Locust Outbreaks and Migration in the Asian Steppe: The Influence of Land Management Practices and Host Plant Nutrient Status

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved March 2012 by the
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May 2012

ABSTRACT

Land management practices such as domestic animal grazing can alter plant communities via changes in soil structure and chemistry, species composition, and plant nutrient content. These changes can affect the abundance and quality of plants consumed by insect herbivores with consequent changes in population dynamics. These population changes can translate to massive crop damage and pest control costs. My dissertation focused on *Oedaleus asiaticus*, a dominant Asian locust, and had three main objectives. First, I identified morphological, physiological, and behavioral characteristics of the migratory ("brown") and non-migratory ("green") phenotypes. I found that brown morphs had longer wings, larger thoraxes and higher metabolic rates compared to green morphs, suggesting that developmental plasticity allows greater migratory capacity in the brown morph of this locust. Second, I tested the hypothesis of a causal link between livestock overgrazing and an increase in migratory swarms of *O. asiaticus*. Current paradigms generally assume that increased plant nitrogen (N) should enhance herbivore performance by relieving protein-limitation, increasing herbivorous insect populations. I showed, in contrast to this scenario, that host plant N-enrichment and high protein artificial diets decreased the size and viability of *O. asiaticus*. Plant N content was lowest and locust abundance highest in heavily livestock-grazed fields where soils were N-depleted, likely due to enhanced erosion and leaching. These results suggest that heavy livestock grazing promotes outbreaks of this locust by reducing plant protein content. Third, I tested for the influence of dietary imbalance, in conjunction with high population density, on migratory plasticity. While high population density has clearly been shown to induce the migratory morph in several locusts, the effect of diet has been unclear. I found that locusts reared at high population density and fed unfertilized plants (i.e. high quality plants for *O. asiaticus*) had the greatest migratory capacity, and maintained a high percent of brown locusts. These results did not support the hypothesis that poor-quality resources increased expression of migratory phenotypes. This highlights a need to develop new theoretical frameworks for predicting how environmental factors will regulate migratory plasticity in locusts and perhaps other insects.

ACKNOWLEDGMENTS

I would like to thank my co-chairs, Jon Harrison and James Elser for their countless hours of invaluable guidance and advice; my committee members, Mike Quinlan, Dale Denardo, and John Sabo for helpful discussion of experimental design and navigating academia; and my Chinese collaborators, Le Kang and Shuguang Hao, for support in the field. Feng Jin, Colleen Ford, Danielle Niren, Dan Flynn, Yuan Kong, Zhantou Fan, Jennifer Esman, Teja Peela, Quansheng Chen, and Guangming Zhang provided exceptional field and lab assistance and support. I would also like to thank Jingle Wu, Michelle McCrackin, Jessica Corman, John Vandenbrooks, James Waters, Jaco Klok, Deborah Lutterschmidt, Spencer Behmer, Tony Joern, Steve Simpson, and Fiona Clissold for helpful discussion as my research has developed; Margaret Coulombe for her incredible mentorship in science media and relations; and the staff, faculty, and students of the School of Life Sciences and ASU for their support in immeasurable ways; and my parents, Bill and Susan Cease, for their unwavering support. Importantly, I want to thank the organizations that have supported my research: Arizona State University (SOLS, Graduate College, GPSA), the National Science Foundation (EAPSI, DDEP to AJC, DEB-0925017 to JJE, and EAR-0746352 to JFH), Sigma Xi, Achievement Rewards for College Scientists (Marley-Webb and Johnston

Foundations), Lisa Dent Memorial Foundation, and P.E.O. (specifically, Frances Burruel and Ambre Leffler of Chapter BA, Scottsdale, Arizona).

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Chapter 1

INTRODUCTION

Socioeconomic and ecological importance of grasslands and dryland ecosystem degradation

Globally, grasslands cover 25% of total land area and store approximately 34% of the total terrestrial carbon (Lal 2001), making grassland nutrient cycling and anthropogenic impacts on grassland functioning and services important areas of ecological research. Most of our understanding about grassland ecosystem functioning comes from Europe or North America whose grasslands have been severely fragmented due to conversion to croplands (Johnson 1994; Samson and Knopf 1994). Unfortunately, many of the world's grasslands have been over-utilized, leading to soil erosion and severe degradation of the ecological diversity and productivity. The FAO describes desertification as, "the degradation of land in arid, semi-arid, and dry sub-humid areas. It occurs because dryland ecosystems, which cover over one third of the world's land area, are extremely vulnerable to over-exploitation and inappropriate land use. Poverty, political instability, deforestation, overgrazing, and bad irrigation practices can all undermine the land's fertility (FAO)." My dissertation research focused on relatively unstudied threatened dry grasslands in Inner Mongolia, China. In northern China and the vast semi-arid region of central Eurasia, 9 million square kilometers of grassland steppes make up the largest continuous grassland in the world (DAHV and CISNR 1994). However, rapid steppe degradation (Tong et al. 2004) has led to reduced biodiversity, decreased productivity and, in some cases, to desertification (Wu and Overton 2002), likely due to anthropogenic factors, especially livestock grazing (Han et al. 2008).

Anthropogenic impacts ↔ plant communities ↔ insect population dynamics

How humans alter plant nutrient content directly has been the topic of many agricultural and ecological studies (Wani, Rupela, and Lee 1995; Reddy et al. 2003; Shaviv and Mikkelsen 1993; McLauchlan 2006; Welbaum et al. 2004). However, we know little of how these changes in plants affect the physiology of insect herbivores, and how these changes potentially feedback into agricultural systems. Insects comprise 56% of all known animal species on Earth (Thomas 2005) and play a critical role in ecosystem functioning. Insects are crucial for most pollination and terrestrial seed dispersal and contribute significantly to ecosystem nutrient cycling; insects are also significant pests and major contributors to global food insecurity (Janzen 1987). Thus, how insects will respond to changes in plant communities and nutrient content will be critical to our long term agricultural systems and sustainability of our natural systems.

Nutrient balance and effects of nitrogen availability on host-plant chemistry

Biologically available nitrogen (N) limits net primary production in most terrestrial ecosystems (LeBauer and Treseder 2008) and, therefore, humans apply approximately 150 Tg of reactive N annually to increase agricultural yield (Smil 2001; Galloway et al. 2004). Conversely, poor agricultural land management practices can lead to soil erosion and N-depletion from ecosystems. These changes in N availability likely affect many insect herbivores via changes in the N concentration in leaves (Throop and Lerdau 2004; Aber et al. 1998).

Since plants in many terrestrial ecosystems are N-limited (Elser et al. 2007), it is often assumed that primary consumers would be as well (White 1993). However, studies performed on the basis of the geometric framework (Raubenheimer and Simpson 1993) have shown that many animals have specific dietary intake targets for protein, carbohydrates, and other nutrients. Interestingly, such studies have found that excess protein decreased lifespan in fruit flies (Lee et al. 2008) and that herbivores and omnivores (including humans) will overeat carbohydrates but are unlikely to overeat protein (Simpson and Raubenheimer 2009). Thus animals may sometimes be impaired by ingesting excess protein (Bernays 1983). Indeed, the Australian plague locust, *Chortoicetes terminifera* (Acrididae: Oedipodinae), grew slowly on one host plant because it obtained excess protein relative to carbohydrate (Clissold, Sanson, and Read 2006). Similarly, my dissertation research showed that high nitrogen and protein content in host plants decreased growth and survival in *Oedaleus asiaticus* (see Chapter 3).

Developmental responses to diet

Nutrition-triggered phenotypic plasticity is a widespread and evolutionarily primitive adaptive phenomenon. For example, in spade-foot toad tadpoles, a single cue – shrimp ingestion – can trigger alternative developmental trajectories (Pfennig 1990). If a tadpole consumes a critical number of shrimp, it will grow into a fast-developing carnivore morph with a large body and mouth morphology enabling it to eat the available prey. One of the most common and fundamentally important alternative phenotypes in animals may be an increased migratory or dispersal capacity (Krug 2001; Lees 1961; Wigglesworth 1961; Southwood 1962; Harrison 1980; Dingle 1985; Roff 1986; Solbreck 1986; Denno and Roderick 1992; Nijhout 1994; Zera and Mole 1994). Development of a phenotype capable of extended migration allows insect herbivores to find and impact widely-spread crops, and survive and flourish despite inferior environmental conditions in their natal region. Diet quality has been shown to influence migratory polyphenism in a number of insects, including the diamondback moth (Campos, Schoereder, and Sperber

2004), planthoppers (Denno, Douglass, and Jacobs 1985), and velvetbean caterpillars (Fescemyer and Erlandson 1993). Aphids are probably the best-studied—high population density tends to increase migration (Johnson 1965), while N-fertilized host plants suppress development of the migratory form (Branson and Simpson 1966).

While nutrition has often been suggested as an environmental factor influencing migratory polyphenism in grasshoppers (Johnson 1969; Uvarov 1966, 1977; van Huis et al. 2008), the direct effects of food quality in triggering grasshopper migratory polyphenism has only been investigated in two species to date. Similar to aphids, in the wing dimorphic, *P. nebrascensis*, high density promoted development of long wings, while N-fertilized plants promoted development of short, flightless wings (Gaines 1989). My dissertation research on *Oedaleus asiaticus* has also shown different diets promote development of alternative (migratory vs. nonmigratory) morphologies and physiologies (see Chapter 4).

Oedaleus "locusts": economically important but poorly studied

Grasshoppers (family: Acrididae) provide excellent model systems to test hypotheses about anthropogenic effects on nutritional quality of host-plants for insect herbivores. They are often used as a model generalist herbivore for testing questions about plant preference and effects of climate change on herbivore communities (e.g. Perez-

Harguindeguy et al. 2003). Arguably, the nutritional ecology and physiology of grasshoppers is better known than that for any other insect (Jonas and Joern 2008; van Huis et al. 2008; Loaiza, Jonas, and Joern 2008; Fielding and Defoliart 2008; Chapuis et al. 2008; Hahn 2005; e.g. Berner, Blanckenhorn, and Korner 2005; Appel and Joern 1998; Chambers, Raubenheimer, and Simpson 1997; Fielding and Brusven 1992; Bernays and Barbehenn 1987; Behmer and Joern 2008; Danner and Joern 2004; reviewed in Chapman and Joern 1990). Furthermore, extensive studies on the ecology and physiology of the plague locusts, *Schistocerca gregaria* and *Locusta migratoria*, has provided a wealth of field and experimental data that generally inform our understanding of grasshopper nutritional ecology (Uvarov 1977; Anstey et al. 2009; Uvarov 1966; Pener and Simpson 2009).

All locusts are grasshoppers (family: Acrididae), and here I use the term “locust” to indicate grasshopper species in which the larval environment triggers development into either a migratory, swarm-forming morph or a sedentary morph (reviewed in Pener and Simpson 2009). Our knowledge of these types of grasshoppers arises almost exclusively from extensive studies on two species: *Locusta migratoria* and *Schistocerca gregaria*. The migratory and non-migratory forms of these species differ in color, behavior, physiology, and morphology (Al-Ajlan 2007; Bouaichi and Simpson 2003; Bouaichi, Simpson, and Roessingh 1996; Deng et al. 1996;

Hagele et al. 2000; Hagele and Simpson 2000; Heifetz 1997; Injeyan and Tobe 1981; Maeno, Gotoh, and Tanaka 2004; Maeno and Tanaka 2004, 2007; McCaffery et al. 1998; Miller et al. 2008; Roessingh, Bouachi, and Simpson 1998; Rogers et al. 2003; Simpson and Miller 2007; Simpson et al. 2002; Tanaka and Maeno 2006; Tanaka and Maeno 2008; Van der Werf et al. 2005; van Huis et al. 2008; Vandersmissen, De Loof, and Gu 2007; Wedekind-Hirschberger, Sickold, and Dorn 1999). Non-migratory forms tend to be green, blending in with the vegetation and prefer to be solitary, avoiding interactions with other individuals. Migratory forms can be conspicuous black or yellow (Faure 1932; Uvarov 1966, 1977; Lorenz 2009), and are more active and prefer to aggregate (Applebaum and Heifetz 1999; Pener, Ayali, and Golenser 1997; Pener and Yerushalmi 1998; Uvarov 1966). Thus, the terms commonly associated with these two forms, solitary and gregarious, are in reference to their distinctive behavior (Uvarov 1921).

Migratory grasshoppers vary in the intensity with which they express associated physiological and morphological characters (Bouaichi, Simpson, and Roessingh 1996; Rogers et al. 2003; Simpson, McCaffery, and Hagele 1999; Wedekind-Hirschberger, Sickold, and Dorn 1999). Migratory locusts tend to have elevated resting, mass-specific metabolic rate (Butler and Innes 1936; Heifetz 1997; Applebaum and Heifetz 1999; Uvarov 1966), perhaps due to the elevated maintenance cost of larger fight muscles (Nespolo, Roff, and Fairbairn 2008). The migratory forms also tend to have a larger body size, increased flight musculature and wing size, increased lipid stores for long-distance flight, and a delayed onset of fecundity (Applebaum and Heifetz 1999; Pener, Ayali, and Golenser 1997; Pener and Yerushalmi 1998; Uvarov 1966); which supports a general model of increased investment in flight-related physiology and morphology at the expense of reproductive capacity. Body color polyphenism can be observed in hatchlings, in this case, color is likely initially determined by maternal environment (Applebaum and Heifetz 1999; Islam et al. 1994; Maeno and Tanaka 2008; McCaffery et al. 1998; Tanaka and Maeno 2006; Tanaka and Maeno 2008). Color can change within the life of an individual, influenced by environmental cues; some evidence suggests that population density, temperature, humidity, and background color of the habitat can all play a role (Applebaum and Heifetz 1999; Maeno, Gotoh, and Tanaka 2004; Sword and Simpson 2000; Sword et al. 2000; Tanaka 2000; Tanaka et al. 2002; Faure 1932; Pener 1991). Other migratory characters, such as behavior and flight muscle, can be altered throughout a given developmental stage (Roessingh and Simpson 1994; Bouaichi, Roessingh, and Simpson 1995; Roessingh, Bouai¨chi, and Simpson 1998; Simpson, McCaffery, and Hagele 1999; Pener 1991; Uvarov 1977). Thus, color cannot be used to definitively identify a migratory phenotype. Migratory locusts such as *L. migratoria* and *S. gregaria* are the world's most destructive insect pests, while the non-migratory, solitary forms are of no economic importance (Walsh 1988).

Oedaleus grasshopper species are sometimes characterized as "locusts", "non-model locusts", or "grasshoppers which exhibit some locust characteristics" (Song 2011). Most *Oedaleus* exhibit green-brown polyphenism. In the two species where it has been tested, *O. asiaticus* (see Chapter 2) and *O. senegalensis*, outbreaks are comprised almost entirely of brown phenotypes, and brown morphs exhibit migratory locust-like characteristics (Cease et al. 2010; Popov 1989). There are 25 species of *Oedaleus*; *Oedaleus senegalensis* is likely the best-studied of all Oedaleus due to its importance as the main pest of the Sahel. For example, in 1986, 1988, and 1989, 1-3.5 million ha were treated for *O. senegalensis* infestations, and in a period of 7 years (1886-1992), 177 million USD was spent on control (Popov 1996; Cheke 1990). This species is known to form migratory marching bands as juveniles, and migratory swarms that make nocturnal migrations as adults (*L. migratoria* and *S. gregaria* swarms are typically diurnal). Locoq (1978) described how migratory swarms of *O. senegalensis* are just as remarkable as *L. migratoria* and *S. gregaria* and can "settle at night in ripening cereal crop fields and completely decimate them in a spectacular way within a few hours (Lecoq 1978)." My dissertation focused on *O. asiaticus* in Inner

Mongolia, China. While documented migrating swarms of this species have had significant socioeconomic impacts throughout Asia for more than a century (DAHV and CISNR 1994), virtually no research has investigated the nutritional ecology and migratory plasticity of *O. asiaticus.*

Chapter 2

ARE COLOR OR HIGH REARING DENSITY RELATED TO MIGRATORY POLYPHENISM IN THE BAND-WINGED GRASSHOPPER, OEDALEUS ASIATICUS?

This chapter has been published in the Journal of Insect Physiology; see Appendix A.

Chapter 3

HEAVY LIVESTOCK GRAZING PROMOTES LOCUST OUTBREAKS BY LOWERING PLANT NITROGEN CONTENT

This chapter has been published in Science; see Appendix B.

Chapter 4

HIGH QUALITY, LOW-PROTEIN DIET ENHANCES PERFORMANCE AND MIGRATORY CHARACTERS IN A POLYPHENIC LOCUST

Abstract

The ability of animals to adaptively relocate has long captivated biologists, and has become an increasingly important topic as we seek to understand how animals will cope with their changing environments due to anthropogenic impact. Locusts present an impressive and well-studied example of phenotypic plasticity in which population density regulates a variety of behavioral, morphological, and physiological traits that often culminate in migratory swarms. *Oedaleus asiaticus*, a north Asian locust, has outbreaks and migratory swarms comprised predominantly of brown morphs. Heavy livestock grazing promotes outbreaks by lowering plant nitrogen content and likely also lowering the protein:carbohydrate ratio to the optimal range for this locust. However, it is unclear if a change in plant quality can modulate the effect of density to either enhance or suppress expression of migratory characteristics. We found that locusts reared at high population density and fed unfertilized plants (i.e. high quality plants for *O. asiaticus*) had the most enhanced migratory characteristics while locusts fed high N plants consistently had decreased expression of migratory characters. These results did not support the

hypothesis that poor-quality resources increased expression of migratory phenotypes.

1. Introduction

The ability of animals to adaptively relocate has long captivated biologists (Dingle 1996), and has become an increasingly important topic as we seek to understand how animals will cope with their changing environments due to anthropogenic impact (Berg et al. 2010). Insect migratory plasticity, where a single genome produces alternative dispersal phenotypes in response to environmental cues, has broad-ranging implications for biodiversity conservation and food security, as well as spanning biological disciplines from functional genomics, developmental biology, and biomechanics to population and ecosystem ecology (Drake and Gatehouse 1995; Whitman and Ananthakrishnan 2009; Roff and Fairbairn 2007; Braendle et al. 2006; Zera and Denno 1997). Locusts present an impressive and well-studied example of phenotypic plasticity called "phase polyphenism," in which population density regulates a variety of behavioral, morphological, and physiological traits that often culminate in migratory swarms that can devastate agriculture (reviewed in Pener and Simpson 2009). The two morphs, or phases, are often termed 'solitarious' and 'gregarious' after their associated behaviors (Uvarov 1966). Here we study the effect of plant nutritional quality, particularly nitrogen (N) status and the protein:carbohydrate ratio, on the tendency for *Oedaleus asiaticus*, a locust of Inner Mongolia, to form migratory phenotypes. Decreasing food quality has been broadly implicated in increasing frequency of migratory phenotypes; however, food quality is often loosely defined and not tested directly for the organism in question.

1.1 What is high quality food?

When considering host plant quality, nitrogen is a currency frequently used by ecosystem ecologists and managers, and allows for connection of multiple interest levels (e.g. ecosystem nutrient cycling to herbivore biology). Current paradigms generally assume there is a positive linear relationship between plant N content and herbivore performance (White 1993). However, extensive studies based on ecological stoichiometry (Sterner and Elser 2002) and the geometric framework (Raubenheimer and Simpson 1993) have demonstrated that animals have an optimal balance of nutrients. Animals select for these optimal ratios by combining multiple food items with complementary nutrient ratios—the ratio of nutrients for which they select is termed the "intake target" (Simpson and Raubenheimer 1995). Protein and carbohydrate are the most strongly regulated nutrient groups in insects (Simpson and Raubenheimer 2000) and insects confined to diets unbalanced (i.e. different than their intake target) in these two macronutrients *in either* *direction* can suffer consequences (e.g., decreased growth rate, increased mortality). In addition to nutrient balance, herbivores may have to balance intake of plant toxins or other plant defenses.

Many studies investigating the effects of food quality on migratory plasticity and other responses often assume that higher nitrogen or protein content is always "high quality" and frequently do not clarify between foods that are "high quality" because they are high protein or "high quality" because they increase growth and viability in the organism of interest. This lack of differentiation can be misleading because the assumption that higher plant protein content is always better quality is likely incorrect. Therefore, for clarity in this paper, we use the terms "high GS (growth and survivorship) quality food" and "low GS quality food" in place of "high quality food" and "low quality food."

For the locust *O. asiaticus* consuming either their natural plant community assemblage, or when confined to particular common plants from their habitat, nitrogen (N) fertilization decreases growth rate, adult size, and survival. Further, this locust has a protein:carbohydrate target of 0.5 on artificial diets, quite low compared to other grasshoppers that have been studied (Cease et al. 2012). These results suggest that high GS quality food is relatively low in nitrogen and protein, in contrast to current N-limitation paradigms for herbivores. In the current study, we test the hypothesis that low GS food (high protein, in this case) favors development of migratory phenotypes in *O. asiaticus*.

1.2 Nutritional effects on migratory polyphenism

In some insects, there is clear evidence that low GS quality plants can trigger development of migratory phenotypes. Denno et al (1985) showed that there was an interactive effect between plant nutrient content and population density in the wing dimorphic planthopper *Prokelisia marginata*. In that case, heavily-fertilized plants (nitrogen, phosphorus, and potassium), which increased adult size and fecundity, suppressed the development of long-winged morphs at high density (Denno, Douglass, and Jacobs 1985). Fescemyer and Erlandson (1993) found that increasing the proportion of foliage (soybean leaves or kudzu) in an artificial diet decreased performance in velvetbean caterpillars, *Anticarsia gemmatalis*, and also increased the percentage of dark, migratory phase insects (Fescemyer and Erlandson 1993). Further, field studies on aphids have shown that N-deficient host plants (low GS quality in this case) can trigger development of winged aphids (e.g. Branson and Simpson 1966). This tendency for insects to develop migratory forms in response to low-GS-quality food has been hypothesized to be an adaptive response that increases fitness in winged individuals able to seek out better quality food.

While the pervasive hypothesis is that poor nutritional environments (low GS quality plants) trigger development of migratory-capable adults, there are conflicting results. In the thrip *Thrips nigropilosus*, for example, Nakao (1994) found no differences in the percentage of each wing form in females reared on four different host plants, despite differences in mortality and development time on the different plants (Nakao 1994). In a review on aphids, Müller et al (2001) addressed the validity of the long-implicated hypothesis that poor nutritional GS quality, as defined by decreased growth performance and survival, increases production of winged morphs. They found that of the 38 studies included, this hypothesis was rejected in 55% of the cases (accepted in 34% of cases). These studies demonstrate that the issue of plant GS quality directing migratory polyphenism is complex and requires more attention. Figure 1 incorporates some hypotheses for when low or high GS quality food might promote development of migratory or non-migratory phenotypes.

As yet, studies testing the effect of food GS quality on the tendency of any locust to form the gregarious/migratory phase have been limited to understanding how distribution of resources and diet quality influence foraging behavior and the subsequent probability of coming into physical contact with conspecifics (and thereby inducing phase change through changes in effective density) (Babah and Sword 2004; Bouaichi, Simpson, and Roessingh 1996; Despland and Simpson 2000). Figure 2 is a simple schematic illustrating factors that regulate locust dynamics and polyphenism. No studies have tested the capacity of food GS quality to directly modify the density response of locusts to develop phase characteristics. However, Gaines (1989) tested the interactive effects of host plant N-fertilization and population density on the non-swarming but wing-dimorphic grasshopper, *Phoetaliotes nebrascensis*. She reared eggs from short-winged adults in a 2x2 factorial experiment (two levels of population density and two levels of fertilized grass). The one combination to produce long-winged offspring (25%) was high density and unfertilized grass (Gaines 1989). Later, Joern and Behmer (1998) demonstrated that diets with increasing N content decreased survival rate in *P. nebrascensis* (but not in another abundant and co-existing acridid, *Melanoplus sanguinipes*) (Joern and Behmer 1998). Therefore, the unfertilized grass in Gaines' 1989 study likely reflected high GS quality food, which, in conjunction with high density, promoted development of long-winged adults; low GS quality food (high N content in this case) suppressed this response to high density.

There is some evidence for a contrary result in the desert locust, *Schistocerca gregaria*. Maeno and Tanaka (2011) tested the effects of plant quality on *S. gregaria* by rearing solitarious and gregarious *S. gregaria* on either fresh cut (high N:C) or old and wilted (low N:C) grass

(*Dactylis glomerata*). They found that locusts reared from hatching on old grass had higher wing:leg length ratios, but decreased performance (lower survival, longer development times, and decreased adult body weight) (Maeno and Tanaka 2011), supporting the hypothesis that low GS quality food promotes the migratory phenotype. However, they did not test for the interactive effects of population density and plant quality directly; nymphs were reared individually. Population density is a key factor regulating locust phase polyphenism and a full suite of phase characteristics will likely not be expressed in the absence of high density (Uvarov 1966; Pener and Simpson 2009). In the current study, we test for the interactive effects of low and high GS quality food on the development of migratory phenotypes in the locust *O. asiaticus*.

1.3 Interaction between density and nutrition?

One of the key open questions concerning the ecological regulation of polyphenism in acridids is the extent to which field nutrition conditions may interact with density to affect polyphenism and migratory characteristics. While it is clear that both density and host plant nutrient status can affect acridid phase polyphenism, no study to date has tested the interactions between these two important factors. *Oedaleus asiaticus* provides an excellent model for testing for such interactions. Outbreaks and migratory swarms are comprised predominantly of brown morphs, which have increased relative investment in their thorax and hind legs as compared to the green, non-migratory morphs (Cease et al. 2010). Our prior studies have shown that the brown form of *O. asiaticus* prefer and perform best on low-N plants from heavily grazed fields of the Inner Mongolian steppe (Cease et al. 2012). Because these low-N plants also support more rapid growth, we infer that heavy grazing increases the population levels of *O. asiaticus* by creating more nutritious, preferred food for this species. What remains unknown is whether the low-N levels can actually induce development of the migratory phenotype.

Here, using a series of lab and field studies, we tested the prediction that low GS quality food (in this case high plant N content), in conjunction with high local population density, would enhance migratory characteristics in the non-model locust, *O. asiaticus*. We conducted two rearing experiments (lab and field cages) where we confined locusts to low or high density and fed them host plants with low or high nitrogen treatments. We measured changes in growth performance, physiology, morphology, and color in the locusts, and changes in chemistry in their host plants. Previously, we demonstrated that *O. asiaticus* prefers low-N plants when given a selection of the six most common grasses. Further, when given pairs of low- and high-N grass blades from its preferred host (*Stipa grandis*), this locust consumed more low-N *Stipa* (Cease et al. 2012). In the current study, we add additional plant choice comparisons among the two most abundant grasses (*Stipa* and *Leymus*) collected from areas with different levels of livestock grazing (this changes plant nutrient content) and different N fertilization treatments. We predicted that *O. asiaticus* would select the lowest protein option when given two choices. In sum, these experiments allowed us to address the question of how plant quality may influence phase polyphenism in this locust.

2. Methods

2.1 Field site

Field experiments were conducted near the Inner Mongolia Grassland Ecosystem Research Station in the Xilin River Basin, Inner Mongolia Autonomous region, China (43°38′ N, 116°42′ E). These studies were carried out over four years: 2008 (lab rearing experiments), 2009 (field cage rearing experiments), 2010 (host plant choice tests). *Oedaleus asiaticus* (*O. decorus asiaticus* Bei-Bienko, 1941) were collected from a population near to the research station. This species hatches in early June, undergoes five juvenile stages, and then molts into adults in mid-July (Li, Wang, and Chen 1987). Inner Mongolia is representative of much of the Eurasion Steppe region and is characterized by dark chestnut soil with relatively homogeneous physiochemical properties and co-dominated by *Stipa grandis* and *Leymus chinensis* (Li, Yong, and Liu 1988; Wu and Loucks 1992). Floristically and ecologically, Inner Mongolia is representative of much of the Eurasian Steppe region. This typical steppe is characterized by dark chestnut soil with relatively homogeneous physiochemical properties and co-dominated by *Leymus chinensis* and *Stipa grandis*. The mean annual precipitation and temperature in the study area are 345 mm and 1.1 ºC, respectively (Chen and Wang 2000).

2.2 Rearing experiments: effects of population density and plant nitrogen on locust phenotype

2.2.1 Lab experiment: effects of density and plant nitrogen on morphology, growth, and respiration (final larval instar)

We collected fourth-instar brown female locust nymphs from field populations and randomly assigned them to control (0 kg N $ha^{-1}yr^{-1}$) or fertilized (175 kg N $ha^{-1}yr^{-1}$) food plant treatment groups and either low (1 locust $cage^{-1}$) or high (8 locusts $cage^{-1}$) density on day one of the fifth and final larval instar. In the fertilized plots, ammonium nitrate (NH_4NO_3; 175 kg N $ha^{-1}yr^{-1}$) was applied once in the late spring, just before a heavy rainfall and at least two weeks before plants were collected (lab experiment) or insects were added to the cages (field experiment: Section 2.2.2). Cages were 10x10x15 cm and made of 1-mm^2 cloth mesh. They were kept in an incubator at the following day:night cycle: 14:10 light:dark, 27°C:25°C, 50%RH:40%RH. Fresh *Leymus chinensis* grass was cut from the either control or fertilized field plots every other day, secured with cotton in glass cylinders containing water, and presented *ad libitum*, similar to Cease et al. (2010).

Specific growth rates (μ) were calculated as $\mu = \ln(M_2/M_1)/dt$, where M_2 and M_1 are the locust body masses at day 1 after molt to adult and at day 1 of the final juvenile instar, respectively, and dt is the number of days spent in the final juvenile instar. Respiration rate was measured when locusts were approximately 75% through the fifth instar, similar to Cease et al. (2010). Within four hours after molt to adult, we isolated treatment grasshoppers in a cage with no food for 24 hours to allow the cuticle to harden. Insects were weighed to the nearest 0.1 mg using a Mettler-Toledo AB204-S/Fact balance, frozen at -20°C, and then dried at 50ºC for three days. We dissected the insects after they had dried by removing their gut and separating the head, wings, legs, thorax, and abdomen. Body components were weighed to the nearest 0.001 mg using a Mettler Toledo MX5 microbalance. We then relaxed the hind wings using a weak vinegar solution, spread them flat for drying, digitally scanned the flat/dry wings, and then measured wing area using Image J software (resolution = 79 pixels/cm; Rasband 2008).

To compare relative allocation to different body components, we first standardized the variables using a Z-score transformation so that all variables would be weighted evenly in our analysis (Gotelli and Ellison 2004). Mass of most body components were correlated (Table 1), so we combined these variables into one linear variable using a maximum likelihood factor analysis (Table 2). Increased values on the migratory index represent increased relative allocation of mass to thorax, wings, and hind legs, as compared to abdomen and head.

For comparison to field-caught locusts, we collected green and brown morphs from the same population two days after we observed the first adults appear in the field population. We followed the same protocol as for the lab-reared insects for drying, dissecting, and weighing body components.

2.2.2 Field experiment: effects of density and plant nitrogen on color and survival (entire larval period)

We constructed 1 m^3 cages with iron rod frames and fine cloth mesh covering over plant communities that were either in control or N-fertilized plots. Spiders and other arthropods were removed from the cages prior to adding locusts. Insects were collected as either first or third instars and randomly assigned to control or N-fertilized plots and either low (20 locusts $cage^{-1}$) or high (100 locusts $cage^{-1}$) density; all locusts for a given cage were added at the same time. The high density level was similar to the field density where we collected the insects. We used cages of either all green or all brown phenotypes to allow assessment of any changes in color and survival rate for the two different color morphs. For an explanation of how we classified these two color morphs see Cease et al. (2010). We surveyed field cages every two weeks until the final larval instar to monitor survival (June-July 2009).

2.3 Host-plant response to locust density and N fertilization

After we removed the locusts from their cages at the end of the field experiment (section 2.2.1), we collected the aboveground vegetation from within a subset of randomly-selected cages (1 m^2 area) and from areas within the control or N-fertilized plots but outside of where the cages were placed (ambient community). All vegetation was collected within one week in mid-July, which is approximately three weeks prior to typical peak aboveground biomass and seeding for this plant community (Bai et al. 2004). We sorted the aboveground vegetation by species, and then isolated the leaves (the part the locusts eat), with the sheaths and stems removed, of *Stipa grandis* and *Leymus chinensis*. Leaves collected from each species were pooled for each 1 m^2 sample, dried, and then ground to a fine powder. We measured carbon and nitrogen content using a Perkin-Elmer model 2400 CHN analyzer, and protein using the Bio-Rad micro assay based on the Bradford assay [0–8 μg IgG (bovine gamma globulin)], with duplicate samples read in triplicate (similar to Clissold, Sanson, and Read 2006; Cease et al. 2012).

We used a semi-quantitative assay for measuring alkaloid content of dried/ground leaf blades by assessing colorimetric response to Dragendorff reagent, which is specific to heterocyclic nitrogen compounds (Wagner, Bladt, and Rickl 1984). This reagent was prepared in two steps, similar to Wagner et al. (2009), using two solutions (1.7 g basic bismuth nitrate in 100 ml water/acetic acid, 4:1 and 40% w/v potassium iodide) added to 29% acetic acid (Wagner, Bladt, and Rickl 2009). Alkaloids were extracted from dried/ground leaf blades using 95% ethanol (24 hour incubation), and 400 uL of supernatant + 400 uL de-ionized water were added to 12x75 mm disposable glass culture tubes. Three blank controls were made with 400uL 95% ethanol + 400 uL de-ionized water. Dragendorff reagent (500uL) was added to each tube and then scored after two minutes by ranking opacity when the tube was positioned directly in front of a dissecting lamp with a typed page containing text ranging from 8-20 point fonts in between the tube and the lamp (Table 3).

We performed this assay to gain a basic insight as to the possible role of endophyte-derived alklaoids—which can be elevated by N-fertilization (Faeth and Fagan 2002)—in the deleterious affect we found of high-N plants on *O. asiaticus* (Cease et al. 2012). Beyond detecting the presence of a reaction to the Dragendorff reagent, we sought a coarse method to quantify this response and generate rough estimates for percent alkaloid in leaves. We generated a standard curve using nicotine (VWR Catalog No TCN0079). We assigned a score to six response ranges (0-5) to the Dragendorff reagent and determined the range of micrograms of nicotine sufficient to elicit each score (Table 3). We then calculated a biologically-relevant standard curve (% of alkaloid in leaves), making the assumption that a microgram of whatever alkaloids were present in the grass tissue would react to the same degree that a microgram of nicotine did to the Dragendorff reagent. This assumption is not unreasonable. Bustamante et al (2006) tested a range of different alkaloids and determined that identical masses of alkaloids from different classes produced similar amounts of precipitate in response to Dragendorff reagent addition (Bustamante, Chacón, and Niemeyer 2006). We were then able to assign each score an average alkaloid percent (Table 4) and we present these percentage values (Fig. 8C). However, while we think these results add value to the discussion of the larger context of this paper, we maintain that this analysis is cursory and that we cannot draw strong conclusions from these results.

2.4 Preference for Stipa *and* Leymus *grasses under different conditions*

We gave locusts a series of paired plant choices to determine their preference for the two co-dominant grasses, *Stipa* and *Leymus*, and how those preferences might change if they were in a protected field versus one heavily grazed by livestock. We used fresh leaves for some trials, and dried/ground leaves for others to control for water content and plant toughness. We used the same protocol as Cease et al. (2012). To compare nitrogen, protein, and alkaloid contents of grasses in protected and grazed fields, we collected 1 m^2 vegetation samples (similar to Cease et al. 2012), and used methods as described in section 2.3.

To investigate how locust density and N-fertilization might influence plant choice, we gave field-caught locusts another set of paired choices using leaves collected from the field cage experiment (section 2.2.1) that were dried/ground for nutrient analyses (section 2.3). Plant samples came from four treatment groups (low density grasshoppers on unfertilized plots, low density grasshoppers on N-fertilized plots, high density grasshoppers on unfertilized plots, high density grasshoppers on N-fertilized plots). We compared the preference of Oedaleus grasshoppers for *Stipa* and *Leymus* collected from these treatment cages separately, such that *Stipa* leaves were only compared with other *Stipa* leaves and the same for *Leymus*. Locusts were collected and treated similarly to the paired plant choice tests from Cease et al. (2012), except the duration of this experiment was up to 36 hours. We extended the duration because many insects refused to eat initially, particularly in the *Leymus* groups.

2.5 Statistics

All data were tested for assumptions of normality and homoscedasticity implicit in parametric tests. All proportion data was arcsine transformed prior to analysis, and remaining datasets were arcsine or log transformed and outliers removed as necessary to meet assumptions for parametric tests. Analyses were performed using Statistica 10 (2010).

3. Results

3.1 Rearing experiments to test the interactive effects of population density and host plant N enrichment on locust phenotype

3.1.1 Lab rearing experiment: morphology, growth, and respiration (final larval instar)

In summary, locusts reared at high density on unfertilized grass exhibited the most enhanced migratory characters, the heaviest adult mass, fastest development time, and among the highest specific growth rates. The combination of high density and N fertilization resulted in locusts with the least enhanced migratory characters, lowest adult mass, slowest development time, and the lowest specific growth rate.

There was a significant interactive effect of locust density and host plant N enrichment on wing area (two-factor ANCOVA $F_{(1, 29)} = 4.97$, $p = 0.03$, covariate means: total dry body mass (mg): 86). Simple main effects analyses showed that when fed unfertilized host plants, locusts reared at high density had a higher wing area than those reared at low density (ANCOVA $F_{(1, 14)} = 7.85$, $p = 0.01$, covariate means: total dry body mass (mg): 89) but there were no differences between high and low density groups when fed N-fertilized plants (ANCOVA $F_{(1, 14)} = 0.10$, $p = 0.76$, covariate means: total dry body mass (mg): 83) (Fig. 3). Note that we analyzed wing area using body mass as a covariate; thus, the figure represents the inverse of wing loading (higher values mean greater wing area per body mass).

Likewise, there was a significant interactive effect of locust density and host plant N enrichment on migratory morphology index (two-factor ANCOVA $F_{(1, 29)} = 20.64$, $p < 0.001$, covariate means: total dry body mass (mg): 86) (Fig. 4A). When fed unfertilized host plants, locusts reared at high density also had a higher migratory morphology index (see section 2.2.1 for a description) than those reared at low density (ANCOVA $F_{(1, 14)} = 16.53$, $p = 0.001$, covariate means: total dry body mass (mg): 89) but there were no differences between high and low density groups when fed N-fertilized plants (ANCOVA $F_{(1, 14)} = 3.45$, $p = 0.08$, covariate means: total dry body mass (mg): 83). Higher values on the migratory morphology index indicate increased relative allocation of body mass to wings, thorax, and hind legs. This is illustrated in Figure 4B where there was a significant interactive effect of locust density and host plant N fertilization on the ratio of (wings+thorax+hind legs mass):(abdomen+head mass) (two-factor ANOVA on ranks $F_{(1, 30)}$ = 12.58, p = 0.001). For comparison, field-caught brown morphs had higher (wings+thorax+hind legs mass):(abdomen+head mass) ratios than field-caught green morphs (Mann-Whitney U Test Z = 2.14, p = 0.03) (Fig. 4B inset).

Simple main effects analyses showed that, when fed N-fertilized plants, locusts reared at higher density attained a smaller adult mass (ANOVA $F_{(1, 15)}$ = 4.86, p = 0.044) and had lower specific growth rates (ANOVA $F_{(1, 15)}$ = 31.81, $p < 0.001$) but there the extension in development time was not statistically significant (ANOVA $F_{(1, 15)}$ = 2.90, p = 0.11) (Fig. 5). When fed unfertilized host plants, locusts reared at high density had a shorter development time than those raised at low density (ANOVA $F_{(1, 15)}$ = 6.62, p = 0.02), but there were no differences in adult mass (ANOVA $F_{(1, 15)}$ = 1.04, p = 0.32) or specific growth rate (ANOVA $F_{(1, 15)}$ = 1.05, p = 0.32). There were a significant interactive effects of locust density and host plant N enrichment on adult mass (two-factor ANOVA $F_{(1, 30)}$ = 5.24, p = 0.03), development time (two-factor ANOVA $F_{(1, 30)}$ = 7.66, p = 0.01), and mass specific growth rate (two-factor ANOVA $F_{(1, 30)}$ = 8.27, p = 0.007).

In contrast to strong effects on morphological and growth parameters, there were no effects on respiration rate due to N fertilization (MANCOVA $F_{(2, 27)}$ = 0.55, p = 0.58, covariate means: total dry body mass (mg): 458) or density (MANCOVA $F_{(2, 27)} = 0.63$, $p = 0.54$, covariate means: total dry body mass (mg): 458), nor an interactive effect of these two factors (two-factor MANCOVA $F_{(2, 27)} = 0.58$, $p = 0.57$, covariate means: total dry body mass (mg): 458), as measured by oxygen consumption and carbon dioxide production (data not shown).

3.1.2 Field rearing experiment: color and survival (entire larval period)

In summary, N fertilization decreased and high density increased the percent of brown morphs when locusts started as green morphs. However, there were no effects of either treatment on the percent of brown morphs with locusts started as brown morphs. High density decreased survival in all conditions. Locusts that started as brown morphs and were reared at low density on unfertilized plots had the highest survival rate (mean: 42%) and N fertilization decreased survival (mean: 27%). However, for locusts that started as green morphs and were reared at low density there was no beneficial effect on survival rate for those reared on unfertilized plots (mean: 32%) as compared to fertilized plots (mean: 35%).

In cages where all individuals were started as green juveniles, there were significant main effects of both N-fertilization, which promoted green morphs (ANOVA $F_{(1, 38)} = 7.52$, $p = 0.009$) and high density, which promoted brown morphs (ANOVA $F_{(1, 38)} = 4.09$, $p = 0.05$), but no significant interaction of fertilization and density (two-factor ANOVA $F_{(1, 38)} = 0.20$, $p = 0.66$) on the percent of brown morphs per cage (Fig. 6A). Comparing the low and high density treatment groups separately using simple main effects analyses showed that, at low density, N fertilization maintained a low percent of brown morphs while locusts fed unfertilized plants exhibited an increase in the percent of brown morphs per cage (ANOVA $F_{(1, 27)} = 9.01$, $p = 0.01$). However, there was no effect of diet treatment (0N vs +N) when locusts were reared at high population density (ANOVA $F_{(1, 10)} = 0.92$, $p = 0.36$).

In cages where all individuals were started as brown juveniles they maintained a high percentage of brown morphs in all cases. There were no significant main effects of either N-fertilization (ANOVA $F_{(1, 44)} < 0.01$, $p = 0.90$) or density (ANOVA $F_{(1, 44)} < 0.01$, $p = 0.93$), nor an interactive effect of these two variables (two-factor ANOVA $F_{(1, 44)} < 0.01$, $p = 0.98$) on the percent of brown morphs per cage (Fig. 6B).

Comparing survival rate in cages where all individuals were started as green juveniles, there was a significant main effect of high density, which decreased survival (ANOVA $F_{(1, 40)} = 11.55$, $p = 0.002$), but no significant main effect of N-fertilization (ANOVA $F_{(1, 40)} = 0.03$, $p = 0.87$) nor a significant interaction of fertilization and density (two-factor ANOVA $F_{(1, 40)} = 0.08$, $p = 0.78$) on percent surviving per cage (Fig. 7A). Comparing the low and high density treatment groups separately using simple main effects analyses revealed no differences between control and N-fertilization treatments at either low density (ANOVA $F_{(1, 28)} = 0.39$, $p = 0.54$) or high density (ANOVA $F_{(1, 11)} = 0.65$, $p = 0.44$).

However, in cages where all individuals were started as brown juveniles, there were significant main effects of N-fertilization (ANOVA $F_{(1, 46)} = 4.62$, $p = 0.04$) and high density (ANOVA $F_{(1, 46)} = 23.57$, $p < 0.001$); both decreased survival but there was no interactive effect of fertilization and density (two-factor ANOVA $F_{(1, 46)} = 1.22$, $p = 0.27$) on percent surviving per cage (Fig. 7B). Comparing the low and high density treatment groups separately using simple main effects analyses showed that, at low density, N fertilization decreased survival rate (ANOVA $F_{(1, 30)} = 4.14$, $p = 0.05$) but there was no significant difference between the fertilization treatment groups when locusts were reared at high density (ANOVA $F_{(1, 16)} = 1.86$, $p = 0.19$).

3.2 Host plant response to locust density and N fertilization

In summary, N fertilization increased N and protein contents in both *Stipa* and *Leymus* but *Leymus* had consistently higher N and protein contents than *Stipa* in all treatments. Higher locust densities increased N content for *Leymus* but not for *Stipa*. *Leymus* had higher alkaloid contents than *Stipa*. While the alkaloid content of *Stipa* did not change in response to any treatments, higher locust densities increased alkaloid content in *Leymus*.

In a statistical model including all treatments (plant species, fertilization treatment, and locust density) there were significant main effects for all three treatments on leaf N content (Fig. 8). *Leymus chinensis* had a higher N content than *Stipa grandis* (ANOVA $F_{(1, 67)} = 50.44$, $p < 0.001$), N fertilization increased leaf N content (ANOVA $F_{(1, 67)} = 244.31$, $p < 0.001$), and high locust density increased plant N content (ANOVA $F_{(2, 67)} = 6.41$, $p = 0.003$). However, there was no significant interaction of plant species, fertilization treatment, and locust density for leaf N content (three-factor ANOVA $F_{(2, 67)} = 0.86$, $p = 0.43$). Separate analyses for *Stipa* and *Leymus* indicated that these species responded differently to fertilization. In *Stipa* (Fig. 8A) there was an overall effect of N fertilization on N content (ANOVA $F_{(1, 34)} = 195.34$, $p < 0.001$) but no overall effect of locust density (ANOVA $F_{(2, 34)} = 2.19$, $p = 0.13$) and no interactive effect between fertilization and density (two factor ANOVA $F_{(2, 34)} = 0.90$, $p = 0.41$). However, in *Leymus* (Fig. 8B), there were overall effects of both N fertilization (ANOVA $F_{(1, 33)} = 88.25$, $p < 0.001$) and locust density (ANOVA $F_{(2, 33)} = 4.18$, $p = 0.02$) but no interactive effect (ANOVA $F_{(2, 33)} = 1.54$, $p = 0.23$).

There were no significant main effects of N fertilization (ANOVA $F_{(1, 67)} = 0.00$, $p = 0.99$), locust density (ANOVA $F_{(2, 67)} = 0.05$, $p = 0.95$), or species on leaf carbon content (ANOVA $F_{(1, 67)} = 1.25$, $p = 0.27$), nor any interactive effect of these three treatments (three factor ANOVA $F_{(2, 67)} = 0.37$, $p = 0.69$). The overall mean for carbon content was 46.23 ± 0.38 SEM (data not shown).

In a model including all treatments (plant species, fertilization treatment, and locust density) there were significant main effects for species and fertilization on leaf protein content (Figs. 8C and 8D). *Leymus chinensis* had a higher protein content than *Stipa grandis* (ANOVA $F_{(1, 58)} = 7.98$, $p = 0.006$), and N fertilization increased leaf protein content (ANOVA $F_{(1, 58)} = 24.80$, $p < 0.001$); there was no overall effect of locust density (ANOVA $F_{(2, 58)} = 0.85$, $p = 0.43$). There was no significant interactive effects of plant species, fertilization treatment, and locust density on leaf protein content (three-factor ANOVA $F_{(2, 58)} = 0.28$, $p = 0.75$). Separate analyses of *Stipa* and *Leymus* indicated that these species responded differently. For *Stipa* (Fig. 8C), there was an overall effect of N fertilization on protein content (ANOVA $F_{(1, 30)} = 14.51$, $p < 0.001$) but no overall effect of locust density (ANOVA $F_{(2, 30)} = 0.95$, $p = 0.40$) and no interaction between fertilization and locust density (two-factor ANOVA $F_{(2, 30)} = 0.40$, $p = 0.67$). For *Leymus* (Fig. 8B), there was also an overall effect of N fertilization on leaf protein content (ANOVA $F_{(1, 28)} = 10.72$, $p = 0.003$) but no overall effect of density (ANOVA $F_{(2, 28)} = 0.59$, $p = 0.56$) and no interactive effect (two-factor ANOVA $F_{(2, 28)} = 0.01$, $p = 0.99$).

In a model including all treatments (plant species, fertilization treatment, and locust density), there were significant main effects of species on leaf alkaloid content (Figs. 8E and 8F). *Leymus* had higher alkaloid contents than *Stipa* (ANOVA $F_{(1, 57)} = 18.87$, $p < 0.001$). However, there were no main effects of N fertilization (ANOVA $F_{(1, 57)} = 2.84$, $p = 0.10$) or locust density (ANOVA $F_{(2, 57)} = 2.29$, $p = 0.11$) nor any interactive effects among plant species, fertilization treatment, and locust density type (three-factor ANOVA $F_{(2, 57)} = 1.04$, $p = 0.36$). Separate analyses of *Stipa* and *Leymus* indicated species-specific responses. For *Stipa* (Fig. 8E), there was no interactive effect between fertilization and density (two factor ANOVA $F_{(2, 29)} = 0.11$, $p = 0.89$) and no main effects of either fertilization (ANOVA $F_{(1, 29)} = 1.18$, $p = 0.29$) or density (ANOVA $F_{(2, 29)} = 0.60$, $p = 0.56$) on alkaloid content. For *Leymus* (Fig. 8F), there was an overall effect of locust density on leaf alkaloid content (ANOVA $F_{(2, 28)} = 5.86$, $p = 0.008$) but no overall effect of fertilization (ANOVA $F_{(1, 28)} = 1.79$, $p = 0.19$) and no interactive effect (two-factor ANOVA $F_{(2, 28)} = 2.04$, $p = 0.15$).

3.3 Preference for Stipa *and* Leymus *grasses under different conditions*

In summary, *O. asiaticus* preferred *Stipa* over *Leymus* and generally preferred plants collected from heavily grazed fields over plants collected from protected fields. When confined to paired choice tests of either *Stipa* or *Leymus* collected from the field cage experiments, there was a much higher mortality and rate of food refusal on *Leymus* than *Stipa*.

Figure 9 displays overall results of field preference tests involving *Stipa* and *Leymus*. For each pair, we assigned a winner and loser if there was a significant difference (represented by W and L in a given line in Fig. 9A) and considered it a tie (represented by T and T in a given row in Fig. 9A) if the difference was not significant. In fresh leaf paired comparisons, *O. asiaticus* preferred *Stipa* over *Leymus* (both collected from a heavily grazed field) ($\chi^2_{(1, N=28)} = 10.29$, $p = 0.001$) and also preferred *Stipa* collected from grazed over ungrazed fields ($\chi^2_{(1, N=11)} = 4.55$, $p = 0.03$). However, the locust had no difference in preference for *Leymus* collected from grazed versus ungrazed plots ($\chi^2_{(1, N=11)} = 0.18$, $p = 0.67$). In dried/ground leaf paired comparisons, *O. asiaticus* preferred *Stipa* collected from grazed over ungrazed fields ($\chi^2_{(1, N=12)} = 6.00$, $p = 0.01$), and *Leymus* collected from grazed over ungrazed plots ($\chi^2_{(1, N=11)} = 8.91$, $p = 0.003$).

There were significant differences in chemical contents among the four plant treatment groups collected from fields either protected or heavily grazed by sheep (*Stipa* + grazing, *Stipa* + no grazing, *Leymus* + grazing, *Leymus* + no grazing). To present a ranked comparison of these four groups alongside locust preference in the paired choice test (Fig. 9A), we used ANOVAs followed by Bonferroni corrected post hoc tests. There were significant differences among the four groups for N content (ANOVA: $F_{(3, 20)} = 14.10$, $p < 0.001$), protein content (ANOVA: $F_{(3, 24)} = 14.39$, $p < 0.001$), and alkaloid content (ANOVA: $F_{(3, 24)} = 7.16$, $p = 0.001$).

We were not able to run statistics for the other paired plant choice experiment using grass collected from the field cage experiment (section 2.2.1), due to insufficient sample size. Our initial sample size was somewhat low and many of the locusts refused to eat and/or died, particularly in the *Leymus* groups. This experiment was still informative, however, because it revealed a striking difference in willingness to eat and mortality between locusts offered *Stipa* and *Leymus* (Fig. 9B). We analyzed these differences using Chi-square contingency tables. Locusts provided with *Leymus* had higher mortality (71%) than those provided with *Stipa* (26%) ($\chi^2_{(1, N=106)} = 21.37$, $p < 0.001$), and a higher proportion refused to eat *Leymus* (65%) than *Stipa* (26%) ($\chi^2_{(1, N=106)} = 16.03$, $p < 0.001$) (Fig. 9B).

4. Discussion

4.1 Summary

In contrast with our original hypothesis that *poor* GS quality host plants promote development of migratory phenotypes, we found that *high* GS quality plants, in conjunction with high locust density promoted development migratory characteristics in *O. asiaticus*. It is important to note that we define high-quality diets as those that improve growth and survival (GS). For *O. asiaticus*, these are relatively low-protein diets. Previously, we showed that heavy livestock grazing promotes outbreaks of *O. asiaticus* by lowering plant N status to a favorable range for the growth and survival of this locust (Cease et al. 2012). Here we show that the combination of low N plants (high GS quality) and high *O. asiaticus* population density—as is found in heavily grazed fields—also promotes development of migratory characteristics (Figs. 3-4). These results suggest that *O. asiaticus*, and perhaps other locusts and insects, fit an alternative hypothesis where access to *high* GS quality food resources may be necessary to support development of a migratory or gregarious phenotype (Fig. 1).

4.2 Does poor or high plant quality promote development of migratory characteristics?

High population density is known as the primary driver for phase change in locusts, but a key unanswered question was if diet quality could directly manipulate this response to density (Fig. 2). Indeed, we found that only *O. asiaticus* reared at high density and fed high GS quality (low-N; Fig. 5) host plants developed the most enhanced migratory characteristics (Figs. 3-4). In contrast, locusts reared at high density but fed poor GS quality (high-N; Fig. 5) host plants had decreased expression of migratory characteristics (Figs. 3-4). Results from our field cage study corroborate these findings. For locusts that started as green, both high density and high GS quality plants (low-N; Fig. 7) promoted a shift to brown morphs (Fig. 6).

As discussed earlier, when determining the role of host plant quality in migratory polyphenism, it is important to define what equates to "low" or "high" food quality. In the lab experiment, locusts reared at high density on unfertilized plants attained the highest adult mass and had the shortest development time (Fig. 5). In contrast, the high-density, high plant-N group had the lowest specific growth rate, longest development time and lowest adult body mass. These results are consistent with the field cage survival rate data (Fig. 7B) and previous research showing that high quality food is relatively low protein food, at least for the brown *O. asiaticus* morphs (Cease et al. 2012). High density, in particular when confined to a cage, may be a stressor (Applebaum and Heifetz 1999), which is why it is hypothesized to induce migratory phenotypes in insects so they can escape to find better resources (Johnson 1969). Indeed, in the field cage study, we found that high density significantly decreased survival (Fig. 7). These results may help explain why animals in the high-density, high plant-N group did so poorly in the lab experiment. High density and high-N plants were likely a dual stressor that decreased overall performance of brown morphs when in combination.

To confirm that N fertilization increased N and protein content in host plants, we measured N, carbon (C), and protein content in the two most common plant species: the perennial bunchgrass *Stipa grandis* and the rhizomatous *Leymus chinensis* (each comprise approximately 40% of the aboveground biomass in the Xilin River basin region where our study took place). *Leymus* is highly palatable for grazing livestock, presumably due to its high nitrogen and protein content, and often used as hay (Zhu, Li, and Yang 1981). However *O. asiaticus* prefers *Stipa* over *Leymus*, and generally over all plants in the community (Cease et al. 2012; Flynn 2011). As predicted, we found that *Leymus* had higher nitrogen and protein contents than *Stipa* under all treatments and N fertilization increased nitrogen and protein content in both *Stipa* and *Leymus* (Fig. 8A-D). Higher densities increased N content of *Leymus* leaves (Fig. 8B). Locust density had no other effects on N or protein content of either grass.

Nitrogen fertilization can change other constituents in grasses that may deter herbivory, such as alkaloids (Bush, Wilkinson, and Schardl 1997; Faeth and Fagan 2002). Our results suggest that alkaloids are not influencing performance or host plant choice in *Stipa*. The alkaloid content of *Stipa* remained low in all grasshopper density and fertilization treatments (Fig. 8E) and in *Stipa* collected from different grazing intensities (Fig. 9A). Our results corroborate those of Wei et al (2006). They sampled a number of grass species across China (including near our field site) and reported a low occurrence of fungal endophytes in *S. grandis* (1%) (Wei et al. 2006) and no grasses have been documented to produce alkaloids without a fungal endophyte (Cheplick and Faeth 2009). In *L. chinensis*, the infection rate was slightly higher (5%). We also found that *Leymus* had higher overall alkaloid levels than *Stipa* (Fig. 8), and that higher locust (Fig. 8F) and livestock (Fig. 9A) densities increased alkaloid content in *Leymus*. This suggests that *Leymus* may use alkaloids as a defense against herbivory and may partially explain *O. asiaticus*' strong aversion to *Leymus* (Fig. 9). However, these data should be interpreted with caution because we used a coarse method that is generally reserved for detecting presence/absence of alkaloids. Alkaloids are diverse and can have different impacts on different organisms (Cheplick and Faeth 2009, and references therein). Therefore, further studies using more specific methods for identifying and quantifying alkaloids are necessary to fully elucidate the influence of *Leymus* or other plant alkaloids on *O. asiaticus* populations.

Fertilization and livestock grazing can change the palatability of *S. grandis* to the locust *O. asiaticus* (Cease et al. 2012; see also Flynn 2011). However, it was unknown how those factors influenced palatability of *L.*

chinensis. We found that, in most cases, *O. asiaticus* selected for the plant with the lowest protein content (Fig. 9A). Although, when given a choice between fresh leaves of *L. chinensis* collected from grazed versus protected fields, locusts showed no preference. Results from our alkaloid assays suggest that sheep grazing increases alkaloid content in *L. chinensis* (but not in *S. grandis*). In this case, *O. asiaticus* may have balanced between two negatives—high protein in protected fields and high alkaloids in grazed fields—resulting in no observed preference. However, when given the same paired choice but with dried/ground leaves to control for leaf toughness and water content, locusts chose *L. chinensis* collected from grazed fields over *L. chinensis* collected from protected fields. This indicates that water content and/or leaf toughness may have modulated host plant selection in fresh leaves. For example, Flynn (2011) found that *L. chinensis* leaves in grazed pastures had higher silica contents than those in protected pastures (Flynn 2011).

It was also unknown how *O. asiaticus* herbivore intensity influenced palatability of grasses to conspecifics. Using dried/ground leaves collected from the locust cages used in earlier experiments (i.e. Fig. 8), we attempted a similar paired comparison, pitting *S. grandis* leaves collected from each of the treatment groups (low or high density and unfertilized or N-fertilized plants) against each other (six paired comparisons). We repeated the design using dried/ground *L. chinesis* leaves (six paired comparisons). We did not compare *Stipa* and *Leymus* leaves against each other because extensive tests had already demonstrated that *Stipa* is strongly preferred over *Leymus* by *O. asiaticus* (Figs. 8A, and Cease et al. 2012; Flynn 2011). Unfortunately, due to unexpected mortality we did not have enough trials from each pair to run statistical comparisons for plant preference. However, this experiment did yield useful information about the willingness of *O. asiaticus* to try dried/ground *Stipa* and *Leymus* leaves and their survival rate when confined to these two options. In contrast to paradigms for N-limitation by herbivores, *O. asiaticus* had a greatly reduced survival rate when confined to high-N *Leymus* leaves as compared to low-N *Stipa* leaves (Fig. 9B). Since locusts were collected from the field and transferred directly to experimental cages, we would predict some level of food refusal due to a novel environment and novel form of plants (dried/ground). Indeed, 26% of locusts refused to eat their preferred host plant, *Stipa*, under these circumstances; all locusts that did not eat died. In contrast, locusts confined to pairs of *Leymus* refused to eat in 65% of the trials and 71% died.

Nevertheless, these data reinforce the conclusion that herbivores are not always N-limited and that different acridid species often have different optimal nutritional profiles for which they select in their habitats (e.g., Joern and Behmer 1998; Behmer and Joern 2008). For *O. asiaticus* and *Stipa*, there is strong evidence that balancing protein intake with other nutrients (likely carbohydrate) is a primary driver for food selection (Cease et al. 2012). Indeed, protein and carbohydrate are the most strongly regulated nutrient groups in insects (Simpson and Raubenheimer 2000) and studies using the geometric framework and ecological stoichiometry approaches have shown that balancing multiple nutrients shapes the decisions of numerous organisms (Raubenheimer, Simpson, and Mayntz 2009; Sterner and Elser 2002). Across *Leymus* treatments, our data suggest that other factors also influence host plant choice of *O. asiaticus*, such as alkaloid content (Fig. 8F) and/or silica content (Flynn 2011). However, *L. chinensis* is highly palatable for sheep (Zhu, Li, and Yang 1981) and is preferred over *S. grandis* for several Inner Mongolian acridid species (Cease and Hao, unpublished data), suggesting that it does not contain toxins or silica to the extent that it is inherently unpalatable. Behmer et al (2002) showed that secondary compounds may only deter feeding by herbivores under certain dietary conditions, indicating complex interactions among nutrition, toxicity, and feeding (Behmer, Simpson, and Raubenheimer 2002).

4.3 Shape of a migratory insect

To answer the question of how diet affects development of migratory phenotypes, we first need to understand what a migratory morph looks like. We found that rearing *O. asiaticus* at high density on low-N grass resulted in morphs that had features characteristic of other migratory insects and similar to characteristics exhibited by brown *O. asiaticus* morphs collected from field populations (Figs. 3-4). For this species, outbreaks and migratory swarms are comprised predominantly of brown morphs, and we previously showed that brown morphs have higher metabolic rates, higher activity levels, and a greater relative investment in thorax and hind legs as compared to green morphs (Cease et al. 2010). This change in body shape is found in other insects. For example, long-winged morphs of the common water strider (*Gerris remigis*) had larger thoraxes but smaller abdomens than short winged morphs (Fairbairn 1992). Roff and Bradford (1998) compared shape differences among long-winged and short winged crickets (*Allonemobius socius*) and found that long-winged crickets had larger femurs and wider rear pronotums than short winged crickets (but narrower front pronotums) (Roff and Bradford 1998). The shape differences between the two morphs of the cricket *A. socius* are similar to those we document for *O. asiaticus*, which also had increased hind leg size in the brown, migratory morphs and in insects reared at high density (Fig. 4 and Cease et al. 2010).

Although we have not tested their functional significance, larger hind legs may be important for jumping into flight and/or escaping predation (Bennet-Clark 1990). Another, but not mutually exclusive, hypothesis is also possible —a "cannibalism avoidance" hypothesis—where larger hind legs better enable locusts and crickets to repel conspecifics and thereby avoid cannibalism under crowded conditions. Cannibalism often occurs in crowded populations of orthopterans (Hansen et al. 2011; Simpson et al. 2006)—and is, in fact, a mechanism that promotes collective movement (Bazazi et al. 2008). Indeed, Simpson et al (2006) observed that immobilized Mormon crickets required hind legs to defend themselves from conspecifics by kicking, and thereby avoiding cannibalism (Simpson et al. 2006). However, to our knowledge the effects of rearing density on leg size or the relationship between leg size and kicking ability have not been investigated in orthopterans. Interestingly, there was an interaction between density and plant fertilization such that the locust group reared at low density and on high-N plants had a higher migratory index value than the locust group reared at high density and on high-N plants (Fig. 4). Further experiments are needed to elucidate the functional significance of this pattern and if it would hold for other comparisons of other phase characteristics. For example, this pattern did not hold for comparing migratory capacity based on wing loading (Fig. 3).

4.4 Color of a migratory insect

In several locusts and several other insects, gregarious, migratory nymphs (and sometimes adults) exhibit dark coloration relative to solitarious, non-migratory nymphs (e.g., *S. gregaria* and *L. migratoria*, but not *C. terminifera*). In our field cage studies, a small percentage of locusts that were collected as brown changed to green but there no differences among any of the treatment groups. Locusts that started as green, however, were equally influenced to change to brown by high density and unfertilized grass (Fig. 6). These results suggest that color is, at least in part, a phase characteristic because brown coloration is associated with enhanced migratory characteristics (Cease et al. 2010) and high density promotes development of green morphs into brown morphs (Fig. 6A). These results also suggest that *O. asiaticus* is more prone to develop into brown morphs, and once a brown morph, there is more resistance to changing to a green morph. Results from the lab study (Figs. 3-4) showed that body shape can change over the course of a single juvenile instar in brown morphs (while color did not change), suggesting that body shape can begin shifting towards a non-migratory, or perhaps solitarious, individual prior to the color shift from brown to green.

4.5 Metabolic rate was not affected over one instar

Metabolic rate is often higher in migratory insects. For example, respiration rates have are higher in gregarious *L. migratoria* (Butler and Innes 1936) and *S. gregaria* (Heifetz 1997) (reviewed in Pener and Simpson 2009) as compared to solitarious morphs and higher in field-caught brown *O. asiaticus* final-instar nymphs than green nymphs (Cease et al. 2010). Therefore, we predicted that high density would increase metabolic rate in *O. asiaticus* but we did not find any significant differences among the different treatment groups in O_2 consumption or CO_2 production. Perhaps this was because this experiment was only conducted over one instar and we measured respiration at ¾ of the way through this final juvenile instar. Higher resting metabolic rates in flying insects have been attributed to elevated maintenance cost of larger flight muscles (Nespolo, Roff, and Fairbairn 2008), thus perhaps a longer-term experiment would allow for greater differences in flight muscle development. Further studies considering the effect of diet and density on respiration rates across the entire developmental cycle are needed for this species.

4.6 Does overgrazing promote formation of the migratory phenotype?

Heavy livestock grazing in Inner Mongolia can result in low-N plants that promote growth and survival of *O. asiaticus* (Cease et al. 2012). These increases in growth and survival likely translate to high density locust populations with access to nutritionally-favorable, low-N food (Fig. 9A). This combination of high density and a low protein:carbohydrate ratio diet produced locusts with the most enhanced migratory morphology in lab studies (Figs. 3-4) and shifted the most green morphs to brown morphs in field cage studies (Fig. 6A). Therefore, it is likely that heavy livestock grazing promotes *O. asiaticus* outbreaks and, as locust density moves closer to reaching the carrying capacity, the combination of high density and high quality food promote development of migratory phenotypes.

Table 1. Correlation matrix of mass allocation to different body components extracted in Table 2, and shown in Figure 2A. (†p<0.10, **p<0.01, ***p<0.001)

variable	head mass (Z)	hind legs mass (Z)	abdomen mass (Z)	thorax mass (Z)	wings mass (Z)
head mass (Z)	--	0.32†	0.29†	-0.07	0.11
hind legs mass (Z)	0.32†	--	0.51**	0.64***	0.81***
abdomen mass (Z)	0.29†	0.51**	--	0.63***	0.60***
thorax mass (Z)	-0.07	0.64***	0.63***	--	0.82***
wings mass (Z)	0.11	0.81***	0.60***	0.82***	--

Table 2. Factor loadings for mass allocation to different body components using a maximum likelihood extraction.

variable	factor loadings
head mass (Z)	0.13
hind legs mass (Z)	0.82
abdomen mass (Z)	0.63
thorax mass (Z)	0.84
wings mass (Z)	0.98
Eigenvalue	2.7
% of total variance	55

Table 3. Scale for scoring alkaloid assay for ranking amount of precipitate formed (opacity) in response to Dragendorff reagent. The middle two columns are the amount of nicotine (ug) sufficient in 800uL of solution to elicit the response in the final column. See methods section 2.3 for a detailed description.

Alkaloid score	min (ug)	max (ug)	response
0	0.00	0.48	indistinguishable from blank control tubes
1	0.71	4.75	slight color change or cloudiness
2	4.99	11.64	cloudy, but can read all letters through the tube easily
3	11.88	21.38	cloudy, can read large letters
4	21.61	32.06	cloudy, can barely see shapes through tube
5	32.06		completely opaque

Table 4. Percent of alkaloid sufficient to elicit each alkaloid score. See methods section 2.3 for a detailed description.

Alkaloid score	min (%)	max (%)	avg (%)
0	0.00	0.00	0.00
1	0.00	0.03	0.02
2	0.03	0.07	0.05
3	0.07	0.12	0.09
4	0.12	0.18	0.15
5	0.18	+	0.21+

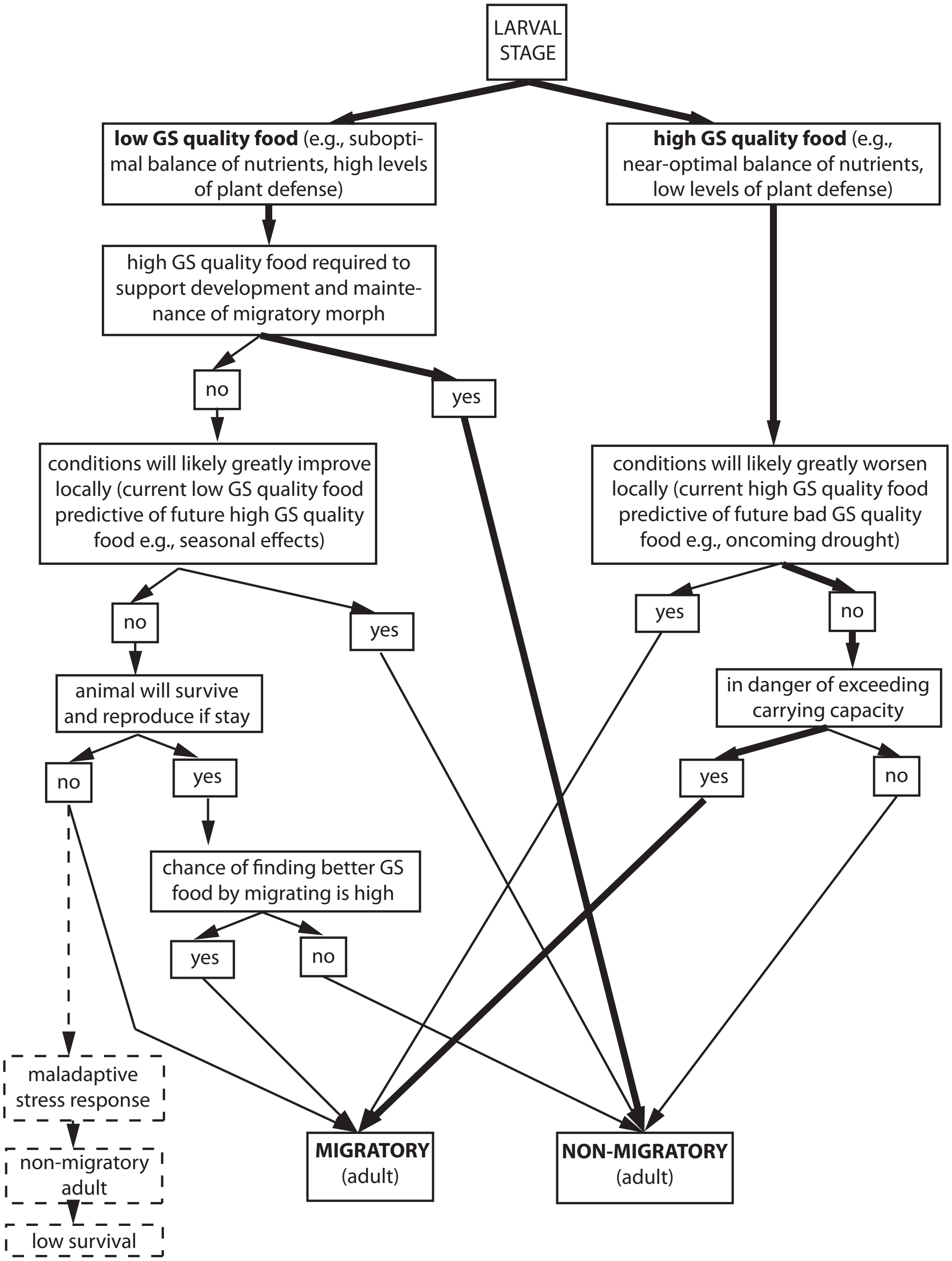


Figure 1. Qualitative model illustrating how low or high GS food quality may affect development of migratory phenotypes. Bolded arrows represent that pathways that may be occuring in O. asiaticus.

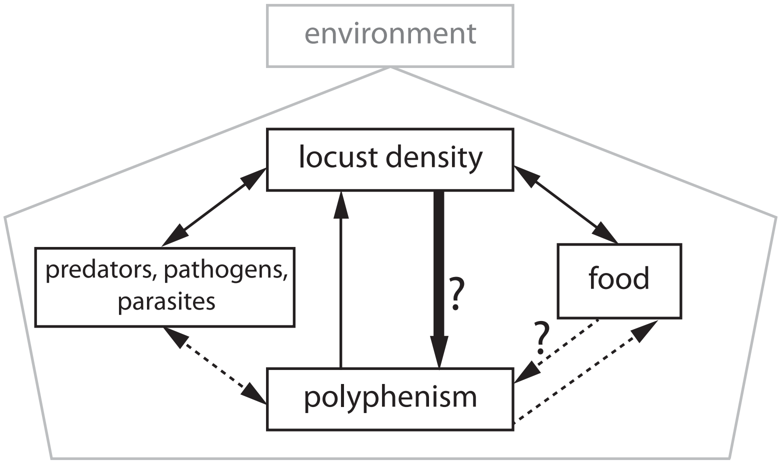


Figure 2. Factors that regulate locust density and polyphenism. Question marks are next to arrows that we tested in this study. Solid arrows represent well-studied interactions; the effects of density on polyphenism (bolded arrow) are particularly well-studied. Dashed arrows represent interactions where very few or no studies have been done. There are likely complex effects of abiotic environmental factors on each of these interactions.

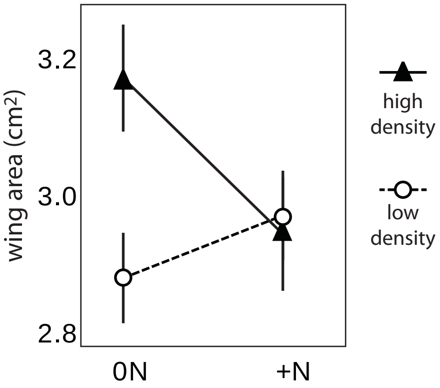


Figure 3. High density and low host plant N content increased wing area. Locusts were reared in lab incubators for the final larval instar at either high or low density and fed either control or N-fertilized grass. We measured area of the hind wing and analyzed differences using mass as a covariate. Thus, higher values represent lower wing loading and likely greater flight capacity.

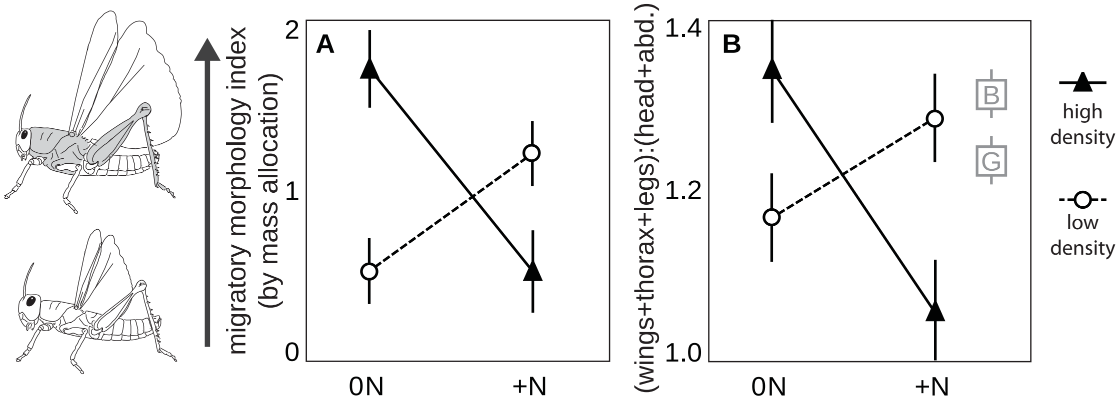


Figure 4. Locusts reared at high density and fed low-N host plants had the highest migratory index. We calculated this index by combining the mass variables for five body components into one linear variable using a maximum likelihood factor analysis (see methods for details). Higher values in A represent increased mass allocation to the thorax, wings, and hind legs, as represented by shaded portions in the locust illustrations, and as illustrated in B. For comparison, field-caught brown (“migratory”) morphs have higher relative mass allocation to the thorax, wings, and hind legs as compared to green (“non-migratory”) morphs (B, inset).

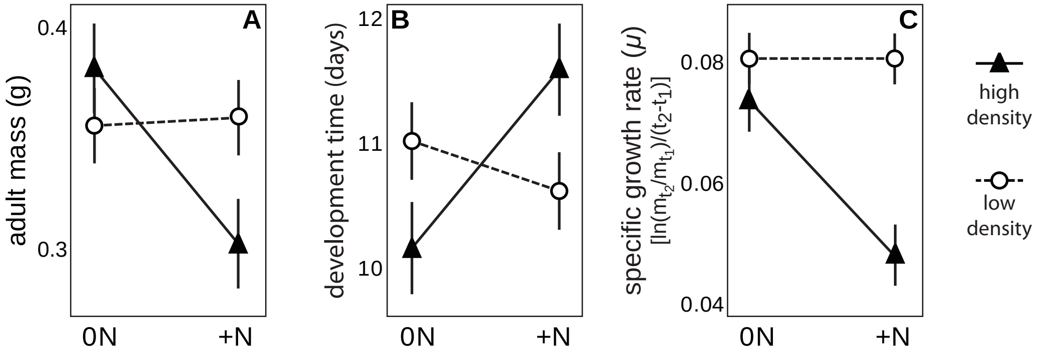


Figure 5. Adult mass (A), development time (B), and specific growth rate (C) in locusts from the lab rearing study. Locusts reared at high density and fed low-N plants had the heaviest adult mass, shortest development time, and among the highest specific growth rates. Conversely, locusts reared at high density and fed high-N plants had the worst performance in each of those categories. There were no differences between locusts reared at low density and fed low-N or high-N plants.

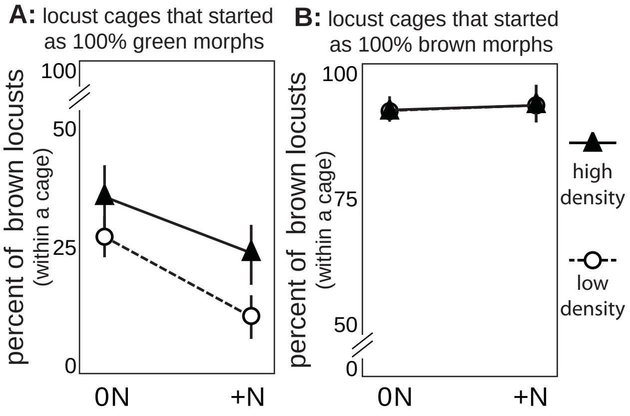


Figure 6. Color change in locusts reared in field cages. High density and low-N plants increased the percent of brown morphs in cages where all locusts started as green morphs (A). There were no effects of diet or density on the percent of brown morphs in cages where all locusts started as brown morphs (B). These results indicate that color can be influenced by both diet and density, but that brown morphs are resistant to changing to green morphs.

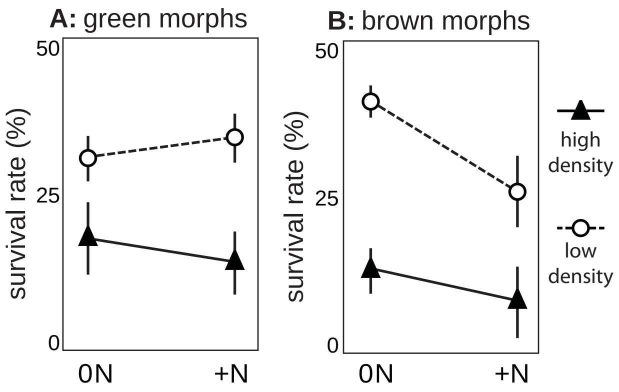


Figure 7. Survival rate in locusts reared in field cages. N fertilization decreased survival rate in brown morphs (B), but had no effect on green morphs (A). Density decreased survival in all cases.

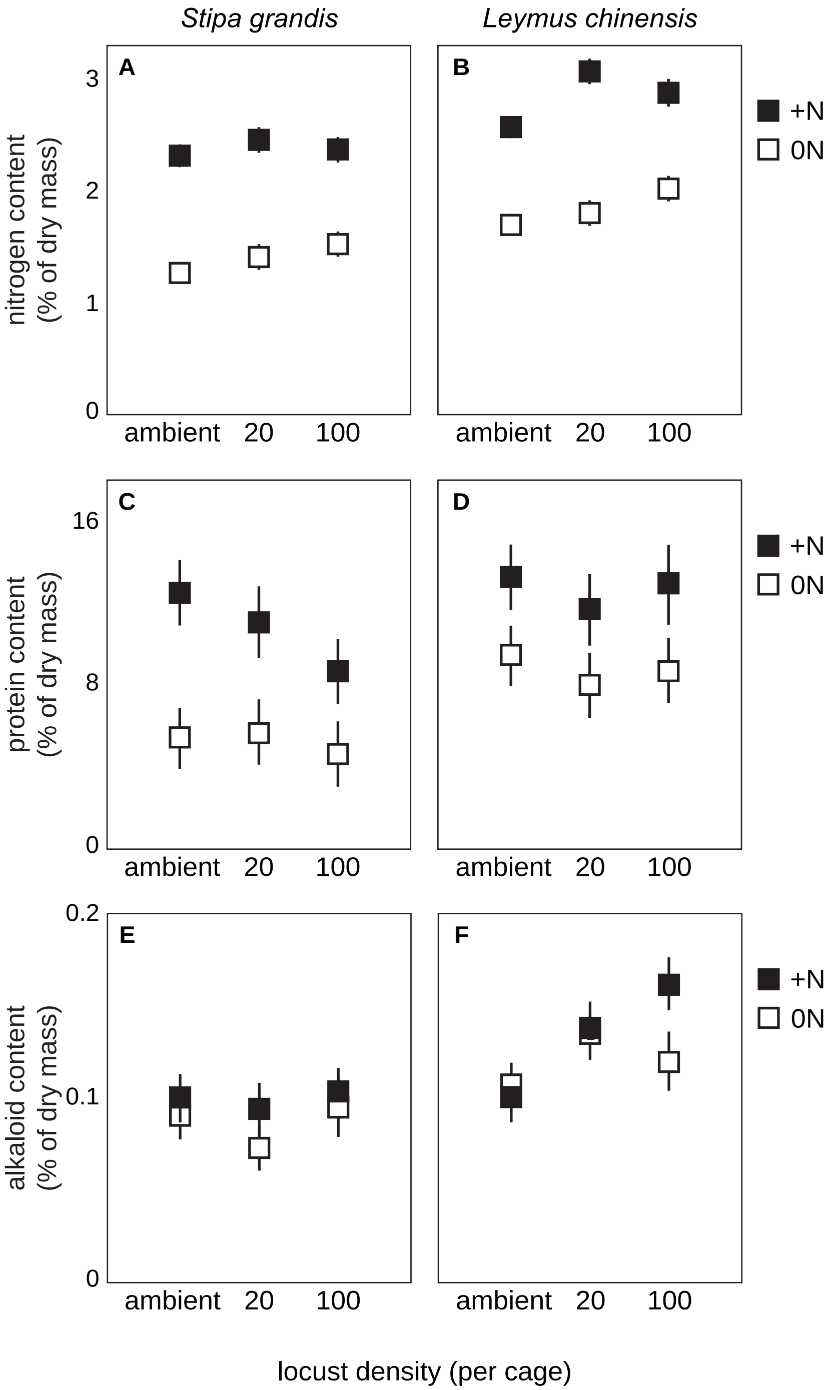
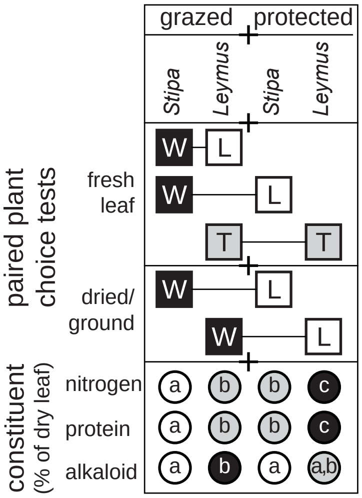


Figure 8. Nitrogen, protein, and alkaloid content in Stipa grandis and Leymus chinensis grasses collected from locust cages. N fertilization increased nitrogen and protein content in all cases. Higher locust densities increased N content in Leymus, but not Stipa. Alkaloid content was higher in Leymus, and increased with increasing locust density. Alkaloid levels remained low in all cases for Stipa.

A: leaves collected from livestock pastures



B: leaves collected from locust cages

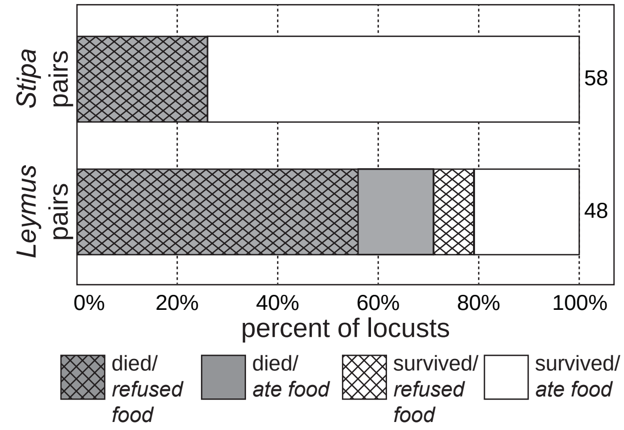


Figure 9. Paired plant preference trials. Locusts were given pairs of either fresh leaves or dried/ground leaves collected from either grazed or ungrazed fields (A) or from the locust cages (B). In pairs where there was a significant difference in preference, the preferred plant (or winner) is denoted with a “W” and the loser with an “L.” Ties are indicated with “T.” In general, locusts preferred plants lowest in protein. Significant post hoc differences within each constituent are indicated by lowercase letters and corresponding shading of each bubble (A). When confined to pairs of Leymus, they had a higher mortality rate and rate of refusal than when confined to pairs of Stipa (B).

Chapter 5

SUMMARY

Locust phase polyphenism—the impressive ability some grasshoppers exhibit, switching from gregarious and migratory to solitary and sedentary morphs—has captivated human interest for more than 2,000 years. While many residents of the U.S. and Europe think of locust plagues as a historical reference, locust outbreaks continue to cause substantial agricultural problems today throughout much of Africa, Asia, and Australia. These problems are compounded in developing countries with lack of transportation infrastructure, access to education, and many other factors, that contribute to food insecurity and famine. As a Peace Corps Volunteer in West Africa, I lived and worked in a small rural community of subsistence farmers and gained insight as to the reality of locust outbreaks in that region. This is was a critical event that has shaped my career path. Although great advancements have been made in locust research since phase polyphenism was first described in 1921, there are many unanswered questions. In particular, there are limited comparative studies that include non-model locusts (i.e. not *Locusta migratoria* or *Schistocerca gregaria*) in developing regions of the world. These species, such as *Oedaleus asiaticus* and *O. senegalensis*, may not produce migratory swarms to the same magnitude, but their chronic annual persistence may have greater overall impacts on agriculture and local ecology. Another important area of research that is limited is how humans and locusts interact, and how anthropogenic impacts on plant communities influence locust outbreaks. As is the case for the locust I studied for my dissertation, land overuse and degradation may be at the heart of many locust swarms.

My research is focused on field ecosystem and community ecology; and organismal nutrition, development, and plasticity at the intersection of plant responses to land management. Agricultural land management practices alter plant communities, which changes insect herbivore growth, behavior, and reproduction with consequent changes in population dynamics, leading to outbreaks. To develop sustainable pest and agricultural land management strategies we must understand the effects of various land management strategies on plant-insect interactions. In my dissertation research in the Chinese grasslands, I found that heavy livestock grazing promotes outbreaks of the locust Oedaleus asiaticus by lowering plant nitrogen (N) content. Heavy livestock grazing enhances erosion and loss of soil N, leading to degraded pastures and low-N plants—a vulnerable state where a locust outbreak could exacerbate degradation. Contrary to paradigms that terrestrial communities are often N-limited, this locust prefers and has the best survival on low-N plants and low-protein artificial diets. Further, I found that a combination of low-N host plants and high density promotes development of migratory characteristics in this locust. Taken together, these data have advanced our understanding of the nutritional influences on this impressive plasticity exhibited by locusts and have shown how land management decisions can modulate locust response through changes in plant nutrient status.

Moving forward, I plan to expand my research in two directions: one is to better understand the mechanisms by which varying host-plant nitrogen is affecting locust outbreaks and plasticity; the second is to incorporate mechanisms of locust outbreaks and plasticity into coupled socioeconomic-ecological models that can be used to guide policy.

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APPENDIX A

CHAPTER 2: ARE COLOR OR HIGH REARING DENSITY RELATED TO MIGRATORY POLYPHENISM IN THE BAND-WINGED GRASSHOPPER, OEDALEUS ASIATICUS? (PUBLISHED MANUSCRIPT)

Journal of Insect Physiology 56 (2010) 926–936

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Are color or high rearing density related to migratory polyphenism in the band-winged grasshopper, *Oedaleus asiaticus*?

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ARTICLE INFO

Article history:
Received 13 March 2010
Received in revised form 21 May 2010
Accepted 24 May 2010

Keywords:
Migratory polyphenism
Density
Insect
Locust

ABSTRACT

Locusts represent an impressive example of migratory polyphenism, with high densities triggering a switch from a solitarious, shorter dispersal range, and sometimes greenish phenotype to a gregarious and sometimes darker form exhibiting behavioral, morphological and physiological traits associated with long-distance migratory swarms. While such polyphenism has been well documented in *Locusta migratoria* and *Schistocerca gregaria*, the extent to which other grasshoppers exhibit this type of migratory polyphenism is unclear. Anecdotally, the Chinese grasshopper, *Oedaleus asiaticus*, forms migratory swarms comprised mostly of a darker, brown-colored morph, but also exhibits a non-migratory green-colored morph that predominates at low densities. In a population in Inner Mongolia not currently exhibiting migratory swarms, we found that while green and brown *O. asiaticus* are found concurrently across our sampled range, only brown grasshoppers were found in high densities. Differences between field-collected brown and green forms matched some but not key predictions associated with the hypothesis that the brown form is morphologically and physiologically specialized for gregarious migration. Controlling for body mass, brown forms had more massive thoraxes, abdomens and legs, and higher metabolic rates, but not more flight muscle or lipid stores. Further, the brown and green grasshoppers did not differ in gregarious behavior, and neither would fly in multiple lab and field trials. Lab or field-rearing at high densities for one-to-multiple juvenile instars caused grasshoppers to exhibit some morphological traits predicted to benefit migration (larger wings and a shift in relative mass from abdomen to thorax), but did not change color or induce flight behavior. One hypothesis to explain these data is that a migratory form of *O. asiaticus* is partially triggered by high field densities, but that existing ecological conditions blocked full expression of such traits (and outbreak swarms). Alternatively, color variation in this species may more tightly linked to other functions in this species such as crypsis or disease resistance, and mechanisms other than late-juvenile rearing density (e.g. genetic variation, maternal effects) may be more critical for promoting variation in color and/or migratory polyphenism.

1. Introduction

In a changing world, developmental plasticity (polyphenism) is critical to enable a single genome to produce alternative morphologies in response to environmental cues (reviewed in Via et al., 1995). One of the most common and fundamentally important alternative phenotypes in animals may be an increased migratory or dispersal capacity, often including increased flight muscles and wing size (Dingle, 1985; Harrison, 1980). One common cue that induces an increased migratory capacity in insects is higher population density (Applebaum and Heifetz, 1999). High population density may increase intraspecific competition and lead to deteriorating local food availability, providing an advantage for migratory behavior (Roffey and Popov, 1968; Uvarov, 1977). In this study, we tested for density-regulated or color-associated migratory characteristics in *Oedaleus asiaticus*, a common band-winged grasshopper from the Inner Mongolia grasslands of China.

Enhanced migratory capacity in macropterous (long-winged) insects is often associated with relatively large wing areas, well-developed flight muscles and increased long-distance flight endurance (Dingle, 1985; Rankin and Burchsted, 1992). Increased lipid stores are also observed, though these can require an increase in metabolic rate during flight due to both added mass and increased drag (Cenedella, 1971; Dudley, 1995). Such variation

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doi:10.1016/j.jinsphys.2010.05.020

between migratory and non-migratory forms has been demonstrated in a diverse array of insects including the milkweed bug *Oncopeltus fasciatus* (Dingle, 1968), the African armyworm moth *Spodoptera exempta* (Parker and Gatehouse, 1985), and the grasshopper *Melanoplus sanguinipes* (McAnelly and Rankin, 1986). Migratory forms of insects often exhibit reduced or delayed reproduction, suggesting that investment in locomotory structures and energy stores has significant costs (Roff and Fairbairn, 2007). Therefore, there are likely to be strong ecological effects on fitness of migratory and non-migratory forms, as well as trade-offs in fitness between these forms, that maintain intra-specific variation in the plasticity of migratory phenotypes (Southwood, 1977).

High density often but not always enhances morphological and physiological characteristics associated with migratory capacity. For example, in both the planthopper *Nilaparvata lugens* (Morooka et al., 1988) and the lygaeid bug *Cavelerius saccharivorus* (Fujisaki, 1989), high density increases the frequency of macropterous as opposed to brachypterous adults. In addition, high density increases dark coloration in *N. lugens* and the body length and mesothorax development in *C. saccharivorus*. Larvae of the African armyworm *S. exempta* heighten food consumption, respiratory rate, and fat content in response to high density (reviewed in Applebaum and Heifetz, 1999). In contrast, no changes in flight behavior or morphology was observed in response to density in the noctuid, *Agrotis ipsilon* (Sappington and Showers, 1992).

Grasshoppers (family: Acrididae) are well known to exhibit phenotypes that vary continuously from gregarious and migratory to solitary and non-migratory (Uvarov, 1921, 1966, 1977). Locust phase polyphenism is best documented in *Locusta migratoria* and *Schistocerca gregaria* where it is directed primarily by larval density (Ellis, 1963; Ellis and Pearce, 1962), but development of phenotypes can also be influenced by other environmental cues and maternal factors (Miller et al., 2008). In *L. migratoria* and *S. americana*, migratory and non-migratory forms of these species can differ in color, behavior, physiology, and morphology during some developmental stages (reviewed in Pener and Simpson, 2009; Pener and Yerushalmi, 1998). Migratory locusts tend to have increased lipid stores for long-distance flight, elevated resting metabolic rate (perhaps due to the elevated maintenance cost of larger fight muscles) and a delayed onset of reproduction (Butler and Innes, 1936; Pener et al., 1997; reviewed in Pener and Yerushalmi, 1998). Newly emerged gregarious (migratory) female *S. gregaria* have relatively larger fat bodies (Schneider and Dorn, 1994). However, solitarious (non-migratory) locusts tend to be larger in body size due to an extra juvenile molt, and thus have an increased average wing length, thorax width, and hind legs by direct comparison (Uvarov, 1966). In *L. migratora* adults and *S. gregaria* juveniles, non-migratory, solitary forms tend to be green, blending in with the vegetation, while migratory forms tend to be conspicuous black or yellow (Pener and Simpson, 2009).

The scant comparative work available suggests that the magnitude of migratory polyphenism varies in grasshopper species, and that there is substantial variation in the degree to which behavior and morphology of grasshoppers is density-dependent (reviewed in Pener and Simpson, 2009). *S. americana*, a North American species that is closely related to *S. gregaria*, expresses locust-like density-dependent changes in behavior (Sword, 2003). Gaines (1989) demonstrated that short-winged adults of *Phoetaliotes nebrascensis* produced long-winged offspring when reared at high but not low densities. The northern Israel grasshopper, *Aiolopus thalassinus*, responds to high population density by increased activity level, hemolymph lipid and carbohydrates, metabolic rate, and food consumption, but without any changes in morphology or coloration (Heifetz and Applebaum, 1995). In sum, these studies suggest that the model documented for *S. gregaria* and *L. migratoria* in which high density promotes plastic formation of a migratory morph likely applies to a wide range of species, but that the suite of correlated traits varies interspecifically.

Key to enhancing migratory theory is understanding characteristics that are correlated with migratory phenotypes and the environmental triggers which may enhance outbreak and migratory phenotypes (Johnson, 1969). We tested the hypotheses that migratory characteristics of *O. asiaticus* are enhanced by population density and associated with color as documented in some other grasshopper species. We studied a population of *O. asiaticus* prone to frequent outbreaks that occasionally develop into migratory swarms in Inner Mongolia, as migrating swarms have had significant socioeconomic impacts throughout Asia for more than a century (Kang et al., 2007; Kang and Zhang, 1996). Anecdotally, these outbreaks and subsequent swarms are comprised predominantly of a brown-colored morph (S. Hao and L. Kang, pers. comm.; Jiang et al., 2003). Color phenotypes are continuous from green to brown in this species. Despite numerous attempts, we have been unable to rear *O. asiaticus* in the laboratory for multiple generations, so it is unknown whether color is genetically or maternally related. Anecdotal observations indicate that color can change at molt for many individuals, suggesting that color might be sensitive to density as in *S. gregaria* and *L. migratoria* (Pener and Simpson, 2009).

First, we surveyed field populations to document general patterns of density and color in field populations of *O. asiaticus*. Second, we collected green and brown females from the same field population and measured multiple morphological, physiological, and behavioral traits that have been shown to differ among migratory and non-migratory morphs of *L. migratoria* and *S. americana*. Third, we experimentally manipulated population density in the lab (during the final juvenile instar) and field (from the first instar) and recorded the degree of plasticity in migratory traits in females. We focused our studies on females as they are predicted to have the greatest degree of trade-offs between migratory capacity and reproduction, and thus, we would expect more detectable differences between migratory and non-migratory females as compared to males (oogenesis-flight syndrome; Johnson, 1969).

2. Materials and methods

2.1. Field site

Oedaleus asiaticus were collected from a field population near the Inner Mongolia Grassland Ecosystem Research Station in the Xilin River Basin, Inner Mongolia Autonomous region, China (43°38′N, 116°42′E). This station is a research facility of the Institute of Botany, Chinese Academy of Sciences (Bai et al., 2004; Wang et al., 2005). Inner Mongolia is representative of much of the Eurasian Steppe region floristically and ecologically (Li et al., 1988; Wu and Loucks, 1992). This typical steppe is characterized by dark chestnut soil with relatively homogeneous physiochemical properties and dominated by *Leymus chinensis*. The mean annual precipitation and temperature in the study area are 345 mm and 1.1 °C, respectively (Chen and Wang, 2000). *Oedaleus asiaticus* begins hatching from egg pods deposited in the ground in the beginning of June, goes through five juvenile instars and then molts into an adult in mid-July. Adults live for 4–9 weeks (Li et al., 1987).

2.2. Experiments

2.2.1. Field patterns in color

To document patterns of density and color in field populations, we surveyed two 750 m transects in two fields that were known to have high density of this species (Transect 1: 43°32′N, 116°32′E,

Fig. 1. Brown and green *Oedaleus asiaticus* females collected from the field population near the Inner Mongolia Grassland Ecosystem Research Station. Populations appear to have a bimodal distribution of brown and green phenotypes, but there is a continuum of grasshoppers expressing green to brown coloration. For our studies, we selected grasshoppers from either end of the color extremes.

July 8, 2009 and Transect 2: 43°37′N, 116°44′E, August 7, 2009. To reduce bias in selection of each sampling site, we sampled at regular intervals along each transect: 75 m (transect 1) and 250 m (transect 2). We used a global positioning unit (Garmin: GPSmap 60CSx; 6.4 m (±3 m) accuracy) to measure distance between sampling points. We collected grasshoppers using a standardized sweep net method of one sweep per step for at total of 10 sweeps at each sampling point. The three people involved in collecting were rotated to minimize collection bias.

Grasshoppers were classified as either green or brown. These green and brown grasshoppers were visually apparent (Fig. 1). While some *O. asitiaticus* are a mixture of both green and brown color components, there were no completely ambiguous grasshoppers collected in samples during the field survey for density patterns and thus we classified them based on their strongest color. For the following experiments, we selected only those at the extreme of either color scale.

2.2.2. *Differences between field-caught green and brown females in adult morphology*

If brown *O. asiaticus* are a migratory phenotype, we predicted that newly emerged adult female brown grasshoppers would have increased lipid stores, relative thorax, wing, and hind leg investment, and increased flight and hind leg muscle mass compared to green grasshoppers. To minimize the time grasshoppers spent in the lab but to ensure that we were comparing animals at the same developmental stage, we collected late 5th instar grasshoppers from the field (July 18, 2008). Grasshoppers were then kept in outdoor cages placed over *Leymus*-dominated plant communities at either 10 (green) or 30 (brown) individuals m^{-2} for approximately 1–2 days until the molt to adult occurred. The field density at that time was approximately 30 *O. asiaticus* m^{-2}, thus we kept brown grasshoppers at the same high density and reduced the density of green grasshoppers. Individuals were collected within 24 h of their molt to the adult stage, and were isolated for 2 days with *Leymus* grass in lab cages until sacrificed or used in behavioral trials. Grasshoppers were kept in cages for 2 days to ensure their cuticle was hardened prior to taking morphological measurements. One subset of animals was weighed to the nearest 1 mg and then frozen at −20 °C. Morphometrics, muscle, and lipid mass were all estimated for this first subset.

2.2.3. *Density and color effects on gregarious adult female behavior*

If *O. asiaticus* exhibit locust phase polymorphism similar to *L. migratoria* and *S. gregaria*, then we would expect high density to increase gregarious behavior (defined in this study as increased activity level and time spent near to a stimulus group). If brown *O. asiaticus* are a migratory phenotype, then we would further predict that brown animals would be more prone to exhibit gregarious behavior than green animals. Fifty green and 50 brown adult females were transported at approximately 25 grasshoppers per 60 cm × 30 cm × 30 cm cage to the Institute of Zoology, Chinese Academy of Sciences, Beijing for behavioral trials; the drive took about 9 h. Because the level of density can alter behavioral phase in *S. gregaria* within 4 h (Bouaichi et al., 1995), once at the institute, brown grasshoppers were kept at high density; while green grasshoppers were transferred to solitary chambers for 24 h before the behavioral testing. All grasshoppers were fed wheat seedlings and wheat bran, *ad libitum*, during those 24 h. We predicted that brown grasshoppers kept at high densities would be more prone to exhibit gregarious behavior than green forms kept at low densities. Note that this experimental design does not allow us to separate the effects of density and color on gregarious behavior.

2.2.4. *Differences in field-caught green and brown juvenile females in gas exchange rates*

Insect migratory phenotypes often have an elevated resting metabolic rate (Candy, 1985). This has been attributed, in part, to the metabolic cost for maintaining larger flight muscles (Rankin and Burchsted, 1992), the development of which can be detected during the final juvenile instar (Ready and Josephson, 2005). Therefore, based on our hypothesis that brown *O. asiaticus* exhibit traits that increase their migratory capacity, we predicted that brown forms would have higher rates of gas exchange in the terminal juvenile instar.

We collected mid-5th instar *O. asiaticus* from the field population (July 12, 2008). Grasshoppers were kept in indoor wire mesh cages with *Leymus* grass overnight; oxygen consumption and carbon dioxide production were then measured the following day between 10 AM and 4 PM using closed system respirometry (see technical methods for description). Pairs of one green and one brown grasshopper were matched for size so there was no difference in average mass between green and brown grasshoppers. To determine if there was a difference between green and brown grasshoppers in movement while in the metabolic chambers that could lead to differences in metabolic rate, we quantified movement patterns for seven green and seven black grasshoppers, counting all changes in location within the respirometer, as well as jumps.

2.2.5. *Effects of color and density on adult flight capacity*

We predicted that brown grasshoppers and grasshoppers reared at high density would have a greater flight capacity (long-distance flight endurance) as compared to green grasshoppers and those reared at low density. We tested flight capacity using two methods: a flight mill (see technical methods for description of mills) and outdoor free flying. For both, grasshoppers (males and females) were tested from 5 AM to 9 PM and at an approximate temperature range of 20–38 °C. Because the grasshoppers tended not to fly when we tested them, we attempted flight trials using the flight mills with multiple protocols based on migratory patterns in other acridids (Farrow, 1990). In some cases, we attempted to induce flight indoors with a light focused on the top-center of the flight mill. We also tried a paper guide wrapped around the exterior, with the bottom portion painted black and the top portion painted white to create a visual horizon (Wilson, 1968). We attempted flight trials during both the day and night, and with the flight mills outdoors on either sunny or rainy days. In each case, grasshoppers tested included a minimum of five representatives from each of the following categories: 1, 5, and 10 days post-molt to adult, green, brown, male, female, field caught, and reared in field cages at high (approximately 20 adults m^3) or low (approximately 7 adults m^3) density. Since none of these variations resulted in more than 30 s of flight on the flight mill, we also tried a free-flying method. This approach involved either dropping a grasshopper from a 2-m height, or tossing it into the air and then measuring the distance it flew before landing. In a few cases, a grasshopper would fly up to 5 m. However, during most of the trials, the grasshopper would open its wings only to break its direct fall to the ground. In multiple cases, the grasshopper never opened its wings before landing on the ground. In sum, flight behavior was minimal for both color morphs and for grasshoppers reared at high and low density.

2.2.6. Effects of density on color, body mass, shape and metabolic rate

Based on our hypotheses that high population density enhances migratory characteristics and that brown grasshoppers are a migratory phenotype, we predicted that high density would increase the proportion of brown grasshoppers, increase the relative investment in thorax, wings, and hind legs, and increase metabolic rate.

2.2.6.1. Lab. We collected fourth-instar brown female nymphs from field populations and kept them in an outdoor arena for 1–2 days until they molted to the 5th instar. Nymphs were randomly assigned to low- (1 grasshopper cage^{-1}) or high- (8 grasshoppers cage^{-1}) density treatment groups ($n = 10$ cages per treatment). Cages (10 cm × 10 cm × 15 cm with 1 mm^2 cloth mesh) were kept in an incubator set to day and night conditions similar to field conditions: 14:10 L:D cycle, 27 °C:25 °C, 50% RH:40% RH. However, due to frequent power outages, the temperature of the incubator fluctuated in the range of 20–35 °C approximately every 3 days; all treatment groups were exposed to the same temperature fluctuations. Fresh *Leymus chinensis* grass was cut from the field plots every other day, secured with cotton in glass cylinders containing water, and presented *ad libitum*. Development time and daily mass gain were recorded over the entire 5th instar. Metabolic rate was measured over a 60-min period when grasshoppers were approximately 3/4 through the 5th instar. Animals were withheld food for 1 h prior to, and during, the gas exchange measurement. Within 4 h after molt to adult, we isolated treatment grasshoppers in a cage with no food available for 24 h. Adults were weighed, frozen at −20 °C and then dried (50 °C for 3 days)

2.2.6.2. Field cages. Cages (1 m^3) were constructed over *Leymus*-dominated plant communities (coordinates) using iron rod frames and fine 1 mm^2 cloth mesh covering. Brown male and female grasshoppers were collected from the field population at first instar (June 7, 2009) and transferred to either high (200 per cage; $n = 5$) or low (40 per cage; $n = 5$) density treatments. We selected the high-density treatment level to be slightly higher than the high-density field population from which they were collected (∼175 first instar *O. asiaticus* m^{-2}). We selected the low-density treatment level to ensure that sufficient animals would be available at the end of the experiment, knowing that there is a high mortality rate during the early instars. Because there were too few green grasshoppers available during the first instar, we collected additional brown and green grasshoppers at approximately mid-third instar (June 16–18) to fill additional treatment cages. At that time, the field density had decreased, as it does naturally due to high mortality in early instars, to approximately 80 *O. asiaticus* m^{-2}. We again selected a high treatment density that was slightly higher than that (100 per cage = high density; 20 per cage = low density). The number of cages for each treatment group was: green, low density (16; all started at 3rd instar), green, high density (6; all started at 3rd instar), brown, low density (25; 5 cages started at 1st instar, 20 cages started at 3rd instar), and brown, high density (8; 5 cages started at 1st instar, 3 cages started at 3rd instar) – 2820 grasshoppers in total. Average densities in field cages several weeks later when grasshoppers were in the 5th instar or adults and during our final cage assessment (July 8) were 7.5 (±0.4 SEM) and 21.7 (±2.8 SEM) for low and high density treatments, respectively. The population density in the field population at that time (mostly brown grasshoppers) was approximately 18 grasshoppers m^{-2}. This suggests that grasshoppers in our field cages suffered similar mortality rates as those in the field population. We surveyed field cages every 2 weeks and recorded the color and instar, but not the sex of each grasshopper in each cage

2.3. Technical methods

2.3.1. Morphology, lipid and muscle mass

Animals were weighed to the nearest 0.1 mg using a Mettler-Toledo AB204-S/Fact balance and then frozen. We measured pronutum and head maximum lateral width and hind femur length and maximum width (dorsal–ventral in approximately the center of the femur) to the nearest 0.01 mm (as described in detail in Dirsh, 1953) with a digital micrometer (Mitutoyo CD-6"BS, Japan). Animals were then dried at 50 °C for 3 days. Dissections occurred post-drying and consisted of gut removal, and separation of the head, wings, legs, thorax, and abdomen. Body parts were weighed to the nearest 0.001 mg using a Mettler Toledo MX5 microbalance. Flight and hind leg muscle mass were determined from the difference in dry masses of thorax and femur masses before and after dissolving all tissue with 1 mol L^{-1} NaOH (Marden, 1987). Abdominal lipid content was determined from the difference in mass of the abdomen before and after lipids were extracted by soaking for 24 h in 2 mL of a 2:1 (v/v) chloroform: methanol solution (similar to Kent and Rankin, 2001). Wings were relaxed using a weak vinegar solution, spread, pinned, and digitally scanned. Area of the hind wings was measured using Image J software (resolution = 79 pixels/cm; Rasband, 1997–2009).

2.3.2. Metabolic rate and behavior in chambers

Animals were placed in a 60-mL plastic syringe covered in tin foil to minimize visual disturbance. The syringe was flushed for 3 min with dry CO_2-free (Drierite, Ascarite) air at 100 mL min^{-1}, which lowered CO_2 levels within the syringe to below 0.1 ppm. The syringe was sealed and placed in a dark incubator (27 °C) for 60 min. Then the syringe was gently removed, and a 25-mL air bolus was ejected from the syringe into an air stream within plastic tubing. Air in the tubing was pulled at approximately 100 mL min^{-1} through a water scrubber (magnesium perchlorate), the CO_2 analyzer (LiCor model LI-6252), a CO_2 scrubber (Ascarite) the O_2 analyzer (Sable Systems Foxbox 2004 model) and then the pump (Sable Systems Foxbox). The analog output of the gas analyzers was digitized (Sable Systems UI2) and continuously recorded using Sable Systems Expedata and a laptop. To calculate the gas exchange rates, we integrated the area under the O_2 and CO_2 peaks produced by the 25-mL bolus injection and converted this to mL CO_2 produced and O_2 consumed h^{-1} as described in Lighton (2008, pp. 31–40).

To record behavior in the metabolic chambers, grasshoppers were placed in the chambers within the 27 °C incubator in the same manner as for measuring gas exchange but without the foil covering. Pairs of green and brown grasshoppers were videotaped for 25 min. Time moving (jumping or walking) and number of jumps were recorded manually by an observer using a stop watch while watching the videos.

2.3.3. Gregarious behavior

Gregarious behavior was measured by a using a behavioral assay similar to Roessingh et al. (1993). The main difference in our assay was the size of each arena (22 cm long × 18 cm wide × 7 cm high as opposed to 57 cm long × 30 cm wide × 10 cm high). Four arenas and trials were filmed simultaneously. Each arena had three sides that were opaque and one that was clear with perforations to allow visual and chemical cues from the stimulus group. Stimulus groups of 20 conspecifics were placed behind the clear perforated partition. Grasshoppers being tested were pre-treated in dark solitary chambers for 2 min. Next, a grasshopper was gently placed in one of the corners furthest from the stimulus group and held in place by a plastic cylinder. The cylinder was then removed and grasshoppers were filmed from above for 10 min. Behavior was analyzed using the computer software Ethovision.

2.3.4. Flight mill

We constructed a flight mill similar to Schumacher et al. (1997). The axis consisted of two magnets placed in a plexiglass frame. A drinking straw was used as a flight arm and a pin pushed through this straw served as an axle held in place by the two magnets, thus minimizing friction. An infrared transmitter/receiver mounted to the frame registered every rotation of the mill. Grasshoppers were attached to the head of a second pin using dental gum. The pin was then pushed through one end of the straw at a 95° angle. The flight mills were tested using colony-reared *S. americana* (subfamily: Cyrtacanthacridinae; average female is 1700 mg) and field-caught *Trimerotropis pallidipenis* (subfamily: Oedipodinae; average female is 500 mg – the same as *O. asiaticus*) collected near Phoenix, Arizona. Most individuals of both species flew for a minimum of 3 min on these flight mills, so they were suitable for grasshoppers in this size range.

2.4. Statistics

Prior to analyses, all data were checked for the assumptions of parametric tests. If data did not meet these assumptions, they were either transformed to meet assumptions or non-parametric tests were performed. For all multivariate tests, to ensure that all response variables were evenly weighted, we standardized variables using a *Z*-score transformation (Figs. 3A and 6A; Gotelli and Ellison, 2004, p. 400). For all covariate analyses, we examined the assumption of slope homogeneity using homogeneity-of-slopes model MANCOVAs. In all cases, the categorical variable (e.g. color) by covariate (e.g. dry mass) interaction was not significant and therefore, we concluded that our assumption was met and proceeded with traditional MANCOVA models. Retrospective power analyses were conducted on non-significant results ($P > 0.05$) to assess likelihood of committing type II errors. We set $\alpha = 0.05$. The power analyses were conducted for a 'medium effect size' given as d (Student's t-test, Mann–Whitney U-test), f (ANOVA/ANCOVA) or f^2 (MANOVA) (Cohen, 1988). While there is no standard, and the usefulness of a retrospective power analysis is controversial (Thomas, 1997), a power of 0.8 is generally considered adequate to accept the null hypothesis, assuming $P > 0.05$ (Cohen, 1988). Initial analyses were performed using Statistica 9 (2009). Power analyses were performed using GPOWER (Erdfelder et al., 1996). Details of statistical analyses performed can be found in the results section and figure legends. Throughout, statistical significance was judged as $\alpha < 0.05$.

3. Results

3.1. Field patterns in color

Both green and brown *O. asiaticus* were found in all collection areas but green animals were never abundant while brown *O. asiaticus* were relatively abundant and their frequency increased as overall population density increased (Fig. 2).

3.2. Differences between field-caught green and brown females in adult morphology

In field populations, 2 days post-molt to adult, brown females were, on average, heavier than green (Fig. 3A). To determine if there was an overall shape difference in these two groups, we standardized all variables (Gotelli and Ellison, 2004, p. 400) and then conducted a multivariate analysis, testing the categorical factor of color (covariate = dry mass; dependent variables = wing, head, hind leg, abdomen, and thorax mass; wing area, femur length, femur max width, head max width and pronotum max width. We followed the MANCOVA by Tukey multiple comparisons tests and found that overall shape was different between the two color morphs (MANCOVA: $F_{(9,28)} = 2.9$, $P = 0.01$; covariate means: dry body mass (mg): 119). When controlling for body mass, brown *O. asiaticus* females had a significantly increased relative investment in hind legs and thorax, and a non-significantly reduced investment in wings and head (Fig. 3B and C).

We then tested for predicted higher levels of abdomen lipid, and thoracic and hind leg muscle masses in brown grasshoppers (covariate = dry mass; dependent variables = abdomen lipid, thorax, and hind leg muscle masses; prediction = brown > green). The masses of these features did not differ between green and brown grasshoppers (MANCOVA: $F_{(3,33)} = 0.49$, $P = 0.69$; covariate means: dry body mass (mg): 119). To further investigate differences, we followed the MANCOVA by separate ANCOVAs (categorical factor = color; covariate = dry mass; dependent variable = either lipid, thorax, or hind leg muscle mass; prediction = brown > green). All ANCOVAs were also non-significant with a Power = 0.33, $f = 0.25$, and covariate means (dry body mass mg) = 119: abdominal lipid mass ($F_{(1,36)} = 0.25$, $P = 0.6$), thoracic muscle mass

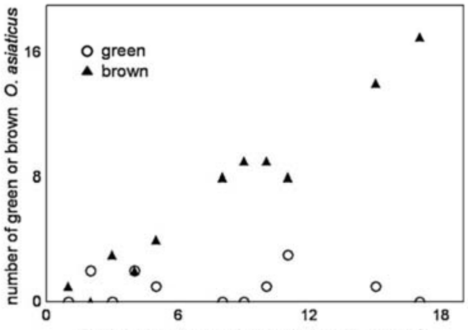

Fig. 2. Field surveys of male and female *O. asiaticus*. Brown and green *O. asiaticus* were found across all field densities surveyed; however only brown grasshoppers were found in high abundance (greater than three grasshoppers per sweep sample).

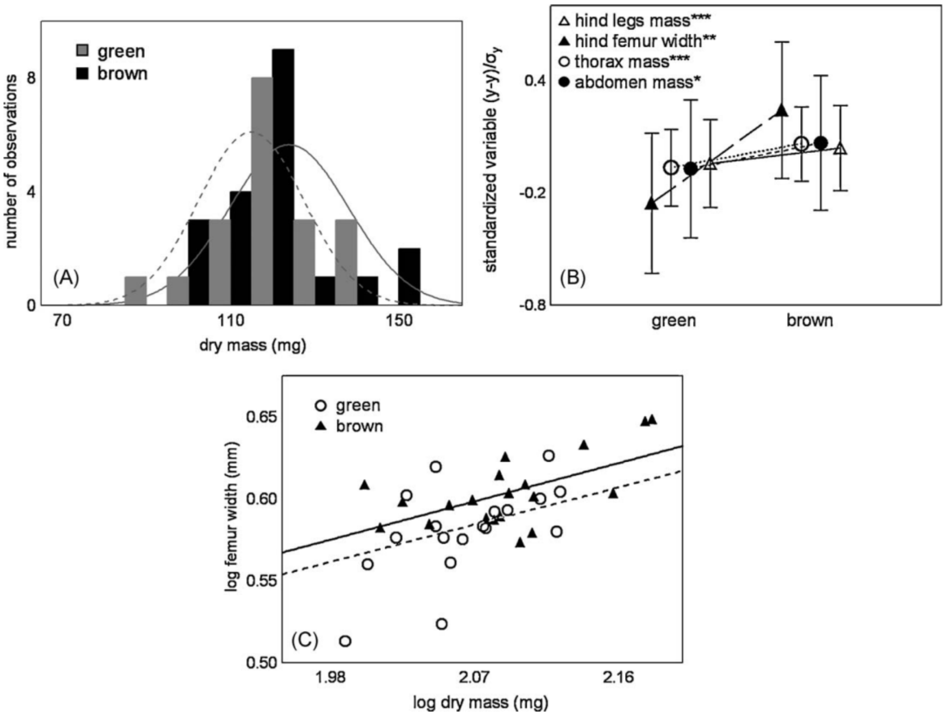

Fig. 3. Brown adult female *O. asiaticus* were heavier, on average, and had an increased relative investment in hind legs, thorax, and abdomen (from a MANCOVA with dry mass as a covariate). Green and brown female grasshoppers were field-caught in late 5th instar and caged until 2 days post-molt to adult. (A) Brown grasshoppers had a heavier dry body mass as compared to green (*t*-test: $t = 2.09$, $P = 0.04$). (B) Asterisks indicate significant differences from Tukey multiple comparisons tests ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). Other variables included in the overall MANCOVA, but that resulted in non-significant differences from Tukey multiple comparisons tests (all within $0.05 < P < 0.3$) are wing area, wing mass, head mass, head width, femur length, and pronotum max width. With the exception of pronotum width, brown had lower means for these six variables (MANCOVA: $F_{(9,28)} = 2.9$, $P = 0.01$; covariate means: dry body mass (mg): 119). Error bars denote 95% confidence intervals. (C) Example regression of a variable from the MANOVA where significant differences were found (femur width).

($F_{(1,35)} = 0.9$, $P = 0.35$), hind legs muscle mass ($F_{(1,35)} = 0.7$, $P = 0.4$). Mean proportions of abdominal lipid, thoracic, and hind legs masses relative to total dry body mass ± SEM were as follows: abdominal lipids (green: 0.079 ± 0.004, brown: 0.075 ± 0.003), thoracic muscle (green: 0.25 ± 0.004, brown: 0.26 ± 0.005), and hind leg muscle (green: 0.127 ± 0.005, brown: 0.133 ± 0.005).

3.3. Density and color effects on gregarious adult female behavior

When all variables were standardized and included in the initial analysis (independent variable = color, dependent variables = average distance from stimulus group, total distance moved, average velocity, turn angle, angular velocity, and meander), there were no differences in gregarious behavior between green and brown adults (MANOVA: $F_{(6,100)} = 1.2$, $P = 0.3$, Power = 0.84, $f^2 = 0.15$). When tested separately, brown grasshoppers kept at high densities tended to have a higher mean angular velocity than green grasshoppers kept at low densities for 24 h (°/s; i.e. the amount of turning per unit time; t test: $t = 2.1$, $df = 94$, $P = 0.036$), and mean meander (°/cm; i.e. the amount of turning per unit distance; *t*-test: $t = 2.02$, $df = 94$, $P = 0.045$).

As another approach to testing for gregarious behavior, we compared the amount of time brown or green grasshoppers spent near the stimulus group of grasshoppers. We divided the arena into four equal-sized zones ranging from closest to furthest from the stimulus group, and compared the percentage of time spent in each zone (independent variable = color, dependent variables = percent time in zone 1, 2, 3, and 4; dependent variables were standardized prior to the analysis). We found no significant differences between brown and green grasshoppers in their tendency to spend time near other grasshoppers (MANOVA: $F_{(3,103)} = 0.9$, $P = 0.46$, Power = 0.89, $f^2 = 0.15$).

3.4. Differences in field-caught green and brown juvenile females in gas exchange rates

Field-caught brown mid-5th instar females had an increased rate of gas exchange as compared to green (Fig. 4; O_2 consumption: $t = -2.37$, $df = 34$, $P = 0.02$; and CO_2 production $t = -2.03$, $df = 34$, $P = 0.05$ from *t*-tests). The difference in gas exchange was more apparent in larger grasshoppers. For brown grasshoppers, both O_2 consumption and CO_2 production were positively correlated with body mass. There was no correlation between these variables in green grasshoppers, suggesting that mass specific metabolic rate decreases with increasing body size in green, but not in brown grasshoppers (Fig. 5A and B).

The differences in metabolic rates between brown and green grasshoppers were not explained by observable differences

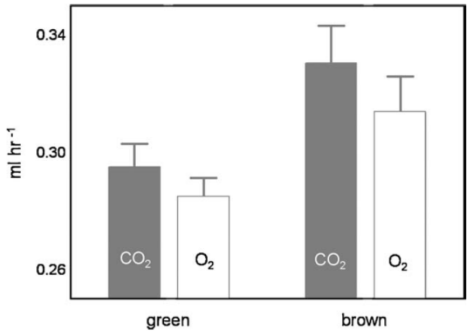

Fig. 4. Brown mid-5th instar female *O. asiaticus* had a higher rate of gas exchange than green. Brown grasshoppers had a higher CO_2 production (*t*-test: $t = -2.03$, $df = 34$, $P = 0.05$) and O_2 consumption (*t*-test: $t = -2.37$, $df = 34$, $P = 0.02$). Green and brown grasshoppers were matched for size so that there was no difference in the average mass between the groups.

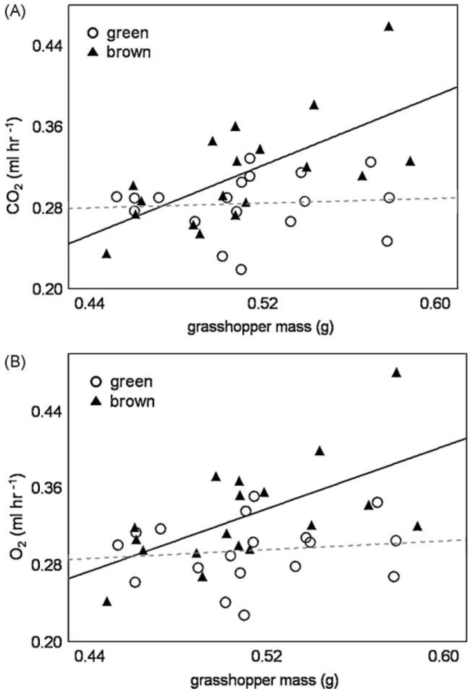

Fig. 5. Brown mid-5th instar female *O. asiaticus* had a higher rate of gas exchange than green at higher body masses. (Homogeneity-of-slopes model MANCOVA: $F_{(2,31)} = 3.6$, $P = 0.04$; covariate means: dry body mass (mg): 511). Brown grasshoppers exhibited a positive correlation between body mass and both (A) CO_2 production ($y = -0.1 + 0.86 \times x$; $R^2 = 0.44$; $P < 0.01$) and (B) O_2 consumption ($y = -0.08 + 0.8 \times x$; $R^2 = 0.37$; $P < 0.01$). Green grasshoppers did not exhibit any correlation between body mass and metabolic rate (CO_2 production: $R^2 < 0.01$; $P = 0.80$; O_2 consumption: $R^2 = 0.01$; $P = 0.60$).

in activity in the respirometry chambers. There were no significant differences between brown and green grasshoppers in the time spent moving in the respirometer (minutes grasshopper was mobile out of a total of 25 min: *t*-test: $t = -1.7$, $df = 12$, $P = 0.12$, Power = 0.14, $d = 0.5$; green 2.4 ± 1.1 SEM; brown 0.4 ± 0.34 SEM; average of all animals was 5.6% time spent moving). There was also no difference in the total number of jumps during 25 min (Mann–Whitney *U*-test: $df = 12$, $P = 0.5$, Power = 0.13, $d = 0.5$; green 1.3 ± 0.7 SEM; brown 0.8 ± 0.8 SEM; average for all animals was 0.03 jumps per minute).

3.5. Effects of color and density on adult flight capacity

There were no differences in flight capacity between any of the groups tested, and *O. asiaticus* individuals generally did not fly at all regardless of how tested.

3.6. Effects of density on color, body mass, shape and metabolic rate

3.6.1.1. Lab

We used the same variables and statistical procedure as for the field-caught grasshoppers, predicting that color morphs would differ in overall shape (categorical factor = density; covariate = dry mass; dependent variables = wing, head, hind leg, abdomen, and thorax mass; wing area, femur length, femur max width, head max width and pronotum max width). Grasshoppers reared at high density during the 5th instar had a different shape as adults than those reared at low density (MANCOVA: $F_{(10,5)} = 4.9$, $P = 0.046$; covariate means: dry body mass (mg): 89). We followed the MANCOVA by Tukey multiple comparisons tests and found that grasshoppers reared at high density had a significantly increased relative investment in hind legs and wings and a non-significantly reduced investment in head and abdomen (Fig. 6A and B).

There was no difference in mass between the two groups (*t*-test: $t = 0.07$, $df = 15$, $P = 0.94$, Power = 0.16, $d = 0.5$; low density 88.79 ± 4 SEM, high density 88.44 ± 2.4 SEM) and no evidence of color change over that time period. There were no differences in rate of gas exchange between the two groups (covariate = body mass; prediction = high density > low density): CO_2 production (ANCOVA: $F_{(1,16)} = 0.0008$, $P = 0.97$; covariate means: dry body mass (mg): 47; Power = 0.17, $f = 0.25$) and O_2 consumption (ANCOVA: $F_{(1,16)} = 0.0001$, $P = 0.99$; covariate means: dry body mass (mg): 47; Power = 0.17, $f = 0.25$).

3.6.1.2. Field cages

While many grasshoppers (10–20%) did change from one color to the other, density had no effect on the average color of grasshoppers. For the grasshoppers that were collected as brown at first instar, there was no difference in the proportion of brown grasshoppers per cage between high (0.93 ± 0.04 SEM) and low (0.86 ± 0.05 SEM) density treatments, though the power was relatively low (Mann–Whitney *U*-test: $df = 6$, $P = 0.23$, Power = 0.09, $d = 0.5$). However, for the grasshoppers that were collected as brown at third instar, there was a non-significant tendency for a density effect opposite to the predicted one, as the proportion of brown in high density cages (0.84 ± 0.08 SEM) was less than the proportion in low density cages (0.93 ± 0.02 SEM; Mann–Whitney *U*-test: $df = 31$, $P = 0.30$, Power = 0.24, $d = 0.5$). For grasshoppers collected as green at the third instar, there was a non-significant tendency for high density to increase the proportion of brown; again the test had relatively low power (high densities: proportion brown: 0.36 ± 0.04 SEM; low densities: proportion brown: 0.26 ± 0.04 SEM; Mann–Whitney *U*-test: $df = 53$, $P = 0.32$, Power = 0.19, $d = 0.5$)

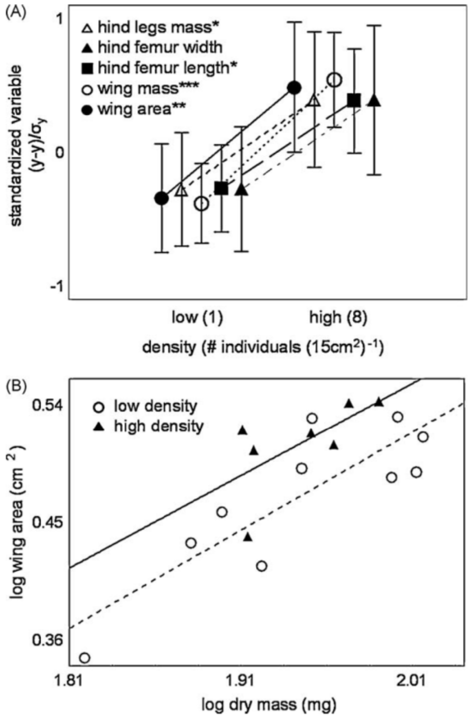

Fig. 6. Grasshoppers reared at high density during the 5th (final) juvenile instar had an increased relative investment in hind legs and wings 1 day post-molt to adult (MANCOVA with dry mass as a covariate). All grasshoppers were collected as and retained brown coloration. (A) Asterisks indicate significant differences from Tukey multiple comparisons tests (no asterisk: $P \leq 0.08$, $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). Other variables included in the overall MANCOVA where high density grasshoppers had higher mean values, but that resulted in non-significant differences ($P < 0.6$) from Tukey multiple comparisons tests were thorax mass, pronotum max width, and head max width. Two remaining variables included in the MANCOVA that resulted in non-significant differences ($0.05 < P < 0.3$) where high density grasshoppers had lower mean values were head mass and abdomen mass (MANCOVA: $F_{(10,5)} = 4.9$, $P = 0.046$; covariate means at a dry body mass of 89 mg). Error bars denote 95% confidence intervals. (B) Example regression of a variable from the MANOVA where significant differences were found (wing area).

4. Discussion

As in the much better studied African plague locusts, grasshoppers with different colors differed in size and shape, and rearing at different densities even for one juvenile instar, induced changes in shape. Most of the significant differences supported the hypothesis that high-density and brown color were associated with improved migratory capacity. There was evidence for larger wings, thorax and legs, and higher metabolic rates being associated with high density rearing or brown color. However, key differences most commonly associated with migratory polyphenism (differences in flight behavior, gregariousness, flight muscle, lipid stores) were not correlated with color or high-density rearing. At present, it is unclear whether these negative results are due to ecological conditions that suppressed migration, our experimental design, or interspecific variation.

4.1. Experimental design issues

Experiments with the African plague locusts have clearly demonstrated that maternal and early-instar effects are important for development of color and migratory polyphenism (refs). Experiments with other grasshoppers and insects have also demonstrated that genetically based variation can contribute to variation in color and physiological and morphological traits related to migration (refs). Tests for maternal or genetically based effects require laboratory rearing, and unfortunately, as yet this has not been accomplished with *O. asiaticus*, despite significant efforts (juveniles die during lab-rearing, possibly because key dietary requirements are missing). Our experiments have necessarily focused on animals collected from the field in the midst of their juvenile development. The possibility of maternal, early-instar, or genetic effects on color or migratory polyphenism must be considered in interpretation of all of the results presented here.

4.2. Brown, but not green, grasshoppers are found in high abundance in the field

Our surveys showed that green *O. asiaticus* are generally present at low density wherever *O. asiaticus* are found and are rarely, if ever, found in high abundance (i.e. in our sampling, we never collected more than two green at any given coordinate). Conversely, brown *O. asiaticus* are found in both high and low abundance. These field observations suggest are consistent with prior anecdotal reports that migratory swarms are largely comprised by the brown phenotype. However, it is important to point out that our experiments do not allow determination of how the differences in field abundances arise. For example, it is possible that regions with high concentrations of brown grasshoppers result from density effects on color, nutritional effects on color, or color-associated genetically based differences in gregariousness.

4.3. Weak connection between color variation and migratory capacity

Brown and green *O. asiaticus* collected from the field had some significant differences in morphology and physiology, but these differences were not clearly linked to migratory polyphenism. Some of the predictions based on the hypothesis that brown morphs are migratory were met; brown grasshoppers had an increased investment in thorax and legs (Fig. 3B and C), and increased metabolic rates (Fig. 4). Larger hind legs may enhance jumping into flight (Katz and Gosline, 1992) and a larger thorax can allow for increased flight muscles. The higher metabolic rates in brown grasshoppers was not related to greater activity in the respirometer, and resulted from higher rates in larger animals (Fig. 5), suggesting that heavier animals might have a higher proportion of metabolically active tissues.

In contrast to these supportive findings, key predictions of the hypothesis that brown grasshoppers have a greater migratory capacity were not met. Thoracic and leg muscle masses and abdominal lipid stores did not differ between green and brown morphs. Mean values were very similar for flight muscle mass and lipid stores, suggesting that the lack of statistical significance was not due to lack of power. Wing size also was not larger in brown grasshoppers (in fact, the mean wing area was greater in the green morphs in the MANCOVA), although wing size does not always correlate with migratory capacity (reviewed in Rankin and Burchsted, 1992). Finally, we found no evidence that brown grasshoppers were more prone to fly; in fact, neither morph could be induced to fly.

There was also no evidence that brown morphs were more gregarious, though this conclusion should be taken with caution. We measured gregarious behavior for green and brown grasshoppers collected from the same population, driven to Beijing for 9 h at relatively high densities, and then subsequently kept in solitary confinement or high density, respectively, for 24 h. While 4 h is sufficient to induce gregarization in solitary-reared locusts, up to 24 h may be necessary to reduce gregarization in a crowd-reared locusts (*S. gregaria*; Roessingh et al., 1993). Conceivably a longer time may be required to eliminate gregarious behavior in this species. If that was the case, we would not be able to detect evidence of gregarious behavior as we have no baseline behavior for solitary-reared *O. asiaticus*. Testing *O. asiaticus* that have been reared for the entirety of their juvenile development at either high density or in solitary cages would provide a more definitive answer to this question.

In sum, the green-brown phenotypes from this field population had some differences in morphology and metabolic rates, but these did not seem to be linked with migratory polyphenism. Thus, our comparisons of field-collected green and brown forms did not support the hypothesis that brown *O. asiaticus* represent a gregarious, migratory form of this grasshopper analogous to those documented for *S. gregaria* or *L. migratoria*. However, it is important to note that color, migratory behavior, morphology and physiology exist on a continuum. It is possible that ecological conditions (e.g. good local forage) precluded development of fully functional migratory *O. asiaticus*, and that under such conditions a clearer correlation between color and migratory phenotype might be observed.

4.4. *Effects of rearing density*

Rearing density did significantly affect the shape of *O. asiaticus*, and the significant differences observed were generally consistent with the hypothesis that high densities promote formation of a migratory form. As predicted by this hypothesis, females reared at high density during the 5th instar had an increased investment in wings and hind legs, and a decreased investment in head and abdomen. The greater wing mass and area per body mass will result in a lower wing loading and could translate into increased flight capacity (Rankin and Burchsted, 1992). However, no *O. asiaticus* would fly for long distances, regardless of rearing density. Also, since we did not measure flight or leg muscle mass in this experiment, and the comparison between color morphs indicated that changes in thorax and leg dimensions do not correspond to differences in flight and leg muscle masses of the morphs, the conclusion that the observed changes in morphology actually improve flight performance must be considered provisional. Further, we found no direct evidence for an effect of density on color suggesting that color, shape, and migration are not necessarily linked in this species. However, high-density rearing induced larger hind legs as found for the brown forms, suggesting that these differences in field-caught brown and green forms could be caused by rearing density. It is possible that longer juvenile rearing at high density, or multi-generational rearing at high density is necessary to elicit a full migratory phenotype that exhibits flight behavior. Alternatively, other ecological conditions may be critical for triggering migratory development and behavior in this species.

The population studied was in the Xilin River basin region where *Oedaleus* have exhibited swarming in other years (S. Hao and L. Kang, unpublished). Thus, this grasshopper can and will fly under some circumstances. However, during the 2 years of this study, the populations of *O. asiaticus* were not swarming, despite exhibiting densities exceeding those previously documented for swarming *O. asiaticus* (Jiang et al., 2003). Perhaps deteriorating local forage conditions are required in addition to high densities to induce swarming and migration in *O. asiaticus*.

4.5. *Potential functional significance of color variation*

While we cannot completely reject the hypothesis that color variation is linked with migratory polyphenism in *O. asiaticus*, our results do suggest that other hypotheses for the significance of color variation should be considered. For example, color may affect likelihood of predation with green forms being more cryptic in more lush microhabitats and brown forms more cryptic in dryer or open habitats (Chapman and Joern, 1990). As an alternative to crypsis, the brown color may be aposematic, reducing deaths to predation in large groups, as suggested for some locusts (Sword et al., 2000). Brown coloration may be related to melanization and resistance to diseases, cannabalism or parasites (Schmid-Hempel, 2005), with a trade-off of increased energetic costs (consistent with the higher metabolic rates in brown forms, Fig. 4).

4.6. *Potential mechanisms causing color variation*

Grasshoppers commonly exhibit three types of color polyphenism: phase color-polyphenism, green-brown color polyphenism, and homochromy where an individual matches the color of the underlying background (described in Pener and Simpson, 2009). These color polyphenisms typically have different underlying mechanisms and some locusts, including *L. migratoria*, exhibit all three hierarchically (e.g. in the absence of the mechanistic triggers for phase and green–brown polyphenism, they exhibit the third type, homochromy).

When crowded, *L. migratoria* exhibit phase color-polyphenism, developing dark, contrasting colors by way of the neurohormone [His^7]-corazonin (Tawfik et al., 1999). In the absence of crowding (i.e. low density), green–brown polyphenism can be regulated by humidity. High humidity, found in wetter seasons or microhabitats, directs development into a cryptic green morph. In the absence of high humidity, many grasshoppers exhibit homochromy and can have a tan or yellow to dark brown coloration depending on dominant environment color (Chapman and Joern, 1990). The high concentrations of brown *O. asiaticus* documented in Fig. 2 occurred in fields subject to heavy livestock grazing (Kang and Chen, 1995; A. Cease et al., personal observation), and these heavily grazed fields have a significantly reduced ground cover and soil water content (Zhao et al., 2007).

Alternatively, brown coloration could be caused by better nutrition, which was supported by the larger size of brown forms (Fig. 3A) and the correlation of brown forms with higher field abundances (Fig. 2). Further experiments will be necessary to distinguish these various hypotheses concerning the mechanism and significance of color variation in this species.

4.7. *Summary and future directions*

Our results show that neither brown color nor high density rearing for one-to-several instars is associated with expression of a clear migratory phenotype, consistent with the lack of migratory swarms observed for *O. asiaticus* during the 2 years of this study. Our results support the general understanding that the type and degree of migratory polyphenism can exist along a continuum in grasshopper species (reviewed in Pener and Simpson, 2009). The lack of flight behavior and migratory swarm formation despite high field densities suggests the importance of other ecological factors in determining migration in this species. Definitive tests of hypotheses regarding the mechanisms and functional significance of color migratory polyphenism in this species will require development of laboratory rearing protocols. In addition to enhancing migratory theory, understanding characteristics that are correlated with migratory phenotypes and the environmental triggers that may enhance outbreak and migratory phenotypes is key to developing sustainable management strategies for pestivorous and swarm-forming grasshoppers.

Acknowledgements

We thank Feng Jin, Colleen Ford, Yuan Kong, Zhantao Fan, Chunjiang Liu and Yingfu Liu, and Jennifer Esman for field and lab assistance. We thank Guangming Zhang for technical assistance in IMG and the three anonymous reviewers for their comments. This work was supported by grants from the National Science Foundation (EAPSI to AJC, DEB-0925017 to JJE, and EAR-0746352 to JFH), the National and ASU Chapter of Sigma Xi, and Achievement Rewards for College Scientists to AJC.

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APPENDIX B

CHAPTER 3: HEAVY LIVESTOCK GRAZING PROMOTES LOCUST OUTBREAKS BY LOWERING PLANT NITROGEN CONTENT (PUBLISHED MANUSCRIPT)

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Acknowledgments: We are grateful to the principal investigators of the CTFS forest plots used in this analysis for the assembly and sharing of unique data on the structure and dynamics of tropical forests: R. Condit, S. Hubbell, R. Foster (Barro Colorado Island, Panama); S. Bunyavejchewin (Huai Kha Khaeng, Thailand); D. Thomas, D. Kenfack, G. Chuyong (Korup, Cameroon); M. Alvarez (La Planada, Colombia); S. Tan, L. H. Seng, S. Davies, T. Yamakura (Lambir, Malaysia); S. Noor, R. Kassim (Pasoh, Malaysia); and R. Valencia (Yasuni, Ecuador). The manuscript benefitted from the insightful and constructive comments of two reviewers. R.E.R. thanks the Alexander von Humboldt Foundation and the Curators of the University of Missouri for financial support. Original data are available at www.ctfs.si.edu.

Supporting Online Material
www.sciencemag.org/cgi/content/full/335/6067/464/DC1
Materials and Methods
SOM Text
Table S1
References (*34–40*)

12 October 2011; accepted 5 December 2011
10.1126/science.1215182

Heavy Livestock Grazing Promotes Locust Outbreaks by Lowering Plant Nitrogen Content

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Current paradigms generally assume that increased plant nitrogen (N) should enhance herbivore performance by relieving protein limitation, increasing herbivorous insect populations. We show, in contrast to this scenario, that host plant N enrichment and high-protein artificial diets decreased the size and viability of *Oedaleus asiaticus*, a dominant locust of north Asian grasslands. This locust preferred plants with low N content and artificial diets with low protein and high carbohydrate content. Plant N content was lowest and locust abundance highest in heavily livestock-grazed fields where soils were N-depleted, likely due to enhanced erosion. These results suggest that heavy livestock grazing and consequent steppe degradation in the Eurasian grassland promote outbreaks of this locust by reducing plant protein content.

Plant nitrogen (N) content has long been linked to the performance and dynamics of herbivorous insects (*1*). Most nitrogen in plants is in the form of protein, and current paradigms generally assume that increased plant N content will enhance herbivore performance by relieving any constraints on growth and reproduction due to limitations of available protein (*2*). Because plants in many terrestrial ecosystems are N-limited (*3*), it is often assumed that primary consumers would be as well (*2*). However, studies performed on the basis of the geometric framework (*4*) have shown that many animals have specific dietary intake targets for protein, carbohydrates, and other nutrients. Interestingly, such studies have found that excess protein decreased life span in fruit flies (*5*) and that herbivores and omnivores (including humans) will overeat carbohydrates but are unlikely to overeat protein (*6*). Thus, animals may sometimes be impaired by ingesting excess protein (*7*). Indeed, the Australian plague locust, *Chortoicetes terminifera* (Acrididae: *Oedipodinae*), grew slowly on one host plant because it obtained excess protein relative to carbohydrate (*8*). Given that humans have substantially altered ecosystem N cycling through fossil fuel combustion, agricultural fertilizer application (*9*), and domesticated animal production (*10*), they may also affect insect dynamics in unexpected ways by altering plant nitrogen supplies. Understanding these anthropogenic impacts is critical to developing sustainable land management practices that minimize economically damaging insect outbreaks.

Oedaleus asiaticus (Acrididae: *Oedipodinae*) is a nonmodel [e.g., (*11*)], economically damaging locust of the north Asian steppe (*12*, *13*), part of the largest grasslands in the world (Fig. 1A). We examined the effects of increases in the N content of host plants due to fertilization inputs of 175 kg N ha^{-1} year^{-1} on growth and viability of this locust in both laboratory and field cage experiments (*14*). This level of N addition is similar to the fertilization rates of most crops (*15*). We then used artificial diets varying in their protein:carbohydrate ratio to assess effects on dietary preference and growth rates.

In contrast to the existing paradigm of limitation of insects by low content of plant protein, our results show that N fertilization and high-protein artificial diets can have consistent negative effects on the performance of the locust. Survival decreased strongly with N fertilization in the field (Fig. 1B). The decreased survival of *O. asiaticus* in N-fertilized field plots could have been due to many possible consequences of fertilization, such as changes in plant structure or chemical content, species composition, predators, or microclimate. However, results from lab experiments that controlled for these factors indicated that growth rate, size, and development rate were all reduced when locusts were fed N-fertilized plants (Fig. 1C), indicating that the field survival results were due to effects associated with plant N status. Furthermore, when offered complementary artificial diets [e.g., (*16*)], locusts selectively consumed a protein:carbohydrate ratio of 0.5, which most correlated with maximal survival (Fig. 1, D and E). Performance (growth rate × survival) was significantly reduced when locusts were confined to artificial diets with a protein:carbohydrate ratio above 1:1 (Fig. 1E), consistent with the hypothesis that the mechanism by which N fertilization reduces survival of this locust in the field is elevation of plant protein content.

In addition, plant N content was closely associated with the relative amounts of different food plants consumed when locusts were offered a palette of the six most common local plants collected from unfertilized plots (five grasses and one sedge) (Fig. 2A). Indeed, contrary to expectations from protein-limitation paradigms, *Stipa grandis*, the grass with the lowest N content, was the most highly consumed over 36 hours [a period long enough for locusts to take multiple meals and regulate nutrient intake on the basis of internal conditions (*17*)]. Direct behavioral observations confirm that low-N *S. grandis* was the most highly consumed plant under field conditions (*18*).

We compared food selection over 8 hours between N-fertilized and unfertilized *S. grandis*, using dried, ground leaves to control for toughness and water content (*8*, *19*). Locusts strongly favored unfertilized *S. grandis* leaves over *S. grandis*

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collected from N-fertilized plots (Table 1). N fertilization increased the protein content of *S. grandis* (Table 2), suggesting that the negative effects of N fertilization are due to changes in the protein: carbohydrate ratio away from the intake target of *O. asiaticus (20)*, although it is also possible that N fertilization increased the concentration of N-rich alkaloids or other allelochemicals (*21*).

In Inner Mongolia, a primary outcome of heavy livestock grazing is the loss of topsoil and organic N due to erosion, resulting in plants with a low N content (*10*). Grazing also results in a shift in the balance between *S. grandis* and *Leymus chinensis*, with *S. grandis* becoming more prevalent in heavily grazed fields (*13*). [Note, however, that there was always a substantial biomass of each species available across our study sites and field cages (table S1).] Together, these grasses comprise roughly 80% of the above-ground biomass in the Inner Mongolian grasslands (*22*); thus, a large locust outbreak must depend on one or, more likely, both of these grasses during its development.

Consistent with a population-level impact of sheep grazing on locust abundance via effects on plant N content, locust density was highest in heavily grazed pastures dominated by plants with the lowest N content (Fig. 2B). Furthermore, locusts preferred low-N *S. grandis* collected from heavily grazed pastures over *S. grandis* from ungrazed plots (on the basis of paired-choice comparisons involving either fresh leaves or dried, ground leaves, to control for toughness and water content) (Table 1). Although the field measurements of locust abundance did not control for other possible effects of livestock grazing [e.g., shifts in predator communities, microclimate, sward structure, plant secondary chemicals, and landscape patchiness (*23*)], these data are consistent with the hypothesis that the high densities of *O. asiaticus* on the heavily grazed plots are due to this locust's preference for low-N plants and its improved growth and survival on such plants.

The hypothesis that low plant N can stimulate locust outbreaks is also supported by observations that *O. asiaticus* population explosions occur exclusively in pastures heavily grazed by livestock (*24*). Our data are in accordance with work showing that some North American Acrididae species (grasshoppers and locusts) also prefer low-N plants, likely due to their long-term adaptation to N-limited grassland environments (*25*). There is mounting evidence that N excess, rather than N limitation, is a critical nutritional factor regulating plant-insect interactions, especially for grasshoppers (*8*, *26*) and other herbivores that have evolved to exploit a relatively poor autotroph nutrient base in nature.

Our results fit well with the emerging paradigm that related animal species can vary dramatically in intake targets and performance responses to dietary variation. Seven cogeneric grasshopper species from one community were tested and all but two had unique target intake ratios, which suggests that each species may fill a unique nutritional niche and that species with extreme intake targets might be most sensitive to environmental variation (*27*). The lowest protein: carbohydrate intake target observed was 0.7, similar to what has been reported for the migratory locust (*L. migratoria*), which shares a subfamily with *Oedaleus* (*28*). We found that *O. asiaticus* had a protein:carbohydrate intake target of 0.5 (Fig. 1D), lower than any grasshopper previously studied (*21*). This finding may explain why dramatic, damaging population outbreaks of *O. asiaticus* occur in association with changes in land management (in this case, grazing intensity).

Our results strongly suggest that heavy livestock grazing promotes outbreaks of this locust by shifting plant nutrient content toward lower N conditions favorable to *O. asiaticus*. Although the precise physiological mechanisms by which high plant N exerts negative effects on this locust in the field remain to be elucidated, data from our artificial diet studies strongly suggest that protein

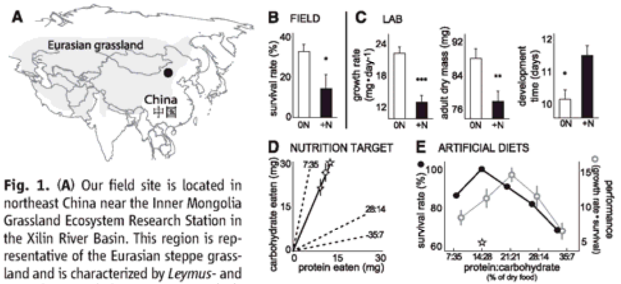

Fig. 1. (**A**) Our field site is located in northeast China near the Inner Mongolia Grassland Ecosystem Research Station in the Xilin River Basin. This region is representative of the Eurasian steppe grassland and is characterized by *Leymus*- and *Stipa*-dominated plant communities (*22*). Rapid steppe degradation (*29*) has led to reduced biodiversity, decreased productivity and, in some cases, desertification (*30*), likely due to anthropogenic factors, especially livestock grazing (*31*). N fertilization of field plots negatively affected *O. asiaticus*, (**B**) reducing survival in field cages and (**C**) growth, size, and development rates when reared in the laboratory (on *L. chinensis* from these plots). All comparisons were made using Student's *t* tests; data for proportion surviving in field cages were arcsine transformed before analysis ($^*P < 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). In artificial diet studies of field-collected animals, (**D**) locusts given pairs of diets complementary in their protein:carbohydrate ratios—7:35 and 35:7 (square) or 7:35 and 28:14 (triangle)—ate similar amounts of carbohydrate and protein (multivariate analysis of covariance: Wilks' lambda = 0.95, $F_{2,29} = 0.72$, $P = 0.50$; initial locust mass covariate means, 244 mg). Locusts fed nonrandomly to achieve a target protein:carbohydrate intake of ~0.5. (**E**) Locusts had 100% survival when fed artificial diets with a 0.5 protein:carbohydrate ratio, the same ratio they selected when given a choice. Survival rate was lowest (68%) in the highest protein diet. Overall performance (i.e., growth rate × survival) decreased above and below a protein:carbohydrate ratio of 1:1 (analysis of covariance: $F_{4,68} = 8.85$, $P < 0.001$; initial locust mass covariate means, 136 mg).

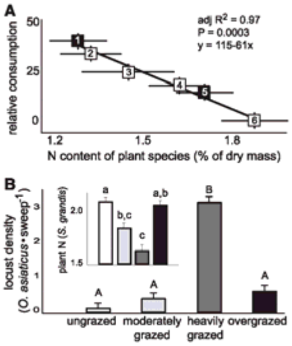

Fig. 2. (**A**) The percentage of consumption of each plant species (see SOM for how this was calculated) was negatively related to the percentage of N of that species (N was measured in leaf blades). Locusts were concurrently offered leaf blades from each of the six most common plants for 36 hours. 1, *S. grandis*; 2, *Carex korshinskyi*; 3, *Agropyron cristatum*; 4, *Cleistogenes squarrosa*; 5, *L. chinensis*; 6, *Achnatherum sibiricum*. Darkened squares are the two most common plants, each responsible for ~40% of aboveground biomass. Remaining species make up <5% each of aboveground biomass. (**B**) *O. asiaticus* was most abundant in heavily grazed fields where the N content of *S. grandis* was lowest during an outbreak year (2009); collected from large (>20 ha) and adjacent pastures [analysis of variance (ANOVA): $F_{3,16} = 64.95$, $P < 0.001$]. The ungrazed field has been fenced and protected since 1979; adjacent fields have been grazed at relatively constant levels for >5 years. Grazing level was determined by differences in ground cover and aboveground biomass [e.g., (*12*)]. Each observation represents the number of *O. asiaticus* collected in sweep-net samples from a given pasture and the percentage of N of a concurrently collected composite of above ground *S. grandis* leaves from a 1-m^2 quadrat (inset) (ANOVA: $F_{3,12} = 14.81$, $P < 0.001$). Letters indicate differences using Scheffe's post hoc comparison tests.

Table 1. Nitrogen enrichment of field plots decreased the palatability of *S. grandis* (the preferred host plant), and heavy grazing increased palatability in paired-choice tests using either dried/ground (8-hour assay) or fresh leaves (single-meal assay, average percentage of leaf area consumed).

Treatment (*S. grandis*)	Number of wins	Amount consumed	Chi- squared *P*
		Ground leaf	
Unfertilized	18	13.8 ± 2*	<0.01
Fertilized	7	8.6 ± 2*	
Heavily grazed	9	4.1 ± 1*	0.01
Ungrazed	3	2.9 ± 1*	
		Fresh leaf	
Heavily grazed	8	28 ± 7†	0.03
Ungrazed	3	15 ± 5†	

Table 2. Nitrogen enrichment of field plots increased the N content (percentage of dry mass) and protein content (percentage of dry mass) of *S. grandis* and *L. chinensis*. All comparisons were analyzed using Student's *t* tests after arcsine transformation of proportional data.

Nutrient	Unfertilized	N-fertilized	*P*	N	Heavily grazed	Ungrazed	*P*	N
			S. grandis					
% C	46 ± 0.8	46 ± 0.8	0.77	24	47 ± 0.2	44 ± 2	0.12	9
% N	1.4 ± 0.05	2.4 ± 0.06	<0.001	24	1.7 ± 0.05	2.1 ± 0.04	<0.001	9
% protein	4.3 ± 0.5	11.1 ± 1.8	<0.01	24				
Protein/N ratio	3.1 ± 0.4	4.6 ± 0.7	0.10	24				
			L. chinensis					
% C	46 ± 1	46 ± 0.8	0.99	24	46 ± 0.3	46 ± 0.3	0.18	8
% N	1.8 ± 0.08	2.9 ± 0.1	<0.001	24	2.4 ± 0.09	2.7 ± 0.03	0.12	8
% protein	8.7 ± 1	11.9 ± 1	0.03	23				
Protein/N ratio	4.9 ± 0.5	4.2 ± 0.4	0.37	23				

excess is playing a role. Furthermore, these findings reinforce the realization that differential responses of herbivore species to plant nutrient content can structure herbivore communities (*27*), providing new insights that may improve livestock and fertilization management strategies to limit the occurrence of economically damaging locust outbreaks.

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Acknowledgments: The authors thank F. Jin, D. Niren, D. Flynn, Y. Kong, Z. Fan, J. Esman, Q. Chen, and G. Zhang for field and laboratory assistance; M. Quinlan, D. Denardo, J. VandenBrooks, C. Klok, S. Behmer, and J. Sabo for helpful discussion of experimental design; J. Wu for funding and scientific support; and M. McCrackin, F. Clissold, and three anonymous reviewers for valuable comments on the manuscript. This work was supported by the National Science Foundation (EAPSI, DDEP to A.J.C., DEB-0925017 to J.J.E., and EAR-0746352 to J.F.H.), Sigma Xi, Achievement Rewards for College Scientists (Marley-Webb and Johnston Foundations), P.E.O. Scholar Award to A.J.C., the Chinese Research Grants of Public Welfare Fund for Agriculture (Project 200903021), and the Foundation of Chinese Academy of Sciences (Project Kscx2-yw-z-1021). A.J.C. designed and performed experiments, analyzed data, and wrote the paper. J.J.E. and J.F.H. designed experiments and wrote the paper. C.F.F. and S.H. designed and performed experiments. L.K. gave scientific support and guidance. Data used in the analyses are available in the Supporting Online Material.

Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6067/467/DC1
Materials and Methods
Fig. S1
Table S1
References (*32–36*)

26 September 2011; accepted 15 November 2011
10.1126/science.1214433

Depth Perception from Image Defocus in a Jumping Spider

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The principal eyes of jumping spiders have a unique retina with four tiered photoreceptor layers, on each of which light of different wavelengths is focused by a lens with appreciable chromatic aberration. We found that all photoreceptors in both the deepest and second-deepest layers contain a green-sensitive visual pigment, although green light is only focused on the deepest layer. This mismatch indicates that the second-deepest layer always receives defocused images, which contain depth information of the scene in optical theory. Behavioral experiments revealed that depth perception in the spider was affected by the wavelength of the illuminating light, which affects the amount of defocus in the images resulting from chromatic aberration. Therefore, we propose a depth perception mechanism based on how much the retinal image is defocused.

Visual systems that accurately and reliably judge distance or depth are valuable. A wide variety of animals, including humans, perform this task with binocular stereoscopic depth perception (*1*). Two types of monocular depth cues also provide absolute depth perception in some animals: accommodation (i.e., focal adjustment) in chameleons and other vertebrates (*2*, *3*)

Supporting Online Material for

Heavy livestock grazing promotes locust outbreaks by lowering plant nitrogen content

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This PDF file includes:

Materials and Methods
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Materials and Methods

Field site

Oedaleus asiaticus locusts and their host-plants were collected from field populations near the Inner Mongolia Grassland Ecosystem Research Station in the Xilin River Basin, Inner Mongolia Autonomous region, China (43°38' N, 116°42' E). Inner Mongolia is representative of much of the Eurasian Steppe region floristically and ecologically (*32, 33*).

Field cage N-enrichment experiment

Cages (1 m^3) were constructed over *Leymus*- and *Stipa*-dominated plant communities using iron rod frames and fine 1 mm^2 cloth mesh covering (Table S1). We removed spiders and other natural enemies from the field cages before adding the locusts. Mesh cages stifled wind flow and sunlight equally among all groups, likely greatly reducing the effects of the small changes in plant biomass and species composition on microclimates, as found in prior studies (*26*). Locusts were collected at either first or third instar and randomly assigned to control (0 kg N $ha^{-1}yr^{-1}$) or fertilized with ammonium nitrate (NH_4NO_3; 175 kg N $ha^{-1}yr^{-1}$) plant treatment groups (20 or 100 locusts per cage). There were no interactive effects of fertilization and locust density on survival rate (two factor ANOVA: $F_{1,46}$=1.22, P=0.28), so we collapsed the groups for further analyses. Ammonium nitrate was applied to plants at one time, in late spring just before a heavy rainfall, and a minimum of two weeks prior to insects being added to cages. Locusts started at first and third instars were put in separate cages, but these cages (of either first or third instars) were distributed equally among fertilization treatment groups. We used brown phenotypes as they are far more prevalent than green phenotypes (*12*). We surveyed field cages every two weeks to monitor survival until the final larval instar (June-July 2009).

Lab N-enrichment experiment

We collected fourth-instar female locust nymphs from field populations and randomly assigned them to control (0 kg N $ha^{-1}yr^{-1}$) or fertilized (175 kg N $ha^{-1}yr^{-1}$) food plant treatment groups on day one of the fifth and final larval instar (n = 6 cages per treatment; 8 locusts per 10x10x15 cm cloth mesh cage). Fresh *Leymus chinensis* grass was cut from the either control or treatment field plots every other day, secured with cotton in glass cylinders containing water, and presented *ad libitum.* The chemical composition of the grass likely changed over the 48 h duration between replacing food plants. Development time and daily mass gain were recorded throughout the fifth instar (July 2008). There was no significant difference in survival rate between the different treatment groups in the lab. Within 4 h after molt to adult, we isolated treatment locusts in a cage with no food available for 24 h. Adults were weighed, frozen at -20°C and then dried (50°C for 3 days).

Artificial diet experiments

We made dry, granular chemically defined foods (based on Dadd 1961 (*34*), as modified by Simpson and Abisgold 1985 (*35*)) that varied in protein and digestible carbohydrate macronutrients. All foods had equal amounts of digestible macronutrients (42% of dry weight). For all experiments, diets were dried to a constant mass before introducing them into the locust cages and again after removal from the cages by sealing them in an airtight container with silica desiccant.

Confined diet experiment: We collected fourth-instar locust nymphs from field populations and kept them in the lab on host-plants collected from the field until the start of the experiments. Animals were individually housed for the entire fifth instar with one of five randomly-assigned artificial diet treatments (7:35, 14:28, 21:21, 28:14, or 35:7, protein:carbohydrate ratio, as a percent of dry weight), with 12-16 animals per treatment (equal numbers of males and females for each group). Cages were 15cm diameter x 10cm height plastic cups with a newspaper bottom. Fresh diets were supplied every three days until day nine, and every five days thereafter; ample water was available in each cage. Locusts were fed ad lib such that there was always remaining food when we changed the dishes. Adult mass was obtained within six hours of molting.

Intake Target Experiment: Locusts were collected from the field as nymphs during mid-5th instar in the late afternoon. They were kept overnight with ample water, but no food, and introduced into the experimental cages the following morning. Locusts were given one of two complementary diet pairings (7:35 & 28:14 or 7:35 & 36:7), plus ample water. We used two pairings to verify that intake targets were not the result of eating randomly between the two dishes. Locusts were allowed to feed for a total of 48h, with food being replaced once after 24 hours. By weighing the amount eaten from each dish, we were able to determine the total amount of protein and carbohydrate consumed for each locust.

Relative consumption of different plant species

All plants were collected in the late afternoon of July 2, 2009 and the experiment was initiated by adding locusts to each cage the following morning. Locusts were originally collected at third instar and reared in outdoor mesh cages placed over ambient plant communities until mid-fourth instar when they were randomly assigned to treatment groups in this experiment. Leaves without herbivory were selected and isolated from sheaths and stems, but otherwise kept intact. Similar bundle-sizes and masses of each plant species were inserted into water-filled petri dishes via individual slits made on the petri dish lid (i.e. six slits total on each lid for six plant species). In all cases, leaves were sufficient such that no locust consumed the entirety of any single plant species. Remaining portions of uneaten leaves were collected after 36 h, dried at 60°C for 24 h and then weighed. Dry mass eaten was calculated by subtracting the dry mass of the uneaten leaves from an estimate of the original dry mass for a given sample. Original dry mass for each plant sample was calculated by multiplying the initial wet mass by the dry:wet mass ratio estimate for a given species. The dry:wet ratio estimate was calculated from additional leaf bundles for each plant species that were treated similarly to our experimental samples, but withholding herbivory. The percentage of consumption of each plant species (relative consumption in Fig. 2A) was calculated as the dry mass consumed of species X, divided by the total dry mass consumed for all plant species during the assay, multiplied by 100, and averaged for all individuals.

Paired *S. grandis* selection experiments

The fresh leaf paired plant preference comparisons were a measure of initial sensory preference by locusts. Leaves (excluding sheath and stems) were collected from the field and two samples of equal size (three, 5 cm long, leaves) and mass were immediately placed into water-filled petri dishes via two slits on the lid. Locusts were placed individually in small plastic cages with the two plant samples. We left the locusts undisturbed until they completed their first meal, or up to six hours. Locusts that did not eat were excluded from the analyses. The outcome was thus a measure of initial plant preference and a score of either win or loss was assigned to each sample for each test. Wins and losses were assigned based on the percent of each sample that was eaten. Insects were closely monitored such that we could account for uneaten and dropped leaf tips. We analyzed the proportion of wins:losses against the null prediction that there would be an equal number of wins and losses for each plant treatment.

The dried, ground leaf paired preference comparisons were conducted by collecting the leaves (excluding stem and sheath) from treatment plots, drying at 60°C for 24 h, and then milling and grinding the leaves to fine powder to pass through a 100 um mesh screen. Field-collected, final larval instar locusts were offered two dishes, each containing 50 mg of a given plant sample, plus a third dish with water, and were permitted to eat from both dishes for eight hours. No locust completely finished any food dish. We conducted this assay to determine a ranked preference among two plant treatment groups (i.e. grazed vs ungrazed and fertilized vs unfertilized), thus, a win or loss was assigned to each paired comparison. Wins and losses were assigned based on the dry mass eaten of each sample. We analyzed the proportion of wins:losses against the null prediction that there would be an equal number of wins and losses for each plant treatment using chi squared tests.

These paired *S. grandis* selection experiments were conducted over two weeks in July 2010, using 4th and 5th instar field-collected locusts. Equal numbers of 4th and 5th instar nymphs and males and females were used in each treatment group. It is important to note that we did not control for nutritional status of the locusts, thus these tests examined preferences of locusts who had recently been feeding on unfertilized plots near the IMGERS field station.

Livestock grazing fields: locust and plant surveys

Samples were collected near 43°32' N, 116°32' E on July 8, 2009 (e.g., *12*). For details see Figure 2 legend.

Plant chemistry

To measure plant chemistry, we collected all aboveground biomass from 1 m^2 patches. All patches were collected within one week of July 22, when field populations of *O. asiaticus* are in the 5th instar, and approximately three weeks prior to peak aboveground biomass and seeding (*36*). Samples from the grazing treatments were all collected on the same day. Samples from the N fertilized and unfertilized plots were all collected within four days of each other. Plants were sorted by species, then by leaf and stem. Leaves (with sheaths and stems removed) for a given species were combined for each 1 m^2 sample, dried, and then ground to fine powder to pass through a 100 um mesh screen. Thus, each sample was a composite of all leaf blades from a given species from a

1-m^2 quadrat. Carbon and nitrogen content were measured using a Perkin-Elmer model 2400 CHN analyzer. To measure protein content, the Bio-Rad micro assay based on the Bradford assay [0–8 μg IgG (bovine gamma globulin)] was used, with duplicate samples read in triplicate (similar to *8*).

Statistical analyses

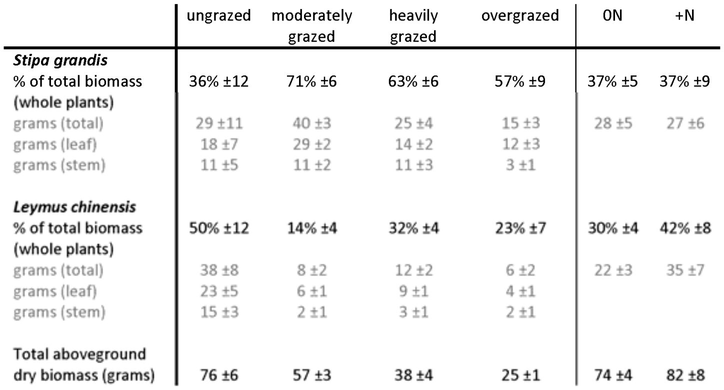
All data were tested for assumptions of normality and homoscedasticity implicit in parametric tests. Arcsine or log transformations were performed as necessary. Analyses were performed using Statistica 10 (2010).

Fig. S1.

Photos from a field heavily grazed by sheep (A; with C. Ford) and a field that has been protected for five years (B).

Table S1.

Relative aboveground dry biomass of *Stipa grandis* and *Leymus chinensis* contained within 1 m^2 vegetation samples. The middle section is the biomass from the samples we used to determine plant nutrient content over the different livestock grazing intensities. The section furthest right is the biomass that remained in the control (0N) and N fertilized (+N) field cages at the end of the experiment. These data illustrate that livestock grazing and N fertilization do influence the balance of these two species, but that both grass species are prevalent in all cases. For all data points, we have included the mean ± standard error.



	ungrazed	moderately grazed	heavily grazed	overgrazed	0N	+N
Stipa grandis						
% of total biomass (whole plants)	36% ±12	71% ±6	63% ±6	57% ±9	37% ±5	37% ±9
grams (total)	29 ±11	40 ±3	25 ±4	15 ±3	28 ±5	27 ±6
grams (leaf)	18 ±7	29 ±2	14 ±2	12 ±3		
grams (stem)	11 ±5	11 ±2	11 ±3	3 ±1		
Leymus chinensis						
% of total biomass (whole plants)	**50% ±12**	**14% ±4**	**32% ±4**	**23% ±7**	**30% ±4**	**42% ±8**
grams (total)	38 ±8	8 ±2	12 ±2	6 ±2	22 ±3	35 ±7
grams (leaf)	23 ±5	6 ±1	9 ±1	4 ±1		
grams (stem)	15 ±3	2 ±1	3 ±1	2 ±1		
Total aboveground dry biomass (grams)	**76 ±6**	**57 ±3**	**38 ±4**	**25 ±1**	**74 ±4**	**82 ±8**

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Acknowledgments: The authors would like to thank F. Jin, D. Niren, D. Flynn, Y. Kong, Z. Fan, J. Esman, Q. Chen, and G. Zhang for field and lab assistance, M. Quinlan, D. Denardo, J. VandenBrooks, C. Klok, S. Behmer, and J. Sabo for helpful discussion of experimental design, and M. McCrackin, F. Clissold, and three anonymous reviewers for valuable comments on the manuscript. Supported by the National Science Foundation (EAPSI, DDEP to AJC, DEB-0925017 to JJE, and EAR-0746352 to JFH), Sigma Xi, Achievement Rewards for College Scientists (Marley-Webb and Johnston Foundations), P.E.O. Scholar Award to AJC; The

Chinese Research Grants of Public Welfare Fund for Agriculture (Project no:200903021) and The Foundation of Chinese Academy of Sciences (Project no:Kscx2-yw-z-1021). A.J.C. designed and performed experiments, analyzed data and wrote the paper. J.J.E. and J.F.H. designed experiments and wrote the paper. C.F.F. and S.H. designed and performed experiments. L.K. gave scientific support and guidance. Data used in the analyses are available in the Supporting Online Material.