Abiotic and Pathogen Factors of *Entomophaga grylli* (Fresenius) Batko Pathotype 2 Infections in *Melanoplus differentialis* (Thomas)

by

DWIGHT KEITH TILLOTSON

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Approved:

David C. Margolies

David C. Margolies

Major Professor

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BACKGROUND AND LITERATURE REVIEW

The differential grasshopper, Melanoplus differentialis (Thomas), occurs most abundantly in the central and southern Great Plains and can cause considerable damage to crops (USDA 1954). This univoltine species hatches in June and occurs as an adult from July until frost.

The application of chemical insecticides is the currently used means of controlling grasshoppers, acting quickly and resulting in rapid reductions in the pest population. However, this approach has its problems. The occurrence of resistance of the insect pest to the insecticide is an ever-present threat. Also, the non-selective nature of the chemicals results in mortality to non-target insects, including predators and parasites of the grasshopper. Chemicals only achieve temporary effects and must be repeatedly applied from season to season and within seasons. Insect resistance and off-target effects are less evident when control is effected by the use of natural enemies. Natural enemies are often very selective, with the possibility of establishment as a permanent mortality factor. Furthermore, the ecosystem is less adversely affected. Natural enemies, though, do not provide the rapid reductions afforded by chemical insecticides.

One natural enemy showing potential for biological control is Entomophaga grylli (Fresenius) Batko (Zygomycetes:Entomophthorales), an obligate fungal pathogen. This fungus occurs worldwide and has been cited for its role in epizootics in Acrididae (Roffey 1968; Chapman & Page 1979; Rockwood 1950; Smith 1933; Pickford & Riegert 1964; Skaife 1925; Milner 1978; Dewhurst 1978). Two pathotypes of E. grylli are recognized in North America (Soper et al. 1983). Pathotype 2 differs from

pathotype 1 in the absence of conidiophores which penetrate the integument to produce external conidia and its ability to produce epizootics under generally drier conditions. Suppression of conidia formation can occur in pathotype 2 if the integument of the host remains intact for some time after host death (Humber & Ramoska 1986). Under these conditions (i.e. low oxygen), thick-walled mature resting spores form that effectively withstand desiccation.

With proper moisture and relative humidity, mature resting spores can produce a germ conidiophores and germ conidia. Germ conidia have been determined to be an infective stage of E. grylli and invade the host through the integument when the relative humidity of the microenvironment is 95-100% (Krueger & Ramoska 1985). Once penetration has occurred, the conidia release protoplasts which proliferate in the host's hemocoel. Exact progress of pathogenesis is somewhat uncertain, but preliminary observations suggested that fat body attrition and nutrient depletion cause host death (Funk 1987). In a field study, Pickford and Riegert (1964) found death to occur about 13 days after infection at a mean temperature of 20°C. In a laboratory study conducted at 25°C:18°C (day:night) and L:D 16:8, mortality occurred at a mean time of 14 days for field collected grasshoppers and 25 days for laboratory reared grasshoppers (Kish 1984). In field observations, researchers have reported a preponderance of E. grylli induced mortality in late afternoon (Pickford & Riegert 1964; Skaife 1925). Laboratory studies have shown that all mortality due to E. grylli occurred betweem 5 pm and 11 pm (Ramoska, personal communication). In the later stages of

the disease, the infected grasshoppers climb plant stems and die with the heads pointing up and legs clasping the stems.

As the host nears death, the fungal parasitic protoplasts form cell walls to become hyphal bodies. These pleiomorphic hyphal bodies bud to form round immature resting spores. Unless the integument of the grasshopper is ruptured, the cell walls of the immature resting spores thicken to form mature resting spores (Funk 1987). However, the abdomens of many infected grasshoppers soften dramatically 1-4 h after death and the intersegmental membranes easily rupture. If this occurs, hyphal bodies and immature resting spores are released (Humber et al. unpublished). With 95-100% relative humidity, these two fungus stages may form conidiophores and conidia that differ from germ conidia. These conidia, called the cryptoconidia (Humber & Ramoska 1986), differ from germ conidia in four ways. First, they are pyriform instead of globose (personal observation). Second, they occur at a different point in the life cycle (Humber & Ramoska 1986). Third, preliminary evidence suggests that germ conidia initiate an epizootic and cryptoconidia sustain it (Humber & Ramoska 1986). Finally, cryptoconidia form and begin to discharge within a few hours following cadaver disruption (Humber et al. unpublished). In laboratory studies, mature resting spores have required at least 3 days in distilled water followed by incubation at 95-100% relative humidity to produce germ conidia (Krueger & Ramoska 1985; Krueger personal communication).

If the integument remains intact after host death, hyphal bodies and immature resting spores develop into mature resting spores. In this stage, the fungus can overwinter and survive hot, dry periods. When ade-

quate moisture and temperature conditions once again occur, the mature resting spore can germinate to produce a second type of conidia, infective germ conidia. It is in this manner that the disease is spread from generation to generation of the host.

Part I

Effects of temperature and photoperiod on percent mortality, time to mortality, and mature resting spore production in *Melanoplus differentialis* infected by *Entomophaga grylli* pathotype 2

INTRODUCTION

The differential grasshopper, Melanoplus differentialis (Thomas), can cause considerable damage to crops in the Great Plains (USDA 1954). Among its natural enemies in this region is the fungal pathogen, Entomophaga grylli (Fresenius) Batko (Zygomycetes:Entomophthorales) which has been known to cause epizootics in species of Acrididae in North America (Rockwood 1950; Pickford & Riegert 1964; Smith 1933; Walton & Fenton 1939) and throughout the world (Roffey 1968; Chapman & Page 1979; Skaife 1925; Milner 1978; Dewhurst 1978). Two pathotypes of E. grylli have been identified using isozyme analysis (Soper et al. 1983). Fungal stages of pathotype 1 cadaver produce conidiophores within the grasshopper that penetrate the integument to form the infective stage, the conidium, on the surface. In grasshoppers killed by pathotype 2, the host integument must be torn open for conidiophores and conidia to form (Humber & Ramoska 1986).

Fungal epizootics can cause significant reductions in grasshopper populations and warrant further study regarding the control of pest species. A fungal epizootic involves the interaction of the host, the pathogen, and the abiotic environment. The influence of the abiotic environment can be subdivided into the effects of moisture, temperature, and light on both the host and the pathogenic fungus.

Moisture may be the most important factor for the fungus in the generation of epizootics, especially for the infection process (Wilding 1981). Once infection has occurred, however, the importance of environmental moisture may lessen because pathogenesis proceeds within the host. Any temperature and photoperiod effects on the temperature process

might be obscured by the dominant effect of environmental moisture (Wilding 1981). When moisture becomes less important, e.g. pathogenesis, the effects of temperature and photoperiod may become more evident.

Many authors have studied the effects of temperature on stages in the life cycles of Entomophthorales species. Of great importance to epizootics, though, are temperatures favorable to development, or sporulation, of the infective stage. Favorable sporulation temperatures for most species of Entomophthorales range from 16° to 30°C (Yendol 1968; Pady et al. 1971; Wilding 1981; Kenneth et al. 1972; Newman & Carner 1975; Shimazu 1977; Milner & Lutton 1983). Some entomophthoralean species, however, have caused epizootics when temperatures ranged from 5° to 25°C (Wilding 1970) and 5° to 12° C (Teernstra-Eeken & Engel 1967).

Temperature influences grasshopper activity, with feeding and movement generally occurring above 20°C (USDA 1954). The lethal limits for grasshopper hosts are broad. For three Melanoplus species these limits occur from -15° (Knipling & Sullivan 1957) to 55°C (Knipling & Sullivan 1958). The upper ambient limit, though, will not commonly exceed 40°-44°C in the central and southern Great Plains. The metabolic rate increases with temperature up to an optimum, which in turn leads to an increased rate of development (Chapman 1982).

Light may affect organisms by its frequency, its energy, and its daily rhythm, with separate effects on E. grylli, M. differentialis, and a host-pathogen interaction. Any attempt to understand the ecology of E. grylli and M. differentialis, however, should include at least the consideration of the daily rhythm, or photoperiod. Wallace et al. (1976) suggested that photoperiod may act as an environmental signal to syn-

chronize the development of the fungus with the season. In three studies involving Entomophthorales species, photoperiod has been shown to significantly affect resting spore germination. Wallace et al. (1976) found that photophases of 14 h or more greatly enhanced resting spore germination in Entomophthora aphidis. In another study (Stoy et al. 1988) the percentage of germinated spores of E. grylli pathotype 2 increased with increased photophase. Kish (1984) also used E. grylli pathotype 2 and found resting spore germination to be significantly higher at L:D 24:0 than L:D 0:24.

An increasing number of insect species have been found to be influenced by photoperiodism with a varied range of effects (Beck 1980; Saunders 1982). It has been demonstrated or reasonably inferred to control diapause in egg (Wardhaugh 1980; Dean 1982) and adult (Norris 1959; Norris 1965; Geldiay 1971) stages of some Acrididae. Other photoperiodic phenomena observed in Acrididae include male sexual behavior (Perez et al. 1971), egg pod shape (Wardhaugh 1977), developmental time (Dean 1982), body size (Dean 1982), and daily growth layers in the endocuticle (Neville 1967).

The neuroendocrine system regulates growth, development, reproduction, and behavior and is in turn influenced by such environmental factors as photoperiod (Tauber et al. 1986). This raises possibilities regarding host-pathogen interactions, since E. grylli protoplasts have been found in the fat body, central nervous system, muscle, and hemolymph in infected M. differentialis (Funk 1987). The presence of E. grylli protoplasts in both the central nervous system and hemolymph

could result in interaction with grasshopper hormones whose production may have been influenced by photoperiod.

Temperature and photoperiod can affect both the host and pathogen. As seasonally varying factors, they could influence the pathogenesis of E. grylli in M. differentialis. The aim of the study was to examine the effects of the seasonally varying abiotic factors, temperature and photoperiod, on host and fungus. Temperature and photoperiod were selected because both fluctuate in a more or less predictable way throughout the grasshopper's growing season. Therefore, it was possible to test whether these factors have a seasonal effect on pathogenesis of E. grylli in M. differentialis. This was determined by examining percent mortality, time to mortality, and mature resting spore production.

MATERIALS AND METHODS

Adults of M. differentialis were obtained from wild populations in the Manhattan, Kans. area. The fungus, E. grylli pathotype 2, was obtained from diseased grasshoppers, M. differentialis and M. bivittatus, in the Tuttle Creek Reservoir area near Manhattan, Kans. in August-September, 1985. Cadavers showing the symptoms of legs wrapped around plant stem and heads pointed up were collected. The fungus was extracted from the grasshoppers by chopping the cadavers into beakers of distilled water. Grasshopper tissue was removed by pouring the beaker contents through cheesecloth. The filtrate was poured into a separatory funnel to allow the fungus to precipitate. The precipitate was collected and the procedure repeated with fresh distilled water until the supernatant was clear. To demonstrate that the precipitate contained viable E. grylli, M. differentialis were injected with it. Field collected fungi thus cleaned and injected produced the other stages in the fungus life cycle as well as the characteristic climbing and clasping behavior on the sides of the cages.

Once cleaned, the fungus was placed in 15% dimethyl sulfoxide, then transferred to 1 ml glass ampules. The ampules were heat sealed, then placed in a freezer for at least 4 h, after which the ampules were transferred to liquid nitrogen for permanent storage. When needed, the ampules were immersed in water at 40°C, then opened. Next, the fungi were rinsed three times in distilled water and transferred to tissue culture flasks (0.1 ml fungi in 5 ml sterile distilled water) for 3 days at 20°C to induce germ tube formation. Sixteen microliters of gentamiacin were added to each tissue culture flask to inhibit bacterial

growth. The fungi were removed from the tissue culture flasks after 3-4 days and pelleted by low speed centrifugation(3200 rpm). They were suspended in 0.8 ml sterile distilled water and spread evenly on 1% agar plates. Streptomycin (0.1%) and penicillin (0.02%) were added to the agar to inhibit bacterial growth. The plates were left uncovered in a laminar flow hood (model VBM 400,Baker Co.,Sanford, Maine) until the water had nearly evaporated.

At that point agar plates were inverted over petri dishes of equal diameter (90 mm) filled with sterile distilled water, which were in turn placed in covered 210 mm diameter clear glass casserole dishes lined with water-soaked paper towels. The casserole dishes were placed at 20°C for 6 h in order to obtain adequate production of the germ conidium, the invasive stage of the fungus.

Fungi comprised of germinated and ungerminated mature resting spores were rinsed from the agar plates after 6 h and suspended in saline/glycerol (30v/70v). Fungal concentration was determined with an improved Neubauer hemacytometer. Adult grasshoppers were anesthetized with carbon dioxide and each was injected with a mixture of approximately 1400 mature resting spores, germinated and ungerminated, using a syringe with a 27.5 ga. needle mounted in a microapplicator (ISCO model M, Instrumentation Specialties Co., Inc.,Lincoln, Nebr.). The grasshoppers were injected in the second or third intersegmental membrane in the ventral region of the abdomen.

Throughout the treatment, all grasshoppers were fed a quantity of washed leaf lettuce that could be consumed in approximately one day.

Excess lettuce was removed the following day. Wheat bran was served in a 5 cm diameter plastic petri dish and replenished as needed.

The grasshoppers were treated under four environmental conditions that varied in temperature and photoperiod. Two temperatures were used: 26.5(±0.5) and 14.0(±0.5)C. These temperatures represented average daily temperatures for mid-July and mid-October. These temperature means represented a 1951-1980 average for Manhattan, Kans. Mid-July to mid-October represented the approximate lifespan of adults of M. differentialis. Two photoperiods were used: L:D 16:8 and 12:12, approximating naturally occurring daylengths for Manhattan, Kans. in mid-July and mid-October respectively; photophases included civil twilight.

The temperatures and photophases were combined to yield four different treatment combinations: 26.5°-16 h (H16), 26.5°-12 h (H12), 14°-16 h (L16), and 14°-12 h (H12). Relative humidity was subject to ambient conditions . The mean relative humidities for H16, H12, L16, and L12 were 47 (SD=7.05), 67 (SD=7.4), 75 (SD=9.9), and 78 (SD=11.0), respectively.

Experimental treatments H12 and L12 were maintained in two Sherer model CEL-25-7 environmental chambers, L16 in a Sherer model 4005-OB environmental chamber (Sherer-Gillett Co., Marshall, Mich.), and H16 in a room measuring 2.6 m x 3.1 m x 4.1 m. Fluorescent lights were used for illumination and had the following intensities in foot-candles for H16, H12, L16, L12 respectively: 195 (SD=6), 246 (SD=79), 200 (SD=115), 515 (SD=77). Grasshoppers were kept in wire mesh cages measuring 45 cm x 31 cm x 31 cm during experimental trials. Each environmental chamber/treatment room represented an experimental unit.

Each of the four treatments contained 200 adult grasshoppers. After an entrainment period of 21 days in the adult stage, all 800 grasshoppers were injected with the fungus. Dead grasshoppers were removed daily. Date and cause of death were recorded. Even though a control group was not included in the experimental design, prior experiments had been carried out in which 50 grasshoppers were injected with 2 microliters of E. grylli suspended in saline/glycerol (30v/70v) while 150 were injected with 2 microliters of the solution only. By the time all 50 of the treated grasshoppers had died from E. grylli infection (18 d post-injection), only 2 (1.3%) of the 150 control grasshoppers had died, suggesting that an injection effect would not significantly affect the results.

To detect the presence of E. grylli, a wire loop was inserted through an incision in the first or second ventral abdominal segment and forward into the thorax to obtain a tissue sample. A wet mount of the tissue sample was examined at 100X on a phase contrast microscope. The presence of parasitic microorganisms was recorded. Whenever detected, E. grylli, regardless of life stage, was assumed to be the only cause of death if other parasites were absent. Grasshoppers dying from E. grylli were collected from each treatment daily and The fungi were extracted as described for field samples. The proportion of mature resting spores was determined if a minimum of 20 fungi appeared on the hemacytometer. A 2% aceto-orcein stain was applied to identify the mature resting spores.

A two sample t-test was applied separately for photoperiod and temperature for each of the three effects: percent mortality, time to mortality, mature resting spore production. Mature resting spore production

was expressed as a proportion for analysis and reporting of results. Temperature and photoperiod data points were plotted on separate charts for each of the three effects prior to t-tests to examine possibilities for interaction between the two factors. An arc sine square-root transformation was carried out on percentages and proportions prior to statistical analysis (Zar 1984).

RESULTS

Mortality was considerably greater at high temperature under both photoperiods than at low temperature. Percent mortality in treatments H16, H12, L16, and L12 was 67, 61.5, 16, and 9.5% respectively. The two-sample t-test between the two temperatures (14.0 and 26.5) ignoring photoperiod showed differences in mortality to be significant ($P=0.01$, $t=9.98$, $df=2$). A plot of the data (Fig. 1) indicated that percent mortality was slightly and uniformly higher at each temperature under long daylength (L:D 16:8) than under short daylength (L:D 12:12). However, the t-test of mortality between photoperiods ignoring temperature showed no significant differences ($P=0.88$, $t=0.17$, $df=2$). A plot of the data (Fig. 2) indicated that percent mortality was considerably and uniformly higher at each photoperiod under high temperature than at low temperature. In both Figs. 1 and 2 the parallel lines suggested the absence of temperature-photoperiod interaction, although it could not be test statistically.

Time to mortality in days post-injection was 13.6, 14.6, 26.0, and 34.2 for treatments H16, H12, L16, and L12 respectively. The possibility of temperature-photoperiod interaction existed because lines were non-parallel (Figs. 3 & 4), lending uncertainty to the results of an analysis of differences. Differences in time to mortality between temperature conditions were non-significant ($P=0.06$, $t=3.87$, $df=2$; two-sample t-test). Time to mortality was not significantly related to photoperiod ($P=0.73$, $t=0.40$, $df=2$; two-sample t-test).

Mean mature resting spore production was considerably greater at high temperature under both photoperiods than under low temperatures.

For treatments H16, H12, L16, and L12 mature resting production was 0.50, 0.49, 0.11, and 0.04 respectively. The t-test for differences between the two temperatures (14.0 and 26.5) was significant (P=0.02, t=7.45, df=2). A plot of the data (Fig. 5) showed that mature resting spore production production was higher at each temperature under L:D 16:8 than under L:D 12:12, but the differences were not uniform. The t-test for photoperiod differences indicated that mature resting spore production at L:D 16:8 did not differ significantly from L:D 12:12 (P=0.86,t=0.20,df=2). A plot of the data (Fig. 6) showed that mature resting spore production was higher for both photoperiods at high temperature than low temperature, but the differences were not uniform. In both cases the occurrence of non-parallel lines suggested the possible absence of temperature-photoperiod interaction, although it could not be statistically analyzed.

DISCUSSION

The significant positive relationship between temperature and mortality was consistent with the general trend of fungal pathogenesis in arthropods, including species of Entomophthorales (Brandenberg & Kennedy 1981; Milner & Bourne 1983; Smitley et al. 1986; Wilding 1970; Stimman 1968).

The effects of temperature on mean disease length, although statistically insignificant at P=0.06, may still have had biological significance. For example, temperature had been found to be inversely related to the infection period for other species of Entomophthorales (Brandenberg & Kennedy 1981; Carner 1976; Milner & Bourne 1983; Milner & Lutton 1983; Smitley et al. 1986; Wilding 1970; Stimman 1968; Eilenberg 1987). The infection period decreased with increasing temperature until a lethal or inhibiting limit to the fungus was reached.

There was a significant positive relationship between temperature and mature resting spore production. Despite the statistically significant results, effects of temperature must be regarded with caution due to the possibility of a temperature-photoperiod interaction. However, increasing temperature appeared to accelerate the rate of development of E. grylli, and thus production of mature resting spores. The very low proportions of mature resting spores in the low temperature treatments (L16 & L12) tentatively suggests that grasshoppers infected in the field under cool conditions prevalent in mid-autumn would be less likely to produce enough resting spores to initiate infections the following spring. Reservoirs of overwintering mature resting spores would more likely come from grasshoppers infected earlier in the growing season

when more favorable temperatures would occur. In order to test whether the lab results apply to a field situation, the ability of an infected grasshopper to thermoregulate in the field would need to be assessed.

Of the two temperatures I tested, 26.5°C appeared to be more favorable for pathogenesis than 14°C because host mortality was higher. Although preliminary, these results suggest that additional trials should be run over a wider range of temperatures to determine optimal temperatures for host mortality and time to mortality. An optimal temperature would provide the basis for further laboratory tests on epizootics of E. grylli, which would lead to field studies. Because optimal temperature for the development of the fungus within the host may not be the same for infection of the host, both of these two aspects need to be tested. Furthermore, field infections by E. grylli may not correlate well with temperatures established in laboratory studies because more important factors, such as moisture, may obscure the effect of temperature. Roberts and Campbell (1977) cited a trend matching temperatures of field epizootics with those found in the laboratory, but evidence also exists to the contrary (Berisford & Tsao 1974; Wilding 1975). Each study needs to be regarded with caution regarding field-laboratory correlations.

Photoperiod did not appear to significantly affect mortality, time to mortality,and production of mature resting spores. Direct exposure of E. grylli pathotype 2 to light resulted in significant effects on fungal growth (Kish 1984). The effects of photoperiod on the fungus after mediation through the host, though, have been little studied in entomogenous fungi. Research on Entomophthora gammae in its host Pseudoplusia includens, the soybean looper, showed that photoperiod had no

effect on the spore in infected larvae (Newman & Carner 1975). In their discussion, the authors proposed the possibility that temperature and photoperiod could alter spore form ratios in the field, where changes "occur subtly as the season progresses and are paralleled by changes in plants and in host insect physiology." They added that temperature and photoperiod effects could act, not only during the time of infection, but also over more than one generation of the fungus.

Photoperiod has been only one of several possible factors affecting pathogenesis and the life cycle of the fungus. Its effect, if present, would require further research. The next step, then, would be the replication of the procedure in this project. The design then could be treated as a 2 x 2 factorial design. The data could be analyzed with an analysis of variance and thus provide a better indication of significant effects as well as test for interaction between temperature and photoperiod.

Part II

Effect of cadaver age on cryptoconidia production in *Melanoplus differentialis* infected by *Entomophaga grylli* pathotype 2

INTRODUCTION

Two pathotypes of Entomophaga grylli (Fresenius) Batko are recognized from North America (Soper et al. 1983). Pathotype 2 attacks grasshoppers of genus Melanoplus, but, unlike pathotype 1, does not form the masses of conidiophores that penetrate the integument to form conidia. Pathotype 2 forms resting spores shortly after the death of the host if the cadaver integument is not ruptured (Humber et al. unpublished; Ramoska personal communication).In this stage the fungus can overwinter or withstand hot dry conditions, even after the integument has disintegrated.

When favorable temperature (20^{o}-30^{o}C) and humidity conditions (90-100% rh) occur, resting spores can then form germ conidia that will discharge and infect a new host (Roberts & Campbell 1977; Pickford & Riegert 1964). If, however, the cadaver integument is disrupted soon after host death, and temperature and humidity conditions are appropriate, hyphal bodies and immature resting spores will produce conidia that will be discharged immediately instead of developing into mature resting spores. The exposure to atmospheric levels of oxygen has been found to stimulate this process (Humber & Ramoska 1986). The term "cryptoconidia" is used for these conidia because they form at sites temporally and spatially removed from those where conidiogenesis would typically occur (Humber & Ramoska 1986).

The identification of the cryptoconidium is potentially significant, since it may account for much or most of the horizontal transmission of E. grylli pathotype 2 during outbreaks (Humber et al. unpublished).

Aspects of this infective stage need to be studied regarding its ability to initiate and sustain epizootics.

This study seeks to focus on aspects relating to the dose of cryptoconidia required to kill a host, looking to the source of the inoculum--the cadaver. The dose produced by the cadaver depends on: (1) the quantity of fungi capable of producing cryptoconidia; that is, hyphal bodies and immature resting spores, (2) the proportion of hyphal bodies and immature resting spores that will produce cryptoconidia (active stages).

A third possible factor which could be related to these two factors is cadaver age. Cryptoconidia production occurs when, given adequate relative humidity, the cadaver integument is torn open (Ramoska personal communication). The integument splits open due to the following factors acting alone or in combination: fungal enzymatic activity, physical motion of the vegetation, grasshoppers landing on the cadaver, cannibalism (Humber et al. unpublished).

The timing of cadaver integument disruption could be significant in its effect on cryptoconidia production by affecting the number of hyphal bodies and immature resting spores produced in the cadaver. As long as the cadaver integument remained intact, hyphal bodies and immature resting spores would turn into mature resting spores, which could not produce cryptoconidia (Humber & Ramoska 1986). The older the cadaver, then, the greater likelihood of a decreasing proportion of hyphal bodies and immature resting spores and an increasing proportion of mature resting spores. In that event, increasing cadaver age could result in decreasing cryptoconidia production.

This study investigated the possible effects of cadaver age on the production of cryptoconidia by M. differentialis infected with E. grylli pathotype 2. This was achieved through the measurement of:

1. number of mature resting spores per cadaver
2. number of hyphal bodies and immature resting spores per cadaver
3. number of cryptoconidia produced per sample
4. number of cryptoconidia produced per sample as a proportion of the number of hyphal bodies and immature resting spores per cadaver.

MATERIALS AND METHODS

To obtain fungi for cryptoconidia production, adult differential grasshoppers, M.differentialis, were infected with injected doses of E. grylli pathotype 2. The fungi were first concentrated by centrifugation, then suspended in 2 microliters of saline/glycerol (30v/70v) and injected into the grasshoppers using a 23 ga. needle mounted in a micro-applicator (model M, Instrumentation Specialties Co., Inc., Lincoln, Nebr.). Each individual was injected with approximately 2940 fungi consisting of mature resting spores (germinated and ungerminated) and germ conidia of E. grylli. The dose per grasshopper was determined with an improved Neubauer hemacytometer.

Infected grasshoppers were kept in wire mesh cages measuring 45 x 31 x 31 cm at 24°C and L:D 16:8. They were maintained until death (13-16 d post-injection) on a diet of leaf lettuce and wheat bran. As soon as death was observed, grasshoppers were removed from cages and the fungus was either immediately extracted by the methods described in Part I, or stored in a sealed petri dish at 20°C. Death was defined as the complete cessation of movement of the grasshopper when prodded.

The fungus was extracted from cadavers either at the time of death (0 h), or 24 h after death. Four grasshoppers were examined at 0 h and 5 at 24 h. Each cadaver was coarsely chopped and immersed in 0.15M sodium chloride. After stirring, the chopped tissue was poured through two layers of cheesecloth and centrifuged. The supernatant was drawn down to 0.7 ml and the remaining pellet of fungus was resuspended. The amount of fungus extracted from each grasshopper was quantified. Another sample, stained with 2% aceto-orcein, was used to determine the propor-

tion of mature resting spores. The number of hyphal bodies and immature resting spores could then be determined by subtracting the number of mature resting spores. An improved Neubauer hemacytometer was used in both quantifications. The fungus was transferred to 1% water agar plates treated with streptomycin (0.01%) and penicillin (0.02%). Excess liquid on the plates was allowed to dry in a laminar flow hood and the plate was inverted over a petri dish of equal diameter (90 mm).

To collect cryptoconidia, five cover slips covering about 40% of the agar plate were equally spaced on the lower dish. The cover slips rested on four layers of paper towels soaked with distilled water to help maintain a saturated atmosphere. In addition, each dish was placed within a transparent glass casserole dish lined with paper towels saturated with distilled water and covered with a fitting lid. Distilled water was added periodically to maintain saturation.

Each dish was placed in an environmental chamber at 20°C under constant fluorescent light. These conditions have been shown to stimulate germination of pathotype 2 resting spores (Kish 1984).

Every hour for 24 h the cover slips for each sample were removed and replaced with five new ones. The exposed cover slips were mounted on glass microscope slides, using lactophenol as the mounting fluid, then sealed around the edges with Permount. The cryptoconidia on each coverslip were counted and the mean was calculated for the five coverslips representing each grasshopper each hour. It was not possible to differentiate primary and secondary cryptoconidia, therefore both were included in the counts of cryptoconidia. A measurement of active hyphal bodies and immature resting spores was determined by comparing the number of

cryptoconidia produced against the total number of hyphal bodies and immature resting spores on the plate and expressing it as a percentage.

The number of cryptoconidia for 0 h cadavers was tabulated over a 24 h period, by which time the hourly counts leveled off (Fig. 7). For the 24 h treatment, hourly counts were only recorded through 15 h for the same reason. Only data through 15 h were used in the statistical analysis of the two treatments.

A one-way analysis of variance for a completely randomized design using the ANOVA procedure (SAS Institute 1982) was used to analyze the following: (1) number of hyphal bodies and immature resting spores extracted from each cadaver, (2) number of cryptoconidia produced from each cadaver; (3) cryptoconidia produced per cadaver as a percentage of the number of hyphal bodies and immature resting spores; (4) proportion of mature resting spores in each cadaver. Percentages and proportions were subjected to arc sine square root transformation prior to data analysis (Zar 1984).

RESULTS

Mature resting spores were found in all five of the 24 h cadavers, resulting in a mean proportion of 0.27 (SD=0.10). This differed significantly from the 0 h treatment, in which none were found in the four cadavers (P=0.001, F=28.19, df=1,7) (Fig. 8). The number of mature resting spores were subtracted from the total, since they do not produce cryptoconidia. This left a mean count of 1,257,631 (SD=132,184) hyphal bodies and immature resting spores . A mean count of 288,688 (SD=89,450) hyphal bodies and immature resting spores were found in the four cadavers in the 0 h treatment. The 0 and 24 h treatments differed significantly for mean counts of hyphal bodies and immature resting spores (P=0.0001,F=155.6,df=1,7) (Fig. 9).

Mean production of cryptoconidia was 17,892 (SD=5,901) for the 0 h treatment versus 50,968 (SD=22,590) for the 24 h treatment. The two means were significantly different (P=0.03,F=7.93,df=1,7) (Fig. 10).

Mean production of cryptoconidia as a percentage of the number of hyphal bodies and immature resting spores produced in the cadaver did not differ significantly between the 0 and 24 h treatments (P=0.11, F=3.37, df=1,7) (Fig. 11). The 0 h treatment resulted in 6.54% (SD=2.26) versus 4.03% (SD=1.75) for the 24 h treatment.

DISCUSSION

As hypothesized, the proportion of mature resting spores was higher in the 24 h cadavers. However, this did not result in lower cryptoconidia production as expected. The 24 h cadavers contained a significantly higher number of hyphal bodies and immature resting spores. This suggests that proliferation of hyphal bodies may occur during at least the first 24 h after death in nature. A mechanism explaining the multiplication of hyphal bodies as well as formation of immature resting spores from hyphal bodies remains unclear. The presence of mature resting spores indicates that at least a portion of the hyphal bodies produced immature resting spores (Fig. 12).

The percentage of cryptoconidia produced did not differ significantly between the two treatments, suggesting that there was also no difference in proportions of active hyphal bodies and immature resting spores.

The findings of this study suggest, then, that cadavers whose integuments are disrupted 24 h after death could more likely initiate or sustain an epizootic due to higher cryptoconidia production. Such an occurrence on a widespread scale at the time of host death, however, could possibly lessen the intensity of an epizootic.

SUMMARY AND CONCLUSION

Seasonally varying abiotic factors as well as features of the pathogen were investigated in regard to their effects on pathogenesis of E. grylli pathotype 2 in M. differentialis.

Temperature and photoperiod were chosen to examine for effects on the post-infection process (pathogenesis). Effects were determined by measuring percent mortality, time to mortality, and production of mature resting spores. Temperature was significantly and positively related both to mortality as well as production of mature resting spores. Because it was not possible to statistically analyze temperature and photoperiod interaction, any significant effects were regarded with caution. Although the effect of temperature on time to mortality was non-significant (P=0.06), the relatively low probability value suggested a possible influence.

Other studies have demonstrated that vegetative growth (McGuire et al. 1987; Stimman 1968), conidia production (Smitley et al. 1986), infection (Wilding 1970), incubation period (Wilding 1970), and mortality (Stimman 1968) in Entomophthorales species are positively related to temperature. In each of the studies, however, an upper limit was reached that was either lethal or inhibiting. Especially relevant were the data on pathogenesis in the study by Wilding (1970), in which pea aphids, Acyrthosiphon pisum infected by Entomophthora aphidis were incubated over a range of 0 to 30°C. The aphids incubated at 30°C died without showing signs of the disease, suggesting lethal or inhibiting effects on the fungus. Based on tentative comparisons between the sporulation range of most Entomophthorales species (16°-30°C) and the activ-

ity range of most grasshoppers (20^{o}-44^{o}C), it is hypothesized that pathogenesis in an infected grasshopper with an internal body temperature significantly higher than the upper range of the fungus could be inhibited. Such an effect could be tested on the pathogenesis of E. grylli in M. differentialis.

Photoperiod did not significant affect percent mortality, time to mortality, or production of mature resting spores. Nonetheless, the potential for interaction with temperature exists, as shown in a study by Stoy et al. (1988). A temperature-photoperiod interaction was indicated in the study of effects of temperature and photoperiod combinations on resting spore germination of E. grylli pathotype 2.

The pathogen, E. grylli pathotype 2, was investigated regarding the production of the infective stage, the cryptoconidium, in grasshopper cadavers. Since production of cryptoconidia does not occur until the cadaver is opened, the effect of time on this event was examined. The studies showed that cadavers opened 24 h after death produced a significantly higher number of cryptoconidia than those opened at the time of death.

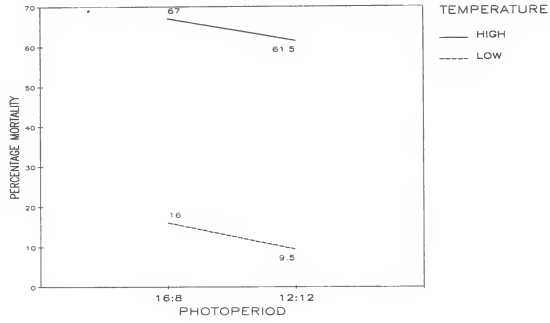
The results of these studies carry implications for the use of E. grylli pathotype 2 in the control of economically important species of the genus Melanoplus. The study on seasonal factors provides information on temperature and photoperiod to be used as a basis for further research on epizootics. Both the infection and post-infection processes would need to be tested over a wide range of temperatures in an attempt to determine an optimum. These findings could then be used as a reference point for subsequent field tests. Further work on photoperiod is

needed to critically assess the role, if any, that this abiotic factor plays in contributing to epizootics.

The effects of the timing of cadaver disruption on cryptoconidia helps create possible scenarios for field epizootics, but also carries implications for artificial application of the fungus. The findings of this study could be integrated into further studies examining cryptoconidia production in which a cadaver age yielding a maximum quantity could be determined. Properly formulated, the cryptoconidia could then be applied under appropriate environmental conditions in either lab or field settings to initiate epizootics.

Fig. 1. Graph of mortality data grouped according to temperature.

Fig. 2. Graph of mortality data grouped according to photoperiod.


TEMPERATURE
HIGH
LOW
PERCENTAGE MORTALITY
70
60
50
40
30
20
10
0
67
61.5
16
9.5
16:8
12:12
PHOTOPERIOD

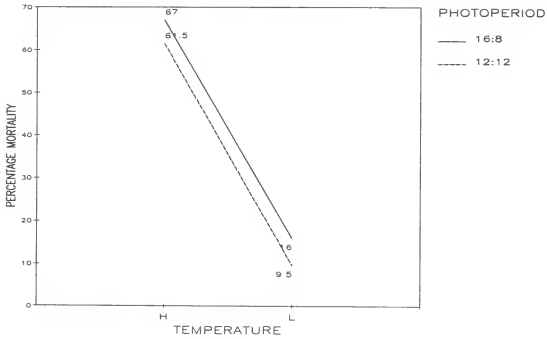
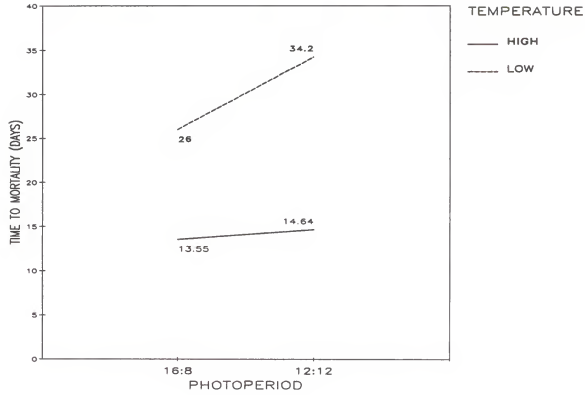

PHOTOPERIOD
16:8
12:12
PERCENTAGE MORTALITY
70
60
50
40
30
20
10
0
67
61.5
16
9.5
H
L
TEMPERATURE

Fig. 3. Graph of time to mortality data grouped according to temperature.

Fig. 4. Graph of time to mortality data grouped according to photoperiod.


TEMPERATURE
HIGH
LOW
TIME TO MORTALITY (DAYS)
40
35
30
25
20
15
10
5
0
34.2
26
14.64
13.55
16:8
12:12
PHOTOPERIOD

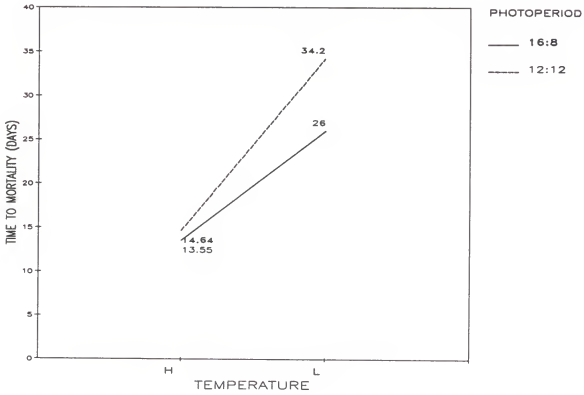
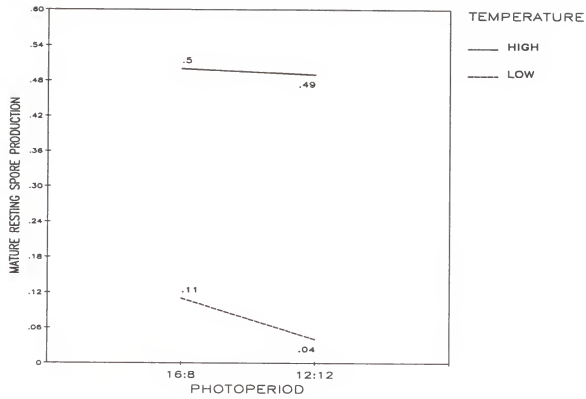

PHOTOPERIOD
16:8
12:12
TIME TO MORTALITY (DAYS)
40
35
30
25
20
15
10
5
0
34.2
26
14.64
13.55
H
L
TEMPERATURE

Fig. 5. Graph of mature resting spore production data grouped according to temperature.

Fig. 6. Graph of mature resting spore production data grouped according to photoperiod.


.60
.54
.48
.42
.36
.30
.24
.18
.12
.06
0
MATURE RESTING SPORE PRODUCTION
.5
.49
.11
.04
16:8
12:12
PHOTOPERIOD
TEMPERATURE
HIGH
LOW

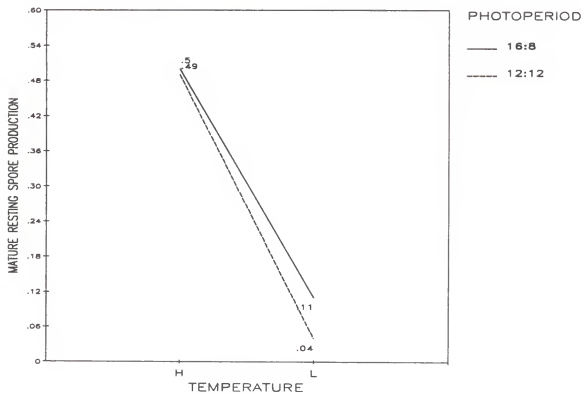

.60
.54
.48
.42
.36
.30
.24
.18
.12
.06
0
MATURE RESTING SPORE PRODUCTION
.49
.11
.04
H
L
TEMPERATURE
PHOTOPERIOD
16:8
12:12

Fig. 7. Mean hourly production of cryptoconidia.

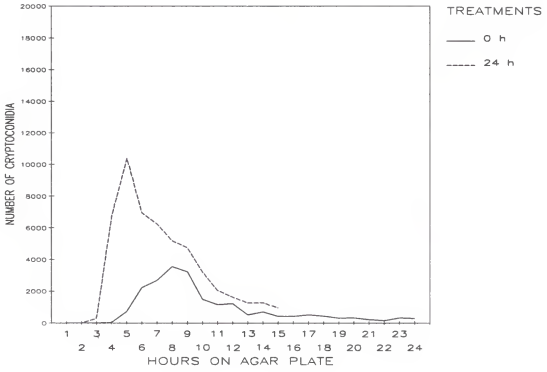

TREATMENTS
0 h
24 h
NUMBER OF CRYPTOCONIDIA
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
1 3 5 7 9 11 13 15 17 19 21 23
2 4 6 8 10 12 14 16 18 20 22 24
HOURS ON AGAR PLATE

Fig. 8. Number of mature resting spores.

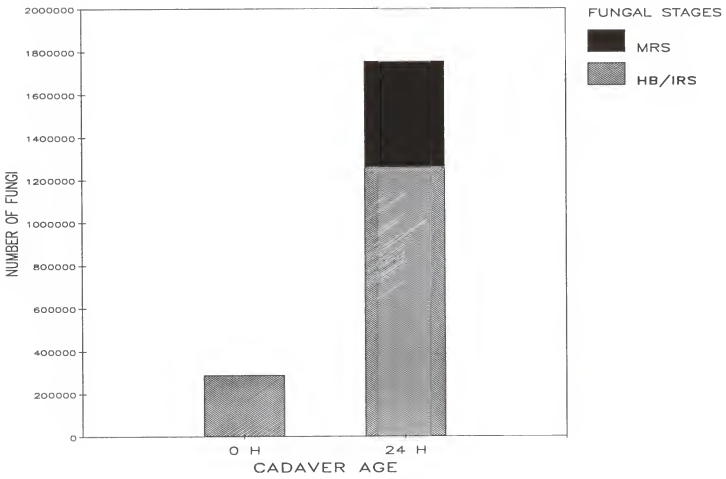

2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
NUMBER OF FUNGI
0 H
24 H
CADAVER AGE
FUNGAL STAGES
MRS
HB/IRS

Fig. 9. Number of hyphal bodies + immature resting spores.

HYPHAL BODIES AND
IMMATURE RESTING SPORES
0
139500
279000
418500
558000
697500
837000
976500
1116001
1255501
1395001
0 HR.
24 HRS.
CADAVER AGE

Fig. 10. Number of cryptoconidia.

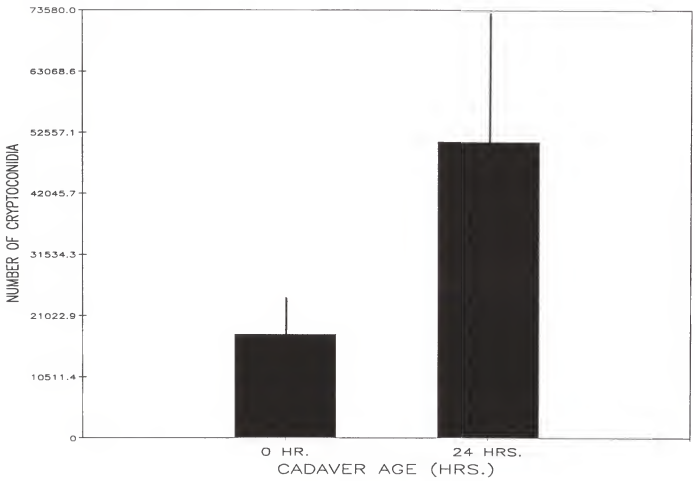
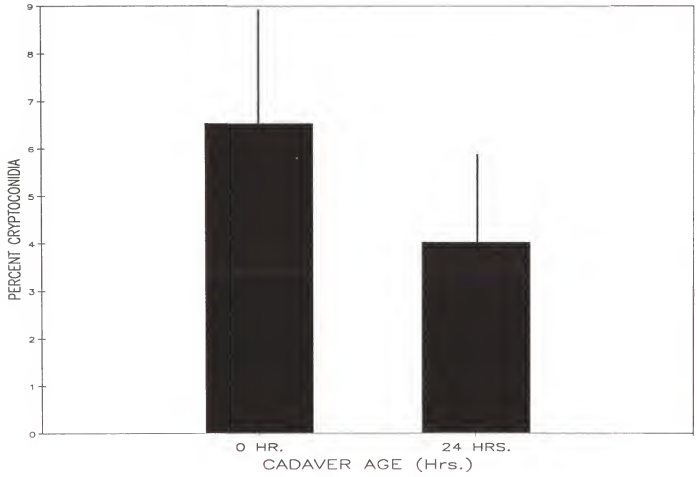
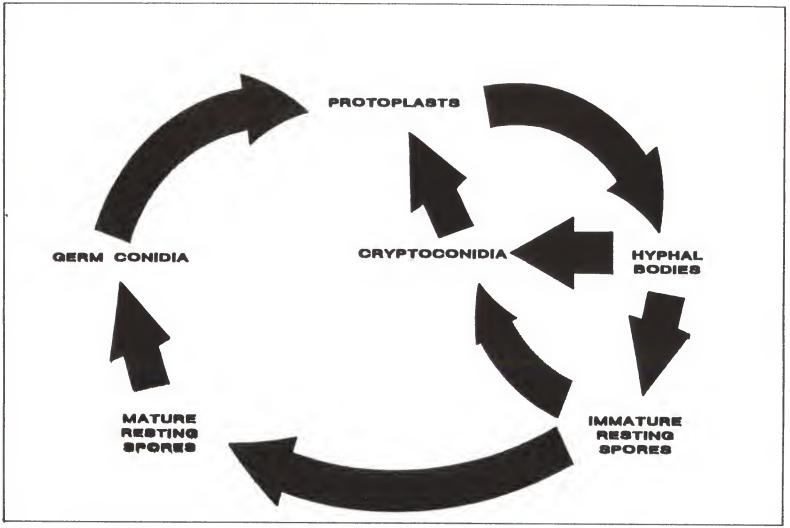

73580.0
63068.6
52557.1
42045.7
31534.3
21022.9
10511.4
0
NUMBER OF CRYPTOCONIDIA
0 HR.
24 HRS.
CADAVER AGE (HRS.)

Fig. 11. Cryptoconidia as a percentage of number of hyphal bodies + immature resting spores on agar plate.


PERCENT CRYPTOCONIDIA
0
1
2
3
4
5
6
7
8
9
0 HR.
24 HRS.
CADAVER AGE (Hrs.)

1P

Fig. 12. Life cycle of Entomophaga grylli pathotype 2.


PROTOPLASTS
CRYPTOCONIDIA
HYPHAL BODIES
IMMATURE RESTING SPORES
MATURE RESTING SPORES
GERM CONIDIA

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Abiotic and Pathogen Factors of *Entomophaga grylli* (Fresenius) Batko Pathotype 2 Infections in *Melanoplus differentialis* (Thomas)

by

DWIGHT KEITH TILLOTSON

B.S., Kansas State University, 1973

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Entomology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1988

ABSTRACT

Abiotic and pathogen factors were investigated in infections of Entomophaga grylli (Fresenius) Batko pathotype 2 in Melanoplus differentialis (Thomas).

Two abiotic factors of temperature and photoperiod were selected and their separate effects were studied in regard to percent mortality, time to mortality and production of mature resting spores for M. differentialis infected by E. grylli.

Using a two-sample t-test, temperature was found to have a significant positive relationship to both mortality and proportion of mature resting spores and a non-significant effect on disease length. Photoperiod did not have a significant effect on any of these three aspects. Because the interaction between temperature and photoperiod could not be analyzed statistically, photoperiod could have some modifying effect on temperature responses that could not be detected in this study.

The effect of cadaver age on cryptoconidia production was studied using fungi extracted from cadavers 0 h and 24 h following death from E. grylli. The quantity of hyphal bodies and immature resting spores extracted per grasshopper was significantly higher in the 24 h. The number of cryptoconidia produced in proportion to the quantity of hyphal bodies and immature resting spores did not significantly differ between the two treatments. But, the overall number of cryptoconidia produced was significantly higher in the 24 h treatment. The timing of cadaver disruption and subsequent production of cryptoconidia could significantly affect the extent of an epizootic.