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Susceptibility to disease across developmental stages: Examining the effects of an entomopathogen on a grasshopper (Orthoptera: Acrididae) pest

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Abstract

An important but under studied component of grasshopper ecology is how pathogens affect grasshopper population dynamics. However, insect population responses to disease may depend upon age demographics which vary temporally. In a field experiment, we varied grasshopper [*Camnula pellucida* (Scudder)] developmental stage (3rd instar, 4th instar, 5th instar, and adults) and fungal pathogen exposure (lab and field) and measured grasshopper mortality and survival. Lab exposed grasshoppers were directly inoculated with the pathogen, while field exposed grasshoppers were passively exposed to naturally occurring spores. Disease increased grasshopper mortality rates and decreased overall survival. However, this effect varied with grasshopper developmental stage and mode of pathogen exposure. Adults were far less susceptible to fungal infection than nymphs. Disease mortality was 52% higher in lab exposed grasshoppers compared to individuals exposed to a natural fungal epizootic in the field. Lab exposure decreased 3rd instar survival rates more than exposure to the pathogen in the field. In contrast, grasshopper survival was invariant with disease exposure for 4th and 5th instars due to peak levels of naturally occurring fungal spores. By August 2010, the field epizootic was declining and food availability became the most important determinant of adult grasshopper survival. Therefore, age demographics may need to be considered when predicting how grasshopper populations will respond to disease.

Key words

Entomophaga grylli pathotype 1, entomopathogen, host-pathogen interaction, epizootic, grasshopper, *Camnula pellucida*, developmental stage

Introduction

Research suggests that predator and food limitation are important components of grasshopper (Orthoptera: Acrididae) population dynamics (Belovsky & Joern 1995; Belovsky & Slade 1993, 1995; Oedekoven & Joern 2000; Branson 2008; Laws & Joern 2013). However, little research has examined the impact of pathogens on grasshopper population dynamics despite the diversity of pathogens that are known to infect grasshoppers (Dempster 1963).

Entomopathogens are common pathogens of insects that can exhibit massive epizootic outbreaks, which dramatically reduce grasshopper populations (Dempster 1963; Hajek & St. Leger 1994) and have been used for the biocontrol of grasshoppers around the world (Goettel *et al.* 1995; Jaronski 2010). However, these pathogens have complex life cycles with epizootics varying spatially and temporally (MacLeod *et al.* 1966; Erlandson *et al.* 1988). For example, massive disease-induced grasshopper die offs often occur under favorable climatic conditions and when the most susceptible developmental stages are abundant (Carruthers *et al.* 1988a; Hajek & St. Leger 1994).

Insect developmental stage can have significant effects on pathogen establishment, development, and fecundity as well as host resistance and mortality (Mackauer 1973). Nymphs are often more vulnerable to infection than adults due to inferior resistance (Hajek & St. Leger 1994; Carruthers *et al.* 1988a). Yet, experimental studies examining how different insect life stages respond to entomopathogens are rare (Hajek & St. Leger 1994). In a field experiment performed in an intermountain prairie, we manipulated entomopathogen (*Entomophaga grylli* pathotype 1) exposure and host developmental stage to assess the susceptibility of the grasshopper *Camnula pellucida* to fungal pathogen infection.

Methods

Study site and organisms.— This research was conducted at the National Bison Range, MT (NBR: 47°21.040 N, 114°10.190 W), at an elevation of 832 m. This site is an intermountain (Palouse) prairie. The study site is dominated by C_3 grasses: *Poa pratensis* L. and *Elymus smithii* (Rydb.) Gould. Common forbs include *Aster falcatus* Lindl., *Achillea millefolium* L., and *Erigeron spp*. The gramnivorous *C. pellucida* (Scudder) (Orthoptera: Acrididae) is common at the site. This grasshopper is an univoltine, egg-overwintering species that hatches in late May through early June (Pfadt 1994). *Camnula pellucida* is an insect pest that undergoes fluctuating population sizes capable of very high densities (Pickford 1963).

Entomophaga grylli pathotype 1, unofficially known as *E. macleodii* (Humber unpubl. data; Casique-Valdez 2012), is one member of a species complex of obligate grasshopper fungal entomopathogens (Goettel *et al.* 1995; Carruthers *et al.* 1997). The entomopathogen *E. macleodii* is endemic to North America and is an obligate pathogen of grasshoppers in the subfamily Oedipodinae (Goettel *et al.* 1995; Carruthers *et al.* 1997; Casique-Valdez *et al*. 2012). Periodically, this pathogen is known to dramatically decrease populations of *C. pellucida* (Pickford & Riegert 1964; Erlandson *et al.* 1988; Carruthers *et al.* 1997) and is common at this site (Kistner, unpubl. data). This entomopathogen overwinters as dormant spores that germinate in the spring and infect grasshoppers by contact (MacLeod *et al.* 1966). Development of the fungus within the grasshopper host depends on environmental conditions and grasshopper developmental stage (Carruthers *et al.* 1988a). The infection leads to death within 7-10 days (Carruthers *et al.* 1997). Just before dying, the infected grasshopper climbs to the top of a plant where it dies grasping the foliage. This posture is characteristic of grasshopper mortality from *E. grylli* and can be easily diagnosed (Pickford & Riegert 1964; Sawyer *et al.* 1997). The cadaver can produce resting spores that transmit the disease in the next year, or conidia which can transmit the disease in the current year (Carruthers *et al.* 1997). Conidia

are highly vulnerable to high temperatures, low humidity, and UV exposure (Carruthers *et al.* 1988b), and tend to be produced under cool, humid conditions, while resting spores are produced under hot, dry conditions (Carruthers *et al.* 1997).

Experimental design.— In order to investigate grasshopper susceptibility to *E. grylli*, we implemented grasshoppers either directly exposed to the pathogen under laboratory conditions or passively exposed to naturally occurring spores in the field. Furthermore, we varied host developmental stage. We recognize that grasshopper susceptibility to disease can be affected by food availability, climate, and population density (Hajek & St. Leger 1994), but we purposely did not design this experiment to match field conditions exactly. Instead, our intent was to assess the ability of *E. grylli* to infect different developmental stages of grasshoppers, and this necessitated a manipulative experiment.

Grasshoppers were added to aluminum window screen cages placed over natural vegetation (basal area = 0.1 m^2; height = 1 m). Each cage had aluminum flashing at its base, which was buried in the ground to prevent insects from entering or leaving (Belovsky & Slade 1995). The cage was secured by wire to wooden stakes, and cage tops were closed using binder clips, which allow easy access but prevent grasshoppers from escaping. Cages were spaced about 2m apart and, as much as possible, each cage was placed over similar vegetation.

Two treatments were used: 1) pathogen exposure (lab or field) and 2) grasshopper developmental stage (stocked with 3rd instars, 4th instars, 5th instars, or adults). Essentially, lab exposed individuals were directly exposed to *E. grylli* under laboratory conditions while field exposed individuals could only contract the disease via naturally occurring *E. grylli*. We used a 2 × 4 completely randomized design with 8 replicate cages for each treatment combination for a total of 64 cages. Treatment combinations were randomly assigned to cages. Based on results from Belovsky and Slade (1995), high grasshopper densities were utilized in this study (10 per cage).

Grasshoppers were collected with insect nets and observed in terraria for 48h prior to stocking to minimize the use of injured individuals. Cages were stocked when particular grasshopper developmental stages were abundant: 3rd instars on July 5th, 4th and 5th instars on July 15th, and adults on August 3rd. To manipulate entomopathogen exposure, grasshoppers were either directly exposed to *E. grylli* conidia in the lab or through a natural epizootic that occurred throughout western MT from June-July 2010. To reduce the risk of unwanted infections confounding the results, lab exposed grasshoppers were collected from a dry, non-irrigated site where *E. grylli* infection rates were less than 3% (Kistner, unpubl. data). In contrast, field exposed grasshoppers were collected near an irrigated hay field where an *E. grylli* outbreak was observed. *Entomophaga grylli* conidia were obtained from infected grasshopper cadavers collected from Charlo, MT in July. Cadavers were stored in individual petri dishes and soaked with deionized water until conidiophores developed (MacLeod & Müller-Kögler 1973). Sporulating cadavers were then placed in a desiccator jar for 12h. Newly formed conidia were collected in deionized water from the bottom of the desiccator jar (Carruthers *et al.* 1988b). *Entomophaga grylli* conidia (~60 conidia/mm^2 in 0.5 ml of water) were topically applied beneath the grasshopper's pronotum (Jaronski pers. comm.). To avoid cross-contamination, lab exposed grasshoppers were kept in a separate room from field exposed grasshoppers. In addition, lab exposed grasshoppers were stocked in the morning while field exposed grasshoppers were stocked during mid-day.

Individuals in cages and cadavers were counted every day (Belovsky & Slade 1993, 1995). Cadavers clinging high in the vegetation or on the sides of the cage were noted as mortality from the disease, as this behavior is uniquely characteristic of the disease. These individuals were left to continue disease transmission, which can occur over time when temperature/humidity is appropriate (Sawyer *et al.* 1997). All other cadavers (mainly those on the ground) were collected, frozen, and later stained with lacto fuchsin (AEML Inc., Pompano Beach, FL) to examine for fungal hyphae, conidia, and spores under a microscope (Sánchez-Peña 2005). The majority of cadavers removed from cages (96%) were not infected with *E. grylli* indicating that this cadaver removal did not affect overall transmission rates. Only individuals whose cause of death could be determined (27% of all experimental individuals were never recovered) were included in the mortality analysis. Mortality due to the disease was the sum of elevated cadavers plus the number of other cadavers found to be infected, and is presented as a proportion relative to all accounted for deaths.

Statistical analysis.—We employed generalized linear modeling (GLM) with a binomial distribution and a logit link function to examine whether disease mortality varied with treatments (Wilson & Grenfell 1997). Grasshopper survival was examined using Cox's Proportional Hazards Model with pathogen exposure and developmental stage as variables (Wilson *et al.* 2002). We used survivorship analysis (Kaplan-Meier) with the Mantel-Cox nonparametric test to compare total mortality (combined pathogen and non-pathogen deaths) between the lab exposed and field exposed treatments. The survivorship analysis was conducted on the first 30 days of each developmental stage treatment, the period before vegetation (food) senesced and cold temperatures caused mortality. All statistics were performed with R 2.10.1 (R Development Core Team 2009).

Results

Grasshopper mortality from *E. grylli* peaked in the third and fourth weeks of July and was no longer detected by mid-August. The majority of fungal deaths (84%) occurred within the first two weeks of the experiment. The disease was fast acting and virulent with less than 5% of disease mortality occurring after an individual transitioned between developmental stages (3rd instar to 4th instar, 4th instar to 5th instar, 5th instar to adult). Disease mortality was significantly higher in lab exposed grasshoppers ($F_{1,62}$ = 8.04, P = 0.018). Only 32% of field exposed grasshoppers died of disease (94 deaths), while 49% of lab exposed grasshoppers died of disease (143 deaths). Overall, the number of grasshopper deaths from *E. grylli* was 52% higher when grasshoppers were exposed to *E. grylli* in the lab (Fig. 1).

Disease mortality rates also varied across grasshopper developmental stages ($F_{3,59}$ = 8.12, P < 0.001). It was clear that 4th and 5th instars exhibited the highest rates of disease mortality as there were much higher numbers of grasshopper cadavers clinging to the vegetation or the sides of the cage with 67% of 4th instar and 53% of 5th instar cadavers compared to 35% of 3rd instar or 13% of adult cadavers. An additional 2% of 4th instar, 2% of 5th instar, and 3% of adult cadavers were determined to have died of fungal infection when microscope examinations revealed the presence of fungal hyphae, conidia, or resting spores. Adults exposed to *E. grylli* in the lab exhibited 60% less disease mortality compared to nymphs. This same trend was seen in adult grasshoppers exposed to natural levels of *E. grylli* in the field. The pathogen exposure × developmental stage interaction was not significant ($F_{3,56}$ = 0.64, P = 0.277).

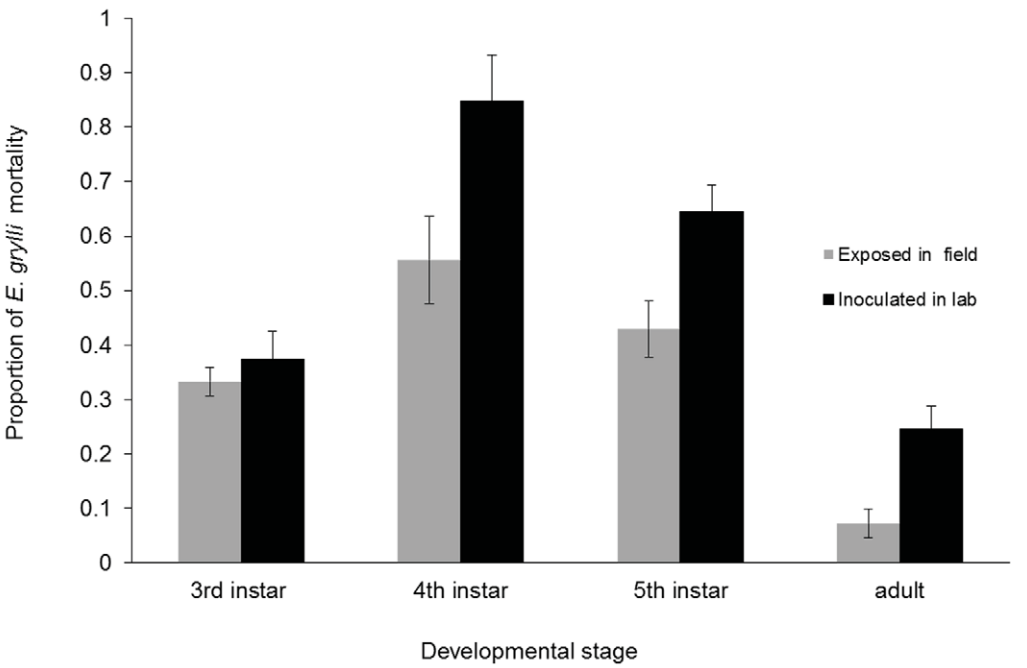

Fig. 1. Proportion of caged grasshoppers that died from *E. grylli* infection (±SE) throughout the duration of the 30 days experiment.

Pathogen exposure and developmental stage affected the survival of experimental grasshoppers, with a significant pathogen exposure × developmental stage interaction (Wald statistic = 106.7, *df* = 7, P < 0.001). Pathogen exposure had the greatest impact on grasshopper survival since the mortality risk for lab exposed grasshoppers was 1.84 times higher than field exposed grasshoppers (95% confidence interval, 1.32-2.58). The entomopathogen reduced grasshopper survival but the effect varied across developmental stages (Fig. 2). Lab exposed 3rd instars exhibited greater reductions than those exposed in the field (Mantel-Cox = 5.750, *df* = 1, P = 0.016). Field exposed 4th instars exhibited greater survival than their lab exposed counterparts, but this trend was not significant (Mantel-Cox = 0.642, *df* = 1, P = 0.423). Survival of 5th instars was not significantly different for those exposed to the pathogen in the lab compared to field exposed individuals (Mantel-Cox = 0.023, df = 1, P = 0.881). Adults exposed to the waning field *E. grylli* epizootic in August survived longer than lab exposed adults (Mantel-Cox = 29.434, *df* = 1, P < 0.001).

Discussion

We documented high rates of disease mortality in this grasshopper-entomopathogen system with responses to disease ultimately dependent upon host developmental stage. Clearly, age demographics were important in this experiment. Our study provides experimental field evidence demonstrating that entomopathogens can reduce grasshopper numbers, but this impact varies across life stages.

Although disease mortality was widespread in all treatment combinations, lab exposed grasshoppers exhibited a two-fold higher disease mortality rate than field exposed grasshoppers. This outcome is unsurprising given that *E. grylli* is highly sensitive to hot and dry conditions and has an estimated 48h life span in the field (Carruthers *et al.* 1988b). Furthermore, lab exposed grasshoppers were directly inoculated with high levels of infectious conidia. However, our results must be approached with caution given that lab exposed grasshoppers may have been exposed to naturally occurring *E. grylli* at the site where they were collected or in the experimental cages themselves. A combination of laboratory and field experiments is required to verify that susceptibility to entomopathogens varies across grasshopper developmental stages. Despite this discrepancy, both lab and field exposed individuals exhibited similar responses to disease across different developmental stages.

Nymphs were highly vulnerable to *E. grylli* infections while adults were far more resistant (Fig. 1). This difference in susceptibility arises as a function of the adult's thicker exoskeleton, making penetration by an entomopathogen more difficult (MacLeod *et al.* 1966; Hajek & Leger 1994). In addition, adults were present when it is hot and dry, conditions that are least favorable for conidia propagation and survival (Carruthers *et al.* 1997).

High rates of *E. grylli* mortality led to steep declines in grasshopper numbers (Fig. 2). Survival rates varied by mode of pathogen exposure with lab exposed grasshoppers having a 1.84 times higher risk of dying than their field exposed counter parts. In addition, this effect varied with developmental stage as indicated by a significant pathogen exposure × developmental stage interaction (X^2 = 9.897, df = 3, P = 0.019). While field exposed 3rd instars exhibited greater survival than their lab exposed counter parts, 4th and 5th instar survival was invariant with mode of pathogen exposure. We suspect this is the result of seasonal variation in *E. grylli* abundance at our site and high stocking densities. Fourth and 5th instar treatments began ten days after 3rd instars treatments, coinciding with peak *E. grylli* disease incidence in the field. We found that 36% of *C. pellucida* grasshoppers collected at our site during the third week of July 2010 were infected with *E. grylli* (Kistner unpubl. data). Furthermore, caged populations were stocked at relatively high densities. High host densities are often linked to increased *E. grylli* transmission which leads to higher host mortality (Carruthers *et al.* 1988a; Carruthers *et al.* 1997; Fig. 1). This result is in agreement with our findings that grasshopper disease mortality increased by 60% when host density levels were doubled (Kistner & Belovsky unpubl. data).

High experimental density also explains the low survival rates of adult grasshoppers despite relatively moderate levels of disease mortality (Fig. 1). This is likely the result of the interplay of density and intraspecific competition for food. Grasshoppers at this site are often food-limited, since the vegetation at our site decreases over the summer due to herbivore consumption and desiccation (Belovsky & Slade 1995). Meanwhile grasshopper food requirements increase as

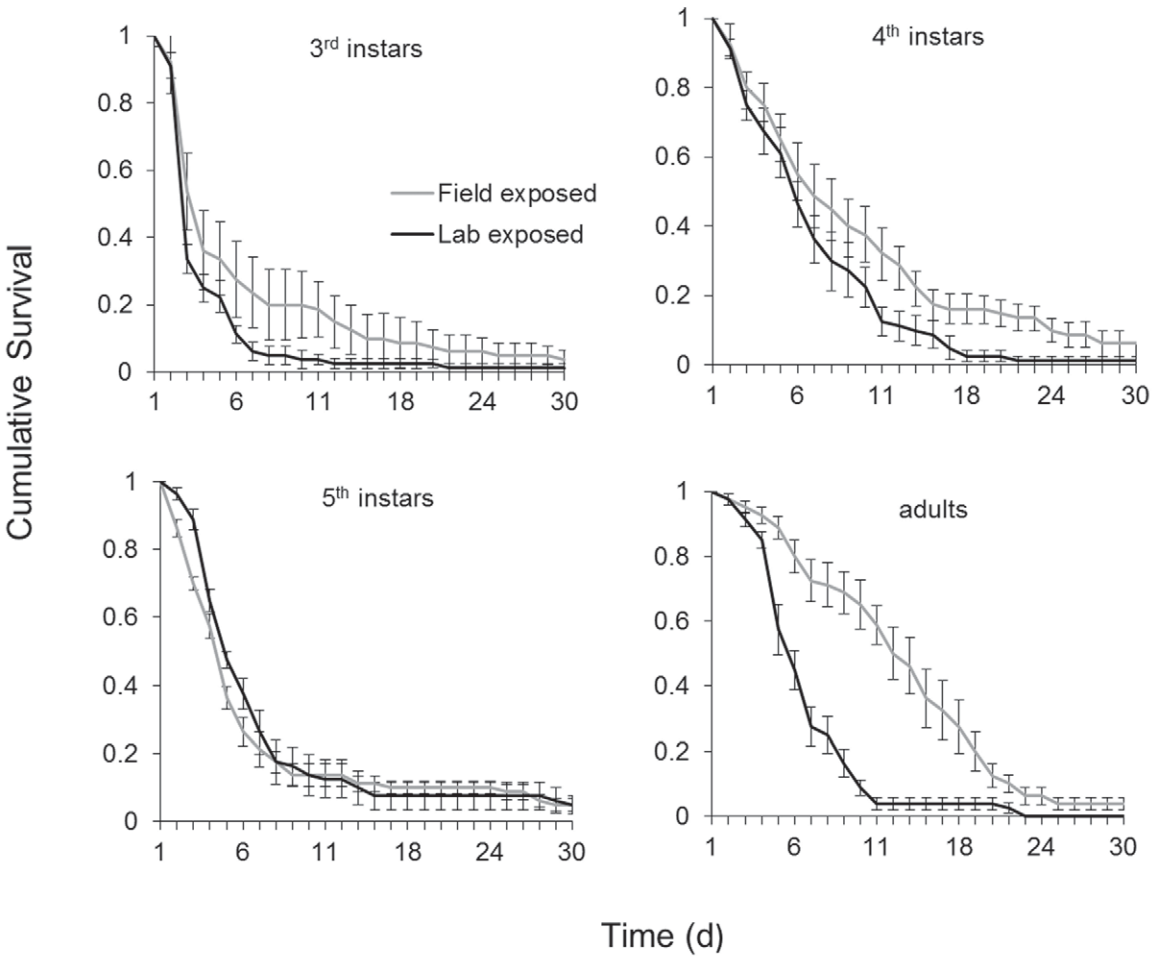

Fig. 2. Grasshopper survival (± SE) for pathogen exposure and developmental stage treatment combinations in caged populations

they mature over the summer (Belovsky & Joern 1995). This became apparent in our caged populations as adult grasshoppers consumed the cadavers of *E. grylli* killed individuals (Lockwood 1988, 1989). This act of cannibalism may have resulted in the steep decline of lab exposed adults due to increased rates of disease mortality (Figs 1-2). While the densities used in this experiment are within the range observed at this site (Belovsky & Slade 1995), future studies should consider using more moderate density levels to reduce confounding variables like density-dependent disease transmission and food limitation.

Taken together, our results suggest that grasshopper susceptibility to entomopathogens is highly dependent upon developmental stage. Our findings are consistent with the work done by Carruthers *et al.* (1988a,b; 1997) which suggests *E. grylli* epizootics are linked to high densities of susceptible nymphs. However, additional research is needed to understand how host dynamics affect grasshopper population responses to entomopathogens.

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