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I, Alina Avanesyan, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Biological Sciences.

It is entitled:

Native versus Exotic Grasses: The Interaction between Generalist Insect Herbivores and Their Host Plants

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Native versus Exotic Grasses: The Interaction between Generalist Insect
Herbivores and Their Host Plants

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Graduate School

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In the Department of Biological Sciences

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by

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ABSTRACT

Although the interaction between native and exotic plants and their insect herbivores has been examined extensively, experimental studies on plant responses to generalist insects as well as feeding preferences of generalist insects on native versus exotic plants have provided inconsistent results. This dissertation addresses this issue by incorporating recent methodological recommendations and by exploring interactions between generalist herbivores and native and exotic plants from both the plant and insect perspectives. Using native (*Andropogon gerardii* and *Bouteloua curtipendula*) and exotic grasses (*Miscanthus sinensis* and *Bothriochloa ischaemum*) with generalist *Melanoplus* grasshoppers, I combined behavioral and molecular approaches to explore (1) plant resistance and tolerance to grasshopper herbivory, and (2) feeding preferences of *Melanoplus* grasshoppers for native and exotic plants. Overall, the results from this dissertation project have demonstrated lower resistance of exotic grasses to generalist grasshopper herbivory compared to native grasses; and similar level of plant tolerance to herbivory in native and exotic grasses. *Melanoplus* grasshoppers demonstrated strong feeding preferences for exotic plants in most of the behavioral experiments and under natural conditions. This suggests that exotic *M. sinensis* and *B. ischaemum* grasses with a lack of coevolutionary history with native generalist *Melanoplus* grasshoppers might have lower physical and chemical defenses than native grasses. Furthermore, generalist *Melanoplus* grasshoppers may provide biotic resistance to these exotic grasses should they invade natural areas. The results from this dissertation project have important applications for predicting the interaction between exotic plants and generalist herbivores in the introduced range, and if plant invasion has already occurred, for developing effective control plans.

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By

Alina Avanesyan

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CHAPTER 1

General Introduction

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According to the National Invasive Species Management Plan (NISMP, 2006), an invasive species is “a non-native species whose introduction does or is likely to cause economic or environmental harm or harm to human, animal, or plant health”. The distribution and abundance of invasive plant species in natural communities often have a huge impact on current biodiversity, as well as on the interaction between invasive and native plant species, and on native herbivores (e.g. Sakai et al., 2001; Thebaud & Simberloff, 2001). For example, once established in a new area, invasive plant species often outcompete native plants; they can grow larger, reproduce more and live longer than they do in their native range (e.g. Thebaud & Simberloff, 2001).

Among other possible reasons, successful establishment and disproportional spread of exotic plants in the introduced range can be explained by their specific interactions with native insect herbivores. A simplified model of interaction between plants and insect herbivores can be described as follows: insect herbivores attack an individual plant, thereby potentially decreasing its fitness, while the plant responds by increasing its defenses to minimize this negative effect. In natural communities, however, this scheme is complicated by many factors which need to be considered. Such factors include, but are not limited to, plant defense strategies and insect feeding preferences; both of these aspects are the focus of this dissertation.

In response to herbivory, plants can invest more resources either in resistance (the ability of a plant to reduce herbivore damage) or in tolerance (the ability of a plant to maintain fitness while sustaining herbivore damage), or maintain both strategies at an intermediate level (Price et al., 2011; Carmona & Fornoni, 2013). Feeding preferences of insect herbivores also might vary, from avoiding feeding on exotic plants (e.g. Siemann & Rogers, 2003) to strong preferences for exotic plants (e.g. Fielding & Conn, 2011).

How do plant defense strategies differ between native and exotic plants? And how can the interaction between native insect herbivores and exotic plants in the introduced range affect plant invasion? To answer these questions, various hypotheses have been proposed. This dissertation primarily focuses on the Enemy Release Hypothesis (ERH), the Evolution of Increased Competitive Ability Hypothesis (EICA), and Biotic Resistance Hypothesis (BRH), which are all commonly accepted hypotheses that are directly related to the interaction between native herbivores and native versus exotic plants.

The ERH explains the success of invasive plant species as escape of these plants from their native specialist herbivores, which are not present in the introduced range; as a result, invasive plants have reduced herbivore damage compared to native plant species, which potentially allows invasive plant species to outperform native species (Keane & Crawley, 2002). An extension of the ERH is the EICA hypothesis, which suggests that introduced, invasive plants reallocate their resources from defense against herbivores to growth and reproduction, which increases their competitive ability comparing to that in native plants (Blossey & Notzold, 1995). On the contrary, the BRH predicts an inability of the exotic plants to defend themselves against evolutionarily novel enemies, and consequently results in a lack of avoidance of exotic plants by native generalist insect herbivores, or even preference of these herbivores for exotic food (Parker & Hay, 2005).

These hypotheses, however, have received mixed support in experimental studies and many authors provided suggestions regarding experimental design, choice of study species, measurements, etc. to improve consistency and accuracy of experimental results (e.g. Bossdorf et al., 2004; Parker & Hay, 2005; Tallamy et al., 2010; Atwood & Meyerson, 2011; Ali & Agrawal, 2012). For example, Bossdorf et al. (2004) emphasized that (1) differences between specialist

and generalist herbivores and (2) distinctions between the resistance and tolerance components of plant defense should receive more attention in the experimental studies. Although the interaction between specialist herbivores and invasive plants has been well explored (see Ali & Agrawal, 2012), the impact of generalists on invasive plants, and consequently plant responses to herbivory by generalist insects, is not easily predicted and needs to be studied further (Bossdorf et al., 2004; Tallamy et al., 2010). According to Parker and Hay (2005), it is important to focus on generalist herbivores, because generalists have stronger impacts on the plant community than specialists. In addition, Atwood and Meyerson (2011) provided methodological suggestions for improving the consistency of results on plant responses and herbivore preferences. These suggestions include but are not limited to considering the effect of experimental conditions on potential results (field, greenhouse, etc.), exploring multiple plant traits (such as resistance and tolerance), and using standard metrics.

Based on these ideas, in my dissertation I explore the interaction between native generalist insect herbivores and native versus exotic host plants from two perspectives: (1) plant response to herbivory, and (2) feeding behavior of generalist insects with regard to the same plant species. Using grasshoppers and tallgrass prairie grasses as a model, I combine behavioral and molecular approaches to study resistance and tolerance of native and exotic grasses to herbivory (Chapters 2 and 3), and grasshopper feeding preferences on these same plants (Chapters 4 and 5).

Chapter 2 focuses on one defensive strategy, plant resistance, by examining leaf damage caused by generalist *Melanoplus* grasshoppers to morphologically and physiologically similar native (*Andropogon gerardii* and *Bouteloua curtipendula*) and exotic (*Miscanthus sinensis* and *Bothriochloa ischaemum*) grasses. In this chapter, I explore the predictions of the EICA and the

BRH hypotheses about lower resistance of exotic plants in the introduced range compared to native plants. I address issues about the inconsistency of results on leaf damage in native and exotic plants by combining a variety of experiments with both intact plants and clipped leaves under field and greenhouse conditions.

In Chapter 3, I explore the prediction of higher tolerance to herbivory in exotic plants compared to native plants, which has also received mixed support in experimental studies. Potential reasons for such mixed results are different methods of estimation of plant tolerance in terms of plant growth and challenges in accurate estimation of plant biomass in grasses. To address these issues, I first determine the best predictors for plant biomass changes in my study species and using these best predictors, I then compare plant growth during herbivory and during a subsequent regrowth period.

Given that grasshoppers are important generalist herbivores in natural communities and are among the main agricultural pests (Hewitt & Onsager, 1983; Belovsky & Slade, 2000), the knowledge of their feeding preferences, especially with regard to native versus exotic plants, is critical. Although there have been many studies on grasshopper feeding, very few have compared feeding on native versus exotic plants (Siemann & Rogers 2003; Jogesh et al., 2008; Zou et al., 2008; Branson & Sword 2009); and the results from these previous investigations are inconsistent. One of the challenges in the experimental studies on insect feeding is to accurately determine food digestion. Consequently, in addition to behavioral experiments, I apply a molecular approach (Chapters 4 and 5), which has shown to be a more accurate method for detecting ingested plants compared to field surveys (e.g. Garcia-Robledo et al., 2013). Because of the lack of existing PCR-based methods for detecting plant food within grasshopper gut contents, I focus exclusively in Chapter 4 on developing a step-by-step protocol, from tissue

preparation to obtaining plant DNA sequences. In Chapter 5, I then demonstrate how this protocol can be applied to estimate grasshopper feeding preferences under natural conditions without experimental manipulations.

Invasive species cause significant environmental problems on different levels: from economic losses and human health problems to changes in natural communities and the loss of biodiversity (Pimental et al., 2005). Playing an important role in accelerating nutrient cycling and providing food for many other organisms (Belovsky & Slade 2000), grasshoppers, as generalist insect herbivores, may provide biotic resistance to plant invasion by impacting plant community composition and particularly, the relative abundance and species richness of native and invasive plants. Consequently, knowledge about the interaction between grasshoppers and native versus exotic, potentially invasive, grasses can provide greater insight into the successful invasion of introduced plant species and is critical for predicting invasions in certain areas and/or developing plans for effective control of invasive plants.

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CHAPTER 2

Resistance of Native and Exotic Grasses to Herbivory by Nymph *Melanoplus*

Grasshoppers¹

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Abstract

The general interaction between plants and specialist insect herbivores has received considerable attention in ecological studies. However, responses of native and exotic plants to herbivory by generalist insects are still poorly understood; experimental studies comparing the resistance of plants to generalist insects have provided inconsistent results. Our study addresses these issues by examining leaf damage caused by generalist *Melanoplus* grasshoppers in morphologically and physiologically similar native (*Andropogon gerardii* and *Bouteloua curtipendula*) and exotic grasses (*Miscanthus sinensis* and *Bothriochloa ischaemum*). In a two year study, we explored whether exotic grasses sustain less damage than native plants, as predicted by some of the commonly accepted invasion hypotheses, and consequently, whether native generalist grasshoppers exert a potential fitness impact on these exotic plants. Using a combination of choice and no-choice feeding experiments with intact plants and with clipped leaves under different (field and greenhouse) conditions, we found that exotic grasses demonstrated equal or lower levels of resistance to grasshopper herbivory than did native plants. Our results suggest that exotic grasses which do not share a coevolutionary history with native generalist *Melanoplus* grasshoppers might have lower physical and chemical defenses than native plants. Consequently, *Melanoplus* grasshoppers may provide biotic resistance to these exotic grasses should they invade natural areas at the study regions. These results have important applications for predicting the interaction between exotic plants and generalist herbivores in the introduced range, which is critical for understanding factors facilitating plant invasions.

Key words: Coevolutionary history, herbivory, leaf damage, plant-insect interaction, plant invasion, plant resistance

Introduction

The interaction between exotic plants and insect herbivores can be an important mechanism which facilitates plant invasion in the introduced range (Keane and Crawley 2002). Specifically, plant responses to herbivory, such as plant resistance (the ability of a plant to reduce herbivore damage) and plant tolerance (the ability of a plant to maintain fitness while sustaining herbivore damage), can affect the establishment of exotic plants (Blossey and Notzold 1995, Rogers and Siemann 2005, Fornoni 2011). The intensity and pattern of these plant responses, as well as their underlying mechanisms, however, could differ between damage caused by specialist and generalist herbivores (Bossdorf et al. 2004, Ali and Agrawal 2012). Bossdorf et al. (2004) suggest that generalist insects in the exotic range may attack invasive plants with a similar intensity compared to that in the plant's native range, and therefore, invasive plants should be well-defended against generalists. Whereas the interaction between specialist herbivores and invasive plants has been well explored (see Ali and Agrawal 2012), the impact of generalists on invasive plants is not easily predicted and needs to be studied further (Bossdorf et al. 2004, Tallamy et al. 2010, Schaffner et al. 2011). To address this concern, we focused on one defensive strategy, plant resistance, which is the first immediate barrier which native generalist insects might encounter in their attempt to feed on introduced plants. According to Rausher (1992), "a resistance trait is any plant character that influences the amount of damage a plant suffers". These resistance traits include the presence of surface wax, spines, trichomes (hairs), and synthesis of chemicals (Price et al. 2011). One of the commonly used measurements for plant resistance is leaf damage: plants with more damage from herbivores are generally considered to have a lower level of resistance to herbivory (Mauricio 2000, Zou et al. 2008).

Several hypotheses have been proposed to explain and/or predict the level of resistance of exotic plants compared to native herbivores in the introduced range. For example, the Evolution of Increased Competitive Ability (EICA) hypothesis states that in the absence of native specialist enemies, invasive plant species reallocate their resources from defenses against herbivores to growth and reproduction (Blossey and Notzold 1995). The Novel Weapon Hypothesis (NWH) predicts that invasive plants possess novel defensive chemicals, which are effective against both native plants and native generalist herbivores (Callaway and Ridenour 2004). The Behavioral Constraints Hypothesis (BCH) also suggests that insect herbivores will avoid sampling novel and potentially toxic food due to their behavioral avoidance constraints (Lankau et al. 2004). In contrast, the Biotic Resistance Hypothesis (BRH) predicts that exotic plants which do not share coevolutionary history with native herbivores, will be less well defended compared to native plants, and native generalist herbivores will prefer to feed on these exotic plants, posing biotic resistance to plant invasions (Parker and Hay 2005). In other words, if native herbivores cause greater damage in exotic plants, they may constrain the spread of exotic plants, limit their abundance and, ultimately, prevent potential negative effects from their invasion.

Recent reviews and meta-analyses used to test these invasion hypotheses have shown some inconsistency in studies comparing leaf damage between native and exotic plants (e.g. Chun et al. 2010, Atwood and Meyerson 2011, Inderjit 2012). Such inconsistency may be due to measurement of only a single plant trait, using non-standard metrics, and/or different experimental designs across studies, such as conducting field experiments, greenhouse experiments, or experiments with clipped leaves alone (Atwood and Meyerson 2011). Moreover, results from laboratory or common garden experiments alone might not reflect all possible plant responses which occur naturally and therefore should be interpreted carefully (Motheral and

Orrock 2010). Using similar feeding experiments under different conditions allows us to control different factors and to explore plant responses from different perspectives (Motheral and Orrock 2010). Han et al. (2008) also suggested that variation of physiological and morphological traits between native and exotic species can affect results of leaf damage more than the location that the plants originated from. We address these concerns in our study, as described below.

Our primary objective was to determine if exotic grasses, which do not share a coevolutionary history with native generalist insect herbivores, exhibit less resistance and, therefore, sustain more leaf damage compared to native grasses. For our experiments, we chose native (*Andropogon gerardii* and *Bouteloua curtipendula*) and exotic, potentially invasive, grasses (*Miscanthus sinensis* and *Bothriochloa ischaemum*), all of which were exposed to herbivory by nymphs of generalist grasshoppers of the *Melanoplus* genus (Orthoptera: Acrididae). Considering that *Melanoplus* grasshoppers do not inhabit any areas in the native range of *Miscanthus* and *Bothriochloa* grasses, and thus do not share co-evolutionary history, we hypothesized that exotic grasses would demonstrate lower resistance to herbivory, and therefore greater leaf damage than native grasses. Using this information, we explored the potential impact of native *Melanoplus* grasshoppers on exotic, potentially invasive grasses in our North American study regions. Confirmation of our expectations of greater leaf damage in exotic grasses would indicate that *Melanoplus* grasshoppers can pose biotic resistance to exotic *Miscanthus* and *Bothriochloa* grasses in Ohio and Maryland, should they escape from ornamental settings or cultivation in grasslands. Consequently, this knowledge about plant responses to herbivory and grasshopper feeding preferences will allow us to better understand and predict interactions between exotic plants and generalist insect herbivores in the introduced range. To explore our objective, we conducted a two year study to compare native and exotic grasses in terms of the

relative amounts of leaf damage caused by generalist grasshoppers. We followed recent suggestions (e.g. Atwood and Meyerson 2011) and explored a set of standard measures of leaf damage for our grass-grasshopper system using a combination of experiments with intact plants and clipped leaves under different (field and laboratory) conditions. In addition, we quantified grasshopper growth rate separately on native and exotic plants as an additional estimate of plant resistance (Beck 1965, Emden 1969, Awmack and Leather 2002).

Methods

Study sites

To examine herbivory levels in the field, we established a common garden ($12 \times 8 \text{ m}^2$) at the Western Maryland Research and Education Center (WMREC, $39^\circ 30.618' \text{ N}$, $077^\circ 44.070' \text{ W}$, Keedysville, MD), in June 2012. The ground was tilled prior to planting and no fertilizer was applied during the period in which the project took place. The chosen plot was surrounded by a corn field and a plot with sunflowers (at a distance of 6 m from all sides), which presumably were attractive to grasshoppers. We removed weeds throughout the experiments and watered the plants when necessary to supplement natural precipitation.

To replicate the field experiment and to explore whether patterns of leaf damage in response to the same grasshoppers species would be consistent across sites, we established another similar common garden in 2013 at the University of Cincinnati Center for Field Studies (UCCFS, $39^\circ 17.134' \text{ N}$, $084^\circ 44.413' \text{ W}$, Harrison, OH), in addition to the study plot in Maryland. This second site was treated as in the same way as described previously.

To control for possible differences in plant biomass and their potential effect on grasshopper feeding choice, we also repeated the experiments in 2013 in the greenhouse at the

University of Cincinnati (UC) using potted plants. Finally, to explore how cutting plants may affect resistance to grasshopper herbivory, we also conducted experiments with clipped leaves at the UC greenhouse in both 2012 and 2013.

Study organisms

We used grasses of the Andropogoneae tribe of the Poaceae family - these consisted of two native species (*Andropogon gerardii* [big bluestem] and *Bouteloua curtipendula* [sideoats grama]) and two exotic, potentially invasive species (*Miscanthus sinensis* [Chinese silver grass] and *Bothriochloa ischaemum* [yellow bluestem]). All species are perennial C₄ grasses. Both *A. gerardii* and *B. curtipendula* are widely distributed throughout most of the United States, where they are dominant species in tallgrass prairie. *Bothriochloa ischaemum* and *M. sinensis* were introduced in the US in late 1800s from Eurasia and Japan respectively, because of their high forage quality (Hickman et al. 2006, Schmidt et al. 2008, Quinn et al. 2010). More recently, *M. sinensis* has also become important as a landscaping plant in the horticultural industry. When escaping cultivation, both exotic species demonstrate high competitive ability and inhibit growth of native grasses, and thus are considered potentially invasive (Schmidt et al. 2008, Quinn et al. 2010). All plant species were reported to be suitable for grasshopper feeding (Alward and Joern 2003, Han et al. 2008, Nabity et al. 2012), and were selected in part because their general morphological similarity in plant structure should provide similar attractiveness for grasshoppers.

For the first year of the study (2012), we used *A. gerardii*, *B. curtipendula*, and two cultivars of *M. sinensis* ('Zebrinus' and 'Gracillimus'). For the purpose of the experiments, we treated *M. sinensis* cultivars separately because of their differences in leaves shape, toughness,

and texture of the leaf surface, which might provide different resistance to grasshopper herbivory. For the experiments, we used potted plants (3.5" grower plugs, 25-30 cm in height) of native and exotic plants obtained from a local plant nursery. We then planted six potted plants of each species in the field (for field experiments) and kept the remainder (four potted plants per species) in the greenhouse for the experiments with clipped leaves, described below.

In 2013, we replaced the two cultivars of *M. sinensis* with a wild type of a non-cultivated variety (to eliminate potential similarity between two cultivars in comparison to other species), and added *Bothriochloa ischaemum*. We obtained seeds for our study plants from Prairie Moon Nursery, Winona, MN (*A. gerardii* and *B. curtipendula*); Outsidepride.com, Inc., Independence, OR (*M. sinensis*), and Warner Brothers Seed Company, Lawton, OK (*B. ischaemum*). We planted seeds in the University of Cincinnati greenhouse, and a month later we transferred potted plants (20-25 cm in height, 3.5" square pot) to the study plots in Maryland and Ohio. Other plants were kept at the greenhouse for the experiments with potted plants and clipped leaves. To more closely simulate grasshoppers' natural food choices, we offered four plant species (two native and two exotic) all together to grasshoppers in feeding experiments.

The generalist herbivore used in our study were nymphs of the *Melanoplus spp.* grasshoppers (Acrididae: Orthoptera), presumably *M. differentialis* and *M. femur-rubrum*. Nymph grasshopper species were identified based on their size, color and stripes pattern using the keys of Pfadt (1994). We did not rear the nymphal grasshoppers for species identification in this study. We however maintained identical nymphs collected from the same study sites for other experiments; the emerging adults were *M. differentialis* and *M. femur-rubrum*. In addition, the same species as adults were observed later in the season at the same collection sites.

Grasshoppers of the *Melanoplus* genus were chosen because of their wide distribution, abundance at both study plots in Ohio and Maryland, as well as their ecological and agricultural importance (Belovsky and Slade 2000, Branson and Sword 2009). Grasshopper nymphs (third and fourth instars) were collected adjacent to the plot and maintained a few hours in an open-air aluminum screen cage at the site prior to the field experiments, and/or a few days in the greenhouse prior to the experiments with potted plants and clipped leaves. During that time grasshoppers were fed a mixture of plants collected from the same location.

Field experiments with intact plants

All experiments were conducted on the fourth week after planting at the field site. Plants were watered every other day during the first week after planting, and whenever the soil was dry during the following three weeks prior to the commencement of the experiments. Plants were not watered during the experiment to simulate natural field conditions.

In 2012, 24 potted plants were arranged and planted in two native/exotic pairs: *A. gerardii* / *M. sinensis* ‘Zebrinus’, and *B. curtipendula* / *M. sinensis* ‘Gracillimus’. We conducted two identical feeding experiments subsequently with each plant pair. For each plant pair, we set up six open air aluminum screen cages (16×16×20" Repti Breeze Aluminum Screen Cage, Zoo Med Laboratories, Inc., California, USA) in two rows with 1 m spacing between them and 2 m spacing between rows. First, two grasshopper nymphs were placed in each cage with *A. gerardii* / *M. sinensis* ‘Zebrinus’ plants for 5 days. On the sixth day, the grasshoppers were removed and all components of leaf damage were measured. The cages were then reinstalled around *B. curtipendula* / *M. sinensis* ‘Gracillimus’ plants, and the same experiment was immediately repeated with this plant pair. For each plant, we determined the following: (1) *the total volume of*

the grazed portion (TGP), calculated as the sum of (length × width × depth of each scar, cm³)

(we called each grazed mark “a scar”); (2) *number of scars* per plant (NS); and (3) *average*

volume of grazed portion (AGP): total volume of grazed portion/number of scars (cm³).

Measurements of the length and the width of scars were taken as maximum values; the depth of scars was estimated as the maximum leaf thickness near the main vein of the leaf, which is often left intact by grasshoppers.

In 2013, 48 potted plants (12 pots of each of four species) were arranged and planted in twelve groups of four plants each: each potted plant was of a different species (*A. gerardii*, *B. curtipendula*, *M. sinensis*, and *B. ischaemum*). We set up one of 12 aluminum cages around each plant group. As we increased the number of plant species exposed to herbivory in each cage (from 2 to 4), we also increased the number of grasshoppers used in feeding experiments: we placed 3 grasshoppers in each cage. To eliminate any potential effect of seasonal changes on plant resistance to grasshopper herbivory, the experiments at both sites were conducted simultaneously. In addition to the measurements described above, we also estimated: (1) *number of missing tips per plant* (MT); (2) *number of damaged leaves* (DL); and (3) *the portion of a plant utilized for grazing* (PUG): [height of the highest scar - height of the lowest scar]/plant’s height.

Greenhouse experiments with potted plants

In 2013, simultaneously with field experiments in Ohio and Maryland, we conducted two types of feeding experiments with potted plants in the greenhouse of the University of Cincinnati: (1) choice experiment (native and exotic plants were both placed in the same cage), and (2) no-choice experiment (native or exotic plants were placed in separate cages). For the

choice experiment, 48 potted plants (the same plant species as those in the field experiments), were similarly arranged in groups of four (one of each species) and were placed in twelve fabric cages (Bioquip, rearing and observation cage, $14 \times 14 \times 24"$, Cat. No. 1466B). For no-choice experiments, potted plants were arranged into two groups: native (*A. gerardii* and *B. curtipendula*) and exotic (*M. sinensis* and *B. ischaemum*). Native and exotic plants were then placed separately in similar fabric cages: two potted plants (one of each species) in each cage. Grasshoppers (n=3 for choice and n=1 for no-choice trials) were placed in each cage to feed for 5 and 10 days respectively. A longer period for the no-choice experiment was needed for estimating grasshoppers' growth rates.

Leaf damage in choice and no-choice experiments was estimated, as described above for the field experiments using the same six measurements. In addition, growth rate of grasshoppers in no-choice experiments was estimated using two measurements: (1) changes in *body weight* (BW): [final weight – initial weight]/initial weight); and (2) changes in *body length* (BL): [final length – initial length]/initial length. Body length was measured as the length from the tip of the head till the tip of the abdomen. Dead (16.6%) and molted grasshoppers (12.5%) in no-choice experiments were excluded from data analysis.

Experiments with clipped leaves

For each choice experiment conducted with leaves in 2012, we clipped approximately 0.3 g of leaves (1-2 leaves of 20-25 cm length) from each plant species and offered them to nymph grasshoppers for consumption. The leaves were arranged in the same native/exotic plant pairs as described above in the field experiments, and two separate identical experiments were subsequently performed (the *A. gerardii* / *M. sinensis* 'Zebrinus' plant pair first, then *B.*

curtipendula / *M. sinensis* ‘*Gracillimus*’). The feeding arena consisted of small plastic containers (7×4.5× 5" All Living Things® Critter Totes, PetSmart, Inc., USA). For each of six plant combinations, the base of the leaves was wrapped with moist filter paper (to keep leaf tissue fresh and attractive for grasshoppers during the experiment) and placed on the bottom of the container. One grasshopper was placed in each container. Another six control containers with leaves but without grasshoppers were similarly prepared. All containers with leaves were transported to the field and kept near the plot in shadow at 25–26°C for 5 hours, after which time the experiment was concluded and plants were measured.

In 2013, to eliminate the potential effect of number of leaves and their different sizes on grasshopper choice, we clipped one leaf of the same length (25 cm) and maximum width of 0.7 cm from each plant species (*A. gerardii*, *B. curtipendula*, *M. sinensis*, and *B. ischaemum*). Then four leaves all together were offered to grasshoppers in the same containers. Twelve containers with grasshoppers and twelve control containers were prepared, similar to our procedure in 2012. The feeding trial lasted 5 hours, during which all containers were kept in the greenhouse at 25–26°C to control conditions of the experiment.

Grasshoppers were starved for 24 hours prior to all feeding trials, and new individuals, which had not been used in the experiments previously, were used for each trial. After grasshoppers were removed from the containers, leaves were weighed, and all measurements of leaf damage were taken. In both 2012 and 2013, we measured the following traits: (1) *the total amount of leaf tissue consumed* (TLC) by grasshoppers, following the formula suggested by Waldbauer (1968): $[(1 - (\text{natural loss of aliquot}/\text{initial weight of aliquot}/2)) \times (\text{weight of food introduced} - (\text{weight of uneaten food (L)} + ((\text{natural loss of aliquot}/\text{final weight of aliquot}) \times L)))]$; (2) *the proportion of the amount of leaf tissue consumed* (PLC): absolute amount of leaf tissue

consumed/initial amount of leaf tissue offered, g/g; and (3) *the proportion of leaf length damaged* (PLL), defined as ([length of leaf offered-length of leaf remained]/length of leaf offered, cm/cm).

Statistical analysis

Prior to data analysis, all measurements of leaf damage in 2012 were averaged within exotic and within native plant pairs across experiments. In 2013, all measurements of leaf damage were averaged within exotic and within native plants for each cage: [e.g., (grazed portion of *A. gerardii* in cage 1 + grazed portion of *B. curtipendula* in cage 1)/2]. All components of leaf damage were analyzed using a generalized linear mixed model (GLMM) in R (v.2.15.2, package *lme4*).

The cage effect was defined as an effect of potential differences in the enclosures (aluminum cages, fabric cages, and plastic containers were examined separately), as well as effect of differences in the location of enclosures, the amount of penetrated light and/or soil quality inside of the enclosures on leaf damage measurements. To account for this potential cage effect, we ran GLMM for each experiment where plant type (native or exotic), site, type of experiment (choice or no-choice), and year were considered as fixed effects, and enclosures were considered as random effect. Then we used the two-way fixed effects ANOVA to determine whether exotic plant species sustained less leaf damage than native plant species. For this analysis, the leaf damage data were grouped either by site (field experiments in 2013) or by type of the experiment (greenhouse experiments in 2013), or by year (experiments with clipped leaves). The field experiment in 2012 was analyzed separately using one-way fixed effects ANOVA due to differences in variables from that we measured in 2013. For each group of tests,

significance levels were adjusted using Bonferroni correction. Normality and homoscedasticity of all data were tested using the Shapiro-Wilk test and Bartlett's test respectively at $\alpha = 0.05$.

To estimate the potential differences among plant species which might affect leaf damage, the Kruskal-Wallis test followed by post-hoc Mann-Whitney's U test with a Bonferroni correction (due to lack of normality of data and lack of damage in about 25% plant individuals) was conducted to compare the leaf damage between plant species within native and exotic pairs. These tests were also used to detect any difference in leaf damage within native and exotic plant groups under field and greenhouse conditions.

Results

Field experiments with intact plants

In field experiments in 2012 in which intact plants were grouped in native/exotic pairs, native (*A. gerardii*, *B. curtipendula*) and exotic (*M. sinensis* 'Zebrinus,' *M. sinensis* 'Gracillimus') grasses did not exhibit significant differences in any leaf damage trait (Table 1; Appendix 1). In addition, there were no differences between plant species when analyzed individually across all species. More than 50% of variation in all leaf damage traits, however, was explained by a cage effect (Appendix 2).

In 2013, when mixed groups of native and exotic species were offered to grasshoppers, all measures of leaf damage except AGP and PUG were greater in exotic grasses at both Maryland and Ohio field sites (Table 1; Appendix 1). In total, the volume of grazed leaf tissue of exotic plants was about six times greater than that of native plants at both field sites. In addition, exotic plants contained four times more scars and five times more damaged leaves and missing tips than native plants (Table 1). There was no or small cage effect (<50% of variation in leaf

damage was explained by a cage effect) on any of these measurements (Appendix 2). In addition, field site as a factor did not have a significant effect on any leaf damage trait. Differences among plant species were significant only for TGP ($\chi^2 = 37.88$, df = 3, p < .0001) and PUG ($\chi^2 = 27.96$, df = 3, p < .0001). For both these traits (TGP and PUG), *M. sinensis* had the greatest damage; all other comparisons among plant species did not reveal a significant difference.

Greenhouse experiments with potted plants

Similar to the results from field experiments in 2013, all measurements of leaf damage except AGP and PUG were greater in exotic grasses in both choice and no-choice experiments (Table 2; Appendix 3). There was also no cage effect on leaf damage (Appendix 3). Differences among plant species, however, were detected for TGP ($\chi^2 = 17.01$, df = 3, p < .001), AGP ($\chi^2 = 16.22$, df = 3, p < .001), and PUG ($\chi^2 = 20.42$, df = 3, p < .0001). For both TGP and PUG, *M. sinensis* sustained the greatest damage; AGP was significantly greater in *M. sinensis* but only compared to *B. ischaemum*. All other comparisons among plant species did not reveal a significant difference. Also, we found a significant effect of type of experiment on leaf damage and a [Plant Type × Experiment] interaction for all measurements except AGP and PUG: exotic plants in choice experiments sustained more damage than in no-choice experiments (Appendix 3). All measurements of leaf damage except AGP were greater in the greenhouse than in the field for both native and exotic plants (Figure 1).

In terms of body mass, the growth rate of grasshoppers did not differ between native and exotic plants; grasshopper body length, however, was greater on exotic plants (Table 2; Appendix 3). Overall, the body length of grasshoppers increased during the experiment (0.05 ± 0.02 cm on native plants and 0.29 ± 0.06 cm on exotic plants), whereas their body weight

decreased on both native and exotic plants (Table 2; Appendix 3). A large amount of variation in grasshopper growth rate (>50% for weight) was explained by cage effect (Appendix 2).

Experiments with clipped leaves

In the experiments with leaves in both 2012 and 2013, we did not observe any differences in leaf damage between native and exotic plants except that the proportion of leaf length damaged (PLL) was greater in native plants than in exotic plants (Table 3; Appendix 4). Also, there was a cage effect on PLL (in 2012) and TLC (in 2013; Appendix 2). There were no other detected differences in leaf damage among plant species.

There was, however, a significant effect of year on TLC data and the [Plant Type × Year] interaction for PLL data (Appendix 4). Overall, grasshoppers consumed a greater amount of leaf tissue in 2012 than in 2013. In 2012, when leaves of equal amount were offered grasshoppers in native/exotic pairs, the total amount of leaves consumed by grasshoppers was 0.07 ± 0.02 g on native plants, and 0.06 ± 0.02 g on exotic plants, which represented about 30% of initial amount of leaves offered to grasshoppers (Table 3). In 2013, when a group of four leaves (each of different species) of the same length were offered at the same time to grasshoppers to consume, the total amount of leaves consumed by grasshoppers was 0.01 ± 0.005 g on native plants, and 0.02 ± 0.005 g on exotic plants; which represented no more than 15% of the initial amount of leaf tissue from both native and exotic plants (Table 3).

Discussion

In spite of their escape from specialist enemies in their native range, exotic plants may still possess effective defenses against generalist insect herbivores in the introduced range

(Bossdorf et al. 2004). The question of whether exotic plant species are less well defended against generalist insects than their native plant counterparts, is however, not easily answered: experimental studies which tested the predictions of different invasion hypotheses generated inconsistent results about differences in leaf damage, a standard measurement of plant resistance, between native and exotic plants (e.g. Chun et al. 2010). In our study, we incorporated recent suggestions about experimental conditions and measurements (e.g. Atwood and Meyerson 2011). We conducted feeding experiments by initially exposing two native/exotic plant pairs, followed then by four plant species (two native and two exotic) together to grasshopper herbivory under both field and controlled conditions (in the greenhouse). The experiments with intact plants were accompanied by similar experiments using clipped leaves.

Overall, our results demonstrated an equal or greater level of leaf damage, and therefore, less resistance, in exotic grasses compared to natives. Our hypothesis was supported in field experiments in 2013, as well as in experiments with potted plants in the greenhouse, in which the amount and intensity of leaf damage were greater on exotic grasses. However, we did not find support for our hypothesis in field experiments in 2012 and in experiments with clipped leaves. In these cases, we observed a comparable level of leaf damage in exotic and native plants. Similarly, grasshoppers' growth rates (in terms of both body weight and body length) on native and exotic plants also suggest that exotic plants did not have a more (if any) inhibitory effect on grasshopper growth than do native plants.

The difference in our results obtained in 2012 (field experiment with plant pairs) and in 2013 (field experiment with plant groups of four) can be explained, among other possible reasons, by the increased number of possible food choices, which provided more natural conditions for our feeding trials. It has been demonstrated that generalist grasshoppers,

consuming a wide range of plants, often switch between plants depending on the insects' state of hydration (Bernays and Chapman, 1994) or chemical composition of plants (Hull-Sanders et al., 2007). As the field experiments in 2013 more closely simulated natural conditions, our results suggest that grasshoppers do not avoid feeding on exotic plants, presumably because of similar or lower level of resistance of exotic plants to herbivory compared to native plants. The lack of avoidance of exotic plants by grasshoppers was supported by the results from the no-choice experiment: when presented separately, both native and exotic plants were similarly utilized by grasshoppers.

Greater resistance of native grasses to grasshopper herbivory compared to exotic grasses in our study can be due to the following: (1) the hairy leaf surface (glandular hairs on the margin of leaf blade in *B. curtipendula*, and more sparse hairs in *A. gerardii*), and (2) noticeably denser leaves of these native species, which can prevent active foraging of grasshoppers, especially nymphs, considering the size of their legs and mouth parts. In contrast, exotic *M. sinensis* and *B. ischaemum* have smoother leaf surfaces and leaves that are less dense, with larger internodes, which presumably make it easier for nymph grasshoppers to move and feed on these plants. Leaf toughness of *Miscanthus* and *Bothriochloa* apparently does not prevent grasshoppers from feeding. For example, it has been demonstrated in greenhouse experiments with *Miscanthus* plants and the American grasshopper *Schistocerca americana*, an important crop pest, that silica in plant tissue influences cell thickness and also increased the consumption rate of this grasshopper species although conversion efficiency was reduced (Nabity et al. 2012). In our study, we also found that whenever differences among plant species were detected, *M. sinensis* sustained the greatest level of leaf damage among plant species. It would be interesting for future

studies to explore whether the same silica component of plant resistance as in *M. sinensis* affects feeding of the *Melanoplus* grasshoppers with regard to *Bothriochloa* plants.

In terms of chemical compounds, Mole and Joern (1994) demonstrated that native North-American grasses of the Poaceae family (including *Andropogon* and *Bouteloua*) possessed neither the strong deterrents nor phagostimulants that could affect grasshopper feeding. A comparison of secondary metabolites of *Miscanthus* and *Bothriochloa* grasses to those found in *Andropogon* and *Bouteloua*, and specifically the effect of these metabolites on grasshopper feeding, would be also helpful to better explain the increased leaf damage in these exotic grasses. We can only speculate that the balance of deterrents and phagostimulants in *Miscanthus* and *Bothriochloa* grasses made them attractive to grasshoppers. It is possible that after removal from the plant, the level of resistance of the clipped portion decreased in native plants, which caused them to be as palatable for grasshoppers in our feeding experiments as were leaves clipped from exotic plants. Equal levels of leaf consumption by grasshoppers suggests that leaves of exotic plants either had not changed resistance after they had been clipped, or their resistance levels had decreased but were not lower than those of native plants.

Given that two different grasshopper species were used in this experiment, it is possible that they might exhibit different preferences for native and exotic grasses. However, for the purpose of our experiments, we were interested in general plant responses to herbivory by generalist species under natural conditions. We have not separated *M. differentialis* and *M. femur-rubrum* nymph grasshoppers in our experiments as they occur in the same habitats and consume plants of the same genus (e.g. Caswell and Reed 1976). Following studies which used combined *Melanoplus* spp. species in feeding experiments (e.g. Berdahl, 1990), we expected

these grasshopper species, especially at nymphal stage, to have similar preferences with regard to native and exotic grasses.

Our discovery of lower or similar levels of resistance in exotic plants compared to native plants are consistent with previous studies, indicating that generalist insect herbivores do not avoid feeding on exotics and when available, readily incorporate these plants in their diet (Agrawal and Kotanen 2003, but see Agrawal et al. 2005, Siemann and Roger 2003, Lankau 2004, Hull-Sanders et al. 2007, Zou et al. 2008, Lind and Parker 2010, Fielding and Conn 2011, and Fan et al. 2013). Our findings are also consistent with results from similar studies on non-insect invertebrates, where generalist herbivores preferred exotic plants over natives (e.g. Morrison and Hay 2011). However, Lankau et al. (2004) demonstrated that generalist grasshoppers, which preferred exotic plants over natives in laboratory and common garden trials, might not recognize exotics as a potential food in nature because of behavioral constraints. The authors studied invasive Chinese tallow tree (*Sapium sebiferum*) – a plant, which morphologically and physiologically differs from grasses. Future investigations should explore natural herbivory of the grasses from the current study to see if grasshoppers demonstrate the same behavioral constraints and avoid exotic grasses under natural conditions.

Our results do contrast with some other studies of interactions between exotic plants and native generalist insects (e.g. Han et al. 2008, Jogesh et al. 2008, Tallamy et al. 2010, Schaffner et al. 2011), as well as with some investigations involving non-insect invertebrates (e.g. Motheral and Orrock 2008, Tomas et al. 2011). Some of these authors used laboratory feeding trials (e.g. Tallamy 2010, Tomas et al. 2011) or focused on congeneric native/exotic plant pairs only (e.g. Jogesh et al. 2008). Congeneric comparisons are critical in understanding differences in resistant traits of native and exotic plants which might facilitate invasion. However, using

morphologically and physiologically similar plants, which share common habitat and are not necessarily closely related, should also provide important insight into plant resistance from the insect's perspective. This way we can observe plant responses under natural feeding behavior of generalist herbivores, as it is unlikely that each feeding choice of generalist grasshoppers in the field is limited by a choice between two congeneric native/exotic plant species.

One limitation of our study involves the procedure used to estimate the portion of the grazed leaf based on maximum measurements of length, width and depth of scars. We originally intended to follow Zou et al. (2008), who calculated the grazed area of leaves from Chinese tallow tree by scanning them and using the ScionImage program. However, this approach proved to be unfeasible for measuring the leaf area of grasses due to the shape, size, and large quantities of leaves per plant. Based on similar patterns of grazed portions among all grasses, we considered our measurements to be the most accurate way to estimate leaf damage within the context of our study.

Conclusions

Invasive plants often cause environmental and economic problems and their control is often costly (Pimentel et al. 2000, 2005). Knowing how exotic, potentially invasive grasses interact with native generalist herbivores, such as grasshoppers, is critical for predicting invasion in certain areas and/or developing plans for effective control of invasive plants. Playing an important role in accelerating nutrient cycling and providing food for many other organisms (Belovsky and Slade 2000), grasshoppers can provide biotic resistance to plant invasion by impacting plant community composition and particularly, the relative abundance and species richness of native and invasive plants. Our study supports the idea that lack of coevolution

between exotic plants and native generalist herbivores can result in decreased resistance of exotic plants to novel native herbivores (Parker et al. 2006), and provides potential support for the EICA, BRH, and BCH hypotheses. However, testing these hypotheses using the proposed grasses-grasshoppers model would require additional investigations, such as exploring plant tolerance and competitive ability of exotic grasses (EICA hypothesis), and/or obtaining additional data on natural herbivory by *Melanoplus* grasshoppers (BCH and BRH). It is also critical to conduct these experiments on naturally co-occurring native and exotic grasses or on established plants at the study plots. Combined results from such studies will provide more information about interactions between native insect herbivores and their novel host plants from both plant and insect perspectives.

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Table 1 Mean values of leaf damage in native and exotic plants caused by nymph *Melanoplus* grasshoppers in field experiments in 2012-2013.

Year	Site	Measurements	Plants		<i>P</i> value	Plant differences
			Native	Exotic		
2012	WMREC	Total volume of the grazed portion (cm ³)	0.01 ± 0.006	0.06 ± 0.01	0.0183	ns
		Average volume of grazed portion (cm ³)	0.003 ± 0.0009	0.008 ± 0.001	0.0249	ns
		Number of scars per plant	2.83 ± 0.87	8.33 ± 2.18	0.0423	ns
2013	WMREC	Total volume of the grazed portion (cm ³)	0.007 ± 0.002	0.04 ± 0.008	0.0001*	*
		Average volume of grazed portion (cm ³)	0.007 ± 0.002	0.01 ± 0.001	0.822	ns
		Number of scars per plant	1.17 ± 0.27	5.63 ± 0.76	3.57e-06*	ns
		Number of missing tips	0.54±0.18	2.54±0.47	0.0009*	ns
		Number of damaged leaves per plant	1.33±0.27	5.63±0.72	3.41e-05*	ns
		Portion of a plant utilized for grazing (cm/cm)	0.10 ± 0.04	0.31 ± 0.05	0.0102	*
UCCFS		Total volume of the grazed portion (cm ³)	0.006 ± 0.002	0.04 ± 0.008	0.0001*	*
		Average volume of grazed portion (cm ³)	0.005 ± 0.001	0.006 ± 0.001	0.822	ns
		Number of scars per plant	1.21 ± 0.29	4.92 ± 0.72	3.57e-06*	ns

plant				
Number of missing tips	0.58 ± 0.18	2.71 ± 0.51	0.0009*	ns
Number of damaged leaves per plant	1.29 ± 0.3	5.42 ± 0.72	3.41e-05*	ns
Portion of a plant utilized for grazing (cm/cm)	0.12 ± 0.04	0.32 ± 0.04	0.0102	*

¹Field experiments were conducted at Western Maryland Research and Education Center (WMREC) and at the University of Cincinnati Center for Field Studies (UCCFS).

²Native plants included *Andropogon gerardii* and *Bouteloua curtipendula* in both 2012 and 2013; exotic plants included two cultivars of *Miscanthus sinensis* ('Zebrinus' and 'Gracillimus') in 2012, and *Miscanthus sinensis* (wild type) and *Bothriochloa ischaemum* in 2013.

³Average volume of grazed portion and portion of a plant utilized for grazing were compared between plants with at least one grazed mark (a scar) found; undamaged plants were excluded from analysis of these characteristics.

⁴All values indicate mean \pm 1 SE. *P* values for the differences which are significant at the unadjusted 0.05 level are in bold; *P* values with asterisks ("*") are significant at the adjusted significance of 0.016 (for 2012) and 0.008 (for 2013). Significant differences between plant species in native and exotic pairs indicated as “*”; differences which are not significant “ns”.

Table 2 Mean values of leaf damage in native and exotic plants caused by nymph *Melanoplus* grasshoppers, as well as grasshopper growth rates on these plants, in greenhouse experiments in 2013.

Experiment	Measurements	Plants		<i>P</i> value	Plant differences
		Native	Exotic		
Choice	Total volume of the grazed portion (cm ³)	0.03 ± 0.009	0.13 ± 0.03	0.0002*	*
	Average volume of grazed portion (cm ³)	0.005 ± 0.001	0.006 ± 0.001	0.449	*
	Number of scars per plant	6.83 ± 1.4	17.83 ± 2.13	1.12e-05*	ns
	Number of missing tips	1.87±0.26	5.45±0.97	0.0003*	ns
	Number of damaged leaves per plant	5.54±0.72	12.25±1.28	1.58e-05*	ns
	Portion of a plant utilized for grazing(cm/cm)	0.4 ± 0.04	0.55 ± 0.05	0.0201	*
No-choice	Total volume of the grazed portion (cm ³)	0.03 ± 0.007	0.04 ± 0.01	0.0002*	*
	Average volume of grazed portion (cm ³)	0.006 ± 0.001	0.005 ± 0.001	0.449	*
	Number of scars per plant	4.83 ± 0.93	7.25 ± 1.26	1.12e-05*	ns
	Number of missing tips	1.12±0.29	2.66±0.75	0.0003*	ns
	Number of damaged leaves per plant	4±0.72	5.87±1.06	1.58e-05*	ns
	Portion of a plant utilized for grazing(cm/cm)	0.32 ± 0.05	0.37 ± 0.05	0.0201	*
	Grasshopper growth rate: weight (g)	-0.25 ± 0.08	-0.04 ± 0.17	0.5230	NA

Grasshopper growth rate: body length (cm)	0.05 ± 0.02	0.29 ± 0.06	0.0042*	NA
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¹ Experiments with potted plants were conducted at the University of Cincinnati (UC) greenhouse.

² Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Miscanthus sinensis* (wild type) and *Bothriochloa ischaemum*.

³ Average volume of grazed portion and portion of a plant utilized for grazing were compared between plants with at least one grazed mark (a scar) found; undamaged plants were excluded from analysis of these characteristics.

⁴ All values indicate mean \pm 1 SE. *P* values for the differences which are significant at the unadjusted 0.05 level are in bold; *P* values with asterisks (“*”) are significant at the adjusted significance of 0.008 (for leaf damage) and 0.025 (for grasshoppers growth rates). Significant differences between plant species in native and exotic pairs indicated as “*”; differences which are not significant “ns”.

Table 3 Mean values of leaf damage in native and exotic plants caused by nymph *Melanoplus* grasshoppers in laboratory experiments with clipped leaves in 2012-2013.

Year	Site	Measurements	Plants		<i>P</i> value	Plant differences
			Native	Exotic		
2012	WMREC	Total amount of leaf tissue consumed (g)	0.07 ± 0.02	0.06 ± 0.02	0.5951	ns
		Proportion of the amount of leaf tissue consumed (g/g)	0.34 ± 0.08	0.28 ± 0.08	0.5119	ns
		Proportion of leaf length damaged (cm/cm)	0.32 ± 0.08	0.06 ± 0.17	0.0112*	ns
2013	UC	Total amount of leaf tissue consumed (g)	0.01 ± 0.005	0.02 ± 0.005	0.5951	ns
		Proportion of the amount of leaf tissue consumed (g/g)	0.1 ± 0.04	0.15 ± 0.04	0.5119	ns
		Proportion of leaf length damaged (cm/cm)	0.06 ± 0.02	0.12 ± 0.03	0.0112*	ns

¹ In 2012, experiments with clipped leaves were conducted at the WMREC. In 2013, they were conducted at the University of Cincinnati (UC) greenhouse.

² Native plants included *Andropogon gerardii* and *Bouteloua curtipendula* in both 2012 and 2013; exotic plants included two cultivars of *Miscanthus sinensis* ('Zebrinus' and 'Gracillimus') in 2012, and *Miscanthus sinensis* (wild type) and *Bothriochloa ischaemum* in 2013.

³ All values indicate mean ± 1 SE. *P* values for the differences which are significant at the unadjusted 0.05 level are in bold; *P* values with asterisks ("*") are significant at the adjusted significance of 0.0016. Significant differences between plant species in native and exotic pairs indicated as “*”; differences which are not significant “ns”.

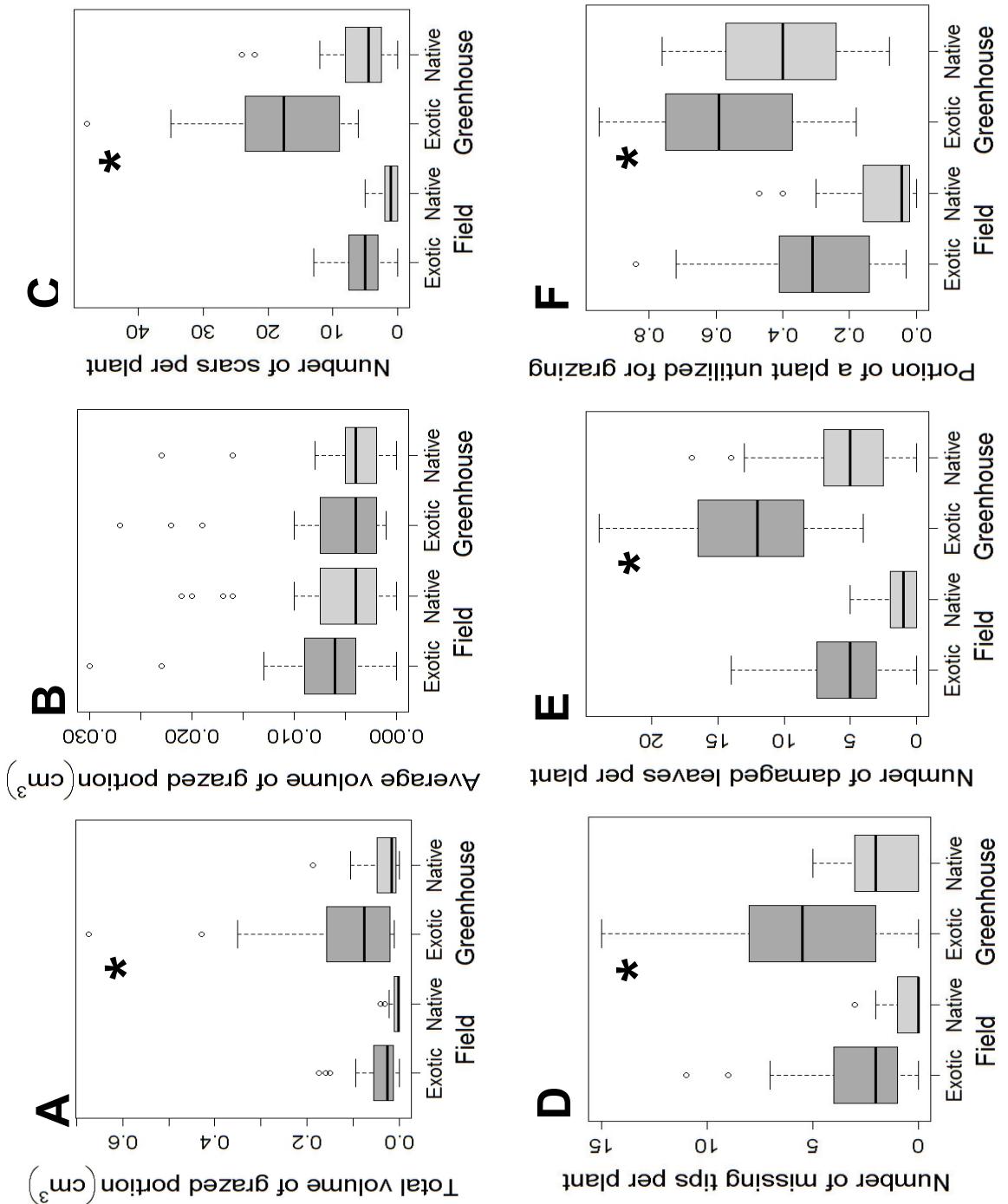


Figure 1 Overall leaf damage of intact exotic *Miscanthus sinensis* and *Bothriochloa ischaemum* and native *Andropogon gerardii* and *Bouteloua curtipendula* plants in the field (dark grey boxplots) and greenhouse (light grey boxplots) experiments in 2013. Field experiments were conducted at Western Maryland Research and Education Center and at the University of Cincinnati Center for Field Studies; boxplots for field data represent results combined across both sites. Asterisks (“*”) indicate significant differences within exotic and native plants under field and greenhouse conditions at the significance level of $P = 0.05$.

Appendix 1

General linear model parameter estimates of resistance of native versus exotic grasses to herbivory by *Melanoplus* grasshoppers in the field experiments with intact plants in relation to location of the experiment (“Site”) and plant origin (“Plant Type”).

Year	Parameter	Fixed effects	Estimate	Std. Error	t value	Pr(> t)
2012	Total volume of the grazed portion	(Intercept)	0.05802	0.01133	5.121	0.00045 ***
	Average volume of grazed portion	Plant Type	-0.04512	0.01602	-2.816	0.01829 *
		(Intercept)	0.008360	0.001086	7.696	0.000117 ***
		Plant Type	-0.004635	0.001629	-2.845	0.024880 *
2013	Number of scars per plant	(Intercept)	8.333	1.672	4.985	0.000549 ***
	Total volume of the grazed portion	Plant Type	-5.500	2.364	-2.326	0.042303 *
		(Intercept)	0.039917	0.005639	7.078	8.78e-09 ***
	Average volume of grazed portion	Site	-0.003417	0.007975	-0.428	0.670449
		Plant Type:Site	0.003250	0.011279	0.288	0.774586
	Number of scars per plant	(Intercept)	0.0073182	0.0011819	6.192	1.78e-06 ***
		Plant Type	0.0004818	0.0021143	0.228	0.822
		Site	-0.0008682	0.0017128	-0.507	0.617
		Plant Type:Site	-0.0004318	0.0033360	-0.129	0.898
	Total volume of the grazed portion	(Intercept)	5.6250	0.5949	9.455	3.65e-12 ***
		Plant Type	-4.4583	0.8413	-5.299	3.57e-06 ***
		Site	-0.7083	0.8413	-0.842	0.404
		(Intercept)	0.039917	0.005639	7.078	8.78e-09 ***

	Plant Type:Site	0.7500	1.1898	0.630	0.532
Number of missing tips	(Intercept)	2.5417	0.3991	6.368	9.69e-08 ***
	Plant Type	-2.0000	0.5644	-3.543	0.000949 ***
	Site	0.1667	0.5644	0.295	0.769165
	Plant Type:Site	-0.1250	0.7982	-0.157	0.876277
Number of damaged leaves per plant	(Intercept)	5.6250	0.6577	8.553	6.58e-11 ***
	Plant Type	-4.2917	0.9301	-4.614	3.41e-05 ***
	Site	-0.2083	0.9301	-0.224	0.824
	Plant Type:Site	0.1667	1.3153	0.127	0.900
Portion of a plant utilized for grazing	(Intercept)	0.32700	0.04046	8.083	4.98e-08 ***
	Plant Type	-0.19700	0.07007	-2.811	0.0102 *
	Site	-0.01089	0.05878	-0.185	0.8547
	Plant Type:Site	-0.06911	0.12212	-0.566	0.5772

¹ Two sites were utilized for the field experiments in 2013: Western Maryland Research and Education Center and University of Cincinnati Center for Field Studies.

² Plants of two types were used in the experiments with intact plants: native and exotic plants. Native plants included *Andropogon gerardii* and *Bouteloua curtipendula* in both 2012 and 2013; exotic plants included two cultivars of *Miscanthus sinensis* ('Zebrinus' and 'Gracillimus') in 2012, and *Miscanthus sinensis* (wild type) and *Bothriochloa ischaemum* in 2013

³ * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

Appendix 2

Model (GLMM) estimates of an effect of grasshopper enclosures (cages) on measurements of plant resistance.

Year	Experiment	Parameters	Random Effects		Fixed Effects		Std. Error	t value
			Groups	Variance	Std. Error	Groups		
2012	Field: Intact plants	Total volume of the grazed portion	Cage (Intercept)	0.00032576	0.018049	(Intercept)	0.05802	0.01133
			Residual	0.00044437	0.021080	Plant Type	-0.04512	0.01217
		Average volume of grazed portion	Cage (Intercept)	2.7589e-06	0.001661	(Intercept)	0.008360	0.001069
			Residual	2.9517e-06	0.0017181	Plant Type	-0.004654	0.0011183
		Number of scars per plant	Cage (Intercept)	13.567	3.6833	(Intercept)	8.333	1.672
	Clipped Leaves		Residual	3.200	1.7889	Plant Type	-5.500	1.033
		Total amount of leaf tissue consumed	Cage (Intercept)	0.00035550	0.018841	(Intercept)	0.062500	0.015368
			Residual	0.0010621	0.032590	Plant Type	0.009167	0.018816
		Proportion of the amount of leaf tissue consumed	Cage (Intercept)	0.0046024	0.067841	(Intercept)	0.28908	0.07459
			Residual	0.0287823	0.169654	Plant Type	0.05525	0.09795
2013	Field: Intact plants	Proportion of leaf length damaged	Cage (Intercept)	0.0189111	0.137518	(Intercept)	0.16550	0.06602
			Residual	0.0072419	0.085099	Plant Type	0.15842	0.04913
		Total volume of the grazed portion	Cage (Intercept)	4.9742e-05	0.0070528	(Intercept)	0.039917	0.005639
			Residual	3.3190e-04	0.0182181	Plant Type	-0.033375	0.007438
		Average volume of grazed portion	Cage (Intercept)	3.0820e-06	0.0017556	(Intercept)	0.0073191	0.0011736
			Residual	1.2121e-05	0.0034815	Plant Type	0.0007010	0.0019383
					Site	-0.0008696	0.0015425	0.362
					Plant Type:Site	-0.0012794	0.00031390	-0.564
						-0.0012794	0.00031390	-0.408
		Number of scars per plant	Cage (Intercept)	0.77683	0.88138	(Intercept)	5.6250	0.5949
			Residual	3.47033	1.86288	Plant Type	-4.4583	0.7605

			Site	-0.7083	0.7605	-0.931
			Plant Type:Site	0.7500	1.0755	0.697
Number of missing tips	Cage (Intercept)	0.19665	0.44346	(Intercept)	0.3991	6.368
	Residual	1.71480	1.30951	Plant Type	-2.0000	-3.741
			Site	0.1667	0.5346	0.312
			Plant Type:Site	-0.1250	0.7560	-0.165
Number of damaged leaves per plant	Cage (Intercept)	1.0249	1.0124	(Intercept)	5.6250	0.6577
	Residual	4.1654	2.0409	Plant Type	-4.2917	8.553
			Site	-0.2083	0.8332	-5.151
			Plant Type:Site	0.1667	0.8332	-0.250
Portion of a plant utilized for grazing	Cage (Intercept)	0.000000	0.000000	(Intercept)	0.32700	0.04046
	Residual	0.016368	0.12794	Plant Type	-0.19700	8.083
			Site	-0.01089	0.05878	-2.811
			Plant Type:Site	-0.06911	0.12212	-0.185
Greenhouse: Intact plants	Total volume of the grazed portion	Cage (Intercept)	0.0000000	0.0000000	(Intercept)	0.12954
	Residual	0.0032859	0.057323	Plant Type	-0.09487	0.01655
			Exp	-0.08595	0.02340	-4.054
			Plant Type:Exp	0.08486	0.03310	-3.673
Average volume of grazed portion	Cage (Intercept)	0.0000e+00	0.0000000	(Intercept)	0.0063750	7.828
	Residual	1.0764e-05	0.0032809	Plant Type	-0.0010750	0.02340
			Exp	-0.0009813	0.02340	-3.673
			Plant Type:Exp	0.0026463	0.03310	2.564
Number of scars per plant	Cage (Intercept)	0.000	0.0000	(Intercept)	0.0009471	6.731
	Residual	29.587	5.4394	Plant Type	-0.0014048	0.0014048
			Exp	-0.0014975	0.0014975	-0.655
			Plant Type:Exp	0.0020965	0.0020965	1.262
Number of missing tips	Cage (Intercept)	0.0000	0.0000	(Intercept)	2.042	1.570
	Residual	5.0232	2.2412	Plant Type	-2.792	2.221
			Exp	-3.583	2.221	-4.954
			Plant Type:Exp	5.458	3.140	-4.766
Number of damaged leaves per plant	Cage (Intercept)	0.000	0.000	(Intercept)	8.542	11.357
	Residual	11.478	3.388	Plant Type	-10.583	2.221
			Exp	-11.000	0.915	-4.954
			Plant Type:Exp	17.833	1.294	-4.766
Number of damaged leaves per plant	Cage (Intercept)	0.000	0.000	(Intercept)	12.250	8.436
	Residual			Plant Type	-6.708	0.915
					1.383	-3.916
						-3.051
						1.578
						12.525
						-4.850

			Exp	-6.375	1.383	-4.609
		Plant Type:Exp	4.833	1.956	2.471	
	Cage (Intercept)	0.000000	0.00000	0.05129	10.334	
Portion of a plant utilized for grazing	Residual	0.023672	0.15386	0.07253	-2.459	
		(Intercept)	0.53000	0.07754	-1.649	
		Plant Type	-0.17833	0.10771	1.241	
		Exp	-0.12786			
		Plant Type:Exp	0.13369			
		(Intercept)	0.10771			
		Plant Type	-0.16787			
		(Intercept)	0.05636	0.15937	0.354	
		Plant Type	-0.14938	0.14938	-1.124	
		(Intercept)	0.10185	0.04964	6.464	
Grasshopper growth rate: weight	Residual	0.010373	0.12937	0.05925	-4.201	
		(Intercept)	0.32091	0.04964	6.464	
		Plant Type	-0.24890	0.05925	-4.201	
		(Intercept)	0.011985	0.005456	3.300	
Grasshopper growth rate: body length	Residual	0.00014363	0.012847	0.005808	-0.358	
		(Intercept)	0.018007	0.005808	-0.358	
		Plant Type	-0.002077	0.005808	-0.358	
		(Intercept)	0.035365	0.04674	2.753	
Total amount of leaf tissue consumed	Residual	0.00016506	0.143569	0.06299	-0.347	
		Plant Type	-0.02184	0.06299	-0.347	
		(Intercept)	0.12869	0.04674	2.753	
		Plant Type	-0.02184	0.06299	-0.347	
		(Intercept)	0.000000	0.00000	0.00000	
Proportion of the amount of leaf tissue consumed	Residual	0.0075048	0.08663	(Intercept)	0.12000	0.02501
		Plant Type	-0.06125	0.03537	-1.732	
		(Intercept)	0.02501	4.798		
Proportion of leaf length damaged	Residual	0.0075048	0.08663	Plant Type	-0.06125	
		(Intercept)	0.03537	-1.732		

¹Plants of two types were used in the experiments with intact plants: native and exotic plants. Native plants included *Andropogon gerardii* and *Bouteloua curtipendula* in both 2012 and 2013; exotic plants included two cultivars of *Miscanthus sinensis* ('Zebrinus' and 'Gracillimus') in 2012, and *Miscanthus sinensis* (wild type) and *Bothriochloa ischaemum* in 2013

Appendix 3

General linear model parameter estimates of plant resistance of native versus exotic grasses to herbivory by *Melanoplus* grasshoppers in the greenhouse experiments with intact plants in relation to the type of the experiment (“Exp”) and plant origin (“Plant Type”).

Parameter	Fixed effects	Estimate	Std. Error	t value	Pr(> t)
Total volume of the grazed portion	(Intercept)	0.12954	0.01655	7.828	7.12e-10 ***
	Plant Type	-0.09488	0.02340	-4.054	0.000202 ***
	Exp	-0.08595	0.02340	-3.673	0.000647 ***
	Plant Type:Exp	0.08486	0.03310	2.564	0.013832 *
Average volume of grazed portion	(Intercept)	0.0063750	0.0009471	6.731	7.42e-08 ***
	Plant Type	-0.0010750	0.0014048	-0.765	0.449
	Exp	-0.0009813	0.0014975	-0.655	0.516
	Plant Type:Exp	0.0026463	0.0020965	1.262	0.215
Number of scars per plant	(Intercept)	17.833	1.570	11.357	1.14e-14 ***
	Plant Type	-11.000	2.221	-4.954	1.12e-05 ***
	Exp	-10.583	2.221	-4.766	2.08e-05 ***
	Plant Type:Exp	8.542	3.140	2.720	0.00931 **
Number of missing tips	(Intercept)	5.458	0.647	8.436	9.61e-11 ***
	Plant Type	-3.583	0.915	-3.916	0.000309 ***
	Exp	-2.792	0.915	-3.051	0.003856 **
	Plant Type:Exp	2.042	1.294	1.578	0.121772
Number of damaged leaves per plant	(Intercept)	12.250	0.978	12.525	4.17e-16 ***
	Plant Type	-6.708	1.383	-4.850	1.58e-05 ***

	Exp	-6.375	1.383	-4.609	3.46e-05 ***
	Plant Type:Exp	4.833	1.956	2.471	0.0174 *
Portion of a plant utilized for grazing	(Intercept)	0.53000	0.05129	10.334	3.11e-11 ***
	Plant Type	-0.17833	0.07253	-2.459	0.0201 *
	Exp	-0.12786	0.07754	-1.649	0.1099
	Plant Type:Exp	0.13369	0.10771	1.241	0.2245
Grasshopper growth rate: weight	(Intercept)	0.05636	0.15740	0.358	0.724
	Plant Type	-0.15303	0.23464	-0.652	0.523
Grasshopper growth rate: body length	(Intercept)	0.32091	0.04959	6.471	4.36e-06 ***
	Plant Type	-0.24202	0.07393	-3.274	0.00422 **

¹ Experiments were conducted in the University of Cincinnati greenhouse.

² Plants of two types were used in the experiments with intact plants: native and exotic plants. Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Miscanthus sinensis* (wild type) and *Bothriochloa ischaemum*.

³ Experiments of two types were conducted: no-choice (native or exotic plants only) and choice (both types of plants).

⁴ * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

Appendix 4

General linear model parameter estimates of leaf damage of native versus exotic grasses by *Melanoplus* grasshoppers in the experiments with clipped leaves in relation to the year (“Year”) and plant origin (“Plant Type”).

Parameter	Fixed effects	Estimate	Std. Error	t value	Pr(> t)
Total amount of leaf tissue consumed	(Intercept)	93.62050	27.16437	3.446	0.00175 **
	Plant Type	20.46450	38.08761	0.537	0.59516
	Year	-0.04650	0.01350	-3.445	0.00176 **
	Plant Type:Year	-0.01017	0.01892	-0.537	0.59521
Proportion of the amount of leaf tissue consumed	(Intercept)	326.40075	167.00649	1.954	0.0604
	Plant Type	155.48225	234.16253	0.664	0.5119
	Year	-0.16208	0.08298	-1.953	0.0605
	Plant Type:Year	-0.07725	0.11635	-0.664	0.5120
Proportion of leaf length damaged	(Intercept)	91.71150	116.19732	0.789	0.4358
	Plant Type	442.12775	164.32783	2.691	0.0112 *
	Year	-0.04550	0.05773	-0.788	0.4364
	Plant Type:Year	-0.21967	0.08165	-2.690	0.0112 *

¹ Experiments were conducted in 2012 and 2013.

² Plants of two types were used in the experiments with intact plants: native and exotic plants. Native plants included *Andropogon gerardii* and *Bouteloua curtipendula* in both 2012 and 2013; exotic plants included two cultivars of *Miscanthus sinensis* ('Zebrinus' and 'Gracillimus') in 2012, and *Miscanthus sinensis* (wild type) and *Bothriochloa ischaemum* in 2013

³ * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

CHAPTER 3

Comparison of Native and Exotic Grasses in Their Tolerance to Herbivory by a Generalist

Insect Herbivore¹

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Abstract

Plant tolerance to herbivory is an important component of plant competitive ability, which can facilitate the plant invasion process (the Evolution of Increased Competitive Ability Hypothesis). The prediction of higher tolerance to herbivory in invasive plants has received mixed support in experimental studies, partially due to differences in methods of estimating plant tolerance in terms of plant growth. Using non-destructive methods to accurately estimate aboveground biomass, a standard measure of plant growth, can be challenging, especially for grasses. In addition, using different proxies for plant biomass may potentially cause mixed results in the estimation of plant tolerance. To address these issues in our study, we conducted field and greenhouse experiments using native *Andropogon gerardii* and *Bouteloua curtipendula*, and exotic *Bothriochloa ischaemum* and *Miscanthus sinensis* grasses, and examined their relative response to nymphal *Melanoplus spp.* grasshoppers. We first determined that in most grass species the product [plant height × number of leaves] and plant height were the best predictors of changes in plant biomass during herbivory and after a subsequent regrowth period. Based on these best predictors in grasshopper herbivory assays, we then found no difference in tolerance to herbivory among our study grasses. These results suggest that exotic grasses which have not yet become invasive at the study sites might not demonstrate strong allocation from defenses to growth and reproduction, at least at the current time in these locations.

Key words: EICA hypothesis, grasshopper herbivory, plant invasion, plant tolerance

Introduction

Plant tolerance to herbivory (the ability to maintain fitness while sustaining damage) is an important plant defense strategy which drives the ecology and evolution of plant-herbivore interactions (Rosenthal and Kotanen 1994, Price et al. 2011). As a complex trait, plant tolerance includes many physiological components, such as growth rate, storage capacity, photosynthetic rates, and nutrient uptake, among which growth rate has been considered fundamental (Rosenthal and Kotanen 1994). Plant tolerance to herbivory is especially important for grasses, and grasslands with high grazing rates and a long grazing history are dominated by tolerant species (Rosenthal and Kotanen 1994, Briske et al. 1996).

The Evolution of Increased Competitive Ability (EICA) hypothesis, proposed by Blossey and Notzold (1995), predicts higher tolerance to herbivory (due to reallocation of plant resources from defenses to growth and reproduction), and consequently increased competitive ability of exotic plants compared to native plants. This prediction however, has received mixed support in experimental studies. For example, results from experiments with the Chinese tallow tree *Sapium sebiferum* (Siemann and Rogers 2003), and plants from the genus *Centaurea* (Jogesh et al. 2008) were consistent with the predictions of the EICA hypothesis, suggesting that invasive ecotypes are herbivory-tolerant. Meanwhile, experiments with invasive *Lythrum salicaria* (Willis et al. 1999), *Hypericum perforatum* (Maron et al. 2004), *Solidago canadensis* (Van Kleunen and Schmidt 2003), and *Alliaria petiolata* (Bossdorf et al. 2004) did not reveal differences in growth and size between introduced and native plant populations in response to herbivory. It has been suggested that variability in studies that tested the EICA hypotheses can be explained by different experimental design parameters (Atwood and Meyerson 2011). Such differences primarily include experimental conditions (outdoor garden or greenhouse) and the actual

measurement of plant tolerance. Atwood and Meyerson (2011) emphasized that using standard metrics and exploring multiple traits which are important in post-establishment evolution of exotic plants could benefit research on plant tolerance to herbivory.

The compensatory growth response of plant is a standard measure of the effect of herbivory (Atwood and Meyerson 2011). Plant growth in terms of aboveground plant biomass is a critical factor in managing grasslands (Leis and Morrison 2011), and it has commonly been used in many ecological studies on grasses, including their tolerance to herbivory (e.g. Anderson and Briske 1995, Hendon and Briske 2002). Accurate estimation of biomass of grasses, however, can be challenging, and the comparisons of plant biomass can be limited, as there is no universal method for biomass estimation which is valid across all plant communities (Redjadj et al. 2012).

Redjadj et al. (2012) indicated that an important requirement for estimating biomass is that the method be non-destructive and easy to implement. Harvesting and weighing of vegetation is a common method of estimating aboveground plant biomass (Catchpole and Wheeler 1992). Although accurate, this method is destructive and typically time-consuming. Recently, many non-destructive methods for estimating aboveground biomass in grasses have been proposed. These methods include, for example, 3D quadrat and the BOTANICAL method (Redjadj et al. 2012), allometric equations (e.g. Oliveras et al. 2013), digital photography technique (Leis and Morrison 2011), and spectral information of an UltraCamTM image (Clevers et al., 2007). Studies on grasses which explored the best predictors for aboveground biomass used variables such as the maximum height, the crown area (for tussock grassland species), basal area (Oliveras et al. 2013), or the number of seedheads (Andariese and Covington 1986). Although these methods are non-destructive, they are not always accessible due to their cost or time needed to implement them. In addition, most of these methods have been developed

for large-scale field measurements of biomass production in grasslands; consequently it might be difficult to implement them in the experiments on herbivory using field or greenhouse enclosures. In addition, certain proxies for plant biomass, such as plant height or number of leaves, may not necessarily work for different and even closely related species, which potentially could cause mixed results in the estimation of plant tolerance.

To address these issues in our study of plant tolerance to grasshopper herbivory, we first were interested in finding the best predictors for aboveground plant biomass in native *Andropogon gerardii* and *Bouteloua curtipendula*, and in exotic *Bothriochloa ischaemum* and *Miscanthus sinensis*. We then used these predictors to compare the tolerance of our study species to herbivory by nymphal *Melanoplus* grasshoppers. These grasses, as well as their congeners, play an important role in tallgrass prairies (e.g. Hendon and Briske 2002, Schmidt et al. 2008, Quinn et al. 2010) and have been examined in experiments on herbivory, including by grasshoppers (e.g. Alward and Joern 1993, Whipple et al. 2009, Nabity et al. 2012). Given that grasshoppers are an important group of insect herbivores in grasslands (e.g. Belovsky and Slade 2000; Scherber et al. 2010), accurate estimation of tolerance of grasses to grasshopper feeding is required. In addition, determining the best predictor for biomass in these grasses is critical for estimating the compensatory plant regrowth after herbivory, an important measure of plant recovery after herbivory (Rosenthal and Kotanen 1994, Scherber et al. 2010).

Our study had two primary objectives. First, we compared three proxies for plant biomass which are commonly used in studies on herbivory: plant height, number of leaves, and [plant height \times number of leaves]. The best predictor which explained the greatest amount of variation in plant biomass was then selected for comparison of plant tolerance among plant species. We conducted separate analyses for plant growth during herbivory by nymphal

Melanoplus grasshoppers and after a subsequent regrowth period. Considering that plant height is often used to characterize plant response to grasshopper herbivory (e.g. Rogers and Siemann, 2005), we hypothesized that this predictor variable would explain the greatest amount of variation in plant biomass during herbivory and during the period of plant recovery. Second, we compared tolerance to grasshopper herbivory among native *A. gerardii* and *B. curtipendula*, and exotic *B. ischaemum* and *M. sinensis* using these best predictor variables for plant biomass. Based on the prediction of the EICA hypothesis of increased tolerance in exotic plants, we hypothesized that exotic *B. ischaemum* and *M. sinensis* would demonstrate greater growth during herbivory and after a subsequent regrowth period compared to native *A. gerardii* and *B. curtipendula* grasses.

Methods

The best predictors for plant biomass

To determine the best predictor for plant biomass, we planted seeds for *A. gerardii*, *B. curtipendula*, *B. ischaemum*, and *M. sinensis* in May 2012 in the University of Cincinnati (UC) greenhouse (seeds were obtained from Prairie Moon Nursery, Winona, MN; Outsidepride.com, Inc., Independence, OR; and Warner Brothers Seed Company, Lawton, OK respectively). In the beginning of July 2013, we planted 120 potted plants (20-25 cm in height, 3.5" square pots; hereafter referred to as plant individuals) grown from these seeds into the field site established at the University of Cincinnati Center for Field Studies (UCCFS, 39°17.134' N, 084°44.413' W, Harrison, OH). Plants were arranged in 4 rows with 0.5 m spacing between rows, and each row consisted of 30 plant individuals of each species.

These plants were grown simultaneously with plants used for grasshopper herbivore assays (described below) and were harvested at three time points: (1) at the beginning of feeding trials, (2) at the end of feeding trials (in 5 days), and (3) 2 weeks after the feeding trials were completed. The feeding trials started 10 days after planting at the field site. At each of the three time points, 10 different plant individuals of each of the 4 species were clipped at the ground, weighed, and air dried at room temperature for 3 weeks. The height and the number of leaves of each plant were also measured.

All analyses were conducted using a generalized additive model (GAM) in R (R v.2.15.2, package *mgcv*). For each plant species, we estimated the following predictor variables: plant height, number of leaves, and [plant height \times number of leaves]. We conducted separate analyses for (1) plant growth during herbivory (day 1-5) and (2) plant compensatory regrowth after herbivory (day 6-19) by nymph *Melanoplus* grasshoppers. For each analysis, the model with a predictor variable which explained the greatest amount of variation in plant biomass and with the lowest Akaike Information Criterion (AIC) was selected as the best model. Consequently, the predictor variable used in the best model was considered as the best predictor for plant biomass.

We estimated the best predictors of plant biomass in terms of both wet weight and dry weight. Two best predictors were chosen: one predictor for estimating biomass changes during plant growth (Predictor 1) and, one predictor for estimating plant biomass after a subsequent regrowth period (Predictor 2, if different from Predictor 1). These best predictors were then used to compare tolerance among the same grass species to grasshopper herbivory under field and greenhouse conditions. To increase the accuracy of our results and minimize the effect of potential seasonal changes in plants on their growth, both herbivory assays and determining the best predictor for plant biomass were conducted simultaneously.

Grasshopper herbivory assays

To compare the tolerance among grasses to grasshopper herbivory, we conducted field and greenhouse experiments in July 2013. We first planted 192 potted plants in the field (96 potted plants at each site): at the UCCFS, and additionally, at the Western Maryland Research and Education Center (WMREC, 39°30.618' N, 077°44.070' W, Keedysville, MD) where we established a similar plot to repeat this field experiment (see Chapter 2 for further description).

At each field site, we planted 96 potted plants in twelve groups of eight: each group contained two plant individuals of each plant species, a control plant and an experimental plant (i.e. a plant which has been exposed to grasshopper herbivory). Around each plant group, we set up an open air aluminum screen cage (16×16×20" Repti Breeze Aluminum Screen Cage, Zoo Med Laboratories, Inc., California, USA), divided by a window screen, separating control and experimental plants (Fig. 1 A-D). Three nymphs of the *Melanoplus spp.* grasshoppers (Acrididae: Orthoptera), presumably *M. differentialis* and *M. femur-rubrum* (Pfadt, 1994), were caught near each plot and were then placed in each cage for 5 days. The grasshoppers were then removed and the plants were allowed to regrow for two weeks (Fig. 1 E).

Simultaneously with the field experiments, we set up a similar greenhouse experiment using potted plants at the University of Cincinnati greenhouse. As we used fabric cages (Bioquip, rearing and observation cage, 14×14×24", Cat. No. 1466B) in which we were not able to separate plants within the cage, we placed control and experimental plants in different cages. In total, we used 12 cages with control plants and 12 cages with experimental plants. We placed 4 potted plants (each pot of a different plant species) in each cage. Nymphal grasshoppers were caught at the UCCFS on a day before the experiment. We then placed three nymph grasshoppers in each

experimental cage for 5 days. On day 6, grasshoppers were removed and plants were allowed to regrow for 2 weeks.

To estimate plant tolerance in terms of plant growth during herbivory and after a subsequent regrowth period (for both field and greenhouse experiments), we measured the height (cm), the number of leaves, as well as [plant height \times number of leaves], of all plants at both the beginning and end of the herbivory period, as well as 2 weeks after grasshopper herbivory ended. For each variable, plant growth (PG) was measured using the method that Siemann and Rogers (2003) applied for plant stem height: [(day 6 measurement - day 1 measurement) / day 1 measurement, cm/cm]. Similarly, we estimated plant regrowth (PR) as [(day 19 measurement - day 6 measurement) / day 6 measurement, cm/cm]. For comparative purposes, we used plant growth rate per day [PG / 5 days, cm/cm/day] and regrowth rate per day [PR / 14 days, cm/cm/day]. As different plant species might grow at different rates, we could not compare plant growth directly. Instead, we estimated the difference in growth and regrowth rates between control and experimental plant individuals for each plant species within each cage as [growth/regrowth rate of a control plant – growth/regrowth rate of an experimental plant]. We then used these values for all comparisons among plant species. To meet the assumptions of normality and homoscedasticity, all data for these predictor variables were square root-transformed.

To account for a potential cage effect on plant tolerance data, we ran a generalized linear mixed model (GLMM) in R (v.2.15.2, package *lme4*). Plant species (*A. gerardii*, *B. curtipendula*, *B. ischaemum*, and *M. sinensis*) and site (UCCFS, WMREC, and UC greenhouse) were considered as fixed effects, and cages were considered as a random effect. When the random effect was minimal or absent, the two-way fixed effects ANOVA (for comparison of plant

tolerance across all sites) and one-way fixed effects ANOVA (for comparison of plant tolerance under field and greenhouse conditions, as well as among sites) were used to estimate differences in plant tolerance to grasshopper herbivory among plant species.

Results

The best predictors for plant biomass

We found that [plant height \times number of leaves] was the best predictor for biomass changes during plant growth during herbivory in *A. gerardii*, *B. curtipendula*, and *B. ischaemum*; this predictor variable explained 71-99% variation of wet plant biomass and 79-99% variation of dry plant biomass (Table 1, Fig. 2 A-C). The product [plant height \times number of leaves] was the second best predictor for the growth of *M. sinensis* (44.6 and 50.3% variation explained in wet and dry biomass respectively) (Fig. 2 D). Plant height was the best predictor for *M. sinensis*; it explained 67.8% and 79.4% variation of wet and dry plant biomass respectively (Fig. 3 A).

We also found that plant height was the best predictor for plant regrowth after herbivory in *B. curtipendula*, *B. ischaemum* and *M. sinensis*; this predictor variable explained 70-98% variation of wet biomass and 78-98% variation of dry biomass (Fig. 4). Plant height was the second best predictor for the regrowth of *A. gerardii* and explained 70% variation of plant biomass; whereas the number of leaves was the best predictor for the regrowth of *A. gerardii* explaining 94% variation of plant biomass (in terms of both wet and dry values) (Fig. 3 B). Consequently, [plant height \times number of leaves] was chosen to compare plant growth (Predictor 1) and plant height was selected to compare compensatory regrowth after herbivory (Predictor 2) among plant species in the subsequent grasshopper herbivory assays.

Grasshopper herbivory assays

There was no significant cage effect on plant growth during grasshopper herbivory, although there was a minimal cage effect (5% of variation in plant regrowth was explained by a cage effect) on plant regrowth after herbivory (Appendix 1). Therefore, we proceeded with direct comparisons of tolerance to herbivory among plant species.

Based on the best predictors we determined for plant biomass (described above), we found no significant differences in plant tolerance (in terms of both plant growth and plant regrowth) among plant species, in both separate and combined analyses across sites (Table 2, Fig. 5).

We also did not observe differences in plant tolerance under field and greenhouse conditions (Growth: $F_{1, 89} = 2.605$, $p = 0.11$; Regrowth: $F_{1, 74} = 0.568$, $p = 0.454$). However, site as a factor had a significant effect on overall plant tolerance combined across plant species (Table 2, Fig. 5). At the UCCFS (OH), plants which sustained herbivory overall demonstrated greater differences in growth and regrowth, compared to control plants, compared to plants at the WMREC (MD) and the UC greenhouse (Fig. 6); consequently, the overall plant tolerance to herbivory at the UCCFS were lower than at other sites. All other comparisons were not significant.

Discussion

The best predictors for plant biomass

Plant growth in terms of plant biomass is an important measurement in many studies on plant responses to herbivory. As it is critical to use non-destructive methods of plant biomass estimation in exploring plant recovery after herbivory, our objective was to determine the best

predictor variable for aboveground biomass changes in our study grasses. Our hypothesis that plant height is the best predictor variable for plant biomass was supported for growth during herbivory of *M. sinensis* and for a subsequent regrowth period of *M. sinensis*, *B. curtipendula*, and *B. ischaemum*. This hypothesis was not supported for growth of *A. gerardii*, *B. curtipendula*, and *B. ischaemum* (in which [plant height × number of leaves] was the best predictor), as well as for regrowth of *A. gerardii* (in which number of leaves was the best predictor).

The possible cause for these results is the difference in growth pattern among grasses over the course of the experiment, and especially the difference in morphological traits at different stages of plant growth during herbivory (Fig. 1 B, C) and after a subsequent regrowth period (Fig. 1 E). Our results demonstrated that plant growth in *A. gerardii*, *B. curtipendula*, and *B. ischaemum* during the first week after planting was similar because of the simultaneous increase of plant height and number of leaves (Fig. 1 A-C). During the subsequent two weeks, the growth of *B. curtipendula* and *B. ischaemum* resulted mostly from an increase in height while the production of new leaves contributed less to the increase of plant biomass (Fig. 1 E). In *A. gerardii*, however, new leaf production apparently became more important than plant height during this regrowth stage (Fig. 1 C, E). As for *M. sinensis*, growth during all 3 weeks of the experiment (for both herbivory and a subsequent regrowth period) apparently resulted from an increase in plant height only. This may be explained by the maximum height of 4-6 m which these plants can reach during their lifetime (Faix et al., 1989). Our experiments were conducted during only a 3-week summer period; it would be interesting for future studies to explore how these predictors contribute to plant biomass changes during the entire summer season.

It is possible that predictor variables, other than those explored in this study, may play important role in growth of grasses. For example, Andarieze and Covington (1986) demonstrated

that plant basal area (i.e. the circle where the harvested grass is bound together) was the best predictor for biomass of grasses *Poa fendleriana*, *Festuca arizonica*, and *Muhlenbergia montana*. Some studies on tallgrass prairie plants used measurements such as tiller number (Hendon and Briske, 2002) and number of seedheads (Andariese and Covington, 1986). Based on tiller number, Hendon and Briske (2002) found that herbivory tolerance in *Sorghastrum nutans*, *Schizachyrium scoparium*, and *Bothriochloa laguroides* was similar. Using tiller number in our study was complicated by a different pattern of tiller development in our study species: *M. sinensis* plants, for example, did not develop many new tillers during the experiment, whereas new tillers in *B. ischaemum* plants grew relatively quickly and by the end of the experiment filled the most space in cages (Figure 4 E). We considered using the number of seedheads in our study, however we were not able to obtain those data due to a small number of seedheads per plant individual (2-3 inflorescences) observed in *A. gerardii*, *B. curtipendula*, and *B. ischaemum*, and no seedheads observed in *M. sinensis* (which matured later in the season).

Overall, the proposed method for estimating plant tolerance to herbivory is beneficial for small-scale herbivory assays which require repetitive measurements of the same plants while keeping them alive. The best predictor variables which we determined for *A. gerardii*, *B. curtipendula*, and *B. ischaemum*, and *M. sinensis*, may be most applicable to grasses whose growth pattern is similar to that of our study species. If the grass species of interest exhibits a growth pattern which is different from our study species, then a researcher should explore other methods to determine the best predictor of biomass of their grass species under specific experimental conditions.

Grasshopper herbivory assays

In our herbivory assays, we implemented methodological considerations from previous studies on plant tolerance (e.g. Rosenthal and Kotanen, 1994; Scherber et al., 2010, Atwood and Meyerson, 2011) and used the best predictor for plant biomass, which we determined earlier, to compare plant tolerance to grasshopper herbivory.

Our hypothesis that there would be greater tolerance to grasshopper herbivory of exotic *B. ischaemum* and *M. sinensis* compared to native *A. gerardii* and *B. curtipendula* grasses was not supported in any of the field and/or greenhouse experiments. Plant growth during both herbivory and a subsequent regrowth period did not differ among individual plant species, using the best predictor variables of [plant height \times number of leaves] (for growth) and plant height (for regrowth). Based on other predictors (data are not shown), differences among plants were still not significant, however the interpretation of results was complicated by a significant effect of the [site \times plant] factor in many comparisons as well as variability of the data. This suggests that to compare plant tolerance among species, it is critical to use the best predictors which explain the most amount of variation in biomass of each plant species; it increases accuracy of data and applicability of results from such studies.

There are at least two factors which might affect our results: (1) the presence of other grass species in the same cage and, therefore, their competitive ability, and (2) experimental conditions (field or greenhouse). For example, Schmidt et al. (2008) demonstrated that *B. ischaemum* might be a superior competitor when grown in close proximity to the native grass species. In their experiments, *B. ischaemum* significantly reduced all growth parameters of the natives, *A. gerardii* and *S. scoparium* and belowground biomass of *B. curtipendula*. Although such competitive ability of *B. ischaemum* most likely did not affect the results from the

experiments for determining the best predictors for plant biomass (as grasses were not grown in the cages and were placed at a certain distance from each other), it might affect growth of other plant species in the cages during the herbivory assays.

Although our results showed that plant tolerance under field and greenhouse conditions did not differ among plant species, greenhouse conditions might affect certain plant traits and consequently, their contribution to biomass. We found significant difference between tolerance of plants used in the greenhouse experiments and plants grown at the UCCFS (but not at the WMREC). It would be beneficial for future studies which use greenhouse herbivory assays to conduct additional experiments to determine the best predictors for plant biomass under greenhouse conditions. An additional suggested modification would be to grow the plants for determining the best predictor of biomass in cages similar to those used for herbivory assays.

Our results of similar plant tolerance to herbivory among native and exotic grasses did not support the prediction of the EICA hypothesis, and are consistent with the results from some experimental studies on other plants (e.g. Bossdorf et al., 2004). Bossdorf et al. (2004) found no difference in tolerance between native and introduced *Alliaria* populations to a generalist insect herbivore. The authors also demonstrated the lack of a trade-off between tolerance and resistance, detailed exploration of which was beyond the scope of our study. We did however demonstrate lower resistance of exotic grasses to grasshopper herbivory compared to native grasses (see Chapter 2); it would be interesting for future studies to explore whether lower resistance in exotic grasses used in our study corresponds to greater tolerance.

Our results, however, are in contrast to some other studies: Rogers and Siemann (2005), for example, used similar grasshopper herbivory assays to demonstrate that the compensatory regrowth of native *Sapium* ecotypes was significantly reduced compared to introduced ecotypes.

Unlike those studies, we used different plant species; it would be interesting for future experiments to compare plant tolerance to grasshopper herbivory between native and exotic populations of *B. ischaemum* and *M. sinensis*.

In our investigation, we explored the potential of exotic grasses to demonstrate a greater tolerance to herbivory compared to natives, should these grasses escape cultivation and become invasive in the introduced range. To test the EICA hypothesis, however, it is important to focus on both predictions of increased tolerance and the decreased resistance of invasive species (Turner et al., 2013). The lack of observed increased tolerance in exotic grasses suggests that these species might not yet demonstrate the strong allocation from defenses to growth and reproduction, and consequently, possess the ability to quickly replace photosynthetic tissues (Briske 1991; Rosenthal and Kotanen 1994). The latter has not been tested in our experiments and in combination with plant resistance, could be a focus for future studies.

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Table 1. Results of generalized additive models for plant height (H), number of leaves (L), and [height × number of leaves] (H×L) which were used as predictor variables for wet and dry biomass of four grass species.

Plant species	Predictor	Plant growth				Plant regrowth			
		Wet biomass		Dry biomass		Wet biomass		Dry biomass	
variables	Dev.	Akaike	Dev.	Akaike	Dev.	Akaike	Dev.	Akaike	Dev.
	expl., information	expl., information			expl., information	expl., information			information
	% criterion	% criterion	% criterion	% criterion	% criterion	% criterion	% criterion	% criterion	criterion
	df	AIC	df	AIC	df	AIC	df	AIC	df
									AIC
<i>Andropogon gerardii</i>	H	86.5	10.1	-36.4	39.4	3	-34.2	70.9	4.1
	L	33.3	3	-18.8	54.9	4.8	-36.4	94.5	10.9
	H×L	87.9	9.7	-39.6	84.1	5.4	-56	55.2	3
<i>Bouteloua curtipendula</i>	H	98.2	10	-35	97.9	9.8	-35.5	85.3	9.6
	L	62.9	3	11.8	62.6	3	8.2	52.5	4.6
	H×L	99.6	9.52	-65.2	99.5	9.9	-63.1	54.9	3
<i>Bothriochloa ischaemum</i>	H	12.1	3	76.8	8.6	3	41.8	70.2	5.8
	L	67.9	3.9	58.5	63.3	3.7	24.8	37.9	6.2
	H×L	71.3	3.7	55.9	79.2	4.5	15.1	69.2	3

<i>Miscanthus</i>	H	67.8	3	-99.1	79.4	5.8	-102.1	98.3	6.3	27.8	98.1	6.5	-3.5
<i>sinensis</i>	L	18.4	3	-80.4	20.1	3.1	-81.2	87.6	7.5	70.3	89.7	7.8	33.7
	H×L	44.6	3	-88.2	50.3	3.9	-89.1	96.9	7.4	42.1	97.8	7.6	2.2

The best predictor variables chosen for comparisons of plant tolerance during herbivory (Plant growth) and after a subsequent regrowth period (Plant regrowth)
are in bold.

Table 2. The results of two-way fixed effects ANOVA for comparison of plant tolerance among four grass species.

Site	Source	Growth				Regrowth			
		df	SS	F	P	df	SS	F	P
(Ohio)	Plant species	3	0.11	0.35	0.79	3	0.36	1.89	1.16
	Residuals	27	2.81			22	1.39		
(Maryland)	Plant species	3	0.09	1.82	0.172	3	0.06	0.7	0.56
	Residuals	22	0.37			18	0.52		
UC	Plant species	3	0.16	0.65	0.59	3	0.05	0.33	0.8
	Residuals	30	1.78			27	1.32		
greenhouse	Plant species	3	0.09	0.44	0.725	3	0.22	1.58	0.2
	Residuals	79	5.2						
Combined	Plant species	3	0.09	0.44	0.725	3	0.22	1.58	0.2
	Site	2	1.73	13.12	1.2e-05	2	0.72	7.87	0.0009
sites	Plant species:Site	6	0.327	0.83	0.55	64	0.32	1.17	0.33
	Residuals	79	5.2						
							2.9		

¹ Native plant species included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plant species included *Miscanthus sinensis* and *Bothriochloa ischaemum*.

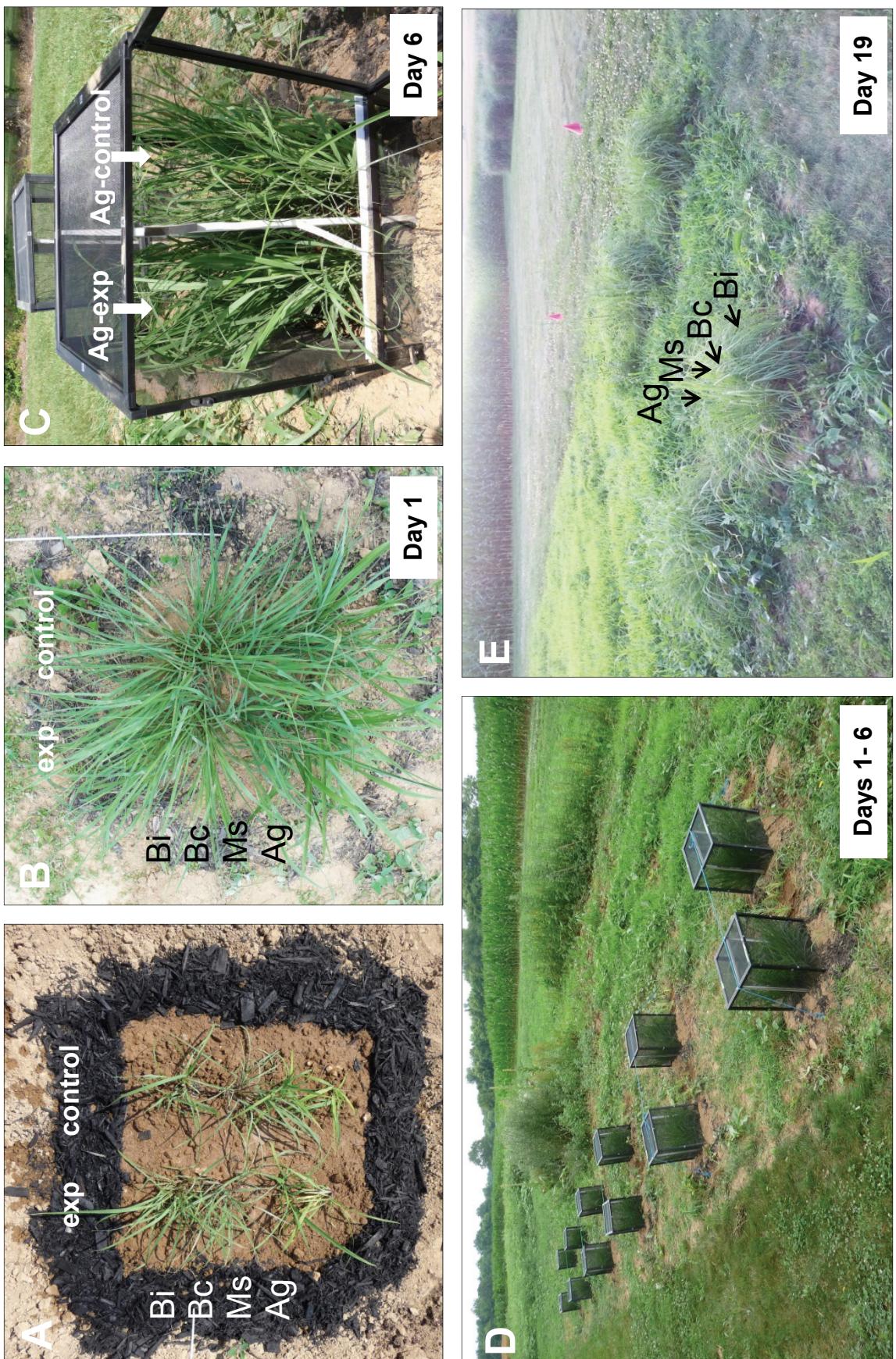


Figure 1. Growth patterns of native *Andropogon gerardii* (Ag) and *Bouteloua curtipendula* (Bc), and exotic *Bothriochloa ischaemum* (Bi) and *Misanthus sinensis* (Ms) during grasshopper herbivory assays at the Western Maryland Research and Education Center. (A) – planted control and experimental (exp) plants; (B, C) – plants at the beginning (day 1) and at the end (day 6) of herbivory assays; (D) – herbivory assays; (E) – plants on day 19 of the regrowth period after herbivory (more than 50% of plant biomass in each plant group belongs to *B. ischaemum*).

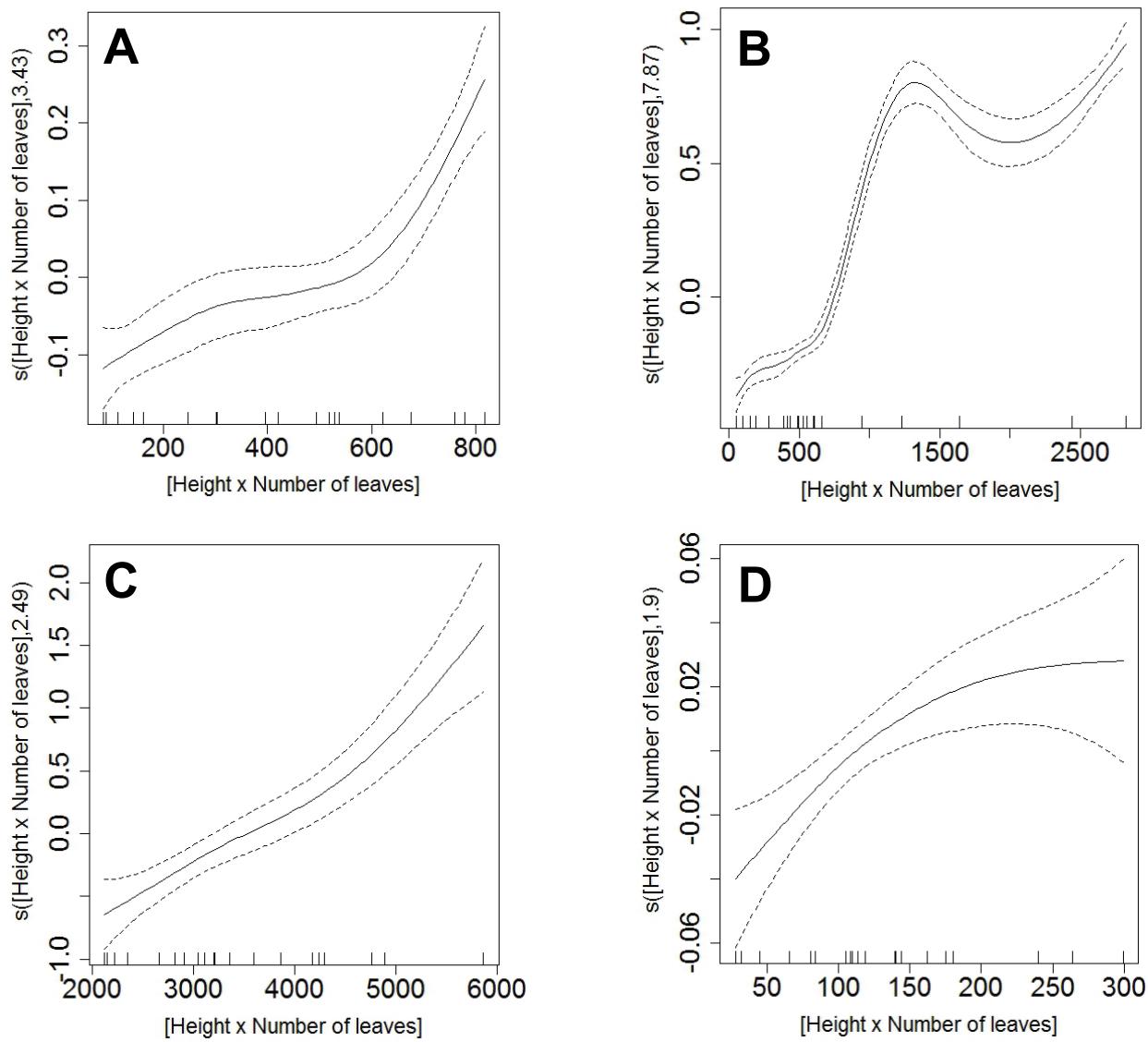


Figure 2. Plots from generalized additive models in which plant biomass changes during herbivory have been modeled as a smooth function of [plant height \times number of leaves]. Results are shown for dry values of plant biomass of native *Andropogon gerardii* (A) and *Bouteloua curtipendula* (B), and exotic *Bothriochloa ischaemum* (C) and *Miscanthus sinensis* (D). The dashed lines are approximate two standard error limits.

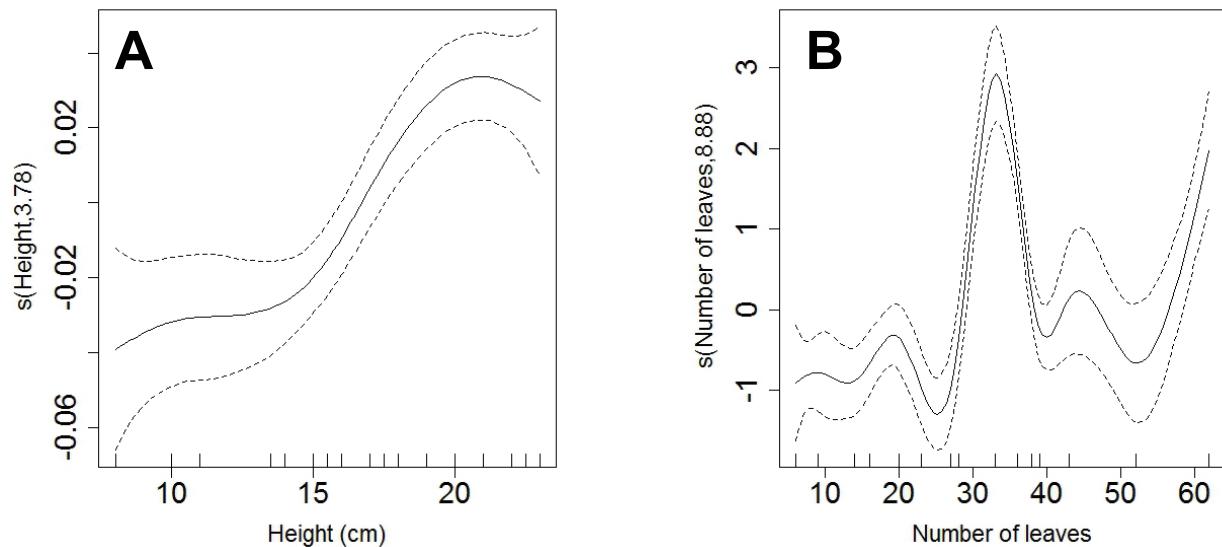


Figure 3. Plots from generalized additive models in which plant biomass has been modeled as a smooth function of (A) the plant height in *Miscanthus sinensis* during herbivory and (B) the number of leaves after a subsequent regrowth period in *Andropogon gerardii*. Results are shown for dry values of plant biomass. The dashed lines are approximate two standard error limits.

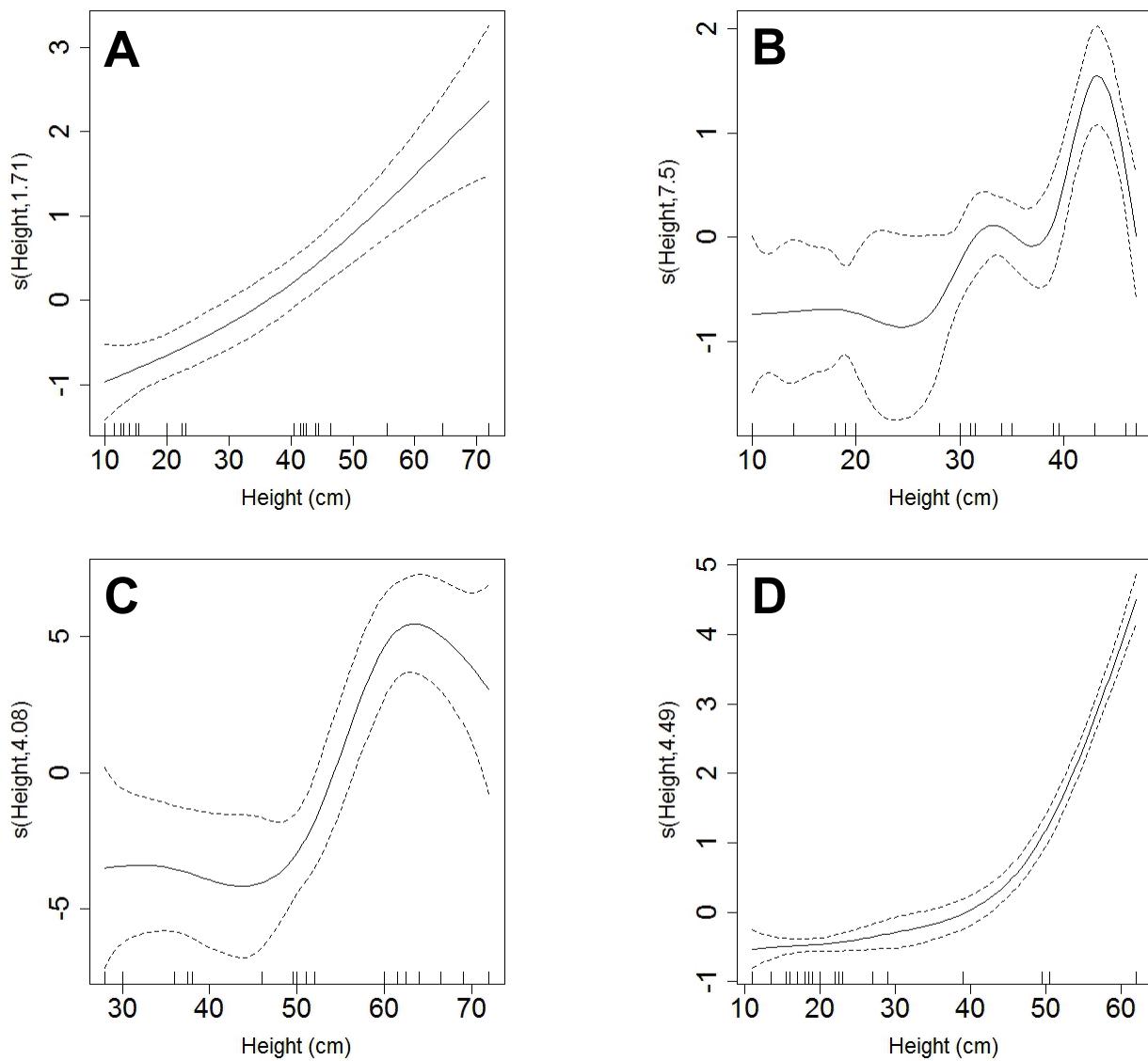


Figure 4. Plots from generalized additive models in which plant biomass changes during plant regrowth after herbivory have been modeled as a smooth function of plant height. Results are shown for dry values of plant biomass of native *Andropogon gerardii* (A) and *Bouteloua curtipendula* (B), and exotic *Bothriochloa ischaemum* (C) and *Miscanthus sinensis* (D). The dashed lines are approximate two standard error limits.

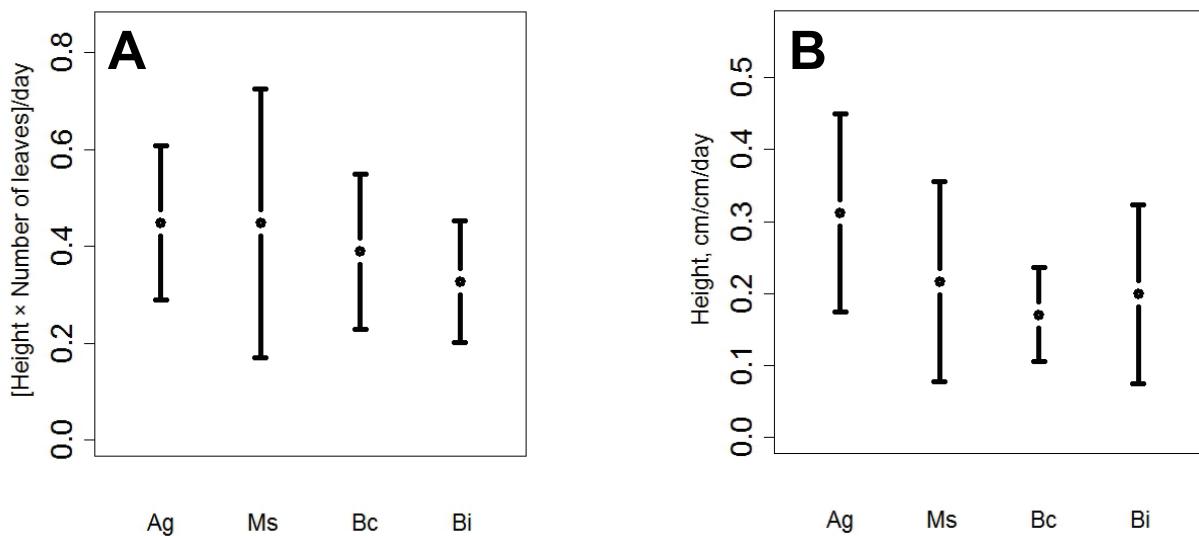


Figure 5. Plant tolerance of native *Andropogon gerardii* (Ag) and *Bouteloua curtipendula* (Bc), and exotic *Bothriochloa ischaemum* (Bi) and *Miscanthus sinensis* (Ms) during herbivory (A) and a subsequent regrowth period (B). All comparisons were conducted using the best predictors for plant biomass changes during those growth periods. The data represent the differences in [plant height \times number of leaves] (A) and in plant height (B) between control (no herbivory) and experimental (exposed to herbivory) plants for each plant species. (A): values on Y-axis correspond to [(plant height \times number of leaves/day of a control plant) – (plant height \times number of leaves/day of an experimental plant)]; (B): values on Y-axis correspond to [(plant height/day of a control plant) – (plant height/day of an experimental plant)]. The vertical lines represent mean values \pm 95% confidence intervals. Plant tolerance in terms of both growth and regrowth after herbivory did not differ among plant species ($P>0.05$).

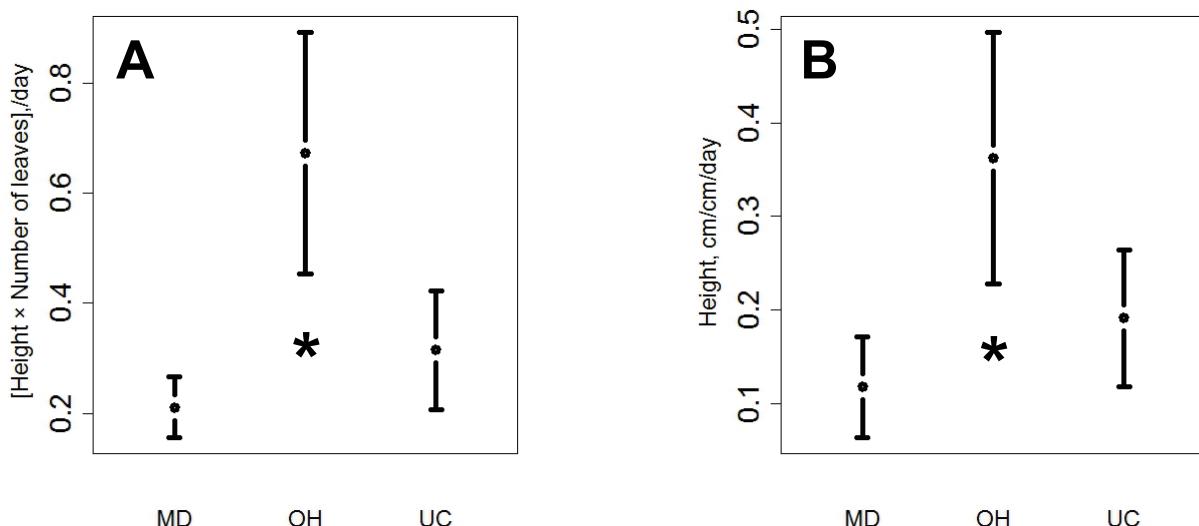


Figure 6. The effect of study site location on plant tolerance to grasshopper herbivory. Plant species included native *Andropogon gerardii* and *Bouteloua curtipendula*, and exotic *Bothriochloa ischaemum* and *Miscanthus sinensis*. The data are combined across plant species. The plots represent the differences in [plant height \times number of leaves] during herbivory (A) and in plant height during a subsequent regrowth period (B) between control (no herbivory) and experimental (exposed to herbivory) plants. For both growth periods, these differences were greater at the University of Cincinnati Center for Field Studies (OH) compared to the Western Maryland Research and Education Center (MD) and the University of Cincinnati greenhouse (UC). The vertical lines represent mean values \pm 95% confidence intervals. Asterisks ("*") indicate significant differences at $P = 0.05$.

Appendix 1

Model (GLMM) estimates of an effect of grasshopper enclosures (cages) on plant growth during herbivory by nymph *Melanoplus spp.* grasshoppers and after a subsequent regrowth period.

Effects	Plant growth				Plant Regrowth							
Random	Random effects:				Random effects:							
effects	Groups	Name	Variance	Std. Dev.	Groups	Name	Variance	Std. Dev.				
	Cage	(Intercept)	0.00000	0.00000	Cage	(Intercept)	0.0026187	0.051173				
	Residual		0.06541	0.25575	Residual		0.0435496	0.208685				
	Number of obs: 71, groups: Cage, 12				Number of obs: 76, groups: Cage, 12							
Fixed	Fixed effects:				Fixed effects:							
effects		Estimate	Std. Error	t value		Estimate	Std. Error	t value				
	(Intercept)	0.436127	0.077163	5.652	(Intercept)	0.38185	0.06291	6.069				
	PlantMs	0.004416	0.081305	0.054	PlantMs	-0.12025	0.06621	-1.816				
	PlantBc	-0.077827	0.083619	-0.931	PlantBc	-0.12148	0.06459	-1.881				
	PlantBi	-0.073495	0.088323	-0.832	PlantBi	-0.08709	0.07207	-1.208				
	SiteOH	0.340289	0.078636	4.327	SiteOH	0.24554	0.06145	3.995				
	SiteUC	0.078200	0.077954	1.003	SiteUC	0.07888	0.06084	1.297				
	Correlation of Fixed Effects:				Correlation of Fixed Effects:							
	(Intr)	PlantMs	PlantBc	PlantBi	SiteOH	(Intr)	PlantMs	PlantBc	PlantBi	SiteOH		
	PlantMs	-0.514				PlantMs	-0.503					
	PlantBc	-0.483	0.464			PlantBc	-0.573	0.474				
	PlantBi	-0.482	0.433	0.420		PlantBi	-0.467	0.417	0.430			
	SiteOH	-0.596	-0.021	-0.032	0.042	SiteOH	-0.555	-0.026	0.073	0.035		
	SiteUC	-0.634	0.058	0.027	0.025	0.592	SiteUC	-0.612	0.074	0.144	0.037	0.555

¹ Plant species included native *Andropogon gerardii* (Ag) and *Bouteloua curtipendula* (Bc), and exotic *Bothriochloa ischaemum* (Bi) and *Miscanthus sinensis* (Ms).

² Three sites were included in comparisons: University of Cincinnati Center for Field Studies (OH), Western Maryland Research and Education Center (MD), and the University of Cincinnati greenhouse (UC).

CHAPTER 4

Plant DNA Detection from Grasshopper Guts: a Step-by-Step Protocol, from Tissue Preparation to Obtaining Plant DNA Sequences¹

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Abstract

- *Premise of the study:* A PCR-based method of identifying ingested plant DNA in gut contents of *Melanoplus* grasshoppers was developed. Although previous investigations have focused on a variety of insects, there are no protocols available for plant DNA detection developed for grasshoppers, agricultural pests that significantly influence plant community composition.
- *Methods and Results:* The developed protocol successfully utilized the noncoding region of the chloroplast *trnL* (UAA) gene and was tested in several feeding experiments. Plant DNA was obtained at seven time points post ingestion (PI) from whole guts and separate gut sections, and was detectable up to 12 hours PI in nymphs and 22 hours PI in adult grasshoppers.
- *Conclusions:* The proposed protocol is an effective, relatively quick, and low cost method of detecting plant DNA from a grasshopper gut and its different sections. This has important applications, from exploring plant “movement” during food consumption, to detecting plant-insect interactions.

Key words: grasshoppers; insect gut content; plant DNA barcoding; trophic interactions.

Introduction

Knowledge of the diet of generalist insect herbivores is critical for understanding insect feeding preferences regarding different plants, as well as for detecting and predicting plant-insect interactions in natural communities. This becomes especially important when the insects of interest are agricultural pests, such as grasshoppers. Grasshoppers cause significant damage to crops and rangelands resulting in serious economic losses in the US and worldwide. For example, in 17 western US states, grasshoppers annually consume 25% of available rangeland forage, which averages about \$1 billion per year (Hewitt and Onsager, 1983). Having an important role in accelerating nutrient cycling, grasshoppers can influence plant community composition and, in particular, alter the abundance and species richness of plant species (Belovsky and Slade, 2000). Consequently, knowledge of the feeding preferences of grasshoppers can be important for control efforts and effective restoration of damaged areas (Branson and Sword, 2009).

The first step in any study on feeding preferences of insect herbivores is an accurate confirmation of food that is consumed. Among various techniques available for food-identification (including direct observation, feeding trails, and microscopical gut content analysis), PCR assays have shown to be an accurate and relatively quick method for detecting ingested plants, features that are especially important for large scale studies (e.g. Jurado-Rivera et al., 2009; Garcia-Robledo et al., 2013). In particular, plant DNA sequences extracted from insect gut contents can provide information about insect feeding choices occurring under natural conditions, which can be hidden from direct observations of insects on plants, or may contradict feeding preferences of insects observed in laboratory feeding trials (e.g. Garcia-Robledo et al., 2013). Therefore, potentially erroneous plant-insect interactions can be corrected.

Previous studies on plant DNA detection from insect guts have been conducted on beetles (e.g. Jurado-Rivera et al., 2009; Wallinger et al., 2013), moths (Miller et al., 2006), flies (Junnila et al., 2011), and hemipterans (Matheson et al., 2008); but only Matheson et al. (2008) included one grasshopper in their study, dissecting it after four hours (h) post ingestion (PI). Studies that used small insects or insect larvae often obtain whole-body DNA extracts (e.g. Staudacher et al. 2011). The extraction of plant DNA from relatively large insects is complicated by the presence of excessive amounts of non-target DNA of the herbivore; in this case isolating the digestive system and preventing contamination of gut contents with possible plant material from the outside surface of the insect (e.g. Matheson et al., 2008) is critical for increasing the yield of target plant DNA. Given that grasshoppers which can reach large sizes as adults are amongst the most important agricultural pests with enormous economic costs (Hewitt and Onsager, 1983), information about their food consumption and in particular, on tissue preparation and subsequent detection of plant DNA from their gut contents, is much needed.

In addition, the availability of a protocol for plant DNA extraction from different parts of an insect gut has many advantages in terms of exploring new aspects of herbivore feeding, and is especially useful for insects of relatively large size. It can allow the researcher to “follow” the plant DNA during food consumption and, for example, (1) to determine the approximate time of food consumption from its location in each compartment of the insect digestive system, or in the case of mixed diet, (2) to infer the sequence of ingestion of different plant species.

In this study I provide, for the first time, (1) an optimized step-by-step protocol for DNA extraction and PCR assay for detecting plant food in grasshoppers; (2) evidence of detectability of ingested plant DNA in nymphs and adult grasshoppers via feeding experiments; and (3) a

step-by-step protocol for dissection and plant DNA detection in different sections of grasshopper guts to follow up the digestive pathway through the gut.

Methods and Results

Sample collection — Adult *Melanoplus femur-rubrum* and *M. differentialis* grasshoppers (Acrididae: Orthoptera), and nymphs of the *Melanoplus* spp. grasshoppers (c.f. *M. differentialis* and *M. bivittatus*) were collected at the Western Maryland Research and Education Center (Keedysville, MD) and Cincinnati Center for Field Studies (New Haven, OH). In addition, 40 different plant species of Poaceae, Asteraceae, Fabaceae, and Plantaginaceae families were collected from the study plots and used for the feeding experiments, described below. Among these species, *Trifolium repens* Linnaeus, *Cichorium intybus* Linnaeus, *Plantago lanceolata* Linnaeus, and *Miscanthus sinensis* Andersson were used for testing primers; voucher specimens for these plants (#AA-0001, #AA-0002, #AA-0003, and #AA-0004 respectively) have been deposited at CINC. Furthermore, *Bouteloua curtipendula* (Michaux) Torrey and *Bothriochloa bladhii* (Retz.) S.T.Blake used in the feeding experiments described below were grown at the University of Cincinnati greenhouse from seeds obtained from Prairie Moon Nursery (Winona, MN) and Plant World Seeds (Newton Abbot, Devon, U.K.), respectively.

Protocol development — To first obtain plant DNA in grasshopper guts, a step-by-step protocol was developed. Following are the most important steps of this protocol; more details are provided in Appendices 1-2.

Step 1: Dissection and tissue preparation — After collection, grasshoppers' bodies and plant leaves (1-2 leaves from each plant species) were immediately frozen separately at -20°C. On the day of dissection, four frozen grasshoppers (two adult *M. femur-rubrum*, one adult *M.*

differentialis, and one nymph) were removed from the freezer and immediately rinsed with 70% ethanol to wash off all possible large, non-host plant debris from the exterior of insects. The grasshopper tissues were relatively soft and easy to dissect, so additional time for thawing was not needed. The hind legs and wings were then removed using fine forceps and fine scissors from a standard dissecting set. The exoskeleton of each grasshopper was then cut along the side and the digestive system was extracted. Whole guts were then stored in 1.5 ml microcentrifuge tubes with 70% ethanol overnight before the DNA extraction (Appendix 1, Video 1).

Step2: DNA extraction – Plant DNA was extracted from four samples of grasshopper gut contents and from *T. repens*, *C. intybus*, *P. lanceolata*, and *M. sinensis*, representing grasshopper host plants (prepared in Step1 above); both plants and grasshoppers were collected from the same study plot. DNA extraction was conducted with QIAGEN DNeasy Plant Mini Kit (Cat. No 69104, QIAGEN, Culver City, California, USA) according to QIAGEN guidelines. Although this kit is generally used for DNA extraction from standard plant tissue, the kit was recommended by QIAGEN Technical Service as useful for isolating plant material inside the insect gut. After isolation, DNA from plants and grasshopper guts was stored at -20⁰C for further PCR amplification.

Step3: Primer testing and PCR amplification – DNA barcodes amplifying the chloroplast *trnL* (UAA) gene and the nuclear ITS 1-2 region were chosen for screening of plant DNA obtained from grasshopper guts because these primers proved successful for detecting ingested plant DNA in a wide range of insect herbivores (e.g. Jurado-Rivera et al., 2009; Staudacher et al., 2011; Pumarino et al. 2011). In contrast, primers suggested by Matheson et al. (2008) that targeted the *rbcL* region did not work in my initial screens and were not pursued further. Four sets of universal primers were tested separately on plants and grasshopper gut contents: three sets

for non-coding regions of the chloroplast *trnL* (UAA) gene (Taberlet et. al., 1991, 2007) and one set for the nuclear ITS region (White et al., 1991). The primer mix was prepared for each primer pair (2 µM of each forward and reverse primer). Each PCR reaction (of 10 µL volume) consisted of the following: 5 µL QIAGEN Master Mix (QIAGEN), 1 µL Primer mix, 3.8 µL of dH₂O, and 0.2 - 0.3 µL DNA. Although other PCR-based protocols sometimes use larger amounts of DNA (e.g. Matheson et al., 2008), the smaller amounts used here were sufficient, as evidenced below. Samples were amplified under the following thermocycler conditions: denaturation of 95°C for 15 min, followed by 35 cycles of 95°C for 15 s, 57°C for 90 s, and 72°C for 60 s, followed by a final extension of 60°C for 30 min. PCR products were then separated in a 1% agarose gel and visualized under a UV transilluminator (Fig. 1A-B).

Step4: DNA sequencing and final primer selection - To confirm the presence and identity of plant DNA isolated from grasshopper guts, PCR products obtained from grasshoppers and from known plant species (from Step 1 above) were sequenced using Sanger sequencing at the Beckman Coulter Genomics facility (Danvers, MA). Sequences were then edited in BioEdit (Hall, 1999) and BLASTed against NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) for plant identification using 98-100% match identity. Following Chen et al. (2010), the quality of sequences for both plants and grasshopper gut contents was estimated using CodonCode Aligner 4.2.5.0 (CodonCode Co., USA) for low, middle, and high quality levels. The highest-quality sequences (quality values higher than 30) were observed for primers *c-d* (Taberlet et al., 1991); consequently, these primers were chosen to demonstrate the utility of this protocol.

To confirm the utility of primers *c-d* for a wide range of grasshoppers' potential host plants and grasshopper gut content samples, DNA extraction, amplification, and sequencing were

repeated with the remaining 36 collected plant species of Poaceae, Asteraceae, Fabaceae, and Plantaginaceae families and also with 26 nymphs of the *Melanoplus spp.* grasshoppers collected from the same study plots. High-quality sequences with quality values higher than 30 (Chen et al., 2010) were obtained for all 36 study plants (100%, $p < 0.0001$, Binomial Test) and for 18 out of 26 (69%) grasshopper guts ($p = 0.03$, Binomial Test). For this analysis, grasshopper gut contents with only single plant DNA were considered. Thus, these results demonstrated that the 500-bp region of the chloroplast *trnL* (UAA) gene, amplified by primers *c-d*, can be reliably detected in grasshopper guts and their potential host plants (Fig. 1C-D).

Testing the protocol — To further demonstrate the effectiveness of this protocol and to determine how long plant DNA remains detectable in the digestive system of grasshoppers of different sizes, three choice experiments and one no-choice feeding experiment with *Melanoplus* grasshoppers were conducted (Table 1, Appendix 2). In no-choice experiments, grasshoppers were fed a single plant species, while in choice experiments grasshoppers were provided with two or more plant species. Grasshoppers were originally collected in the field and their weights ranged from 0.11-1.66 g. Following Siemann and Rogers (2003), grasshoppers were starved for 24 h prior to all feeding experiments to make sure that no previously digested plants were present in the gut. Nymph grasshoppers (Experiment 1, choice) and adult *M. differentialis* grasshoppers (Experiment 2, choice) were offered leaves from both *Bouteloua* and *Bothriochloa* grasses for 3.5 hours; *M. femur-rubrum* grasshoppers were fed a mixture of plants for two days (Experiment 3, choice), and additionally, another group of *M. femur-rubrum* grasshoppers were fed leaves from *Bothriochloa bladhii* grass for 3.5 hours (Experiment 4, no-choice). After feeding, grasshoppers were transferred to new containers which did not contain food. Grasshoppers were then frozen separately at -20°C at several time intervals after feeding (one grasshopper at each

time point); plant DNA was then extracted and sequenced from each grasshopper using the protocol described above.

The results demonstrated that plant DNA can be detectable up to 12 h PI in the guts of nymph *Melanoplus spp.* grasshoppers (Table 2, Fig. 2A) and adult *M. femur-rubrum* grasshoppers (Table 2, Fig. 2B,C), as well as up to 22 h PI in *M. differentialis* grasshoppers, which were the largest in size (Table 2, Fig. 2D). Because of the difference in size, and consequently weight of grasshoppers, the DNA extraction step (*Step 2*) of the protocol was adjusted. To meet the requirements for sample weight according to the QIAGEN kit (≤ 100 mg wet weight), I used whole bodies of nymph grasshoppers, whole guts of *M. femur-rubrum* grasshoppers, or two parts of a gut of *M. differentialis* grasshoppers (foregut and combined midgut+hindgut, Appendix 1).

Choice feeding experiments with *M. differentialis* grasshoppers were also used to illustrate the utility of the proposed protocol for detection of plant DNA in different parts of the grasshopper digestive system. In this case, the tissue preparation step (*Step 1*) of the described protocol was also adjusted: after isolating the grasshopper gut from the body, the foregut and combined midgut+hindgut parts were separated (Appendix 1; Video 1). These parts were then stored separately in 70% ethanol, and plant DNA was then extracted from each section of the digestive system. The results of PCR amplification and obtained sequences of ingested plant DNA demonstrated that a researcher can “follow” the plant DNA in the process of food consumption up to 22 h PI and can make conclusions about the feeding behavior of an insect, specifically, the order of ingested plants. For example, in this study, the pattern of PCR amplification for foregut and combined midgut+hindgut sections at 3 h PI (Fig. 2D) suggested that *M. differentialis* grasshoppers consumed different plant species sequentially, and did not switch often between grasses which have been offered in the choice experiments.

Conclusion

Considering the high agricultural significance of grasshoppers (e.g. Hewitt and Onsager, 1983) and their impact on plant communities (e.g. Belovsky and Slade, 2000), there is a major need for an effective protocol for detecting grasshopper interactions with host plants. The utility of the chloroplast *trnL* (UAA) gene for detecting plant DNA from some coleopteran species has been demonstrated in similar studies (e.g. Jurado-Rivera et al., 2009; Staudacher et al., 2011). Using the developed protocol, I also demonstrated the utility of the chloroplast *trnL* (UAA) gene for PCR-based work with grasshoppers; 500-bp fragments of ingested plant DNA were successfully amplified and sequenced within grasshopper guts across multiple time intervals post ingestion. The developed protocol was also effective for detecting plant DNA from different sections of grasshopper guts, which has not yet been reported as previous studies on large insects used whole guts for plant DNA extraction (e.g. Matheson et al., 2008).

The protocol described here has many applications. For example, researchers can sacrifice a small subsample of grasshoppers to accurately determine the time of starvation needed to make sure that no other previously digested plant fragments are present in gut contents. In addition, researchers can follow the “movement” of plant DNA during the food consumption process to better understand the feeding behavior of herbivore insects.

The main advantages of this protocol are as follows: (1) it includes a relatively quick DNA extraction step (less than three hours); (2) it results in high resolution of the *trnL* gene for plant identification at the genus and, often at the species level; and (3) it capitalizes on the low cost of PCR and sequencing procedures, which are advantageous for small labs without access to next generation sequencing technologies. Potential difficulties of using this protocol include the following: (1) occasionally low resolution of the *trnL* in species discrimination (3 out of 40 cases

in this study); and (2) detection of multiple plant DNA in some gut contents (6 out of 26 samples in my study). When critical, the former can be addressed by amplifying additional loci; the latter requires additional molecular techniques, such as cloning (Garcia-Robledo et al., 2013), or less labor-intensive methods, such as computational analysis of mixed sequencing chromatograms (Kommedal et al., 2008; Chang et al., 2012). Overall, this is a convenient protocol for detecting plant-insect interactions, and although it was developed specifically for grasshoppers, it can potentially be extended to other plant and insect species to explore different aspects of insect herbivory.

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I would like to thank my research advisor Dr. Theresa Culley for valuable suggestions during the experiments, helpful comments on this manuscript, and for financial support of the molecular analysis.

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TABLE 1. The feeding experiments used in the study for plant DNA detection from grasshopper gut contents. PI – post ingestion; h – hours; SE – standard error.

Grasshopper species	Life stage	Weight (g), Mean±1SE	Type of feeding experiment	Plant species used for feeding	Total time of feeding	Tissues for DNA extraction
<i>Melanoplus spp.</i>	Nymph	0.11 ± 0.02	Choice	<i>Bouteloua curtipendula</i>	3.5 h	Whole body
<i>Melanoplus differentialis</i>	Adult	1.66 ± 0.27	Choice	<i>Bouteloua curtipendula</i>	3.5 h	Foregut and combined midgut+ hindgut separately
<i>Melanoplus femur-rubrum</i>	Adult	0.36 ± 0.01	Choice	Plant mixture	2 d	Whole gut
		0.35 ± 0.02	No-choice	<i>Bothriochloa bladhii</i>	3.5 h	

TABLE 2. Plant DNA detectability in grasshopper gut contents across several time intervals post ingestion in different grasshopper species. A single grasshopper was tested for each time point in all experiments.

Grasshopper species	Life stage	Type of feeding	Time intervals post ingestion (hours)							
			0	1	2	3	4	6	8	10
experiment										
<i>Melanoplus spp.</i>	Nymph	Choice	+	+			+	+	+	+
	Adult	Choice	+	+			+	+	+	+
<i>Melanoplus differentialis</i>										+
<i>Melanoplus femur-rubrum</i>	Adult	Choice	+		+		+	+	+	-
	No-choice	+		+			+	+	+	+

Note: + = plant DNA was successfully amplified and sequenced; — = plant DNA was not detected.

^a Empty cells indicate the cases where data was not available for this specific time interval.

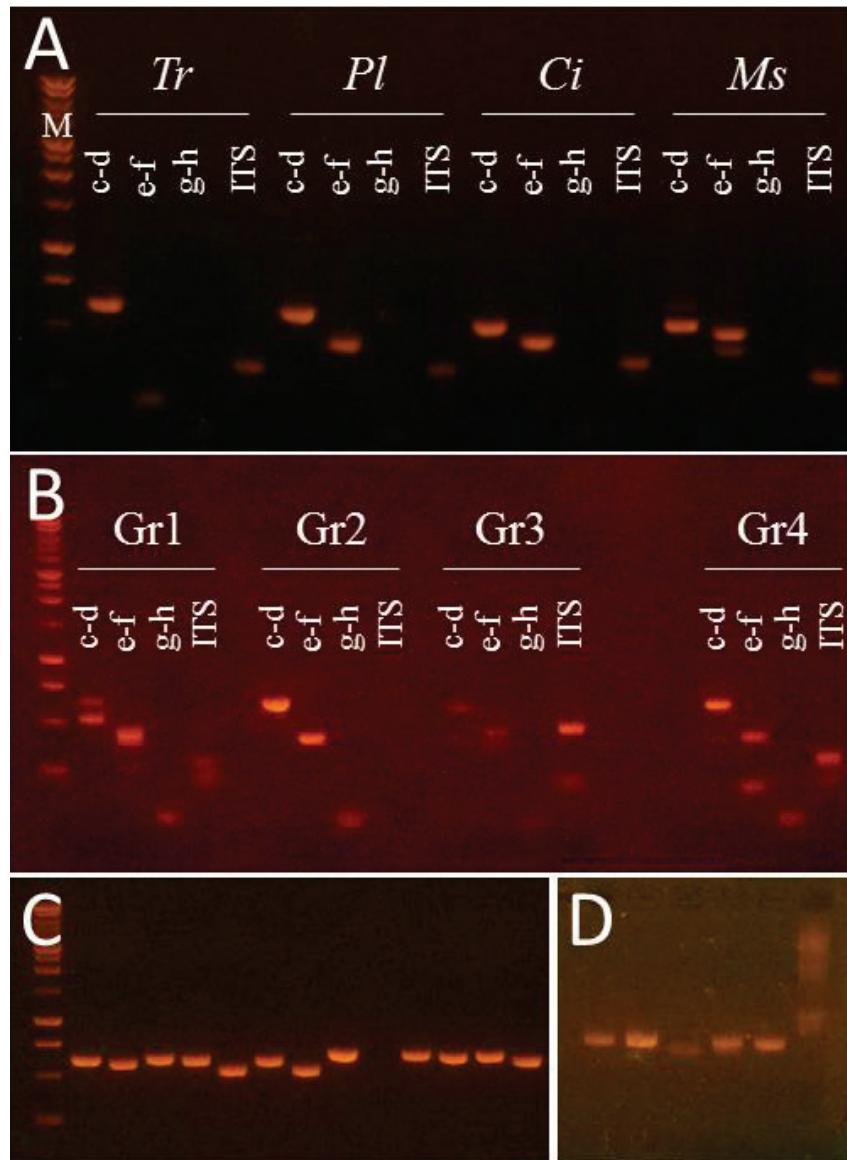


Fig. 1. PCR amplification of three fragments of the *trnL* gene (primers *c-d*, *e-f*, and *g-h*) and the ITS gene from four test plants (A) and from ingested plants within gut contents of four grasshopper individuals (B). *Ci*: *Cichorium intybus*; *Ms*: *Miscanthus sinensis*; *Pl*: *Plantago lanceolata*; *Tr*: *Trifolium repens*. Each group of four lanes for each plant species represents one plant individual. Gr1: *Melanoplus differentialis*, Gr2: nymph *Melanoplus spp.* grasshopper; Gr3-4: *M. femur-rubrum* grasshoppers; M - molecular marker (1kb DNA ladder). Each group of four lanes for each grasshopper species represents one individual. Primers *c-d* successfully amplified

fragments of the chloroplast *trnL* (UAA) gene in several other test plants (C) and in ingested plants in gut contents of several nymph individuals of the *Melanoplus spp.* grasshoppers (D). Each lane represents a different plant individual of Poaceae, Asteraceae, Fabaceae, and Plantaginaceae families (C) and a different grasshopper individual (D).

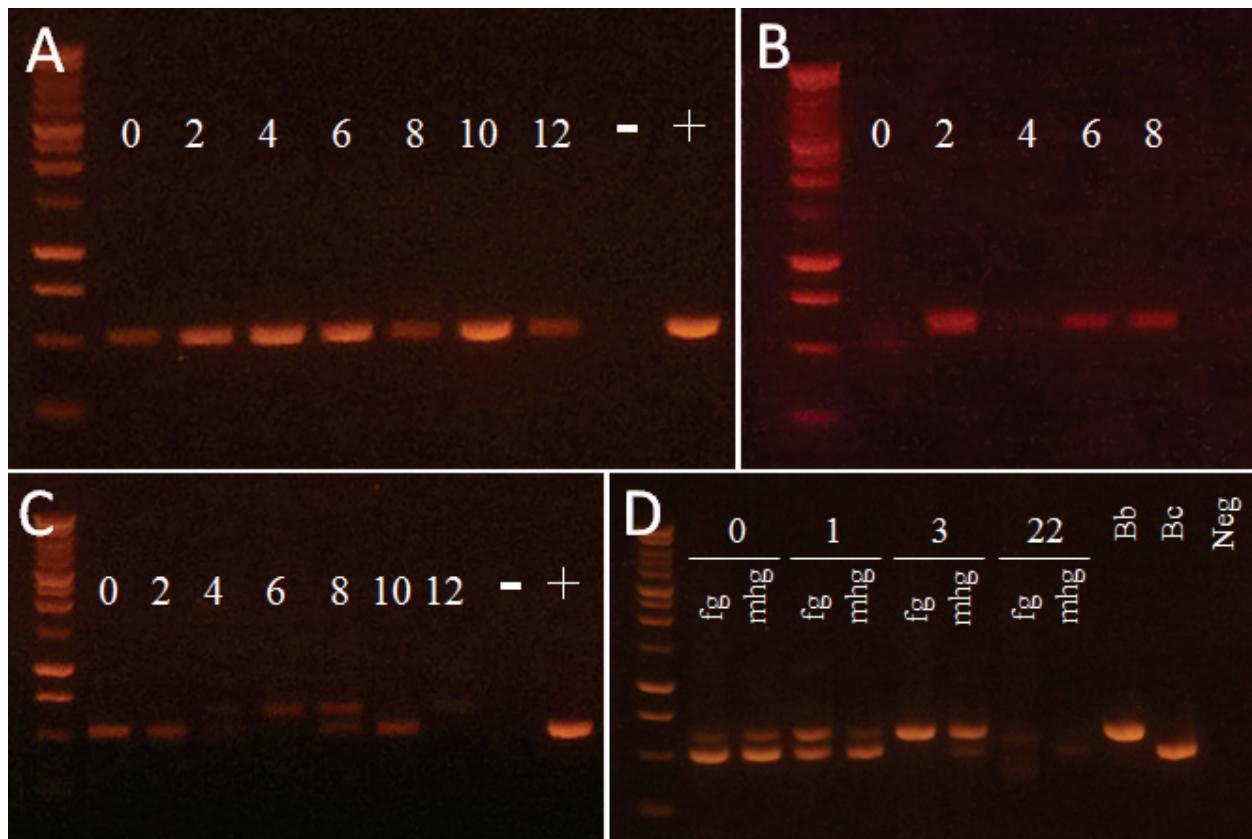


Fig. 2. PCR amplification of the *trnL* gene at different time intervals post ingestion (PI) after feeding experiments with grasshoppers. The numbers correspond to hours post ingestion (h PI). One grasshopper individual has been dissected at each time point. “-”: Negative control (DNA from grasshoppers legs’ muscle tissue); “+”: Positive control (plants offered for feeding). Each lane (A-C) represents a different grasshopper individual. (A) No-choice feeding experiment with adult *Melanoplus femur-rubrum* grasshoppers and *Bothriochloa bladhii* plants. The plant DNA was present in the grasshopper guts up to 12 h PI. (B) Choice feeding experiment with adult *M. femur-rubrum* grasshoppers and a mixture of plants. The plant DNA was present in the grasshopper guts up to 8 h PI. (C) Choice feeding experiment with nymph *Melanoplus spp.* grasshoppers. The plant DNA was present in the grasshopper guts up to 12 h PI. (D) Choice feeding experiment with adult *M. differentialis* grasshoppers. Bb: *Bothriochloa bladhii* (positive

control 1); Bc: *Bouteloua curtipendula* (positive control 2); Neg: Negative control; fg: foregut; mhg: combined midgut+hindgut. Each of the two lanes (foregut and combined midgut+hindgut) at each time point represents the same grasshopper individual. The plant DNA was present in both foregut and combined midgut+hindgut parts up to 22 h PI.

Appendix 1

Protocol for dissecting grasshoppers and tissue preparation.

Developed by A. Avanesyan

The details for isolating a gut and preparing foregut and combined midgut+hindgut parts are presented in Video 1 (<https://www.youtube.com/watch?v=AVMOHDILrtE>)

Part I. Isolation of grasshoppers' guts (for *M. femurrubrum* grasshoppers*, Fig. A1A-E, Video 1):

1. Take a frozen grasshopper from the freezer and rinse it with 70% ethanol.
2. Use forceps and scissors to carefully remove hind legs and wings.
3. Put the grasshopper on its side and use insect pins to anchor it to the dissecting pad.
4. Use scissors to cut the exoskeleton along the side. Start with the last segment of the abdomen and move slowly toward the head.
5. Carefully pull out the digestive system (if dissecting a female, remove bright yellow ovaries and fat bodies which are in the abdomen).
6. Place the whole gut in 1.5 ml microcentrifuge tube with 70% ethanol and store it overnight before the DNA extraction. Skip this step if you immediately proceed with DNA extraction (rinse the gut with 70% ethanol for 10s).

Part II. Preparing foregut and combined midgut+hindgut parts (for *M. differentialis* grasshoppers, Video 1):

1. Place an isolated gut on the dissecting pad (Fig. A1E).

2. Review a scheme of the internal structure of the grasshopper to match the main parts of the digestive system (Fig. A2).
3. Find the border between foregut and combined midgut+hindgut parts (Fig. A1E).
4. Use a scalpel to separate foregut and combined midgut+hindgut parts.
5. Place foregut and combined midgut+hindgut parts separately in 1.5 ml microcentrifuge tubes with 70% ethanol and store them overnight before the DNA extraction (Fig. A1F). Skip this step if you immediately proceed with DNA extraction (rinse the gut's parts with 70% ethanol for 10s).

* Use the whole body of a nymph grasshopper (due to its size) in DNA extraction. Remove hind legs if necessary.

Supplies check list:

Small vinyl dissecting pad (11 3/4 x 8 in, Carolina Biological Supply Company)

Standard dissecting set (fine scissors, straight, 4-1/2"; fine forceps, straight, 4-1/2"; fine forceps, curved, 4-1/2"; scalpel)

Insect pins (black enamel insect pins, size 2, pkg. of 100, BIOQUIP)

70% ethanol

1.5 ml Microcentrifuge tubes (FISHERBRAND, Cat. No. 05-408-129)

Scheme of internal structure of a grasshopper (Fig. 2)

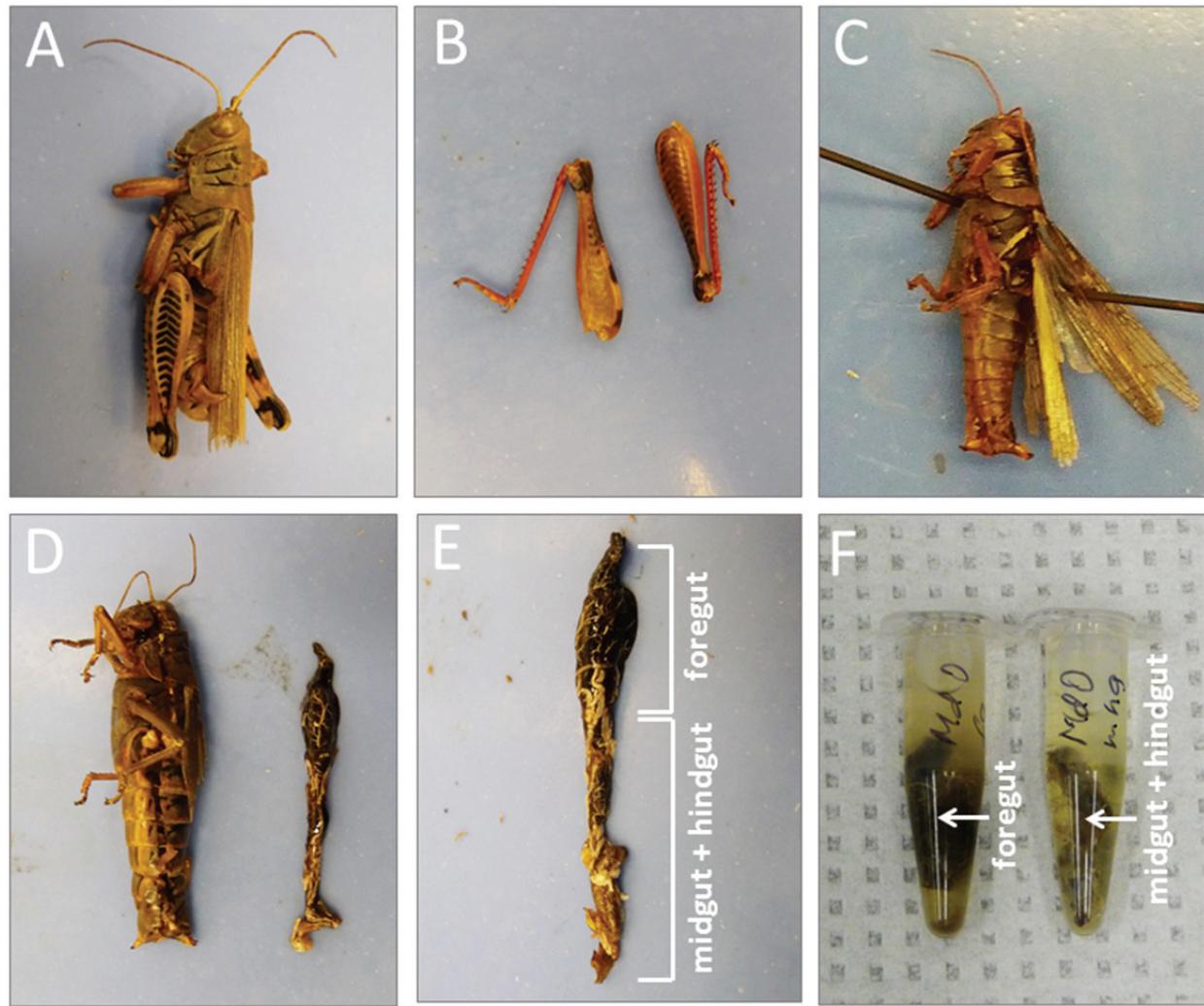


Fig. A1. Basic steps of dissecting grasshoppers and preparing their guts: removing hind legs and wings (A-C); cutting the exoskeleton along the side and pulling out the digestive system (D); separation of foregut and combined midgut+hindgut parts (E); storing different parts of the gut in 70% ethanol (F). Step F is not needed if the dissection is immediately followed by DNA extraction (Images by A. Avanesyan; Video 1).

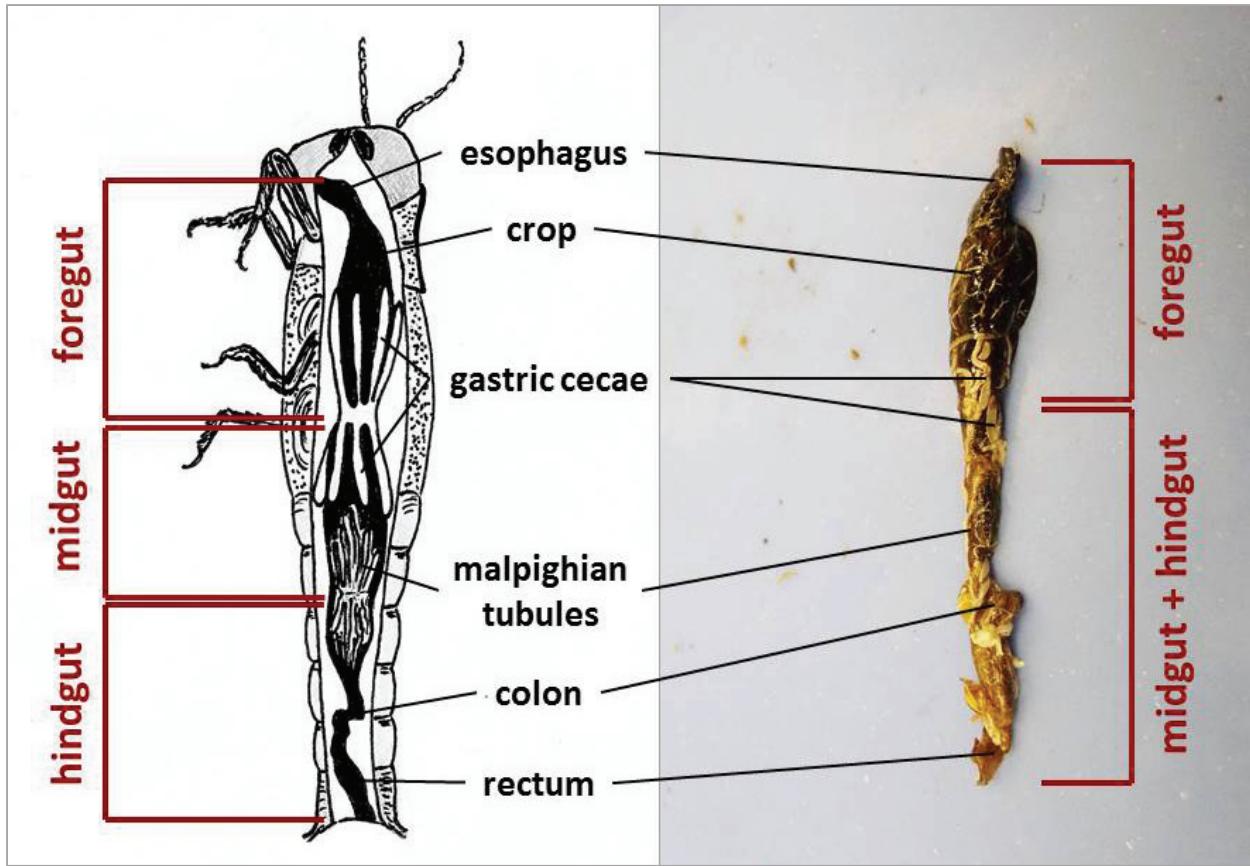


Fig. A2. Scheme of a grasshopper's digestive system (on the left) and corresponding parts in the gut pulled out from the grasshopper (on the right) (Images by A. Avanesyan).

Appendix 2

Protocol for feeding experiments.

Developed by A. Avanesyan

All grasshoppers were starved for 24 hours prior to all feeding experiments, which consisted of the following four types.

1) Feeding experiment with nymph grasshoppers:

1. Place twelve¹ nymph grasshoppers individually in small plastic containers.
2. Clip equal number of leaves (~ 0.3 g total weight) from plants which will be offered to grasshoppers. For example, in this study, *Bouteloua curtipendula* and *Bothriochloa bladhii* plants were used.
3. Put leaves together and wrap the clipped ends of leaves with moist filter paper.
4. Place leaves on the bottom of each container and let grasshoppers feed for 3.5 hours.
5. Randomly choose seven nymphs which ate the most of leaf tissue and place them separately in new containers. Other grasshoppers should be continued to be maintained in the laboratory for other feeding experiments.
6. Randomly take one of the selected grasshoppers, put it in a plastic bag, and freeze it immediately at -20⁰C.
7. Similarly freeze the rest of the grasshoppers in 2, 4, 6, 8, 10 and 12 hours (h) post ingestion (PI) at -20⁰C in separate plastic bags.
8. Freeze samples of leaf tissue (~2.5 cm²) from both plant species at -20⁰C for genetic analysis.

2) No-choice feeding experiments with adult *M. femur-rubrum* grasshoppers:

1. Place 12 grasshoppers individually in small plastic containers.
2. Clip equal number of leaves (~ 0.3 g total weight) from *Bothriochloa bladhii* plants.
3. See steps 3-8 above (in the experiments with nymphs).

3) *Choice feeding experiments with adult M. femur-rubrum grasshoppers:*

1. Place seven grasshoppers in the same aluminum cage.
2. Prepare a mixture of plants² collected on the study plot and place them in a glass vial with water.
3. Place container with plants in the cage with grasshoppers.
4. Let grasshoppers feed on this mixture of plants for two days.
5. Randomly take one grasshopper, put it in a plastic bag, and freeze it immediately at -20⁰C.
6. Take other grasshoppers out from the cage and place them separately in small plastic containers.
7. Similarly freeze the rest of the grasshoppers in 2, 4, 6, 8, and 10 h PI at -20⁰C in separate plastic bags.

4) *Feeding experiment with adult M. differentialis grasshoppers:*

1. Place six grasshoppers individually in small plastic containers.
2. See steps 2-6 in the experiments with nymphs.
3. Freeze the rest of grasshoppers in 1, 3, 8, 10, and 22 hours PI at -20⁰C in separate plastic bags. (Two grasshoppers in my study did not eat, so I froze the other four grasshoppers in 0, 1, 3, and 22 hours PI at -20⁰C).

¹ Seven nymphs were actually frozen for the DNA extraction; a minimum of seven nymphs (12 were used in this study) need to be used in the feeding experiments in case some nymphs do not eat. There can be any number of extra grasshoppers.

² In this study, several collected plants of the Poaceae, Asteraceae, Fabaceae, and Plantaginaceae families were used. To simulate natural feeding in the field, plant stems with leaves were placed in a glass vial with water to keep plants hydrated. The vial with plants was then placed in the cage with grasshoppers.

Supplies check list:

Plastic containers (7×4.5×5" All Living Things® Critter Totes, PetSmart, Inc., USA)

Aluminum cage (16×16×20" Repti Breeze Aluminum Screen Cage, Zoo Med Laboratories, Inc., California, USA)

Small Ziplock plastic bags for freezing

CHAPTER 5

Feeding Preferences of *Melanoplus femur-rubrum* Grasshoppers on Native and Exotic Grasses: Behavioral and Molecular Approaches¹

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Abstract

Generalist insect herbivores, such as grasshoppers, may either avoid feeding on exotic plants, potentially enabling these plants to become invasive in the introduced range, or insects may incorporate exotic plants into their diet; contributing to the biotic resistance of native communities and potentially preventing plant invasions. Although there has been some investigation of grasshopper feeding on native versus exotic plants, the results from these studies are inconsistent and consequently, the feeding preferences of grasshoppers are still poorly understood, especially in grasses. To address this issue, we combined behavioral and molecular approaches to accurately compare food consumption of the polyphagous red-legged grasshopper, *Melanoplus femur-rubrum*, on native (*Andropogon gerardii* and *Bouteloua curtipendula*) and exotic, potentially invasive grasses (*Miscanthus sinensis* and *Bothriochloa ischaemum*). We found that *M. femur-rubrum* grasshoppers demonstrated strong feeding preferences toward exotic grasses in experiments with intact plants under both field and greenhouse conditions, but they showed no preference in experiments with clipped leaves. Additionally, we sampled the gut contents of *M. femur-rubrum* collected in the field and identified the ingested plant species based on DNA sequences for the noncoding region of the chloroplast *trnL* (UAA) gene. We found that exotic plants were prevalent in the gut contents of grasshoppers collected at study sites in Ohio and Maryland. These results suggest that the generalist herbivore *M. femur-rubrum* does not avoid feeding on exotic grasses with which they do not share coevolutionary history. In addition, by demonstrating greater food consumption of exotic plants, these grasshoppers can potentially provide biotic resistance should these grasses escape cultivation and become invasive in the introduced range.

Key words: DNA barcoding, grasshoppers, herbivory, *Melanoplus femur-rubrum*, plant invasion

Introduction

Understanding the feeding preferences of generalist insect herbivores is critical for determining plant-insect trophic interactions - especially with regard to exotic plants which potentially can become invasive in the introduced range. For example, evidence of avoidance of exotic plants by generalist insect herbivores may provide a better understanding of mechanisms facilitating invasion, such as the release of exotic plants from their native enemies (the enemy release hypothesis, Keane & Crawley, 2002). On the contrary, if generalist insect herbivores do not avoid feeding on exotic plants and/or even show a preference for them over native plants (due in part to an inability of the exotic plants to defend themselves against evolutionarily novel enemies), such insect feeding behavior can contribute to biotic resistance of native communities to exotic plants and potentially prevent plant invasion (the biotic resistance hypothesis, Parker & Hay, 2005, Parker et al., 2006). Consequently, determining the trophic interactions among generalist insect herbivores, and native and exotic plants is important for a better understanding of the mechanisms underlying the process of plant invasion.

The red-legged grasshopper *Melanoplus femur-rubrum* (Acrididae: Orthoptera) is a polyphagous insect herbivore that is a convenient organism with which to study insect feeding preferences: it is relatively easy to collect, identify and maintain under laboratory conditions. This grasshopper species is widely distributed throughout North America, including Ohio and Maryland, and is a known crop pest. *Melanoplus femur-rubrum* successfully survives in a variety of habitats (Preston-Mafham & Preston-Mafham, 1990), although areas with thick vegetation are most preferable (Capinera, 2004). It consumes a wide range of plants including grasses and broad-leaved plants such as legumes, dandelion, chicory, goldenrod, Kentucky bluegrass, barley, oats, smooth brome, and timothy (Pfadt, 1994; Lamp et al., 2007), but it prefers herbs to grasses

(Preston-Mafham & Preston-Mafham, 1990). In addition, *Melanoplus femur-rubrum* tends to choose a mixed diet; it has been demonstrated, for example, that a mixture of corn, lettuce and radish increased grasshoppers' survival and egg production, whereas a sole diet of alfalfa resulted in high nymphal mortality (Pfadt, 1994). Acridid grasshoppers, including *Melanoplus* species, actively feed on warm season grasses such as *Andropogon gerardii* (Whipple et al., 2009; Loaiza et. al., 2011), *Bouteloua gracilis* (e.g. Alward & Joern, 1993), *Bothriochloa bladhii* (e.g. Avanesyan, 2014), *Miscanthus sinensis* (Nabity et al., 2012), as well as on cold season grasses such as *Bromus inermis* and *Poa pratensis* (Whipple et al., 2009). Consequently, grasshoppers can contribute to leaf damage in these important tallgrass prairie plants and affect plant community composition in grasslands (Belovsky & Slade, 2000). It has been demonstrated that the food selection of grasshoppers depends not only on chemicals produced by plants but also on physical characteristics of plant leaves, such as presence of trichomes and leaf toughness (Chapman, 1974). Grasshoppers often choose the most abundant host plant in the area, even when other plants are more acceptable for feeding (Chapman, 1974; Boys, 1981; Bernays & Chapman, 1994). Food selection by grasshoppers is also greatly influenced by the suitability of host plants for grasshopper survival, growth and reproduction (Mulkern, 1967).

Although there have been many studies on grasshopper feeding, only some have compared feeding on native versus exotic plants (Porter & Redak, 1997; Siemann & Rogers, 2003; Jogesh et al., 2008; Branson & Sword, 2009; Fielding & Conn, 2012; Fan et al., 2013). The results from these investigations are inconsistent: some studies demonstrated grasshoppers' preferences towards native plants (Porter & Redak, 1997; Siemann & Rogers, 2003), but others showed strong preferences of grasshoppers for exotic plants (Whipple et al., 2009; Fielding & Conn, 2012; Fan et al., 2013). The question which still remains unanswered is whether grasshoppers

actively select native plants (Branson & Sword, 2009). These inconsistencies in the literature can partially be explained by differences in the experimental conditions; some researchers conducted experiments solely with potted plants, clipped leaves, or with plants planted in the field. Each type of experiment controlled for specific factors (i.e. plant biomass, temperature) and revealed grasshopper feeding behavior under these specific experimental conditions; grasshopper feeding behavior, however, may or may not remain the same if these conditions change. The quality of plants could also affect grasshopper behavior; therefore, it is critical to conduct feeding experiments with plants of the same stage of growth and maturity (Mulkern, 1967). Similarly, the physiological state of grasshoppers might influence their food choice; so it is important to use grasshoppers of similar age and stage of development in feeding experiments (Mulkern, 1967). Consequently, a combined approach which utilizes different experimental conditions, as well as feeding trials that control for possible differences in plants and grasshoppers, is needed to provide more insight on grasshopper feeding preferences (Mulkern, 1967; Harvey & Fortuna, 2012).

Although field experiments and observations of natural grasshopper herbivory are informative of grasshoppers' food choices under natural conditions, it is critical to accurately confirm food ingestion, for example, by an analysis of insect gut contents (Garcia-Robledo et al., 2013). Analysis of grasshopper gut contents has an advantage compared to feeding experiments as grasshoppers do not sustain outside influences in their food selection, such as manipulations or the presence of a researcher (Mulkern 1967). A few studies on grasshopper feeding used combined feeding experiments with intact plants and clipped leaves (e.g. Lankau, 2004; Fielding & Conn, 2011), some studies conducted microscopical examination of gut contents (e.g. Joern, 1979), but to our best knowledge, there have been no studies on grasshopper feeding preferences

that utilized molecular approaches and which could provide more accurate information about ingested plants (Garcia-Robledo et al., 2013). Using a previously developed, PCR-based method to detect plant meals from grasshopper gut contents (Avanesyan, 2014), we investigated the prevalence of native and exotic plants in the gut contents of grasshoppers collected in the field.

Here we report the results of a study on grasshopper feeding preferences on native and exotic plants using behavioral and molecular approaches. In this study, we were interested whether generalist insect herbivores, such as *M. femur-rubrum* grasshoppers, may contribute to the biotic resistance of native communities to plant invasion by consuming and damaging exotic grasses. Our main research questions were: Do *M. femur-rubrum* grasshoppers incorporate exotic grasses in their diet? If so, do they prefer to feed on exotic than on native grasses? In our study, we used two native (*Andropogon gerardii* and *Bouteloua curtipendula*) and two exotic, potentially invasive, grasses (*Miscanthus sinensis* and *Bothriochloa ischaemum*). We hypothesized that *M. femur-rubrum* grasshoppers would not avoid exotic grasses and might even prefer to feed on them. As such, we expected that gut contents of *M. femur-rubrum* grasshoppers collected in the field would contain a similar or greater proportion of exotic plants compared to native plants. To explore our research questions, we conducted a one-year study which utilized field and greenhouse feeding experiments with intact plants and clipped leaves, as well as detecting plant DNA within grasshopper gut contents to more accurately determine grasshopper food choices under experimental and natural conditions.

Materials and methods

Feeding experiments with intact plants

To examine grasshopper feeding preferences on intact plants, we obtained seeds for *A. gerardii* and *B. curtipendula* (Prairie Moon Nursery, Winona, MN), *M. sinensis* (Outsidepride.com, Inc., Independence, OR), and *B. ischaemum* (Warner Brothers Seed Company, Lawton, OK); we planted them in the University of Cincinnati greenhouse in May 2013. Our choice of grasses was influenced by known grasshopper attractiveness (especially that of *M. femur-rubrum*) to tall vertical patterns which correspond to upright blades of grasses (Mulkern, 1967); thus we could stimulate the feeding activity of grasshoppers in the enclosures. Also, to minimize the effect of any potential physical and physiological differences among plant species on grasshopper behavior, we chose morphologically and physiologically similar, closely related C₄ warm-season grasses.

We then established two field study plots of 90 m² area at the University of Cincinnati Center for Field Studies (UCCFS, 39°17.134' N, 084°44.413' W, Harrison, OH) and at the Western Maryland Research and Education Center (WMREC, 39°30.618' N, 077°44.070' W, Keedysville, MD). In the end of July 2013, we arranged 144 potted plants grown from these seeds (plants were of 20-25 cm in height in 3.5" square pots) in three groups of 48 (each group of 48 contained 12 pots of each plant species): two groups were transferred to the field plots (one to each field site), and one group was kept in the greenhouse. In addition, extra plants of each species were grown separately in the greenhouse to be used for experiments with clipped leaves (described below). In the field at each site, the potted plants were arranged and planted in twelve groups of four with approximately 1 m spacing between the groups; each potted plant within the group represented a different species (two native and two exotic species per group). Plants were

watered only during the first two days after planting, and then plants were allowed to grow under natural field conditions.

All experiments were conducted in September 2013, on the fifth week after transplanting plants to the field. On the day before the experiments, we set up an open air aluminum screen cage (16×16×20" Repti Breeze Aluminum Screen Cage, Zoo Med Laboratories, Inc., California, USA) around each plant group. Male *M. femur-rubrum* grasshoppers were collected near the study plots on the first day of the experiment and were immediately weighed and placed in the cages (2 males per cage). We intentionally did not starve grasshoppers before the feeding trials, as it has been demonstrated previously that the longer grasshoppers are starved, the more likely they are to choose unpalatable food (Bernays & Chapman, 1994). In addition, as our study grass species were not present on the collection sites, the feeding choices of grasshoppers in the experiments were not influenced by their previous encounters and feeding on these species.

In the greenhouse experiment, plants were watered daily. On the day before the experiment, grasshoppers were collected at the UCCFS and were kept overnight in the greenhouse in an open air aluminum screen cage and fed a mixture of plants collected on the same collection site. On the first day of the experiments, 48 potted plants were arranged in twelve groups of four, as described previously for the field experiment; each plant group and two male grasshoppers were then placed in a fabric cage (Bioquip, rearing and observation cage, 14×14×24", Cat. No. 1466B) in the greenhouse. Field and greenhouse experiments were conducted simultaneously to eliminate any potential effects of seasonal changes on grasshopper behavior. Each experiment lasted 5 days. On the sixth day, all grasshoppers were removed from the cages and released.

To estimate food consumption, we measured on each plant *the total volume of the grazed portion (GP)*: [sum of (length × width × depth of each scar), cm³]. Each grazed mark was called “a scar”. All measurements of each scar’s volume were taken as maximum values. Feeding activity was estimated in terms of both grasshopper movement (*frequency of scarring (FS)*) as [number of scars/number of leaves]) and intensity of feeding (*feeding rate (FR)*) as [total volume of grazed portion per unit weight of grasshopper (g) per day]. To calculate grasshoppers feeding rate, we followed Farrar et al. (1989) and used each grasshopper’s mean weight at the beginning of the experiment.

Differences in grasshopper feeding on native and exotic plants were assessed using a generalized linear mixed model (GLMM) in R (v.2.15.2, package *lme4*). Field and greenhouse experiments were analyzed separately. Mean values for native and exotic plants averaged within each cage were used for all analyses. GLMMs were fitted on each measured variable using site (Ohio or Maryland) and plant origin (native or exotic) as fixed effects. The cages were set as random effects to account for the potential effect of differences in environmental conditions (such as cage location, distances between plant individuals within a cage, etc.) on measured variables. When the random effect was minimal or absent, the two-way fixed effects ANOVA (for field experiments) and the one-way fixed effects ANOVA (for the greenhouse experiment), using general linear model (GLM), were then used to determine whether food consumption and feeding activity of grasshoppers were greater on exotic plant species than on native plant species. Within each field and greenhouse experiment, significance levels of 0.05 were adjusted using a Bonferroni correction. All data were square root-transformed to meet the assumptions of normality and homoscedasticity (based on the Shapiro-Wilk test and Bartlett’s test respectively). Plants without any evidence of grazing were excluded from analyses of the measurements of

frequency of scarring and feeding rate. A Kruskal-Wallis test followed by a post-hoc Mann-Whitney's U test (due to lack of normality of data) with a Bonferroni correction were used to estimate potential differences among plant species which might affect grasshopper feeding behavior. These tests were also used to detect any difference in grasshopper feeding on native and exotic grasses under field and greenhouse conditions, as well as on different field sites.

Feeding experiments with clipped leaves

To estimate food consumption and food assimilation of grasshoppers on native and exotic plants, experiments with clipped leaves were conducted in the greenhouse in September 2013 in small plastic containers ($7 \times 4.5 \times 5"$ All Living Things® Critter Totes, PetSmart, Inc., USA). We clipped one leaf of 25 cm length from each plant species, weighed it (weight ranged from 0.07 - 0.16 g), and placed either all four leaves (in a choice experiment) or two leaves from either native or exotic plants only (in separate no-choice experiments) in each container. To keep leaves hydrated during the experiments, we wrapped their bases with a moist paper towel. Following Siemann and Rogers (2003), grasshoppers were starved for 24 hours prior to feeding on clipped leaves. Unlike experiments with intact plants, it was critical in the experiments with clipped leaves to use a short period of starvation which would not affect palatability of plants for grasshoppers but would eliminate any plant contents from grasshopper guts. This allowed us to accurately estimate digestibility and assimilation of the ingested food, as well as to stimulate simultaneous feeding of all grasshoppers during the short experimental period. One male grasshopper was then placed in each container for 3.5 hours. The same number of "control" containers (without grasshoppers) were similarly prepared for choice (n=10) and no-choice (n=9) experiments. All containers were kept in the greenhouse during the experiments at 25-26°C.

In 3.5 hours after the beginning of the experiment, all grasshoppers were removed and the wet weight of remaining leaves was measured and leaf damage (if any) was quantified. The remaining leaves and any grasshopper fecal material, as well as the control leaves, were air dried at room temperature for several weeks. Leaf dry weight and dry weight of feces were then measured. Food consumption of clipped portions of leaves was estimated in terms of (1) *the total volume of the grazed portion* [sum of (length × width × depth of each scar), cm³], (2) *food intake* (g) following Waldbauer (1968), (3) *feeding rate* [food intake per g of insect per hour] (Delvi & Pandian, 1972), and (4) *the fresh-weight consumption index* (Waldbauer, 1968). We also calculated food assimilation in terms of (5) *the amount of assimilated food* [dry weight of food ingested - dry weight of feces, (g)] (Matsumoto, 1971), (6) *assimilation rate* [food assimilated per g of insect per hour] (Delvi & Pandian, 1972), (7) *the approximate digestibility* [the amount assimilated/dry weight of food ingested] (Bailey & Mukerji, 1976), and (8) *feces production* (g). Similar to the experiments with intact plants (see above), we used the weight of grasshoppers obtained at the beginning of the experiment for these calculations.

Differences in grasshopper food consumption and food assimilation on native and exotic plants were assessed by fitting GLMMs on each measured variable. Similar to the experiments with intact plants, all measurement values were averaged for native and exotic plants. Type of experiment (choice or no-choice), and plant origin (native or exotic) were used as fixed effects. A random cage effect was used to account for a potential effect of location of feeding arena, amount of light and other potential differences in environmental conditions on measured variables. When the random effect was minimal or absent, a two-way fixed effects ANOVA (for comparison of all food consumption variables across choice and no-choice experiments) and a one-way fixed effects ANOVA (for food assimilation variables in no-choice experiment) using

GLM were used to determine grasshopper feeding preferences. For each set of ANOVAs involving multiple comparisons, significance levels of 0.05 were adjusted using a Bonferroni correction. The food consumption data were square root-transformed to meet the assumptions of normality and homoscedasticity (based on the Shapiro-Wilk test and Bartlett's test respectively). Leaves without any evidence of grazing were excluded from analyses of the measurements such as consumption index, assimilation rate, feces production and the approximate digestibility. Similar to the experiments with intact plants with non-normally distributed data, a Kruskal-Wallis test followed by a post-hoc Mann-Whitney's U test with Bonferroni correction were used to estimate potential differences among different plant species which might affect grasshopper feeding behavior in choice experiments.

Molecular confirmation of diet

To estimate the prevalence of exotic and native host plants in grasshopper gut contents under natural conditions, we collected both *M. femur-rubrum* grasshoppers and reference plants (one individual per plant species was clipped at the ground) from two natural field sites in Ohio ($18 \times 10 \text{ m}^2$, $39^\circ 17.266' \text{ N}$, $084^\circ 44.426' \text{ W}$, Harrison, OH) and Maryland ($60 \times 12 \text{ m}^2$, $39^\circ 30.783' \text{ N}$, $077^\circ 43.968' \text{ W}$, Keedysville, MD) which were located about 100 m from the study plots on which we conducted our feeding experiments. Both sites were adjacent to corn and soybean fields (approximately 5 m distant) and presumably were attractive for grasshoppers.

Plants and grasshoppers were immediately frozen at -20°C prior to DNA extraction. The fragments (~500 bp) of the noncoding region of the chloroplast *trnL* (UAA) gene were isolated from grasshopper gut contents and from reference plants using a previously developed protocol (Avanesyan, 2014). All PCR products were sequenced using Sanger sequencing at the Beckman

Coulter Genomics facility (Danvers, Massachusetts, USA). DNA sequences obtained from reference plants were then BLASTed against the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) and plant species were identified using 98-100% match identity. The local reference plant database for each site was created using BioEdit (Hall, 1999). DNA sequences obtained from ingested plants were then BLASTed against the local plant database which we had previously created; ingested plants were then identified using the highest possible (93-100%) match identity. If an ingested plant did not match any of the reference plants (presumably due to grasshopper feeding on a territory adjacent to the study site), the corresponding sequences were blasted directly against NCBI GenBank database for species identification. The origin of ingested plants (native or exotic) was determined using the United States Department of Agriculture's PLANTS Database (<http://plants.usda.gov>).

The prevalence of native and exotic plants in grasshopper guts was determined using a Binomial test. Only grasshopper gut contents with only a single plant species DNA were considered for this analysis. Grasshoppers with mixed plant DNA (11% of all collected grasshoppers) were excluded from the analysis, as the analysis of mixed DNA would require additional molecular techniques, such as cloning (e.g. Garcia-Robledo et al., 2013), or computational analysis of mixed sequencing chromatograms (e.g. Chang et al., 2012), which was beyond the scope of this study. The chi-squared test was used to estimate the difference between the observed proportion of native and exotic plant species within grasshopper gut contents and the proportion of native and exotic plant species in the field (expected proportion).

Results

Feeding experiments with intact plants

In both the field and greenhouse experiments, a cage effect was minimal (<14% of variation in leaf damage was explained by a cage effect) for all the measured variables except for frequency of scarring at the WMREC (>50% of variation was explained by a cage effect) (Appendix 1), so we proceeded with direct comparisons of the main effects of plant origin (native vs. exotic) on grasshopper herbivory.

Under field conditions, the total volume of the leaf tissue portion grazed by grasshoppers was 7.5 times greater for exotic plants than native plants (Table 1, 2), whereas the frequency of scarring did not differ between native and exotic plants (Table 2). Native plants in two cages at the WMREC, as well as both native and exotic plants in one cage at the UCCFS, were not grazed upon at all. Consequently, those plants were excluded from the comparison of grasshopper feeding rate on native and exotic plants; grasshopper feeding rate was four times greater on exotic plants (Table 1, 2). Site location (WMREC or UCCFS) did not have an effect on any of the measured variables (Table 2). However a significant “Plant origin × Site” interaction was detected in the total volume of the grazed portion (Table 2): native grasses were grazed more frequently at the UCCFS than at the WMREC ($\chi^2 = 4.82$, df = 1, p = 0.0281), whereas grazing on exotic plants did not differ between sites ($\chi^2 = 1.29$, df = 1, p = 0.2568). The smallest volume of the grazed portion and the lowest feeding rate of grasshoppers at both sites were detected on *B. curtipendula*, which was not grazed at all at the UCCFS (consequently, these plants were excluded from comparisons of frequency of scarring and feeding rate). All other comparisons among plant species did not reveal a significant difference.

When potted plants were offered to grasshoppers in the greenhouse, the total volume of the leaf tissue portion grazed by grasshoppers was four times greater on exotic plants than on native plants (Table 1, 2). In contrast to the results from the field experiments, the frequency of scarring by grasshoppers in the greenhouse, on average, was three times greater on exotic plants than on native plants (Table 1, 2), whereas the feeding rate of grasshoppers did not differ between native and exotic plants (Table 2). Plant species differences were observed for the frequency of scarring only ($\chi^2 = 12.14$, df = 3, p = 0.007): *B. ischaemum* and *A. gerardii* were grazed upon more often than *B. curtipendula*; all other comparisons among plant species did not reveal a significant difference between plant species (Appendix 2).

Comparisons of grasshopper herbivory under field and greenhouse conditions revealed that food consumption and feeding activity of grasshoppers on exotic plants were greater in the greenhouse than in the field (GP: $\chi^2 = 7.44$, df = 1, p = 0.0063; FS: $\chi^2 = 5.69$, df = 1, p = 0.017; FR: $\chi^2 = 9.003$, df = 1, p = 0.0027; Figure 1A-C). As for native plants, grasshopper feeding rate only was higher in the greenhouse than in the field ($\chi^2 = 8.03$, df = 1, p = 0.0045); all other measured variables did not differ significantly under field and greenhouse conditions (Figure 1D-F).

Feeding experiments with clipped leaves

When clipped portions of leaves were offered to grasshoppers under laboratory conditions, food consumption in terms of both food intake and the total volume of the grazed portion did not differ between native and exotic plants (Table 3, 4). We also did not observe any differences in feeding rate, consumption index, amount of assimilated food, assimilation rate, and fecal production between native and exotic plants (Table 3, 4). The approximate digestibility

did not differ between plants at the adjusted significance of $P = 0.006$; however, digestibility was greater on native plants than on exotic at the unadjusted significance of $P = 0.05$ (Table 3, 4).

The type of experiment (choice or no-choice), as well as interaction of “Type of experiment \times Plant origin”, did not significantly affect any of the measured variables (Table 4). We also did not find a cage effect concerning any of the variables in the no-choice experiment; however, there was a cage effect on food intake in the choice experiment (~ 63% of variation in food intake was explained by a cage effect) (Appendix 3). No differences in leaf damage among different plant species were observed.

Molecular confirmation of diet

DNA sequences for 500bp-fragments of the noncoding region of the chloroplast *trnL* (UAA) gene were obtained from plants ingested by grasshoppers (24 plant species for UCCFS and 19 for WMREC) and from reference plants collected at the study sites (10 for UCCFS and 20 for WMREC) (Table 5, Figure 2A-D). Plants sampled at the UCCFS consisted of 1 native and 10 exotic species, while 7 native and 19 exotic species were identified at the WMREC (Table 5). Seven plants which were found in grasshopper gut contents were not present in field surveys at the local study site (1 at the UCCFS and 6 at the WMREC).

The analysis of the prevalence of exotic and native plants in grasshopper guts demonstrated greater numbers of exotic plant species for grasshoppers collected from both the UCCFS ($p < 0.0001$, Binomial Test) and the WMREC ($p = 0.0003$, Binomial Test) study sites. The difference between the observed proportion of native and exotic plant species within grasshopper gut contents and the proportion of native and exotic plant species in the field was not significant for both sites (UCCFS: $\chi^2 = 0$, $df = 1$, $p = 1$; WMREC: $\chi^2 = 0.59$, $df = 1$, $p = 0.4431$). Among all

ingested plants at the UCCFS, the exotic *Cichorium intybus* was the most common; DNA of this species was found in the gut contents of 37.50 % of all dissected grasshoppers at that site (Table 5). At the WMREC, most of the collected grasshoppers (52.63%) ingested the exotic *Hordeum vulgare* (Table 5).

Discussion

To more accurately investigate feeding preferences of the *Melanoplus femur-rubrum* grasshopper on native and exotic grasses, we incorporated methodological recommendations from previous studies (e.g. Mulkern, 1967; Harvey & Fortuna, 2012; Garcia-Robledo et al., 2013) and used a combination of behavioral and molecular approaches.

Our expectations of lack of avoidance of exotic plants by this grasshopper species were confirmed in all feeding experiments: grasshoppers consumed exotic plants in the field and in the greenhouse experiments with intact plants, as well as in the experiments with clipped leaves. We also detected the presence of exotic plant DNA in gut contents of grasshoppers collected in the field. Our hypothesis of relatively greater food consumption of exotic grasses compared to native grasses was supported overall in the field experiments with intact plants (in Ohio and Maryland) and in the greenhouse experiments with potted plants. Plant DNA detection in grasshopper gut contents also revealed greater prevalence of exotic plant species compared to native plant species. Our hypothesis, however, was not supported in the laboratory feeding trials with clipped leaves: grasshopper food consumption and food assimilation did not differ on the leaves clipped from native and exotic grasses. The only major difference in plant attractiveness for grasshoppers was complete avoidance of native *B. curtipendula* by grasshoppers in Ohio in the field experiment. Based on all other comparisons, we observed minor differences among plant species

in the experiments with intact plants and no differences among leaves from different plant species in the experiments with clipped leaves.

Our observation of greater food consumption of grasshoppers on exotic plants in the field enclosures and in the greenhouse was consistent with the results of similar experiments conducted by Lankau et al. (2004): in their study, *M. angustipennis* grasshoppers fed preferentially on exotic *Sapium* seedlings. Meanwhile, the authors observed a natural low load of grasshopper herbivory on introduced *Sapium*. Lankau et al. (2004) suggested this resulted from behavioral constraints of grasshoppers toward novel (and potentially toxic) food under natural feeding conditions and the grasshoppers' inability to recognize this suitable food source. Based on the results from our molecular confirmation of grasshopper food choice, we did not observe behavioral avoidance of exotic plants by *M. femur-rubrum* under natural feeding conditions. Our study grasses were not present on the collection sites, although they are present within Ohio and Maryland. In general, *M. femur-rubrum* is a common species in grasslands where closely related native and non-native plants, including our study species, are abundant (Han et al., 2008; Whipple et al., 2009). Therefore, we suggest that these grasshoppers do not avoid feeding on these exotic grasses under natural conditions. Further studies on natural grasshopper herbivory on *Bothriochloa* and *Miscanthus* individual plants in grasslands would be helpful.

Our findings of grasshopper feeding preferences for exotic plants in the field and in the greenhouse were also consistent with the results of similar choice feeding experiments with *M. borealis* grasshopper and the invasive weed *Crepis tectorium* along with several native plants (Fielding & Conn, 2011). In addition, Fan et al. (2013) used experiments with potted plants to show that leaf biomass of invasive *Alternanthera philoxeroides* was significantly reduced by herbivory from the *Atractomorpha sinensis* grasshopper, compared to the native plant congener

Alternanthera sessilis. We support the authors' view that native herbivores, such as grasshoppers, do not exhibit feeding preferences toward native plants and can potentially limit the negative effects from successfully established invasive plants. Future studies might examine how grasshopper density affects the suppression of the spread of invasive plants.

Greater feeding activity of *M. femur-rubrum* on exotic plants, in terms of both intensity of feeding and movement, can consequently be explained in part by greater attractiveness of these plants compared to native plants. It has been shown that grasshoppers move more actively in areas of favorable host plants (Mulkern, 1967). Frequency of scarring (our proxy for grasshopper movement on a leaf) in our greenhouse experiment and feeding rate (our proxy for intensity of feeding) in our field experiments were relatively greater on exotic plants. As grasshopper feeding rate in the greenhouse experiment did not significantly differ between native and exotic plants, greater leaf consumption on exotic plants apparently could be due to the fact that grasshoppers moved faster on these plants and therefore damaged more leaves. Under field conditions, where grasshopper movement (frequency of scarring) did not differ between native and exotic plants, greater feeding rate on exotic plants may result in a greater volume of exotic leaf tissue consumed by grasshoppers. Further exploration of the relationships between leaf tissue consumption and frequency of scarring and feeding rate would be helpful to better understand what components of feeding behavior have the greatest effect on grasshoppers' food choice.

Given that *M. femur-rubrum* does not occur in the native range of *B. ischaemum* and *M. sinensis*, one explanation for our results is that grasshopper greater use of exotic plants reflects a lack of coevolutionary history in this plant-insect system, and consequently, a lower level of defenses of exotic plants (primarily resistance) to this novel herbivore compared to native plants (Parker et al., 2006). In our study, we used native and exotic grasses that exhibited similar

morphological and physiological traits. Consequently, differences in physical defense between these grasses were minimal and they did not play a major role in attractiveness or deterrence of *M. femur-rubrum* grasshoppers. In the current study, we used adult grasshoppers only, whose feeding could be much less affected by plant physical traits (e.g. hairy leaf surfaces) compared to nymphs (Chapman, 1974). Chemical defenses more likely affected the palatability of plants for these grasshoppers. However, due to a lack of coevolution of *M. femur-rubrum* grasshoppers with exotic grasses used in this study, chemical defenses of exotic grasses might not be as effective against novel grasshoppers as those that may occur in native plants. The minimal attractiveness, and possibly greater chemical defenses, were observed in *B. curtipendula* in our field experiments with intact plants. It has been demonstrated that although gustation in grasshoppers influences their food choice and regulates feeding, actual food ingestion depends on the balance between food phagostimulants and deterrents (Medeiros et al., 2008). Generalist insect herbivores, unlike specialists, do not use specific feeding cues: most of the plants can be consumed unless plant defenses deter generalist insects (Parker et al., 2006). Neither the strong deterrents nor phagostimulants have been demonstrated for native *Andropogon* and *Bouteloua* plants (Mole & Joern, 1994); consequently, we can speculate that the balance between these two types of chemicals might be shifted toward attractiveness in *Miscanthus* and *Bothriochloa* grasses, although further investigation is necessary.

The observed lack of grasshopper feeding preferences in our experiments with clipped leaves differs from the findings of Lankau et al. (2004); in their choice experiments with clipped leaves, *M. angustipennis* grasshoppers preferred to feed on exotic plants. Similarly, our results contrast with the findings of Whipple et al. (2009) who demonstrated that *Arphia xanthoptera* and *Dichromorpha viridis* grasshoppers also consumed more clipped leaves of exotic grasses.

Whipple et al. (2009) used native warm-season (*A. gerardii* and *B. curtipendula*) and exotic cool-season grasses (*Poa pratensis* and *Bromus inermis*). The authors indicated that phylogenetic and physiological difference between those grasses might affect grasshopper food choice more than plant origin (native or exotic). For this reason, we intentionally used warm-season grasses only which were morphologically and physiologically very similar.

Differences in our results between the experiments with intact plants and the experiments with clipped leaves can also be explained by manipulations such as cutting, which might cause release of secondary chemicals from the cut portion of the plant and thereby affect plant attractiveness and accessibility for herbivores (Mulkern, 1967; Motheral & Orrock, 2010). It is of note, that in our experiments with clipped leaves, the approximate digestibility of native plants at the unadjusted level of significance was greater than that of exotic plants, unlike other measurements of feeding behavior which did not differ between native and exotic plants. Further exploration of digestibility of native and exotic plants with presumably different level of plant resistance would allow us to better understand how clipping of leaves affects their digestibility in insects; specifically, a molecular approach to this question would be effective (Barkhordar et al., 2013).

The molecular analysis of grasshopper gut contents also demonstrated grasshoppers' higher relative use of exotic plants. Most of the grasshoppers collected on the study sites contained DNA from a single plant species in their guts, which could be explained by pauses during grasshopper feeding. It has been demonstrated, for example, that each feeding period of *Locusta* species lasted about 10 minutes and was separated by 1 hour (or more) intervals (Chapman, 1974). In our previous experiments on plant DNA detection within different sections of the grasshopper digestive system, we also demonstrated that *M. differentialis* grasshoppers

consumed different plant species sequentially; while observing leaf damage in two plant species in a feeding trial, we recorded a single plant DNA only in a combined midgut+hindgut section of a grasshopper in 3 hours post ingestion (Avanesyan, 2014). Thus, in the present study, plants which had been ingested before the last feeding period could be digested by the time of collection, rendering such plant DNA indetectable. Consequently, each sample of grasshopper gut contents analyzed represents a “snapshot” of the last plant species which had been fed on by a grasshopper.

In addition to the lack of coevolutionary history of native *M. femur-rubrum* grasshopper and exotic plants, the results of the gut content analysis can possibly be explained by the abundance of exotic plants on the study sites. It has been demonstrated previously that grasshoppers often chose the most abundant host plant in the area, even when other plants were more acceptable for feeding; as a result, the more frequently ingested plants may not necessarily be the most preferred for grasshoppers (e.g. Mulkern, 1967; Boys, 1981). For example, *M. femur-rubrum* frequency of ingestion of exotic *Poa pratensis* was proportional to its abundance (Mulkern, 1967). Given that exotic plants composed more than 50% of all our reference plants, grasshoppers’ food choice might be the consequence of their habitat (Mulkern, 1967). In this study, we found that the proportion of ingested native and exotic plant species within grasshopper gut contents was similar to the proportion of native and exotic plant species in the field. We, however, estimated the number of native and exotic plant species only; further investigations are needed to explore the effect of plant coverage in the field on the proportions of ingested plants within grasshopper gut contents.

In conclusion, lack of avoidance of exotic plants and feeding preference of *M. femur-rubrum* grasshoppers toward exotic plants, especially under natural field conditions,

suggest that these grasshoppers can potentially provide biotic resistance to exotic *Bothriochloa* and *Miscanthus* should these grasses escape cultivation and become invasive. Given that these grasshopper species are among common species in tallgrass prairie, we believe that these generalist insect herbivores would be less likely affected by invasion of exotic grasses. This is also important for predicting the impacts of invasive grasses on numerous trophic interactions which are associated with invasive plants in the introduced range (Harvey & Fortuna, 2012). Considering that *M. femur-rubrum* is one of the important agricultural pests, our study can be extended to explore feeding preferences of this grasshopper species on crop plants compared to grasses growing at field edges to develop more effective means to suppress crop damage.

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Table 1 Mean (\pm 1 SE) values of leaf tissue consumption and feeding activity of *Melanoplus femur-rubrum* grasshoppers on native and exotic intact plants.

Site	Parameter	Plants		<i>P</i> value
		Native	Exotic	
WMREC	Total volume of the grazed portion (cm^3) ¹	0.002 \pm 0.001	0.015 \pm 0.003	0.0016*
	Frequency of scarring	0.04 \pm 0.008	0.067 \pm 0.01	0.180
	Feeding rate ($\text{cm}^3/\text{g/day}$)	0.002 \pm 0.001	0.008 \pm 0.002	0.0152*
UCCFS	Total volume of the grazed portion (cm^3)	0.01 \pm 0.003	0.013 \pm 0.005	0.0016*
	Frequency of scarring	0.045 \pm 0.009	0.111 \pm 0.034	0.180
	Feeding rate ($\text{cm}^3/\text{g/day}$)	0.009 \pm 0.003	0.013 \pm 0.007	0.0152*
UC greenhouse	Total volume of the grazed portion (cm^3)	0.013 \pm 0.004	0.052 \pm 0.016	0.011*
	Frequency of scarring	0.065 \pm 0.02	0.192 \pm 0.036	0.0056*
	Feeding rate ($\text{cm}^3/\text{g/day}$)	0.015 \pm 0.003	0.048 \pm 0.014	0.056

¹ Feeding experiments with intact plants were conducted in the field at Western Maryland Research and Education Center (WMREC) and at the University of Cincinnati Center for Field Studies (UCCFS), as well as at the University of Cincinnati (UC) greenhouse.

² Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Misanthus sinensis* and *Bothriochloa ischaemum*.

³ Frequency of scarring and feeding rate were estimated for plants with at least one grazed mark ("a scar"); undamaged plants were excluded from analysis of these variables.

⁴ *P* values for the differences which are significant at the unadjusted 0.05 level are in bold; *P* values with asterisks ("**") are significant at the adjusted significance of 0.016.

Table 2 General linear model parameter estimates of leaf tissue consumption and feeding activity of *Melanoplus femur-rubrum* grasshoppers on intact plants in relation to location of the experiment (“Site”) and plant origin (“Plant Type”).

Experiment	Parameter	Fixed effects	Estimate	Std. Error	t value	Pr(> t)
Field	Total volume of the grazed portion	(Intercept)	0.11654	0.01558	7.481	7.76e-09 ***
		Plant Type	-0.07531	0.02203	-3.418	0.00158 **
		Site	-0.02318	0.02203	-1.052	0.29970
		Plant Type:Site	0.06811	0.03116	2.186	0.03539 *
	Frequency of scarring	(Intercept)	0.25049	0.02851	8.787	4.85e-10 ***
		Plant Type	-0.05866	0.04276	-1.372	0.180
		Site	0.05399	0.04142	1.304	0.202
		Plant Type:Site	-0.04091	0.06028	-0.679	0.502
	Feeding rate	(Intercept)	0.08657	0.01230	7.036	1.8e-07 ***
		Plant Type	-0.04799	0.01846	-2.600	0.0152 *
		Site	0.01387	0.02009	0.690	0.4962
		Plant Type:Site	0.03259	0.02907	1.121	0.2726
Greenhouse	Total volume of the grazed portion	(Intercept)	0.20614	0.02689	7.666	4.48e-07 ***
		Plant Type	-0.10784	0.03803	-2.836	0.011 *
	Frequency of scarring	(Intercept)	0.41541	0.04090	10.157	7.02e-09 ***
		Plant Type	-0.18176	0.05784	-3.142	0.00563 **
	Feeding rate	(Intercept)	0.19991	0.02543	7.862	1.68e-06 ***
		Plant Type	-0.08009	0.03844	-2.083	0.056

¹ Two sites were utilized for the field experiments: Western Maryland Research and Education Center and University of Cincinnati Center for Field Studies. Greenhouse experiments were conducted at the University of Cincinnati greenhouse.

² Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Miscanthus sinensis* and *Bothriochloa ischaemum*.

³ Plants of two types were used in the experiments with intact plants: native and exotic plants.

⁴ * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Table 3 Mean (± 1 SE) values of food consumption and food assimilation of *Melanoplus femur-rubrum* grasshoppers on leaves clipped from native and exotic plants.

Experiment	Parameter	Plants		<i>P</i> value
		Native	Exotic	
Choice	Total volume of the grazed portion (cm ³)	0.002 \pm 0.001	0.003 \pm 0.001	0.504
	Food intake (g)	0.011 \pm 0.004	0.011 \pm 0.004	0.982
	Feeding rate (g/g/hour)	0.018 \pm 0.007	0.019 \pm 0.005	0.753
	Consumption index	0.018 \pm 0.007	0.034 \pm 0.006	0.09
	Total volume of the grazed portion (cm ³)	0.005 \pm 0.003	0.003 \pm 0.001	0.504
	Food intake (g)	0.011 \pm 0.003	0.012 \pm 0.004	0.982
No-choice	Feeding rate (g/g/hour)	0.011 \pm 0.003	0.016 \pm 0.004	0.753
	Consumption index	0.028 \pm 0.006	0.036 \pm 0.006	0.09
	The amount of assimilated food (g)	0.014 \pm 0.003	0.008 \pm 0.032	0.134
	Assimilation rate (g/g/hour)	0.018 \pm 0.000	0.009 \pm 0.000	0.109
	The approximate digestibility (g/g)	0.87 \pm 0.005	0.567 \pm 0.039	0.029
	Feces production (g)	0.002 \pm 0.000	0.003 \pm 0.000	0.192

¹ Feeding experiments with clipped leaf portions were conducted at the University of Cincinnati under laboratory conditions.

² Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Miscanthus sinensis* and *Bothriochloa ischaemum*.

³ Feeding rate, consumption index, assimilated food, and assimilation rate were estimated for grasshoppers which made at least one grazed mark (="a scar") on leaves; undamaged leaves were excluded from analysis of these variables.

⁴ All *P* values were not significant at the adjusted significance of 0.006. *P* values for the differences which are significant at the unadjusted 0.05 level are in bold.

Table 4 General linear model parameter estimates of food consumption and food assimilation of *Melanoplus femur-rubrum* grasshoppers on clipped leaves in relation to a type of experiment (“Exp”) and plant origin (“Plant Type”).

Parameter	Fixed Effects	Estimate	Std. Error	t value	Pr(> t)
Total volume of the grazed portion	(Intercept)	0.04132	0.01090	3.790	0.000588 ***
	Plant Type	-0.01041	0.01542	-0.675	0.504091
	Exp	0.01049	0.01584	0.663	0.512095
	Plant Type:Exp	0.01415	0.02240	0.632	0.531753
Food intake	(Intercept)	0.0816533	0.0206807	3.948	0.000507 ***
	Plant Type	0.0007267	0.0312663	0.023	0.981627
	Exp	0.0142121	0.0312663	0.455	0.653066
	Plant Type:Exp	-0.0068641	0.0448177	-0.153	0.879413
Feeding rate	(Intercept)	0.134632	0.020013	6.727	1.18e-06 ***
	Plant Type	-0.009474	0.029684	-0.319	0.753
	Exp	-0.016378	0.028303	-0.579	0.569
	Plant Type:Exp	-0.018502	0.039775	-0.465	0.647
Consumption index	(Intercept)	0.180679	0.024428	7.397	2.83e-07 ***
	Plant Type	-0.064249	0.036232	-1.773	0.0907
	Exp	0.005276	0.034546	0.153	0.8801
	Plant Type:Exp	0.032351	0.048549	0.666	0.5124
The amount of assimilated food	(Intercept)	0.007510	0.003101	2.422	0.0277 *
	Plant Type	0.006812	0.004385	1.553	0.1399

Assimilation rate	(Intercept)	0.03162	0.01298	2.436	0.0288 *
	Plant Type	0.03139	0.01836	1.710	0.1093
The approximate digestibility	(Intercept)	0.56704	0.08826	6.424	1.59e-05 ***
	Plant Type	0.30328	0.12482	2.430	0.0292 *
Feces production	(Intercept)	0.003500	0.000742	4.717	0.00033 ***
	Plant Type	-0.001437	0.001049	-1.370	0.19228

¹ Experiments of two types (choice and no-choice) were conducted at the University of Cincinnati greenhouse.

² Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Miscanthus sinensis* and *Bothriochloa ischaemum*.

³ * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

Table 5 Food consumption of adult *Melanoplus femur-rubrum* grasshoppers based on DNA sequences of ingested plants based on their gut contents.

Site	Plant species	Origin	Presence at study plot	Grasshopper feeding choices	
				Number	%
UCCFS	<i>Alopecurus pratensis</i>	Exotic	+	4	16.67
	<i>Cichorium intybus</i>	Exotic	+	9	37.50
	<i>Lolium multiflorum</i>	Exotic	+	2	8.33
	<i>Plantago lanceolata</i>	Exotic	+	5	20.83
	<i>Pyrus pyrifolia</i>	Exotic	+	-	-
	<i>Setaria sphacelata</i>	Exotic	+	-	-
	<i>Setaria viridis</i>	Exotic	+	-	-
	<i>Sorghum bicolor</i>	Exotic	+	-	-
	<i>Stellaria media (L.) Vill.</i>	Exotic	-	2	8.33
	<i>Symphytum novi-belgii</i>	Native	+	2	8.33
	<i>Trifolium repens</i>	Exotic	+	-	-
WMREC	<i>Allium ampeloprasum L.</i>	Exotic	+	-	-

<i>Amaranthus spinosus L.</i>	Native	+	-	-	-
<i>Arcium lappa L.</i>	Exotic	+	-	-	-
<i>Bromus arvensis L.</i>	Exotic	-	1	5.26	
<i>Conyza sumatrensis (Retz.) E. Walker</i>	Native	+	-	-	
<i>Erigeron annuus (L.) Pers.</i>	Native	+	-	-	
<i>Glycine max (L.) Merr.</i>	Exotic	-	1	5.26	
<i>Hordeum vulgare L.</i>	Exotic	+	10	52.63	
<i>Lamium amplexicaule L.</i>	Exotic	+	-	-	
<i>Lobelia kalmii L.</i>	Native	+	-	-	
<i>Morus rubra L.</i>	Native	+	-	-	
<i>Oxalis corniculata L.</i>	Exotic	+	1	5.26	
<i>Panicum dichotomiflorum Michx</i>	Native	+	-	-	
<i>Physalis heterophylla Nees</i>	Native	+	-	-	
<i>Poa pratensis</i>	Exotic	-	1	5.26	
<i>Rhamnus davurica Pall.</i>	Exotic	-	3	15.79	
<i>Setaria viridis (L.) P. Beauv.</i>	Exotic	+	-	-	

<i>Sorghum bicolor (L.) Moench</i>	Exotic	+	-	-
<i>Veronica arvensis L.</i>	Exotic	-	1	5.26
<i>Veronica persica Poir.</i>	Exotic	-	1	5.26

¹ Presence of plants on the site is denoted as “+”. Absence of plants on the site, as well as plants which DNA were not detected in grasshopper gut contents are denoted as “-”.

² The origin of reference plants (native or exotic) was determined using The PLANTS Database (<http://plants.usda.gov>).

³ Grasshopper feeding choices are displayed in terms of the number of grasshoppers (“Number”) and the proportion of grasshoppers (“%”) consumed a particular plant.

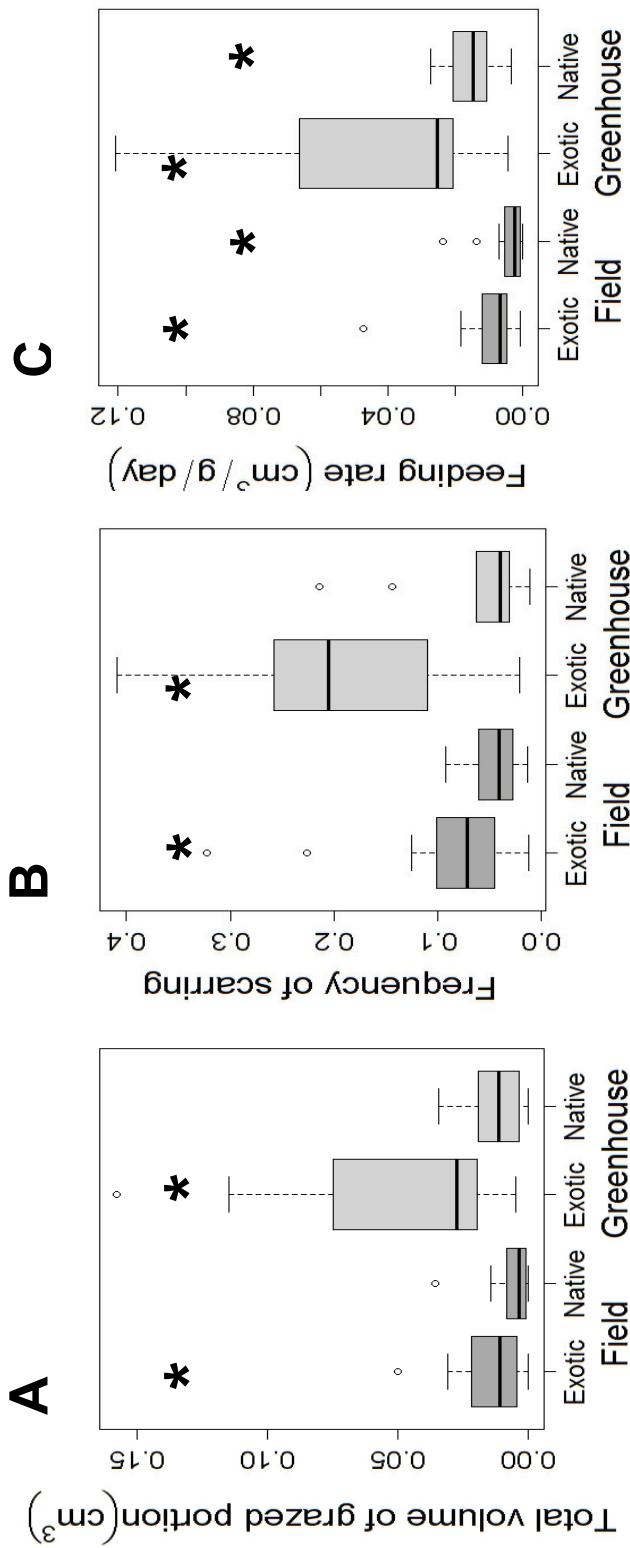


Figure 1 Food consumption (A) and feeding activity (B, C) of *Melanoplus femur-rubrum* grasshoppers on intact exotic *Miscanthus sinensis* and *Bothriochloa ischaemum* and native *Andropogon gerardii* and *Bouteloua curtipendula* plants in the field (dark grey boxplots) and greenhouse (light grey boxplots) experiments. Field experiments were conducted at Western Maryland Research and Education Center and at the University of Cincinnati Center for Field Studies; boxplots for field data represent results combined across both sites. Asterisks (“**”) indicate significant differences within exotic and native plants under field and greenhouse conditions at the significance level of $P=0.05$.

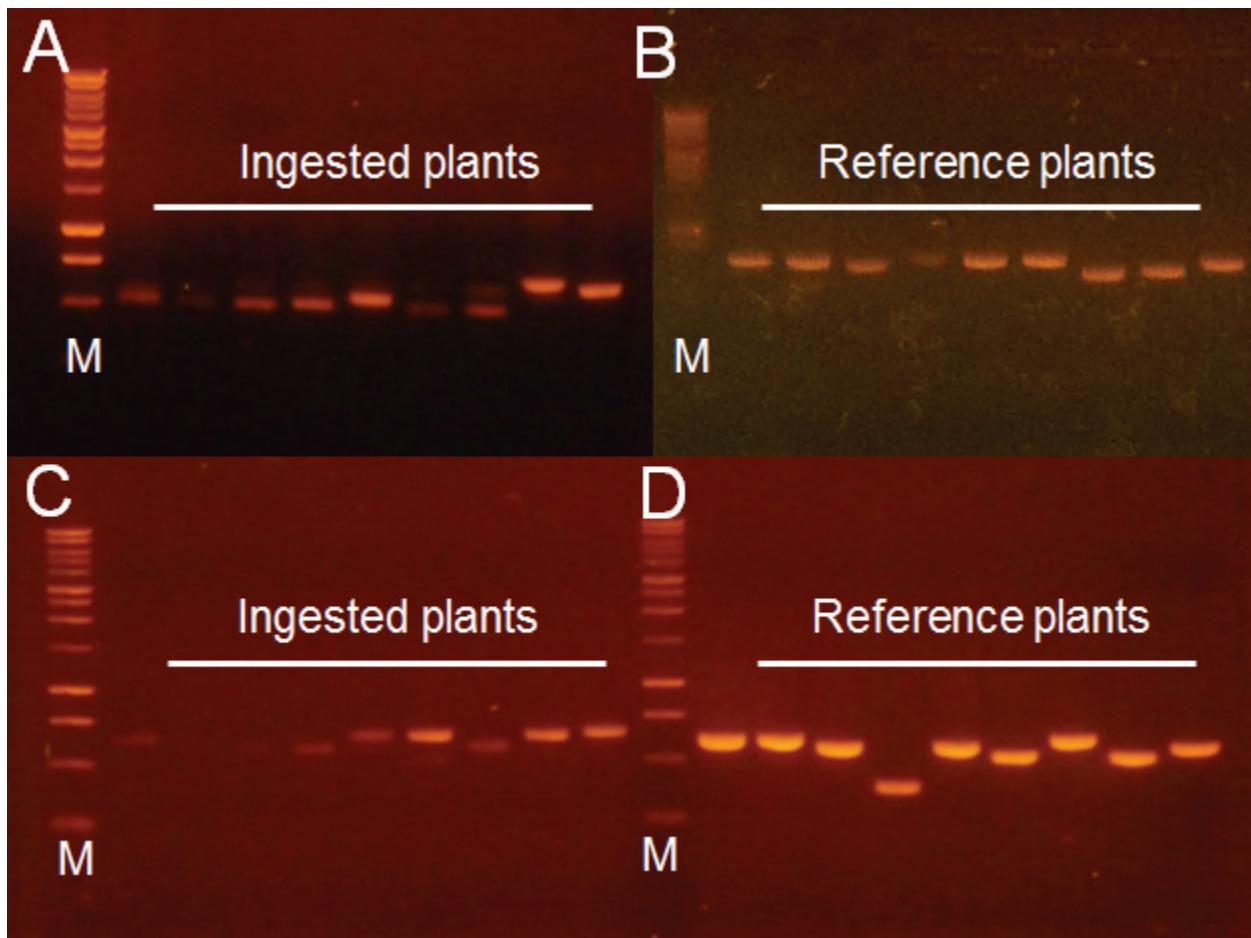


Figure 2 PCR amplification of the 500bp fragments of the chloroplast *trnL* gene from reference plants and from plant material ingested by *Melanoplus femur-rubrum* grasshoppers. Both plants and grasshoppers were collected from the same study sites; (A-B): University of Cincinnati Center for Field Studies (UCCFS); (C-D) Western Maryland Research and Education Center (WMREC). The results of PCR amplification are shown for 9 samples of reference plants and 9 samples of ingested plants in grasshopper gut contents. Each lane represents one plant individual of reference plants (A, C) and one grasshopper individual of ingested plants (B, D). M: molecular marker (1-kb DNA ladder, Invitrogen).

Appendix 1

Model (GLMM) estimates of an effect of grasshopper enclosures (cages) on leaf tissue consumption and feeding activity of *Melanoplus femur-rubrum* grasshoppers on intact plants in relation to plant type (native or exotic).

Conditions	Site	Parameter	Random effects		Fixed effects		Estimate	Std. Error	t value
			Groups	Variance	Std. Dev.	(Intercept)			
Field	WMREC	Total volume of the grazed portion	Cage (Intercept) Residual	6.7744e-06 3.9977e-05	0.0026028 0.0063227	(Intercept) Plant Type	0.015160 -0.012460	0.002162 0.002828	7.012 -4.407
		Frequency of scarring	Cage (Intercept) Residual	0.00062347 0.00029179	0.024969 0.017082	(Intercept) Plant Type	0.067177 -0.035490	0.009567 0.008404	7.022 -4.223
	Feeding rate	Total volume of the grazed portion	Cage (Intercept) Residual	2.0676e-06 1.6268e-05	0.0014379 0.0040333	(Intercept) Plant Type	0.008376 -0.006521	0.001354 0.001925	6.186 -3.387
		Frequency of scarring	Cage (Intercept) Residual	4.7418e-05 1.2938e-04	0.0068861 0.0113747	(Intercept) Plant Type	0.012631 -0.002964	0.004205 0.005087	3.004 -0.583
UCCFS		Total volume of the grazed portion	Cage (Intercept) Residual	0.0000000 0.0054306	0.0000000 0.073693	(Intercept) Plant Type	0.11093 -0.06563	0.02456 0.03474	4.516 -1.889
		Frequency of scarring	Cage (Intercept) Residual	5.2056e-05 1.2759e-04	0.007215 0.011296	(Intercept) Plant Type	0.013067 -0.004352	0.005472 0.006522	2.388 -0.667
	Feeding rate	Total volume of the grazed portion	Cage (Intercept) Residual	0.00016539 0.00113248	0.012860 0.033652	(Intercept) Plant Type	0.05196 -0.03875	0.01139 0.01505	4.561 -2.575
		Frequency of scarring	Cage (Intercept) Residual	0.00090933 0.0077697	0.030155 0.088187	(Intercept) Plant Type	0.19233 -0.12740	0.02947 0.03944	6.526 -3.230
Greenhouse									

	Feeding rate	Cage (Intercept)	6.3086e-05	0.00079427	(Intercept)	0.04814	0.01109	4.342
	Residual	1.0429e-03	0.0322934	0.0322934	Plant Type	-0.03262	0.01633	-1.997

Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Miscanthus sinensis* and *Bothriochloa ischaemum*.

Appendix 2

Mean (± 1 SE) values of leaf tissue consumption and feeding activity of *Melanoplus femur-rubrum* grasshoppers on native and exotic plant species.

Site	Parameter	Plants			
		Native	Exotic	<i>Andropogon</i>	<i>Bouteloua</i>
WMREC	Total volume of the grazed portion (cm ³)	0.005 ± 0.002	0.001 ± 0.000*	0.091 ± 0.025	0.083 ± 0.016
	Frequency of scarring	0.051 ± 0.015	0.012 ± 0.005	0.003 ± 0.001	0.014 ± 0.003
	Feeding rate (cm ³ /g/day)	0.003 ± 0.001	0.0005 ± 0.000*	0.024 ± 0.009	0.002 ± 0.001
UCCFS	Total volume of the grazed portion (cm ³)	0.019 ± 0.006	0.000 ± 0.000*	0.082 ± 0.018	0.000 ± 0.000
	Frequency of scarring	0.016 ± 0.007	0.000 ± 0.000	0.022 ± 0.013	0.002 ± 0.001
UC	Total volume of the greenhouse grazed portion (cm ³)	0.017 ± 0.007	0.009 ± 0.005	0.069 ± 0.029	0.035 ± 0.017

Frequency of scarring	0.114 ± 0.040*	0.016 ± 0.006*	0.216 ± 0.067	0.169 ± 0.045*
Feeding rate (cm ³ /g/day)	0.014 ± 0.006	0.010 ± 0.006	0.058 ± 0.024	0.039 ± 0.020

¹ Feeding experiments with intact plants were conducted in the field at Western Maryland Research and Education Center (WMREC) and at the University of Cincinnati Center for Field Studies (UCCFS), as well as at the University of Cincinnati (UC) greenhouse.

² Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Miscanthus sinensis* and *Bothriochloa ischaemum*.

³ Frequency of scarring and feeding rate were estimated for plants with at least one grazed mark (="a scar"); undamaged *Bouteloua curtipendula* plants at the UCCFS were excluded from analysis of these variables.

⁴ Asterisks ("**") indicate significant differences at the adjusted significance of 0.008 in pairwise comparisons between plant species.

Appendix 3

Model (GLMM) estimates of an effect of grasshopper enclosures (containers) on food consumption and food assimilation of *Melanoplus femur-rubrum* grasshoppers in the experiments with clipped leaves in relation to plant type (native or exotic).

Experiment	Parameter	Random effects				Random effects			
		Groups	Name	Variance	Std. Dev.	Estimate	Std. Error	t value	
Choice	Total volume of the grazed portion	Container (Intercept)	3.4193e-07	0.00058474	(Intercept)	0.0026270	0.0006989	3.759	
		Residual	4.5432e-06	0.00213148	Plant Type	-0.0010460	0.0009532	-1.097	
Food intake	Container (Intercept)	7.6104e-05	0.0087238	(Intercept)	0.0103523	0.0036078	2.869		
		Residual	4.5670e-05	0.0067580	Plant Type	0.0001733	0.0036966	0.047	
Feeding rate	Container (Intercept)	5.4797e-06	0.0023409	(Intercept)	0.019408	0.005414	3.585		
		Residual	1.7044e-04	0.0130553	Plant Type	-0.001249	0.007939	-0.157	
Consumption index	Container (Intercept)	2.5624e-19	5.0620e-10	(Intercept)	0.034187	0.006472	5.282		
		Residual	2.5134e-04	1.5834e-02	Plant Type	-0.015823	0.009600	-1.648	
No-choice	Total volume of the grazed portion	Container (Intercept)	0.00000e+00	0.0000000	(Intercept)	0.003407	0.002122	1.605	
		Residual	4.0535e-05	0.0063667	Plant Type	0.001731	0.003001	0.577	
Food intake	Container (Intercept)	0.00000000	0.000000	(Intercept)	0.012133	0.003813	3.182		
		Residual	0.00010179	0.010089	Plant Type	-0.001473	0.005222	-0.282	
Feeding rate	Container (Intercept)	0.00000e+00	0.0000000	(Intercept)	0.015628	0.004015	3.893		
		Residual	9.6718e-05	0.0098345	Plant Type	-0.004947	0.005311	-0.931	
Consumption index	Container (Intercept)	0.00000000	0.000000	(Intercept)	0.035785	0.006138	5.830		
		Residual	0.00022605	0.015035	Plant Type	-0.007719	0.008120	-0.951	
The amount of assimilated food	Container (Intercept)	0.00000e+00	0.0000000	(Intercept)	0.007511	0.003098	2.425		
		Residual	8.6371e-05	0.0092936	Plant Type	0.006822	0.004381	1.557	

Assimilation rate	Container (Intercept)	0.0000000	0.000000	(Intercept)	0.009038	0.003703
	Residual	0.00010972	0.010475	Plant Type	0.008975	0.005237
The approximate digestibility	Container (Intercept)	0.000000	0.00000	(Intercept)	0.56704	0.08826
	Residual	0.0062321	0.24964	Plant Type	0.30327	0.12482
Feces production	Container (Intercept)	0.0000e+00	0.0000000	(Intercept)	0.003500	0.000742
	Residual	4.4042e-06	0.0020986	Plant Type	-0.001437	0.001049
						-1.370

Native plants included *Andropogon gerardii* and *Bouteloua curtipendula*; exotic plants included *Miscanthus sinensis* and *Bothriochloa ischaemum*.

CHAPTER 6

General Conclusions

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In this dissertation, I explored the interaction between generalist insect herbivores and native versus exotic plants, which is critical for understanding the mechanisms facilitating plant invasion. By incorporating recent methodological recommendations (e.g. Atwood & Meyerson, 2011; Garcia-Robledo et al., 2013), I applied a combination of behavioral and molecular approaches to explore the interactions in a grass-grasshoppers system from both plant and insect perspectives. Specifically, I investigated the main components of plant responses to herbivory (plant resistance and plant tolerance) and grasshopper feeding preferences on native versus exotic plants.

To achieve these goals, I first explored resistance of native (*Andropogon gerardii* and *Bouteloua curtipendula*) and exotic grasses (*Miscanthus sinensis* and *Bothriochloa ischaemum*) to herbivory by generalist *Melanoplus* grasshoppers under field and greenhouse conditions, as well as in the experiments with intact plants versus clipped leaves (Chapter 2). Using the same experimental design, I then compared tolerance to herbivory among these grass species (Chapter 3). As plant regrowth after sustaining herbivory is one of the main components of plant tolerance, this project required a non-destructive method of estimating plant biomass, which is challenging especially for grasses. In addition, using different proxies for plant biomass may potentially cause mixed results in the estimation of plant tolerance. To address these issues in Chapter 3, I first compared several standard proxies for plant biomass. I then used [plant height × number of leaves] and plant height for comparisons of plant tolerance, as these variables were the best predictors for biomass changes during herbivory and after a subsequent regrowth period. Overall, exotic plants demonstrated greater damage and thus lower resistance to grasshopper herbivory than native plant species in most experiments (Chapter 2), while they tolerated the herbivory similar to native plant species (Chapter 3). Consequently, greater tolerance of exotic

plants compared to native plants, as predicted by the EICA hypothesis (Blossey & Notzold, 1995), was not observed for exotic grasses in this study; future studies might focus on exploring whether the degree of invasiveness might affect the trade-off between plant resistance and plant tolerance to herbivory.

To address the previous inconsistency of results from published studies on grasshopper feeding preferences on native and exotic plants, I conducted a variety of choice and no-choice feeding trials with intact plants and with clipped leaves under field and greenhouse conditions (Chapters 4 and 5). In addition, following recommendations of many authors (e.g. Garcia-Robledo et al., 2013), I applied a PCR-based method for identifying ingested plants, which I have developed specifically for grasshoppers (Chapter 4). Results from both behavioral experiments and molecular confirmation of diet were consistent: grasshoppers preferred to feed on exotic plants. These results were also consistent with the results of lower resistance in exotic plants compared to natives that we observed earlier (Chapter 2). Given that *Melanoplus* grasshoppers do not occur in the native range of *B. ischaemum* and *M. sinensis*, the overall results from this dissertation project suggest that a lack of coevolutionary history in this plant-insect system resulted in a lower level of defenses of exotic plants (primarily resistance) to this novel herbivore in the introduced range, as compared to native plants. These results also suggest that *Melanoplus* grasshoppers, as generalist insect herbivores, may contribute to the biotic resistance of native communities that potentially may prevent plant invasions (Parker & Hay, 2005).

Although testing invasion hypotheses was beyond the scope of my dissertation project, the results from feeding experiments were consistent with the prediction of the EICA hypothesis about lower resistance of exotic plants and with the prediction of the BRH about feeding

preferences of generalist herbivores towards exotic plants. The results from this dissertation, however, did not demonstrate complete escape of exotic grasses from their enemies in the introduced range, as predicted by the ERH; future studies might focus on adding specialist herbivores and pathogens to this grass-grasshopper model to fully explore the predictions of this hypothesis.

In addition, the combined experimental approach that I used in my dissertation, demonstrated its utility for minimizing inconsistency in results from the experiments on the interaction of generalist insect herbivores with native versus exotic plants. The results from this dissertation project revealed certain consistency between plant responses to grasshopper herbivory and grasshopper feeding preferences on native and exotic grasses. This approach, consequently, can be applied to other plant-insect models, especially highly invasive species, to effectively control them.

This dissertation proposed two important methodological tools: (1) a non-destructive method for estimating plant biomass in grasses, and (2) a PCR-based protocol for plant DNA detection within grasshopper gut contents (Avanesyan, 2014). The proposed non-destructive method for plant biomass estimation in grasses will be especially beneficial for herbivory assays which focus on plant recovery after herbivory, as well as for determining the best predictor for biomass of grass species of interest under specific experimental conditions in small-scale studies. The PCR-based method for detecting plant DNA within grasshopper guts is important for exploring plant “movement” during food consumption, and detecting plant-insect trophic interactions.

The results from this dissertation project provide greater insight into the mechanisms of potential invasion of introduced plant species and its consequences for natural communities; this

information has economical, agricultural, medical and ecological importance (Pimental et al., 2005). Specifically, these results are important for predicting the impacts of invasive grasses on trophic interactions which are associated with invasive plants in the introduced range (Harvey & Fortuna, 2012). Given that *Melanoplus* grasshoppers are among common species in the tallgrass prairie, the results from this study demonstrated that these insect herbivores would be less likely affected by invasion of exotic *B. ischaemum* and *M. sinensis*. Considering that *Melanoplus* grasshoppers are also among important agricultural pests, this study can be extended to exploring feeding preferences of grasshoppers on crop plants, as well as resistance and tolerance of crop plants to grasshopper herbivory. This will help agricultural and landscape managers to develop more effective programs for pest control and restoration of certain areas after plant invasions.

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