0.005
56-H	0.704	0.002	0.779	**0.791**	0.111	0.039	0.674	***1.000***	0.023
112-H	0.412	0.007	0.607	0.615	0.010	0.014	**0.834**	***1.000***	0.015
168-H	0.577	0.000	0.000	0.000	0.000	0.000	***1.000***	***1.000***	0.000
224-H	0.492	0.000	0.000	0.000	0.000	0.000	***1.000***	***1.000***	0.000
MHR3									
56-L	0.029	0.147	0.033	0.179	***1.000***	0.002	0.011	**0.188**	0.003
112-L	0.107	0.075	0.111	0.177	***1.000***	0.002	0.016	**0.191**	0.003
168-L	0.013	0.013	0.014	0.037	***1.000***	0.013	0.002	**0.039**	0.001
224-L	0.023	0.029	0.024	0.054	***1.000***	0.004	0.004	**0.058**	0.001
56-H	0.065	0.115	0.069	0.180	***1.000***	0.004	0.006	**0.185**	0.003
112-H	0.058	0.121	0.062	0.183	***1.000***	0.007	0.014	**0.195**	0.003
168-H	0.003	0.005	0.004	0.022	***1.000***	0.016	0.002	**0.024**	0.000
224-H	0.004	0.002	0.004	**0.010**	***1.000***	0.004	0.001	**0.010**	0.000
MHR4									
56-L	0.048	0.066	0.049	0.113	***1.000***	0.002	0.003	**0.115**	0.002
112-L	0.022	0.005	0.022	0.035	***1.000***	0.011	0.004	**0.038**	0.001
168-L	0.028	0.083	0.031	0.117	***1.000***	0.006	0.008	**0.124**	0.002
224-L	0.002	0.000	0.003	0.015	***1.000***	0.013	0.001	**0.016**	0.000
56-H	0.030	0.046	0.031	0.077	***1.000***	0.003	0.004	**0.081**	0.001
112-H	0.025	0.025	0.026	0.055	***1.000***	0.006	0.003	**0.057**	0.001
168-H	0.027	0.018	0.028	0.053	***1.000***	0.010	0.004	**0.056**	0.001

224-H	0.058	0.001	0.064	0.228	***1.000***	0.188	0.022	**0.247**	0.004

[a]estab = seedling establishment rate, q = shape parameter in asymptotic density dependent seedling survival function, z = shape parameter in asymptotic density dependent seedling survival function, sppmax = maximum seed production per plant, a = shape parameter in asymptotic density dependent seed production function, i = shape parameter in asymptotic density dependent seed production function, osss = over summer seed survival, owss = over winter seed survival, pred = seed predation

[b]HS1 = herbicide susceptible population 1, MHR3 = multiple herbicide resistant population 3, MHR4 = multiple herbicide resistant population 4

[c]56 = 56 kg ha^{-1} N, 112 = 112 kg ha^{-1} N, 168 = 168 kg ha^{-1} N, 224 = 224 kg ha^{-1} N, L = low spring wheat seeding rate (67 kg ha^{-1}), H = high spring wheat seeding rate (101 kg ha^{-1})

Table C3. Seedbank elasticity under *Triticum aestivum*-fallow cropping system.

Treatment	estab[a]	Fall_a	Fall_i	Fallsppmax	kill	q	z	sppmax	i	a	osss	owss	pred
HS1[b]													
56-L[c]	0.197	0.000	0.005	0.126	***1.000***	0.001	0.089	0.170	0.097	0.192	0.042	**0.316**	0.005
112-L	0.046	0.000	0.001	0.034	***1.000***	0.001	0.026	0.029	0.005	0.004	0.015	**0.054**	0.001
168-L	**0.003**	0.000	0.000	0.002	***1.000***	0.000	0.001	0.001	0.000	0.000	0.002	**0.003**	0.000
224-L	**0.002**	0.000	0.000	**0.002**	***1.000***	0.000	0.000	0.000	0.000	0.000	**0.002**	**0.002**	0.000
56-H	0.006	0.000	0.000	0.005	***1.000***	0.000	0.003	0.003	0.000	0.001	0.003	**0.007**	0.000
112-H	0.002	0.000	0.000	0.002	***1.000***	0.000	0.001	0.001	0.000	0.000	0.002	**0.003**	0.000
168-H	0.001	0.000	0.000	**0.002**	***1.000***	0.000	0.000	0.000	0.000	0.000	**0.002**	**0.002**	0.000
224-H	0.001	0.000	0.000	**0.002**	***1.000***	0.000	0.000	0.000	0.000	0.000	**0.002**	**0.002**	0.000
MHR3													
56-L	0.050	0.050	0.004	0.036	0.218	0.156	0.022	0.178	0.002	***1.000***	0.017	**0.226**	0.004
112-L	0.152	0.097	0.019	0.094	**0.482**	0.092	0.082	0.166	0.002	***1.000***	0.025	0.274	0.004
168-L	0.018	0.012	0.003	0.013	**0.068**	0.020	0.010	0.037	0.011	***1.000***	0.005	0.053	0.001
224-L	0.036	0.029	0.003	0.022	**0.115**	0.034	0.018	0.053	0.003	***1.000***	0.006	0.078	0.001
56-H	0.073	0.061	0.005	0.046	**0.236**	0.143	0.036	0.178	0.003	***1.000***	0.011	0.231	0.004
112-H	0.076	0.044	0.014	0.057	**0.292**	0.142	0.038	0.180	0.006	***1.000***	0.025	0.256	0.004
168-H	0.007	0.005	0.001	0.006	**0.032**	0.006	0.003	0.023	0.016	***1.000***	0.003	0.030	0.000
224-H	0.007	0.005	0.001	0.005	**0.028**	0.003	0.004	0.010	0.004	***1.000***	0.001	0.016	0.000
MHR4													
56-L	0.045	0.024	0.004	0.028	0.139	0.089	0.023	0.111	0.002	***1.000***	0.006	**0.143**	0.002
112-L	0.051	0.008	0.007	0.033	**0.171**	0.019	0.027	0.050	0.008	***1.000***	0.009	0.088	0.001
168-L	0.048	0.031	0.003	0.031	**0.178**	0.090	0.022	0.115	0.005	***1.000***	0.012	0.155	0.003
224-L	0.007	0.000	0.000	0.005	**0.052**	0.001	0.003	0.013	0.010	***1.000***	0.003	0.020	0.000
56-H	0.039	0.022	0.004	0.025	**0.134**	0.056	0.019	0.076	0.003	***1.000***	0.008	0.106	0.002
112-H	0.032	0.018	0.003	0.020	**0.105**	0.036	0.016	0.054	0.005	***1.000***	0.005	0.078	0.001
168-H	0.045	0.025	0.003	0.027	**0.147**	0.026	0.023	0.054	0.008	***1.000***	0.006	0.083	0.001

224-H	0.016	0.000	0.000	0.012	**0.123**	0.000	0.006	0.032	0.029	***1.000***	0.008	0.049	0.001

[a]estab = seedling establishment rate, Fall_a = shape parameter in asymptotic density dependent fallow seed production function, Fall_i = shape parameter in asymptotic density dependent fallow seed production function, Fall_sppmax = maximum fallow seed production per plant, kill = fallow glyphosate application, q = shape parameter in asymptotic density dependent seedling survival function, z = shape parameter in asymptotic density dependent seedling survival function, sppmax = maximum seed production per plant in crop, a = shape parameter in asymptotic density dependent in crop seed production function, i = shape parameter in asymptotic density dependent in crop seed production function, osss = over summer seed survival, owss = over winter seed survival, pred = seed predation

[b]HS1 = herbicide susceptible population 1, MHR3 = multiple herbicide resistant population 3, MHR4 = multiple herbicide resistant population 4

[c]56 = 56 kg ha^{-1} N, 112 = 112 kg ha^{-1} N, 168 = 168 kg ha^{-1} N, 224 = 224 kg ha^{-1} N, L = low spring wheat seeding rate (67 kg ha^{-1}), H = high spring wheat seeding rate (101 kg ha^{-1})