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MODE OF INFECTION OF *SIMULIUM* BY *ERYNIA CONICA*

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The four conidial types of *Erynia conica* were examined to see which could function as infective propagules. Of these only the secondary aerial globose conidium was seen penetrating the cuticle of adult *Simulium*. Attempts to infect other stages in the life-cycle of the blackfly by the globose or any other of the conidial types were unsuccessful.

The mode of infection by Entomophthoraceae has been investigated by many authors (Rees, 1932; Sawyer, 1933; Speare, 1912; Harris, 1948; Rockwood, 1950; Krejzova, 1972; Carner, 1976; Brobyn & Wilding, 1977; Kramer, 1980, 1982; Milner & Bourne, 1983; Glare, Chilvers & Milner, 1985; Yendol & Paschke, 1965). These investigations were on members of the Entomophthoraceae that sporulate in air and are found in a terrestrial environment. These fungi rarely have more than two kinds of conidia as potential infective propagules, and the hosts have no more than one or two stages in their life-cycle potentially susceptible to infection.

The discovery of tetraradiate aquatic conidia (Webster, Sanders & Descals, 1978) led Descals *et al.* (1981) to suggest that the immature aquatic stages of Simuliidae might be infected by *Erynia conica* (Nowak.) Remaudière & Hennebert. Whilst *E. conica* is known to sporulate in the ovipositing female population (Descals *et al.*, 1981; Hywel-Jones & Ladle, 1986) it was not known if it could infect the immature stages. Hywel-Jones (1984) found no evidence to support this hypothesis.

Tetraradiate conidia of *E. conica* have been found in foam samples (Ingold, 1967, 1968; Webster *et al.*, 1978; Descals *et al.*, 1981; Hywel-Jones, 1984) showing that they are present in river water and therefore might infect the submerged larval or pupal stages of the blackfly (Fig. 1 of Descals *et al.*, 1981). Hywel-Jones (1984) observed conidia of *E. conica* in river water but found no evidence of infection of any of the immature stages of Simuliidae by them. Descals *et al.* (1981) considered five pathways for the infection of adult *Simulium*. The results of investigations on these and other pathways are presented in this paper.

METHODS

All experiments used field-collected flies as the source of conidia. Most of the flies were *Simulium argyreatum* Meigen or *S. variegatum* Meigen from sites on the R. Teign in Devon, South West England. Other species used included *S. ornatum* Meigen from the R. Teign and other rivers, *S. reptans* Linn. from the R. Teign and *S. equinum* Linn. from East Devon rivers and Dorset chalk streams.

Maintenance of adults

Rearing dishes were made from plastic 9 cm Petri dishes with a 2 cm hole in the lid through which the flies could be put. This hole was closed with the disc of plastic which had been removed held in place with adhesive tape. The dishes contained a folded piece of tissue paper wetted with distilled water. A square of tissue paper soaked in 3% sucrose solution was the food supply for the flies.

Attempted infection of adults by primary aerial cornute conidial. Petri dishes with a disc of wet filter paper in the lid were used as infection chambers. Healthy flies were taken from mass rearing chambers and anaesthetized with natural gas (Hywel-Jones, 1984) before transfer to the infection dishes where they were exposed to a shower of primary cornute conidia for 6–18 h. After exposure, flies were removed from the infection chambers without anaesthetization using a pooter and were put into the rearing dishes (10–20 per dish). The rearing dishes were examined twice a day for evidence of diseased flies until all the flies had died. No attempt was made to examine the flies for attached conidia or to dissect them and look for hyphal bodies. Several runs of this experiment were carried out at 10–12° and at 15°.

Attempted infection of adults by primary and secondary aerial conidia. Conidia of *E. conica* need free water to survive and germinate (Hywel-Jones, 1984). Attempts were, therefore, made to maintain a saturated atmosphere to ensure that primary aerial conidia could survive long enough to germinate or produce secondary conidia.

Primary aerial conidia were discharged for 24 h at 20° from infected flies fixed into the lids of 9 cm Petri dishes either on to moist filter paper or on to squares of wet cellophane. The inoculum was removed by replacing the Petri dish lids containing the inoculum with empty fresh lids. The dishes were then inverted. Healthy flies were anaesthetized and placed on the dry floor of the infection chambers and allowed to recover before the dishes were again inverted so that conidia were beneath the flies. Flies were kept in these chambers overnight for 12–15 h at either 15 or 20°. As many as half the flies were, at times, drowned in the confined space as they became trapped by their wings in the surface water film. Those that were still alive were removed and transferred to rearing dishes and kept at either 15 or 20°. The dishes were examined twice a day for dead flies which were removed and either incubated overnight to see whether *E. conica* would grow out or dissected for hyphal bodies. Flies that were incubated were also dissected if there were no external signs of infection by *E. conica* after 24 h.

Infection of adults by tetraradiate conidia. The discovery of large numbers of tetraradiate conidia in the surface water films on the boulders used by the females for oviposition (Hywel-Jones, 1984) suggested that tetraradiate conidia might be involved in the infection of this stage.

Field-collected infected flies were submerged in aerated water for 6 h to encourage the development of aquatic conidia. An undetermined number of the two types of tetraradiate conidia (Descals *et al.*, 1981) were seeded on to 4 cm squares of wet cellophane. Dead flies bearing aquatic coronate conidia were then removed and placed in the centre of wet 4 × 4 cm cellophane squares in a 9 cm Petri dish lid. The squares and inoculum were incubated at 20° for a further 24 h, after which the inoculum source was removed. Adults from field-collected pupae reared in the laboratory were anaesthetized and dropped on to the wet cellophane bearing conidia. The Petri dish lids were then replaced and the dishes gently shaken until the flies were wet. The flies were then picked off the cellophane, and transferred to rearing dishes, placed at 15° and examined for tetraradiate conidia. As flies died they were incubated and examined for external and internal signs of infection.

Moss substratum infection chamber. Infected female *Simulium* found along the weirs at Fingle Bridge and Steps Bridge are often attached by rhizoids to the moss covering the boulders forming the weir. Observation in the field showed that some of the flies with wet wings crawl through and over the moss before coming to rest on the downstream surfaces of the oviposition sites. Similarly, some ovipositing flies land on the downstream face of boulders before migrating to the oviposition sites by walking and/or making hopping flights over the moss (Hywel-Jones & Ladle, 1986).

A vertical glass tube (18 × 2 cm) was packed loosely with washed moss collected from Fingle Bridge. De-ionized water was dripped slowly through the moss and four infected flies collected from the weir were put on to the moss. A flask was placed below the tube to collect the outflow. The set-up was left for 24 h at 20° to allow sufficient time for the conidia to develop. The glass tube was then inserted through the hole in the side of the clear plastic sandwich container (Fig. 1) containing moist paper towelling and filter paper soaked in 3% sucrose.

One hundred blackflies of undetermined species, and sex (approximately 1:1), were anaesthetized and put into the glass tube (Fig. 1). A length of aluminium foil was wound tightly round the tube

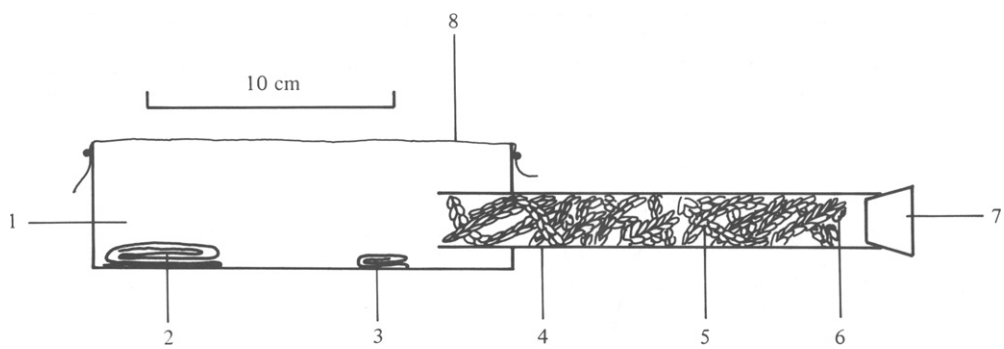


Fig. 1. Chamber used for infecting *Simulium* adults with conidia of *Erynia conica*. 1, sandwich container; 2, tissue paper soaked with water; 3, tissue paper soaked with a 3% sucrose solution; 4, glass tube wrapped with aluminium foil; 5, moss; 6, inoculum of infected flies; 7, rubber bung; 8, muslin cloth held in place with an elastic band.

so that the only light entering the tube was from the end which opened into the plastic sandwich container. On recovering from the anaesthetic the blackflies were attracted to the light and crawled through the moss before emerging into the container. The flies in the container were examined twice a day. Dead flies, removed from the container and from the glass tube, were dissected and examined for attached conidia and/or hyphal bodies.

Examination of flies for infecting conidia of E. conica

The abdomen and wings were examined for attached conidia of *E. conica*. Wings were removed and mounted in lactofuchsin and examined microscopically. The abdomens of the flies were removed and placed on microscope slides. A tear was made along the length of the abdomen from posterior to anterior and just below the tergites. The abdominal organs were removed and the remains were mounted in a drop of lactofuchsin with the subcutaneous tissue uppermost. Two insects pins with their tips bent at 45° were used to scrape away the subcutaneous layers. The cuticle was then remounted with the exterior surface uppermost and examined microscopically. The abdominal contents were also mounted in lactofuchsin and examined for hyphal bodies. Several flies were also examined by SEM.

RESULTS

Conidial shower method

Over three hundred flies were exposed to aerial conidia. The flies were a mixture of both sexes and of four species *S. argyreatum/variegatum*, *S. ornatum* and *S. reptans*. In none of these was there any external evidence of infection by *E. conica* after death. No hyphal bodies were found in any of the flies which were dissected.

Cellophane substratum method

There was no evidence that flies subjected to these treatments died as a result of infection by *E. conica*. On several occasions dead flies with swollen abdomens were found. In all cases this swelling was probably due to excessive sugar feeding (Davies, 1953) and not to infection. No evidence of hyphal bodies was found neither in flies that were dissected immediately after death nor in flies that had been incubated for a further 24 h after death.

Infection with tetraradiate conidia

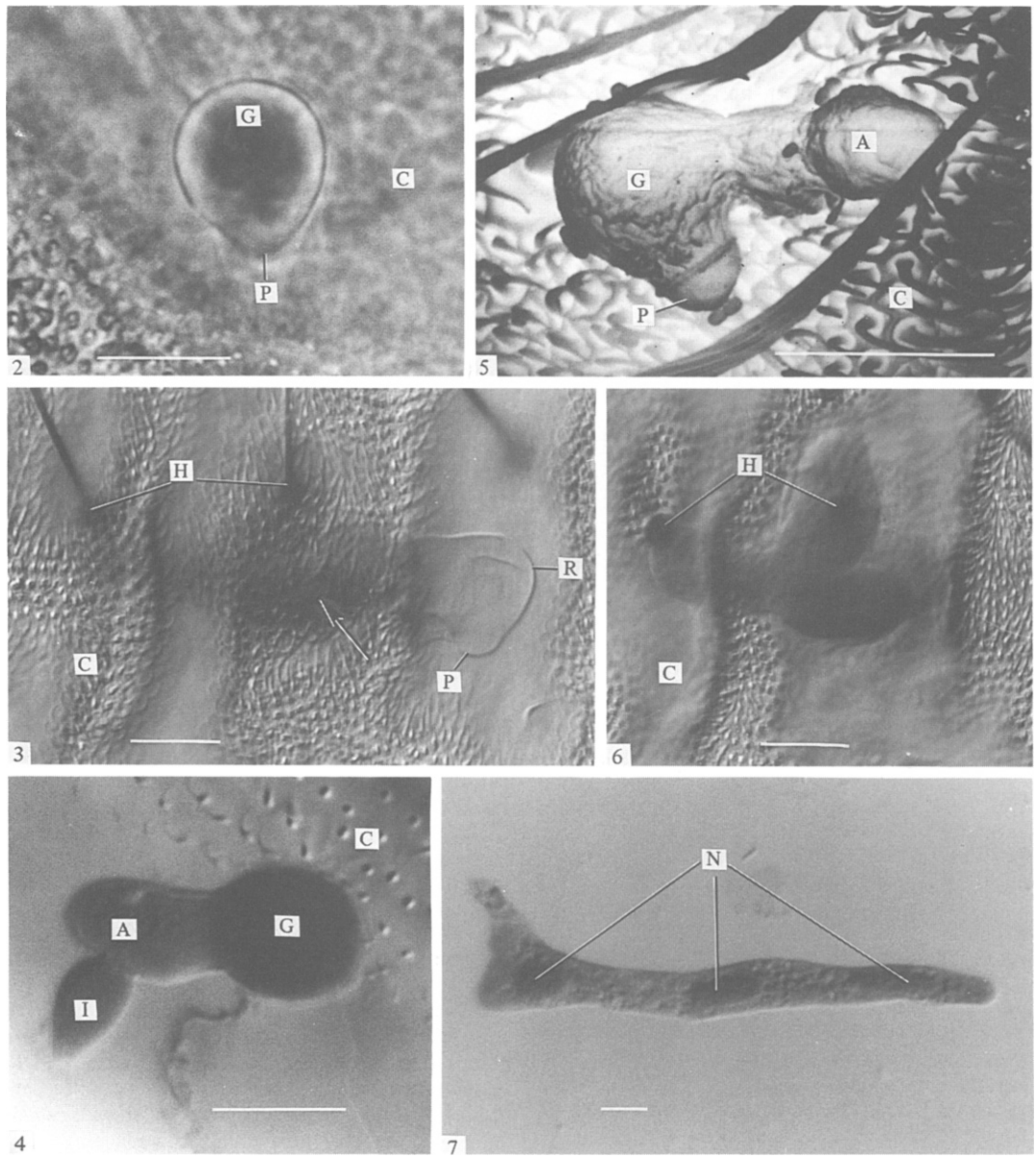
Few of the flies recovered from this treatment. Of 150 flies only 23 were still alive by the following day. Those that died were examined for attached tetraradiate conidia. Both primary coronate and secondary stellate conidia were found trapped amongst the body hairs, mainly of the legs. No evidence of penetration of the cuticle was found. Those tetraradiate conidia which had germinated were producing 'secondary' aerial cornute conidia. Each day, after exposure to the conidia, five flies were selected at random, removed and killed in Formalin Acetic Alcohol (FAA). The flies, examined externally for conidia, were then dissected and examined for hyphal bodies. On the first day after inoculation all the flies had at least one of the tetraradiate conidia visible on its exoskeleton, mainly among hairs on the legs. None of the conidia were infecting the flies. When examined internally no hyphal bodies were seen. On subsequent days no conidia were seen on the exoskeleton and no hyphal bodies were found in the insects.

Moss substratum method

When anaesthetized flies were put into the glass tube they recovered within a minute. Within 10–20 min the first flies emerged into the sandwich container. Twenty four hours after the flies were put into the glass tube it was removed and the hole in the sandwich container closed. The tube was examined for dead flies or flies that had become trapped by their wings to the glass. Four males and three females which were still alive were removed. One of the flies (a male *S. reptans*) had become trapped by its wings beneath one of the sporulating flies. When this individual was examined under the dissection microscope at a magnification of 50× it was found to be coated with aerial cornute and globose conidia. The fly was killed with FAA and dissected. Fourteen globose aerial conidia were attached to the cuticle of the abdomen. The aerial cornute conidia were washed off during preparation. Some coronate and stellate conidia were seen under the cover slip but none were found attached to the cuticle.

The remaining 6 flies were also killed and dissected. All had at least one globose conidium attached to the abdomen and/or wings. One was identified as *S. equinum* and the others were either *S. argyreatum* or *S. variegatum*.

Some of the globose conidia attached to the cuticle contained cytoplasm (Fig. 2) whilst others were empty (Fig. 3). The globose conidia that developed in the moist environment of the moss



Figs 2–7. *Erynia conica*: germination, germ tube penetration and hyphal body development.
Fig. 2. A globose conidium containing cytoplasm adhering to the cuticle of *Simulium argyreatum*. No appressorium has formed at this stage.
Fig. 3. An empty globose conidium. Note the round hole (arrowed) made by the penetrating germ tube. The shadowing is due to the stained hyphal body developing beneath the cuticle. Compare this micrograph with fig. 6.
Fig. 4. A germinating globose conidium dissected from the cuticle of an adult to show the appressorium and infection peg.
Fig. 5. A SEM of a globose conidium germinating on an adult *Simulium*.
Fig. 6. The branched hyphal body produced by the globose conidium of Fig. 3. The two cuticular hairs can be used as reference points;
Fig. 7. A branching hyphal body of *E. conica* from the abdomen of an adult *Simulium*.
The scale bars in the figures are 10 μm. A, appressorium; C, cuticle; G, globose conidium; H, cuticular hair; I, infection peg; N, nucleus; P, papilla; R, remains of spore wall.

were attached to and had germinated and penetrated the abdominal cuticle of the flies. Even globose conidia apparently lacking germ-tubes adhered to the cuticle (Fig. 2) whereas the other three conidial types were easily washed off during preparation.

Having attached to the abdomen a small germ-tube developed; usually within 24 h. One such germinating globose conidium was dissected from the abdomen of a female *S. argyreatum* at an early stage of penetration (Fig. 4). The short, fat germ-tube was appressed to the insect's cuticle and appeared to function as an appressorium (Fig. 5). The base of the appressorium was flattened against the cuticle (Fig. 4).

A tube, 3 μm diam, developed beneath the appressorium and penetrated the cuticle. On one occasion the penetration peg was seen developing from the tip of the appressorium where it was pressed against a wing vein. No evidence was seen of cuticular tearing such as the triradiate fissures described for *Entomophthora muscae* Fres. by Webster (1980). Once the peg had passed through the cuticle it began to swell (Fig. 4). The infection body grew to 75 × 18 μm within 24 h of invading the fly. On several occasions the infection body was seen to bud soon after development (Fig. 6).

Flies were removed from the sandwich container each day and examined for signs of infection. Forty eight hours after removing the tube and inoculum source no evidence was found of conidia of any kind adhering to the cuticle of the flies. However, hyphal bodies which were usually branched and multinucleate but never numerous (Fig. 7) were found. After three days, bacteria were seen in increasing numbers in the insects and infection by *E. conica* did not develop further. All flies that subsequently died contained many bacteria. Occasionally hyphal bodies of *E. conica* were seen in these dead flies.

DISCUSSION

Whereas some species of Entomophthoraceae can infect their hosts with different conidial types (Table 1). *E. conica* would seem to be limited to one type of infective conidium namely the aerial globose. No evidence was found of primary cornute or tetraradiate aquatic conidia being the infective agents. Descals *et al.* (1981) considered 10 possible ways of infection; 5 aerial infecting the adult and 5 aquatic, infecting the larva. In the course of this work and that of Hywel-Jones (1984) all of these pathways were studied and only one resulted in successful infection.

Glare *et al.* (1985) concluded that 'primary conidia are undoubtedly the main infective agents in entomophthoran species which lack capilliconidia'. This does not appear to be the case for *E. conica* which does not produce capilliconidia. We suggest that the primary conidia may act as a dispersal stage with the secondary conidia acting as the main infective agent in most species of Entomophthoraceae. Where primary conidia have been described as the infective agents this has often been on species whose primary and secondary conidia are similar. Empty spore coats of primary conidia of *T. fresenii* were found near germinating capilliconidia suggesting development of the latter from the former (Brobyn & Wilding, 1977; Bitton *et al.*, 1979). Similarly, primary aerial conidia of *E. conica* were found giving rise to 'secondary' globose conidia on the insect's cuticle. Matanmi & Libby (1975) considered that forcible discharge of conidia enhances adhesion prior to germination and penetration. Glare *et al.* (1985) found that the passively discharged capilliconidia have an 'attaching sac' or viscid droplet which aids attachment.

Webster (1980) described an appressorium in the development of infecting conidia of *Ent. muscae* but Brobyn & Wilding (1983) considered this structure to be a pad which may merely be a part of the protoplasm discharged with the conidium and therefore not an appressorium ('a swelling on a germ tube or hypha, especially for attachment in an early stage of infection') in the sense of Hawksworth, Sutton & Ainsworth (1983). However, Brobyn & Wilding (1977) described the development of an appressorium by the capilliconidia of *T. fresenii*. It would appear that *E. conica*

Table 1. *Infective stages of species of Entomophthoraceae*

Conidiobolus coronatus (Cost.) Batko	Primary & microconidia	Prasertphon (1963)
C. coronatus	Primary	Prasertphon & Tanada (1968)
C. thromboides Drechsler	Primary & secondary	Brobyn & Wilding (1977)
C. thromboides	Primary	Matanmi & Libby (1975)
Entomophthora planchoniana Cornu	Primary & secondary	Brobyn & Wilding (1977)
E. lampyridarum (Thaxter) MacLeod & Muller-Kogler	Capilliconidia	Carner (1980)
Triplosporium fresenii (Nowakowski) Batko	Capilliconidia	Brobyn & Wilding (1977)
T. floridanum (Weiser & Muma) Weiser	Capilliconidia	Carner & Canerday (1968)
T. floridanum	Capilliconidia	Carner (1976)
Zoophthora phalloides	Capilliconidia	Glare *et al.* (1985)
Erynia conica	Secondary globose	This study

infects its host with 'secondary' conidia producing appressoria in much the same way as *T. fresenii*. Appressoria are therefore present in at least two genera of the Entomophthoraceae and are produced by two different types of infective propagule.

Gabriel (1968), who studied the histochemistry of penetration of the insect cuticle by *Conidiobolus coronatus* (Cost.) Bakto, was able to demonstrate the presence of lipase activity and concluded that chitinolytic activity was very weak. Protein hydrolysis occurred around the invading hypha. Black spots on the insect's cuticle were due to a melanin reaction by the host. No such spots were found around penetrating germ tubes of *E. conica* although they were produced in response to attack by parasitic mites (Hywel-Jones, 1984). Penetration is by enzymatic and mechanical means in *Ent. muscae* (Brobyn & Wilding, 1977, 1983). Small triradiate (occasionally tetraradiate) fissures are associated with the penetration tube of *Ent. muscae* (Webster, 1980; Brobyn & Wilding, 1983). These were not seen around the germ tubes of invading *E. conica* 'secondary' globose conidia. The smallness of the hole made by the infection peg and the lack of cuticular tearing suggests that *E. conica* might penetrate primarily by enzymatic rather than mechanical means. The appressorium of *E. conica*, however, also provide an attachment for the growing infection peg to act against and the penetration process possibly involves a combination of enzymic digestion and mechanical pressure. The importance of each of these will depend of the species of fungus. Once a germ tube from the conidium has penetrated the cuticle all the cytoplasmic contents move into the advancing germ peg. This appears to be general for all entomopathogenic species of the Entomophthoraceae (see introduction). The infection peg of *E. conica* grows into either a long sausage-shaped body or into a lobed structure within 24 h of infection. Forty eight hours after infection multibranched hyphal bodies are seen in the haemolymph. The further development of these is prevented by rapid bacterial contamination.

There is a large difference in the proportions of infected females to infected males in the field-collected flies (Descals *et al.*, 1981; Hywel-Jones & Ladle, 1986). This imbalance is not evident in the laboratory-infected population of *Simulium* and both sexes appear to be susceptible to infection by *E. conica*. Although the conidia came from field-collected *S. argyreatum/variegatum* they infected two other species, *S. reptans* and *S. equinum*, neither of which have previously been reported as hosts for *E. conica*. However, further discussion of the host specificity of *E. conica* must wait until the complete cycle of infection has been demonstrated in the laboratory.

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