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PHYSIOLOGICAL ECOLOGY OF ERYNIA CONICA AND ERYNIA CURVISPORA (ZYGOMYCETES: ENTOMOPHTHORALES) ATTACKING BLACK FLIES (DIPTERA: SIMULIIDAE) IN QUEBEC.

by

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A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the degree of
Doctor of Philosophy

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A Chantale,

et à cette complicité qui nous lie
ABSTRACT

Study of the Entomophthorales infection in selected black fly populations from two outlets of a lake was conducted at the Réserve Faunique du Saint-Maurice (Québec). *Entomophaga near limoniae* infected *Simulium verecundum/rostratum*, whereas *Erynia curvispora* predominantly parasitized *Simulium decorum*, and *Erynia conica* infected *Simulium venustum* and the *Simulium verecundum/rostratum* and *Simulium vittatum* complexes. This is the first report of the presence of *Erynia conica* and *Entomophaga near limoniae* in black flies represents a new association.

A successful method for rearing *Simulium rostratum*, and the *in vitro* production of fungal conidiospores, produced a sufficient supply of material to study the cuticular invasion process of *Erynia conica* in the laboratory. Both *Erynia* species exhibited a diurnal periodicity in the formation of secondary conidiospores in darkness *in vitro*. This mode of development was influenced by the pH of the medium. The cyclic pattern in secondary conidia formation by *Erynia conica* disappeared with exposure to light, which suggests that photoperiod may be a factor contributing to host infection. Only secondary type 2 conidia of *Erynia conica* produced germ tubes that invaded the cuticle of the proper host, *Simulium rostratum* as opposed to the primary conidia. The infective unit exhibited delayed germination and formed neither appressoria nor invaded the cuticle of the non-host, *Simulium decorum*, which may explain the host specificity observed in the field.
study. Cuticular lipids triggered appressoria formation and penetration pegs on the host black fly and did not seem to inhibit fungal invasion on the non-host.

The effects of selected physical factors on the development of the infective unit of *Erynia conica* were determined. Germination and sporulation *in vitro* were influenced by medium pH, environmental temperature and charge of the substrate, but both processes were independent of substrate hydrophobicity. Germination and sporulation occurred throughout the pH and temperature ranges tested, with both achieving maximum at pH 7.5-8.0 and 10-20 °C. Temperature range of the germination of the conidiospores *in situ* was similar that *in vitro*, which corresponded to the temperature variation in the field. Production of invasive structures was noted for *in situ* experiments only and was more temperature sensitive than was germination.
RÉSUMÉ

Nous avons étudié à la Réserve Faunique du Saint-Maurice (Québec), des champignons entomopathogènes de l'ordre des Entomophthorales s'attaquant à deux populations d'adultes de mouches noires. *Entomophaga*, apparenté à *limoniae* infecte le complexe *Simulium verecundum*/*rostratum*, alors qu'*Erynia curvispora* infecte surtout *Simulium decorum* et qu'*Erynia conica* attaque les complexes *Simulium venustum*, *Simulium verecundum*/*rostratum* et *Simulium vittatum*. C'est la première mention d'*Entomophaga* apparenté à *limoniae* et d'*Erynia conica* en Amérique du Nord. C'est également la première fois qu'*Erynia curvispora* est observée au Canada.

Afin d'étudier au laboratoire le mode de pénétration de la cuticule de l'insecte par les champignons, nous avons développé une méthode d'élevage de *Simulium rostratum*, et produit *in vitro* les conidiospores des deux espèces d'*Erynia*. Nous avons observé une périodicité, durant la journée, au niveau de la formation dans l'obscurité des conidiospores secondaires de type 2 chez les deux espèces d'*Erynia*. Cette formation de conidies secondaires est également influencée par le pH du milieu. Toutefois, chez *Erynia conica*, la périodicité disparaît lors d'une exposition à la lumière. L'influence de la photopériode sur le cycle de production des conidies de type 2 d'*Erynia conica*, en relation avec la transmission du pathogène chez l'hôte est discutée. Lors de l'étude du mode de pénétration de l'hôte *Simulium rostratum* par *Erynia conica*, nous avons constaté que seulement les conidies secondaires de type 2, comparativement aux primaires, forment des appressoria et des tubes pénétrants. Ces derniers permettent le passage du champignon...
à travers la cuticule de l'insecte. Lorsque les conidies de type 2 sont projetées à la surface de l'espèce résistante, la *Simulium decorum*, la germination des spores est ralentie, et il y a inhibition des structures infectieuses. Ainsi le champignon ne traverse pas la barrière cuticulaire de l'insecte. La spécificité observée dans les conditions de terrain peut s'expliquer par cette incapacité d'*Erynia conica* d'infecter *Simulium decorum*. Les lipides cuticulaires favorisent la formation des appressoria et des tubes pénétrants chez l'hôte, alors qu'ils ne semblent pas impliqués lors de l'inhibition de l'invasion cuticulaire chez l'espèce de mouche noire résistante à l'invasion.

Nous avons également étudié les effets de certains paramètres physiques sur le développement des conidies infectieuses d'*Erynia conica*. La germination et la sporulation *in vitro* sont influencées par la température, le pH du milieu et la charge du substrat, alors qu'elles sont indépendantes de l'hydrophobicité du substrat. La germination ainsi que la sporulation des conidies sont observables à chaque valeur de pH et température testée, avec des maximums à pH 7.5 à 8.0 et entre 10 et 20 °C. Les résultats concernant l'effet de la température sur la germination des conidiospores *in vitro* sont comparables aux résultats *in situ*. Toutefois, la formation des structures infectieuses n'est notée que lors des expériences *in situ*. Cette formation des structures infectieuses est plus affectée par la température environnante que ne l'est la germination.
SHORT TITLE

Erynia conica and Erynia curvispora on adult black flies.
ACKNOWLEDGEMENTS

This thesis is the result of years of work on the road of knowledge that required generous support of many people to whom I must express my gratefulness.

First, I would like to thank my supervisors, Dr. Gary Dunphy and Dr. Jacques Boisvert, for their precious guidance along this work. I am especially grateful to Dr. Dunphy, who accepted me in his laboratory and dealt with my French accent and other language problems. I thank members of my supervisory committee, Dr. David Lewis and Dr. Manfred Rau for their helpful comments. I also thank Dr. Bernard Papierok (Institut Pasteur, France) and Dr. Peter Adler (Clemson University, South Carolina, U.S.A.) for generous donating expertise in entomophthoralean fungi and black flies, respectively. I acknowledge also, Mr. Gilles Proteau from Ministère de l'Environnement et Faune du Québec, who provided facilities during my field season at the Réserve Faunique du Saint-Maurice. I thank Mrs. Guy Rimmer and Pierre Langlois who supplied technical assistance in part of this work.

I also appreciated help and support of my companions of the laboratory, Dr. Barry Jewell, Adla Halwani and Philip Maxwell.

Je tiens à remercier toute la famille Nadeau, mes parents, Marie-Paule et Charles-Henri, mes sœurs, Charline, Pauline, Louise, Luce, Julie, Suzanne, et mon frère Mario, ainsi que les membres de leur famille respective pour le soutien moral et financier. A ma
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Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g. in appendices) to allow a clear precise judgement to be made of the importance and originality of the research reported in the thesis.

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In Chapter 2, experiment design, data collection were carried out by the author of the thesis. Dr. Bernard Papierok and Dr. Peter Adler, confirmed identification of fungal and black fly species. I have received comments and help in discussions with the supervisors.
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW.
INTRODUCTION

Adult black flies (Diptera; Simuliidae) are an important worldwide pest. In the tropics this bloodsucking insect serves as the vector of the filarial nematode *Onchocerca volvulus* (Leuckart) Railliet and Henry, the aetiological agent of human onchocerciasis, or "river blindness" which severely impairs the health and economic performance of its host. The biology of the parasite and the development of onchocerciasis are summarized by Nelson (1991). In Canada and the Northeastern United States black flies do not transmit human pathogens (Wood, 1985; Molloy, 1990). Nevertheless, they elicit allergic reactions to their bite. The pest status of the insects has been based on their annoyance effect on the vertebrate host; for cattle this translates into reduced milk and beef production, and for humans, black flies limit outdoor activities such as mining, lumbering and tourism and thus have an adverse economic impact (Fredeen, 1977; 1985). Therefore, the control of black flies has received considerable attention.

Environmentally unacceptable pest control based on synthetic chemical insecticides and the development of host resistance to these insecticides have been conducive to the development of biological control agents as potential alternative strategies (Lacey and Undeen, 1987). The variety of natural enemies known to attack black flies has been reviewed by Crosskey (1990).
The successful development of a biological control agent is represented by the commercialization of the entomopathogenic bacterium *Bacillus thuringiensis* Berliner subsp. *israelensis*. Today formulations of this pathogen are used in Canada and elsewhere to control black fly populations (Mason and Kusters, 1991; Davies, 1994). However, resistance of biting flies to these bacteria is possible (Goldman et al., 1986), although unlikely, where used infrequently (Molloy, 1990). Dependence on one microbial insecticide may not be prudent, and the success obtained with *B. thuringiensis* subsp. *israelensis* has promoted research on the development of other biological pathogens for the long term control of black flies.

Entomopathogenic fungi are known to infect larval and adult black flies, and may be candidates for development as mycoinsecticides (Molloy, 1987). The fungi *Coelomycidium simulii* Debaïsieux and *Culicinomyces clavisporus* Couch, Romney and Rao infect black fly larvae (Gaugler and Jaronski, 1983). However, the limited ability of both fungi to infect these insects reduces their acceptability as possible mycoinsecticides (Jännback, 1973; Sweeney and Roberts, 1983).

Fungi of the order of Entomophthorales attacking the adult stage of black flies have been reported over the last century. Nowakowski (1883) in Europe and Thaxter (1888) in North America described *Erynia curvispora* (Nowakowski) Nowakowski on *Simulium latipes* Meigen and *Entomophthora culicis* (Braun) Fresenius on *Simulium molestum* (= *S. venustum* Say), respectively. Today, six species of Entomophthorales are known to infect black flies including *Entomophaga papillata* (Thaxter) Keller, *En. culicis*, *Erynia conica* (Nowakowski) Remaudière and Hennebert, *E. curvispora*, *Erynia ovispora* (Nowakowski) Nowakowski and *Erynia rhizospora* (Thaxter).
Remaudière and Hennebert (Gustafsson, 1965; Rubtzov, 1967; Descals et al., 1981; Kramer, 1983; Keller, 1987; Keller, 1991; Papierok and Dumas, 1992). At times these fungi cause epizootics in insect populations (Shemanchuk and Humber, 1978; Keller, 1987). Not only do these fungi reduce the host population level through direct mortality but also, as evident by mycoses caused by *E. conica* and *E. curvispora* in female black flies, they indirectly lower host population levels by reducing the egg producing capability of the insect (Rubtzov, 1967; Kramer, 1983; Hywel-Jones and Ladle, 1986). These entomophthoralean fungi are established in insect populations in temperate climate (Shemanchuk and Humber, 1978). Additional reasons to study this fungal group as potential mycoinsecticides include the ability to grow some species *in vitro*, infection is by surface contact, and they have recycling potential (defined as the ability to increase above the initial inoculum) (Humber, 1986; Papierok and Brey, 1990). Amplification of the fungi in the field may occur by the production of infective units on mummified cadavers. Mummies and the formation of resting spores allow the survival of the fungi under unfavorable environmental conditions. However, there is little information about the life cycle and the infective stage(s) of these fungi, even in situations where laboratory transmission is possible. Kramer (1983) infected *Simulium vittatum* Zetterstedt and *Simulium pictipes* Hagen with an undefined stage of *E. curvispora* isolated from *Simulium decorum* Walker. Secondary type 2 conidia (globose shape) of *E. conica* may be the infective unit on adult simuluids (Hywel-Jones and Webster, 1986a).

The discovery of the entomophthoralean mycoses in populations of adult black flies from different sites at the Réserve Faunique du Saint-Maurice (Nadeau, 1990), provided an opportunity to study the relationship between the Entomophthorales and
the black fly host and the role of environmental factors on the host range of the fungi, the infection mechanism, and factors associated with the transmission of the pathogen. The goals of this thesis were to examine the above aspects with a view to determine the potential of Entomophthorales as biological control agent of black flies.

Chapter 1 introduces the rationale and objectives of the study, as well as the black fly host and the fungi (Entomophthorales) associated with these insects.

Chapter 2 examines the Entomophthorales in two selected black fly populations at the Réserve Faunique du Saint-Maurice in Quebec during the season 1990 in terms of the identification of both the Entomophthorales and host black fly species and possible host specificity.

Chapter 3 presents the laboratory methods for rearing of the host of *Erynia conica*, i.e. the black fly *Simulium rostratum*, which is a sibling species of the *Simulium verecundum* Stone and Jamnback complex.

Chapter 4 reports on the periodicity of the *in vitro* production of the conidiospores of *Erynia conica* and *Erynia curvispora* and the influence of pH on the development of primary conidia for both fungal species. Also examined is the daily pattern of replicative conidiospore production of *Erynia conica* under continuous dark and light regimes.

Chapter 5 describes the invasion process of *Erynia conica* on the wings and abdominal cuticle of the host, *Simulium rostratum* and the non host, *Simulium*
decorum using two types of aerial conidiospores. Furthermore, the effects of the sex of Simulium rostratum and Simulium decorum and the role of lipids of the two insect species in this process were examined to explain the difference in fungal development observed between host male and female and the absence of the fungus on S. decorum reported in Chapter 1.

Chapter 6 assesses the effects of environmental factors such as pH, temperature, surface hardeness, wettability and charge in vitro and in situ on the development of infective conidiospores of Erynia conica.

Chapter 7 presents an overall summary and a general discussion of the results obtained in Chapters 2 to 6.
THE SIMULIIDS

The simuliiids (black flies) are insects of the order Diptera in the family Simuliidae that are associated with running water and found throughout the world (Crosskey, 1990). They are holometabolous insects with traditional egg, larval, pupal and adult stages. The eggs are laid by the female on a substrate (rock, logs, leaves) emerging from the stream. Oviposition on the water surface is rare (Golini and Davies, 1987). Incubation time prior to eclosion varies between insect species, ranging from 2 to 7 days, and in some cases, the egg stage undergoes a period of dormancy (diapause) with hatching occurring after several months.

Black fly larvae are adapted to a lotic (= a running water) habitat with a fusiform body fixed to the substratum by sticky silk and the hooks on the abdominal proleg. The larva can trail passively in the stream current attached to a silk thread. They may also move along the surface of the substrate using their thoracic and abdominal prolegs. Larval development comprises 6 to 9 instars, the insect increasing in size between each moult. The larvae feed by filtration of particles (e.g. detritus, diatoms, algae and animal matter) in suspension in the water and by scaping materials from the substrate. The larval stage generally lasts 1 to 2 weeks, however, as with the eggs in some species, the larvae may have a dormancy period of several months.
The last larval instar transforms to a pupa enclosed within a cocoon made by the larva before metamorphosis. The pupae are motionless and do not feed for a period of 2 to 10 days, after which the adult black flies emerge from the cocoon in an air bubble; males emerge before the females (Crosskey, 1990).

The adults of both sexes feed on the nectar of flowers, while females are hematophagous, feeding on homeothermic vertebrates. The host's blood stimulates egg maturation for anautogenous species. Some black fly species are autogenous or primiparously autogenous. Mating occurs in flight when females penetrate the swarm of males which are generally located closed to an oviposition site. Females seek a blood-meal after mating. Depending of the species, black flies are either univoltine or multivoltine. In anautogenous, multiparous species, females complete subsequent gonotrophic cycles without mating (Anderson, 1987), but each laying must be preceded by a blood meal. The life span of adult black flies is estimated to be 2 to 4 weeks. Table 1 presents selected biological characteristics of black fly species discussed in this thesis. The abundance and the activities of adults black fly are periodic; bloodfeeding occurs during the day light hours, whereas oviposition usually occurs around sunset.

The black fly species described in this thesis originated from the outlets of Inman Lake (46°59'N, 73°07'W) and Eveline Lake (46°55'N, 73°01'W) in the Réserve Faunique du Saint-Maurice (Quebec). The Nearctic black flies at these sites were composed of more than one species and, at other locations these species coexisted in space and time (Imhof and Smith, 1979). Carlsson et al. (1977) suggest that the high
density of black flies found at the outlet of similar lake systems was due to the increased quality and quantity of food to support the population of insect larvae.

The identification of black flies to species is difficult, particularly for sibling species. Sibling species are very similar morphologically but they differ in other biological aspects and are reproductively isolated (Rothfels et al., 1978). Adults of *Simulium venustum* Say and *Simulium verecundum* Stone and Jamnback mentioned in this thesis belong to a complex of sibling species that includes *Simulium rostratum* (*S. verecundum* cytospecies ACD) (Rothfels et al., 1978). The adults of each species complex cannot be separated on the basis of morphological characters. Larvae of the two complexes (*venustum* and *verecundum*) can be differentiated at the species level only by means of cytotaxonomy using polytene chromosome banding patterns from salivary glands (Rothfels, 1979; McCreadie et al., 1994), and will be referred to by external morphological characters to as the *S. venustum/verecundum* complex.

**ENTOMOPHTHORALES**

Fungi of the order Entomophthorales are members of the class Zygomycetes. The entomopathogenic genera of the order include *Conidiobolus, Entomophaga, Entomophthora, Erynia, Neozygites, Strongwellsea, Tarichium and Zoophthora* (Keller, 1987; 1991). The distinctive feature of the entomophthoralean fungi is the active projection of conidiospores ( = conidia, the asexual spore) (Eilenberg et al., 1986).
Entomophthorales, like other fungal pathogens of insects and plants, invade their hosts through the external surface. The insect cuticle may represent the primary barrier to fungal infection by preventing either spore germination or cuticular penetration by the fungal germ tube (this will be considered in the section on host specificity). The insect cuticle is composed of three principal layers built on the epidermis: the endocuticle, the exocuticle, and the outer epicuticle. The epicuticle is structurally divisible into an inner and outer epicuticle, a wax layer and a cement layer (Hepburn, 1985). Biochemically the epicuticle is composed of waxes (hydrocarbon, fatty acid esters of alcohols), lipoprotein (outer epicuticle) and polyphenol protein complex (inner epicuticle) (Hepburn, 1985). The procuticle (endocuticle plus exocuticle) is composed of chitin (a polymer of N-acetyl glucosamine), associated with protein and lipids. Sclerotization (a cross-linking protein by aromatic compounds) occurs in the exocuticle. The conidium (primary conidium) discharged from the conidiophore (the fungal hyphae bearing conidia) adheres to the insect cuticle and subsequently germinates and either penetrates the insect cuticle or give rise to replicative conidia, the secondary conidia (Brobyn and Wilding, 1977; Glare et al., 1985; Brey et al., 1986; Wraight et al., 1990; Galaini-Wraight et al., 1992).

The attachment of the conidiospores of some mycoinsecticidal Deuteromycetes to the host cuticle may be passive and non-specific in that conidia adhere to both host and non-host cuticles (Boucias et al., 1988; Boucias and Pendland, 1991; Hegedus et al., 1992). This adhesion may mediated by the hydrophobic properties of the external wall of the conidia, since chemical treatments that neutralize conidial hydrophobicity result in significant reductions in conidia adhering to the cuticle (Boucias et al., 1988). The component is responsible for the attachment of the conidia were identified as a
resilient layer of well-organized fascicules of rodlets of the fungal wall. However, removing the hydrophobic layer by chemical treatment, resulted in a partial reduction of the hydrophobicity of the conidia which implies that materials responsible for conidial hydrophobicity are located throughout the cell wall of conidia (Boucias et al., 1988; Boucias and Pendland, 1991). In the case of hydrophilic entomopathogenic fungal conidia (including most entomophthoralean fungi) an amorphous mucus covering the fungal conidiospore facilitates the passive adhesion of the spores to substrata (Brey et al., 1986; Eilenberg et al., 1986; Latgé et al., 1986; Murrin and Nolan, 1987; Wraight et al., 1990; Boucias and Pendland, 1991; Villacarlos and Wilding, 1994).

The germination of the conidium is characterized by the emergence of a germ tube. Germination of entomophthoralean fungi does not require exogenous nutrients as shown by their germination on coverslips, water, and water agar (Yendol, 1968; Kerwin, 1982; Sampedro et al., 1984; Van Roermund et al., 1984; McGuire et al., 1987; Glare and Milner, 1991). This contrasts with the entomopathogenic Deuteromycetes Nomureae rileyi (Fallow) Samson (Boucias and Pendland, 1984), Beauveria bassiana (Balsamo) Vuillemin (Woods and Grula, 1984) and Metharizium anisopliae (Metschnikoff) Sorokin (St-Leger et al., 1989). However, lipoidal compounds of the insect cuticle may stimulate conidial germination as evident by cuticular extracts from Acyrthosiphon pisum Harris and Anticarsia gemmatalis Hubner enhancing germination of Conidiobolus obscurus (Hall and Dunn) Remaudière and Keller by 30 to 50% (Latgé et al., 1987; Boucias and Latgé, 1988). Similarly, oleic acid stimulates the germination of Erynia variabilis (Thaxter) Remaudière and Hennebert (previously misidentified as En. culicis; Kerwin, 1984).
Abiotic parameters, principally relative humidity (RH) and temperature, also influence conidial germination of pathogenic fungi. Saturated or near saturated air or a water-film is necessary for spore germination in the vast majority of fungi including many of the Entomophthorales (Hall and Papierok, 1982). However some Entomophthorales can germinate at lower RH. Kramer (1980) reported that the conidia of *Entomophthora muscae* (Cohn) Fresenius germinate at 48% RH. Conidia of the Entomophthorales germinate over a wide range of temperatures (0-40°C) (Yendol, 1968; Sampedro *et al*., 1984; Van Roermund *et al*., 1984; Carruthers and Haynes, 1986; Glare *et al*., 1986; McGuire *et al*., 1987; Hajek *et al*., 1990; Glare and Milner, 1991; Galaini-Wraight *et al*., 1992) and pH (3-11) (Callaghan, 1978; Van Roermund *et al*., 1984; Sampedro *et al*., 1984). Osmotic pressure, a parameter rarely considered, once in excess of a critical level depressed germination of *Zoophthora radicans* (Brefeld) Batko and *C. obscurus* (Sampedro *et al*., 1984; Magalhaes *et al*., 1991). Increased oxygen concentration also reduced germination of *C. obscurus* (Sampedro *et al*., 1984). Germination of conidia of *Z. radicans*, *Entomophaga maimaiga* Humber, Shimazu and Soper, and *Neozygites fresenii* (Nowakowski) Remaudière and Hennebert was delayed or suppressed by light (Callaghan, 1978; Sampedro *et al*., 1984; Van Roermund *et al*., 1984; Hajek *et al*., 1990; Uziel and Kenneth, 1991). However, among some strains of *C. obscurus* germination of conidia was stimulated by light whereas with other strains it was not (Sampedro *et al*., 1984).

The fungi of the Entomophthorales, like those of other entomopathogenic fungi or plant pathogenic fungi, penetrate the host either directly or by forming a specialized structure termed the appressorium (Brobyn and Wilding, 1977). The appressorium is defined as a swollen structure at the tip of the germ tube from where penetration can
occur (Emmet and Parbery, 1975). In *M. anisopliae* (Deuteromycetes) and some plant pathogenic fungi, DNA synthesis and nuclear division are required for differentiation of invasive structures (Staples and Macko, 1980; St-leger *et al*., 1989). However, for *Z. radicans*, conidial germination and appressoria formation occur independently of DNA replication and nuclear division but require RNA and protein synthesis (Magalhaes *et al*., 1991). According to St-Leger (1993) the appressorium represents a structural adaptation for concentrating physical force and enzymes in a small area to facilitate penetration of the insect body. Differentiation of appressoria in pathogenic fungi has been related to surface topography (thigmotrophic response) (Mims, 1991; St-Leger *et al*., 1991). Appressorium formation by *Z. radicans* and *M. anisopliae* was also influenced by low concentrations of nutrients (St-Leger *et al*., 1989; Magalhaes *et al*., 1990). The absence of nutrients, however, initiated the formation of replicative conidia (Van Roermund *et al*., 1984; McGuire *et al*., 1987; Magalhaes *et al*., 1990). In entomophthoralean fungi replicative sporulation occurs in the absence of factors stimulating the development of infective structures (Kerwin, 1984; Latgé *et al*., 1987; Glare and Milner, 1991) and may represent an adaptation favoring the contact of more susceptible substrate (Mullens and Rodriguez, 1985; Wraight *et al*., 1990; Steinkraus and Slaymaker, 1994).

Studies of the abiotic factors influencing appressorial formation have been limited to the interaction of temperature, water and light on the induction of appressorial formation by plant pathogenic fungi (Emmett and Parbery, 1975). Temperature also influences appressorial formation by *M. anisopliae* (St-Leger *et al*., 1989) and *Z. radicans* (Magalhaes *et al*., 1991; Galaini-Wraight *et al*., 1992).
Uniquely, St-Leger et al. (1989) reported that mildly alkaline pH enhanced development of appressoria of *M. anisopliae*.

The penetration of the insect cuticle is accomplished by mechanical force, as shown by depression of the cuticle, and by enzymatic degradation, as evidenced by a digested area around the penetrating germ tube or pegs (Gabriel, 1968; Brey et al., 1986; Goettel et al., 1989). Entomophthoralean fungi, as well as other pathogenic fungi, produce a series of enzymes including lipases, proteases and chitinases that degrade the constituents of the cuticle (Urbanczyk et al., 1990; 1992; Eilenberg et al., 1992). Charnley and St-Leger (1991) have reviewed the cuticle-degrading enzymes of entomopathogenic fungi.

After passing through the insect cuticle, the Entomophthorales multiply inside the insect as either yeast-like cells or as multinucleate, variously shaped hyphal bodies (Prasertphon and Tanada, 1968; Brobyn and Wilding, 1977; Latgé and Beauvais, 1987). In some entomophthoralean species a phase devoid of a cell wall, the protoplast stage, is the early vegetative phase inside the insect (Macleod et al. 1980; Butt et al., 1981; Latgé and Beauvais, 1987; Funk et al., 1990). *In vitro* production of insecticidal mycotoxins has been reported for both species of the Entomophthorales as well as Deuteromycetes (Prasertphon and Tanada, 1969; Dunphy and Nolan, 1982a; Gillespie and Claydon, 1989; Papierok et al., 1993), however, their role *in vivo* is unknown. The hyphal bodies and protoplasts multiply in the hemocoel of the insect, and in many Entomophthorales the internal tissues are invaded by hyphal bodies (Brobyn and Wilding, 1977; Tomiyama and Aoki, 1982; Brobyn and Wilding, 1983). In general the insect infected with Entomophthorales dies from proliferation of the mycelium in
the internal body; physiological starvation appears to be the ultimate cause of the host's death (Samson et al., 1988; Papierok and Dumas, 1992).

When the humidity and temperature are adequate or nutrients have been exhausted, hyphae or hyphal bodies in some species differentiate into rhizoids and conidiophores which emerge to the outside through the insect's integument (Hywel-Jones and Webster, 1986b; Butt et al., 1990). The rhizoids emerge ventrally, anchoring the insect to the substrate. Certain Entomophthorales produce emerging cystidia (sterile hyphae) from internal fungal elements after the rhizoids and before the conidiophores, which by breaking the abdomen, help the conidiophores to emerge (Brobyn and Wilding, 1977; Hywel-Jones and Webster, 1986b). Conidiophores sporulate by discharging the conidia into the air (Tanada and Kaya, 1993).

Interestingly, species of Entomophthorales may exhibit periodicity in conidial production and discharge (Wilding, 1970; Newman and Carner, 1974; Aoki, 1981; Yamamoto and Aoki, 1983; Mullens and Rodriguez, 1985; Samuels et al., 1988). Sporulation periodicity has been related to the relative humidity, light, pH and temperature, and is believed to maximize the transmission of the pathogen within the host population (Wilding, 1970; Yamamoto and Aoki, 1983; VanRoermund et al., 1984; Mullens and Rodriguez, 1985; Glare et al., 1986; Samuels et al., 1988).

Under adverse environmental conditions many of the Entomophthorales form resting spores which are either produced by gametangial copulation (zygospores) or asexually (azygospore) from hyphal bodies (Latgé et al., 1982). The resting spores are characterized by a thick cell wall and a lipidic droplet in the cytoplasm (Remaudière et al., 1976; Glare et al., 1989). However, resting spores were not observed in all
entomophthoralean species, and the fungi may possibly endure harsh conditions by fungus-induced mummification of the insect (Kenneth et al., 1972; Wilding, 1973).

**Specificity**

The specificity of entomopathogenic fungi has been defined as the expression of reciprocal adaptations and affinities between a pathogenic organism and its host species (Fargues and Remaudière, 1977). Therefore, the insect may be resistant or susceptible to the fungi, and the fungi may exhibit either a broad or narrow host range. Susceptibility of a strain of the pea aphid A. pisum to a pathotype of C. obscurus indicates that specificity of Entomophthorales may be intraspecific (Papierok and Wilding, 1979). Such extreme specialization may have resulted from coevolution of the pathogen and its insect host (Fargues and Remaudière, 1977). Some problems arise when we attempt to assess the host specificity of entomophthoralean fungi. As stated by Papierok (1986), it is often difficult to identify the host to species due to the mummification of the insect. Also, because host range is mainly established in the laboratory, some insect species can be infected in the laboratory but not in the field.

Factors influencing the specificity of fungi can be divided into three areas: (1) the ecological distribution of the pathogen and the host species, (2) the ability of the infective unit to adhere to, germinate on, and penetrate the cuticle and (3) the interaction of the immune system of the host with the virulence mechanisms of the pathogen.

The temporal coincidence between Entomophthorales species and aphid host has been reported to account for their specificity (Fargues and Remaudière, 1977).
Entomophthora phalloides Batko and Conidiobolus osmodes Drechsler are found only during cool weather whereas N. fresenii occurs during warm weather. The pest aphid, Myzus ascalonicus Doncaster, on its principle host En. phalloides, is found predominantly during winter months, whereas, Aphis fabae Scopoli attacked by N. fresenii is present during early summer.

Lack of germination of the conidial inoculum was the most important factor limiting host infection with Z. radicans (Galaini-Wraight et al., 1992). Inhibition of germination of conidia of B. bassiana was attributed to short fatty acids present at the surface of the insect cuticle (Smith and Grula, 1981; 1982; Saito and Aoki, 1983). Lack of germination of conidia of M. anisopliae was related to mycostatic microorganisms associated with the epicuticular surface of the host (Fargues and Vey 1974; Schabel, 1978). Interestingly, the relative composition and levels of fatty acids of the adult stage and pupa of the fly Fannia canicularis (Linnaeus) were suggested to play a role in susceptibility to E. variabilis (Kerwin, 1984). However, in Erynia neoaphidis Remaudière and Hennebert, two aphid biotypes, the first resistant and the other susceptible, both allowed the germination of fungal conidia but penetration was not achieved in the resistant aphid (Milner, 1982). Failure to establish a fungal infection in insects may result from the absence of enzymatic activity; chitinase-negative and lipase-negative strains of the Deuteromycete Beauveria brongniartii (Saccardo) Petch were not able to infect the cockchafer, Melolontha melolontha (Linnaeus) (Paris and Ferron, 1979). However, St-Leger (1993) concluded that cuticle degrading enzymes from entomopathogenic Deuteromycetes were secondary determinants of pathogenicity and contributed more to the virulence of the pathogen than to host specificity. Interspecific morphological differences in the cuticles of
insects may predispose to infection by entomopathogenic fungi; the scales that cover the body and wing veins of the mosquito *Aedes aegypti* (Linnaeus) are a protective barrier against the conidia of *En. muscae* (Steinkraus and Kramer, 1987).

At the level of the cuticle or in the haemocoel cellular and/or humoral defence mechanisms protect the insect against fungal attack. For the immunological reactions of insects during fungal attack see Gotz and Vey (1986) and Vey and Gotz (1986). However, Entomophthorales generally escape the immunological response of the insect including melanization at the level of cuticle, epidermis or in the hemolymph of the insect (Brey et al., 1986; Butt et al., 1988). *Z. radicans* produces two infection pegs from a single appressorium, apparently in response to a strong host melanization (Wraight et al., 1990). The protoplasts of Entomophthorales often fail to elicit host immune responses and multiply rapidly in the hemolymph (Dunphy and Nolan, 1982b; Butt and Humber, 1989; Butt et al., 1990).

**Description of *Erynia* species**

Work in this thesis concentrates on two species of entomophthoralean fungi of the genus *Erynia*: *E. conica* and *E. curvispora* (Figs. 1, 2). Both species were characterized by the presence of branched conidiophores which emerge between abdominal segment of the adult black flies. The cystidia were observed between the among conidiophores, and ventral rhizoids fixed the insect to the substrate. Within the insect numerous hyphal bodies were noted. Conidiophores were observed emerging from the hyphal bodies and conidia were formed apically from the conidiophores. These primary conidia were bitunicate and uninucleate in both fungal species (Figs. 1.1, 1.2; Figs. 2.1, 2.2). Secondary sporulation occurred to produce two forms of
secondary conidiospores; one was identical to the primary conidia (type 1 conidia) and the other form differed in shape (the globose type 2 conidia; Figs. 1.3, 1.4; Figs. 2.3, 2.4).

Both the primary conidia of *E. conica* produced on the insect (*in situ*) and on artificial media (*in vitro*) although variable in shape, were generally elongated, curved, and pointed at the apex (Fig. 1.1). Young resting spores (zygospores) were formed by gametangial copulation (Fig. 1.5). Webster *et al.* (1978), Descals *et al.* (1981) and Keller (1991) described additional aquatic stellate and coronate conidia from *E. conica*. 

*In situ* and *in vitro* conidia of *E. curvispora* were longitudinally curved and rounded at both ends (Fig. 2.1). No resting spores were noted in the material collected in this study. However, zygospore were recorded by Nowahowski (1883). The morphometric data of conidiospores are presented in Table 2. For other descriptive data, the reader is referred to Nowakowski (1883), Thaxter (1888), Gustafsson (1965), Webster *et al.* (1978), Descals *et al.* (1981), Dumas (1990) and Keller (1991) for *E. conica* and for *E. curvispora*: Nowakowski (1883), Gustafsson (1965), Rutzov (1967), Kramer (1983), Dumas (1990) and Keller (1991).
FIG. 1. *Erynia conica*. 1) Bitunicated primary *in vitro* conidia. 2) Primary conidia showing stained nuclei (arrow) and lipid droplet (double arrows) *in situ*. 3) Empty primary conidium with type 1 secondary conidium on a short conidiophore on distilled water agar. 4) Primary *in vitro* conidium bearing secondary type 2 conidium. 5) Resting spore (zygospore) formed by gametangial conjugation in infected adult *Simulium* sp. Bar scale = 10 μm.
FIG. 2. *Erynia curvispora*. 1) Bitunicate primary *in vitro* conidia with lipid droplet (arrow). 2) Primary *in vitro* conidia with stained nuclei (arrow). 3) Secondary type 1 conidium with cytoplasmic migration from primary *in vitro* conidium on distilled water agar. 4) Primary *in vitro* conidium bearing secondary type 2 conidium. Bar scale = 10 µm.
TABLE 1

Biological characteristics of simulid species collected at the Réserve Faunique du Saint-Maurice in 1990.

<table>
<thead>
<tr>
<th>Species or complex</th>
<th>Reproductive strategy</th>
<th>Number of ovarian cycles</th>
<th>Winter stage</th>
<th>References</th>
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<td>Multivoltine</td>
<td>Eggs</td>
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<td>Walker</td>
<td>Anautogenous</td>
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<td>(4) (5)</td>
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<td>complex</td>
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<td></td>
<td>(3)</td>
<td>(4)</td>
</tr>
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<td>Say</td>
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<td>Eggs</td>
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<td>complex</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Zetterstedt</td>
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<td>Larvae</td>
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(1) Davies et al. (1962)
(2) Wolfe and Peterson (1959)
(3) Imhof and Smith (1979)
(4) Abdelnur (1968)
(5) Davies and Peterson (1956)
TABLE 2

Morphometric data of primary and secondary type two conidiospores of *Erynia conica* and *Erynia curvispora* isolated from adult simuliids at the Réserve Faunique du Saint-Maurice in 1990. a

<table>
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<th>Secondary conidia in vivo</th>
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<tr>
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<td>9.0 - 14.0</td>
<td>30</td>
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<td></td>
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<td></td>
<td>(21.0 - 39.9)</td>
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a Data are presented as the range of the mean values in μm with extreme values in bracket from 50 measures per sample.
b Fungal strain growing on GEMYSA medium at 20 °C and dark in 5 cm Petri dish.
c Number of samples.
LITERATURE CITED


(Zygomycotina: Entomophthorales) in the pea aphids *Acyrthosiphon pisum*.


*Bacillus thuringiensis* subspecies *israeilensis* in field and laboratory populations 

Golini, V.I., and Davies, D.M. 1987. Oviposition of black flies. *In "Black flies, 
ecology, population, management, and annotated world list."* (K.E. Kim, and 

Gotz, P., and Vey, A. 1986. Humoral encapsulation in insects. *In "Hemocytic and 
& Sons, New York.

Gustafsson, M. 1965. On species of the genus *Entomophthora* Fres. in Sweden I. 

Hajek, A.E., Carruthers, R.I., and Soper, R.S. 1990. Temperature and moisture 
relations of sporulation and germination by *Entomophaga maimaiga* 
(Zygomycetes: Entomophthoraceae), a fungal pathogen of *Lymantria dispar* 
(Lepidoptera: Lymantriidae). *Environ. Entomol.* 19, 85-90.

of agricultural and medical importance. *Parasitology.* 84, 205-240.

A comparison of the virulence, stability and cell-wall-surface characteristics of


Molloy, D.P. 1990. Progress in the biological control of black flies with *Bacillus thuringiensis israelensis* with emphasis on temperate climates. In "Bacterial control of mosquitoes and black flies" (H. de Barjac, and D.J. Sutherland Eds.). pp. 161-186. Rutgers University Press, New-Brunswick.


Nadeau, M. 1990. Isolation et évaluation de mycètes pour le contrôle biologique des moustiques (Diptera: Culicidae) et des simulies (Diptera: Simuliidae) en zone...
tempérée. Mémoire de maîtrise, Université du Québec à Trois-Rivières. Trois-Rivières, Québec.


Entomophaga. 38, 299-312.


Hilgardia. 39, 580-600.

Entomophaga. 21, 163-177.


CHAPTER 2

ENTOMOPATHOGENIC FUNGI OF THE ORDER ENTOMOPHTHORALES (ZYGOMYCETES) IN ADULT BLACK FLY POPULATIONS (DIPTERA; SIMULIIDAE) IN QUEBEC.

A version of this chapter has been published in the Canadian Journal of Microbiology. 40, 682-686. (1994).
ABSTRACT

Infection of selected adult black fly populations by entomopathogenic fungi of the order Entomophthorales was studied at the outlets of two lakes in the Réserve Faunique du Saint-Maurice (Québec). *Entomophaga* near *limoniae* infected *Simulium verecundum/rostratum* in June only, *Erynia curvispora* predominantly parasitized *Simulium decorum* from the second week of July until September whereas *Erynia conica* attacked the *Simulium venustum* complex, *Simulium verecundum/rostratum* and the *Simulium vittatum* complex throughout May until September. The fungi were not detected on adults of *Prosimulium* species. This is the first report of the presence of *Erynia conica* and *Entomophaga* near *limoniae* in black flies in North America and *Erynia curvispora* in Canada. Although the fungal species and host black flies coexisted in time and space, some evidence of host specificity is presented.
INTRODUCTION

Black flies (Diptera: Simuliidae) are ubiquitous pests in most of Canada and the northeastern United States, but generally do not transmit disease to mammals (Wood, 1985; Lacey and Undeen, 1986). However, their painful bites cause losses in the cattle industry and discourages local tourism (Fredeen, 1985). The environmental risks of chemical insecticides has encouraged the development of biological control methods as an alternative approach for pest control including the use of entomopathogenic fungi. Species of entomopathogenic fungi with broad host ranges including entomophthoraceous fungi, are known to infect larval or adult black flies, and thus are potential candidates for mycoinsecticide development (Molloy, 1987).

Entomophthoralean fungi observed in populations of adult black flies in North America include *Erynia curvispora* (Nowakowski) Nowakowski in *Simulium decorum* Walker and *Entomophthora culicis* (Braun) Fresenius in *Simulium venustum* Say and *Simulium vittatum* Zettersted (Thaxter, 1888; Shemanchuk and Humber, 1978; Kramer, 1983a). In Europe, *Erynia conica* (Nowakowski) Remaudière and Hennebert attacked populations of *Simulium argyreatum* Meigen and *Simulium varegiatun* Meigen, *E. curvispora* infected *Simulium erythrocephalum* (=Boophthora erythrocephala) DeGeer and *Simulium latipes* Meigen, *Erynia ovispora* (Nowakowski) Nowakowski was found on *Simulium morsitans* Edwards, while *Erynia rhizospora* (Thaxter) Remaudière and Hennebert infected *S. argyreatun* (Nowakowski, 1883; Rubtzov, 1967; Descals et al., 1981; Hywel-Jones and Ladle,
$E.\ conica$ and $E.\ curvispora$ species either kill adult black flies directly and or prevent or reduce egg production prior to insect death (Rubtzov, 1967; Kramer, 1983a; Hywel-Jones and Ladle, 1986). Although these pathogens occur in temperate climates, they are resistant to adverse environmental conditions, and are capable of recycling (Shemanchuk and Humber, 1978), little information exists on the ecology of these fungi in black flies. To address this we have examined several species of black flies for infection by fungi of the Entomophthorales from two locations on the Réserve Faunique du Saint-Maurice in Quebec. We report host and locality records and the seasonal presence of three entomophthoralean pathogens.

**MATERIALS AND METHODS**

**Sampling black flies**

The outlets of Inman Lake (46°59'N, 73°07'W) and Eveline Lake (46°55'N, 73°01'W) in the Réserve Faunique du Saint-Maurice were sampled for black fly adults, larvae, and eggs biweekly for 3-5 days from May until October 1990. Uninfected adults, larvae and eggs were kept in 70 % ethanol.

Adhesive green polyester tissue strips used to trap the adult stages mimicked the partially submerged ovipositional substrate of black flies (Golini and Davies, 1975). Traps were placed in slowly running water to collect ovipositing females. The strips (2.5 cm x 25 cm) were sprayed on both sides with Scotch-Guard (3M Canada Inc., London, Ontario) to render them water proof and coated on one side with sticky Tangle-trap (Tanglefoot Company, Grand Rapids, Michigan). Two strips were
attached 8 cm apart to floating polystyrene foam block (Styrofoam SM) (18 cm x 12 cm x 5 cm); attached to rocks in the stream. Traps were placed at each outlet at sunset when black flies were observed to lay eggs (Imhof and Smith, 1979). The strips were removed the next morning. Insects showing signs of infection by entomophthoralean fungi (presence of external conidiophores) were removed, and strips were incubated at room temperature for 24 h to allow the external development of the fungi necessary for species identification.

**Isolation of Entomophthorales**

At each stream outlet, individual adult black flies found attached to the natural substratum or to sticky strip traps and showing external signs of entomophthoralean infections were collected and placed on moist paper on the bottom of the wells of a 96 well tissue culture plate. In the laboratory, fungi were isolated for identification and culture by showering actively discharging conidia from individual cadavers onto microscope slides, in darkness at room temperature, in a humidity chamber. The chamber consisted of a Petri dish where the lid was lined with filter paper moistened with sterile distilled water (Papierok, 1986; Glare and Milner, 1991).

Cadavers were affixed to the moist filter paper with the dorsal surface facing down to facilitate conidial discharge from emerging conidiophores. The terminology of Webster and co-workers (1978) and Descals and co-workers (1981) was used to describe conidial type. Conidia formed on conidiophores are considered to be primary conidia, and conidia produced from the primary conidia are secondary conidia. Secondary conidia are either morphologically identical to (type 1) or different from (type 2) primary conidia. Some primary conidia collected as described above were
allowed to sporulate in a humidity chamber to obtain secondary conidia for use in fungal identification (Ben-Ze'ev and Kenneth, 1982). A sterile slide was used subsequently to collect primary conidia for cultivation of the isolate. The conidia were transferred to GEMYSA medium (0.4 g glucose, 18 ml egg-yolk, 10 ml milk, 0.2 g yeast extract, 1.8 g Sabouraud dextrose and 0.4 g agar in 100 ml of distilled water). Cultures were incubated in the dark at 20 °C. Primary and secondary conidia were also obtained from a seven mm diameter section of sporulating mycelium. The conidia produced on the insect were termed *in situ* conidia, and those produced on GEMYSA medium were termed *in vitro* conidia.

Conidial dimensions were measured from preparations mounted in lactophenol-cotton blue, and the number of nuclei determined using lactophenol-aceto-orcein stain. Giemsa stain was used to stain nuclei in *Entomophaga* near *limoniae* Keller (Punithalingam, 1989). Primary and secondary conidial measurements are presented as the range of arithmetic mean values (with extreme values in brackets) for the length and width of 50 conidia according to Keller (1987). Measurements of both conidial types produced *in situ* and *in vitro* are necessary to identify this group of fungi (Papierok, 1986). The insects were subsequently preserved in 70 % ethanol.

**Black fly identification**

Black flies adults were identified to the level of species or species complex on the basis of morphological characteristics (Davies *et al.*, 1962; Wood *et al.*, 1963). Identification was subsequently confirmed by Dr. Peter Adler (Clemson University, South Carolina). During identification, the abdomens of the adults were teased apart
and examined for the presence of eggs and fungal hyphae, hyphal bodies, and resting spores. Black fly eggs and larvae were examined for similar fungal elements.

**Abiotic parameters**

The pH and conductivity of the streams were measured using a Hanna pH meter at each sampling time. The maximum and minimum air temperatures were supplied by the Quebec environmental station of Saint-Joseph-de-Mékinak located in close proximity to the Réserve, whereas water temperatures were recorded at sampling time using a thermometer.

**RESULTS**

**Locality and characterization of Entomophthorales species**

Three Entomophthorales species, *E. conica*, *E. curvispora* and *Entomophaga nr. limoniae* were observed in adults black fly populations at the Réserve Faunique du Saint-Maurice. All Entomophthorales species were detected at the outlet of Inman Lake, while only *Erynia* species were noted at the Eveline Lake outlet. Female and three male black flies infected with entomophthoralean fungi from both sites were observed on both the sticky strip traps and natural substrates (e.g., floating vegetation, partially immersed stones, logs and twigs) where the humidity was high.

Infected insects were covered by a white mass of conidiophores and cystidia (*Erynia* species) growing between the abdominal intersegmental membranes, thoracic
pleural membranes, the frons and clypeus of the head, and between the leg segments. Flies infected by *Erynia* species exhibited rhizoids that fixed the insect to the substrate. Some infected insects contained eggs (7%, n=228); most, however, contained hyphal bodies and no eggs. Resting spores were seen only in insect infected by *E. conica* collected on June 21 at the Inman Lake (9%, n=35). Black fly eggs and larvae did not reveal resting spores or other fungal entomophthoralean structures. All fungal species were successfully isolated on GEMYSA medium. Secondary type 2 conidia were produced on this medium by *E. conica* and *E. curvispora* from discharged primary conidia but at a low incidence (8.5% (n=59) and 17.2% (n=58) for *E. conica* and *E. curvispora*, respectively).

*Entomophaga* nr. *limoniae* emerged from the host by production of simple conidiophores (Fig. 1, 1) and lacked cystidia and rhizoids. The multivacuolated, multinucleate in situ primary conidia were pyriform to subspherical in shape and unitunicate with a conical or rounded papilla at the base (Fig. 1, 2a and 2b). These conidia [48.8-58.9 μm (31.5-73.5 μm) x 41.0-51.3 μm (29.4-64.0 μm)] were similar in dimension to the in vitro conidia [54.2-58.6 μm (42.0-73.5 μm) x 48.2-51.0 μm (34.6-71.4 μm)]. The number of nuclei per conidium [68(9-128)] and size of the nuclei [3.7 μm (2.9-4.8 μm)] varied substantially. Both in situ and in vitro conidia produced type 1 secondary conidia only (Fig. 1, 4). Polymorphic hyphal bodies but no resting spores were noted in the fly abdomens (Fig. 1, 5).

**Host species and seasonal records**

Based on geographic distribution of the *Simulium verecundum* Stone and Jamnback complex species, the material in this study may be *S. verecundum* and/or
*Simulium rostratum* (*S. verecundum* cytospecies ACD) (P. Adler, personal communication). Thus, the adults materials were identified as belonging to the *S. verecundum*/*rostratum* complex.

Three species of Entomophthorales were noted infecting *Simulium* species from the middle of May to middle of September (Fig. 2). *E. conica* was found in *S. verecundum*/*rostratum* and the *S. vittatum* and the *S. venustum* complexes, but never *S. decorum*. *E. curvispora* was routinely isolated from *S. decorum* and occasionally from the *S. vittatum* complex from mid July until September. In June a new fungus-black fly association was detected which involved *Entomophaga* nr. *limoniae* in the *S. verecundum*/*rostratum* complex. Only one *S. verecundum*/*rostratum* adult was infected by both *E. conica* and *Entomophaga* nr. *limoniae*. After mid-September no mycoses were noted at either field site.

**Abiotic parameters**

The pH range of the stream water over the season was 6.2-6.8 at the Inman Lake outlet and 5.9-6.7 at the Eveline lake outlet. Similar levels of water conductivity were noted at both sites (20.1-28.1 μS). The mean air temperature varied between 2.5 to 28.0 °C, while mean water temperature varied between 10.0-27.0 °C (Fig. 3).

**DISCUSSION**

Although *E. conica* has been recorded on black flies in Europe and *E. curvispora* both in Europe and United States (Nowakowski, 1883; Rubtzov, 1967;
This is the first report of their presence on simuliids in Canada. *S. verecundum/rostatum* and the *S. venustum* and *S. viatum* complexes are new hosts for *E. conica*; thus, the fungus appears to have a broad host range including chironomids, chaoborids, Trichoptera, tipulids and simuliids (Nowakowski, 1883; Thaxter, 1888; Descals et al., 1981; Keller, 1991; Cuesbas-Incle, 1992; Papierok and Dumas, 1992; this study). *Entomophaga* nr. *limoninae* is closely related to *Entomophaga limoninae* Keller on the basis of morphology (Keller, 1987). It differs, however, in terms of the species of host insect and the number of nuclei per conidium, which, in conjunction with the lack of rhizoids and resting spores, precludes its positive identification (B. Papierok, personal communication).

Aerial conidia of both type 1 and 2 described for *E. conica* and *E. curvispora* (Descals et al., 1981; Kramer, 1983a; Keller, 1991) were detected in this study. Secondary type 2 conidia of *E. conica* produced germ tubes that have been reported to penetrate the host cuticle (Hywel-Jones and Webster 1986). However, the primary conidia of both species floated on water and eventually formed secondary conidia; thus, the primary conidia may be the dispersive stage and the secondary type(s) the invasive unit. Laboratory infection studies with the different types of conidia will be required to confirm their specific role(s). The low number of replicative conidia observed to develop from primary conidia may be explained by the inadequate humidity of the environment in that many of the Entomophthorales need saturated relative humidity for conidial discharge (Millstein et al., 1983; Glare et al., 1986). The most critical environmental factor influencing development of most entomopathogenic fungi is relative humidity (Hall and Papierok, 1982). In this study, adult black flies
killed by the fungi were always found at the air-water interface, fixed on a substratum splashed by water (Shemanchuk and Humber, 1978; Kramer, 1983a; Hywel-Jones and Ladle, 1986), and under conditions where daily temperature variations caused dew or fog formation. This ensured that a consistently high humidity was available for fungal development. The stellate and coronate conidia described for *E. conica* by Webster *et al.* (1978), Descal *et al.* (1981) and Keller (1991) were not detected in the present study. Although environmentally resistant resting (zygospores) were observed for *E. conica* only, their absence for *E. curvispora* and *Entomophaga* nr. *limoniae*, implies that the survival of the latter two species in temperate climates might reflect fungal mummification of the host as reported for entomophthoralean infections of terrestrial insects (Kenneth *et al.*, 1972; Wilding, 1973), the adult black fly being regarded as a terrestrial host.

Dead female flies with the abdomen filled with eggs and infected by *E. conica* and *E. curvispora* were commonly detected, which is in consonance with Rubtzov (1967), Kramer (1983a) and Hywel-Jones and Ladle (1986). With the sticky strip traps several infected flies laid only a part of their egg clutch. Therefore, the fungi may have reduced simuliiid populations by limiting egg laying, although it is also possible that egg production would also be lower. It is not known if the eggs were viable.

Although ecological barriers may limit host availability for infection (Kramer, 1983b) the fungal species and species of black flies were observed to be associated in time and space (natural substrate, strip trap) similar to a report by Imhof and Smith (1979) precluding such barriers and yet there appeared to be host specificity. *E. conica* was never observed to infect *S. decorum* and *E. curvispora* was predominantly
associated with *S. decorum*. Insect morphology and cuticular composition and physiology may influence host susceptibility to infections by *E. conica* and related species (Mullens, 1989; Cuevas-Incle, 1992). However, in recognition of the limitations of the small sample size, due principally to the difficulty of host black fly identification to the species level (a result of insect mummification), the report of the absence of *E. conica* in *S. decorum* is tentative. Attempts will be made to confirm host specificity by infecting *S. decorum* and *S. verecundum*rostratum with both conidial types of *E. conica* and *E. curvispora* in the laboratory.

In summary, this is the first report of the presence of *E. conica* and *Entomophaga* nr. *limoniae* in black flies in North America and *E. curvispora* in Canada. *S. verecundum*rostratum, *S. venustum*, and *S. vittatum* are new host species for *E. conica*. Studies in two black fly populations have shown that host specificity for *E. conica* may exist.

**ACKNOWLEDGMENTS**

The author thanks Dr. Bernard Papierok of the Pasteur Institute, France, and Dr. Peter Adler, Clemson University of South Carolina, for their help in fungal and black fly species characterization, and Mr. G. Proteau of the Ministère de l'Environnement et Faune du Québec for facilities at the Réserve Faunique du Saint-Maurice.
FIG. 1. *Entomophaga* near *limoniae*. 1) Simple conidiophores emerging from abdominal intersegmental membranes of infected adult *Simulium verecundum*/*rostratum* and showing apical sporulation. 2) *In situ* primary conidium with (A) conical rounded tip and (B) rounded papilla. 3) Multinucleate *in vitro* primary conidium. 4) Primary *in vitro* conidium on distilled water agar bearing type 1 conidium. 5) Polymorphic hyphal bodies infecting adult of *Simulium verecundum*/*rostratum*. Bar scale = 10 μm.
FIG. 2. Presence of Entomophthorales infections in the adults of black fly species and species complexes collected at the outlets of Inman Lake and Eveline Lake during the 1990 season at the Réserve Faunique du Saint-Maurice. The line represents the presence of black fly species during specific periods of collection, and the squared pattern the infected species by the following fungal species: Entomophaga near limoniae(□), Erynia conica (□) and Erynia curvispora (■).
INMAN LAKE

Prosimulium mixtum
Simulium decorum
S. venustum complex
S. verecundum/rostratum
S. vittatum complex

EVELINE LAKE

Prosimulium mixtum
Simulium decorum
S. venustum complex
S. verecundum/rostratum
S. vittatum complex
FIG. 3. Minimum and maximum air temperature (A), and the minimum and maximum of water temperature at the Inman Lake (B) and Eveline Lake (C) recorded throughout the season in the Réserve Faunique du Saint-Maurice in 1990.
LITERATURE CITED


CONNECTING STATEMENT 4

In Chapter 5, organic molecules influenced the morphogenesis of the type 2 conidia of *Erynia conica*. The effect of abiotic factors such as pH, temperature and substratum hardness, charge and hydrophobicity on conidial development were examined in Chapter 6 to assess possible environmental effects on field mycoses.
CHAPTER 6

EFFECTS OF PHYSICAL FACTORS ON THE DEVELOPMENT OF SECONDARY CONIDIA OF *ERYNIA CONICA* (ZYGOMYCETES: ENTOMOPHTHORALES).
ABSTRACT

The development of secondary type 2 conidia of *Erynia conica* was studied *in vitro* and *in situ* in relation to selected physical factors. Conidia *in vitro* germinated on various inert surfaces and both germinated and formed replicative tertiary conidia on water agar and charged beads. The germination and secondary sporulation occurred without exogenous nutrients, was not influenced by substratum hydrophobicity and was influenced by pH, temperature, and possibly the hardness and surface charge of the substrata. Germination and sporulation occurred throughout the pH range of 5.5 to 8.0, and temperatures ranging between 5 and 30 °C. Both germination and sporulation had the same optimum pH (7.5-8.0) and optimum temperature (10-20 °C). The effect of temperature on the pattern of germination of conidia was similar *in vitro* and *in situ*. The infective stages of appressorial formation and penetration, which were observed only *in situ*, were more affected by temperature than was conidial germination.
INTRODUCTION

*Erynia conica* (Nowakowski) Remaudière and Hennebert is an entomopathogenic fungus reported to infect the adults of numerous black fly species (Diptera: Simuliidae) in Europe, and more recently in Canada (Descals et al., 1981; Hywel-Jones and Ladle, 1986; Hywel-Jones and Webster, 1986; Keller, 1991; Papierok and Dumas, 1992; Nadeau et al., 1994). The fungus produces a globose conidiospore, the secondary type 2 conidia, which is the infective stage for simuliids (Hywel-Jones and Webster, 1986) including *Simulium rostratum* (= *Simulium verecundum* Stone and Jamnback, cytospecies ACD). The infective conidium germinates on the surface of the cuticle of *S. rostratum* and either forms an appressorium followed by a penetrating germ tube that passes through the integument of the insect, or produces a tertiary type 1 conidium by replicative sporulation (Chapter 5).

It has been reported that germination of the primary conidia of entomophthoralean spores, including *E. conica*, do not require exogenous nutrients, as evident by their germination on coverslips, water, or water agar (Yendol, 1968; Kerwin, 1982; Sampedro et al., 1984; Van Roermund et al., 1984; McGuire et al., 1987; Glare and Milner, 1991; Chapter 4). This is in contrast with entomopathogenic Deuteromycetes such as *Beauveria bassiana* (Balsamo) Vuillemin (Smith and Grula, 1981) and *Nomureae rileyi* (Farlow) Samson (Boucias and Pendland, 1984). Although physical factors such as relative humidity and temperature influence conidial germination of primary conidia of entomophthoralean fungi, pH, osmotic pressure, light, and oxygen also have an effect.
(Yendol, 1968; Callaghan, 1978; Sampedro et al., 1984; Van Roermund et al., 1984; Glare and Milner, 1991; Chapter 4). Very few studies have reported on the effect of these physical parameters on the mode of germination of secondary conidia; for *Entomophthora muscae* (Cohn) Fresenius germ tubes developed from secondary conidia incubated at relative humidity ranging from 48-100 %, with high levels observed at nearly saturated relative humidity (Kramer, 1980; Carruthers and Haynes, 1986). *Zoophthora radicans* (Brefeld) Batko (= *Erynia radicans*), *Zoophthora phalloides* Batko (= *Erynia phalloides*) and *Neozygites fresenii* (Nowakowski) Remaudière and Hennebert the secondary capilliconidia germinated also at low relative humidity (Uziel and Kenneth, 1991). Carruthers and Haynes (1986) reported that the germination of secondary conidia of *En. muscae* occurred over a temperature range of 4-27 °C. According to Uziel and Kenneth (1991), light reduced germination of the capilliconidia of *Z. radicans*.

The formation of appressoria and cuticular penetration by entomopathogenic fungi are also influenced by environmental factors, including surface topography, nutrient stimuli and physical parameters (St-Leger et al., 1989; Wraight et al., 1990; Magalhaes et al., 1990, 1991; Galaini-Wraight et al., 1992). Appressorial formation by the fungi *Z. radicans* and *Metarhizium anisopliae* (Metschnikoff) Sorokin is stimulated by low concentrations of nutrients (St-Leger et al., 1989; Magalhaes et al., 1990). Conidia of *Z. radicans* also form appressoria and penetrate the aphid *Empoasca kraemerii* Ross and Moore at temperatures ranging from 5-30 °C, with maximum infection at 23 °C (Galaini-Wraight et al., 1992). Magalhaes et al. (1991) reported appressorial formation for *Z. radicans* at pH 6-9.
Knowledge of the factors involved in fungal development is necessary to understand not only the invasion process of the pathogen but also to ensure the effectiveness of the fungus as a mycoinsecticide. Herein we report the effects of various physical factors on the development of the infective unit of *E. conica*.

**MATERIALS AND METHODS**

**Fungal isolate and inoculum**

Strain 512 of *E. conica*, originally isolated from adults of the *Simulium verecundum* Stone and Jamnback complex collected at Inman Lake of the Réserve Faunique du Saint-Maurice (46°59'N, 73°07'W), was maintained on GEMYSA medium [0.4 g glucose, 18 ml egg yolk, 10 ml milk, 0.2 g yeast extract, 1.8 g Sabouraud dextrose, and 0.4 g agar in 100 ml distilled water, (B. Papierok, personal communication)] at 5 °C. Fungi were subcultured every two months as described in Chapter 4, incubated at 20 °C for 10 days and then returned to 5 °C for storage.

Secondary type 2 (globose) conidia were obtained from primary conidia that had been showered onto water agar from a sporulating mycelial mat. A seven mm diameter sample of two week old fungal culture previously grown on GEMYSA in the dark at 20 °C was aseptically placed on distilled water agar in a Petri dish (35 x 10 mm). The dish was inverted and incubated in the dark at 20 °C (RH 95 %) to allow the primary conidia to discharge. When primary sporulation commenced the inverted dish was placed over another containing distilled water agar for 1.5 h during which the dish was rotated by 90 ° at 15 minutes intervals to allow uniform conidial deposition. The dish of agar with
primary conidia was then inverted and incubated in darkness at 20 °C (RH 95 %) until the secondary spores began to discharge. The secondary conidia were then showered onto designated substrates for 2 h, always in darkness at 20 °C and high humidity. The procedure produced variation in the concentration of spores adhering to the targets between replicates, but generally a uniform concentration occurred within replicates.

Experimental insects

Isolated wings of laboratory reared S. rostratum were used as the cuticular target surfaces for in situ experiments. Wings were selected because infection structures were readily detected on this surface. Materials storage and preparation were described in Chapter 5.

Experimental protocol

We studied the in vitro effects of the following abiotic parameters on the development of secondary type 2 conidia of E. conica: source of water, pH, temperature and surface hardness, surface wettability and charge. The effect of temperature in situ was also determined.

We first established the time course of the development of secondary type 2 conidia in vitro on distilled water agar (1.5 % w/v). Secondary conidia were showered onto water agar in a Petri dish (35 x 10 mm) and after a period of incubation (3, 6, 26, and 48 h) the development of the conidia was recorded.
The effect of water source was determined using water collected at the site where *E. conica* was isolated from the simuliiids (e.g. the outlet of the Inmann Lake of the Réserve Faunique du Saint-Maurice), and distilled water. Both types of water were mixed with 1.5% (w/v) partially purified agar (BDH).

The effect of pH on the development of secondary type 2 conidia was examined using selected buffers in distilled water agar onto which the conidia were showered. The pH ranged from 5.5 to 8.0 in increments of 0.5 units and encompassed the pH of the stream water recorded at the field site (6.2-6.8). The following buffers were used: 2-(N-morpholino)ethanesulfonic acid (MES, pH range: 5.5 to 6.5) and 3-(N-morpholino)propanesulfonic acid (MOPS, pH range: 7.0 to 8.0). The possible effect of the type of buffer on conidiogenesis was tested by overlapping pH values for each buffer (pH 6.5 and 7.0). The buffer was added to distilled water containing agar forming a 5mM buffer concentration, the pH adjusted with 0.2 M NaOH or 1 M HCl and the medium sterilized by autoclaving. Two ml of the agar were poured into a Petri dish. Sampedro et al. (1984) noted that a high concentration of buffer can inhibit the germination of the conidia of the entomophthoralean fungus *Conidiobolus obscurus* (Hall and Dunn) Remaudière and Hennebert. To confirm the possible effect of the concentration of the buffer at the molarity used, a control without buffer was made at pH 7. The buffer molarity used in the present study prevented a change in the pH medium at the end of the experiment.

The effect of the temperature was measured by showering conidia onto the surface of distilled water agar medium buffered at pH 6.5 (MES, 5 mM), the optimum pH for spore germination and development (see results in pH section), and incubating the spores...
at 5, 10, 15, 20, 25 and 30 °C for 24 h. The dishes with the medium were preincubated for one hour at a given temperature before being inoculated with conidia. In the in situ study, each replicate was composed of groups of five wings of *S. rostratum* placed radially in the center of a distilled water agar dish. The dish without the lid was placed in a laminar air flow for 5 minutes to evaporate the water from the surface of the cuticle that was acquired when the substrata were placed on the medium. For this series of experiments the time of incubation was 9 h, as reported in Chapter 5.

To evaluate the effect of surface hardness on the development of secondary type 2 conidia we used two types of surfaces: one was distilled water agar containing 1.5, 3.0 and 6.0 % (w/v) agar adjusted at pH 6.5 (MES, 5 mM), and the other was a glass coverslip (18 mm²) (Fisherbrand).

The effect of the hydrophobicity was studied by showering the conidia onto the following materials representing a range of wettability: glass coverslips (Fisherbrand), glass microscope slides (Fisherbrand), polystyrene Petri dishes (Fisherbrand), transparency film (cellulose acetate) (Avery), wax paper (Country cup board), membrane filters composed of mixed esters of cellulose acetate and cellulose nitrate (Millipore, Bedford, type: HAWP 025 00) and cellophane (Plastic wrap, Reynolds). The wettability of the surface of each substrate was calculated according to Gerhart et al. (1992). A drop of 5 μl from a series of methanol solution (0, 10, 20, 30, 40, 60, 80, 100 % (v/v) in distilled water) was placed on each surface, and the average diameter of the drop measured to the nearest millimeter. Triplicate measurements of drop spreading were made for each surface. Drop measurements were reduced to a single number scale from 0 to 100 by calculating the percentage average of the drop using the following equation:
\[
\% \text{ wettability} = \left( \frac{8}{\frac{1}{w_{100}} + \frac{1}{w_{80}} + \frac{1}{w_{60}} + \frac{1}{w_{40}} + \frac{1}{w_{30}} + \frac{1}{w_{20}} + \frac{1}{w_{10}} + \frac{1}{w_{0}}} \right)^{-4} / 16 \times 100
\]

where

- \( w_{100} \) = mm drop spread at 100% water
- \( w_{80} \) = mm drop spread at 80% water in methanol
- \( w_{60} \) = etc.
- 8 = number of methanol solutions used
- 4 = minimum possible drop measurement in millimeters
- 16 = maximum range of drop size in millimeters

We compared the effect of the surface charge on the development of secondary conidia by showering conidia onto the following substrates: weakly charged beads including amide-modified and carboxylate-modified latex (polystyrene) beads; neutral latex particles (Sigma) and the strongly charged beads including the basic anion (Dowex, 1 x 8-200) and acidic cation (Dowex, 50 x 8-200) ion-exchange resins (Aldrich). The beads were suspended in MES buffer (pH 6.5, 5 mM) and washed five times with buffer by centrifugation (8 160 xg, 20°C) for two minutes to remove the storage solution. Between each centrifugation the supernatant was removed and fresh buffer added. All bead suspensions were kept overnight in the refrigerator (5°C). Microscopic observation of beads in original solutions and after preparation in the buffer and refrigeration showed that the beads looked identical in both liquids; the exception was with the amide-modified latex particles where some bead aggregation occurred. One hundred \( \mu l \) of the latex bead suspensions and 500 \( \mu l \) of strongly charged Dowex beads were placed in the center of a MES-buffered (pH 6.5, 5 mM) water agar surface. A fine paintbrush was used to spread the beads into a single layer on the agar surface. A portion of the buffer solution was
removed by absorption with a piece of filter paper (Whatman # 1) and by incubation for 2
minutes under a laminar air flow prior to showering the particle surfaces with conidia.
Germination controls consisted of conidia that were showered onto distilled water agar
and coverslips.

In all tests, the materials exposed to the secondary type 2 conidia of *E. conica*
were incubated in darkness at the optimum temperature for germination in a humidity
chamber (RH = 95 %) adapted from Rutschke and Grunewald (1984). The concentration
of conidia was determined as the estimation of the average of the number of spores per
mm² by light microscopy. Primary conidia on the water agar surface produced both
secondary type 2 conidia and secondary type 1 conidia although the latter produced less
than the former (Chapter 4). Therefore, before each incubation, the percentage of
secondary type 1 conidia was recorded as the number of type 1 spores/total secondary
spores x 100. At designated times the development of 100-200 conidia from 3-5 replicates
was determined using a light microscope (200 x). Where appropriate, the data were
recorded as the number of germinating spores (G) [conidiospores were regarded as
germinating when the length of the germ tube exceeded half the minor diameter of the
spore (Anonymous, 1943)], the number of conidia forming an appressorium (A), the
number forming appressoria and a penetrating tube (AP), and the number of spores
forming tertiary type 1 conidia (T).

**Presentation of data and statistical analysis**

Calculations were made of the percentage of total conidial germination [= 
\[(G+A+AP+T)/N, \text{ where } N= \text{ total of conidia counted}\], conidia forming appressoria [= 
\[(A+AP)/N\] or penetrated (= AP/N), or forming tertiary type 1 conidia (= T/N). The data
were analysed by ANOVA followed by Student-Newman-Keuls test (SNK-test), or Student's t-test of data transformed by arcsin $p^{1/2}$. Regression analysis was used to compare the relationship between wettability of various surfaces and conidial germination. An ANOVA for repeated measures was also done for in vitro and in situ total germination of secondary conidia test on the simuliiid wings and coverslip. A 0.05 level of significance was used for all statistical tests.

RESULTS

Development of secondary type 2 conidia

Discharged secondary type 2 conidia of *E. conica* were always surrounded by a halo of cytoplasmic material regardless of the substratum on which they landed. On distilled water agar the conidia germinated and the fungus grew vegetatively with hyphal growth or the germ tubes gave rise a tertiary type 1 conidia. Similar results occurred on the cuticle of *S. rostraturn* (Fig. 2 of Chapter 5). The other mode of germination, which included appressoria formation, was observed for conidia in situ only. Germination in vitro, on the surface of distilled water agar was apparent after 6 h of incubation, with a maximum of 30 % germination being obtained at 26 h of incubation (Fig. 1). Therefore 24 h was selected for subsequent experiments using distilled agar as a support medium. Tertiary sporulation peaked (5 %) at 26 h of incubation. Discharge of tertiary conidia was recorded at 24 h on distilled water agar, the discharge being based on the number of empty secondary type 2 conidia. This number of empty secondary conidia (3-10 %) was included in the calculation of the percentage of tertiary conidia formation. Tertiary and secondary type 1 conidia formed quaternary and tertiary type 2 conidia, respectively, *al*
biet at consistently very low levels (< 1 %) and thus we considered that they did not significantly affect the results of the experiments.

**Effect of the pH on conidial development**

The type of biological buffer selected for the study did not significantly affect the development of secondary type 2 conidia of *E. coli*ica whereas medium pH influenced total germination, pH 6.5 being optimum (Table 1); also the buffer concentration at 5 mM did not influence germination or sporulation when compared to unbuffered distilled water agar at pH 6.8-7.0 (Table 2). No significant difference in spore activity was detected using distilled water or river water in terms of either germination or tertiary conidia formation. An average of 25.5 % and 14.9 % conidia germinated in distilled water (pH 6.8) and river water (pH 7), respectively. Tertiary conidia formation with 6.8 % and 7.3 % sporulation occurred on distilled water and river water respectively.

Secondary type 2 conidia germinated and sporulated at all pH values tested, with a maximum at pH 7.5-8.0, when 37.2 % of the conidia germinated and 21.4 % sporulated (Table 2). Germination of conidia was significantly reduced at the lower pH values. Similarly, sporulation was significantly lower at pH 5.5-7.0 than at pH 7.5-8.0.

**Effect of temperature on conidial development**

The temperature *in vitro* significantly influenced both germination and sporulation of secondary type 2 conidia (Table 3). Both stages of development peaked between 10 and 20 °C, with an average of 21 % and 10 % for germination and sporulation, respectively, while at lower (5 °C) and higher (25-30 °C) temperatures, these events were
significantly reduced. Low temperature produced a static effect as opposed to toxic effect on the conidial development. Thus, after returning the conidia from 5 °C to 20 °C, 20% germinated and 4% formed replicative type 1 conidia.

The in situ study using wings of S. rostratum showed that temperature had a significant effect on the duration of various stages of the infection including total germination, appressorial formation, cuticular penetration, and replicative sporulation, with a maximum of 86.8%, 14.5%, 8.6% and 3.4%, respectively occurring at 20 °C (Table 4). Germination was noted at all temperatures, with high levels occurring at 15-25 °C, while maximal appressorium formation, cuticular penetration and sporulation were observed at 15-30 °C, 15-25 °C and 20-25 °C, respectively. After returning the spores incubated at 10 °C to 20 °C the level of total germination increased and appressoria developed.

Effect of substratum hardness on conidial development

Increased concentration of agar in the distilled water agar medium did not change the development of secondary type 2 conidia of E. conica (Table 5). Interestingly, using coverslips, germination was significantly higher than on distilled water agar, with 62% of the conidia germinating on the latter compared to 34% on the former. The germination on coverslips was not only more extensive but also faster than on distilled water agar in that the advent of germination on coverslip was seen immediately after the conidial shower, whereas at this time no germination occurred on the surface of the distilled water agar. However, germination on the coverslips was limited, the germ tube never elongating beyond the cytoplasmic halo area surrounding the conidia, while on the distilled water agar the germ tube grew over this area and formed a hypha. Analysis of
conidial activity *in situ* (on wings of *S. rostratum*) and *in vitro* (coverslip) materials did not reveal a significant difference in total germination by 6 h, but initial (2-4 h) germination was significantly stimulated on coverslips (Table 6). No appressoria, penetrating tubes or replicative sporulation was seen on coverslips or water agar.

**Effect of substratum wettability on conidial development**

The type of material tested significantly affected the level of germination, with maximum germination occurring on coverslips, polystyrene and cellulose acetate (Table 7). However, germination was not dependent of the wettability of the surface implying that other factors might be important ($R^2=0.439$, ANOVA $P=0.4561$). On these substrates neither sporulation nor the development of appressorium occurred.

**Effect of substratum charge on conidial development**

Secondary type 2 conidia of *E. conica* germinated and sporulated equally well on the surface of both weakly negatively-charged beads and neutral substrates, as opposed to weakly cationic beads and strongly cationic and anionic resins (Table 8). Latex beads revealed that a positive charge significantly reduced the development of the conidia when compared to a neutral or negative charge: 19.7 % and 5.5 % of conidia germinated and sporulated on aminated latex beads compare to 30.0-33.7 % and 14.9-18.8 % for and neutral and carboxylated latex beads, respectively. The level of charge also influenced the development of conidia with the strongly charged material more extensively inhibiting germination. Water agar allowed conidial germination to the same extent as the mildly charged and neutral beads whereas the coverslips produced the most extensive results.
DISCUSSION

Secondary type 2 conidia of *E. conica* germinated on distilled water agar. The observation of the *in vitro* experiments on water agar and on inert supports such as coverslips, show that secondary conidia of *E. conica* do not need exogenous nutrients for germination; the conidia may possess endogenous reserves. Hajek *et al.* (1990) indicate that primary conidia of *Entomophaga maimaiga* Humber, Shimazu and Soper germinate on distilled water agar but not on coverslips and suggest that the spores need saturated or near-saturated RH for germination. For some primary conidia of *Entomophthora* species, and the conidia of the Deuteromycete *M. anisopliae* germination was achieved on both surfaces but always at higher level (percentage) on distilled water agar than on glass (Yendol, 1968; Schabel, 1978). Contrary to these studies, the conidia of *E. conica* germinated on glass coverslip with a higher rate than on distilled water agar, suggesting that secondary conidia of *E. conica* may have a preference for lower relative humidity. A low humidity requirement has been reported for secondary conidia of *En. muscae* and the secondary capilliconidia of *N. Fresenii* and *Zoophthora* species (Kramer, 1980; Uziel and Kenneth, 1991). The projected conidia of *E. conica* were always surrounded by a halo of cytoplasmic material, and on coverslips as well as other artificial surfaces the germ tube never developed beyond of this halo. Therefore, conidia may acquire some water from the halo for germination. A study of the effect of relative humidity should be done to confirm the importance of this parameter on the development of the infective unit of *E. conica*.

This study established that physical factors (pH, temperature and the charge) affect the development of globose conidia of *E. conica*. The maximum pH at which
Germination of secondary conidia of *E. conica* and replicative spore formation were dependent on the incubation temperature, these activities occurring over a temperature range corresponding to the range of field temperatures at which the fungus was active in black fly populations (Chapter 2). Similar effects were noted by Carruthers and Haynes (1985, 1986) who indicated that secondary conidia of *En. muscae* germinate over a temperature range of 4-27 °C, with maximum germination near 20 °C. The *in situ* study with *E. conica* indicated that appressoria formation and cuticular penetration were more affected by temperature than was germination, with a preference for moderate temperatures. Galaini-Wraight et al. (1992) noted that for conidia of *Z. radicans* on *Em. kraemeri*, appressoria and cuticular penetration occurred at a range of 5-30 °C with maximum infection at 23 °C. In the present study, lower temperatures (<10 °C) inhibited the formation of infective structures. Magalhaes et al. (1991) observed complete *in vitro* suppression of appressoria of *Z. radicans* conidia at lower temperatures. Differences between the *in situ* and *in vitro* results may be explained by differences in quality of the nutrients that might influence appressorial formation.

Interestingly, the level and rate of germination on coverslips were very similar to that *in situ* on the surface of the wings, suggesting that common physical characteristics of coverslip and wings such as surface hardness or hydrophobicity are involved in conidial germination. Increased concentration of agar did not change the pattern of conidial germination, thus hardness does not seem to contribute to germination. Due to the small surface of the wings, it was not possible to measure the wettability of the wing
surface by the solvent drop-spread technique to compare with the value obtained with coverslips. Using different materials with a range of wettability, we established that germination was independent of hydrophobicity as was reported for *M. anisopliae* (St-Leger *et al.*, 1989). However, the hydrophobicity measurement involved substrates that differed in chemical composition which may have influenced the results; for example, the microscope slides and the coverslip were composed of boron silicate and sodium silicate glass which included chemical elements that qualitatively or quantitatively differed in concentration. This may explain the difference in conidial germination observed on these surfaces. Difference also existed in the hardness of these two glass surfaces, slides being harder than coverslips.

Reproductive sporulation has been reported from entomophthoralean fungi for primary conidia at the surface of distilled water agar including *E. conica* (Callaghan, 1978; Kerwin, 1982; Sampedro *et al*., 1984; Van Roermund *et al*., 1984; McGuire *et al*., 1987; Hajek *et al*., 1990; Chapter 1). Reproductive sporulation of secondary type 2 conidia (i.e. tertiary type 1 conidia formation) was observed *in vitro* but only on distilled water agar from conidia suspended in liquid, and *in situ* on wings of *S. rostratum*. In these situations such sporulation occurred after a period of incubation at 95% relative humidity. Therefore, reproductive sporulation seems to require saturated or near-saturated relative humidity.

Appressorial formation and cuticular penetration by *E. conica* was recorded only during *in situ* study and may reflect a need for nutrients. *M. anisopliae* does not form appressoria on water agar (Schabel, 1978), but does produce invasion structures when a low concentration of complex nitrogenous nutrients and a hard surface are provided (St-
Leger et al., 1989). Nitrogen requirements for appressoria have been reported for the entomophthoralean Z. radicans (Magalhaes et al., 1990). Therefore, even though germination of secondary type 2 conidia of E. conica did not require exogenous nutrients, the appressoria may have had such requirements, although alternative cues such as a receptor binding site or topographical signal may also be important.

Strongly charged particles and mildly charged positive surfaces affected the germination and sporulation of secondary type 2 conidia of E. conica, while mildly negatively charged or neutral material did not. St-Leger et al. (1989) found that germination as well as appressoria formation of the entomopathogenic fungus M. anisopliae was independent of charge, based on exposing conidia to various plastic surfaces that differed in chemical composition.

In summary, germination and sporulation of the secondary type 2 conidia of E. conica, the infective unit of the black fly S. rostratum, was dependent on the physical factors in the environment surrounding the conidia such as pH, temperature and charge. Germination occurred on various hard surfaces and may be nonspecific and independent of exogenous nutrients. Infective structures developed only in situ and, like germination in vitro, the structures were influenced by environmental temperature.
FIG. 1. The *in vitro* development of secondary type 2 conidia (globose conidia) of *Erynia conica* on distilled water agar (incubated in darkness at 20 °C and 95 % RH) in terms of germination (△) and replicative sporulation which consists of the formation of tertiary type 1 conidia (○). Each point represents the mean and standard error of the mean of three groups based on 200 observations. The concentration of the conidia and the percentage of secondary type 1 conidia for the replicate varied between 5.8-13.1 spores/mm² and 0.7-2.5 %, respectively.
### TABLE 1

Effect of buffer type on the development of secondary type 2 conidia of *Erynia conica in vitro*.  

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Total germination (%)</th>
<th>Percentage of tertiary type 1 conidia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td>6.5</td>
<td>30.0 (28.6, 37.8)</td>
<td>11.2 (12.5, 26.7)</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>17.7 (17.5, 32.2)</td>
<td>4.8 (9.0, 16.5)</td>
</tr>
<tr>
<td>MOPS</td>
<td>6.5</td>
<td>39.2 (31.0, 46.6)</td>
<td>17.2 (15.3, 37.7)</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>11.9 (14.6, 25.8)</td>
<td>2.5 (1.3, 15.8)</td>
</tr>
</tbody>
</table>

*a* Incubation on distilled water agar buffered with 5 mM MES (2-(N-morpholino)ethanesulfonic acid) or MOPS (3-(N-morpholino)propanesulfonic acid) in darkness at 20 °C, RH = 95%, for 24 h.

*b* Decoded means of arcsin p/2 transformed data (with 95% confidence limits of the transformed data) from five groups based on 100 observations. The concentration of conidia and the percentage of secondary type 1 conidia varied between 3.8-7.8 spores/mm² and 9.0-13.9%, respectively. For each pH, means within columns are not significantly different according to the Student's t-test (α = 0.05).
TABLE 2

Effect of pH on the development of secondary type 2 conidia of *Erynia conica* in vitro. a

<table>
<thead>
<tr>
<th>pH</th>
<th>Percentage <em>b</em></th>
<th>Percentage <em>b</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total germination</td>
<td>tertiary type 1 conidia</td>
</tr>
<tr>
<td>5.5</td>
<td>1.1 (0.0, 10.7) a</td>
<td>0.7 (0.0, 16.2) a</td>
</tr>
<tr>
<td>6.0</td>
<td>4.7 (0.0, 42.3) ab</td>
<td>0.4 (0.0, 19.8) a</td>
</tr>
<tr>
<td>6.5</td>
<td>19.0 (8.1, 43.5) bc</td>
<td>1.8 (3.1, 12.5) ab</td>
</tr>
<tr>
<td>7.0</td>
<td>11.1 (15.7, 23.3) ab</td>
<td>4.2 (6.7, 16.9) ab</td>
</tr>
<tr>
<td>7.5</td>
<td>20.8 (17.1, 37.2) bc</td>
<td>13.0 (14.8, 27.5) c</td>
</tr>
<tr>
<td>8.0</td>
<td>37.2 (27.3, 47.9) c</td>
<td>21.4 (22.4, 32.6) c</td>
</tr>
<tr>
<td>Control</td>
<td>17.8 (4.8, 45.4) bc</td>
<td>5.7 (4.3, 23.3) b</td>
</tr>
<tr>
<td>Distilled water c</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Incubation on distilled water agar buffered with 5 mM MES (2-(N-morpholino)ethanesulfonic acid) or MOPS (3-(N-morpholino)propanesulfonic acid), in darkness at 20 °C, RH= 95 %, for 24 h.

*b* Decoded means of arcsin p1/2 transformed data (with 95 % confidence limits of the transformed data) from three groups based on 100 observations. The concentrations of conidia and the percentage of secondary type 1 conidia varied between 2.4-8.8 spores/mm² and 4.7-11.5 %, respectively. Means within columns by the same letter are not significantly different according to the SNK-test (α= 0.05).

*c* pH before and after experimentation: 7.0, 6.8.
TABLE 3

Effect of temperature on the development of secondary type 2 conidia of *Erynia conica in vitro*.  

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Percentage total germination</th>
<th>Percentage tertiary type 1 conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.7 (3.3, 11.8) a</td>
<td>0.0</td>
</tr>
<tr>
<td>10</td>
<td>8.3 (15.8, 17.7) b</td>
<td>2.3 (0.0, 27.8) ab</td>
</tr>
<tr>
<td>15</td>
<td>16.2 (3.3, 44.2) b</td>
<td>10.1 (11.6, 25.5) b</td>
</tr>
<tr>
<td>20</td>
<td>21.4 (23.8, 31.3) b</td>
<td>10.4 (15.6, 22.1) b</td>
</tr>
<tr>
<td>25</td>
<td>2.7 (4.3, 14.7) a</td>
<td>0.1 (0.0, 9.1) a</td>
</tr>
<tr>
<td>30</td>
<td>5.0 (10.3, 15.5) a</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*a* Incubation on distilled water agar buffered at pH 6.5 with 5 mM MES (2-(N-morpholino)ethanesulfonic acid), in darkness at 20 °C, RH= 95 %, for 24 h.

*b* Decoded means of arcsin p^{1/2} transformed data (with 95 % confidence limits of the transformed data) from three groups based on 100 observations. The concentrations of conidia and the percentage of secondary type 1 conidia varied between 2.0-3.4 spores/mm² and 6.5-14.6 %, respectively. Means within columns by the same letter are not significantly different according to the SNK-test (α= 0.05).

*c* Kruskall-Wallis oneway ANOVA on rank test.
TABLE 4

Effect of temperature on the development of secondary type 2 conidia of *Erynia conica* on the wings of *Simulium rostratum*. *

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Percentage <em>b</em></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total germination</td>
<td>appressoria</td>
<td>cuticular penetration</td>
<td>tertiary type 1 conidia</td>
</tr>
<tr>
<td>5</td>
<td>6.7 (11.3, 18.8) a</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>10</td>
<td>8.9 (14.6, 20.2) a</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>15</td>
<td>58.8 (34.4, 65.7) b</td>
<td>3.0 (0.0, 31.7) ab</td>
<td>0.3 (0.0, 16.7) a d</td>
<td>0.0</td>
</tr>
<tr>
<td>20</td>
<td>86.8 (63.2, 74.3) c</td>
<td>14.5 (16.9, 27.9) b</td>
<td>8.6 (9.8, 24.5) b</td>
<td>3.4 (6.5, 14.9) a e</td>
</tr>
<tr>
<td>25</td>
<td>69.7 (46.9, 66.3) b</td>
<td>3.1 (0.0, 32.1) ab</td>
<td>0.4 (0.0, 18.8) a</td>
<td>0.2 (0.0, 14.0) b</td>
</tr>
<tr>
<td>30</td>
<td>15.3 (9.10, 37.0) a</td>
<td>0.5 (0.0, 7.2) a</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*a* Incubation at the surface of the wings of the *Simulium rostratum* in darkness at 20 °C, RH=95 %, for 9 h. 
*b* Decoded means of arcsin p^{1/2} transformed data (with 95 % confidence limits of the transformed data) from three groups of five wings based on a minimum of 75 observations. The concentration of conidia and the percentage of secondary type 1 conidia varied between 2.0-8.7 spores/mm^2 and 0.5-8.3 %, respectively. 
Means within columns by the same letter are not significantly different according to the SNK-test (*α*= 0.05). 
*c* After incubation for another 10 hours at 20 °C, 42.0 % of secondary type two conidia were recorded to germinate and 1.4 % formed appressoria. 
*d* Kruskall-Wallis one way ANOVA on rank test. 
*e* Student's t-test.
### TABLE 5

Effect of the hardness of water agar with different agar levels on the development of secondary type 2 conidia of *Erynia conica* in vitro.  

<table>
<thead>
<tr>
<th>Percentage of agar (w/v)</th>
<th>Percentage total germination</th>
<th>Percentage tertiary type 1 conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar 6.0 %</td>
<td>20.2 (8.5, 44.5) a</td>
<td>9.2 (11.7, 23.7) a</td>
</tr>
<tr>
<td>Agar 3.0 %</td>
<td>32.4 (22.6, 46.8) a</td>
<td>15.3 (12.0, 33.0) a</td>
</tr>
<tr>
<td>Agar 1.5 %</td>
<td>33.9 (32.2, 39.0) a</td>
<td>13.0 (20.0, 22.2 a)</td>
</tr>
<tr>
<td>Coverslip</td>
<td>62.0 (41.9, 62.0) b</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*a* Incubation on distilled water agar buffered at pH 6.5 with 5 mM MES (2-(N-morpholino)ethanesulfonic acid), in darkness at 20 °C, RH= 95 %, for 24 h, excepted for coverslip where data was collected after conidia shower.

*b* Decoded means of arcsin p^{1/2} transformed data (with 95 % confidence limits of the transformed data) from three groups based on 100 observations. The concentrations of conidia and the percentage of secondary type 1 conidia varied between 2.5-5.0 spores/mm^2 and 0.0-0.6 %, respectively. Means within columns by the same letter are not significantly different according to the SNK-test (α= 0.05).
TABLE 6

The development of secondary type 2 conidia of *Erynia conica* in vitro (coverslip) and in situ (cuticular wings of *Simulium rostratum*) in terms of the time of incubation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (hour)</th>
<th>Cumulative percentage $^b$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total germination</td>
<td>appressoria</td>
<td>cuticular penetration</td>
<td>tertiary type 1 conidia</td>
</tr>
<tr>
<td>Wings of <em>Simulium rostratum</em></td>
<td>0</td>
<td>57.8 (31.7, 67.2) a</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>74.3 (55.4, 63.7) a</td>
<td>8.4 (0.0, 36.2)</td>
<td>2.3 (6.2, 11.1)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>83.4 (53.0, 78.9) a</td>
<td>6.3 (0.0, 45.7)</td>
<td>3.1 (0.0, 32.3)</td>
<td>0.6 (0.0, 23.5)</td>
</tr>
<tr>
<td>Coverslip</td>
<td>0</td>
<td>82.9 (55.2, 75.9) b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>86.1 (66.9, 69.3) b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>84.7 (63.7, 70.3) a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Incubation in darkness at 20 °C, RH= 95%.

$^b$ Decoded means of arcsin $p^{1/2}$ transformed data (with 95% confidence limits of the transformed data) from three groups of 5 wings based on a minimum of 75 observations, or three groups for coverslip based on 100 observations. The concentration of conidia and the percentage of secondary type 1 conidia varied between 2.9-5.6 spores/mm$^2$ and 0.9-5.5%, respectively. For each time means within columns by the same letter are not significantly different according to the Student's t-test ($\alpha= 0.05$).
TABLE 7

Effect of surface wettability on the germination of secondary type 2 conidia of *Erynia conica* in vitro.  

<table>
<thead>
<tr>
<th>Material</th>
<th>total germination</th>
<th>Percentage</th>
<th>wettability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coverslip (glass)</td>
<td>77.2 (50.1, 72.8)</td>
<td>c</td>
<td>41.0 ± 3.2</td>
</tr>
<tr>
<td>Petri dish (polystyrene)</td>
<td>68.6 (53.9, 58.0)</td>
<td>bc</td>
<td>0</td>
</tr>
<tr>
<td>Cellulose acetate</td>
<td>68.4 (45.4, 66.2)</td>
<td>bc</td>
<td>52.0 ± 3.7</td>
</tr>
<tr>
<td>Wax paper</td>
<td>50.4 (28.8, 61.7)</td>
<td>b</td>
<td>66.1 ± 6.6</td>
</tr>
<tr>
<td>Filter (millipore)</td>
<td>5.2 (10.2, 16.3)</td>
<td>a</td>
<td>100</td>
</tr>
<tr>
<td>Cellophane</td>
<td>0.7 (0.0, 24.6)</td>
<td>a</td>
<td>38.9 ± 6.0</td>
</tr>
<tr>
<td>Microscopic slide (glass)</td>
<td>0.2 (0.0, 14.0)</td>
<td>a</td>
<td>95.2 ± 6.7</td>
</tr>
</tbody>
</table>

a In darkness at 20 °C, after showering with conidia over the surface for 2 h.

b Decoded means of arcsin p^{1/2} transformed data (with 95% confidence limits of the transformed data) from three groups based on 100 observations. The concentrations of conidia and the percentage of secondary type 1 conidia varied between 3.2-7.9 spores/mm² and 5.2-10.5 %, respectively. Means within columns by the same letter are not significantly different according to the Kruskal-Wallis oneway ANOVA on rank test (α= 0.05).

c Scale from 0-100 (0= least wettable, 100= most wettable). Standard deviation is presented for the mean of each material.
TABLE 8
Effect of charge on the development of secondary type 2 conidia of *Erynia conica in vitro*.  

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Percentage</th>
<th></th>
<th>tertiary type 1 conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total germination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak forces</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carboxylated latex beads</td>
<td>33.7 (26.7, 44.4) c</td>
<td>18.8 (14.9, 36.5) b</td>
<td></td>
</tr>
<tr>
<td>aminated latex beads</td>
<td>19.7 (25.9, 26.8) b</td>
<td>5.5 (2.8, 24.4) a</td>
<td></td>
</tr>
<tr>
<td>neutral latex beads</td>
<td>30.0 (21.6, 44.8) c</td>
<td>14.9 (18.0, 27.5) b</td>
<td></td>
</tr>
<tr>
<td>Strong forces</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>basic anion beads</td>
<td>9.0 (11.7, 23.2) a</td>
<td>5.1 (12.2, 13.8) a</td>
<td></td>
</tr>
<tr>
<td>acidic cation beads</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water agar</td>
<td>29.4 (24.8, 41.2) c</td>
<td>16.4 (16.9, 30.9) b</td>
<td></td>
</tr>
<tr>
<td>Coverslip</td>
<td>72.4 (53.5, 63.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Incubation in darkness at 20 °C, RH = 95 %, during 24 hours, except for coverslip where data collected after conidial shower. Latex beads, resin and agar were prepared in buffered solution at pH 6.5 with 5 mM MES (2-(N-morpholino)ethanesulfonic acid).

* Decoded means of arcsin p^{1/2} transformed data (with 95 % confidence limits of the transformed data) from three groups based on 100 observations. The concentrations of conidia and the percentage of secondary type one conidia varied between 5.0-10.4 spores/mm² and 6.4-14.4 %, respectively. Means within columns by the same letter are not significantly different according to the SNK-test (α = 0.05).

* Did not observe.


CHAPTER 7

GENERAL DISCUSSION AND
CONTRIBUTIONS TO ORIGINAL KNOWLEDGE.
GENERAL DISCUSSION

The principal goal of this research was to examine the relationship between species of fungi of the order Entomophthorales and selected black fly species to further determine the potential of the fungi as biological control agents of black flies. Therefore, in Chapter 2, we reported the seasonal presence of three species of Entomophthorales on two selected black fly populations at the Réserve Faunique du Saint-Maurice (Québec). However, the nature of the impact of the Entomophthorales on black fly populations is unknown. Future studies should address this in terms of not only the number of insects killed by mycoses but also in terms of the number of eggs laid by infected females as well as egg viability.

Two *Erynia* species were detected on the multivoltine *Simulium* genera. Host specificity may have been detected since *Erynia conica* (Nowakowski) Remaudière and Hennebert was never observed on *Simulium decorum* Walker. What are the factor(s) that may explain such host specificity? Both *Erynia* species and their respective species of black fly hosts were observed in the same time and space at two separate sites. Thus, specificity may reflect physiological rather than ecological factor(s). We hypothesized that host specificity could be explained in terms of the susceptibility or resistance of the host *Simulium rostratum* (*S. verecundum* Stone and Jamnback cytospecies ACD) and non-host (*S. decorum*) to fungal infection by *E. conica*. A similar study for *Erynia neoaphidis* Remaudière and Hennebert reported the absence of cuticular penetration on the resistant aphid, *Acythosiphon pisum* Harris (Milner, 1982). Such infection studies required the production of conidiospores of *E. conica in vitro* (Chapter 4) and the rearing of
S. rostratum in the laboratory (Chapter 3). Once these objectives were achieved it was possible to determine which type of conidiospores was infective, the mode of infection and the consequence of exposing the infective unit to the non-host. We found that the secondary type 2 conidium of E. conica was the invasive unit on S. rostratum cuticle (Chapter 5) as evident by appressorial formation and the development of penetration tubes on wing material. Invasive conidia may have also formed aerial tertiary (type 1) conidia or may have germinated to form hyphae (vegetative growth) in lieu of appressoria and penetration tubes. While invasive conidia of E. conica on the non-host, S. decorum, did germinate there was no evidence of cuticular penetration by hyphae or the formation of appressoria, confirming the host-pathogen specificity suspected in the course of the field study. Thus, host specificity of E. conica appeared, in part, to be related to the cuticular characteristic(s) of the insect which favor appressorial formation and subsequent penetration by an infection peg as opposed to the inhibition of spore germination.

Microscopic observation of the wings (used as cuticular source in the invasion study) of susceptible and resistant hosts showed that resistant as well susceptible material have similar topography, which may exclude topographical cuticular differences influencing the host specificity as suggested by Steinkraus and Kramer (1987) for Entomophthora muscae (Cohn) Fresenius. Did the chemical composition of insect cuticle may play a role? Kerwin (1984) suggested that composition and levels of fatty acids was the basis of susceptibility of the fly Fannia canicularis (Linnaeus) to Erynia variabilis (Thaxter) Remaudière and Hennebert by the adult stage as opposed to the pupal stage. The removal of lipids from the cuticle of the proper host, S. rostratum, precluded the formation of appressoria by E. conica and cuticular penetration (Chapter 5). With the non-host S. decorum, we did not observe the development of any infective structure from the infective unit as was seen with undelipided cuticle. Therefore lipids played a role by triggering appressorium formation
on the host, but did not seem to act as inhibitory factor(s) on the non-host. Research should be addressed to determine the type(s) of lipid(s) and their level(s) in terms of their influence on the formation of infective structures by *E. conica*.

Laboratory experimentations in Chapters 4 and 5 suggested a potential role for the primary conidia of *E. conica* and the significance of replicative sporulation in the transmission of fungal infection. The primary conidia of *E. conica* floated on the surface of water and formed and projected secondary infective conidia. Similar behavior was observed when we exposed primary conidia to the host surface. This supports a dispersive role for these conidiospores as proposed by Hywel-Jones and Webster (1986). Infective conidiospore formation under the light and dark regimes, suggested a regulatory role of the photoperiod in the diurnal periodicity of infective conidia production and may be a strategy of the pathogen to optimize host infection in that more infective conidia would be produced at the time when the black fly host arrives at the oviposition site, as postulated by Hywel-Jones (1986). The primary conidia of *Erynia curvispora* (Nowakowski) Nowakowski, like these of *E. conica*, floated on the water surface and formed secondary conidia similar to the secondary invasive conidia of *E. conica*. Periodicity in secondary sporulation was also observed for *E. curvispora* (Chapter 4). However we do not know if this type of conidium is the infective unit, and therefore we cannot suggest that the periodicity observed in their production during the day would optimize host infection as for *E. conica*. The infective conidia of *E. conica* may give rise to tertiary conidia that have the same shape as the primary conidia, in the absence of factors triggering infection such as the cuticle of the non-host. The tertiary conidia may form quaternary conidia with shapes similar to the secondary infective conidia (type 2) (Chapter 6). We believe that this replicative behavior will increase the probability of the
fungi contacting a susceptible insect as was suggested for the sporulation of other entomophthoralean fungi (Mullens and Rodriguez, 1985; Wraight et al., 1990; Steinkraus and Slaymaker, 1994). However, the infective capabilities of this quaternary conidium remains to be determined.

In Chapter 6 we also demonstrated that different modes of development of the infective unit of *E. conica* were influenced by physical factors, mainly pH, temperature and the charge of the substratum. Interestingly, infective structures were more affected by temperature than was conidial germination. The optimum formation of infective structures at moderate temperatures suggests that the fungus is adapted to temperate climates and that the development of infective structures would be a better indicator than germination in the evaluation the fungi as a potential mycoinsecticide. The interaction between infection stage, fungal nutrition and physical parameters is not known. However in view of the effects of these interactions on *Zoophthora radicans* (Brefeld) Batko (Magalhaes et al., 1991) such a study is warranted.

The interest in the members of the Entomophthorales as mycoinsecticides stems from their natural activity, specificity and recycling potential (Steinkraus, 1990; Papierok and Brey, 1990). In his review of vector control by the Entomophthorales, Steinkraus (1990) concluded that until basic questions concerning the life cycle, dosage, host range, and environmental and nutritional requirements of the fungi were answered, the Entomophthorales would have poor commercial prospects as mycoinsecticide. Furthermore, the fragility of the conidia (Glare and Milner, 1991; this study), the fact that only the adults were susceptible to the infection, and the loss of the ability of the fungi to sporulate and grow in vitro (as was reported for *E. curvispora* (Kramer, 1983), Chapter
4) restricts the Entomophthorales of this study for the present, as a choice for biological control against black flies.
CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Three Entomophthoralean fungal species, *Erynia conica*, *Erynia curvispora* and *Entomophaga near limoniae* were observed in adult black fly populations at the Réserve Faunique du Saint-Maurice (Québec). This is the first report of the presence of *Erynia conica* and *Entomophaga near limoniae* in North America and *Erynia curvispora* in Canada. A new fungus-black fly association was detected which involves *Entomophaga near limoniae* in *Simulium verecundum/rostratum* complex. *Simulium verecundum/rostratum* and *Simulium venustum* and *Simulium vittatum* complexes are new hosts of *Erynia conica*.

2. A successful method for rearing *Simulium rostratum* in the laboratory is presented. This is the first time that this species has been reared in the laboratory.

3. The two types (1 and 2) of aerial conidiospores of *Erynia conica* and *Erynia curvispora* were produced *in vitro*.

4. This is the first report of *Erynia conica* and *Erynia curvispora* exhibiting a diurnal periodicity for secondary conidial production in darkness. Evidence is presented for the first time that light eliminated the periodicity of *Erynia conica*.

5. Uniquely, this thesis documented that secondary type 2 conidia formation of *Erynia conica* and *Erynia curvispora* was influenced by the pH of the medium.
6. A unique approach was developed to follow the different modes of germination of infective units of *Erynia conica* over time *in situ*. For the first time each mode was quantified.

7. Both sexes of *Simulium rostratum* were equally susceptible to the invasion by *Erynia conica*.

8. *Erynia conica* neither formed appressoria nor invaded the cuticle of the non-host, *Simulium decorum*. This confirms host specificity suggested by field studies. Conidial germination was also delayed, and tertiary sporulation was higher for the infective conidia exposed to *Simulium decorum* than to *Simulium rostratum*. Therefore, specificity of infection may be related to the invasion of the cuticle as opposed to the inhibition of spore germination.

9. Cuticular lipids play a role by triggering appressorial formation of *Erynia conica* on the proper host *Simulium rostratum* but do not seem to be implicated as inhibitory factors on the non-host, *Simulium decorum*.

10. *In vitro* observations on water agar and on inert support such as a coverslip have revealed that secondary conidia of *Erynia conica* do not seem require exogenous nutrients for germination.

11. The germination and sporulation of the invasive unit of *Erynia conica* was dependent on the physical factors surrounding the conidia including pH, temperature and the surface charge of the substrata.
12. The temperature range of the germination of infective conidia of *Erynia conica in situ* was similar to germination *in vitro* and corresponded to temperatures recorded in the field.

13. The *in situ* study on secondary type 2 conidia of *Erynia conica* indicated that appressorial formation and penetration were more affected by the temperature than was germination.
LITERATURE CITED


**TABLE 1**

Diurnal development of primary conidia of *Erynia conica* as a function of the age of the culture during two consecutive days in darkness.  

<table>
<thead>
<tr>
<th>Age of culture (hour)</th>
<th>Percentage $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total germination</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.2 (12.1, 19.1)</td>
</tr>
<tr>
<td>2</td>
<td>29.8 (27.2, 39.0)</td>
</tr>
<tr>
<td>4</td>
<td>37.2 (34.2, 41.0)</td>
</tr>
<tr>
<td>6</td>
<td>65.6 (52.0, 56.2)</td>
</tr>
<tr>
<td>8</td>
<td>82.8 (61.0, 70.0)</td>
</tr>
<tr>
<td>10</td>
<td>58.0 (42.9, 56.3)</td>
</tr>
<tr>
<td>24</td>
<td>1.0 (4.0, 7.2) a</td>
</tr>
<tr>
<td>26</td>
<td>5.1 (9.3, 16.9) b</td>
</tr>
<tr>
<td>28</td>
<td>7.9 (14.1, 18.5)</td>
</tr>
<tr>
<td>30</td>
<td>29.3 (24.8, 40.8)</td>
</tr>
<tr>
<td>32</td>
<td>63.8 (49.0, 57.0)</td>
</tr>
<tr>
<td>34</td>
<td>89.6 (66.9, 75.5)</td>
</tr>
</tbody>
</table>

$^a$ Incubation during three hours at 20 °C, RH= 95 

$^b$ decoded means of arcsin $p^{1/2}$ transformed data (with 95 % confidence limits of the transformed data) from five replicates based on a minimum of 400 observations. The concentration of conidia varied between 30-70 spores/mm$^2$. Means within columns by the same letter are not significantly different according to SNK-test ($\alpha = 0.05$).
TABLE 2

Diurnal development of primary conidia of *Erynia curvispora* as a function of the age of the culture during two consecutive days in darkness.  

<table>
<thead>
<tr>
<th>Age of culture (hour)</th>
<th>Percentage (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total germination</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>53.5 (35.1, 56.9)</td>
</tr>
<tr>
<td>2</td>
<td>96.2 (72.2, 85.4)</td>
</tr>
<tr>
<td>4</td>
<td>98.8 (76.5, 91.1)</td>
</tr>
<tr>
<td>6</td>
<td>98.5 (81.1, 84.7)</td>
</tr>
<tr>
<td>8</td>
<td>88.7 (65.8, 75.2)</td>
</tr>
<tr>
<td>10</td>
<td>57.6 (41.0, 57.8)</td>
</tr>
<tr>
<td>24</td>
<td>35.5 (27.5, 45.7)</td>
</tr>
<tr>
<td>26</td>
<td>76.3 (55.3, 66.5)</td>
</tr>
<tr>
<td>28</td>
<td>83.7 (68.8, 82.6)</td>
</tr>
<tr>
<td>30</td>
<td>98.6 (79.7, 86.9)</td>
</tr>
<tr>
<td>32</td>
<td>98.7 (81.4, 85.4)</td>
</tr>
<tr>
<td>34</td>
<td>80.9 (57.2, 71.0)</td>
</tr>
</tbody>
</table>

\(^a\) Incubation during three hours at 20 °C, RH = 95 %

\(^b\) Decoded means of arcsin \(\pi/2\) transformed data (with 95 % confidence limits of the transformed data) from five replicates based on a minimum of 400 observations. The concentration of conidia varied between 20-60 spores/mm\(^2\). Means within columns by the same letter are not significantly different according to SNK-test (\(\alpha = 0.05\)).
TABLE 3

Daily development of primary conidia of *Erynia conica* as a function of the age of the culture during two consecutive days in darkness. *a*

<table>
<thead>
<tr>
<th>Age of culture (hour)</th>
<th>Percentage <em>b</em></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>secondary germination</td>
<td>type 1</td>
<td>type 2</td>
</tr>
<tr>
<td></td>
<td>germination</td>
<td>discharged</td>
<td>type 1</td>
<td>type 2</td>
</tr>
<tr>
<td>0</td>
<td>10.2 (11.3, 25.2) a</td>
<td>0.0</td>
<td>3.7 (0.0, 29.7) a</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>27.1 (13.1, 49.7) ab</td>
<td>0.1 (0.0, 4.2)</td>
<td>16.3 (3.6, 43.9) b</td>
<td>0.1 (0.0, 6.8)</td>
</tr>
<tr>
<td>8</td>
<td>62.4 (30.3, 74.1) c</td>
<td>0.2 (0.0, 7.3)</td>
<td>54.9 (23.3, 72.3) c</td>
<td>0.8 (0.0, 10.3)</td>
</tr>
<tr>
<td>12</td>
<td>26.8 (12.4, 50.0) ab</td>
<td>0.0</td>
<td>25.6 (11.3, 49.5) b</td>
<td>0.0</td>
</tr>
<tr>
<td>16</td>
<td>24.4 (15.3, 43.9) ab</td>
<td>0.0</td>
<td>17.5 (9.5, 39.9) b</td>
<td>0.0</td>
</tr>
<tr>
<td>20</td>
<td>73.0 (49.7, 67.7) c</td>
<td>0.4 (0.0, 10.7)</td>
<td>66.6 (44.9, 64.5) c</td>
<td>1.1 (3.1, 9.1)</td>
</tr>
<tr>
<td>24</td>
<td>68.9 (47.8, 64.4) c</td>
<td>0.2 (0.0, 12.2)</td>
<td>58.2 (46.1, 53.3) c</td>
<td>1.3 (0.0, 17.6)</td>
</tr>
<tr>
<td>28</td>
<td>28.1 (24.6, 39.4) ab</td>
<td>0.0</td>
<td>24.8 (23.6, 36.2) b</td>
<td>0.3 (0.6, 5.8)</td>
</tr>
<tr>
<td>32</td>
<td>31.3 (31.4, 36.6) b</td>
<td>0.1 (0.0, 5.0)</td>
<td>21.0 (23.1, 31.5) b</td>
<td>1.2 (0.0, 25.6)</td>
</tr>
</tbody>
</table>

* Incubation during three hours at 20 °C, RH= 95%.
* Decoded means of arcsin p1/2 transformed data (with 95% confidence limits of the transformed data) from triplicates based on a minimum of 400 observations. The concentration of conidia varied between 20-50 spores/mm².
* Means within columns by the same letter are not significantly different according to SNK-test (α= 0.05).
* Kruskall-Wallis one way ANOVA on ranks test.
TABLE 4

Daily development of primary conidia of *Erynia conica* as a function of the age of the culture during two consecutive days in light. *a*

<table>
<thead>
<tr>
<th>Age of culture (hour)</th>
<th>Percentage <em>b</em></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total germination</td>
<td>secondary sporulation</td>
<td>secondary discharged</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>type 1</td>
<td>type 2</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>78.4 (46.1, 78.5)</td>
<td>0.0</td>
<td>65.6 (31.7, 76.5)</td>
<td>4.0 (4.3, 18.7)</td>
</tr>
<tr>
<td>4</td>
<td>63.6 (37.2, 67.4)</td>
<td>0.02 (0.0, 4.2)</td>
<td>47.9 (32.8, 54.8)</td>
<td>2.6 (3.3, 15.3)</td>
</tr>
<tr>
<td>8</td>
<td>62.6 (38.6, 72.8)</td>
<td>0.02 (0.0, 4.2)</td>
<td>49.8 (18.7, 71.1)</td>
<td>3.2 (7.4, 13.2)</td>
</tr>
<tr>
<td>12</td>
<td>78.0 (56.7, 67.3)</td>
<td>0.3 (0.0, 8.0)</td>
<td>63.4 (41.9, 63.7)</td>
<td>5.4 (10.4, 16.4)</td>
</tr>
<tr>
<td>16</td>
<td>80.5 (51.2, 76.4)</td>
<td>0.02 (0.0, 4.2)</td>
<td>65.1 (39.0, 68.6)</td>
<td>4.5 (2.2, 22.2)</td>
</tr>
<tr>
<td>20</td>
<td>85.0 (63.3, 70.8)</td>
<td>0.1 (0.0, 5.1)</td>
<td>65.6 (43.8, 64.4)</td>
<td>5.0 (1.3, 24.6)</td>
</tr>
<tr>
<td>24</td>
<td>76.2 (45.7, 75.9)</td>
<td>1.7 (0.0, 18.8)</td>
<td>56.4 (30.4, 67.0)</td>
<td>4.1 (4.9, 18.5)</td>
</tr>
<tr>
<td>28</td>
<td>62.4 (41.3, 63.1)</td>
<td>0.0</td>
<td>44.9 (27.1, 57.1)</td>
<td>4.5 (0.0, 27.0)</td>
</tr>
<tr>
<td>32</td>
<td>69.0 (44.2, 68.2)</td>
<td>0.02 (0.0, 4.2)</td>
<td>47.4 (29.4, 57.6)</td>
<td>9.6 (11.3, 24.9)</td>
</tr>
</tbody>
</table>

*a* Incubation during three hours at 20 °C, RH = 95 %

*b* Decoded means of arcsin p1/2 transformed data (with 95 % confidence limits of the transformed data) from triplicates based on a minimum of 400 observations. The concentration of conidia varied between 20-50 spores/mm². Means within columns by the same letter are not significantly different according to SNK-test (α = 0.05).


CONNECTING STATEMENT 3

With the development of an adult black fly rearing system (Chapter 3) and the production of different types of *Erynia conica* conidiospores (Chapter 4) it is possible to examine the modes of conidial development on the host and non-host black fly species (Chapter 5) to examine suspected fungal specificity detected in the field study (Chapter 1).
CHAPTER 5

DEVELOPMENT OF *ERYNIA CONICA* (ZYGOMYCETES: ENTOMOPHTHORALES) ON THE CUTICLE OF ADULT BLACK FLIES *SIMULIUM ROSTRATUM* AND *SIMULIUM DECORUM* (DIPTERA: SIMULIIDAE).
ABSTRACT

Aerial conidia of *Erynia conica* were showered on to the cuticular wing surfaces of the black flies, *Simulium rostratum*, the proper host, and the non-host species, *Simulium decorum*. Secondary type 2 conidia invaded the wings of *Simulium rostratum* only. The invasion process on both sexes of the host species was rapid with 40% of the conidia germinating shortly after contact with the substratum. Approximately 24% of total spores developed appressoria close to the parental spore and 19% of total spores penetrated the cuticle by 9 h post-inoculation. On *Simulium decorum* invasive conidia exhibited delayed germination and formed neither appressoria nor did they invade the wings, but rather produced high levels of tertiary type 1 spores (26%). Cuticular lipids played a role in the infection process by triggering appressorium formation on the host, but did not seem to act as an inhibitory factor(s) on the non-host.
The entomopathogenic fungus *Erynia conica* (Nowakowski) Remaudière and Hennebert (Entomophthorales: Entomophthoraceae) attacks the adult stage of aquatic insects such as chaoborids, chironomids, tipulids, Trichoptera and simuliids (Nowakowski, 1883; Thaxter, 1888; Descals *et al.*, 1981; Keller, 1991; Cuebas-Incle, 1992; Papierok and Dumas, 1992; Nadeau *et al.*, 1994). The fungi produce primary conidia on conidiophores and secondary conidia from discharged primary conidia. The latter conidia may differ in morphology from primary conidia and are then referred to as type 2 conidia whereas secondary conidia with similar morphology as the primary spores are called type 1 conidia (Descals *et al.*, 1981). The infection process of conidia of the Entomophthorales, like those of other entomopathogenic fungi, consist of conidial germination on the cuticular surface of the insect followed by the direct penetration of the cuticle by the germ tube or by the formation of an appressorium before penetration by an infection peg (Brobyn and Wilding, 1977). The secondary type 2 conidia of *E. conica* may be the unit that infects adult simuliids (Hywel-Jones and Webster, 1986).

In Quebec, *E. conica* has been reported to infect almost exclusively the female simuliids of the *Simulium venustum* Say, *Simulium vittatum* Zetterstedt and the *Simulium verecundum* Stone and Jamnback complex, but not *Simulium decorum* Walker, implying some degree of host specificity (Nadeau *et al.*, 1994). We hypothesized (see Chapter 2) that host specificity could be explained by the non
susceptibility of the non and/or susceptibility of the proper host insect to fungal penetration. To this end, laboratory cross infection studies were attempted exposing the respective host and non-host black fly species to conidia of *E. conica* and *Erynia curvispora* (Nowakowski) Nowakowski. The studies were difficult due to strains of *E. curvispora* ceasing conidial production and growth after limited subculturing on artificial media (Chapter 4). However, strains of *E. conica* continued to grow *in vitro* and sporulated sufficiently to allow (Chapter 4) the study of the early stages of fungal invasion in relation to specificity for *E. conica*. The survival of the adult black flies under laboratory conditions was too short (Chapter 3) to permit detailed studies on host-pathogen association. This limited studies to the early stages of invasion on selected cuticular surfaces.

Host specificity by insect pathogenic Deuteromycetes has been related to either the physicochemical properties of the insect cuticle and the surface topography, both of which influence the formation of appressoria (St-Leger *et al*., 1989), or related to differences in the biochemical composition of the cuticle which preclude conidial germination on the resistant hosts (Saito and Aoki; 1983). However, for *Erynia neoaphidis* Remaudière and Hennebert cuticular penetration was prevented after conidial germination on resistant biotypes of the pea aphid, *Acythosiphon pisum* Harris (Milner, 1982).

This report describes the development of primary and secondary aerial conidiospores of *E. conica* on both sexes of the host black fly *Simulium rostratum* (*S. verecundum* cytospecies ACD) and explains the absence of the fungus on the non-host, *S. decorum*. 
MATERIALS AND METHODS

Fungal isolate and inoculum

Strain 512 of *E. conica* [originally isolated from adults of the *S. verecundum* complex collected at Inman Lake of the Réserve Faunique du Saint-Maurice (46°59'N, 73°07'W)] was maintained on GEMYSA medium [0.4 g glucose, 18 ml egg yolk, 10 ml milk, 0.2 g yeast extract, 1.8 g Sabouraud dextrose, and 0.4 g agar in 100 ml distilled water (B. Papierok, personal communication)] at 5 °C. Fungus was subcultured every two months as described in Chapter 4, incubated at 20 °C for 10 days and then returned to 5 °C for storage.

Depending on the experiment, one of two types of aerial conidiospores were used as the inoculum: the primary (elongate conidia) and secondary type 2 (globose) conidia, of which the latter were produced directly from primary germinating conidia (Webster *et al.*, 1978; Descals *et al.*, 1981). It was not possible to prepare a liquid suspension of spores for use in these studies due to their adhesive and fragile nature, an attribute which they share with other entomophthoralean fungal spores (Glare and Milner, 1991). Primary conidiospores were obtained from a sporulating mycelial mat that discharged the conidia in a moist environment onto the target. A seven mm diameter sample of two week old culture previously grown on GEMYSA in the dark at 20 °C was aseptically placed on distilled water agar in a Petri dish (35 x 10 mm). The dish was inverted and placed in the dark at 20 °C to allow the primary conidia to discharge onto the target surface. Secondary type 2 conidia were produced from primary conidia that had been previously showered onto water agar. The dish of agar was then inverted and the secondary conidia were allowed to shower onto the
designated targets. This procedure produced variation in the concentration of the two types of spores adhering to the targets between replicates. The concentrations of primary conidia on the target substrate ranged between 10-20 spores/mm² and the secondary type 2 spores varied between 2.0-7.0 spores/mm².

**Experimental insects**

Isolated abdomens and wings of adult black flies were used as the cuticular target surfaces. The host black fly adults, *S. rostratum* were obtained by laboratory rearing (Chapter 3). Cytological analysis was used to identify the larval species as *S. rostratum* (= *S. verecundum* cytospecies ACD) (P. Adler, South Carolina University, personal communication). The adults of the non-host species, *S. decorum*, were collected by netting at the field site. Adult insects of both species were stored at -20 °C to accumulate sufficient amounts of material for experimentation.

To prepare the abdominal cuticle, the adults were dissected under a stereomicroscope (100 x). Wings and legs were extirpated and two transverse lacerations were made; one at the level of tergite 8 to remove the terminalia, and the other at the line thoracic/abdominal junction (between tergite 2-3) to remove the head and thorax. A longitudinal cut was made along the ventral abdomen and the internal tissue and organs were scraped away with a glass rods. The abdominal cuticles were washed in sterile distilled water and, like the wings, the abdominal sections were disinfected by incubating for two minutes in 70 % (v/v) ethanol. Disinfection was necessary to preclude microbial contamination acquired during rearing from influencing fungal development, as was reported for Deuteromycetes (Fargués and Vey, 1974; Schabel, 1978). Cuticular samples were subsequently placed in sterile
distilled water in a microcentrifuge tube and frozen at -20 °C. The effects of ethanol treatment and prolonged storage of cuticle segments on fungal development was assessed (see section; Experimental protocols).

**Extraction of cuticular lipids**

Wings rendered lipid free (here, thereafter referred to as delipided wings) that had been recoated with the lipid extracts and untreated wings (controls) of the both black fly species were used to determine the contribution of lipids to the development of invasive structures of the fungus. Lipids were extracted by immersing the wings three times in 200 μl of chloroform: methanol mixture (2: 1 v/v) at 20 °C for 15 seconds. The lipid solutions were stored at 4 °C overnight (Gilby, 1980). Two or more short-term immersions in the organic solvent are more efficient for removing lipids from the cuticle of insects than one long-term immersion (Buckner, 1993). Furthermore, whereas prolonged (five minutes) solvent extractions are known to modify the integrity of the insect cuticle of the pea aphid, (A. pisum), extraction for 30 seconds effectively removes all classes of lipids without distortion of the topography of the cuticle (Brey et al., 1985). Scanning electron microscopy was used to observe the effect of the extraction process on the topography of the wings of the simuliiid species in the present study with comparison with the non-treated control group. The specimens were fixed overnight in 1.5 % (v/v) glutaraldehyde at 4 °C, rinsed twice in distilled water, postfixed with 1 % osmium tetroxide, washed three times in distilled water and dehydrated for 45 minutes through an ethanol series of 20-100 % (v/v), and critical point dried. The material was mounted and coated with carbon and gold and observed under a scanning electron microscope (Cambridge, model S 600).
Replacement of extracted lipids onto the surface of the wings was initially attempted by immersing a delipided wing in the extract from one wing and flushing with nitrogen gas to remove the solvents. Another series consisted of evaporating the solution of lipid extracts from one wing placed on a delipided wings in a drop-wise manner using a glass syringe.

**Experimental protocols**

On the first two series of infection experiments, the wings and abdomens of the host *S. rostratum* were exposed to both the primary and secondary type 2 forms of conidia of *E. conica* to determine which type of conidia was infective and whether both types of substrata were susceptible to invasion by the fungus. To determine if the invasion process was influenced by the sex and species of the insect, the invasive conidia were showered onto the wing cuticular surface of the male and female of *S. rostratum* and female of *S. decorum*. Wings were selected because infection structures were readily discernible only on the wings but not the abdomen. The effects of ethanol on infection and the effects of prolonged storage of the wings were determined with a total of 10 wings of *S. rostratum*. The last two experiments compared the effects of lipid extractions of the proper host and non-host black fly species on conidial germination to determine the involvement of lipoidal compounds in the development of invasive conidia.

Five abdominal sections or wings were placed on distilled water agar in the center of a Petri dish (35 x 10 mm). The open dish was placed in a laminar flow of air for five minutes to evaporate the water from the surface of the cuticular substrata that was acquired during their placement on distilled water agar. The distilled water agar
maintained sufficient relative humidity to support spore germination. Conidia were showered onto the cuticular surfaces for times that varied with the type of conidia; one hour for primary conidia and 2 h for secondary type 2 conidia. During this time the sporulating cultures were rotated by 90° at 15 minute intervals to allow uniform conidial dispersion. The concentration of conidia on the cuticular surfaces was estimated by light microscopy as the average of the number of spores per mm². Primary conidia on the water agar surface produced secondary type 2 but also secondary type 1 conidia (Chapter 4). Therefore, for experiments involving secondary type 2 conidia the proportion of secondary type 1 was also recorded. The treated surfaces were incubated in darkness at 20 °C in a humidity chamber adapted from Rutschke and Grunewald (1984) (relative humidity = 95 %). At designated times the development of 100 conidia was determined using a light microscope (250 x).

Where appropriate, the data were recorded as the number of germinating primary (PG) and secondary spores (SG). The conidiospore was regarded as germinating when the length of the germ tube exceeded half the minor diameter of the spore (Anonymous, 1943). The number of primary conidia forming secondary type 1 (S1) or type 2 (S2) conidia, the secondary type 2 conidia forming an appressorium (A), [defined as a swollen structure at the tip of germ tube where penetration can be achieved (Emmett and Parbery, 1975)], and the level of appressoria forming penetration tubes (AP), or forming a tertiary type 1 conidia (T) were also determined in triplicate.
Presentation of data and statistical analysis

The percentages of following behaviors were calculated: total conidial germination\[\text{primary} = (PG + S1 + S2) / N\], or secondary type 2 = (SG + A + AP + T) / N, where N= total of conidia counted], primary forming secondary type 1 (= S1 / N) or type 2 (= S2 / N), secondary type 2 forming appressoria [= (A + AP) / N] or penetrated (= AP / N), or forming tertiary type 1 conidia (= T / N). The data from experiments on the effect of sex, proper and non-hosts, and lipids on the development of the infection stages of the conidia were analysed by ANOVA followed by Student-Newman-Keuls test (SNK-test), or Student's t-test of data transformed by arcsin p^{1/2}. Data of the total germination on these experiments were analyzed also by the ANOVA for repeated measurements. The data for the time storage and ethanol treatment experiments, recorded as a percentage from one replicate, were compared using a contingency table analysis and Chi^2 test. A 0.05 level of significance was used for all tests.

RESULTS

Development of primary and secondary conidia on cuticular surfaces of Simulium rostratum

Primary conidial germination was observed 2 h after inoculation on both the abdominal and wing surfaces of S. rostratum with secondary formation occurring 2 h later (Fig. 1). The level of germinating type 2 conidia was greater than type 1 conidia on both cuticular sources. Marked differences were observed between the two insect
substrates, with a higher percentage of each stage of fungal development occurring on the abdomen than on the wings. Secondary conidia on both sources of cuticle were discharged by 9 h post-inoculation as was evident by the presence of empty spore envelopes of the primary conidia. Primary conidia of *E. conica* were never observed to form appressoria or to penetrate the cuticle of either the wings or the abdominal segments.

The invasion of the wings of the host, *S. rostratum*, was observed for germinating secondary type 2 conidia only. These conidia were surrounded by a clear layer of cytoplasmic materials and exhibited several modes of development on the wings following initial germ tube formation. One mode of development consisted of the formation of appressoria in which the appressorium formed close to the conidia (Fig. 2.2). Appressoria formed occasionally at the terminal end of an elongated germ tube. Infection tubes emerged from the appressorium, penetrating and ramifying throughout the integument (Fig. 2.2). The other modes of germination consisted of the elongation of the germ tube with either the cytoplasmic contents of the spore migrating into the tube to give rise an aerial tertiary type 1 conidia (Fig. 2.3) or the tube continuing to elongate to form hyphae (vegetative growth) (Fig. 2.1). All modes of development was seen on the same individual wing.

Type 2 conidia germinated rapidly on the wings with 40 % producing germ tubes just after termination of the 2 h showering period (corresponding to zero hour on the graph; Fig. 3). It was common to observe more than one nascent germ tube per conidium, but characteristically only one continued to develop into a true germ tube before the previously described stages of development were detected. Appressoria (8
were also noted at the beginning, with maximum appressorial formation by 9 h post-inoculation. Penetration of the wings occurred 2 h later. A maximum of 70 % total germination and 24 % appressorial formation was observed by 6 h and 19 % penetration observed by 9 h post-incubation. A peak of 3 % tertiary sporulation was recorded by 6 h. Germ tubes, some exhibiting vegetative growth and many exhibiting sporulation in excess of those on the wings (formation of tertiary type 1 conidia) were observed on abdominal cuticle. However, no appressoria or penetration were noted. There was also considerably less germination on the abdomen during the initial 2 h of contact than on the wings, the rate increasing thereafter to reach levels similar to that on the wings (a maximum of 71 % by 6 h). On both substrata, the vegetative hyphae degenerated by 6 to 9 h post-inoculation.

Experiments with wings of *S. rostratum* stored at -20 °C established that the time of storage did not significantly affect the development of the invasive stages (Table 1). Decontamination of the wings of *S. rostratum* with 70 % ethanol also did not significantly affect the development of the invasive unit of *E. conica*. Furthermore, there was no evidence of storage-ethanol interaction (Table 2). Similar results were anticipated for *S. decorum*.

**Development of invasive conidia in terms of sex and specificity**

The sex of *S. rostratum* did not significantly influence the invasion process of secondary type 2 conidia of *E. conica*, no difference being observed between wings of the males and females in terms of conidial germination, appressoria formation, cuticular penetration or tertiary type 1 formation (Table 3). Also, there was no difference in the size of the appressoria on wings of the males (21.9 ± 3.2 x 12.6 ±


and the females (20.7 ± 3.8 x 13.4 ± 1.5 μm, n = 13). However, on the wings of females of the non-host, S. decorum, the conidia neither formed appressoria nor produced germ tubes that invaded the wings. Tertiary sporulation was significantly higher on the non-host (26%) than on proper host wings (10%) by 9 h. Germination was also initially significantly lower on the wings of S. decorum than on those of S. rostratum, although by 9 h post-inoculation the germination levels were similar for both species. The rate of germination over time was not significantly different between the type wings of either species.

**Development of invasive conidia on lipid modified wings**

Compared with the non-extracted control wings that contained lipid, the total germination on delipided wings of S. rostratum was significantly lower from 0-4 h post-inoculation after which comparable levels of germination were achieved in both groups (Table 4). The germination over time was significantly different between these two groups. There was no discernible appressoria formation or penetration on delipided wings, but there was a significant increase in tertiary conidiogenesis.

Recoating of delipided wings with lipids by the two methods (see Materials and methods) did not restore conidial germination or subsequent development to the levels of the control wings. Wings of the non-host S. decorum, regardless of treatment, did not significantly alter any of the previously described modes of germination of secondary type 2 conidia, including the rate of germination over time (Table 5). Because the recoating experiments did not restore the measured parameters, cross-recoating studies between host and nonhost were not done. Few changes were observed in the topography of wings of the both black fly species after treatment with the solvent mixture. The type and amount of lipids removed were not determined.
Scanning electron microscopy showed that the surface remained lightly convoluted. However, hairs were modified in terms of lessening of their curved shape in \textit{S. decorum}, and their becoming thicker on the wings of both black fly species (Fig. 4).

\textbf{DISCUSSION}

The primary conidia are the infective units of \textit{Entomophthora planchoniana} Cornu, \textit{Neozygites fresenii} (=\textit{Entomophthora fresenii}) (Nowakowski) Remaudière and Hennebert (Brobyn and Wilding 1977), \textit{Entomophaga aulicae} (Reichardt) Humber (Murrin and Nolan, 1987), \textit{Conidiobolus obscurus} (Hall and Dunn) Remaudière and Hennebert (Brey \textit{et al.}, 1986), \textit{Erynia blunckii} (Lakon ex Zimmermann) Remaudière and Hennebert (Tomiyama and Aoki, 1982) and \textit{Entomophthora muscae} (Cohn) Fresenius (Tu and Singh, 1993). Additionally, there are reports of both primary and secondary conidia of \textit{E. neoaphidis} (Butt \textit{et al.}, 1990) and \textit{Zoophthora radicans} (Brefeld) Batko (=\textit{Erynia radicans}) (Wright \textit{et al.}, 1990; Pell \textit{et al.}, 1993) being the infective units. However, for \textit{Zoophthora phalloides} Batko (Glare \textit{et al.}, 1985), \textit{Entomophthora sepulchralis} (Thaxter) Remaudière and Hennebert (Kramer, 1980), \textit{Zoophthora aphidis} (Hoffmann) Batko (=\textit{Entomophthora aphidis}) (Brobyn and Wilding 1977), and \textit{N. fresenii} (Steinkraus and Slaymaker, 1994) the secondary conidia are infective. This study established that secondary type 2 conidia of \textit{E. conica} were the infective units on the wings of \textit{S. rostratum} as evident by appressorial formation and the development of penetrating tubes. Hywel-Jones and Webster (1986) reported that the primary conidia of \textit{E. conica} never penetrated the cuticle, but gave rise to secondary conidia that formed appressoria and penetration pegs. Thus, they
proposed that primary conidia had a role in fungal dispersal. Cuebas-Incle (1992) was able to kill the adults stage of the mosquitoes *Aedes aegypti* (Linnaeus) and *Culex restuans*. Theobald using primary conidia of *E. conica* isolated from chaoborid, tipulid and chironomid cadavers, leading them to propose that primary conidia were the infective units. However, the long incubation time in their experiments may have allowed the formation and discharge of secondary conidia, which may have caused the mortality. They did not directly examine the flies to observe the actual infection unit.

The conidia of entomophthoralean fungi exhibit different modes of development when exposed to insects including penetration, vegetative growth and replicative sporulation similar to that recorded herein for *E. conica* (Brobyn and Wilding, 1977; Glare *et al.*, 1985; Brey *et al.*, 1986; Wraight *et al.*, 1990; Galaini-Wraight *et al.*, 1992). Interestingly, the penetration of the cuticle by infection pegs from the appressoria of secondary type 2 conidia of *E. conica* was rapid, occurring within 2-4 h of inoculation, much like that reported for *Z. radicans* (Wraight *et al.*, 1990). Formation of the appressoria before penetration of the cuticle is not an absolute rule for entomophthoralean fungi, with direct penetration being reported for hyphae of *Z. aphidis*, *En. planchoniana*, *En. muscae*, *E. blunki* and *C. obscurus* (Brobyn and Wilding, 1977; Tomiyana and Aoki, 1982; Brobyn and Wilding, 1983; Brey *et al.*, 1986). There was no evidence of direct penetration of *S. rostratum* by *E. conica*. Approximately 20% of conidia produced infective stages that penetrated the cuticular wings of *S. rostratum*. A similarly low percentage of penetration (10-20%) has been reported for *Z. radicans* on the potato leafhopper *Empoasca fabae* (Harris) and *C. obscurus* on pea aphid, *A. pisum* (Brey *et al.*, 1986; Wraight *et al.*, 1990). It is not known if this percentage was dose dependent, as was reported for *Z. radicans* on *En.*
Each mode of development of infective conidia was seen on the same wing and was not restricted to any one part of the wing. This may reflect a uniformity of the chemical composition of the surface of the wing, and/or genetic heterogeneity in the fungal spores. The mode of germination of secondary type 2 conidia of *E. conica* was influenced by the segment of the host body to which it was applied. Thus, the abdomen was not conducive to the formation of invasive fungal structures, posing the question as to the actual infection sites on the host insect. The difference between wings and the abdomens may be explained by surface topography (thigmotrophic response) as proposed for both plant pathogenic fungi (Mims, 1991) and the entomopathogenic fungi *Metarhizium anisopliae* (Metschnikoff) Sorokin (St-Leger *et al.*, 1991) and *Z. radicans* (Wraith *et al.*, 1990). The topography of the abdomen of the black flies was more convoluted than the wings. Hywel-Jones and Webster (1986) observed appressoria and penetration of the abdomens of the black flies of *Simulium argyreatum* Meigen and *Simulium variegatum* Meigen by *E. conica*. However in their study, they used live insects that may have presented different areas of the sclerites or their smooth surface intersegmental membranes to the infective conidia during normal movements. The sex of black flies (*S. rostratum*) did not influence the development of *E. conica* and thus the susceptibility of host wings to cuticular invasion. Therefore, the difference observed in the field, where the number of infected females was higher than that of infected males may be explained primarily by the site where infected insects were found, eg. the oviposition site (Nadeau *et al.*, 1994). The males may have died of *E. conica* mycosis, but at another location.
Results from the wings and abdominal segments of the *S. rostratum* host suggest that the unsuitability of the abdomen for the invasion structures may not have been due to a mycocidal effect because the same level of total germination occurred on both wings and abdomens. The effect may represent limited mycostatic activity since germination was initially lower on the abdomens than on the wings. Tertiary sporulation increased in the absence of infection structures on the abdomens. Tertiary sporulation may indicate a lack of nutrients as reported by Van Roermund and co-workers (1984) for *Z. radicans*. Replicative sporulation was suggested to represent an adaptation favoring the contact of more suitable substrates (Mullens and Rodriguez, 1985; Wraith *et al.*, 1990; Steinkraus and Slaymaker, 1994). The resistance of *S. decorum* initially gave lower levels of type 2 germination and induced higher replicative sporulation of type 2 conidia than on susceptible *S. rostratum*. Delay in germination was reported from the entomopathogenic fungus *Beauveria brongniartii* (Saccardo) Petch on the resistant *Ostrinia nubilalis* Hubner (Lecuona *et al.*, 1991). Therefore, both the parts of the host body and the species of insect influence the germination of *E. conica*, and the lack of host susceptibility to infection may reflect an inadequate milieu for infection due to the absence of a minimum level or type of nutrient(s).

The invasive unit of *E. conica* on the cuticle of *S. decorum* did not show evidence of cuticular invasion by appressorial mediation or direct penetration, confirming host specificity as recorded on field studies (Nadeau *et al.*, 1994) in which *S. decorum* was infected with *E. curvispora* but not with *E. conica*. Ecological barriers, interspecific morphological differences and cuticular composition may influence insect susceptibility to infection by entomopathogenic fungi (Saito and Aoki,
1983; Steinkraus and Kramer, 1987; Mullens, 1989; Cuevas-Inclet, 1992). The ecological barriers and morphological difference are not applicable in the present study, because the proper and improper simuliids host species of *E. conica* coexisted in the same environment (Imhof and Smith, 1979; Nadeau et al., 1994) and because insect wings of the both black fly species exposed to the infective type 2 conidia have similar topography. Differences in the chemical composition of the cuticles may explain the patterns of susceptibility to *E. conica* of the simuliids species tested.

Lipids associated with insect cuticle or plant surfaces have been shown to play a role in the development of fungal pathogens (Podila et al., 1993). Lipoidal compounds from insects were reported to affect principally the germination of entomopathogenic fungi with short-chain fatty acids inhibiting conidial germination of *Beauveria bassiana* (Balsamo) Vuillemin (Smith and Grula, 1981; 1982; Saito and Aoki, 1983) and long-chain fatty acids, sterols, diacylglycerol and/or polar lipids stimulating spore germination of *B. bassiana, Nomuraea rileyi* (Farlow) Samson, *C. obscurus* and *Erynia variabilis* (Thaxter) Remaudière and Hennebert (Smith and Grula, 1981; Boucias and Pendland, 1984; Kerwin, 1984; Boucias and Latgé, 1988). The total cuticular lipid extract of *A. pisum* delayed germination of non-aggressive strains of *C. obscurus* (Latgé et al., 1987). This influence was related not only to the types of lipids but also to lipid concentrations (Kerwin, 1984; Latgé et al., 1987; Bidochka and Khachatourians, 1992). Removing lipids from the wings of *S. rostratum* caused a slower rate of germination and precluded appressorial formation. This may be equivalent to removing chemical stimuli (e.g. lipoidal compounds as nutrients) or exposing inhibitors of appressorium formation and penetration present beneath the lipid layer on the surface of the host cuticle. The minor morphological
differences observed on the wings after treatment with solvent mixture in the present study, may not contribute to a topographical effect. Differentiation of germ tubes of *M. anisopliae* and *Z. radicans* into appressoria was affected by the nutrient level of the environment (St-Leger et al. 1989; Magalhaes et al., 1991). Appressorium formation by the latter species required a minimum level of nutrients. When wings of *S. rostratum* were recoated with lipids the speed of germination or appressorial formation was not restored. It is possible that the lipids were concentrated in one place at the surface of the wing after solvent evaporation, and thus modified the concentration at the surface of the wings (chemical structure) when compared with control wings, the concentration of lipids may have been too high in a given area or, alternately, spores may have landed on a lipid-free area.

Delipidated wings of *S. decorum*, the non-host of *E. conica*, did not support appressorial formation or hyphal penetration. Thus, the inhibiting factors on this insect species may not be lipids. It is also conceivable that the solvent lowered the concentration of the nutrients or other stimulating factors and therefore caused the same effect on germination rate and appressoria formation. The inhibition of infective structures on the non-host *S. decorum* may be explained by the concentration of compounds or the type of nutrients present (different lipids composition) compared to the cuticle of the *S. rostratum* host. This stresses the need for the identification and quantification of extracted lipids before reaching any conclusion about the involvement of lipids in the invasion process of black fly cuticle by *E. conica*.

In summary, only the aerial secondary type 2 conidia of *E. conica* invaded the cuticle of *S. rostratum*. Specificity of infection observed in the field may therefore be
related to the invasion of the cuticle as opposed to the inhibition of spore germination; *E. conica* cannot pass through the cuticle of the resistant species *S. decorum* because the formation of the infective structures was inhibited. Cuticular lipids may play a role by triggering appressorium formation. The inadequate milieu of the non-host or the milieu of various body parts of the proper host may be a factor delaying the germination of the invasive unit and inducing replicative conidial formation.

**ACKNOWLEDGMENTS**

We thank Dr. Peter Adler from South Carolina University for cytological analysis of the *S. rostratum* species and Mr. Guy Rimmer for technical assistance with electron microscopy. This study was supported by N.S.E.R.C. to Dr. Gary Dunphy and Dr. Jacques Boisvert.
FIG. 1. Development of primary conidia of *Erynia conica* on the wings (A) and abdominal sections (B) of *Simulium rostratum* (incubated in darkness at 20 °C and 95 % RH), in terms of the cumulative percentage of germinating conidia (△) and secondary type 1 (○) or type 2 (□) conidial formation. Each point represents the mean and standard error of the mean from triplicate samples representing a minimum of 100 observations. The concentration of conidia for the replicates varied between 12-20 and 11-16 spores/mm² for wings and abdomens, respectively.
**FIG. 2.** Photomicrograph of secondary type 2 conidia of *Erynia conica* on the wings of *Simulium rostratum* (incubated in darkness at 20 °C and 95 % RH). (1) Vegetative germination showing septum (arrow) and cytoplasmic migration at the tip (double arrows). (2) Appressoria formation (arrow) and penetration with fungal ramification after invasion (double arrows). (3) Formation of rare tertiary type 2 conidia. Scale bar = 10 μm.
FIG. 3. Development of secondary type 2 conidia of *Erynia conica* on the wings (A) and abdominal segments (B) of *Simulium rostratum* (incubated in darkness at 20 °C and 95 % RH), in terms of germinating (Δ), appressorial formation (○), penetration of the cuticle (□), and tertiary type 1 conidial formation (Θ). Each point represents the mean and standard error of the mean from triplicate samples and represents a minimum of 75 observations. The concentration of conidia and the percentage of secondary type 1 conidia for the replicates varied between 2.5-4.3 spores/mm² and 0-1.4 %, respectively, for experiments with wings, and 4.6-6.3 spores/mm² and 8-17 %, respectively, for experiments with abdominal cuticle.
FIG. 4. Scanning electron micrograph of wings of *Simulium rostratum* (1,2) and *Simulium decorum* (3,4) controls (1,3) and wings extracted with a chioroform: methanol solvent (2,4). Scale bar = 10 μm.
### TABLE 1

Effect of the storage time of the wings of the proper black fly host *Simulium rostratum* at -20 °C on the stages of invasion of secondary type 2 conidia of *Erynia conica. a*

<table>
<thead>
<tr>
<th>Storage time of the wings</th>
<th>Time (hour)</th>
<th>Cumulative percentage b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total germination</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 month</td>
<td>0</td>
<td>56.6 a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>65.8 a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>69.8 a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>78.9 a</td>
</tr>
<tr>
<td>6 months</td>
<td>0</td>
<td>67.4 a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>71.5 a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>81.9 ab</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>85.9 a</td>
</tr>
<tr>
<td>12 months</td>
<td>0</td>
<td>62.5 a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>70.6 a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>85.7 b</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>86.5 a</td>
</tr>
</tbody>
</table>

a Incubation in darkness at 20 °C, RH= 95 %.

b Each value represents a minimum of 120 observations from 10 wings. The concentration of total conidia and the percentage of secondary type 1 conidia was 4.3 spores/mm² and 0.0-1.0 %, respectively. For each time percentages within columns for the same time by the same letter the are not significantly different according to the Chi² test ($\alpha = 0.05$).
TABLE 2

Effects of four months of storage of wings of the proper black fly host *Simulium rostratum* at -20°C and after decontamination with 70% ethanol on the stages of invasion of secondary type 2 conidia of *Erynia conica.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hour)</th>
<th>Total germination</th>
<th>Apressoria</th>
<th>Cuticular penetration</th>
<th>Tertiary type 1 conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cumulative percentage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Storage at -20°C</td>
<td>0</td>
<td>45.7 a</td>
<td>10.9 a</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>No ethanol</td>
<td>2</td>
<td>65.9 a</td>
<td>13.6 a</td>
<td>4.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>76.2 a</td>
<td>19.0 a</td>
<td>9.5 a</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>81.8 a</td>
<td>21.2 a</td>
<td>12.1 a</td>
<td>6.1 a</td>
</tr>
<tr>
<td>Storage at -20°C</td>
<td>0</td>
<td>61.4 a</td>
<td>8.9 a</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ethanol 70%</td>
<td>2</td>
<td>66.1 a</td>
<td>10.8 a</td>
<td>3.1</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>73.2 a</td>
<td>8.9 a</td>
<td>5.4 a</td>
<td>1.8 a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>79.2 a</td>
<td>15.1 a</td>
<td>5.7 a</td>
<td>5.7 a</td>
</tr>
<tr>
<td>No storage</td>
<td>0</td>
<td>42.8 a</td>
<td>9.5 a</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ethanol 70%</td>
<td>2</td>
<td>51.3 a</td>
<td>7.7 a</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>88.6 a</td>
<td>14.3 a</td>
<td>8.6 a</td>
<td>11.4 b</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>80.0 a</td>
<td>15.0 a</td>
<td>12.5 a</td>
<td>17.5 a</td>
</tr>
</tbody>
</table>

* Incubation in darkness at 20°C, RH = 95%.

* Each value represents a minimum of 60 observations from 10 wings. The concentration of total conidia and the percentage of secondary type 1 conidia varied between 2.0-3.4 spores/mm² and 4.5-15.0%, respectively. For each time percentage within columns for the same time by the same letter are not significantly different according to the Chi² test (α = 0.05).
### TABLE 3

Development of the infection stages of secondary type 2 conidia of *Erynia conica* on wings of both sexes of the proper black fly host *Simulium rostratum*, and the wings of females of the non-host *Simulium decorum*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (hour)</th>
<th>Cumulative percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wings of female <em>Simulium rostratum</em></td>
<td>0</td>
<td>3.7 (0.0, 22.1) a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.7 (1.8, 37.9) a c</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>11.4 (4.8, 34.8) a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>12.3 (13.4, 27.6) a</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>12.9 (6.3, 25.7) a</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>4.2 (7.5, 16.3) a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11.4 (15.4, 24.0) a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.6 (5.1, 31.0) a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>12.9 (5.4, 36.8) a</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>10.3 (3.7, 28.6) a</td>
</tr>
<tr>
<td>Wings of female <em>Simulium decorum</em></td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.0</td>
</tr>
<tr>
<td>Wings of male <em>Simulium rostratum</em></td>
<td>0</td>
<td>16.9 (16.3, 32.3) c</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34.0 (22.1, 49.2) b</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>51.5 (30.6, 61.1) b</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>50.6 (23.6, 67.1) b</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>69.6 (44.6, 68.5) a</td>
</tr>
</tbody>
</table>

* Incubation in darkness at 20 °C, RH = 95%.
* Decoded means of arcsin $p^{1/2}$ transformed data (with 95% confidence limits of the transformed data) from three groups of 5 wings based on a minimum of 100 observations. The concentration of conidia and the percentage of secondary type 1 conidia varied between 2.5-6.8 spores/mm² and 13-13.4 %, respectively. For each time means within columns for the same time by the same letter are not significantly different according to SNK-test ($\alpha = 0.05$).
* Mann-Whitney rank sum test.
### TABLE 4

Effect of the extraction of lipids from the wings of the proper black fly host *Simulium rostratum* on the development of secondary type 2 conidia of *Erynia conica*. $^a$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hour)</th>
<th>Cumulative percentage $^b$</th>
<th>Germination</th>
<th>appressoria</th>
<th>cuticular penetration</th>
<th>tertiary type 1 conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>12.3 (0.0, 49.4) a</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Delipided with chloroform:methanol (2:1 v/v)</td>
<td>2</td>
<td>34.8 (17.9, 54.3) a</td>
<td>0.0</td>
<td>0.0</td>
<td>33.4 (19.8, 50.8) a $^c$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>71.4 (51.3, 64.0) a</td>
<td>0.0</td>
<td>0.0</td>
<td>56.7 (22.7, 75.0) a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>95.5 (72.3, 83.2) a</td>
<td>0.0</td>
<td>0.0</td>
<td>55.8 (26.8, 69.8) a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>95.7 (76.1, 79.9) a</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delipided with chloroform:methanol (2:1 v/v) and recoated with the extract</td>
<td>0</td>
<td>2.0 (0.0, 25.7) a</td>
<td>0.0</td>
<td>0.0</td>
<td>34.6 (21.2, 50.8) a $^d$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.2 (1.8, 42.6) a</td>
<td>0.0</td>
<td>0.0</td>
<td>54.3 (23.0, 71.8) a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>67.4 (40.4, 70.0) a</td>
<td>0.0</td>
<td>0.0</td>
<td>56.4 (31.1, 66.2) a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>72.8 (37.5, 79.5) b</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>81.4 (61.9, 67.0) b</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>51.0 (39.9, 51.2) b</td>
<td>2.4 (7.5, 10.3)</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>71.9 (37.0, 78.9) b</td>
<td>6.4 (2.6, 26.3)</td>
<td>0.2 (0.0, 14.8)</td>
<td>0.5 (0.0, 20.9) a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>80.5 (49.8, 77.5) a</td>
<td>7.3 (1.8, 29.6)</td>
<td>2.7 (0.0, 19.9)</td>
<td>7.2 (6.5, 24.5) a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>86.5 (60.5, 76.3) ab</td>
<td>9.7 (6.3, 29.9)</td>
<td>5.9 (0.0, 33.1)</td>
<td>10.9 (0.0, 37.3) b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>90.3 (62.3, 81.5) c</td>
<td>9.7 (8.5, 27.7)</td>
<td>6.7 (3.1, 26.6)</td>
<td>10.0 (0.0, 37.6) b</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Incubation in darkness at 20 °C, RH= 95 %.

$^b$ Decoded mean of arcsin $p^{1/2}$ transformed data (with 95 % confidence limits of the transformed data) from three groups of 5 wings each with a minimum of 100 observations per group. The concentration of conidia and the percentage of secondary type 1 conidia varied between 2.0-5.4 spores/mm$^2$ and 1.5-6.3 % respectively. For each time means within columns for the same time with the same letter are not significantly different according to SNK-test ($\alpha = 0.05$).

$^c$ Kruskall-Wallis one way ANOVA on ranks test.

$^d$ Mann-Whitney rank sum test.
TABLE 5

Effect of the extraction of lipids from the wings of the non-host black fly *Simulium decorum* on the development of secondary type 2 conidia of *Erynia conica*. a

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hour)</th>
<th>Cumulative percentage b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total germination</td>
</tr>
<tr>
<td>Delipided with chloroform:methanol (2:1 v/v)</td>
<td>0</td>
<td>0.0 (6.8, 26.7) a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>36.6 (12.2, 62.3) a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>69.8 (48.8, 64.5) a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>83.8 (60.1, 72.3) a</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>87.9 (67.0, 72.1) a</td>
</tr>
<tr>
<td>Delipided with chloroform:methanol (2:1 v/v) and recoated with the extract</td>
<td>0</td>
<td>4.0 (9.5, 13.8) a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.4 (17.7, 28.6) a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>60.2 (44.5, 57.2) b</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>87.6 (64.4, 74.4) a</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>90.4 (67.1, 76.8) a</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>16.3 (0.0, 47.2) a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>42.5 (13.0, 68.3) a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>73.4 (57.3, 60.6) a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>84.4 (61.5, 71.9) a</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>90.4 (58.1, 85.6) a</td>
</tr>
</tbody>
</table>

a Incubation in darkness at 20 °C, RH = 95 %.
b Decoded mean of arcsin p^{1/2} transformed data (with 95 % confidence limits of the transformed data) from three groups of 5 wings each with minimum of 100 observations per group. The concentration of conidia and the percentage of secondary type 1 conidia varied between 2.8-4.7 spores/mm² and 12.3-15.4 %, respectively. Means within columns for the same time with the same letter are not significantly different according to SNK-test (α = 0.05).
LITERATURE CITED


of the pea aphid *Acyrthosiphon pisum* (Harris). Comp. Biochem. Physiol. 82, 401-411.


Steinkraus, D.C., and Slaymaker, P.H. 1994. Effect of temperature and humidity on formation, germination, and infectivity of conidia of Neozygites fresenii