THE WHEAT-HESSIAN FLY INTERACTION: CO-EVOLUTION AND ECOLOGY IN AN ECONOMICALLY IMPORTANT PLANT-INSECT SYSTEM

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The study of wheat's *H*-gene mediated resistance to Hessian flies examined the cost of the constitutively expressed *H*-gene which functions in the plant's surveillance system and the cost of the downstream induced response. For the constitutively-expressed *H*-gene, some measures indicated costs, but a greater number indicated benefits. For the induced resistance, plants showed benefits of being attacked. It is expected that fitness costs play a role in determining the rate at which plant defense evolves, and it is important for agriculture as plant breeders decide whether to pyramid resistance genes into a single cultivar to prevent the evolution of pest virulence.

Before plant breeders undertake the effort to transfer resistance into crop cultivars, it must be asked: is the pest a sufficient threat to warrant the effort? To answer this question, the recently discovered female-produced sex pheromone of the Hessian fly was used to explore the pest potential for populations in the Upper Great Plains. Methods for pheromone trapping were established and trapping data were used to explore geographic distribution, phenology, and insect density. It was concluded that the Hessian fly is a risk to wheat in the Upper Great Plains and it was predicted that global warming and the increased cultivation of winter wheat will add to this risk.

If Hessian flies are a sufficient threat to the region's wheat crop, which of the 33 known resistance gene(s) should be used? To answer this question, traditional biotyping and an assay of all available *H*-genes were used to provide information on the virulence of a population of Hessian flies from the Upper Great Plains. The results were surprising as far more virulence was encountered than was expected. Using traditional virulence testing thirteen of the 16 possible

Hessian fly biotypes were present in the North Dakota population, and in the assay of all available *H*-genes few gave 100% protection. In addition to information on Hessian fly virulence, the studies explored aspects of the wheat-Hessian fly interaction providing details on the fate of the Hessian fly and the wheat plant that have not been examined by other research on Hessian fly virulence.

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CHAPTER 1. THE WHEAT-HESSIAN FLY INTERACTION

The co-evolutionary interaction between plant and parasite is sometimes portrayed as an arms race (Whittaker and Feeny 1971, Rausher 1992). In this relationship, the parasite has virulence traits that allow the plant to be attacked and consumed (Karban and Agrawal 2002). This imposes selection for evolution of traits that confer plant resistance, i.e. improved plant fitness in the face of parasite attack (Kareiva 1999). Plant resistance then imposes selection for the evolution of additional virulence traits (Rausher 2001). Simply put, changes in virulence in the parasite are followed by changes in resistance in the host plant, thus allowing both plant and parasite to survive over considerable periods of time (Agrios 1997). The rates at which the plant resistance traits and insect virulence traits evolve depends on many factors, including the benefits and costs of the traits.

For some plant-parasite systems the co-evolutionary interaction between parasite and plant can be explained by the gene-for-gene hypothesis. The gene-for-gene hypothesis was first proposed to explain the relationship between flax and flax rust (Flor 1956). In the gene-for-gene concept, plants have *resistance* (*R*) genes that confer resistance to parasites. For every *R* gene there is a matching *avirulence* (*avr*) gene in the parasite that confers avirulence or the inability to attack and colonize the plant. At the biochemical level, when a parasite attacks a host plant, parasite effectors help suppress plant defenses and promote parasite virulence (Bent and Mackey 2007). However, in the case of plants with *R* gene-mediated resistance, a subset of the parasite's effectors are detected and interact, directly or indirectly, with the plant's *R* protein receptors that then induce defenses that prevent virulence (Bent and Mackey 2007). Because most parasite effectors were identified based on their avirulence activity, effectors that elicit a plant defense response are called *avr* genes (Bent and Mackey 2007). Plant parasites can evolve and avoid

recognition by *R* protein products through mutations in *avr* genes that encode effector proteins (Bent and Mackey 2007). Because of this, the parasite's *avr* product no longer functions as an elicitor of resistance. The result is that the *R* gene-mediated resistance fails to detect the parasite and the parasite can successfully utilize the host plant. The gene-for-gene hypothesis was first proposed to explain the interaction between a plant and a fungal pathogen. In the years since Flor's ground-breaking study, evidence for gene-for-gene relationships has also been found for nematodes, mites and insects (Agrios 1997). The genetic interaction between wheat (*Triticum aestivum*) and the Hessian fly (*Mayetiola destructor*) appears to conform to the gene-for-gene relationship (Hatchett and Gallun 1970).

In recent years many details of the interaction between the Hessian fly and wheat have been revealed (Barnes 1956, Ratcliffe and Hatchett 1997, Harris et al. 2003). The wheat-Hessian fly interaction begins with the mated female fly selecting an oviposition site. Females use a combination of olfactory, chemical and tactile cues when selecting a place to lay their eggs (Harris and Rose 1990, Foster and Harris 1992). Upon hatching, the neonate larvae migrates down the leaf blade to the base of the plant attacking epidermal cells on the abaxial surface of the adjacent younger leaf (Harris et al. 2006). The first instar larvae use paired mandibles to pierce into the cell wall of individual plant cells (Harris et al. 2006, 2010). It has been hypothesized that salivary secretions travel down the grooved internal lateral surface of the mandibles and pass into, or just below, the cell wall (Harris et al. 2006). Within the saliva are proteins that are presumed to act as insect virulence effectors (Chen et al. 2006). In the case of a compatible interaction, where the Hessian fly larvae are virulent and the plant is susceptible, it is thought that these effector proteins initiate biochemical changes in the plant (Zhu et al. 2008, Saltzmann et al. 2009, Liu et al. 2013). Effector proteins up-regulate genes in the wheat plant that suppress

plant defenses and activate wheat susceptibility pathways (Liu et al. 2013). This in turn leads to changes in the plant such as the formation of nutritive tissue (Harris et al. 2006). Nutritive tissue is a group of plant cells that have been reprogramed to act as a nutrient sink, which benefits the growth of the developing larvae (Harris et al. 2006). The nutritive tissue competes for resources that would normally be used for plant growth and reproduction (Anderson and Harris 2006, 2008). Ultimately, attack by Hessian fly larvae leads to wheat seedlings that are stunted and dark green in color. The primary shoot often dies. Plants attacked at later growth stages tend to lodge, and have fewer smaller seed heads (Berzonsky et al. 2003).

In the case of an incompatible interaction, where the Hessian fly larvae are avirulent and the plant is resistant, it is believed that the Hessian fly *H*-gene product in the wheat plant detects the Hessian fly *avr* effector protein in the larvae's saliva. This suggests that effectors that usually are virulence factors are now being recognized by the host and serve an avirulence function (Bent and Mackey 2007). Recognition of Hessian fly elicitors by the plant's *H*-gene mediated surveillance system leads to an induced defense response by the wheat plant (Harris et al. 2010). In response to larval attack, many genes are up-regulated (Liu et al. 2007). Up-regulated genes are assumed to function in defense related processes, which may include the production of insecticidal toxins (Giovanini et al. 2006). At the cellular level signs of an induced resistance response can be seen (Harris et al. 2010). Localized cell death, cell wall fortification and numerous subcellular changes that are similar to plant responses to fungal attack have been observed. Signs of susceptibility (i.e. nutritive tissue) were not seen in plants that have an induced response to larval attack. Resistant plants that have been attacked show little outward evidence of Hessian fly attack. Only the leaves that were actively growing at the time of larval

attack exhibit small growth deficits (Anderson and Harris 2006). The serious growth effects exhibited by susceptible plants do not occur in resistant genotypes.

To further our understanding of the wheat-Hessian fly interaction, it is helpful to know their history. The recorded history of the Hessian fly in the United States dates back about 200 years (Ratcliffe and Hatchett 1997, Pauly 2002). The Hessian fly was first discovered in wheat fields in New York, New Jersey and Connecticut in the late 1770's during the Revolutionary War. It was shortly after its introduction into North America that it received its common name. The inspiration for the name was based on the British use of mercenary soldiers from the German state of Hesse. For early Americans the name Hessian was the "most opprobrious term our language affords" (Pauly 2002). Since its introduction the Hessian fly has spread to all wheat producing areas of the United States.

The domestication of wheat can be traced back about 10,000 years. The earliest types of wheat were likely diploid einkorn (*Triticum monococcum*) with the AA genome and tetraploid emmer (*Triticum turgidum* ssp. *dicoccum*) with the AABB genomes (Shewry 2009). These early forms of wheat were probably landraces, which are plants selected by farmers from wild populations. About 1,000 years later hexaploid bread wheat (*Triticum aestivum*) with the AABBDD genomes made its appearance (Shewry 2009). Bread wheat arose from a hybridization event of tetraploid wheat and *Aegilops tauschii,* which has the DD genome. This hybridization probably happened spontaneously a number of times with farmers selecting and cultivating the hexaploid plants due to their superior qualities (Shewry 2009).

Despite their relatively short recorded histories, it seems that the co-evolutionary relationship between wheat and Hessian fly is ancient. Two lines of evidence support this idea. The first is that they share the same center of origin. It is generally believed that the Hessian fly

and wheat originated in Southwest Asia where they have co-existed for thousands of years (Barnes 1956, Ratcliffe and Hatchett 1997). When wheat production expanded around the world the Hessian fly followed. The second line of evidence is in their respective genomes. The selection pressure by Hessian flies on the grasses in the tribe Triticeae is evident by the large number of Hessian fly resistance genes that have been discovered in wheat and its wild and domestic relatives (Ratcliffe and Hatchett 1997, Harris et al. 2003). Currently 33 *H*-genes have been identified that confer resistance to Hessian flies (Stuart et al. 2012). The source materials for these *H*-genes are bread wheat, durum wheat (*Triticum turgidum* ssp. *durum*), *Aegilops tauschii* and rye (*Secale cereale*) (Ratcliffe and Hatchett 1997). This abundance of resistance genes has in turn placed selection pressure on the Hessian fly. This is most apparent at their shared center of origin in the Fertile Crescent. The virulence diversity in Syrian Hessian fly populations closely matches the resistance diversity in wheat (El Bouhssini et al. 2009). The result is only two of the known *H*-genes (*H25* and *H26*) provide effective resistance to the highly virulent Syrian Hessian fly population.

While this ancient relationship between wheat and Hessian fly may seem far removed from the farm field, the co-evolutionary arms race between these two species has impacts on agriculture (Berzonsky et al. 2003). Plant breeders introduce a new resistant wheat variety. The efficacy of the new variety leads to it being widely grown. The selection pressure placed on the fly population drives the evolution of virulence to this resistance trait (Foster et al. 1991, Gould 1986). The result is a resistant variety that is no longer effective and the resistance is said to have "broken down". Thus far the model for deploying *H*-genes has been as single gene introductions. This deployment strategy has been considered successful and has provided decades of reliable and economical Hessian fly management (Ratcliffe and Hatchett 1997, Cambron et al. 2010).

However, the reliability of using single gene introductions is the greatest concern. The span of time that a particular *H*-gene remains effective can be quite short. The deployment of the *H3*, *H5* and *H6* genes was followed by Hessian fly adaptation 15, 9 and 22 years later respectively (Foster et al. 1991). However, when only years when a specific gene was greater than 50% of the wheat acreage were counted (Gould 1998), times until fly adaptation are 8, 7, and 3 years respectively. It should be noted that we may be able to recycle defeated *H*-genes. Virulence is expected to have a cost (Zhang et al. 2011) and therefore should disappear from the population once selection pressure is removed. This occurred for the defeated *H6* gene, which was successfully redeployed after a 10 year absence from agricultural fields (Foster et al. 1991).

Moving into the future it will be necessary to deploy our most effective *H*-genes in the most thoughtful manner possible. Monitoring Hessian fly populations for virulence is critical for selecting the most effective *H*-gene for a region (Stuart et al. 2012). As well as monitoring the evolution of virulence once new resistant cultivars are deployed. Stacking or pyramiding *H*-genes is a deployment strategy often recommended (Harris et al. 2003, Cambron et al. 2010, Stuart et al. 2012). In theory, deployment of two or more *H*-genes in a single wheat cultivar should be more durable than a single gene deployment (Gould 1986). Another alternative deployment strategy would be to deploy *H*-genes that allow some avirulent larvae to survive on resistant plants (El Bouhssini 2009). This should apply less selection pressure for virulence within the Hessian fly population. Finally, deploying *H*-genes with an interspersed refuge of susceptible wheat should lessen the selection pressure for virulence by providing overwhelming numbers of avirulent flies in comparison to virulent flies. The interspersed refuge strategy is currently being used in Canada with the *Sm1* resistance gene for management of the orange wheat blossom midge, *Sitodiplosis mosellana* (Smith et al. 2004). For the refuge strategy to work

insect virulence has to be an independent, simply inherited, recessive trait. In this way, the small numbers of virulent individuals that develop will likely be mated by avirulent individuals produced in the refuge and any resulting offspring will be phenotypically avirulent because avirulence is the dominant trait.

In a review by Harris et al. (2003), the case was made for using gall midges (including Hessian fly) as useful systems for studying plant defense. The dramatically different phenotypes associated with plant resistance and susceptibility, the easily quantifiable interaction between gall midge larvae and their host plants, relatively simple genetics, their economic importance, and the opportunity to integrate plant and insect genetics and the ecology of co-evolution were some of the strengths of gall midge-plant interaction noted (Harris et al. 2003). More recently, Stuart et al. (2012) renewed the case for using the wheat-Hessian fly system as a useful model for studying plant-insect interactions. They argued that unlike many other plant-parasite interactions the Hessian fly-wheat system was increasingly amenable to genetic and genomic analysis (Stuart et al. 2012). Additionally, the Hessian fly's virulence effectors and wheat's *H*-gene mediated resistance are remarkably similar to what occurs in pathogen-plant interactions (Stuart et al. 2012). Due to these similarities, the Hessian fly-wheat system has relevance for both plant pathology as well as entomology.

Because the wheat-Hessian fly interaction is such a useful system for studying plant-insect interactions, I conducted a series of studies examining co-evolutionary and economically important aspects of plant defense and insect virulence. In the second chapter of my dissertation I explored fitness costs for wheat's *H*-gene-mediated resistance to Hessian flies. The study of fitness costs is important to both agricultural scientists as well as evolutionary biologists. For agriculture, fitness costs mean a loss of yield and quality. For ecology and evolution, costs have

implications for how fast plant resistance traits evolve. My study of fitness costs examined the cost of the constitutively expressed *H*-gene, which functions in the plant's surveillance system, and the cost of the downstream induced responses. My results are of interest to plant breeders who wish to stack *H*-genes to improve resistance durability, and it is of interest to evolutionary biologists who expect fitness costs to play an important role in the evolution of resistance traits.

Before wheat breeders embark on the effort to introduce individual or stacked resistance traits into regionally adapted germplasm, there is another question, are Hessian flies a sufficient threat in this region to warrant the effort? In the third chapter of my dissertation that question is addressed. I first established methods for conducting pheromone trapping using the recently discovered female-produced sex pheromone (Anderson et al. 2012). These methods were then used to study the geographic distribution of Hessian fly in North Dakota, fly phenology during the growing season, and insect density.

If it is determined that Hessian flies are a sufficient threat to the region's wheat crop, the introduction of a resistance trait can be justified. But what *H*-gene is right for our region? In the final chapter of the dissertation I studied this question using a North Dakota Hessian fly population. Assessing the virulence status of the regions Hessian fly population assures that the most effective *H*-genes are deployed in regionally adapted wheat cultivars. To my knowledge no population of Hessian flies from the Upper Great Plains has ever been evaluated for virulence. I initially used the traditional Hessian fly biotyping protocol to determine the biotype composition of the North Dakota population (Gallun 1977). In the case of the Hessian fly there are 16 recognized biotypes that can only be distinguished by their ability to survive on four different resistance genes. I then developed the study further by evaluating the efficacy of all available Hessian fly *H*-genes when tested with a North Dakota Hessian fly population. By adding

measures of insect and plant survival to the virulence assay protocol I was also able to explore the consequences of Hessian fly adaptation for the insect and for the plant.

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CHAPTER 2. NO FITNESS COST FOR WHEAT'S *H*-GENE MEDIATED RESISTANCE TO HESSIAN FLY (DIPTERA: CECIDOMYIIDAE)[1]

Abstract

Resistance (*R*) genes have a proven record for protecting plants against biotic stress. A problem is parasite adaptation via *Avirulence* (*Avr*) mutations, which allows the parasite to colonize the *R* gene plant. Scientists hope to make *R* genes more durable by stacking them in a single cultivar. However, stacking assumes that *R* gene-mediated resistance has no fitness cost for the plant. I tested this assumption for wheat's resistance to Hessian flies. My study included ten plant fitness measures and four wheat *genotypes*, one susceptible, and three expressing either the *H6*, *H9* or *H13* resistance gene. Because *R* gene-mediated resistance has two components, I measured two types of costs: the cost of the constitutively-expressed *H*-gene, which functions in plant surveillance, and the cost of the downstream induced responses, which were triggered by Hessian fly larvae rather than a chemical elicitor. For the constitutively-expressed *H*-gene, some measures indicated costs, but a greater number of measures indicated benefits of simply expressing the *H*-gene. For the induced resistance, instead of costs, resistant plants showed benefits of being attacked. Resistant plants were more likely to survive attack than susceptible plants, and surviving resistant plants produced higher yield and quality. I discuss why resistance to the Hessian fly has little or no cost and propose that tolerance is important, with compensatory growth occurring after *H*-gene-mediated resistance kills the larva. I end with a caution: Given that plants were given good growing conditions, fitness costs may be found under conditions of greater biotic or abiotic stress.

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Introduction

Policy-makers are becoming increasingly concerned about food security. Over the next 10 to 20 years, food production must increase anywhere from 50 to 100% (Baulcombe 2010). To achieve this goal, scientific innovation will be critical (Godfray et al. 2010). One promising area of innovation is plant breeding, which is being transformed by the revolution in genetics and molecular biology. Scientists now are acquiring the knowledge and tools to create superior crops, both through conventional-breeding and genetic transformation.

Resistance (*R*) genes have a proven record for helping plants resist biotic stress. For decades they have been used to control many of agriculture's most dreaded plant diseases, nematodes, insects and mites (Agrios 1997, Smith 2005). Single *R* genes can be moved into a susceptible cultivar through traditional breeding techniques, but also will be used to genetically engineer plants. Typically, the R product plays an important role in a plant surveillance system that detects parasite attack and therefore is considered to be part of the plant immune system (Dodds et al. 2006, Jones and Dangl 2006, Ellis et al. 2009). Once attack is detected, an induced response is triggered that directly harms or kills the parasite (Nimchuk et al. 2003). The *R* gene component of *R* gene-mediated plant resistance has received far more attention than the induced component (Brown 2002). It is easier to study and by itself can transform a susceptible plant into a resistant plant.

The rapid response associated with *R* genes is important when the parasite's first task is to suppress the plant's basal immunity (Bent and Mackey 2007). The longer the plant waits to respond, the harder it is to mount an effective response (Nimchuk et al. 2003). Examples of suppression of plant immunity come from a range of plant parasites including fungi, oomycetes, bacteria, nematodes, and insects (Musser et al. 2002, Bent and Mackey 2007, Zhu et al. 2008,

Will et al. 2007, Stergiopoulos and De Wit 2009, Bird et al. 2009, Hogenhout et al. 2009). The parasite's secreted effectors play a critical role in suppressing basal immunity (Hogenhout et al. 2009), but also are important for manipulating resource allocation within the plant, including redirecting cell development to create a more nutritious food (Williams 1997, Agrios 1997, Sandstrom et al. 2000, Williamson and Gleason 2003).

Unfortunately, *R* genes can be defeated by parasite adaptation. The simplest example of this is when the *R* gene-mediated surveillance system depends on the detection of a specific effector encoded by a matching parasite *Avirulence* (*Avr*) gene (Bent and Mackey 2007, Ellis et al. 2007). The surveillance fails if the parasite fails to produce the effector (Ellis et al. 2009), a cause for this being *Avr* null or loss of function mutations (Stergiopoulos and De Wit 2009). *R/Avr* interactions were discovered 50 years ago in flax/ flax rust interactions (Flor 1942, 1955, 1956). Today agricultural scientists study *R/Avr* interactions because they hope to make *R* gene resistance more durable (Brown 2002, Bent and Mackey 2007). Evolutionary biologists are interested for a different reason: *R/Avr* interactions are rare examples of straightforward plant-parasite co-evolution (Michelmore and Meyers 1998, Burdon and Thrall 1999, Bergelson et al. 2001, Rausher 2001, Thompson 2005, Allen et al. 2004).

R genes have obvious benefits, but less is known about their costs, that is, whether the resistance they confer is associated with lower yields or quality (Brown 2002). For agriculture, a clear understanding of costs is particularly important today because plant breeders plan to "stack" or "pyramid" multiple *R* genes in a single elite cultivar. Stacking is only now being made possible because it relies on the discovery of molecular markers closely associated with individual *R* genes (Edwards and Batley 2010). The reason for stacking is to create a plant that is beyond the evolutionary capacity of the parasite to adapt (Bent and Mackey 2007), essentially

turning a host, for which parasite adaptation is possible, into a non-host, for which adaptation is impossible (Heath 2000). Stacking is commonly invoked as a solution for creating durable plant resistance to emerging diseases, e.g. the Ug99 strain of wheat stem rust (personal communication, Steven Xu, USDA-ARS), and to the insect pests that are controlled by the insecticidal toxins produced by *Bacillus thuringiensis* (Bt), with Bt toxins now stacked in transgenic crop plants (Bravo and Soberón 2008). Evolutionary biologists are interested in fitness costs because the cost associated with *R* gene and the cost associated with the adapted parasite's "jettisoned" *Avr* gene are thought to play important roles in maintaining *R* and *Avr* polymorphisms in co-evolutionary interactions (Bergelson and Purrington 1996, Karban and Baldwin 1997, Thompson 2005).

Costs of resistance can be difficult to measure. This is especially true when the insect damages the plant before (or even during and after) induced resistance. Now the cost of the induced resistance is hard to separate from the cost of the damage that triggered the induced resistance. To get around this, entomologists have mimicked insect attack using chemical inducers of plant resistance, such as methyl jasmonate (Thaler 1999). However, using chemical elicitors raises other questions. Do insect attack and the chemical elicit the same plant responses? Does the chemical have effects on the plant other than eliciting resistance? If the answer is yes, do these other effects have costs? Costs of constitutive resistance are easier to measure because they occur in the absence of attack (Purrington 2000). If a single gene confers the constitutive resistance, near-isogenic plant lines (NILs) are useful tools (Purrington 2000): the susceptible and resistant NILs can be almost identical genetically if they are created via a sufficient number of backcrosses (Xu et al. 2011).

Conversations about costs of *R* gene-mediated resistance tend to ignore the induced component, that is, the part of the resistance that actually harms the organism that attacks the plant (Brown 2002). Given that ecologists view induced plant resistance as more costly than constitutive resistance (Smedegaard-Peterson and Stølen 1981, Baldwin 1998, Heil and Baldwin 2002, Stauss et al. 2002) this omission is surprising. On the other hand, if induced resistance is costly, it has an important advantage over constitutive resistance. Because it is only deployed when the plant is attacked, there are benefits to offset the costs.

I developed a method to simultaneously measure the fitness costs of the constitutive and induced components of *H*-gene-mediated resistance to the Hessian fly, *Mayetiola destructor* (Say), one of a handful of plant-insect systems with well-documented *R/Avr* interactions (Harris et al. 2003, Stuart et al. 2008). The preferred host of the Hessian fly is wheat, *Triticum aestivum* L., a crop that provides the largest proportion of the world's food calories, as well as being the world's most traded agricultural commodity (Fisher 2009, Gustafson et al. 2009). Over 32 *H*-genes have been discovered in wheat and its wild and domesticated relatives and ancestors (Berzonsky et al. 2003, Harris et al. 2003, Porter et al. 2009). Genetic markers are now being developed for individual *H*-genes to allow stacking of the most effective *H*-genes (Yu et al. 2009). Stacking has been promoted to improve the durability of *H*-genes, which are threatened by the proven ability of the Hessian fly to evolve virulence (Gould 1986, 1998).

My study addressed several questions about stacking *H*-genes. Is there a cost associated with the expression of a single *H*-gene? Is there a cost associated with the induced resistance triggered by the *H/Avr* interaction? Do *H*-genes all show the same pattern of costs? My study included ten fitness measures and four wheat genotypes, one susceptible control, and three resistant, the latter each expressing a different single *resistance* gene (*H6*, *H9* and *H13*). *H6* and

H9 come from durum wheat, *Triticum turgidum*, while *H13* comes from wheat's wild ancestor *Aegilops tauschii* (Table 1). Single *H*-genes were available as near-isogenic lines (NILs), with each resistant line >99% genetically identical to the susceptible control line (Xu et al. 2011). Because *H*-gene-mediated resistance is so effective at killing larvae (Harris et al. 2010), I was able to use larvae rather than a chemical elicitor to trigger induced resistance.

Methods and Materials

Plants. Four near-isogenic winter wheat *Triticum aestivum* L. lines were used (Table 1), one susceptible containing no known *H*-genes ('Newton') and three resistant ('Flynn', 'Iris' and 'Molly') containing a single known *H*-gene from one of two donors, both being ancestors of *T. aestivum* (Table 1). The four lines were developed by Patterson et al. (1994) by backcrossing donor plants containing the *H*-gene to the susceptible parent 'Newton', with each resistant genotype being the result of six backcrosses into 'Newton'. Each resistant near-isogenic line has a similar genetic background with susceptible 'Newton': tests using target region amplification polymorphism (TRAP) markers showed differences of less than 1% (Xu et al. 2011). The genetic residues of the *H*-gene donor parent that were found in individual resistant NILs did not appear to be due to linkage drag.

Plant-Insect Interactions. Plants were started in February in a greenhouse maintained at 20° ± 2° C, with an ambient relative humidity (30-60% RH) and a 16:8 light-dark photoperiod. Hours of natural light were extended and enhanced by high-pressure sodium lamps. Individual seeds were planted in Ray Leach Cone-tainers™ (4 cm diam x 21 cm deep, Stuewe & Sons, Inc., Corvallis, WA) held in racks (RL98). Plants grew in potting soil (SB100 Professional Growing Mix, Sungro Horticulture, Bellevue, WA) and were watered daily and fertilized weekly (Jack's Professional 20:20:20 N:P:K Fertilizer®, J.R. Peters Inc., Allentown, PA).

Table 1. Susceptible and resistant wheat *Triticum aestivum* L. (genome AABBDD) genotypes used to study costs and benefits of gene-for-gene resistance to Hessian flies.

Wheat Genotype	*H* Gene	Relationship to Susceptible	Species origin of *H*-gene and its genome	Chromosome location of gene
'Newton' susceptible	none	-----	-----	-----
'Flynn' resistant	*H6*	near-isogenic with 'Newton'	*Triticum turgidum* ssp. *durum* (AABB)	5A Gallun & Patterson (1977)
'Iris' resistant	*H9*	near-isogenic with 'Newton'	*Triticum turgidum* ssp. *durum* (AABB)	1A (short arm) Kong et al. (2005) Liu et al. (2005)
'Molly' resistant	*H13*	Near-isogenic with 'Newton'	*Aegilops tauschii* Cosson (DD)	6D (short arm) Liu et al. (2005)

Insects attacking test plants were from a Hessian fly colony reared in the greenhouse on susceptible hard red spring wheat (cultivar 'Reeder'). The colony originated from ca. 5000 puparia obtained in 2000 from the USDA-ARS Laboratory at Purdue University. Insects in this colony are avirulent on plants expressing any of the 32 known *H*-genes, including *H6*, *H9* and *H13* (Anderson and Harris 2006, 2008, Harris et al. 2010). Larvae die 3-5 days after initial attack and do not grow before dying (Harris et al. 2010).

In my experiment, the timing of plant attack was designed to mimic attack of winter wheat during the autumn when plants are in the seedling stage. Each of the four genotypes was subjected to two insect treatments: plants attacked by larvae and plants not attacked by larvae. Plants were treated similarly except that attacked plants were exposed to gravid Hessian fly adult females (approximately 1 female for each plant) for twenty-four hours. If a plant assigned to the attacked treatment had no eggs, it was discarded. Three days after exposure to adult females,

plants were moved to a high humidity (70-80% RH) plant growth chamber. For plants with eggs, this facilitated egg hatch and larval migration down the leaf lamina to attack sites at the base of the plant. After egg hatch, plants were examined again, scored for larvae that died during migration, and discarded if no larvae survived to attack the plant. All plants remained in the greenhouse for 3 weeks, the time it took for virulent larvae on the susceptible genotype to complete feeding and form a puparium (the overwintering stage). Following this, plants were placed in a cold room for vernalization (2.5° ± 2° C). Artificial lighting was provided by cool white fluorescent lamps with a 12:12 light-dark photoperiod. Forty-four days later, plants were removed from the vernalization chamber. The vernalized plants were segregated by genotype and treatment and held in outdoor cages until adult Hessian flies stopped emerging from attacked susceptible plants. Adult males, which emerge in the early evening, and adult females, which emerge in the early morning (Bergh et al. 1990), were removed from plants before mating could occur. Plants, now at the tillering stage, were taken to the field where they were planted (see section on experimental design), fertilized (Scotts Osmocote Plus 15:9:12 N:P:K Controlled Release Fertilizer, Scotts-Sierra Horticultural Products Company, Marysville, OH), and protected from insects (imidacloprid systemic insecticide, Marathon®, Olympic Horticultural Products, Mainland, PA) and diseases (propiconazole fungicide Tilt®, Syngenta Crop Protection, Inc., Greensboro, NC). Transplanting started on May 16 at Prosper, continued on May 17 at Casselton, and finished at Glyndon on May 18. Plots were covered with fine plastic netting supported by a metal frame, which protected plants from deer, rabbits and birds. Plants were watered for the first two weeks after being transplanted. At maturity (ten weeks after being transplanted to the field) plants were harvested over a period of seven days July 25-August 1).

Experimental Design. The treatment structure was a two-way factorial, factor one being the four wheat genotypes (Susceptible, *H6*, *H9*, and *H13*), and factor two being the insect treatment (attacked or non-attacked). At each of the three field sites, I employed a split-plot design structure with whole-plots having a randomized complete block design (Milliken and Johnson, 1992). Each block consisted of one row, with 15 rows in total spaced one-foot apart. Each row contained four pairs (whole-plots) of growing locations (sub-plots). Genotype was the whole-plot treatment and insect attack was the sub-plot treatment. The three field sites were Glyndon, MN (46º 55'10" N, 96º 39'18" W), Casselton, ND (46º 52'53" N, 97º 14'48" W), and Prosper, ND (47º 0'2" N, 97º 7'12" W). Each site was visited several times a week to monitor plant growth.

Time of Reproduction, Plant Height, Heads, and Seeds. A seed head was scored as 'produced' when 50% of its length had emerged from the boot (Zadok's growth stages 54-55, Zadok et al. 1974). Treatments were compared for timing of head production using data from the middle of the nine day heading period, this measure being the percentage of the total heads produced by the plant that had emerged by day five. Plant height at maturity (just prior to harvest) was measured from ground level to the tip of the tallest seed head, excluding awns. Each plant was harvested individually by hand, with the number of heads and seeds per plant recorded.

Kernel Weight, Total Seed Weight, and Protein. One thousand kernel weight (1000 KWT) was measured by calculating the mass of a grain sample containing 1000 intact wheat kernels. This indicator of grain size and flour milling yield is commonly used by cereal scientists, millers and plant scientists (American Association of Cereal Chemists 1983 and Personal communication, Kelly McMonagle food technician, NDSU Plant Science Department). Total seed weight was measured as the mass of all the seed produced by and individual plant.

Grain protein of the whole-meal flour was determined by Near-Infrared Reflectance (NIR) measured by a Technicon Infralyzer, model 300 (Technician Industrial Systems, Tarrytown, NY).

Germination and Seedling Growth. Criteria for seed testing were those of the Association of Official Seed Analysts (Copeland 1981). Each seed sample (20 undamaged seeds/plant) was randomly selected, rolled in damp K-22 Kimpak® germination paper (Seedburo Equipment Co., Chicago, IL) and placed in a vertical position in a germination chamber (20° ± 2° C; no light). Seven days later, the samples were removed and each seed was scored for production of a normal seedling (i.e. germination) and the length of the coleoptile.

Statistical Analysis. Treatments were first compared for survival. For this analysis, one non-attacked *H13* plant and one attacked *H13* plant from Glyndon, Minnesota were excluded because they died prior to heading due a disease. One "attacked" *H9* plant from Casselton, North Dakota was also excluded because subsequent examination of hatched eggs indicated that no larvae survived egg hatch and migration down the leaf blade to attack at the base of the plant.

For all other analyses, the eight treatments were compared using only the plants that survived to maturity. This meant that the susceptible plants that failed to survive attack were excluded from analyses. Because counts were positive integers and their means often related to variances, discrete distributions were used to model count data (Chapter 4 in Agresti, 2002). Head counts were modeled by the Poisson distribution. Seed counts had problems with over-dispersion (prevent use of the Poisson distribution). Variances were stabilized by square-root transformation, with transformed data then modeled using the normal distribution (page 287 in Snecdecor and Cochran, 1991). Timing of reproduction and germination were modeled by the binomial distribution. Seedling growth, 1000 kernel weight, total seed weight, protein content,

and plant height (all viewed as continuous responses) were modeled using the normal distribution. The Generalized Linear Mixed Model (GLMM) to account for the different distribution models (either the Poisson, normal, or binomial distributions) and the experimental design (two-way factorial split-plot design) was applied. The link functions associated with the Poisson, normal and binomial distributions were chosen to be the log function $g(\mu) = \log(\mu)$, the identity function $g(\mu) = \mu$, and the logit function $g(\mu) = \log(\frac{\mu}{1-\mu})$, respectively. In other words, GLMM assumed an additive (linear) treatment effect on the log mean of head counts, the mean of transformed seed counts, the logit of germination percentage, and the mean of average length of seedlings. The term "mixed" in GLMM corresponds to the fact that the location effect and the block-nested-within-location effect were treated as random. This approach allowed me to generalize my inference, rather than restrict my conclusions within the three locations. Because there are only two degrees of freedom to evaluate the location effect, the GLMM is unstable and some of the estimation algorithms failed to converge. This problem was solved by combining the location and block-nested-within-location random effects into a location × block effect with forty-four degrees of freedom. For head counts and germination counts, treatment effects are estimated by the Generalized Estimating Equation (GEE) method. For the transformed seed counts and average length of seedlings, treatment effects are estimated by the Residual Maximum Likelihood (REML) method. Statistics analyses were run via PROC GLIMMIX (SAS, 2006) and PROC MIXED (SAS, 2004).

Results

Plants of the four plant genotypes assigned to the 'attacked' treatment had similar numbers of larvae attacking the plant ($F_{3, 174} = 0.36$, $P = 0.7837$), an average of 51.38 ± 1.52 (SEM) larvae per plant.

Resistant plants were more likely to survive attack than susceptible plants (Table 2). This pattern was consistent across the three field sites. For the nine remaining measures of plant fitness, susceptible plants that did not survive attack were excluded from analyses.

Table 2. Survival to reproduction.

		% Survival by Location				
Genotype	Larva*	Glyndon MN	Casselton ND	Prosper ND	Total % Surviving	Two-tailed Fisher exact test
Susceptible	(-)	100%	100%	100%	100%	P < 0.0001
	(+)	47%	73%	47%	56%	
H6	(-)	100%	100%	100%	100%	P = 1.000
	(+)	100%	100%	100%	100%	
H9	(-)	100%	100%	100%	100%	P = 1.000
	(+)	100%	100%	100%	100%	
H13	(-)	100%	100%	100%	100%	P = 1.000
	(+)	100%	100%	100%	100%	

* (-) indicated plants not attacked by Hessian fly larvae, (+) indicated plants attacked by Hessian fly larvae

Time of Reproduction (Fig. 1, Table 3). Measured as the percentage of total heads produced by the plant that had emerged midway through the heading period, was influenced by plant genotype ($F_{3, 131} = 64.54$, $P < 0.0001$) but not larval attack ($F_{1, 154} = 0.77$, $P = 0.383$). There was no genotype x attack interaction ($F_{3, 154} = 1.63$, $P = 0.184$). For non-attacked plants, *H13* produced heads at the same rate as susceptible plants ($P = 0.390$), while *H6* and *H9* produced heads faster than the susceptible genotype ($P < 0.0001$). For the within-genotype comparison of attacked versus non-attacked plants, larval attack of both susceptible and resistant genotypes did not influence rate of head production ($P = 0.215$, 0.830, 0.274 and 0.112 for susceptible, *H6*, *H9* and *H13*, respectively).

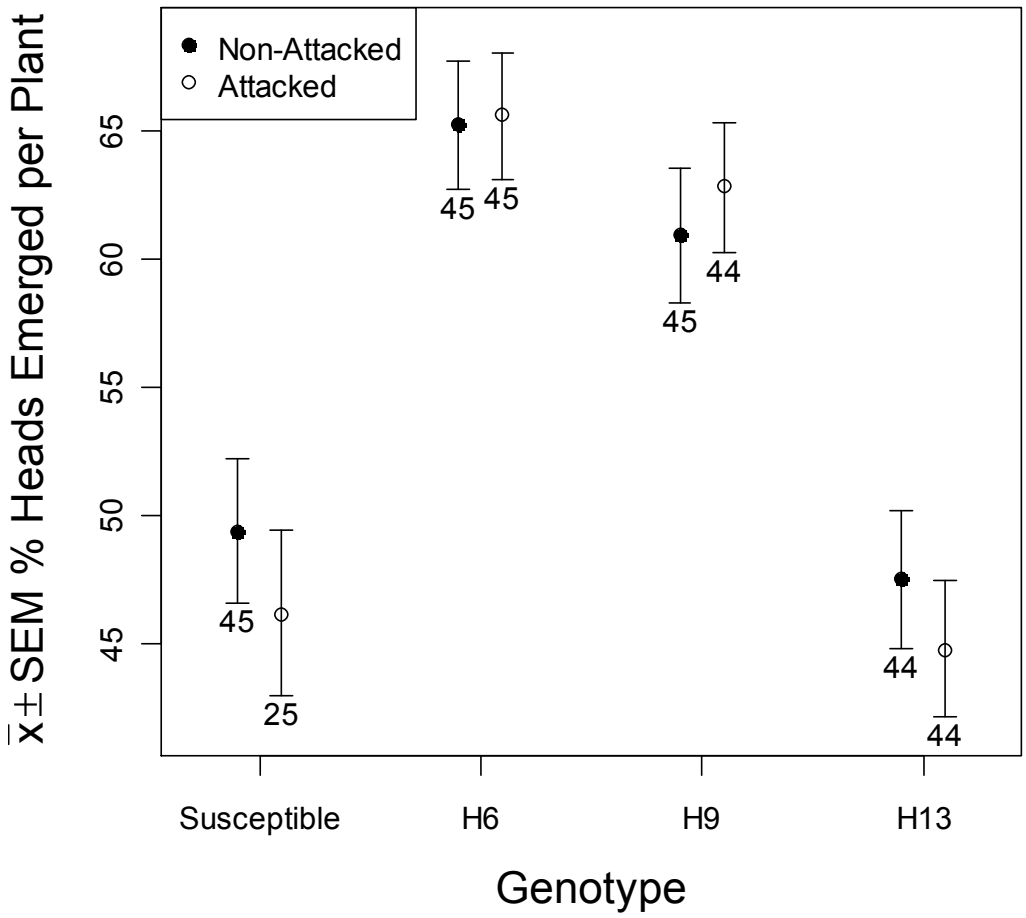

Fig. 1. Mean percentage of emerged seed heads (± SEM) produced by non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. The seed heads that had emerged from the boot (at least 50% of length emerged) were counted midway through the 10 day heading period. This number was then divided by the total heads produced by the plant. Paired bars that are accompanied by an asterisk(s) are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). The number at the bottom of the bar indicates sample size.

Plant Height (Fig. 2, Table 3). Plant height was influenced by plant genotype ($F_{3, 287} = 26.25$, $P < 0.0001$) but not larval attack ($F_{1, 287} = 0.38$, $P = 0.538$). The genotype x attack interaction was significant ($F_{1, 287} = 2.89$, $P = 0.036$). For non-attacked plants, a comparison of each resistant genotype to the susceptible genotype, showed that *H6* plants were shorter ($P = 0.018$), *H9* plants the same height ($P = 0.317$), and *H13* plants taller ($P < 0.0001$). For the

within-genotype comparison of attacked versus non-attacked plants, larval attack had significant effects for the susceptible genotype (at $P = 0.055$), with attacked plants being shorter than non-attacked plants, and for *H6* resistant plants ($P = 0.027$), with attacked plants being taller than non-attacked plants. No differences were found between attacked and non-attacked plants of *H9* ($P = 0.351$) or *H13* ($P = 0.654$).

Table 3. A summary of significant ($P < 0.05$) negatives and positives associated with constitutive and induced components of *H*-gene mediated resistance to Hessian flies. Means from Figures 1-9 were used to calculate % differences.

***H*-gene**	**Constitutive component**[1] Non-attacked plants with/without *H*-gene	**Induced component**[2] Non-attacked vs. attacked *H*-gene plants
H6	**Negatives:** 3% shorter plants 7% slower seedling growth **Positives:** 16% faster reproduction 19% more heads	**Negatives:** None **Positives:** 3% taller plants
H9	**Negatives:** None **Positives:** 12% faster reproduction 17% more heads	**Negatives:** None **Positives:** 14% more heads
H13	**Negatives:** 8% lower 1000 kernel wt. **Positives:** 5% taller plants 36% more heads 19% more seeds 33% greater total seed wt.	**Negatives:** None **Positives:** 13% more heads 7% more seeds 16% greater total seed wt.

[1]This was determined by comparing *H6*, *H9* or *H13* to the susceptible in the absence of Hessian fly attack.
[2]This was determined by comparing non-attacked versus attacked resistant plants (*H6*, *H9* and *H13).*

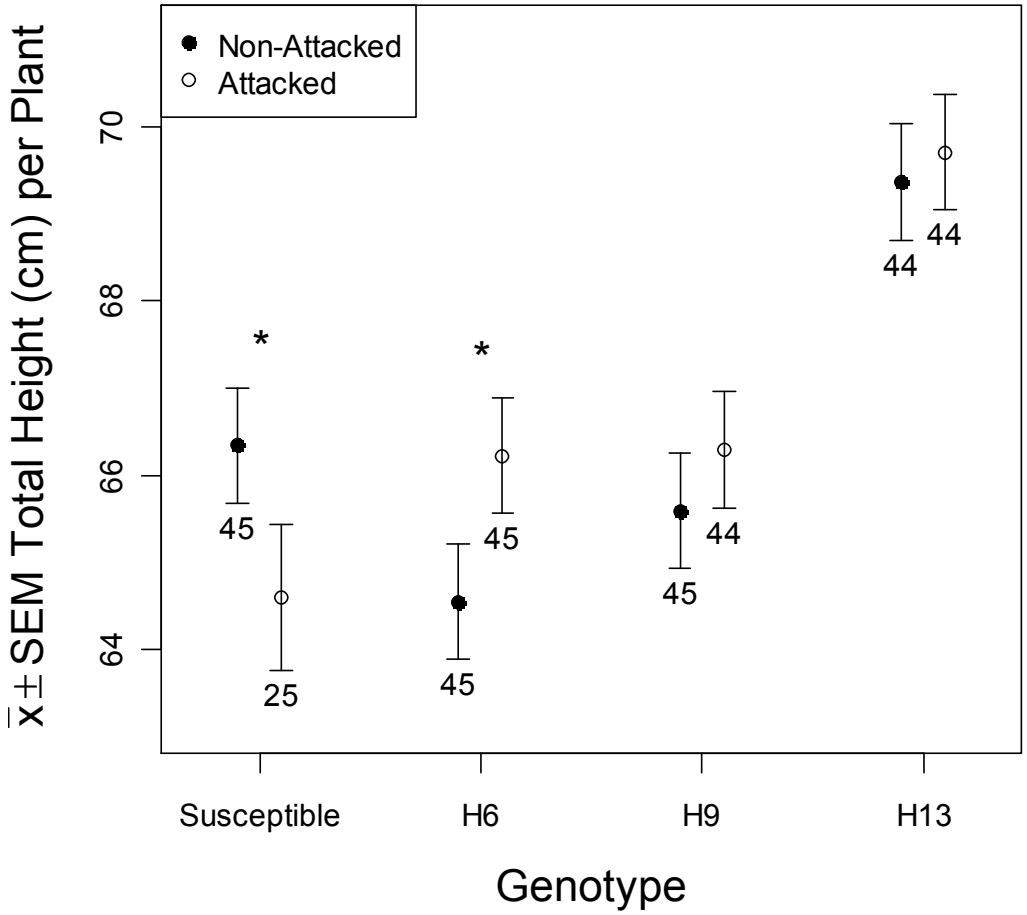

Fig. 2. Mean plant height (± SEM) of non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. Paired bars that are accompanied by an asterisk are significantly different at *, $P < 0.05$. The number at the bottom of the bar indicates sample size.

Seed Heads per Plant (Fig. 3, Table 3). Heads per plant was influenced by plant genotype ($F_{3, 131} = 63.02$, $P < 0.0001$) and larval attack ($F_{1, 153} = 4.23$, $P = 0.042$). The genotype x attack interaction was significant ($F_{3, 153} = 7.29$, $P = 0.0001$). For non-attacked plants, the three resistant genotypes, *H6*, *H9* and *H13*, all produced more heads than the susceptible genotype ($P < 0.0001$). For the within-genotype comparison, attacked *H6* plants produced a similar number of seeds when compared to non-attacked *H6* plants ($P = 0.359$). *H9* and *H13* produced more heads when attacked ($P < 0.001$), while the susceptible did the opposite, producing fewer heads when attacked ($P = 0.013$).

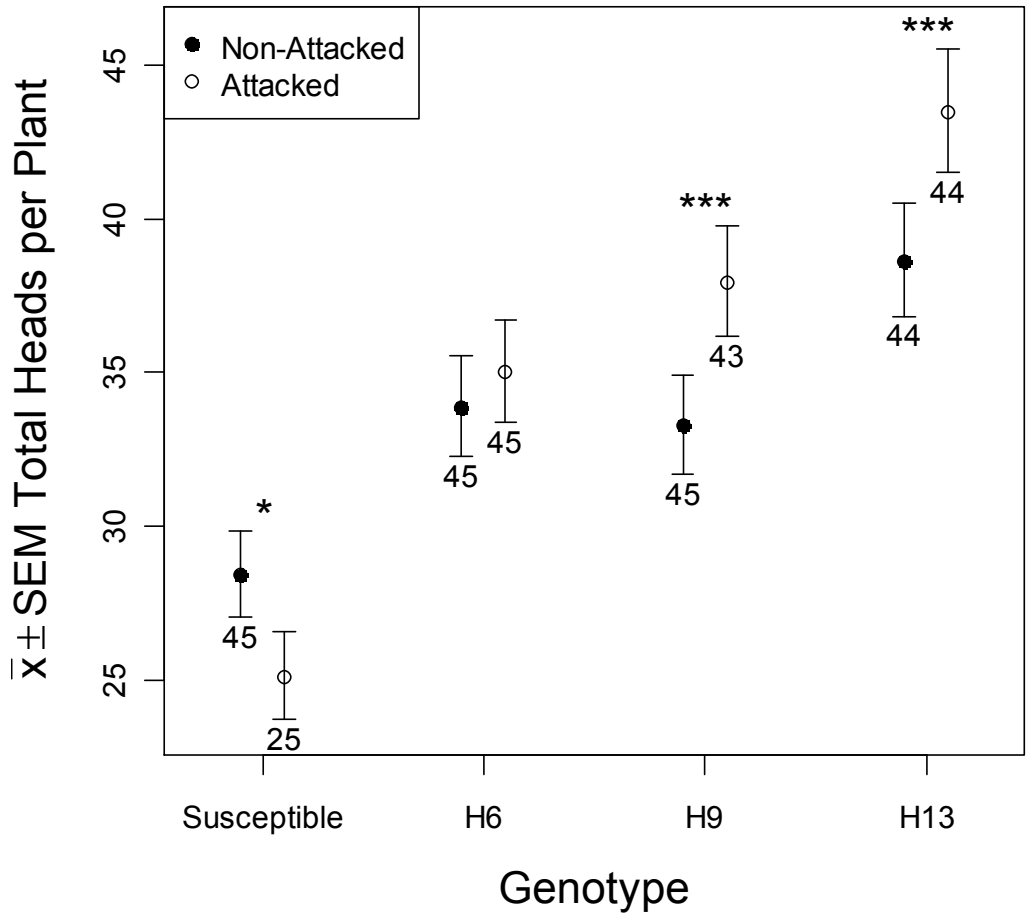

Fig. 3. Mean heads produced per plant (± SEM) of non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. Paired bars that are accompanied by an asterisk(s) are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). The number at the bottom of the bar indicates sample size.

Seeds Produced per Plant (Fig. 4, Table 3). Seeds produced per plant was influenced by plant genotype ($F_{3, 131} = 47.62$, $P < 0.0001$) but not by larval attack ($F_{1, 154} = 1.50$, $P = 0.222$). The genotype x attack interaction was significant ($F_{3, 154} = 3.74$, $P = 0.012$). For non-attacked plants, *H6* and *H9* produced similar seeds compared to the susceptible ($P = 0.196$ and $P = 0.218$, respectively) while *H13* produced more seeds ($P < 0.0001$). For the within-genotype comparison, *H13* produced more seeds when attacked than when not attacked ($P = 0.006$), while the susceptible did the opposite, producing fewer seeds when attacked ($P = 0.053$). *H6* and *H9* produced similar numbers of seeds, regardless of attack ($P = 0.509$ and $P = 0.142$, respectively).

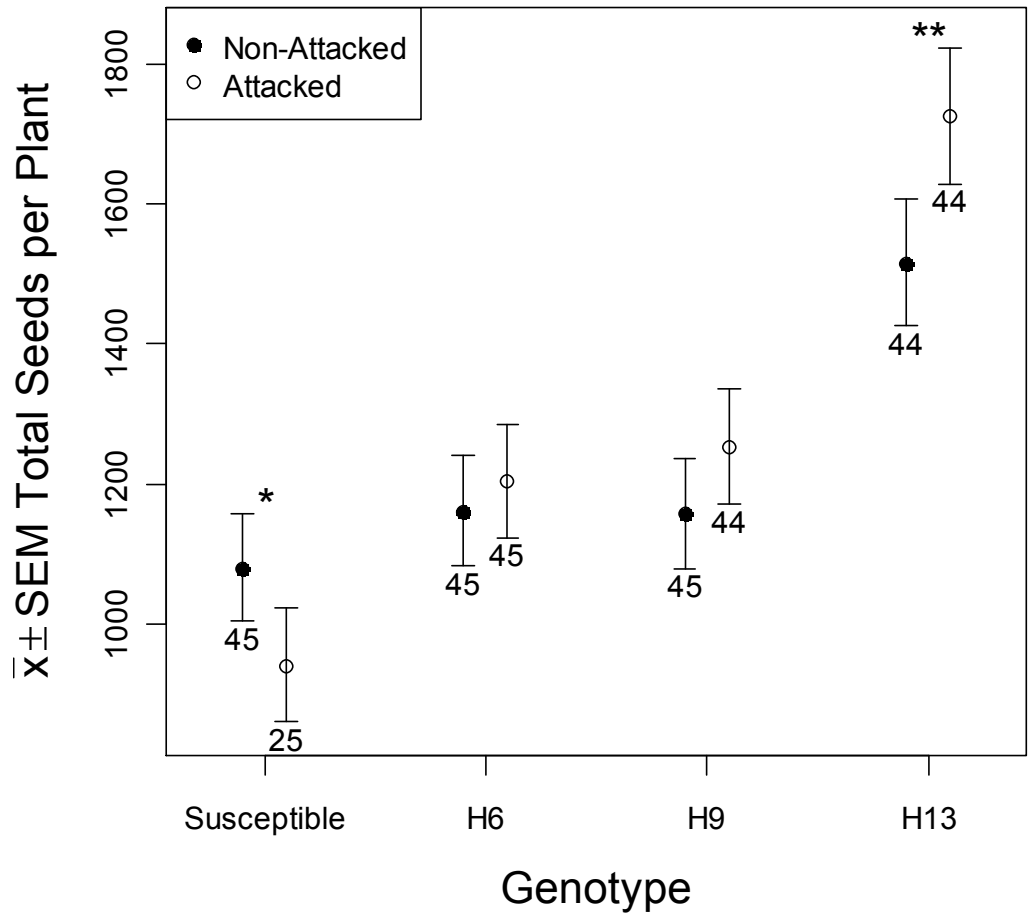

Fig. 4. Mean seeds (± SEM) of non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. Paired bars that are accompanied by an asterisk(s) are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). The number at the bottom of the bar indicates sample size.

Seed Weight (Fig. 5, Table 3). Seed weight was influenced by plant genotype ($F_{3, 129} = 13.25$, $P < 0.0001$) but not by larval attack ($F_{1, 171} = 1.71$, $P = 0.192$). The genotype x attack interaction was not significant ($F_{3, 167} = 0.71$, $P = 0.55$). For non-attacked plants, *H13* produced lighter seeds than the susceptible genotype ($P = 0.001$) while *H6* and *H9* produced seeds of similar weight ($P = 0.204$ and $P = 0.380$). For the within-genotype comparisons, seeds produced by attacked plants were similar in weight to seeds produced by non-attacked plants, regardless of plant type ($P = 0.334$, 0.293, 0.556, and 0.260, respectively for susceptible, *H6*, *H9* and *H13*).

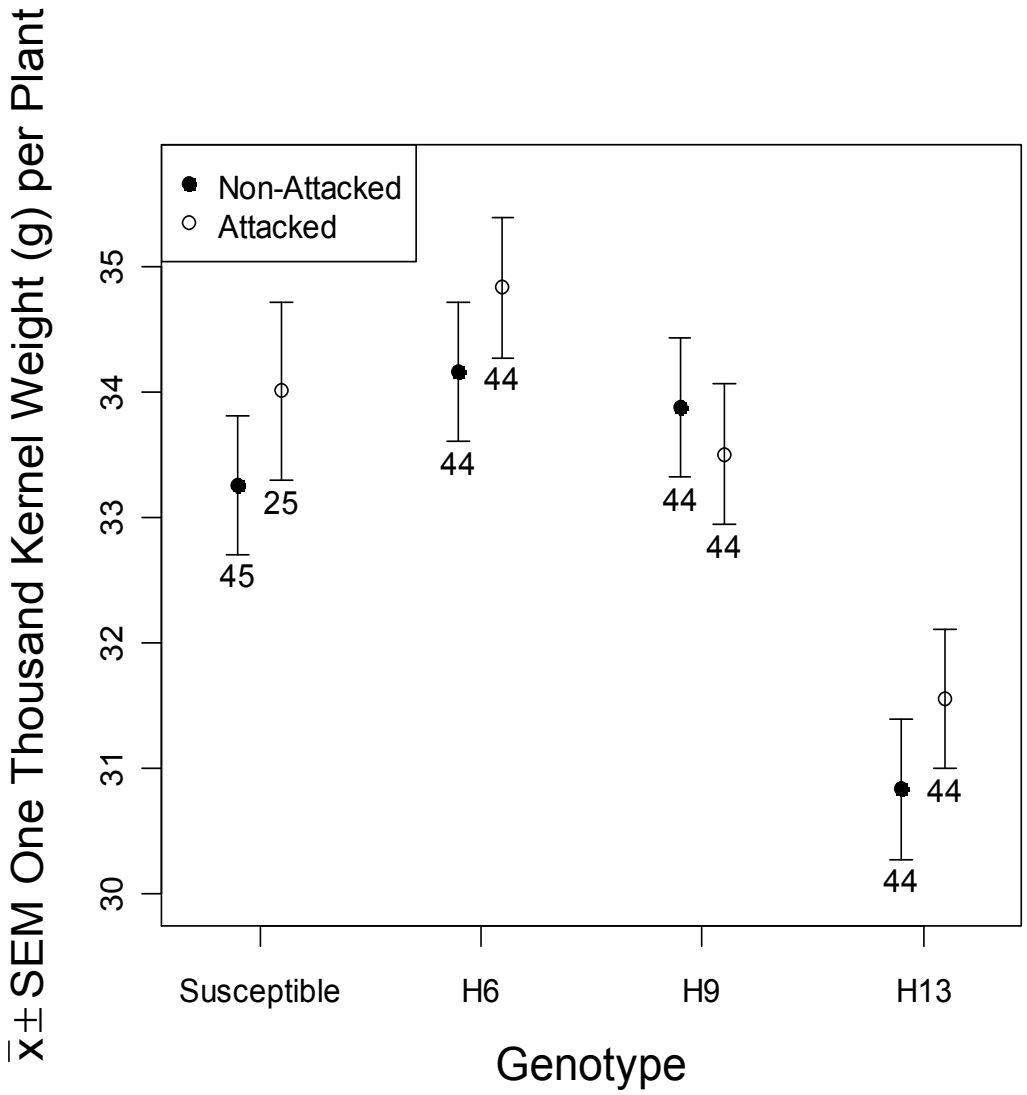

Fig. 5. Mean 1000 kernel weight (± SEM) of non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. Paired bars that are accompanied by an asterisk(s) are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). The number at the bottom of the bar indicates sample size.

Total Seed Weight (Fig. 6, Table 3). Total seed weight was influenced by plant genotype ($F_{3,135} = 30.58$, $P < 0.0001$) but not by larval attack ($F_{1,179} = 2.93$, $P < 0.089$). The genotype x attack interaction was significant ($F_{3,176} = 3.38$, $P < 0.020$). For non-attacked plants, *H13* had heavier total seed weights when compared to the susceptible ($P < 0.001$) while *H6* and *H9* were not significantly different from the susceptible ($P = 0.068$ and $P = 0.076$). For the

within-genotype comparisons, total seed weights for attacked plants were similar to non-attacked plants for the susceptible, *H6* and *H9* ($P = 0.162$, $P = 0.310$, $P = 0.322$ respectively). *H13* produced more seeds when attacked than when not attacked ($P = 0.001$).

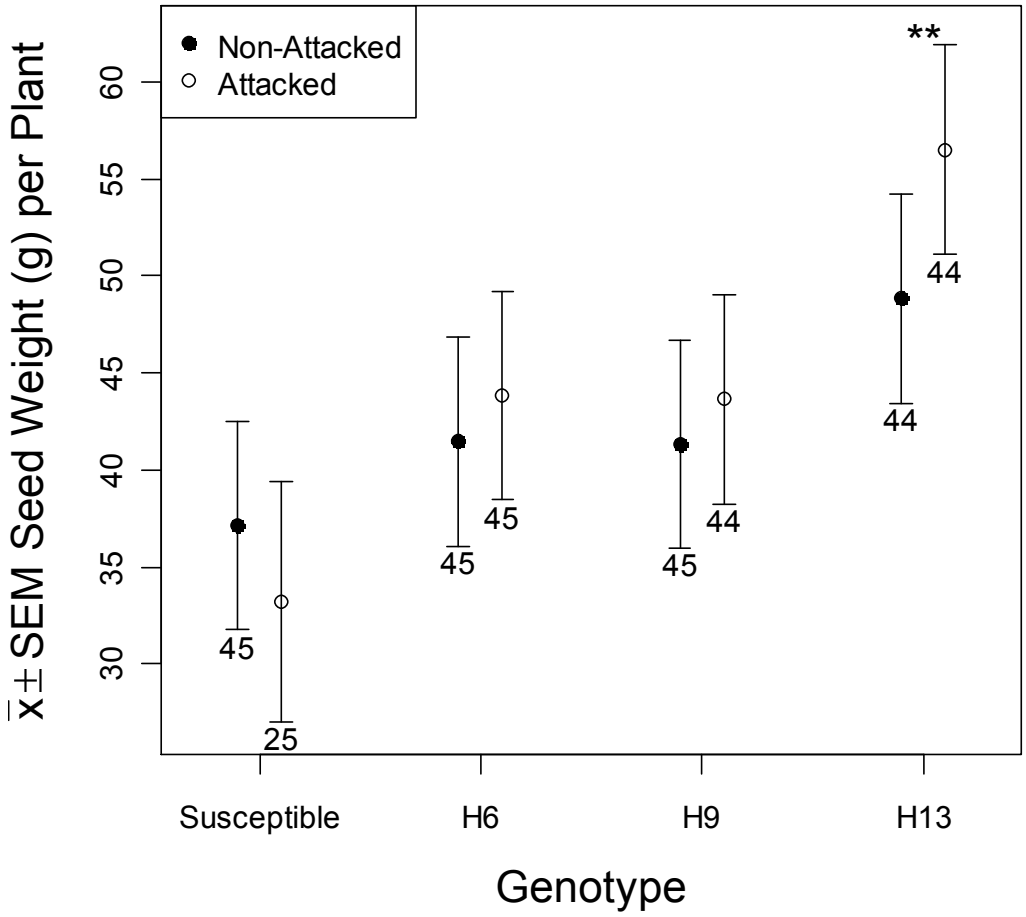

Fig. 6. Mean total seed weight (±SEM) of non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. Paired bars that are accompanied by an asterisk(s) are significantly different at *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$. The number at the bottom of the bar indicates sample size.

Seed Protein (Fig. 7, Table 3). Protein was not influenced by plant genotype ($F_{3, 132} = 1.19$, $P = 0.315$) or larval attack ($F_{1, 169} = 0.90$, $P = 0.334$). The genotype x attack interaction was not significant ($F_{3, 166} = 0.44$, $P = 0.724$). For non-attacked plants, the three resistant genotypes *H6*, *H9* and *H13*, all had similar seed protein contents when compared to the susceptible ($P =$ 0.533, 0.175 and 0.917 respectively). For the within-genotype comparisons, seed protein content

of attacked plants were similar to seed protein found in non-attacked plants, (P = 0.293, 0.391, 0.738 and 0.847 respectively for the susceptible, *H6*, *H9* and *H13* genotypes).

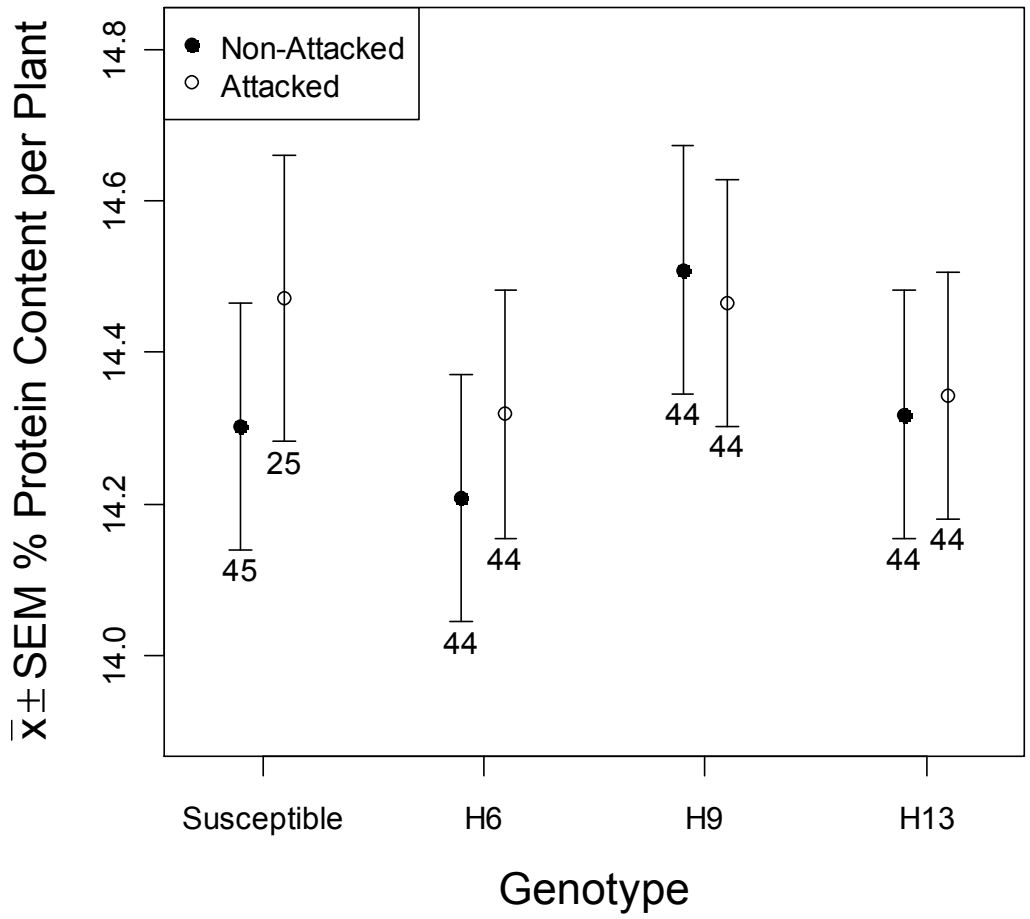

Fig. 7. Mean seed protein (± SEM) of non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. Paired bars that are accompanied by an asterisk(s) are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). The number at the bottom of the bar indicates sample size.

The Percentage of Normal Seeds Produced by the Plant (Fig. 8, Table 3). Percent normal seeds was influenced by larval attack ($F_{3,131} = 5.08$, $P = 0.026$), but not plant genotype ($F_{3,154} = 0.29$, $P = 0.833$), or the genotype x larvae interaction ($F_{3,154} = 0.56$, $P = 0.643$). For non-attacked plants, the percent normal seeds produced by the *H6*, *H9* and *H13* genotypes was similar to the susceptible (P = 0.230, 0.195 and 0.557 respectively). For the within-genotypes

comparisons, larval attack reduced the percent of normal seeds produced by susceptible plants (P = 0.046). The resistant genotypes produced the same percent normal seeds regardless of plant type (P = 0.533, 0.478 and 0.338 respectively for the *H6*, *H9* and *H13* genotypes).

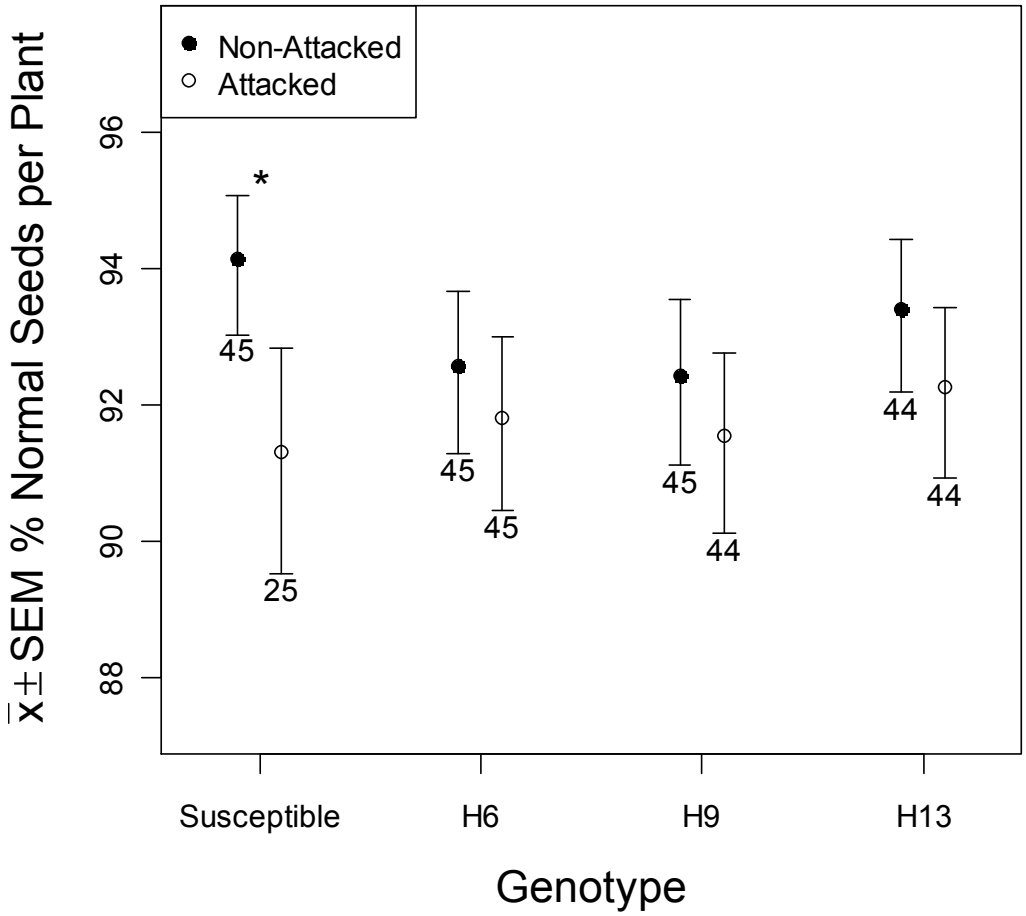

Fig. 8. Mean normal seeds (± SEM) produced by non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. Paired bars that are accompanied by an asterisk(s) are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). The number at the bottom of the bar indicates sample size.

Seedling Growth (Fig. 9, Table 3). Seedling growth was influenced by plant genotype ($F_{3,122} = 2.58$, $P = 0.057$) but not by larval attack ($F_{3,163} = 0.00$, $P = 0.960$) or the genotype x larvae interaction ($F_{3,160} = 0.51$, $P = 0.679$). For non-attacked plants, *H6* plants produced seedlings that grew more slowly than seedlings produced by susceptible plants (P = 0.003), while *H9* and *H13* produced seedlings that grew like susceptible seedlings (P = 0.198, and 0.094,

respectively). For within-genotype comparisons, seedling growth of seeds of attacked plants was similar to seedling growth of seeds of non-attacked plants, (P = 0.497, 0.317, 0.864, 0.812 respectively for the susceptible, *H6*, *H9* and *H13* genotypes).

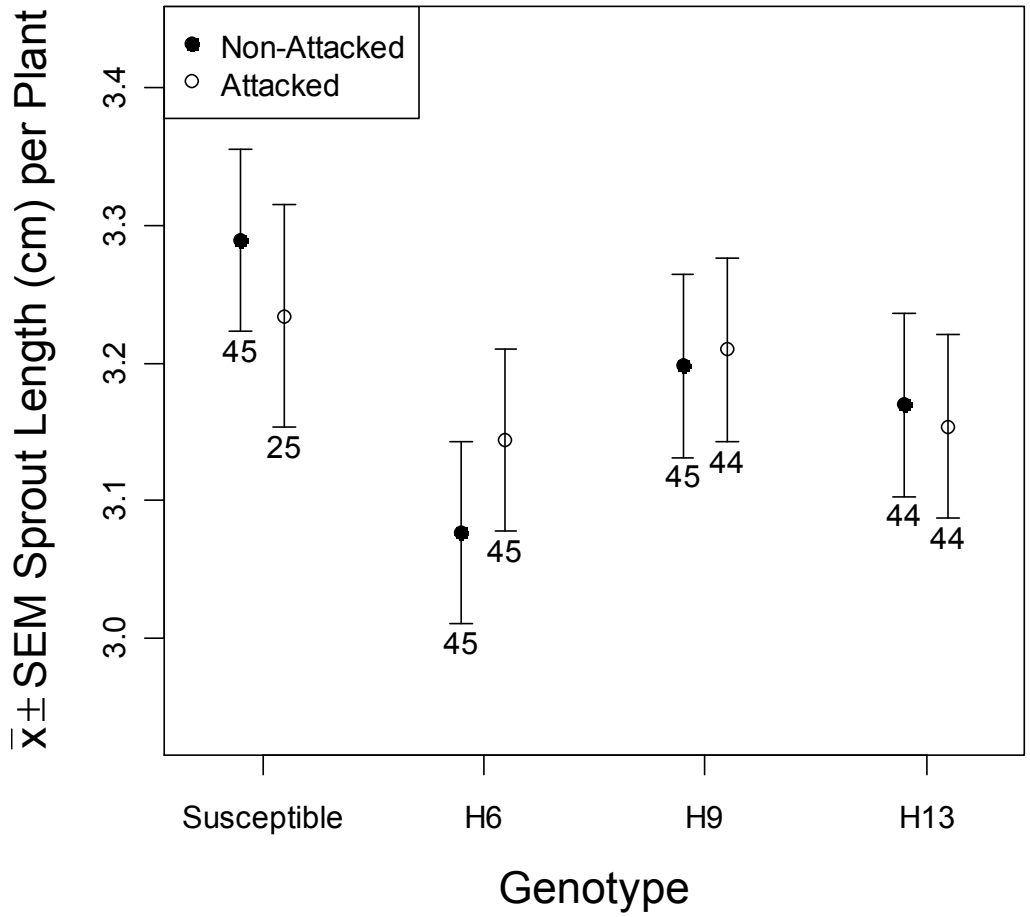

Fig. 9. Mean growth of seedlings (± SEM) produced by non-attacked versus Hessian fly attacked wheat plants of susceptible versus resistant lines expressing the *H6*, *H9*, or *H13* resistance gene. Paired bars that are accompanied by an asterisk(s) are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). The number at the bottom of the bar indicates sample size.

Discussion

The benefits conferred by a plant resistance trait create the context in which costs are assessed (Agrawal 2000, Zavala et al. 2004). In the case of *H*-gene-mediated resistance, there are significant benefits for protecting against the Hessian fly. Most important is survival during the

seedling stage, which increases from 56% for attacked susceptible plants to 100% for attacked resistant plants (Table 2). However, even if susceptible plants survive attack, they continue to have problems, producing fewer seed heads ($P = 0.013$, Fig. 3) and fewer normal seeds ($P = 0.046$, Fig. 8) than susceptible non-attacked plants. They also tend to be shorter ($P = 0.055$, Fig. 2) and produce fewer overall seeds ($P = 0.053$, Fig. 4). The significant benefits of gene-for-gene resistance are one of two reasons why *R* genes are so popular with plant breeders, the other being ease of moving the *R* gene into elite cultivars (Agrios 1997, Pedigo 2002, Smith 2005, Bent and MacKay 2007).

Costs of resistance create tradeoffs for the plant (Strauss et al. 2002, Heil and Baldwin 2002). An example of such a tradeoff comes from the *ACD6* allele of *Arabidopsis*, which, in contrast to *R* gene-mediated resistance, confers a non-specific resistance to a range of diseases (Todesco et al. 2010): the plant benefits from the allele when it is attacked but pays a cost when it is not attacked, with this cost manifested as reduced leaf growth relative to *Arabidopsis* genotypes without the allele. Fitness tradeoffs have also been found for mammalian immune systems, with stronger immunity associated with lower reproductive success (Graham et al. 2010).

There appears to be no tradeoff for plants expressing a single *H*-gene (Figs. 1-9). In each paired comparison of a non-attacked resistant line to the non-attacked susceptible line, the resistant lines had some measures that indicated costs of expressing the *H*-gene ("negatives") but other measures that indicated benefits of expressing the *H*-gene ("positives"). *H6* plants had two negatives, being shorter (Fig. 2) and producing offspring that grew more slowly (Fig. 9) but also had two positives, maturing faster (Fig. 1) and produced more heads (Fig. 3). *H9* plants had no negatives but two positives, maturing faster (Fig. 1) and producing more heads (Fig. 3). *H13*

plants had one negative, producing lighter seeds (Fig. 5) but four positives, being taller (Fig. 2) and producing more heads (Fig. 3), more seeds (Fig. 4), and greater total seed weight (Fig. 6).

This overall pattern of positives and negatives supports three lines of evidence that suggest little or no cost for the constitutive expression of a resistance gene. The first comes from the observation that, if *R* genes had significant costs, plant breeders would not continue to include breeding lines carrying *R* genes in their breeding programs (Brown 2002). The second line of evidence comes from numerous experimental studies showing that *R* genes have no cost (Bergelson and Purrington 1996, Brown 2002), an exception being the study by Tian et al. (2003) which found a 9% decrease in seed production by *Arabidopsis* plants genetically engineered with *RPM1*, a gene which codes for the ability to recognize *Pseudomonas syringae* pathotypes carrying *AvrRpm1* or *AvrB*. The third line of evidence comes from plant genomes, which have large numbers of *R* genes (e.g. Arabidopsis Genome Initiative 2000, Devos 2010). Surely *R* genes must carry little or no cost if a single plant can afford to express large numbers of *R* genes?

A cost of induced resistance creates a different tradeoff, referred to as an allocation cost (Heil and Baldwin 2002). Here the resources allocated to induced resistance mean there are fewer resources for growth and reproduction, or for defense against other enemies. We found no evidence of allocation costs for growth or reproduction of *H6*, *H9* or *H13* (Figs. 1-9). Rather than attacked resistant plants being less fit than non-attacked resistant plants, attacked plants were more fit. Thus, each resistant line showed a number of positives when attacked: attacked *H6* plants were taller (Fig. 2), attacked *H9* plants produced more heads (Fig. 3), and attacked *H13* plants produced more heads (Fig. 3), seeds (Fig. 4) and a greater total seed weight (Fig. 6). Although we did not compare the three *H*-genes statistically, it appears that *H13* had greater

benefits than *H6* and *H9*. This was a surprise: when resistance is triggered by an *R/Avr* interaction, the downstream induced responses are expected to be the same, regardless of which *R*/*Avr* interaction triggers the induced resistance (Nimchuk et al. 2003). Thus, in theory, the downstream, induced resistance triggered by *H6*, *H9* and *H13*, as well as their costs and benefits, should all be the same.

The best-known study of allocation costs of *R* gene-mediated resistance comes from plant pathology (Smedegaard-Peterson and Stølen 1981). There were significant costs of barley resistance to powdery mildew: attacked resistant plants had reduced grain yield (down 7%), reduced seed weight (down 4%), and reduced seed protein (down 11%). At least two reasons can explain why I failed to find similar costs. One is duration of attack. Smedegaard-Peterson and Stølen (1981) continuously exposed barley plants to attack by powdery mildew, starting at the 5-leaf seedling stage and continuing through maturation of seeds. My wheat plants were attacked by large numbers of larvae (ca. 50 larvae per plant) but were attacked for a much shorter period (only during the seedling stage). This short period of Hessian fly attack is realistic for northern regions, such as North Dakota, where winter wheat seedlings are attacked in late autumn by the season's final Hessian fly generation (Berzonsky et al. 2003, Porter et al., 2009). If the wheat is resistant and the Hessian fly population is avirulent, no Hessian flies will emerge in the spring to re-attack plants. This leaves wheat plants free of the Hessian fly over the months in the spring and early summer when they grow to maturity. However, in other parts of the USA, winter wheat is attacked throughout the winter (e.g. Georgia, Porter et al. 2009). Here it would be useful to know if repeated Hessian fly attack during the seedling stage, or during both the seedling and stem elongation phase of wheat development, incurs greater allocation costs.

A second possible reason why Smedegaard-Peterson and Stølen (1981) found greater costs of induced resistance is their study took place in conditions (i.e. a growth chamber) that are less optimal for plant growth. Our wheat plants were attacked as seedlings in the greenhouse but achieved most of their growth in the field where excellent conditions produced high yields and excellent seed quality. However, I conducted an identical study of costs of *H6*, *H9* or *H13* in 2004 under presumably less optimal greenhouse conditions and again found no cost of induced resistance (Anderson unpublished results). Here my reluctance to believe that the induced resistance has no cost led me to repeat the study under field conditions (hence the study reported here).

Bergelson and Purrington (1996) suggested the varying costs of plant resistance found in different plant-parasite study systems might make more sense if more was known about resistance mechanisms. Thus, some resistance mechanisms may simply be less costly than others. This may be true for my study system. Only recently discovered to be a gall-maker (Harris et al. 2006), the Hessian fly larva attacks cells that are rapidly expanding, that is, a small portion of the cells that comprise the seedling wheat plant (an area of approximately 60 mm^2 at the base of a single leaf). These cells are presumably chosen because they are only ones that can be manipulated to produce gall nutritive cells, the cytoplasmically-enriched epidermal and mesophyll cells that feed the sessile 2^{nd} instar larva (Harris et al. 2010). Rapid larval growth occurs at the expense of plant growth: the newly initiated leaves of the seedling stop growing (Anderson and Harris 2006, 2008) and the plant's carbon-nitrogen metabolism shifts dramatically (Zhu et al. 2008).

The small scale of Hessian fly attack may allow the scale of induced resistance to also be small. Histological analysis of Hessian fly attack sites showed death of a small number of cells

alongside wall fortification of living adjacent cells (Harris et al. 2010). Fortification was accompanied by elaboration of the Golgi and endoplasmic reticulum, with vesicles bringing secreted materials to the cell periphery (and perhaps also to the larva). Toxins (e.g. lectins) are another important part of the response (Subramanyan et al. 2006). The relatively small scale of the induced response is suggested by microarray data, which showed that the plant changes far less during induced resistance than during induced susceptibility (Zhu et al. 2008). Induced resistance is very effective at stopping attack: avirulent Hessian fly larvae die 3-5 days after initiating attack of *H6*, *H9*, *H13*, and *H26* plants and fail to grow before dying (Harris et al. 2010).

Cell death, which is considered a costly defense, has long been seen as the primary resistance mechanism of race-specific *R/Avr*-triggered immune responses to plant pathogens (Agrios 1997). This idea is changing with the discovery that *R* gene-mediated resistance can occur in the absence of the hypersensitive response, instead being associated with wall reinforcement of a relatively small number of cells (Bulgarelli et al. 2010). A low cost, cell-wall based resistance is presumably possible for any plant parasite that attacks, and then tries to manipulate, individual plant cells, with this including many small insects, mites, nematodes, and plant pathogens (Agrios 1997, Shorthouse and Rohfritsch 1992, Williams 1994).

I propose tolerance as another reason why I failed to find allocation costs. If the plant is tolerant, there can be an initial allocation cost for induced resistance but this cost will disappear over time if compensatory growth occurs. Compensatory growth is made possible by a number of mechanisms, including diversion of carbon to parts of the plant that are not accessible to the insect (e.g. inaccessible roots for a foliage feeder) and redeployment of carbon for plant growth after the insect is gone (Schwachtje et al. 2006). One line of evidence for tolerance to the

Hessian fly comes from the susceptible 'Newton', the genotype used in the current study and the genetic background of all three resistant genotypes. Its only chance of survival is initiating growth (tillers) from an axillary coleoptile meristem, a growth zone that presumably is not accessible to the Hessian fly larva (Anderson and Harris 2006). The second line of evidence comes from the compensatory growth of resistant plants which contributed to the "positives" exhibited by attacked plants, including taller plants for *H6* plants, more seed heads for *H9* and *H13* plants, and more seeds and greater total seed weight for *H13* plants. Recently it was suggested that the compensatory growth triggered by insect-produced elicitors of plant resistance might provide a way to improve crop yields (Poveda et al. 2010). This might also be possible for plants resistant to the Hessian fly: elicitors of *H*-gene-mediated resistance are the Hessian fly *Avr* effectors produced in the salivary glands (Chen et al. 2008).

The idea that plant defense against the Hessian fly includes both resistance and tolerance goes against the oft-cited hypothesis that plants must choose between the two strategies (Herms and Mattson 1992). One reason why plants must choose is functional redundancy: why would a plant have both resistance and tolerance if each achieves the same thing? However, tolerance is not redundant if the resistance is easily overcome by parasite adaptation. This is the case for plant enemies targeted by *R* genes, which adapt relatively easily via null or loss-of-function mutations in matching *Avr* genes (Stergiopoulos and de Wit 2009). Mutations rapidly spread through parasite populations under *R* gene selection (Bent and Mackey 2007). When the Hessian fly adapts, the advantage of having the *H*-gene is lost: the mutant *Avr* larva succeeds in producing the nutritive tissue and the plant stops growing (Harris unpublished results). Many others have challenged the idea that resistance and tolerance are mutually exclusive defense

strategies, albeit for quite different reasons (Mauricio et al. 1997, de Jong and van der Meijden 2000, Leimu and Koricheva, 2006).

While I found no costs of *H*-gene-mediated resistance, it is possible that costs will be found under other conditions. The *H*-genes that I tested originated from two ancestors of hexaploid bread wheat (AABBDD), the tetraploid *T. turgidum* (donor of AABB genomes and *H6* and *H9*) and diploid *A. tauschii* (donor the DD genome and *H13*). This means there may be costs when the *H*-gene is expressed in its original genetic background. Ploidy may be important here and is known to influence host use by gall midges (Arvanitis et al. 2010). Costs might also be found when *R* genes are stacked rather than deployed singly: certain combinations of *R* genes trigger hybrid necrosis, a mechanism that may contribute to reproductive isolation and speciation in plants (Bomblies 2010). Costs also might have been found if my test had been conducted under conditions of greater abiotic or biotic stress (Valverde et al. 2003, Hawkes and Sullivan 2001). My plants produced excellent yields and seed quality, having been grown under non-stressful conditions, which included protection against weeds, fungi, larger herbivores (deer, rabbits, and birds) and insects other than the Hessian fly.

The benefit/cost ratio of *H*-gene-mediated resistance to the Hessian fly is potentially interesting for co-evolutionary studies. The Near East is the center of origin of wheat and its ancestors (Salamini et al. 2002, Devos 2010) and also appears to be the center of origin of the Hessian fly (Barnes 1956). Today this region contains some of the world's most virulent Hessian fly populations (El Bousshini et al. 2008) and also contains many host and non-host grass species. If *H*-genes have no costs, they may have played a role in the evolution of durable non-host resistance (Heath 2000), evidence for this coming from non-host grasses that are closely related to host grasses. A parallel question for the Hessian fly is: are there fitness costs for

adapting to *H*-genes? We are beginning to explore this question experimentally (Zhang et al. in revision) and expect new insights from the soon-to-be published Hessian fly genome which has revealed large numbers of Hessian fly *avirulence* genes under diversifying selection (Chen et al. 2010).

R genes are important tools for plant breeders (Jones 2001, Tester and Langridge 2010). Their deployment in agriculture will benefit from ideas from ecology and evolution, as well as more experimental data (Rausher 2001, Bent and Mckey 2007). My results indicate that three different *H*-genes targeted at the Hessian fly, *H6*, *H9* and *H13*, have no cost and therefore can be stacked to produce a resistance that is more durable. Stacking is now possible for several *H*-genes that have good genetic markers, including *H9*, *H13* and *H26* (Liu et al. 2005, Yu et al. 2009). For highly effective resistance genes that have not yet been deployed, deployment through stacking is probably a better use of the gene than serial deployment (Gould 1986, 1998). One such gene is *H26*, which is effective against all known Hessian fly populations, including the world's most virulent populations (El Bousshini et al. 2008).

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CHAPTER 3. USING SEX PHEROMONE TRAPPING TO EXPLORE THREATS TO WHEAT FROM HESSIAN FLY (DIPTERA: CECIDOMYIIDAE) IN THE UPPER GREAT PLAINS[2]

Abstract

Before embarking on the 5-10 year effort it can take to transfer plant resistance (*R*) genes to adapted crop cultivars, a question must be asked: is the pest a sufficient threat to warrant this effort? I used the recently discovered female-produced sex pheromone of the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), to explore this question for populations in the Upper Great Plains. Methods for pheromone trapping were established and trapping data were used to explore geographic distribution, phenology, and density. The pheromone lure remained attractive for up to 10 days and only attracted male Hessian flies. Traps placed within the crop canopy caught flies but traps placed above the crop canopy did not. Hessian flies were trapped throughout North Dakota starting in the spring and continuing through the summer and autumn. Densities were low in the spring but increased greatly during the early part of the summer, with peak adult emergence taking place at a time (July/August) when spring wheat was being harvested and winter wheat had not yet been planted. In the autumn, adults were found at a time when winter wheat seedlings are growing. The discovery of flies on Conservation Reserve Program (CRP) land supports the idea that pasture grasses serve as alternate hosts. I conclude that the Hessian fly is a risk to wheat in the Upper Great Plains and predict that global warming and the increasing cultivation of winter wheat will add to this risk.

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Introduction

When the only tactic available to control an insect pest is plant resistance, implementation can take 5-10 years. The first question that must be answered: is the pest a sufficient threat to warrant breeding resistance traits into the elite crop cultivars that are adapted for the region? A second question is: will plant breeders be willing to add the resistance (*R*) gene(s) to their breeding programs, making resistance as much of a priority as yield, quality and resistance to other agents of biotic stress, such as pathogens? A third question arises when multiple *R* genes are available for controlling the insect: which should be chosen and how should they be deployed? This will depend on at least two things, which genes are most effective for the pest populations that occur in the region and which genes are easiest to move into elite cultivars.

The most effective tactic for controlling the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), is plant resistance conferred by *H*-genes (Ratcliffe and Hatchett 1997, Berzonsky et al. 2003, Harris et al. 2003, Wise et al. 2006, Porter et al. 2009). Many of the >32 identified *H*-genes are very effective. Larvae die within 3-5 days of attacking resistant wheat, *Triticum aestivum* (L.), and do not grow (Harris et al. 2010). Little or no damage occurs during the 3-5 days that precede larval death, and attacked resistant plants produce the same, or even better, yields and quality as non-attacked plants (Anderson et al. 2011). *H*-genes are attractive for plant breeders because the resistant plant pays no fitness cost (Anderson et al. 2011).

A problem for *H*-gene mediated resistance is parasite adaptation (Bent and MacKay 2007). A mutation in the Hessian fly's matching *Avirulence* (*Avr*) gene makes the fly 'virulent' and able to survive on the *H*-gene-protected plant (Gallun 1977, Harris et al. 2003, Stuart et al. 2012), albeit with a fitness cost (Zhang et al. 2011). Virulence within Hessian fly populations means that choosing the right *H*-gene is critical for achieving durable resistance (Cambron et al.

2010). The *H*-gene that is chosen for the region is the one for which there is the least virulence within populations. Only a few *H*-genes provide this all-inclusive resistance, for example the *H26* gene discovered in an ancestor of wheat, *Aegilops tauschii* (Cosson) (Cox et al. 1994, Yu et al. 2009). Stacking *R* genes also is expected to provide durable resistance but it requires reliable molecular markers, such as those for *H9*, *H13*, *H18*, *H26*, and *H32* (Dweikat et al. 1997, Liu et al. 2005, Wang et al. 2006, Yu et al. 2009, Yu et al. 2010).

A question for wheat grown in the Upper Great Plains: is the Hessian fly a sufficient threat to warrant breeding resistance traits into regionally adapted elite cultivars? The presence of the Hessian fly in North Dakota has been known for almost 100 years (Webster 1915). In the 1940's the extension entomologist at North Dakota Agricultural College (now North Dakota State University) reported on an outbreak and briefly described its lifecycle (Butcher 1946). Over subsequent decades, sporadic outbreaks in North Dakota and neighboring states suggested that the Hessian fly is widely distributed and capable of rapid population growth. Significant outbreaks occurred in northeast South Dakota in 1978 (Walgenbach et al. 1978), in southeast North Dakota in 1991 (Nelson et al. 1991) and in northeast North Dakota in 2003 (Glogoza 2004). A survey of stem-feeding insects of wheat showed that 38% of fields were infested with Hessian flies in a survey area encompassing parts of Montana, North Dakota, South Dakota, Nebraska and Wyoming (Shanower and Waters 2006).

Two predicted changes complicate the pest status of the Hessian fly in the Upper Great Plains. The first is a changing climate (Badh et al. 2009, Dunnell and Travers 2011). Starting in the late 1800's, each decade has seen an average of 1.2 days added to North Dakota's growing season (Badh et al. 2009). Longer growing seasons create the potential for additional Hessian fly generations, and more rapid population growth (Barnes 1956). The second change concerns

cropping systems. Traditionally winter wheat is rarely grown. For example in 2010 only 134,000 hectares of winter wheat were planted in North Dakota as compared to 2.6 million hectares of spring wheat (USDA-NASS 2011). But this is now changing. One push to increase winter wheat comes from hunters who appreciate the cover it provides for wildlife, e.g. migratory birds, during nesting in the spring (Prairie Grains Magazine 2009). A second reason is climate change, which is expected to drive the northward expansion of winter wheat (Ortiz et al. 2008). Together a longer growing season and expanded cultivation of winter wheat seem likely to increase the pest potential of the Hessian fly in the Upper Great Plains.

I used the recently identified female-produced sex pheromone of the Hessian fly (Andersson et al., 2009) to explore the threat the Hessian fly poses to wheat in the Upper Great Plains. Studies on the Hessian fly sex pheromone began in the early 1920's, when Cartwright (1922) showed, in the field, that caged virgin females attracted male flies over distances of at least 3 meters. In subsequent studies it was shown that males are receptive to calling female flies throughout their short one-day lifespan (McKay and Hatchett 1984, Bergh et al. 1990). In 1991, the major component of the sex pheromone was identified (Foster et al. 1991). Its failure to attract males in the field led to >15 more years of bioassay-driven isolation and identification of four other pheromone components (Andersson et al. 2009). The five-component blend attracted Hessian fly males in the laboratory, small plots, and harvested Kansas wheat fields (Andersson et al. 2009). I expanded on these studies by establishing methods for using the sex pheromone to monitor Hessian fly populations, asking: How many days does the sex pheromone lure remain attractive? Which of two pheromone release substrates, polyethylene caps or rubber septa, is better for trapping males? What is the optimal height for placing traps in wheat fields? Does the trap attract other insects that might be confused with the Hessian fly? I then deployed sex

pheromone-baited traps in various regions of North Dakota (Fig. 10) to explore threats posed by the Hessian fly to wheat in the Upper Great Plains.

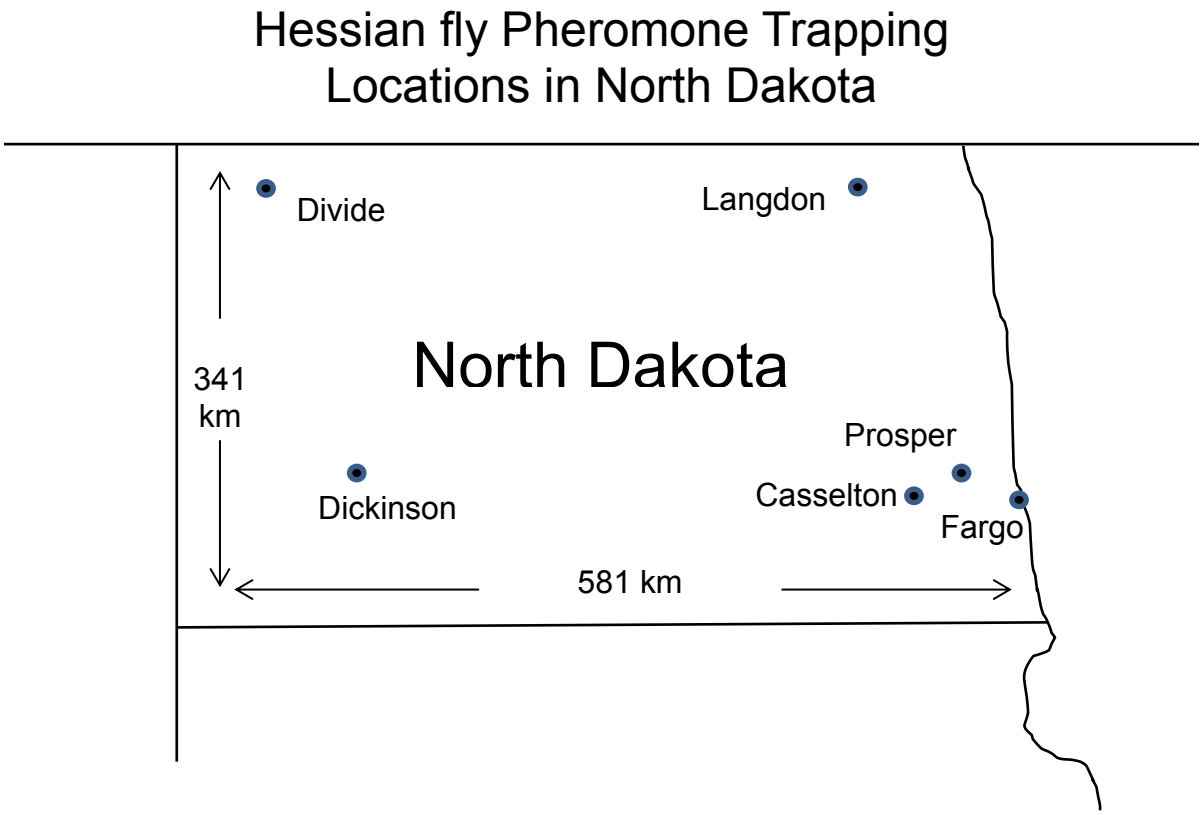

Fig. 10. Locations in North Dakota where Hessian fly sex pheromone traps were deployed. In 2008 and 2009, there were three trapping sites at each of the six locations. In 2010, the Dickinson location was not used, and at the remaining locations there was only one trapping site.

Methods and Materials

Pheromone Lures, Dispensers and Delta Traps. The Hessian fly sex pheromone used in the studies was the five-component blend described in Andersson et al. (2009). The components were: (2*S*)-tridec-2-yl acetate, (2*S*, 10*E*)-10-tridecen-2-yl acetate, (2*S*, 10*E*)-10-tridecen-2-ol, (2*S*, 8*Z*, 10*E*)-8, 10-tridecadien-2-yl acetate, (2*S*, 8*E*, 10*E*)-8, 10-tridecadien-2-yl acetate. The components were applied at a ratio of 10:100:10:10:10 µg per dispenser (100 µg of the main component (2*S*, 10*E*)-10-tridecen-2-yl acetate and 10 µg of the others). The pheromone blend dose and ratio of components were the same in all three years of testing (2008-2010). The

sex pheromone was loaded on either 10 mm (inside diameter) polyethylene (PE) dispensers, (Semadeni®, Ostermundigen, Switzerland) or 9 mm (inside diameter) rubber septa dispensers (PheroNet, Alnarp, Sweden). The sex pheromone dispenser was stapled to the inside of a delta trap (PheroNet, Alnarp, Sweden) so that it was positioned at the top center of the trap. A sticky card insert (9 x 15 cm PheroNet, Alnarp, Sweden) was placed on the bottom of the trap in all experiments.

Pheromone Trapping Protocol. All pheromone traps were placed 30 cm above the soil surface, the exception being the study to determine optimal trap height. The delta trap was attached to either a 1m long x 1 cm diameter metal pole (lure response and seasonal abundance study) or a 1.5 m long x l.3 cm diameter metal pole (lure longevity and optimal trap height study). In all instances the delta trap was attached to the pole with a plastic cable tie. In studies to determine seasonal abundance and distribution of the Hessian flies, pheromone traps were deployed in the field for the entire growing season. In all years, sticky cards were replaced every week and delta traps and pheromone lures were replaced every two weeks. Used sticky cards were placed individually in 1-gallon Ziploc® plastic bags and held in a freezer (-20 ± 2°C) until being examined in the laboratory. Using a stereomicroscope (6-12X), we recorded the presence of male Hessian flies, other Diptera belonging to the same suborder as the Hessian fly (Nematocera) and Hymenoptera (eg. possible parasitoids of the Hessian fly). A sub-sample of specimens from the sticky cards was sent to Dr. Raymond Gagné at the USDA-Systematic Entomology Laboratory, Beltsville, MD to confirm my identifications.

Locations of Field Tests. Trapping locations (Fig. 10) were chosen to represent the major climatic and cropping regions of the state. Climate data for each location was provided by the North Dakota Agricultural Weather Network (NDAWN), and information on cropping

systems for each area was provided by the UDSA-National Agricultural Statistics Service (USDA-NASS 2011). The three locations representing the southeast part of North Dakota are Fargo (46º 53' 50" N, -96º 48' 44" W), Prosper (47º 0' 6" N, -97º 6' 53" W), and Casselton (46º 52' 49" N, -97º 14' 53" W). They have a relatively long frost-free period and abundant rainfall throughout the season. The dominant commodities in the cropping system are spring wheat, corn, soybean and sugar beet. The northeastern location was represented by Langdon, North Dakota (48º 45' 25" N, -98º 20' 28" W). Langdon has one of the highest precipitation rates in the state but a relatively short growing season. To accommodate this region's shorter season, spring wheat, canola and soybean are commonly grown. The northwest location was Divide County, North Dakota (48º 42' 59" N, -103º 32' 2" W). Divide is the northwestern-most county in the state and typically has a semi-arid climate with a short growing season. Spring wheat, durum wheat, flax and pulse crops are commonly grown in this northern location. The southwestern location was Dickinson, North Dakota (46º 53' 42" N, -102º 48' 47" W). Dickinson experiences relatively long warm growing seasons, and like the Divide County location, evapotranspiration exceeds growing season precipitation. Half of the land area in southwest North Dakota is under introduced and native grass hay and pasture while the remaining land is devoted to spring wheat, sunflower, corn, pulse crops, flax, oat and canola.

Pheromone Lure Longevity. The activity period of the lures was evaluated at the North Dakota State University Langdon Research Extension Center in July/August 2009. The large number of second generation Hessian flies observed at the center's wheat research plots in 2008 was the reason Langdon was chosen. Tests were conducted in a 10 hectare field of 'Faller' hard red spring wheat that had matured to early milk development, Zadok's stage Z73 (Zadok et al. 1974). A total of eight treatments were evaluated: PE dispensers loaded with the 2009 batch of

the sex pheromone and either not pre-aged or pre-aged outdoors for 5, 12 or 20 days, PE dispensers loaded with the 2008 batch of the sex pheromone and not pre-aged, rubber septa dispensers loaded with the 2009 batch of the sex pheromone and either not pre-aged or pre-aged outdoors for 20 days, and an un-baited control. To pre-age the pheromone lures, individual lures were placed in delta traps and hung from poles at an outdoor location on the NDSU campus. While being aged the traps were exposed to weather conditions typical for the field. Prior to the start of this study all lures had been stored in sealed foil-lined pouches in the freezer (-20 ± 2° C). The treatments were evaluated in a randomized complete block design, with a total of five blocks and each treatment represented once per block. Spacing was 3 meters between treatments within a block, and 3 meters between blocks. The first block was approximately 10 meters from the field margin and the remaining blocks extended toward the center of the field parallel with the field edge. The spacing of 3 meters between traps was selected based on field observations that indicated that males are attracted to the female sex pheromone from 3 meters away with a zone of attraction particularly strong within 1 meter of the calling female (Cartwright 1922). Because traps were in the field for a total of five days, a lure that had not been pre-aged was now aged five days by the end of the test. The evaluation began on July 29th and ended on August 3rd 2009, a time period that coincided with significant adult Hessian fly emergence.

Optimal Height of Traps. The best height at which to place the pheromone baited traps was evaluated at the North Dakota State University Langdon Research Extension Center in July/August 2009. Evaluations were conducted in the same 10 hectare field of 'Faller' wheat used in the sex pheromone longevity study. The trap height study was conducted at the same time as the pheromone longevity study but was placed approximately 100 meters away. Four treatments were evaluated: 15, 30, 60 or 120 cm above the soil surface. Lures were PE

dispensers loaded with the 2009 batch of the sex pheromone. As with the previous study, the treatments were evaluated in a randomized complete block design, with a total of five blocks and each treatment represented once per block. There was 3 meters spacing between treatments within a block, and 3 meters spacing between blocks. The first block was approximately 3 meters from a vehicle trail running through the wheat field. The remaining blocks extended toward the center of the field parallel with the trail. The test began on July 30th and ended on August 3rd 2009.

Seasonal Abundance. During the three field seasons (2008-2010) sex pheromone baited traps were placed at the six pre-selected locations previously described (Fig. 1). Traps were placed in or immediately adjacent to cropland in the spring and were maintained until the end of the growing season. In 2008, at each of the six locations there were three pheromone baited traps. The distance between traps ranged from 100 to 400 meters. In 2009, at the same locations, there were six pheromone traps which consisted of three pairs of traps, one baited with the sex pheromone lure and the other was left un-baited. The paired traps were separated by 1.5 meters, while each pair was separated by at least 100 m. In 2010 only one pheromone trap was placed at each location and only the pheromone treatment was deployed.

Hessian Flies on Conservation Land. In 2009, Hessian flies were monitored on a 300 x 800 meter field of Conservation Reserve Program (CRP) land. The CRP site was located in Divide County approximately 2 km from the other Divide County pheromone trapping location (48º 42’ 37” N, -103º 32’ 43” W). The site was established as CRP in 1991 and is predominately crested wheatgrass, *Agropyron cristatum* (L.), and smooth bromegrass, *Bromus inermis* (L.). A north to south transect of three pheromone baited traps paired with un-baited traps was placed in the CRP on May 2nd and was maintained until October 7th. Native prairie bordered the CRP site

on the east and west approximately 150 m from and parallel to the transect line, and wheat fields bordered on the north and south. The transect was approximately 800 meters long with the north and south trapping sites approximately 100 meters from the nearest wheat field and the middle site approximately 400 meters from wheat.

Statistical Analysis. Data were analyzed using JMP version 4 (SAS Institute 2001), and SAS version 9.1.3 (SAS Institute 2003). Since the response variables were count data, a negative binomial model with a log-link was used to test for significant effects (Agresti 2002). For analysis of pheromone lure longevity and optimal lure placement, the effects of pheromone treatment and block were tested. When the F-test showed significant effects, means were compared using Tukey-Kramer HSD at $P < 0.05$.

Results

Pheromone Lure Longevity. The number of male Hessian flies captured over a five day period (Fig. 11) was influenced by the pheromone treatment ($F_{7,28}=59.22$, $P < 0.0001$) but not by the effect of blocking ($F_{4,28}=1.39$, $P=0.262$). The most attractive lures were those that were deployed immediately after being stored in the freezer or lures deployed after pre-aging for five days. A significant decrease in PE lure attractiveness occurred for the lures pre-aged for 12 and 20 days before deployment. Pheromone loaded onto rubber septa lures attracted few males, regardless the age of the lure, but did attract more males than controls without the pheromone. The attractiveness of PE lures pre-aged 20 days was not significantly different from that of the controls without pheromone (Fig. 11). The year of lure preparation (2008 or 2009) had no effect and resulted in nearly identical trap catches, 124.0 ± 10.1 males for the 2008 lure treatment versus 118.2 ± 15.9 males for the 2009 lure treatment (mean for 2008 lure not shown in Fig. 11).

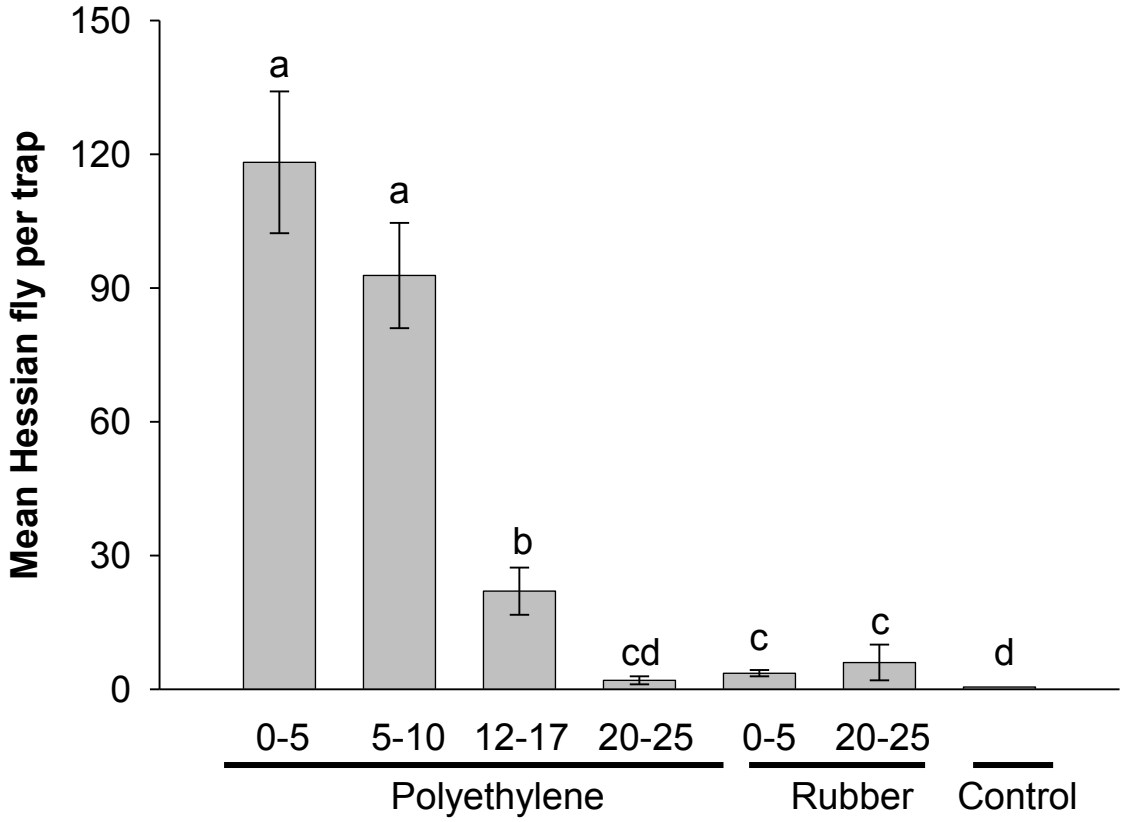

Fig. 11. Mean number of male Hessian flies (± SEM) captured during the sex pheromone longevity study at Langdon, ND. Two dispenser types, polyethylene (PE) and rubber septa (rubber) were compared over four age ranges, 0-5 days, 5-10 days, 12-17 days and 20-25 days old. A trap without a sex pheromone lure served as the control. Means accompanied by the same letter are not significantly different (Tukey-Kramer HSD, P>0.05).

Optimal Height of Traps. The number of male Hessian flies captured (Fig. 12) was influenced by trap height ($F_{3, 12}$=15.96, P=0.0002) but not by the effect of blocking ($F_{4, 12}$=3.03, P=0.061). Traps that were placed within the crop canopy (which was ca. 90 cm tall) at 15, 30 and 60 cm, were similar in flies captured (Fig. 12). Traps placed at 120 cm, approximately 30 cm above the crop canopy, captured only one male.

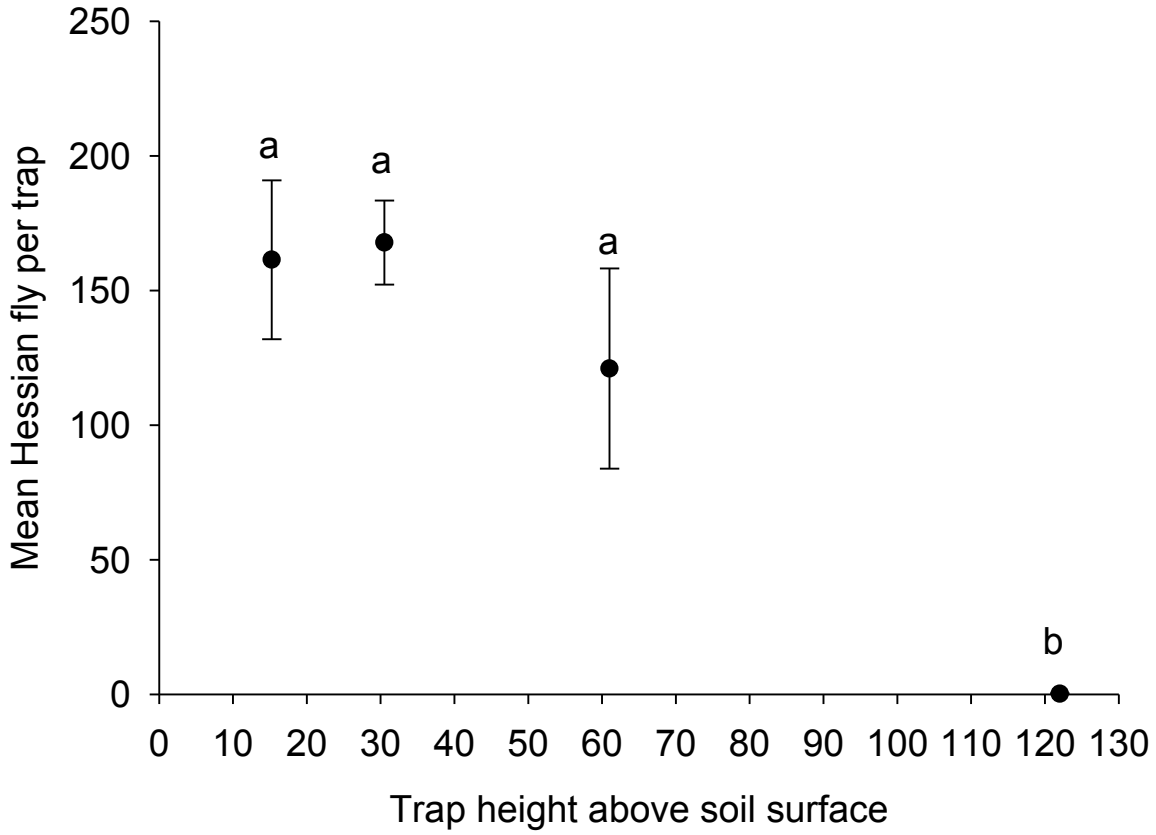

Fig. 12. Mean number of male Hessian flies (± SEM) captured when sex pheromone traps were placed at different heights in a wheat field (post-anthesis) in Langdon, ND. Means accompanied by the same letter are not significantly different (Tukey-Kramer HSD, P>0.05). The average height of the crop canopy was 90 cm.

Pheromone Specificity. Pheromone traps attracted only male Hessian flies (Fig. 13). When the paired traps with and without the sex pheromone were deployed at the six state-wide locations in 2009, significantly more Hessian fly males were attracted to the pheromone traps than the control traps ($F_{1, 750}$=237.95, P < 0.0001) with only six male Hessian flies collected in the control traps. In contrast, Hymenoptera, and other dipterans that might be confused with the Hessian fly (Suborder: Nematocera) (Fig. 13), were captured equally by the pheromone and control traps (Hymenoptera $F_{1, 750}$=0.047, P=0.493; Diptera $F_{1, 750}$=0.01, P=0.903).

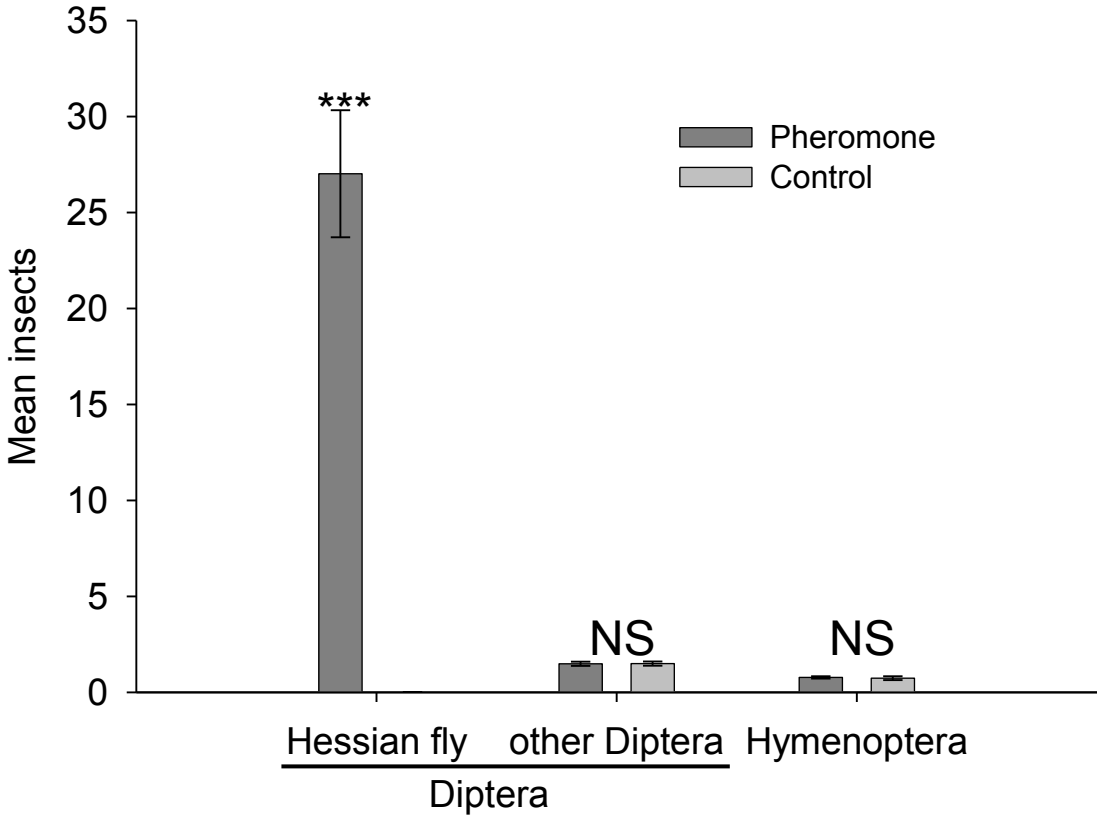

Fig. 13. Insects captured at six North Dakota sites (2009) in traps baited with the Hessian fly sex pheromone (dark bar) versus un-baited control traps (light bar). Data are given for male Hessian flies, flies in the sub-order Nematocera that might be confused with the Hessian fly and Hymenoptera, which could be parasitoids of the Hessian fly. The pair of bars that are accompanied by three asterisks are significantly different at $P < 0.001$.

Seasonal Abundance. Data from three seasons and the six trapping locations indicated that Hessian fly males were present throughout the growing season (Fig. 14). During the years of the study the Langdon location in northeastern North Dakota trapped the most flies (note scale for Langdon is different in Fig. 14). The Prosper location in southeastern North Dakota was next in abundance. Peak trap catches of male Hessian fly usually occurred in July (Fig. 14). However, peak dates as early as late June or as late as early September were not uncommon (Table 4). Hessian fly pheromone traps collected Hessian flies early in the spring (Table 4), often in the very first trap deployed that year.

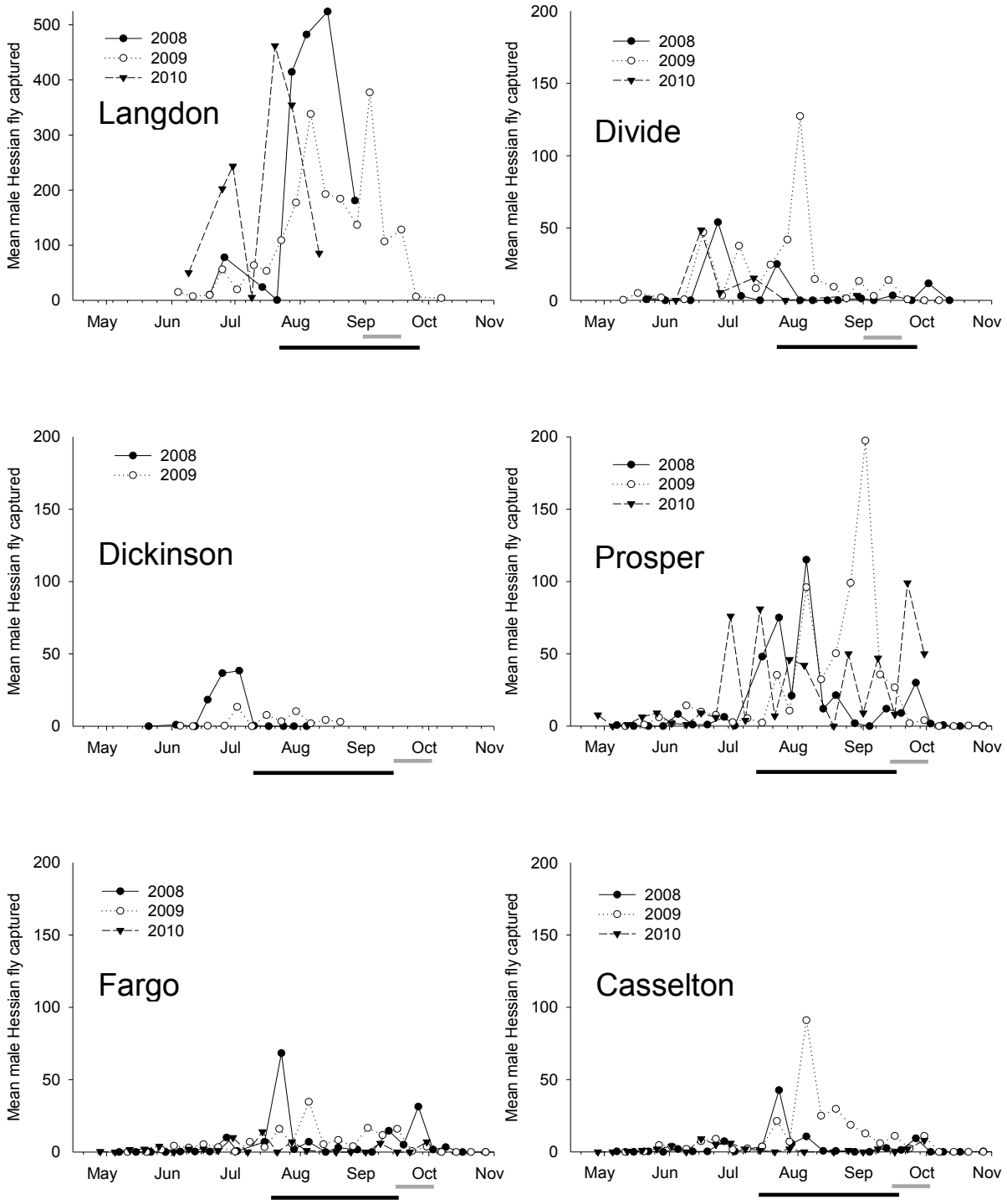

Fig. 14. Seasonal abundance of male Hessian flies at six North Dakota locations for 2008, 2009 and 2010. Black bars beneath the x-axis denotes the harvest period for spring wheat in North Dakota while the grey bar beneath the x-axis denotes the optimal planting period for winter wheat.

In Fig. 15 trapping data are shown in greater detail for the spring and fall. In the spring at the Fargo location (Fig. 15A) flies were found on June 5th in 2008, on May 27th in 2009, and on April 28th in 2010. In the fall at the Prosper location the latest fly captures occurred in late October (Fig. 15B).

Table 4. Dates for Hessian fly emergence events in 2008, 2009 and 2010 at six North Dakota locations determined by monitoring traps baited with a Hessian fly sex pheromone. Dates listed in the table represent the day the trap was collected after an average seven day trapping period. Under first and last Hessian flies captured, dates that appear in italics represent the first or last time the trap was placed in the field.

	First Hessian Flies Captured			**Peak Hessian Fly Emergence**			**Last Hessian Flies Captured**		
Location	**2008**	**2009**	**2010**	**2008**	**2009**	**2010**	**2008**	**2009**	**2010**
Prosper	*May 7*	May 20	*April 28*	Aug. 5	Sept. 2	July 14	Oct. 28	Oct. 21	*Sept. 30*
Casselton	*May 7*	May 20	May 12	July 23	Aug. 5	Aug. 19	Oct. 28	Sept. 30	*Sept. 30*
Fargo	June 5	May 27	*April 28*	July 23	Aug. 5	July 14	Oct. 9	Sept. 30	*Sept. 30*
Divide Co	*May 21*	*May 10*	*May 22*	June 24	Aug. 2	June 16	Oct. 2	Sept. 22	*Aug. 29*
Dickinson	June 3	*June 5*	NA	July 3	July 2	NA	July 10	*Aug. 20*	NA
Langdon	*June 19*	*June 4*	*June 9*	Aug. 4	Sept. 3	July 20	*Aug. 27*	*Oct. 7*	*Aug. 10*

Hessian Flies on Conservation Land. All sex pheromone baited traps on the North-South transect captured flies (Fig. 16). Over the entire season, the northern-most trapping site captured 305 Hessian flies, the middle site captured 97, and the southern-most site captured 260 Hessian flies. First collection of Hessian flies occurred on May 10th. Peak Hessian fly trapped coincided for all three sites in early August. The last Hessian flies collected at the CRP site were collected on October 7th.

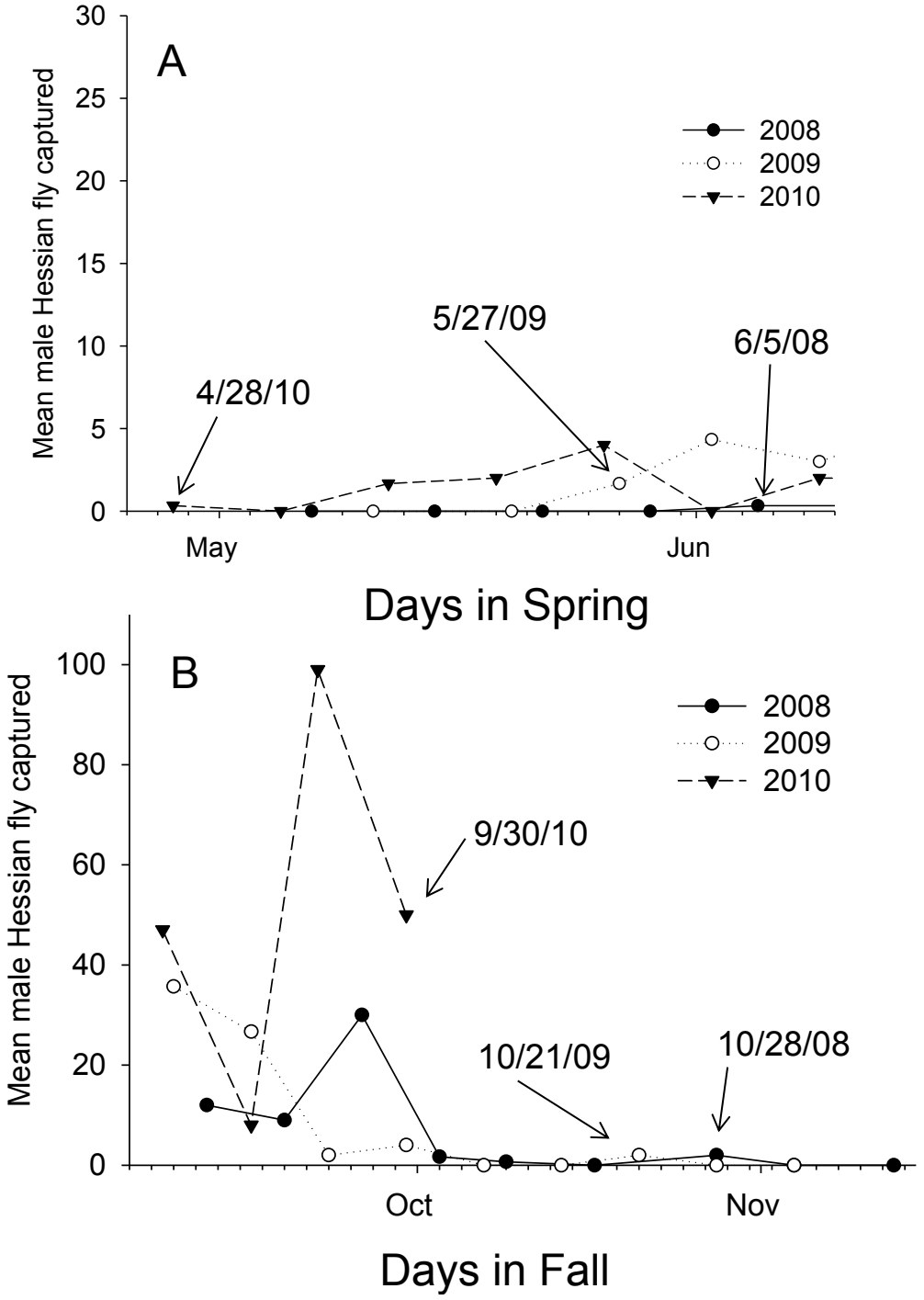

Fig. 15. Details of early and late captures of Hessian fly males. Mean number of male Hessian flies captured in (A) the spring of 2008, 2009 and 2010, at Fargo, ND and (B) in the fall of 2008, 2009 and 2010 in Prosper, ND. Dates and arrows indicate first day (A) and last day (B) male Hessian flies were captured in sex pheromone baited traps. Black bar under the x-axis on Fig. 15B denotes the suggested planting period for winter wheat in this part of North Dakota.

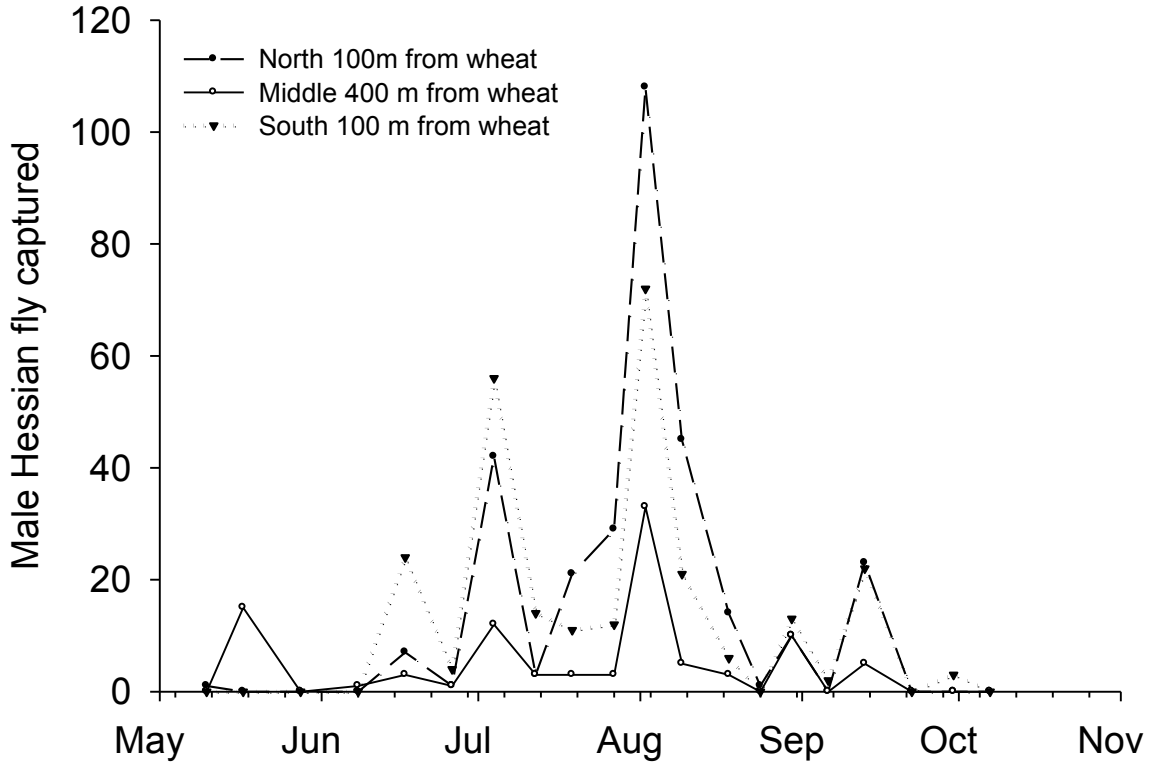

Fig. 16. Male Hessian flies captured in the 2009 growing season using sex pheromone baited traps on Conservation Reserve Program (CRP) land in Divide County, ND. The North to South running transect consisted of a north, middle and south trapping sites.

Discussion

My study provides methods for using the Hessian fly sex pheromone for population monitoring. I first determined how often lures need to be changed to continuously trap males. Lures were effective for up to 10 days, but after that their ability to attract males was greatly reduced (Fig. 11). It was hoped that rubber septa lures would provide a more long lasting release system than the polyethylene dispensers. Yet, rubber septa lures loaded with the sex pheromone barely attracted more males than the un-baited control. Why the rubber septa performed so poorly is unknown. Possibly the pheromone components were not released at a high enough

dosage. Thus loading the rubber septa with a higher dose might make them more attractive and longer lasting, as is done for some other gall midge species (Hall et al. 2012). On the other hand, the cost of this might be prohibitive.

Proper placement of traps relative to the crop is an important factor in the effective use of sex pheromones (McNeil 1991, Hall et al. 2012). My results demonstrated that even when Hessian flies are relatively abundant, as was the case at the Langdon site, an improperly placed trap will not catch males. While traps placed within the crop canopy at 15, 30, and 60 cm captured similar numbers of male Hessian flies, traps placed 30 cm above the canopy (at 120 cm) trapped almost nothing (Fig. 12). The maturity of the wheat field will be important for trap placement. In my test the wheat plants were at or near their maximum height (90 cm). Thus, the pheromone traps at 120 cm were the only traps not sheltered by the crop canopy. My recommendation is to place pheromone traps at 30 cm above the soil preferably in a sheltered location (i.e. within the crop canopy or in a grassy field margin). At 30 cm the traps will be low enough to be in the zone where male flight occurs, but also high enough to not have wind-blown dirt and debris fill the sticky surface of the trap. A sheltered microclimate is important. The Hessian fly is not a strong flier (Withers and Harris 1997). The observation that gall midges fly near ground level or within the crop canopy has also been made for other gall midges (Wall et al. 1991, Pivnick 1993, Hillbur et al. 2005, Cross and Hall 2009).

Together my studies in wheat fields in North Dakota and the original study in harvested wheat fields in Kansas (Andersson et al. 2009) demonstrate that pheromone trapping is a reliable and easy method for monitoring Hessian fly populations. Hessian fly males responded readily to the five-component pheromone lure, as demonstrated by the capture of males at all locations in North Dakota during the three years of my study (Fig. 14). The overwhelming number of males

captured in pheromone traps (nearly 11,000) as compared to the small number of males captured in un-baited control traps (six) confirms that the pheromone is attracting the Hessian fly (Fig. 13) and that very few males end up in traps as a result of random encounters. One thing that complicates monitoring with pheromones is the presence of insects on the sticky card that can be confused with the targeted insect. Insects that could be confused with the Hessian fly were not found in pheromone traps deployed in North Dakota (Fig. 13) or Kansas (Andersson et al. 2009). There also was no indication (Fig. 13) that the sex pheromone attracts egg parasitoids (e.g. *Platygaster hiemalis* Forbes, Barnes 1956), which are plentiful in North Dakota. This can occur in host-parasitoid interactions if the sex pheromone comprises a reliable signal to parasitoids for the presence of host eggs (Zuk and Kolluru 1998). This is not the case for the Hessian fly inasmuch as mating and oviposition can take place in very different locations (Harris and Foster 1999).

Sex pheromone trapping was useful for documenting Hessian fly phenology, including precise information on when egglaying takes place. This is because eclosion of adult males and females is synchronized, mating follows soon after eclosion, oviposition begins 1-2 hours later, eggs are deposited on plants within a period of 3-4 hours, and most adults are dead within 24 hours of eclosion (Gagné 1989, Harris and Foster 1999, Harris et al. 2003). Initial appearance of adult males in the spring was variable, ranging from late April to early June (Table 4). Trapping in Fargo typified this variability (Fig. 15A). Males appeared as early as April 28th in 2010 (the first date a trap was placed in the field) and as late as June 5th in 2008 and May 27th in 2009 (not the first date for trap placement). This coincided with year-to-year variation in the arrival of spring in the Upper Great Plains (Table 5).

Table 5. Average monthly soil temperatures for the 2008, 2009 and 2010 growing seasons at six locations used for trapping Hessian flies. Soil temperature data retrieved from North Dakota Agricultural Weather Network (NDAWN) weather stations.

Average monthly soil temperature (ºC) 2008[a]							
Location[b]	**April**	**May**	**June**	**July**	**Aug.**	**Sept.**	**Oct.**
Divide Co	4.2	11.1	16.9	23.1	22.2	13.7	6.1
Dickinson	7.8	13.6	18.7	25.2	24.3	16.0	7.2
Fargo	3.6	11.3	17.1	22.1	22.0	16.0	8.5
Langdon	3.0	9.3	16.2	21.1	20.9	14.4	6.8
Prosper & Casselton	4.1	13.2	18.6	24.7	24.0	16.3	8.6
Average monthly soil temperature (ºC) 2009 [a]							
Location[b]	**April**	**May**	**June**	**July**	**Aug.**	**Sept.**	**Oct.**
Divide Co	4.0	11.5	17.9	20.5	19.5	18.8	4.1
Dickinson	4.8	14.1	18.9	22.7	21.7	19.6	4.4
Fargo	3.3	10.7	17.2	20.7	20.3	19.2	6.3
Langdon	1.3	8.0	15.1	19.4	18.6	18.0	4.8
Prosper & Casselton	3.2	11.7	20.0	24.1	21.3	19.0	6.0
Average monthly soil temperature (ºC) 2010 [a]							
Location[b]	**April**	**May**	**June**	**July**	**Aug.**	**Sept.**	**Oct.**
Divide Co	6.8	11.1	19.2	23.1	21.9	13.0	8.6
Dickinson	8.3	12.5	20.8	24.5	24.6	14.3	10.7
Fargo	8.4	13.2	19.1	23.5	23.6	14.8	10.4
Langdon	6.1	11.4	17.9	22.4	21.3	12.6	8.8
Prosper & Casselton	10.8	14.8	21.5	24.8	24.1	14.9	10.8

[a] Soil temperature measured on bare soil at a depth of 10 cm.

[b] The Langdon and Prosper locations were approximately 7 miles apart, weather data from the Prosper site was used for the Langdon site as well.

While soil moisture was abundant in all years of the study, the spring of 2010 was noteworthy for arriving early, with the growing season continuing to be warmer than average.

Fargo's average April soil temperature was 8.4º C in 2010, in contrast to 3.6º C and 3.3º C in 2008 and 2009, respectively (Table 5). Spring emergence of the Hessian fly depends on both temperature and moisture conditions (Cartwright 1922, McColloch 1923, Barnes 1956, Wellso 1991).

The large increases in trap catches that took place from the first brood (April/May/June) to the second brood (July/August) indicate that the Hessian fly has significant potential for population growth, this being especially true in northeastern North Dakota at Langdon (Fig. 14). The peak occurring in mid-summer usually was the largest (Table 4). This peak was delayed in 2009, the year that experienced the coldest growing season (Tables 4 and 5). I found no evidence of a generation that aestivates during the hottest part of the summer, a phenomenon that occurs in populations to the south (Wellso 1991). This finding generally agrees with the suggestion that summer aestivation of Hessian fly populations only occurs below 50º N latitude (Criddle 1915, Wellso 1991). My study sites ranged from 47º to 49º N latitude.

Peak eclosion of Hessian fly adults is poorly timed relative to susceptibility of crop hosts, being too late to impact cereals sown in the spring, and too early to affect winter wheat sown in the autumn. Female Hessian flies prefer to oviposit on the young leaves of seedlings or tillers, with the early stages of stem elongation being a second-best option (Barnes 1956, Harris et al. 2003). Hessian fly larvae, being gall-makers (Harris et al. 2006), have specific requirements for colonization, requiring relatively undifferentiated epidermal cells that can be manipulated to form nutritive gall tissue. They are not found attacking wheat seed heads. The timing of peak adult eclosion relative to crop development could explain why, despite tremendous numbers of flies in traps at Langdon at mid-summer (Fig. 14, 703 males trapped from 7/21-7/28/2008), no crop loss was reported.

What then are Hessian fly females laying eggs on during peak emergence in mid-summer? Late-planted wheat and volunteer wheat can serve as hosts for flies in July and August (Barnes 1956). Native and introduced grasses other than wheat also may serve as hosts. The Hessian fly can reproduce successfully on grasses belonging to 17 genera and two tribes, Triticeae and Bromeae (McColloch 1923, Jones 1936, Barnes 1956, Harris et al. 2003). In North Dakota, native and introduced grasses are often found growing in close proximity to wheat fields. Native grasses, such as Canada wildrye, *Elymus canadensis* (L.), and western wheatgrass, *Pascopyrum smithii* (Rydb.), and introduced grasses such as intermediate wheatgrass, *Thinopyrum intermedium* (Host), and crested wheatgrass, *Agropyron cristatum* (L.), are common in pastures and are known hosts of the Hessian fly (Jones 1936).

The idea that populations use non-crop grasses as hosts was supported by the discovery of Hessian flies on Conservation Reserve Program (CRP) land. Currently North Dakota has nearly 1 million hectares of CRP (USDA-FSA 2011), making it a significant part of the agricultural landscape. At the time of my study in 2009, the CRP trapping site in northwestern North Dakota (Divide County) had been enrolled continuously for almost twenty years. Given the distance between the nearest wheat field and the 'middle' trapping site on CRP land (Fig. 16, 400 meters), it seems unlikely that the captured males were migrants from wheat fields. Male gall midges move very little except when stimulated by the female's sex pheromone (Bergh et al. 1990, Harris and Foster 1999). Moreover, the majority of the flies collected in the early spring were captured at the 'middle' site, which was at the greatest distance from neighboring wheat fields (Fig. 16). Crested wheatgrass, a known host of the Hessian fly (Jones 1936), is one of the dominant species in this CRP planting. The use of non-crop grasses growing in pastures and

CRP, as well as along roadsides, is important because it means that the Hessian fly has refuges where populations can be maintained at low levels until superior crop hosts become available.

Hessian fly activity in the autumn suggests that, should winter wheat become a more important crop in the Upper Great Plains, the Hessian fly will be there to attack it. The last males that we captured were found in traps deployed in late September into October (Table 4), but in many cases this was the last trapping date of the season. This means that I have probably underestimated the extent of Hessian fly activity late in the season. At the Prosper location (Fig. 15B), the last fly was collected on Oct. 28th, Oct. 21st and Sept. 30th in 2008, 2009 and 2010, respectively. The presence of adults this late in the season raises the question: are their offspring capable of developing to the required stage before winter comes? The Hessian fly overwinters as a non-feeding third instar larva, which is encased in a puparium referred to as a 'flaxseed' (Gagné 1989). Studies on thermal requirements indicate that Hessian fly eggs oviposited at the onset of cold weather may not develop (Foster and Taylor 1975, Buntin and Chapin 1990). However, the discovery of 2nd instar larvae attacking volunteer wheat on October 18th, 1946 near New England, North Dakota (Butcher 1946), provides anecdotal evidence that, if host plants are available and the weather is mild, a late overwintering generation can develop on wheat. The presence of a late generation is a concern for farmers growing winter wheat. In North Dakota the recommended planting time for winter wheat is from September 1-15 in the northern half of the state and from September 15-30 in the southern half of the state (Peel and Riveland 1997). Based on trapping data and the rate at which seedlings grow, it is likely that Hessian fly adults emerge late enough in the autumn to attack winter wheat in the Upper Great Plains.

I began by asking whether the Hessian fly is a sufficient threat to consider breeding *H*-gene-mediated resistance into regionally adapted elite cultivars. The answer 'yes' is based on the

Hessian fly's wide distribution in North Dakota, presence throughout the growing season, and ability to greatly increase numbers in a single generation, coupled with the state's changing cropping systems and expanding growing season due to climate change (Badh et al. 2009, Dunnell and Travers 2011). It should be noted that determining threats from the Hessian fly would have been extremely difficult without the recently identified Hessian fly sex pheromone (Harris and Foster 1999, Andersson et al. 2009). In the past, estimates of Hessian fly populations have come from the destructive sampling of thousands of wheat plants, the aim being to find tiny larvae, which are hidden within the encircling leaf sheaths at the base of the plant (Berzonsky et al. 2003). Having a pheromone trapping methods eliminates this tedious sampling of wheat plants and also allows populations to be monitored when wheat is not present, i.e. to sample populations on non-crop hosts.

Sex pheromone trapping also will benefit the next step towards durable plant resistance. This is a determination of the virulence status of the region's populations to the currently available set of *H* resistance genes (Porter et al. 2009). In a recent study (Cambron et al. 2010), virulence frequencies for southeastern Hessian fly populations were scored by laborious rearing of numerous Hessian fly populations on a variety of wheat genotypes, each carrying a single *H*-gene. But now that the Hessian fly *Avirulence* (*Avr*) genes matching the *H* resistance genes are being identified (Stuart et al. 2012), it is expected that DNA tests will streamline the scoring of virulence in the future. The role the sex pheromone will play in this new technology is to make it easier to find the Hessian flies that are subjected to the DNA testing.

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CHAPTER 4. EXPLORING VIRULENCE TO *H* RESISTANCE GENES IN A NORTH DAKOTA HESSIAN FLY POPULATION

Abstract

The most successful strategy for managing Hessian flies, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), has been through the use of wheat (*Triticum* spp.) cultivars bred for resistance. Currently there are 33 different Hessian fly resistance genes in wheat. To assure that the most effective *H*-genes are being deployed in an area, regional Hessian fly populations are assessed for virulence. My study is the first to provide this information for a population of Hessian fly from the Upper Great Plains. Through the use of traditional biotyping and an assay of all available *H*-genes, virulence frequencies within a North Dakota Hessian fly population were established. Virulence frequencies were higher than was expected, with traditional biotyping using four *H*-genes revealing 13 of the 16 possible Hessian fly biotypes. Biotyping using 30 *H*-genes showed that only a few gave 100% protection. Why this virulence exists in a Hessian fly population that has never knowingly been exposed to *H*-genes is difficult to say. Accidental deployment of *H*-genes into the regional agro-ecosystem, environmental factors affecting *H*-gene expression, and virulence crossing from wild hosts of Hessian fly are possible factors. In addition to providing virulence information I expanded my studies further and explored aspects of the Hessian fly-wheat interaction, providing details on the fate of both the Hessian fly and the wheat plant that have not been examined by other research on Hessian fly virulence.

Introduction

The Hessian fly*, Mayetiola destructor* (Say), was introduced into the United States in the late 1700's at the time of the American Revolution (Pauly 2002). Since that time the Hessian

flies has spread to nearly all wheat, *Triticum aestivum* L., growing regions of the country. The presence of Hessian fly in the Upper Great Plains has been known for nearly 100 years (Webster 1915). Outbreaks were reported in South Dakota in 1978 (Walgenbach et al. 1978), and in North Dakota in 1991 and 2003 (Nelson et al. 1991, Glogoza 2004). Recently, a survey of stem-feeding insects of wheat revealed that 38% of fields in an area encompassing North and South Dakota, Montana, Nebraska and Wyoming were infested with Hessian flies (Shanower and Waters 2006). Despite these indicators many people still question if the Hessian fly is a sufficient threat to breeding resistant wheat in the Upper Great Plains. The recent introduction of a Hessian fly sex pheromone lure (Andersson et al. 2009) has aided in answering this question. A study using this pheromone lure was conducted in North Dakota from 2008 to 2010 (Anderson et al. 2012). This research determined that Hessian flies were distributed statewide, trapped throughout the growing season, and present late into the autumn when winter wheat seedlings were available to ovipositing females (Anderson et al. 2012). Furthering the pest potential of the Hessian fly is the changing agricultural landscape in the Upper Great Plains. One factor contributing to this change is a longer growing season (Dunnell and Travers 2011, Badh et al. 2009), which creates the potential for additional generations of Hessian fly and more rapid population growth. A second factor is rapidly changing cropping systems that have resulted in an increase in the acreage of winter wheat grown in the Northern Plains (Ortiz et al. 2008). Increased acreage is important as winter wheat is a superior overwintering host of Hessian fly relative to wild grass hosts. Altogether, there is a strong case that the Hessian fly is a credible threat to wheat production in the Upper Great Plains and that wheat grown in this region would benefit from resistance to Hessian fly.

Plant resistance has been the most successful strategy for managing Hessian flies (Ratcliffe and Hatchett 1997, Berzonsky et al. 2003, Harris et al. 2003). Hessian fly larvae attacking resistant wheat die within 3-5 days and do not grow before dying (Harris et al. 2010). Currently there are 33 identified *H*-genes (Table 6). The sources of Hessian fly resistance are numerous including *Triticum aestivum* L., *T. turgidum* L. ssp. *durum*, *Aegilops tauschii* (Cosson), and *Secale cereale* L. (Ratcliffe and Hatchett 1997).

Table 6. Hessian fly resistance genes numbered *H1-H32,* accession names, and corresponding chromosome location (Ratcliffe and Hatchett 1997, Tan et al. 2013).

H-gene	Source of *H*-gene	Accession Name	Chromosome location
H1/H2	*Triticum aestivum*	Dawson	Unknown
H3	*Triticum aestivum*	Carol	1A
h4	*Triticum aestivum*	Java	Unknown
H5	*Triticum aestivum*	Erin	1A
H6	*Triticum turgidum* ssp. *durum*	Flynn	1A
H7/H8	*Triticum aestivum*	Seneca	5D
H9	*Triticum turgidum* ssp. *durum*	Iris	1A
H10	*Triticum turgidum* ssp. *durum*	Joy	1A
H11	*Triticum turgidum* ssp. *durum*	Karen	1A
H12	*Triticum aestivum*	Lola	1A
H13	*Aegilops tauschii*	Molly	6D
H14	*Triticum turgidum* ssp. *durum*	921676A3-5	1A
H15	*Triticum turgidum* ssp. *durum*	81602C5-3-3-8-1	1A
H16	*Triticum turgidum* ssp. *durum*	921682A4-6	1A
H17	*Triticum turgidum* ssp. *durum*	921680D1-7	1A
H18	*Triticum turgidum* ssp. *durum*	Marquillo	2B
H19	*Triticum turgidum* ssp. *durum*	84702B14-1-3-4-3	1A
H20	*Triticum turgidum* ssp. *durum*	Jori	2B
H21	*Secale cerale*	Hamlet	2B
H22	*Aegilops tauschii*	KS85WGRC01	1D
H23	*Aegilops tauschii*	KS89WGRC3	6D
H24	*Aegilops tauschii*	KS89WGRC6	3D
H25	*Secale cerale*	KS92WGRC17	6B
H26	*Aegilops tauschii*	KS92WGRC26	3D
H27	Renumbered *H29*		
H28	*Triticum turgidum* ssp. *durum*	PI59190	1A
H29	*Triticum turgidum* ssp. *durum*	PI422297	1A
H30	*Aegilops triuncialis*	TR-3531	Unknown
H31	*Triticum turgidum* ssp. *durum*	921696A1-15-2-1	5B
H32	*Aegilops tauschii*	W-7984	3D
H. dic	*Triticum turgidum* ssp. *dicoccum*	KS99WGRC42	1A

The long-term practice for utilizing *H*-genes has been to deploy a single *H*-gene in a single wheat cultivar which is planted on a large scale. While this tactic has given good short-term protection (6-8 years), it has also places selection pressure on Hessian fly populations, which leads to adaptation to the *H*-gene (Foster et al. 1991, Ratcliffe et al. 1994, Chen et al. 2009). Virulence in the Hessian fly population is conferred by a mutation in the Hessian fly's *Avirulence* (*Avr*) gene (Stuart et al. 2012). The Hessian fly's avirulence gene functions as part of a gene-for-gene interaction that states that for every *Resistance* (*R*) gene in a plant, there is a corresponding *avr* gene in the insect parasite (Flor 1955). Avirulence proteins function as elicitors of plant resistance but have a different function in the insect acting as virulence effectors. Effectors are defined as parasite proteins and molecules that alter host-cell structure and function, helping the parasite modulate plant development (Hogenhout et al. 2009). A mutation in the *avr* gene means that the insect no longer elicits a resistance response from the plant (Bent and MacKey 2007). The insect lacking the *avr* effector could pay a fitness cost by not being able to as effectively colonize the plant (Zhang et al. 2011, Stuart et al. 2012).

To assure that the most effective *H*-genes are deployed in an area, regional Hessian fly populations are assessed for virulence prior to choosing the *H*-gene. Traditionally this has been done by evaluating the response of individual Hessian fly females to the four wheat varieties that contain the *H3*, *H5*, *H6* and *H7/H8* resistance genes (Gallun 1977, Ratcliffe et al. 1994, 1996, 1997, 2000). These four cultivars were selected because at the time they were the only Hessian fly *H*-genes being utilized (Gallun 1977). The combinations of resistant and susceptible reactions displayed by the differential set creates a total of 2^4 or 16 possible biotype combinations (Table 7), the 16 biotypes are labeled GP (Great Plains) and the remaining 15 by the letters A to O

(Gallun 1977). In the years since the 16 Hessian fly biotypes were designated, the number of Hessian fly *H*-genes have been identified. This increase in *H*-genes has made the labor-intensive traditional method unfeasible and somewhat obsolete as it provides no knowledge of the effectiveness of the newer more recently released H-genes (Chen et al. 2009, Cambron et al. 2010). If the set of *H*-genes used for biotyping was expanded to the 33 currently identified genes, this would create 2^{33} or 8.6 X 10^{9} possible biotypes. To address this situation the traditional biotyping protocol has either been modified with additional *H*-genes (Chen et al. 2009), or eliminated altogether in favor of a virulence analysis of selected groups of *H*-genes evaluated against a population of Hessian flies (Ratcliffe et al. 1996, 2000, Chen et al. 2009, Cambron et al. 2010).

To my knowledge no population of Hessian flies from the Upper Great Plains has ever been evaluated for virulence to the Hessian fly *H*-genes. To fill in this missing piece of information, I used the traditional biotyping protocol to ask: Is there virulence in a North Dakota Hessian fly population to the four differentials (*H3*, *H5*, *H6* and *H7/H8*) used for traditional biotyping? I then developed the study further by evaluating the efficacy of all available Hessian fly *H*-genes (n=30) when tested with a North Dakota Hessian fly population. I asked: Which are the most effective *H*-genes for this region? Which are the least effective *H*-genes? Since I also measured insect and plant survival and growth, I was able to explore the consequences of Hessian fly adaptation for Hessian fly fitness and plant growth.

Methods and Materials

Insects. The colony originated from approximately 500 gravid females collected in 2003 from an infested spring wheat field at the North Dakota State University Research Site (47º 0’2” N, 97º 7’12” W) located near the town of Prosper, North Dakota. At the time the tests were

conducted, the greenhouse population had been in culture less than five generations. Insects were reared under semi-natural conditions in the greenhouse on susceptible hard red spring wheat 'Reeder'.

Plants. Plants used in experiments were grown either in a greenhouse or in a plant growth chamber. Individual seeds were planted in Ray Leach Conetainers™ (4 cm diam x 21 cm deep, Stuewe & Sons, Inc., Corvallis, WA) held in racks (RL98). Plants grew in potting media (SB100 Professional Growing Mix, Sungro Horticulture, Bellevue, WA) and were watered daily and fertilized weekly (Jack's Professional 20:20:20 N:P:K Fertilizer®, J.R. Peters Inc., Allentown, PA). In the greenhouse, plants were maintained at 20º ± 2º C, with an ambient relative humidity (30-60% RH) and a 16:8 light-dark photoperiod. The natural light was supplemented by high-pressure sodium lamps. In plant growth chambers, plants were maintained at 20º ± 2º C, with an ambient relative humidity (30-60% RH) and a 16:8 light-dark photoperiod. Lighting was provided by cool white fluorescent lamps.

Traditional Hessian fly Biotyping (Four Differential Cultivars). The four cultivars routinely used as a differential set for evaluating biotype composition of Hessian fly populations, Monon (*H3*), Magnum (*H5*), Caldwell (*H6*) and Seneca (*H7/H8*) were obtained from the USDA-ARS National Small Grains Collection at Aberdeen, Idaho. The four differential cultivars and the susceptible genotype Newton were initially grown in the greenhouse. At the 2-leaf stage seedlings were moved to a plant growth chamber, where I tested 10 females at a time. The test arenas were constructed from a 30.5 cm diameter x 27 cm deep plastic greenhouse pot containing potting soil. Two plants of each of the four differential cultivars, plus two plants of the susceptible genotype Newton, were placed in the arena (total of 10 plants). The plants were arranged in the pot so they formed a circle at the perimeter of the arena with plants of the same

cultivar placed on opposite sides of the circle. Most of the length of each Conetainer™ was buried in loose potting soil with only the seedling exposed. The test arena was covered with a 23 cm high cylindrical cover made of clear acetate topped with fine nylon mesh. The plants were exposed to a single gravid female for twenty-four hours and then examined for the presence of eggs. Three days after oviposition, the humidity in the plant growth chamber was increased to facilitate egg hatch and larval migration down the leaf lamina to attack sites at the base of the plant. After egg hatch, plants were examined again and scored for eggs that did not hatch and larvae that died during migration.

In order to establish the female's biotype at least one plant of each of the differential cultivars had to be attacked by larvae. If any of the differential cultivars failed to be attacked, that female was removed from any further evaluation. Twenty one days after exposure to the adult female, the general health and appearance of the plants was qualitatively assessed by visual examination and any symptoms of Hessian fly injury were noted. Immediately after assessing the plant's appearance single plants of each differential cultivar and Newton were dissected. With the aid of a stereo microscope, the number and size (small, medium, large) and of larvae were recorded. Also at 21 days the remaining differentials and Newton were removed from their potting soil, trimmed of their leaves and roots and placed in 10 dram snap-cap vials to capture any emerging adults. The vials were kept in a plant growth chambers at 20º ± 2º C, with an ambient relative humidity (30-60% RH) and a 16:8 light-dark photoperiod. For the next 30-40 days, individual vials were checked daily for adult emergence and adults were placed in vials of 85% ethanol. The biotype of an individual female was determined by assessing the resistant/susceptible reaction of the plants based on the information gathered on plant appearance, the results of the plant dissections and the emergence of Hessian fly adults.

My biotyping procedure differs from the traditional protocol (Ratcliffe et al.1994) in a few ways. By having two plants of each genotype I was able to dissect one plant to look for living and dead larvae and save the other plant to see if adult flies would emerge. I also included the susceptible genotype Newton to serve as a control.

***H*-Gene Virulence Assay.** Soft white winter, soft red winter, hard red winter, hard red spring and durum wheat cultivars with *H*-genes conferring resistance to Hessian flies were obtained from Kansas State University, Purdue University, the USDA-ARS Crop Protection and Pest Control Research Unit at Purdue University, and the USDA-ARS National Small Grains Collection at Aberdeen, Idaho (Table 6). The cultivars that were evaluated have *H*-genes numbered *H1* through *H32* with the exception of *H30*, which was unavailable, and *H27*, which had been renumbered *H29*. In addition to the *H*-numbered genotypes, I also evaluated the susceptible hard red winter wheat Newton, un-numbered hard red winter wheat designated *H. dicoccum* with resistance derived from emmer wheat (*Triticum turgidum* ssp. *dicoccum*), and the resistant hard red winter wheat Kawvale whose genetic basis of resistance has not determined but may possess tolerance traits (Berzonsky 2003).

Each wheat genotype was subjected to two insect treatments: insect attack and no-insect attack. For each genotype in each experimental block, usually two seedlings were randomly assigned to the attacked treatment (2 plants x 31 genotypes = 62 attacked plants/block) and one seedling to the non-attacked treatment (1 plant x 31 genotypes = 31 control plants/block). This arrangement was then repeated for a total of 5 blocks. All seedlings in the experiment were treated the same except that attacked seedlings were exposed to gravid Hessian fly adult females (approximately one gravid female per seedling). Seedlings assigned to each treatment group were arranged in a completely randomized design (Steele et al. 1997). Two-leaf stage plants

assigned to the attacked treatment were exposed to Hessian fly females for twenty-four hours. Two-leaf stage plants assigned to the non-attacked treatment were kept free of Hessian fly eggs. The next day all attacked plants were examined for eggs. If a plant assigned to the insect attack treatment had no eggs it was discarded. The average number of eggs/plant for blocks 1 to 5 was 51.1 ± 3.4, 50.8 ± 5.3, 34.3 ± 6.8, 84.3 ± 5.7 and 71.2 ± 9.8 respectively. Three days after oviposition, all plants were moved to a high humidity (70-80% RH) growth chamber to facilitate egg hatch and larval migration on plants assigned to the insect attack treatment. After two nights in the high humidity chamber, the insect attack plants were examined again and scored for eggs that did not hatch and larvae that died during migration. Plants were removed from the experiment if it was determined that all larvae had died on the leaf lamina. All remaining plants were then returned to the plant growth chamber for two weeks, the time it took any virulent larvae to complete larval development. At the end of the two weeks, the plants were evaluated. Each leaf was measured from its base to the tip of the leaf blade. Each plant was then examined for stunting and other signs of Hessian fly damage, and was dissected with the aid of a stereo microscope for the presence of living and dead larvae at the base of the plant. At the time of my dissections, larvae that have successfully established on wheat plants should be molting to the third and final instar (Gagné and Hatchett 1989). All other small larvae were considered to be dead.

Statistical Analysis. Data were analyzed using JMP version 4 (SAS Institute 2001). Prior to ANOVA, homogeneity of variance was tested using O-Brien's test at $P < 0.05$. When variances were heterogeneous data were transformed and again tested. If variances were still heterogeneous a Welch ANOVA at $P < 0.05$ was used. Evaluations of larval mortality measured for the *H*-gene virulence assay (*H1-H32*) were made with the Wilcoxon/Kruskal-Wallis rank

sum test at $P < 0.05$ (SAS Institute 2001). The nonparametric Wilcoxon/Kruskal-Wallis test was used because the larval mortality data for the *H*-gene virulence assay was not normally distributed. Comparisons of plant growth on attacked versus non-attacked plants for the *H*-genes that had 100% larval mortality (*H4*, *H15*, *H21*, *H23*, *H26* and *H29*) were made using a t-test at $P < 0.05$ (SAS Institute 2001).

Results

Traditional Biotyping (Four Differential Cultivars). The biotype of 97 individual females of a North Dakota Hessian fly population were determined (Table 7).Thirteen of the 16 recognized Hessian fly biotypes were present in the North Dakota population. Great Plains (GP) was the most common biotype in the population making up 35% of the total (Table 7).

Table 7. Biotypes found in a North Dakota Hessian fly population based on individual females virulent/avirulent response to four wheat differentials (N=97). The reaction of the differential cultivars was scored either R (resistant) or S (susceptible) in response to attack by Hessian fly larvae.

	Differential Cultivars and *H* genes				
Biotype	Monon *H3*	Magnum *H5*	Caldwell *H6*	Seneca *H7/H8*	% in North Dakota population
Great Plains	R	R	R	R	35.1
A	R	R	R	S	4.1
B	S	R	R	S	1.0
C	R	R	S	S	11.3
D	S	R	S	S	2.1
E	S	R	R	R	3.1
F	R	R	S	R	24.7
G	S	R	S	R	6.2
H	R	S	R	R	4.1
I	R	S	R	S	2.1
J	S	S	R	S	0
K	R	S	S	S	0
L	S	S	S	S	0
M	S	S	R	R	1.0
N	R	S	S	R	3.1
O	S	S	S	R	2.1

The GP biotype is the least virulent strain and cannot survive on any of the *H*-genes present in the four differentials. Biotype F was the next most common biotype in the population. Biotype F has virulence to *H6* but is unable to survive on the other three differentials. Biotype L, the most virulent biotype capable of living on all the *H*-genes in the differential set was not present in the North Dakota population (Table 7). Within the North Dakota population *H6* was the resistance gene for which virulence was found most commonly (Fig. 17). Among the 97 females evaluated 48 produced offspring that were virulent on *H6*. Females in the North Dakota population were least likely to be virulent on *H5* (Fig. 17). Only 12 of the 97 females evaluated had offspring with virulence to *H5*.

Fig. 17. Number of female Hessian flies producing virulent offspring on the differential genotypes having the *H3*, *H5*, *H6* or *H7/H8* resistance genes (N=97).

A total of 424 adult offspring were produced by the 97 biotyped females. The susceptible Newton was responsible for 311 of the offspring, while the differential genotypes *H3*, *H5*, *H6* and *H7/H8* produced 10, 7, 54 and 42 offspring respectively. More females were produced than males, 248 females versus 176 males.

***H*-Gene Virulence Assay.** Larval mortality varied across the 30 resistant genotypes and susceptible Newton ($\chi^2_{30,123} = 101.03$; $P < 0.0001$). Genotypes with *H4*, *H15*, *H21*, *H23*, *H26* or *H29* resistance had 100% larval mortality (Fig. 18). The genotypes with *H14*, *H16*, *H17*, or *H18* resistance genes and the genotype Kawvale had less than 10% mortality (Fig. 18). The remaining 19 genotypes had larval mortality rates that ranged from 20% to over 90% (Fig. 18).

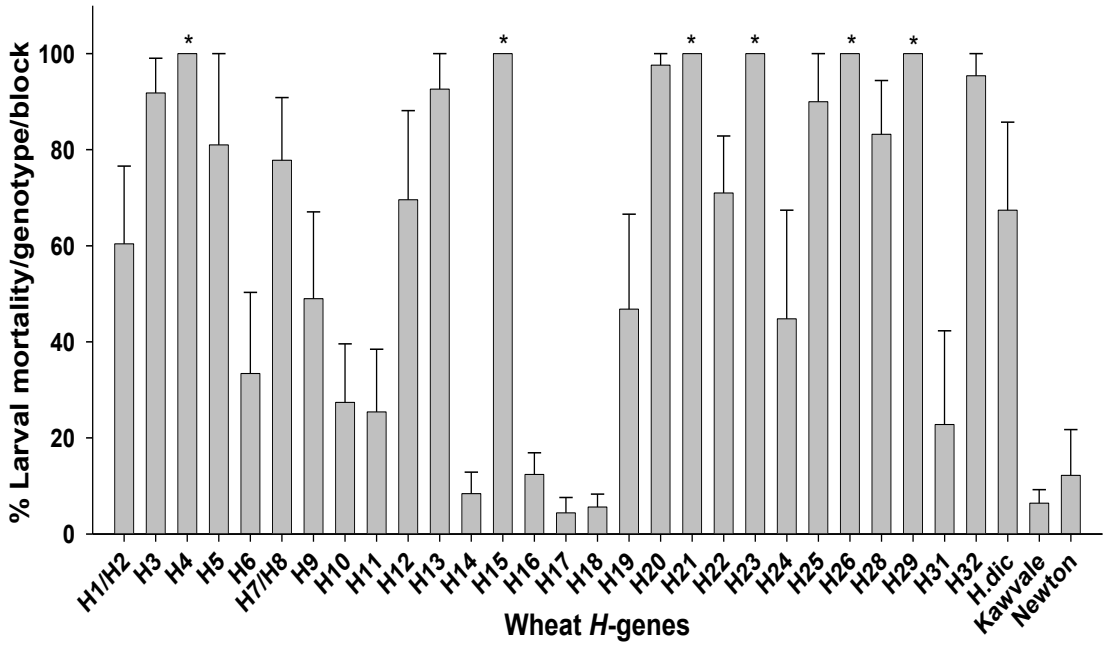

Fig. 18. Hessian fly larval mortality (±SEM) for 30 wheat genotypes with Hessian fly resistance and the susceptible Newton. For each genotype in each experimental block, two seedlings were randomly assigned to the attacked treatment (2 plants x 31 genotypes = 62 attacked plants/block). Means presented are the product of a study with five experimental blocks. The average larval mortality was calculated for each genotype for each block, therefore N=5. An asterisk shows which genotypes had 100 percent larval mortality.

The relationship between eggs and larval mortality (Fig. 19) was not significant ($F_{1,29}$ = 0.217; P = 0.645). The effectiveness of the *H*-gene at causing larval mortality explained less than one percent (R^2 = 0.007) of the variance in egg laying.

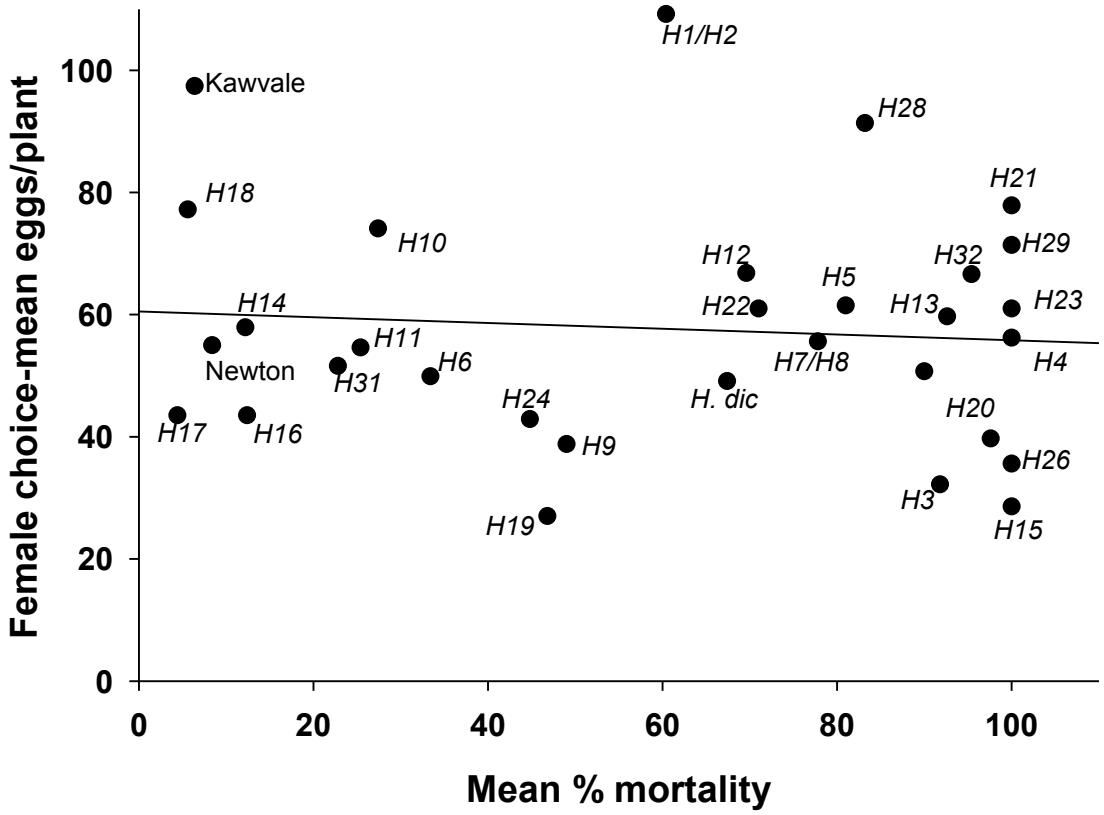

Fig. 19. Relationship between larval mortality per genotype and eggs laid per plant per genotype based on the female Hessian fly's choice of oviposition site. Means presented are the product of a study with five experimental blocks. The average eggs per plant and the average larval mortality was calculated for each genotype for each block, therefore N=5.

There was a negative relationship between the percent of larvae found at the time of plant dissection and percent larval mortality ($F_{1,29}$ = 142.76; P < 0.0001, Fig. 20). The mortality imposed on larvae by the 30 *H*-genes explained more the 80% of the variance in the percentage of larvae that were recovered (R^2 = .83).

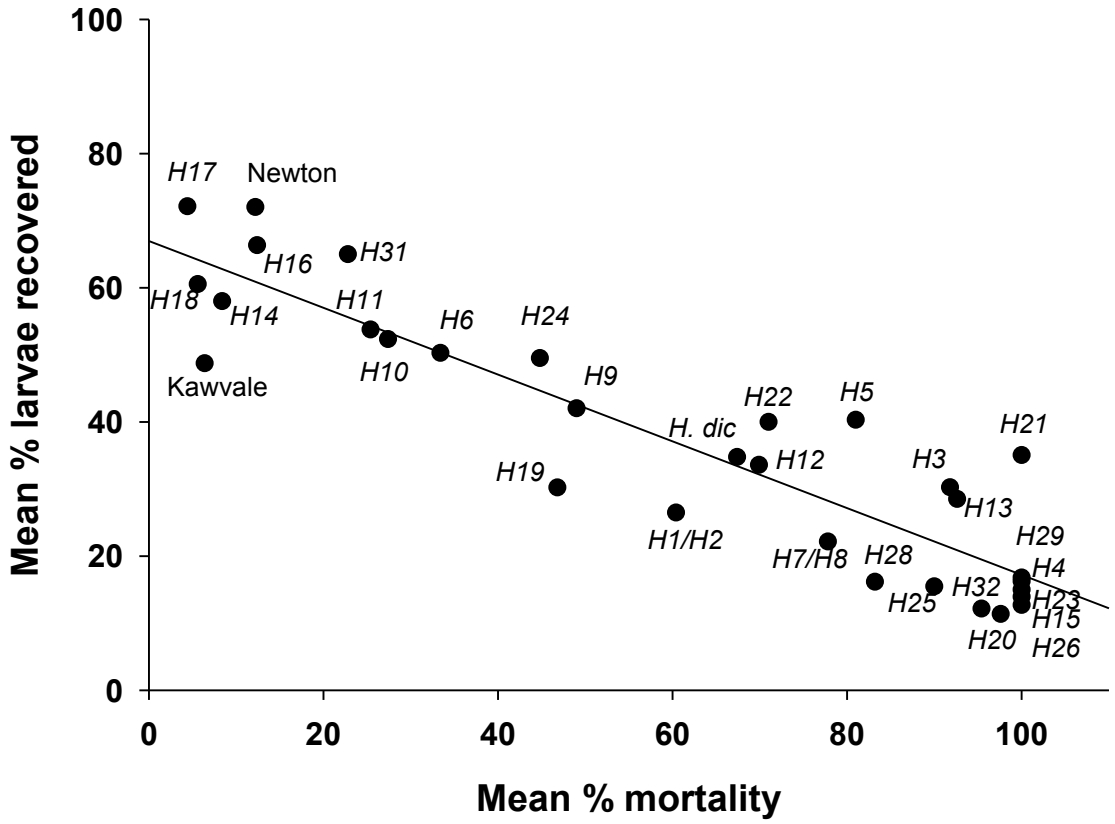

Fig. 20. Relationship between larval mortality per genotype and the total number of larvae recovered at the time the plants were dissected (two weeks after oviposition). Means presented are the product of a study with five experimental blocks. An average number of larvae recovered per plant and larval mortality was calculated for each genotype for each block, therefore N=5.

The growth of attacked plants was expressed as a percentage of non-attacked plant growth and compared to percentage larval mortality ($F_{1,29} = 379.49$; $P < 0.0001$, Fig. 21). Larval mortality explained more than 90% of the variance in plant growth ($R^2 = 0.93$). Growth deficits in the attacked plants were observed even for the *H*-genes that caused 100% larval mortality (Fig. 22). All six genes that caused 100% larval mortality exhibited a growth reduction in combined length of leaves three and four, i.e. the leaves that are most directly impacted by larval attack. However, among the six genotypes the growth differential between attacked and non-

attacked control plants was significant only for *H21* ($T_{1,15}$ = -2.362; *P* = 0.032) and for *H26* ($T_{1,13}$ = -2.510; *P* = 0.026).

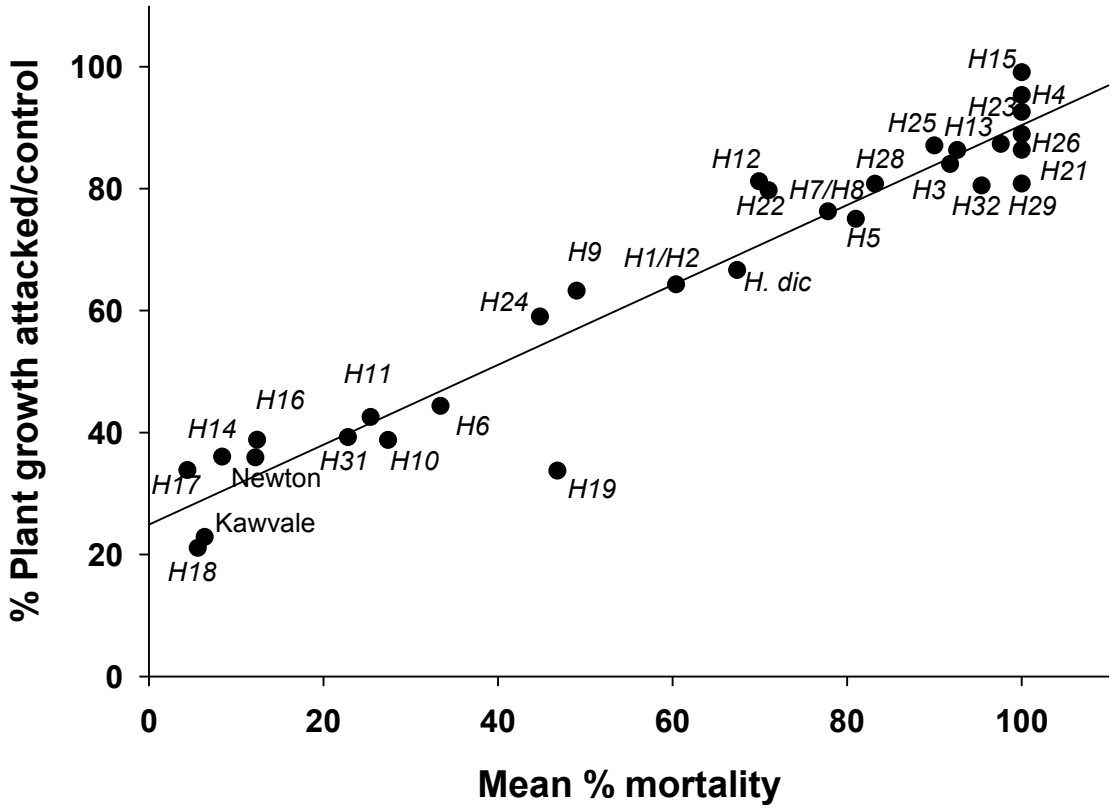

Fig. 21. Relationship between average larval mortality per genotype and the plant growth of attacked plants. Plant growth measurements were the combined lengths of leaf three and four. A % plant growth was calculated for each genotype by dividing the plant growth of attacked plants by the plant growth of non-attacked control plants. Means presented are the product of a study with five experimental blocks. The average larval mortality was calculated for each genotype for each block, therefore N=5.

Discussion

My study is the first to provide virulence information on Hessian flies living in the Upper Great Plains. Through the use of traditional biotyping and an assay of all available *H*-genes (n=30) we were able to assess the level of virulence within a North Dakota Hessian fly population. This information is essential if wheat breeding programs in the Upper Great Plains want to acquire and maintain durable resistance to the Hessian fly. I then expanded my studies further and explored aspects of the Hessian fly wheat interaction providing details on the fate of

both the Hessian fly and the wheat plant that have not been examined by other research on virulence in Hessian fly populations.

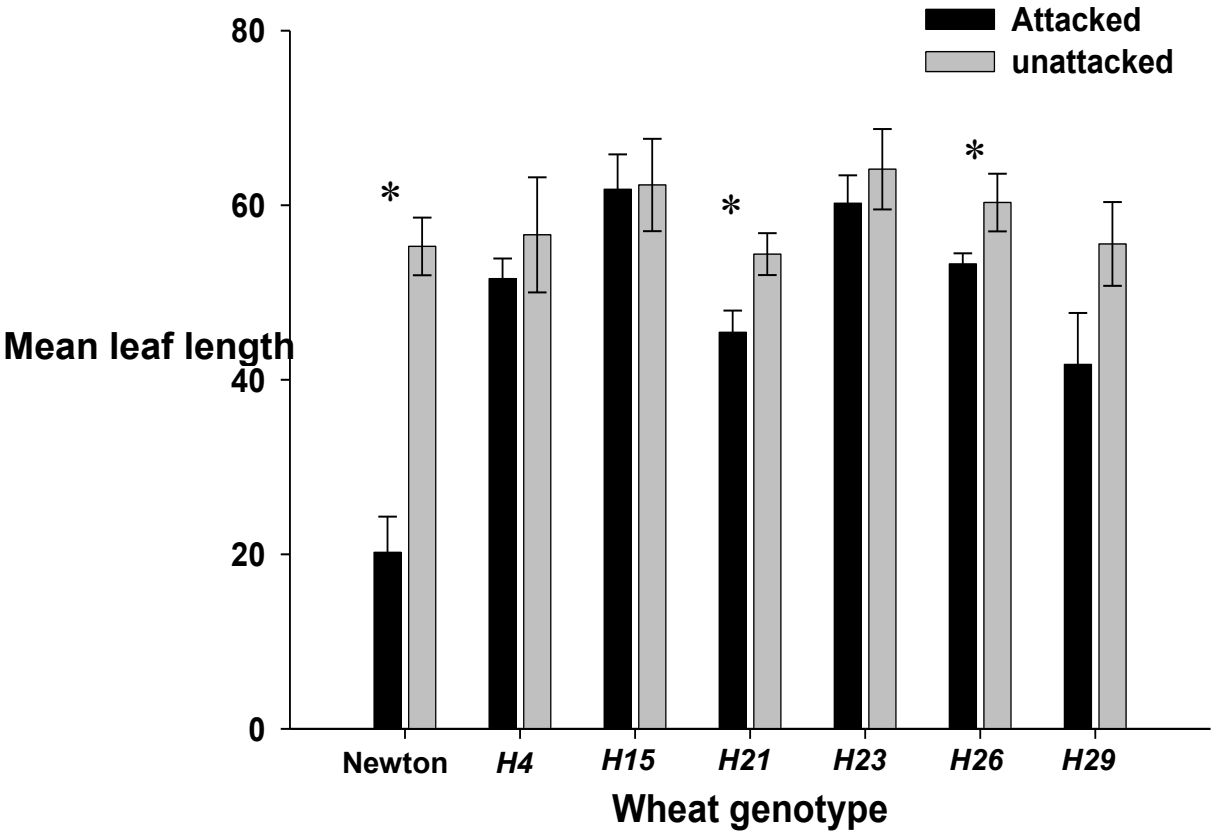

Fig. 22. Combined leaf growth of leaf 3 and 4 for Hessian fly attacked (black bars) and non-attacked control plants (grey bars) for six resistant genotypes with the most effective *H*-genes (i.e. 100% larval mortality) and the susceptible genotype Newton. Leaf three and four were used because they are the most directly impacted by Hessian fly attack. Paired bars that are accompanied by an asterisk are significantly different at $P < 0.05$.

I began my virulence study using the techniques of traditional biotyping (Gallun 1977). In the Northern Great Plains traditional biotyping is still worthwhile because unlike many other wheat growing areas, *H*-genes including the biotyping differentials (*H3*, *H5*, *H6*, *H7/H8*) have never knowingly been deployed in North Dakota and still may be useful. The result of biotyping 97 females from a North Dakota population was somewhat surprising. Far more virulence was

encountered than was expected. While the most virulent biotype, biotype L, was not present, 13 of the 16 other biotypes were (Table 7), and taken as a group ND Hessian flies showed some degree of virulence to all four differential cultivars. Of the 97 females biotyped, 15 had virulence to *H3*, 12 had virulence to *H5*, 48 had virulence to *H6* and 20 had virulence to *H7/H8* (Fig. 17).

Why this virulence exists in a population that has never knowingly been exposed to *H*-genes is difficult to say. It is possible that regional wheat breeding programs, by using germplasm from other parts of the country where *H*-genes are an important of breeding programs, have inadvertently introduced and released these Hessian fly resistance genes in locally adapted wheat cultivars and that has resulted in selection pressure that has given rise to virulence to these differential *H*-genes. Because the *H3*, *H5*, *H6* and *H7/H8* genes were some of the first genes identified and were widely utilized this scenario is possible. Ultimately this level of virulence to the four *H*-genes precludes their reliable use in our region.

Larval mortality across the 30 genotypes with *H*-genes varied (Fig. 18). Six genotypes with *H4*, *H15*, *H21*, *H23*, *H26* or *H29* resistance had 100% mortality. When examined, these genotypes appeared healthy and showed few, if any, signs of Hessian fly attack. When they were dissected only dead neonate larvae were found. I would recommend these genotypes as good candidates for inclusion in a wheat breeding project for resistance to the Hessian fly. In contrast the genotypes with the *H14*, *H16*, *H17* or *H18* gene and the resistant genotype Kawvale had mortality rates of less than 10% per plant (Fig. 18). When examined, these plants tended to show the characteristic stunting, dark green leaves, and seedling death which are all characteristic of a compatible interaction between Hessian fly larvae and a susceptible wheat plant. When dissected, these plants had many large healthy fly larvae. I would not recommend incorporating these genes into locally adapted cultivars as their ability to deter Hessian flies is compromised.

For the remaining 19 genotypes, larval mortality rates and plant symptoms were quite variable. It was not unusual to see some of the genotypes with a mix of healthy plants with dead larvae and unhealthy plants showing signs of larval feeding and many healthy larvae. I feel that some of these *H*-genes might be useful regionally, but I would suggest not using *H*-genes that had < 90% mortality.

Just as with the virulence seen in the traditional biotyping, I was surprised that so many *H*-genes did so poorly. Within the North Dakota population only six of the 30 resistant entries gave 100% protection from the North Dakota Hessian fly population that I evaluated (Fig. 18). This is significant because to the best of my knowledge no effort to deploy any of these resistance genes have ever been made in this region. The poor performance of some *H*-genes is particularly noteworthy. The *H14*, *H16*, *H17* and *H18* genes and the genotype Kawvale had low larval mortality rates similar to the susceptible genotype Newton (Fig. 18). More surprising was the relatively poor 23% larval mortality rate of the *H31* gene. The *H31* gene is one of the most recently described *H*-genes and to my knowledge has not been knowingly deployed (Williams et al. 2003).

The high level of virulence that was observed in the North Dakota population is not unique. Many other studies have described high levels of virulence within Hessian fly populations and how there are few *H*-genes available that give effective crop protection. Similar findings have been reported in the Southern Plains of the United States (Chen et al. 2009), the southeastern United States (Cambron et al. 2010) and the Middle East (El Bouhssini et al. 2009). Some of the virulence is likely the result of selection pressure from wide spread *H*-gene deployment in wheat cultivars. This selection pressure for virulence is in response to both the deliberate and accidental deployment of *H*-genes into the agro-ecosystem. While the purposeful

deployment of *H*-genes is obvious, the presence of *H*-genes unknowingly in germplasm used for plant breeding is certainly possible as well. Because the plant incurs no fitness cost for carrying the *H*-gene (Anderson et al. 2011) there is no incentive to identify and remove these traits. While some of the virulence is the result of selection pressure from wide spread *H*-gene deployment in wheat cultivars, the level of virulence observed cannot be explained by this alone. There are other factors that could possibly contribute to the virulence encountered. In Cambron et al. (2010) it is suggested that the efficacy of the resistant genotypes and the level of gene expression in the resistant genotypes may be inadequate. It was also suggested that environmental factors may play a role in *H*-gene gene expression. The *H18* gene is known to be temperature sensitive and loses effectiveness above 20°C (Cambron et al. 1995). The wild hosts of the Hessian fly are another possible source of virulence. The Hessian fly is known to reproduce successfully on grasses belonging to 17 genera and two tribes, Triticeae and Bromeae (Jones 1936, Barnes 1956, Harris et al. 2003). It has been theorized that virulence to *H*-genes may be selected for during generations when the Hessian fly population is living on wild grass species because crop hosts are not available (Zhang et al. 2011). In effect, Hessian fly populations are adapting to Hessian fly resistance genes found in wild grasses. Then these virulent flies move to cultivated wheat and are found to be virulent on wheat with newly deployed *H*-genes. Whatever the reason for the high level of virulence in the North Dakota population, the virulence described in this assay needs to be addressed when selecting candidate resistance genes for incorporating into regionally adapted wheat varieties.

In my virulence assay the relationship between the number of eggs laid on a genotype and the resulting larval mortality was not significant (Fig. 19). At first glance one might say that Hessian flies are not discriminating in where they lay eggs. But one should be careful drawing

that conclusion. It is well established that the choice of where the female insect places her eggs is important. In the case of the Hessian fly, females are noted for being selective (Harris and Rose 1990, Ganehiarachchi et al. 2013). Because Hessian fly larvae are limited in movement their survival is largely based on the female finding a suitable host for her offspring. In my assay all genotypes received eggs in all five blocks. While some genotypes received more eggs than others, it appears that the Hessian fly found all the genotypes to be suitable hosts. Ultimately, the females in my assay selected oviposition sites based on plant stimuli such as olfactory signals and tactile cues (Harris et al. 2003). However, the resulting performance of the offspring was based on the insect's ability to live on the *H*-genes, meaning that good offspring performance was due to adaptation to the resistance genes and poor performance was due to effective resistance genes. For the Hessian fly to be able to exhibit optimal host selection, the female would not only need to have information on which *H*-genes are in the plant being attacked, but also the virulence status of her offspring relative to all *H*-genes (Harris et al. 2003). Previous research has shown that adult females do not discriminate against plants carrying *H*-genes (Harris et al. 2003; 2006). Hessian fly females are just as likely to lay eggs on plants with *H*-genes as on plants without.

The relationship between being able to find living and dead larvae at the time of plant dissection and larval mortality was significant (Fig. 20). Two weeks after oviposition the plants were dissected. We observed that the most effective *H*-genes (> 80% mortality) tended to have a low rate of larval recovery (< 20%), while the least effective *H*-genes (< 25% mortality) had a relative high rate of larval recovery (> 60%). What happens at the time of insect attack and in the subsequent days determines success in recovering larvae two weeks later during dissection. I hypothesize that the poor larval recovery on the most effective *H*-genes is because avirulent

Hessian fly larvae are not able to establish themselves on the plant and they do not grow. The dead neonate larvae are less than 0.5 mm in length (Gagné and Hatchett 1989, Harris et al. 2011). These small larvae can be quite difficult to see and may be missed at the time of plant dissection. Larval behavior may also be a factor in the low rate of larval recovery. Avirulent larvae on resistant plants have been observed to be actively moving on the leaf surface days after virulent larvae have settled and begun feeding (Subramanyam et al. 2008, Ganehiarachchi et al. 2013). Orientation of the larvae on the plants also differs between virulent and avirulent larvae. Virulent larvae orient themselves parallel to the veins of the leaf sheath, while avirulent larvae are often found perpendicular to the leaf venation (Subramanyam et al. 2008). Avirulent larvae also display a writhing and head rearing behavior not seen in virulent larvae. It is suggested that these behaviors may be a stress response to the defensive chemicals targeting the Hessian fly larvae or a reaction to starvation (Subramanyam et al. 2008). The inability of avirulent larvae to settle into a feeding site may mean that they are more difficult to find due to being widely dispersed on the plant. Some of these larvae may even find themselves pushed out of the plant by leaf elongation (Anderson and Harris 2006). During my plant dissections it was common to find dead avirulent larvae on the abaxial side of leaf blades and sheaths well above the normal feeding sites at the base of the plant.

The relationship between plant growth (i.e. length of leaf 3 and 4, the two leaves directly impacted by Hessian fly larval feeding) and larval mortality was significant (Fig. 21). Plants attacked by Hessian fly larvae that had an effective resistance gene demonstrated growth rates that in some cases were nearly the same as their respective non-attacked control plants, while plants with the least-effective *H*-genes and the susceptible Newton demonstrated a poor rate of plant growth for attacked plants relative to their non-attacked controls (Fig. 21). I observed that

attacked plants with the least-effective *H*-genes (*H14*, H16, *H17*, *H18* and Kawvale) had plant growth responses that were similar to the susceptible genotype Newton. This is not surprising as I can conclude from the virulence assay that the North Dakota Hessian fly population has adapted to these five resistant genotypes. The loss of effectors encoded by an *Avirulence* gene has rendered these genotypes completely ineffective. This means that the plant does not recognize the attack of Hessian fly larvae and therefore no induced defense response take place.

While I observed that plants with effective *H*-genes had similar plant growth to their respective non-attacked control plants, some growth reduction was still evident two weeks after oviposition when the plants were measured (Fig. 22). In the case of the *H21* and *H26* genotypes the growth differential between attacked and non-attacked plants was significant (Fig. 22). The growth deficits observed for these highly-effective *H*-genes likely occurred during the initial larval attack (Anderson and Harris 2006). However the more serious larval-induced growth deficits did not occur. It is likely that these resistant plants were successful in preventing the larvae from establishing nutritive gall tissue at the base of the plant (Harris et al. 2011).

Because of the virulence within Hessian fly populations, selecting the proper *H*-gene is vital to achieving durable resistance. In the past 25 years there have been a number of studies that have investigated biotype composition and screened resistance genes in several regions of the country (Ratcliffe et al. 1994, 1996, 1997, 2000, Chen et al. 2009, Cambron et al. 2010). My study is the first of its kind in the Upper Great Plains. Through traditional biotyping and a virulence assay of all available *H*-genes we now have a good understanding of the virulence within our regional Hessian fly population. When asked, I can provide information on the *H*-genes that work best in our region as well as the *H*-genes to which our regional fly population has become adapted. Beyond just providing information on virulence my studies have provided

details on the Hessian fly-wheat interaction that have not been examined by other projects studying Hessian fly virulence. My virulence assay allowed me to explore several other aspects of the wheat-Hessian fly interaction. Besides providing valuable virulence information, my work drew three conclusions. First, I showed that female Hessian fly found all genotypes to be suitable hosts and laid eggs on all genotypes. Second, the more effective the *H*-gene was the less likely I was to recover dead larvae. I hypothesize that the behavior of larvae on resistant plants may be the key factor in the "findability" of larvae. Third, the defeated *H*-genes displayed extensive growth deficits. This leads me to believe the North Dakota Hessian fly population is completely virulent to these resistance genes. Meanwhile, the most-effective genotypes also show growth deficits. These deficits were likely caused during the initial larval attack and are not the more serious larval-induced growth deficits seen in susceptible plants.

In the future it will be necessary to deploy our most effective *H*-genes in the most thoughtful manner possible. Monitoring Hessian fly populations for virulence and selecting the most effective *H*-gene for a region is a good start. But to maintain durable resistance, we will need to monitor for virulence evolution. In addition to monitoring several gene deployment strategies have been proposed to prevent virulence evolution from occurring. Stacking or pyramiding *H*-genes is often suggested (Harris et al. 2003, Cambron et al. 2010, Stuart et al. 2012). In theory, a gene combination should be more durable than a single gene deployment (Gould 1986). Another alternative deployment strategy would be to deploy *H*-genes that allow some avirulent larvae to survive on resistant plants (El Bouhssini 2001). This should apply less selection pressure for virulence within the Hessian fly population. Finally, deploying *H*-genes with an interspersed refuge of susceptible wheat should lessen the selection pressure for virulence by providing an overwhelming number of avirulent flies in comparison to virulent

flies. The interspersed refuge strategy is currently being used in Canada with the *Sm1* resistance gene for management of the orange wheat blossom midge, *Sitodiplosis mosellana* (Smith et al. 2004).

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